Rational Metabolic Engineering of Escherichia Coli for High-yield L-serine Production

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Abstract

Background: L-serine is widely used in the food, cosmetic and pharmaceutical industries, and direct fermentation of L-serine from glucose is an attractive technique. However, L-serine producers have historically been developed via classical random mutagenesis due to the complicated metabolic network and regulatory mechanism of L-serine production, leading to un-optimal productivity and yield of L-serine and thus limiting its large-scale industrial production.

Result: In this study, a high-yield and high-productivity *Escherichia coli* strain was constructed by a defined genetic modification methodology for L-serine production. First, L-serine-mediated feedback inhibition was removed and L-serine biosynthetic pathway genes (*serA*, *serC* and *serB*) associated with phosphoglycerate kinase (*pgk*) were overexpressed. Secondly, L-serine conversion pathway was further examined by introducing a *glyA* mutation (K229G) and deleting other degrading enzymes based on deletion of initial *sdaA*. Finally, the L-serine transport system was rationally engineered to reduce the uptake and accelerate the export of L-serine. The optimally engineered strain produced 35 g/L L-serine with a productivity of 0.98 g/L/h and yield of 0.42 g/g glucose in a 5-L fermenter, the highest productivity and yield of L-serine from glucose reported to date. Transcriptome and intermediate metabolite were analyzed to further understand the regulatory mechanism of L-serine production.

Conclusion: These results demonstrated that combined metabolic and bioprocess engineering strategies can improve L-serine productivity and yield, thus providing basic principles for rationally designing of high-yield production strains and paving the way for towards a simple and economical process for industrial L-serine production.

1. Background

L-serine (L-Ser) is a vital amino acid in metabolism in humans and other animals and is widely used in the food, pharmaceutical and cosmetic industries[1]. Additionally, L-Ser has been identified as one of the top 30 most interesting biochemical building block. Currently, L-Ser production relies on enzymatic or direct fermentation[2, 3], and the global L-Ser production capacity (350 tons per year) is well below the expected market demand (3000 tons per year)[4]. Therefore, it is necessary to develop a more effective L-Ser production method. Furthermore, demand exists for developing a direct fermentation approach that can be implemented with low-cost substrates and will can simplify operational purification procedures and reduce pollution, as enzymatic conversion always utilizes the expensive precursors glycine and methanol[5].

L-Ser production by microbial fermentation has been extensively studied in *Corynebacterium glutamicum*. In 2007, Petra Peters-Wendisch et al. engineered a *C. glutamicum* strain with an industrial production capacity 36 g/L L-Ser[6–8]. The strain was constructed by overexpressing L-Ser pathway genes, deleting the L-Ser dehydratase *sdaA*, and reducing the expression of serine hydroxymethyltransferase (SHMT) encoded by *glyA* and was cultured with an external folate supply[6–8]. Replacement of folate with corn steep liquor led to the production of an L-Ser titer of 43 g/L in 96 h by another *C. glutamicum* strain with random mutation and minimization of by-product synthesis[9]. However, as a host, *Escherichia coli* has attracted attention due to its well-characterized genetic background, amenability to genetic manipulation, and faster growth rate, higher fermentation intensity and better utilization rate of glucose in direct fermentation[10]. More importantly, L-Ser can be produced from glucose by fermentation with a higher theoretical yield (approximately 1.34 g/g glucose) in *E. coli* than in *C. glutamicum* (1.16 g/g)[11].

Recently, Hemanshu Mundhada et al. developed a strain of *E. coli* with a production capacity of 11.7 g/L L-Ser via ultraviolet radiation on a 1 L scale[1]. Then, this group increased the production capacity to 37 g/L by adaptive laboratory evolution[12]. Subsequently, translation initiation optimization led to the production of an L-Ser titer of 50 g/L with a yield of 0.36 g/g from glucose in 60 h by strain ALE-5 via evolution engineering this L-Ser production capacity is the highest of any strains to date[13]. However, the application of many random mutagenesis events makes the description of efficient L-Ser synthesis mechanisms difficult. Furthermore, previous studies have shown that repeated random mutation often leads to unknown mutations at some locations in the genome together with targeted mutagenesis[14]. The impact of these unknown mutations is difficult to determine. Moreover, whether the unknown mutations affect universally industrialized fermentation. All these factors limit the application of these strains in industrial production.

In this study, an L-Ser-producing strain was constructed from *E. coli* W3110 by introducing a series of defined genetic manipulations (Fig. 1). A basic production strain was constructed by strengthening the L-Ser biosynthesis. Then L-Ser degradation and conversion pathways were optimized. Higher production was further achieved by engineering the L-serine transporter system. The rational design strategies described here significantly improved L-serine productivity and yield in fed-batch fermentation.

2. Results And Discussion

2.1 Construction of the L-serine production strain from *E. coli* W3110

As a prerequisite for L-Ser production, the activity of the branch pathway leading to L-Ser biosynthesis, which involves *serA*, *serC* and *serB*, was enhanced. PGDH, encoded by *serA*, catalyzes the initial reaction in L-Ser biosynthesis and the catalytic activity of PGDH can be regulated by feedback inhibition by L-Ser in *E. coli*[23]. The feedback inhibition was overcome by mutation of two residues (344 and 346) to alanine, as previously described, which would remove the hydrogen bonds between L-Ser and the regulatory binding domain. This led to the mutated gene named *serA* [24]. The feedback resistance of the enzyme PGDH, encoded by *serA*, was investigated by overexpressing these genes in BL21(DE3) via the pT7-7 vector. The activity of *serA* could be sustained at 95% with 80 mmol/L L-Ser, whereas the activity of the wild-type enzyme remained at only 10% (Fig. 2A). Then, *serA*, *serC*, and *serB* were overexpressed in the low copy number pSC vector containing the PR or PL promoter with resulting in SP-01, SP-02 and SP-03 (Fig. 2B).
To produce L-Ser, the *sdaA* gene encoding the L-Ser-specific dehydratase was first deleted from *E. coli* W3110 to construct the SSW-01 strain. Subsequent deletion of *glyA*, encoding SHMT, which converts L-Ser to glycine, resulted in the double knockout SSW-02 strain. To evaluate the L-Ser production capacity, the resulting plasmids SP-01 (SP-*serA*B), SP-02 (SP-*serA*B*Cpgk*) and SP-05 (SP-*serA*B*Cpgk*, *pgk*) were transformed into SSW-02. As shown in Fig. 2C, strain SSW-02/SP-01 was grown in M9-yeast medium supplemented with 50 mmol glucose, and the final concentration of L-Ser was 155 mg/L after 15 h in a shake flask. An L-Ser concentration of 220 mg/L, 42% higher than that obtained by culturing SSW-02/SP-01, was obtained by culturing SSW-02/SP-02/SP-05. SSW-02/SP-05 attained the highest L-Ser concentration, 270 mg/L, which exhibited a 1.74-fold increase compared to only overexpressing *serA*B. The L-Ser accumulation profile shown in Fig. 2C, indicates that the production of L-Ser increased as more biosynthetic genes were overexpressed.

Furthermore, previous studies have shown that only 15% of the carbon assimilated from glucose is directed into the L-Ser biosynthetic pathway in *E. coli*. Hence, SP-*serA*B*Cpgk* (SP-08) was then constructed to increase the carbon flux from glucose to L-Ser and improve L-Ser productivity via amplification of phosphoglycerate kinase encoded by *pgk* (Fig. 2B). Flag culture of the recombinant SSW-02/SP-08 strain produced a final L-Ser concentration of 311 mg/L, 15% higher than that obtained by culturing SSW-02/SP-05 (Fig. 2C). Thus, overexpression of *pgk* improved the L-Ser production capacity of the strain. To further examine L-Ser production of SSW-02/SP-08, fed-batch fermentation was performed in a 5-L fermenter. The highest L-Ser concentration, 17.7 g/L, was observed at 32 h with a yield of 24% from glucose (Fig. 2D).

### 2.2 Influence of mutations in *glyA* on L-serine production and cell growth

A previous study showed that attenuation of *glyA* transcription resulted in increased L-Ser accumulation, a decrease in the purine pool, poor growth and cell elongation (Fig S1, Additional file 1) [25, 26]. The same phenomenon was observed in this study; SSW-02 cells were elongated and exhibited unstable growth at the early stage of fermentation. We reprogrammed the predominant one-carbon source metabolism with suppressed SHMT activity to increase the stability of the strains. A series of error-prone PCRs were employed to construct a *glyA* mutation library [27]. Different reductions in SHMT activity were obtained and examined by transforming the recombinant plasmids harboring *glyA*mut into BL21(DE3). As shown in Table 4, SHMT encoded by *glyA*mut (K229G) showed an activity of 0.13 U, which decreased by 41% compared to wild type. The mutant K229G was modeled by SWISSMODEL based on the wild type SHMT (PDB ID, 1DFO). As shown in Fig. 3A, close view of the SHMT K229G mutant compared with the wild type SHMT complexed with cofactor LPL (pyridoxal 5'-phosphate) and TFHA (PDB ID, 1DFO). The side chain of the K229, which involved the degradation of L-Ser, was removed to obtain the mutant K229G [28]. Sequentially, the *glyA* gene in SSW-01 was replaced with the appropriate *glyA*mut(K229G) via CRISPR/Cas9 to generate SSW-03 (△*sdaA glyA*mut). Then, the L-Ser biosynthesis plasmid SP-08 was transformed into SSW-03, and cell growth and L-Ser production were evaluated. As shown in Fig. 3B, *glyA*mut introduction resulted in a 24% increase in biomass, and cultured cells maintained stable growth throughout repeated experiments. SSW-03/SP-08 produced 21.6 g/L of L-Ser, an increase of 22% compared to SSW-02/SP-08.

### 2.3 Influence of *sdaB, ilvA, tdcB* and *tdcG* deletion on L-serine production

The L-Ser production capacity of *E. coli* was significantly increased by overexpression of *serA*B*, *serB*, *serC* and *pgk* via knockout of the *sdaA* and mutation of *glyA*. The four genes other than *sdaA* and *glyA* i.e., *sdaB*, *ilvA*, *tdcB* and *tdcG*, have been reported to transform L-Ser to pyruvate in *E. coli* [29, 30]. However, previous studies focused mainly on increasing the degradation of L-Ser by deleting all of these genes simultaneously, and few studies have systematically investigated the individual contribution of these degradation genes to L-ser production. To prevent the degradation and improve the production of L-Ser, *sdaB, ilvA, tdcB* and *tdcG* were knocked out individually in the SSW-03 background to generate strains SSW-05, SSW-06, SSW-07 and SSW-08 (Fig. 4A). The plasmid SP-08 was transformed into these mutant strains to produce L-Ser. As shown in Fig. 4B, strain SSW-05/SP-08, which had *sdaB* deletion, showed the highest L-Ser production of 26.5 g/L, an increase of 23% compared to SSW-03/SP-08. This result was expected, because the SSW-05/SP-08 biomass was also increased by 16%, and SSW-05/SP-08 showed an L-Ser productivity of nearly 0.87 g/L/h at 28 h. While the *ilvA* gene was knocked out, the growth of the strains was severely inhibited, and production could not be induced during fermentation of SSW-07/SP-08 (Fig. 4C). The growth restriction of SSW-06/SP-08 may be due to disruption of branched chain amino acid synthesis by deletion of *ilvA* [31]. Regarding the *tdcB* gene, the marginal difference in the L-Ser titer and biomass between the SSW-03/SP-08 and SSW-07/SP-08 strains indicated that deletion of *tdcB* is insufficient to improve L-Ser production (Fig. 4D). However, fermentation of deletion of *tdcG* exhibited unexpected results. This *tdcG* gene knockout strain, SSW-08/SP-08, showed a same biomass and 42% lower L-Ser production than the SSW-03/SP-08 (OD600 ~ 36, 21.6 g/L) (Fig. 4B and Fig. 4E). The complex phenomenon associated with SSW-09/SP-08 may be caused by regulation of the expression of the interrupted operon tdcABCDEFG by deletion of *tdcG*. These results suggested that only deletion of *sdaB* improved L-ser production, increasing the L-Ser titer by 23%; thus, SSW-05 with only deletion of *sdaB* was selected for the following experiment, which would avoid severely affected in cell growth by knockout all L-Ser dehydratases.

### 2.4 Effect of engineering L-serine transport system on strain productivity

Moreover, engineering amino acid transport system is also important to further improving strain productivity by blocking reuptake of amino acid and reducing futile cycles [32, 33]. In *E. coli*, four genes, *srt7* [34], *cycA* [35], *sdaC* [36] and *tdcC* [37], have been reported to be involved in L-Ser uptake. Notably, *sdaC* is the only gene described as a highly specific serine transporter, and deletion of *sdaC* was found to improve L-Ser production in our recent studies [38]. Thus, the highly specific L-Ser uptake gene *sdaC* was deleted from SSW-05 to reduce the unwanted futile cycles caused by L-Ser reuptake; this deletion resulted in strain SSW-10. As shown in Fig. 5A, the SSW-10/SP-08 produced 30 g/L of L-Ser with a yield of 0.37 g/g from glucose, 16% higher than that of SSW-05/SP-08. In addition, the final L-Ser productivity of SSW-10/SP-08 was approximately 0.84 g/L/h, which was almost 1.15-fold that of SSW-05/SP-08. Efflux pump is an important component of amino acid transport system and it is known to increase strain tolerance by accelerating the export of amino acid from cells. However, no research to date has reported well-characterized L-Ser exporters in *E. coli*. *ThrE* has been identified as an L-Ser/L-threonine
exporter in *C. glutamicum* [39]. And *thrE* family identified as amino acid exporters in select bacteria, archaea and eukaryotes, but no homologues were found in *E. coli* [40]. Here, heterologous expression of *thrE* was performed to verify if it works in *E. coli*. Thus, *thrE* was cloned into the constructed expression vector SP-08 adjacent to the PR promoter, resulting in the plasmid SP-09 (Fig. 5B). This recombinant plasmid was then transformed into SSW-10. Figure 5B shows the fermentation process of SSW-10/SP-09. Overexpression of *thrE* resulted in a 9% decrease in the final OD₆₀₀₀ value and an 16% increase in L-Ser production compared to those of SSW-10/SP-08. Although the L-Ser production by the final strain SSW-10/SP-09 (35.1 g/L) was lower than L-Ser production by the strains constructed by Maja Rennig (50 g/L), the yield (42%) of L-Ser from glucose of strain SSW-10/SP-09 was higher than that of the strains constructed by Maja Rennig (36%) [13]. Moreover, strain SSW-10/SP-09 exhibited highest productivity and yield of L-Ser from glucose observed to date.

### 2.5 Transcriptomic analysis of of *E. coli* W3110 and SSW-10/SP-09

To investigate the effect of L-Ser fermentation on intracellular metabolism, transcriptomic analyses of *E. coli* W3110 and SSW-10/SP-09 were performed in the exponential phase. A total of 1679 transcripts were found to be significantly different under two criteria (p-value < 0.05 and fold change > 2.0). 

Transcription levels in central carbon metabolism, including glycolysis, tricarboxylic acid (TCA) cycle and amino acid pathways related L-Ser synthesis, were compared.

Expression of the genes related to most reactions in the glycolysis such as *pgi*, *fabAB*, *tpiA*, *eno*, and *pyk* was downregulated in SSW-10/SP-09, while that of *pgk*, encoding phosphoglycerate kinase, was upregulated due to its expression in plasmid SP-09 (Fig. 6A). In the TCA cycle, expression of most genes were also downregulated in SSW-10/SP-09 (Fig. 6B). As a main machinery for adenosine triphosphate (ATP) synthesis, TCA cycle could produce 12.5 ATP molecules per pyruvic acid (PYR) molecule with important intermediates such as oxaloacetate (OAA) and acetyl-CoA (AccoA) [41]. Downregulation of TCA cycle might cause inferior growth with less energy supply. However, the *mqa* gene encoding malate dehydrogenase that convert malate with quinone to oxaloacetate and reduced quinone was upregulated. Reduced quinone could significantly decrease global DNA methylation level cells, and cause acute oxidative damage [42]. Reduced quinone rise in SSW-10/SP-09 may be another reason for biomass decrease. In this study, the OD₆₀₀₀ of SSW-10/SP-09 was 24, a decrease of 35% compared to that of *E. coli* W3110 (OD₆₀₀₀ = 37). Gene *sdhC*, encoding succinate dehydrogenase (ubiquinone) cytochrome b₅₆₀ subunit, was related to in oxygen availability and upregulated in SSW-10/SP-09 [43].

Next, we analyzed changes in the expression of genes related to L-Ser production in SSW-10/SP-09 (Fig. 6C). The expression levels of *serA*, *serC* and *serB* increased in varying degrees. Expression of the gene *glnA* related to conversion from L-glutamic acid (L-Glu) to L-glutamine (L-Gln), which provided NH₄⁺ for L-Ser biosynthesis, was upregulated. It caused damping reaction in L-Glu, L-Gln and 2-oxoglutarate (2-OXO) such as *gltB* and *gltD*. Expression of the *dsdA* encoding D-Ser ammonia-lyase was upregulated. However, expression of *cysEK*, *ilvA* and *tpAB* involved in L-cysteine (L-Cys) and L-tryptophan (L-Trp) biosynthesis were not change. Likewise, SSW-10/SP-09 showed downregulation of glycine cleavage (Gcv) system genes such as *gcvT*, *gcvP* and *gcvH* due to less intracellular glycine (Gly) (Fig. 6D). It could result in a decreased amount of one-carbon units and poor growth [44]. However, *metF*, encoding 5,10-CH₂-THF reductase, involved in one-carbon metabolism drastically increased, which could compensate for one-carbon unit [45]. Expression of the *betB* encoding the enzymes that converts betaine aldehyde to betaine was upregulated. Betaine could regulate intracellular osmotic pressure and provide methyl [46]. With supplement betaine, production of L-threonine, cobalamin and L-lactate were increased [47]. Expression of genes related to metabolism of L-threonine (L-Thr), a downstream amino acid of L-Ser, was analyzed (Fig. 6E). The expression levels of *ilvA*, which was involved in both L-Thr and L-Ser dehydration, was decreased.

In addition to the above genes, there were still large number of differentially expressed genes. Among the upregulated genes in "pyrimidine metabolism" of SSW-10/SP-09, five gene sets *rutA* (expression ratio 2⁵.⁵³), *rutB* (expression ratio 2⁴.⁵⁵), *rutO* (expression ratio 2⁴.⁰⁹), *rutD* (expression ratio 2⁵.³⁹) and *rutE* (expression ratio 2².⁴⁴). Rut pathway may be proposed to enhance the rate of hydrolysis of aminoacrylate, a toxic side product of L-Ser degradation [48, 49]. Among the downregulated genes, genes in the category "Galactose metabolism" were enriched in SSW-10/SP-09. This category includes *gatZ* (expression ratio 2⁻⁵.⁸⁹), *gatA* (expression ratio 2⁻⁶.⁶⁶), *gatB* (expression ratio 2⁻⁶.⁴⁵), *gatC* (expression ratio 2⁻⁶.⁵⁵), *gatD* (expression ratio 2⁻².⁶⁸) and *gatR* (expression ratio 2⁻².⁵²). Genes *gatZABCDR* related to dihydroxyacetone phosphate synthesis from galactitol and galactosamine were highly involved in biofilms and downregulation of the operon may have connection to cell density decrease [50].

### 2.6 Intermediate metabolite analysis of of *E. coli* W3110 and SSW-10/SP-09

As shown in Fig. 7A, a set of 17 intracellular metabolites, including glycolytic intermediates, intermediate metabolite in TCA cycle and amino acid related L-Ser, were measured. A score plot of the PCA model using 17 intracellular metabolites showed the discrimination of metabolite profiles depending on different strains (Fig. 7B). In the PCA model, the intracellular metabolite profiles of *E. coli* W3110 and SSW-10/SP-09 were clearly discriminated. Along the axis of PC1 of the score plot, the metabolite profiles of *E. coli* W3110 were located on the positive side, while the metabolite profiles of SSW-10/SP-09 were located on the negative side. Intracellular glucose-6-phosphate (G6P) concentration of SSW-02/SP-08 increased. It may be caused by downregulated of most downstream genes such as *pgi*, *fabAB* and *eno* in glycolysis (Fig. 8A and Fig. 9A). Intracellular PYR concentration decreased due to weak glycolysis and efficient carbon flux on L-Ser. In the TCA cycle, 2-OXO concentration and malic acid (MAL) concentration showed no significant changes between SSW-10/SP-09 and *E. coli* W3110. Intracellular L-Ser concentration was 472.5 µg/L/g, which was 32-fold of control. Consumption of L-Gln, pitch into the second step of L-Ser biosynthesis, caused damage of its precursor L-Glu. High intracellular L-Thr concentration was in favor of maintaining L-Gly concentration [12, 51]. It also was the reason for the lessened concentration of L-valine (L-Val), L-leucine (L-Leu) and L-isoleucine (L-Ile). Higher intracellular L-Thr concentration also caused downregulation of *thrABC* (encoding homoserine dehydrogenase I, homoserine kinase, and threonine synthetase), which was consistent with the result showed in Fig. 8C, due to its feedback inhibition [21]. Intracellular L-phenylalanine (L-Phe) concentration
of SSW-10/SP-09 increased 182% when compared to it of E. coli W3110. While there was no distinct relationship reported between L-Phe and L-Ser production to date.

3. Conclusions

In this study, a systematic investigation was performed in E. coli to construct an L-serine-producing strain with defined genetic modification. Pure rational metabolic engineering of L-serine-producing strain would provide clearly information for the further improvement, which are difficult to be applied on the random mutagenesis strains because of unknown mutations in their genomes. The key genes (serA, serC, serB and pgk) for L-serine biosynthesis were overexpressed. The transformation pathways were optimized by introducing a glyA mutation (K229G) and deleting sdaA and sdaB. Furthermore, L-Ser uptake gene sdaC was deleted and L-serine/L-threonine exporter ThrE was overexpressed. L-serine production of 35 g/L with the highest productivity of 0.98 g/L/h and yield of 0.42 g/g glucose was finally achieved. The analysis of transcriptome and intermediate metabolites was performed to further understand the regulatory mechanisms of L-serine production. The fermentation-based process described herein provides an important step towards the industrial production of L-serine directly from glucose. Moreover, further strain development can be achieved through genetic optimization of SSW-10/SP-09 with completely defined genomic traits.

4. Materials And Methods

4.1 Strains, media and materials

Wild-type E. coli W3110 was used as the parent strain for serial engineering of L-Ser production. E. coli 5a was used for cloning and propagation of plasmids. E. coli BL21(DE3) was used for enzyme assays. Further strains constructed in this study are shown in Table 1. For strain construction, cultures were grown at 30 °C or 37 °C in Luria-Bertani medium (LB; 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) and supplemented with antibiotics as appropriate.

For L-Ser production, minimal M9 medium (6.8 g/L Na2HPO4, 3 g/L KH2PO4, 0.5 g/L NaCl, 1 g/L NH4Cl, 0.015 g/L CaCl2·2H2O, 0.49 g/L MgSO4·7H2O and 2.8 × 10−4 g/L MgSO4·7H2O) supplemented with 2 g/L yeast extract and 9 g/L glucose was used in a shake flask. Fed-batch cultures contained 3 g/L MgSO4·7H2O, 0.017 g/L CaCl2·2H2O, 1 g/L NaCl, 5 g/L (NH4)2SO4, 0.07 g/L FeSO4·7H2O, 0.11 g/L Na-citrate·2H2O, 2 g/L yeast extract, 8 g/L glucose and 1.5 mL/L 1000 x mother liquor of a composite additive of trace elements (7 g/L CoCl2·6H2O, 2.5 g/L CuSO4·5H2O, 25 g/L H3BO3, 16 g/L MnCl2·4H2O, 1.5 g/L Na2MoO4·2H2O, and 3 g/L ZnSO4·7H2O).

Plasmid DNA was isolated using a Plasmid Mini-Prep Kit (BIO Basic Inc) Chromosomal DNA from E. coli W3110 was isolated by using a Genomic DNA Isolation Kit (BIO Basic Inc). Agarose gel purification of DNA fragments was performed using a TaKaRa Agarose Gel DNA Purification Kit Ver. 2.0 (TAKARA BIOTECHNOLOGY CO). Restriction enzymes, ligases and other DNA manipulation enzymes were used according to the manufacturer’s manuals (TAKARA BIOTECHNOLOGY CO). All plasmid constructs were verified by DNA sequencing (BIO Basic Inc). Plasmid DNA was transformed into competent E. coli cells by electroporation.

4.2 Construction of gene knockout mutants

Genes were knocked out singly or in combination using the method reported by Kirill A. Datsenko and Barry L. Wanner[15]. The primers used for amplification of the kanamycin cassette from the plasmid pKD4 are shown in Table S1, Additional file 2. The plasmids helper pKD13, pKD46 and pCP20 were used for construction of knockout mutants. All gene knockout strains were verified by sequencing with primers shown in Table S2, Additional file 3.

4.3 Construction of the glyA mutant library

Random mutagenesis was induced by error-prone PCR, and the mutation rate was controlled at 0.66%. Genomic DNA of E. coli W3110 was utilized as the template with the primers glyA4F/R (Table 3). PCR reagents were mixed in a volume of 50 µL according to the following composition: 10 x reaction buffer, 10 pmol each primer, 2 µmol MnCl2, 2 µmol MgCl2, 1 µmol Taq DNA polymerase and unbalanced dNTPs. PCR products were purified and digested with NdeI and Hind III and were then subcloned into the expression vector pT7-7. BL21(DE3) cell transformed with these expression vectors grew in LB at 30 °C. Sequentially, the glyA sequence in pT7-7-glyA was replaced with these different glyA mutation constructs using site-directed mutagenesis with primers shown in Table S3, Additional file 4.

4.4 Chromosomal integration of glymut constructs

Pcas and PtargetF[16] were synthesized by GenScript (Nanjing, China). The sgRNA primer, including N20 sequences followed by the protospacer adjacent motif (PAM) sequence and donor DNA primer, glyA4D, used in this study are shown in Table 3. Genes were replaced using the method reported by Yu Jiang et al[16]. All gene knockout strains were verified by sequencing.

4.5 Plasmid construction for overexpression of L-serine biosynthetic pathway components

All plasmids used for plasmid construction are described in Table 2. The low copy number vector SP is a laboratory stock plasmid and contains the temperature-sensitive lambda repressor cII857 gene and the lambda PR and PL promoters. The vector SP was used as the backbone for all plasmids constructed in this study. The L-Ser biosynthetic genes serA, serB and serC were amplified from E. coli W3110 using the primers shown in Table 3.
serA<sup>fr</sup> mutant was generated by mutating two residues in serA, His344 and Asn346, to alanines by site-directed mutagenesis with the primers shown in Table 3. SerAp1 and serAp2 were used to clone serA<sup>fr</sup> into the Xba I/Nhe I site in SP under the control of the PL promoter, yielding the plasmid SP-01. This plasmid was later used to clone serC, generating SP-02. The gene serB was cloned into the SP-02 vector at the Bgl II and Sca I site, generating SP-05. Subsequently, the gene pgk, encoding phosphoglycerate kinase, was cloned into the SP-05 vector backbone, yielding the vector SP-08. The gene thrE, encoding the L-Ser/L-threonine exporter, was amplified from C. glutamicum ATCC 13032. And the resulting 1.7-kb fragment was cloned into the corresponding restriction sites in SP-08, generating in the vector SP-09.

4.6 PGDH and SHMT enzyme assays

BL21(DE3)/pT7-7-serA<sup>fr</sup> cells were harvested at mid-exponential growth phase through centrifugation after induction by isopropyl-beta-D-thiogalactopyranoside (IPTG), and crude extracts were obtained using ultrasonication. PGDH in crude extracts was purified by ion exchange chromatography (AKTA) on a Sepharose Fast Flow column, and diethylaminoethyl dextran gel (DEAE) was used as the anion exchange agent[17]. PGDH activity was determined by determination of α-ketoglutarate (α-KG) reductase activity instead of glyceral acid-3-phosphate dehydrogenase activity. The 1-mL reaction system contained 40 mmol/L potassium phosphate buffer (pH = 7.5), 1.0 mmol/L DL-dithiothreitol (DTT), 0.25 mmol/L NADH, 5 mmol/L α-KG and 10–30 μg of the purified crude extract[18].

BL21(DE3)/pT7-7-glyA<sup>mut</sup> growth was induced by IPTG at an OD<sub>600</sub> of 0.5, and the culture was centrifuged to obtain bacterial cells at an OD<sub>600</sub> of 4. SHMT activity was determined by a continuous spectrophotometric assay using DL-3-phenylserine hydrate and phosphopyridoxal as substrates[19]. Hydrolysis of DL-3-phenylserine hydrate by SHMT was monitored spectrophotometrically at 279 nm to assess the formation of benzaldehyde. The standard curve was generated with a benzaldehyde concentration gradient by spectrophotometry 279 nm in the dark. The assay buffer contained 1 mg of centrifuged bacterial cells and 1 mL of substrate (50 mmol/L DL-3-phenylserine hydrate, 30 μmol/L phosphopyridoxal, pH = 8.0) at 37 °C. After culture at 30°C, 200 rpm for 1 h, the assay buffer was centrifuged at 5000 rpm for 10 minutes, and the supernatants were evaluated at 279 nm. The production of 1 mol of benzaldehyde per hour with 1 g wet weight of the cell in 1 L assay buffer buffer was defined as one unit.

4.7 Shake flask and fed-batch fermentation

For shake flask studies, a single clone was first grown in 5 mL of LB for 12–14 h, and 5 mL of the culture was transferred to 100 mL of M9 medium with supplemented 2 g/L yeast extract and 9 g/L glucose for culture in a 500-mL shake flask at 30 °C and 200 rpm. Each culture was induced after 3 h by heating to 38 °C. The shake flask studies were repeated at least three times.

Fed-batch fermentation was conducted in a 5-L bioreactor (Biostat A Plus, Sartorius Stedim, Germany). A single clone was precultured in 50 mL of LB and shaken at 33 °C and 200 rpm for 12 to 14 h. The culture was inoculated into 2.5 L of fermentation medium at a 1:20 (v/v) inoculum ratio at an initial temperature of 33 °C. L-Ser production was induced at an OD<sub>600</sub> of 20 by heating to 38 °C. The agitation, air supplementation and feed rate were changed to maintain the dissolved oxygen (DO) concentration above 30% saturation. The pH was controlled at 6.8 using 30% (w/v) NH<sub>3</sub>/H<sub>2</sub>O. The DO-stat feeding strategy was employed to supply exhausted nutrients to the fermenter. The feeding solution contained 40% (w/w) glucose.

4.8 Sample preparation and extraction for intermediate metabolite analysis

Bioreactor-grown cells were harvested at exponential growth phase after induction. 5 mL of culture was injected into the 20 mL quenching solutions (glycerol/saline, 60/40, v/v) and directly centrifuged at 12,00 rpm at − 20 °C for 3 min. After the removal of the supernatant, cell pellets were resuspended by 5 mL saline and cells were collected by centrifugation at 12,00 rpm. The culture was centrifuged to obtain bacterial cells at an OD<sub>600</sub> of 4. Carbohydrate analysis was performed by the phenol/sulfuric acid method. Glucose concentration was analyzed using a glucose meter (YSI 2000, Yellow Springs Instrument, OH). The cell debris was removed by centrifugation for 5 min at 12,000 rpm.

4.9 Analytical methods

Bacterial growth was monitored by measuring the OD<sub>600</sub> in a spectrophotometer (Beckman Germany), and the glucose concentration was analyzed using an SBA sensor machine (Institute of Microbiology, Shandong, China). The L-Ser from fermentation solution was determined by determination of α-ketoglutarate (α-KG) reductase activity instead of glyceric acid-3-phosphate dehydrogenase activity. The 1-mL reaction system contained 40 mmol/L potassium phosphate buffer (pH = 7.5), 1.0 mmol/L DL-dithiothreitol (DTT), 0.25 mmol/L NADH, 5 mmol/L α-KG and 10–30 μg of the purified crude extract[18].

Amino acids within the cell were determined using AB SCIEX QTRAP 5500 system with Agilent InnityLab Poroshell 120 HILIC-Z (2.1 × 100 mm, 2.7 µm) at 35 °C. Conditions of mass spectrometer were as follows: curtain gas, 35 psi; ion source gas 1, 60 psi; ion source gas 2, 60 psi; source temperature, 550°C; polarity, positive; ionspray voltage, 5500 V. Mobile phase A was 20 mM ammonium formate in water at pH 3 whereas mobile phase B consisted of 20 mM aqueous ammonium formate or ace tone/acetamide/water (9:1, V/V). The flow rate of the mobile phase was 0.25 mL/min. The gradient time course was as follows: initial condition: A (5.95, v/v); 15 minutes, A (35.65, v/v); 17 minutes, A (35.65, v/v); 18 minutes, A (5.95, v/v); 20 minutes, completed elution.
Organic acids within the cell were determined using AB SCIEX QTRAP 5500 system with WATERS T3 column (4.6 × 150 mm, 3 µm) at 40 °C. Conditions of mass spectrometer were as follows: curtain gas, 35 psi; ion source gas 1, 55 psi; ion source gas 2, 55 psi; source temperature, 550 °C; polarity, negative; ionspray voltage, 4500 V. Mobile phase A was 0.1% formic acid in acetonitrile whereas mobile phase B consisted of 0.1% formic acid in water. The flow rate of the mobile phase was 0.3 mL/min. The gradient time course was as follows: initial condition, A:B (2:98, v/v); 2 minutes, A:B (2:98, v/v); 6 minutes, A:B (98:2, v/v); 9 minutes, A:B (98:2, v/v); 9.1 minutes, A:B (2:98, v/v); 13.5 minutes, completed elution.

4.10 Transcriptome data sets

For transcriptome analysis, we only used the transcriptome data sets that were obtained at the exponential growth phase after induction. The samples were frozen immediately in liquid nitrogen and sent to Sangon Biotech (Shanghai, China) for transcriptome sequencing. Differentially expressed genes (DEGs) were identified according to the following rules: a log2 fold change (FC) > 2 and a p value < 0.05.[22]

List Of Abbreviations

Glc_{ex}, extracellular glucose;
PEP, phosphoenolpyruvate;
PYR, pyruvic acid;
ATP, adenosine triphosphate;
ADP, adenosine diphosphate;
G6P, glucose-6-phosphate;
F6P, fructose-6-phosphate;
F-1,6-BP, fructose-1,6-bisphosphate;
GA3P, glyceraldehyde-3-phosphate;
1,3-PG, 1,3-bisphosphoglycerate;
3-PG, 3-phosphoglycerate;
2-PG, 2-phosphoglycerate;
AcCoA, acetyl-CoA;
Cit, citric acid;
cis-Aco, cis-aconitic acid;
ICit, isocitric acid;
2-OXO, 2-oxoglutarate;
SucCoA, succinyl-CoA;
Suc, succinic acid;
Fum, fumaric acid;
Mal, malic acid;
OAA, oxaloacetic acid;
NAD^{+}, oxidized nicotinamide adenine dinucleotide;
NADH, reduced nicotinamide adenine dinucleotide;
NADP, oxidized nicotinamide adenine dinucleotide phosphate;
NADPH, reduced nicotinamide adenine dinucleotide phosphate.
3-PHP, 3-phosphonoxyypyruvate;
L-Ser, L-serine;
D-Ser, D-serine;
L-Leu, L-leucine;
L-Ile, L-isoleucine;
L-Val, L-valine;
Gly, glycine;
L-Gln, L-glutamine;
L-Glu, L-glutamate;
THF, tetrahydrofolate;
MTHF, 5,10-methylene tetrahydrofolate;
LPL, lipoylprotein;
DHP, dihydrolipoylprotein;
amDHP, aminomethyldihydrolipoylprotein.

Declarations
Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Availability of data and material
All data generated and analyzed during this study are included in this published article and its supplementary information files.

Competing interests
The authors declare that they have no competing interests.

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Author’s contributions
Chenyang Wang carried out the genes deletion and L-serine uptake activity assay. Chenyang Wang, Xiaojia Chen and Qinyu Li measured enzymes activities. Lei Wang helped analysis structure of enzyme. Chenyang Wang, Li Li and Xinxin Ma performed L-serine fermentation. Chenyang Wang, Junjun Wu and Zhijun Zhao analyzed the data. Chenyang Wang, Junjun Wu, Zhijun Zhao and Jiping Shi conceived the study and reviewed the manuscript.
All authors read and approved the final manuscript.

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Tables
Table 1
Strains used in the experiment

| Strain       | Relevant characteristics | Source      |
|--------------|--------------------------|-------------|
| *E. coli* DH5α | Cloned strains           | TaKaRa      |
| *E. coli* BL21(DE3) | Expression strains      | ATCC 26003  |
| *E. coli* W3110 | Parental strains         | ATCC 23275  |
| SSW-01       | W3110 ΔsdaA              | This work   |
| SSW-02       | W3110 ΔsdaA glyA         | This work   |
| SSW-03       | W3110 ΔsdaA glyA\text{mut} | This work |
| SSW-05       | W3110 ΔsdaA glyA\text{mut} sdaB | This work |
| SSW-06       | W3110 ΔsdaA glyA\text{mut} ilvA | This work |
| SSW-07       | W3110 ΔsdaA glyA\text{mut} tdcB | This work |
| SSW-08       | W3110 ΔsdaA glyA\text{mut} tdcG | This work |
| SSW-10       | W3110 ΔsdaABC glyA\text{mut} | This work |

Table 2
Plasmids used in the experiment

| Plasmid     | Relevant characteristics                      | Source            |
|-------------|----------------------------------------------|-------------------|
| pT 7–7      | amp marker, T7 promoter                      | Takara            |
| pSC         | Low copy number, kan marker, p15A replicon, lambda PR and PL promoters | Lab stock         |
| pKD13       | amp and kan markers                          | [15]              |
| pKD46       | amp marker, temperature-sensitive            | [15]              |
| pCP20       | amp and chl makers, temperature-sensitive    | [15]              |
| pMD19-T simple | amp maker, TA cloning vector, 2692 bp      | Takara            |
| Pcas        | repA101(Ts) kan Pcas-cas9 ParaB-Red lacIq Ptrc-sgRNA-pMB1 | [16]              |
| PtargetF    | pMB1 aadA                                    | [16]              |
| PtargetF-glyA | pMB1 aadA sgRNA-glyA                      | This work         |
| pT- serA     | pT 7–7 derivative, carrying serA             | This work         |
| pT- serA\text{fr} | pT 7–7 derivative, carrying serA         | This work         |
| pT-glyA      | pT 7–7 derivative, carrying glyA             | This work         |
| pT-glyA\text{mut} | pT 7–7 derivative, carrying glyA\text{mut} | This work         |
| SP-01        | SP derivative, carrying serA\text{fr}       | This work         |
| SP-02        | SP derivative, carrying serA^6, serC         | This work         |
| SP-05        | SP derivative, carrying serA^6, serC and serB | This work         |
| SP-08        | SP derivative, carrying serA^6, serB, serC and pgk | This work         |
| SP-09        | SP derivative, carrying serA^6, serB, serC, pgk and thrE | This work         |

*Kan*, Kanamycin Monosulfate;*Amp*, Ampicillin; *Chl*, Chloramphenico
| Primer  | Sequence (5’-3’) a  | Restriction enzyme |
|---------|---------------------|--------------------|
| serA- p1 | TCTAGAAGAAGGAGATATACCATGGCAAGGTATCGCTGGAG  | Xba I |
| serA- p2 | GAGCTCGTGAGTAAGGGTAAGGGAGGATTG  | Sac I |
| serB- p1 | AGATCTAAGAAGGAGATATACCATGCCCTAATTACCTGCTGGAG  | Bgl II |
| serB- p2 | AGTACTGGCTGATATCGGAGATTTCTGGAC  | Sca I |
| serC- p1 | AGATCTAAGAAGGAGATATACCATGGCTCAAATCTTCAATTTAG  | Bgl II |
| serC- p2 | CAGCTGTAGATCGTCTGAAACGCAAGCTATTAG  | pvu II |
| pgk- p1 | TCTAGAAGAAGGAGATATACCATGGCAAGGTATCGCTGG  | Xba I |
| pgk- p2 | GAGCTCGTGGAGTAAGGGTAAGGGAGGATTG  | Sac I |
| thrE- p1 | AGTACTAAGAAGGAGATATACCATGGCAAGGTATCGCTGG  | Xba I |
| thrE- p2 | AGATCTAAGAAGGAGATATACCATGGCAAGGTATCGCTGG  | Xba I |

Site directed mutagenesis primers used for mutation of serA

| Primer  | Sequence (5’-3’) a  | Restriction enzyme |
|---------|---------------------|--------------------|
| serA- p3 | GATGCACATCGCAGAAGCA  | Ndel  |
| serA- p4 | GCCCGGACGTGCTTCTGCGATGTCATC  | Hind III |

glyA primers

| Primer  | Sequence (5’-3’) a  | Restriction enzyme |
|---------|---------------------|--------------------|
| glyA- F | GGAATTCCATATGTTAAAGGCTGAATGAAC  | Ndel  |
| glyA- R | CCCAAGCTTTATGCGTAAACCGGGTAC  | Hind III |

sgRNA- F

| Primer  | Sequence (5’-3’) a  | Restriction enzyme |
|---------|---------------------|--------------------|
| sgRNA- F | TGGCAACCCCCTTCAGCCACCTGATTATTGACCTACGTGACCCCGATTAG  | |

sgRNA- R

| Primer  | Sequence (5’-3’) a  | Restriction enzyme |
|---------|---------------------|--------------------|
| sgRNA- R | GCCGTTCCGACGGCAGATGTTAAAGGCTGAATGAAC  | |

glyA-D

| Primer  | Sequence (5’-3’) a  | Restriction enzyme |
|---------|---------------------|--------------------|
| glyA-D- F | TGGCAACCCCCTTCAGCCACCTGATTATTGACCTACGTGACCCCGATTAG  | |

glyA-D- R

| Primer  | Sequence (5’-3’) a  | Restriction enzyme |
|---------|---------------------|--------------------|
| glyA-D- R | GCCGTTCCGACGGCAGATGTTAAAGGCTGAATGAAC  | |

The underlines indicate homology extensions of a target gene.
Table 4
SHMT activity of various mutants.

| Position | Sequence change | Protein change          | SHMT activity |
|----------|-----------------|-------------------------|---------------|
| -        | -               | -                       | 0.65±0.033    |
| 24       | T→G             | I8M(ATT→ATG)            | 0.63±0.022    |
| 88       | A→T             | I30F(ATC→TTC)           | 0.34±0.005    |
| 145      | T→A             | S49T(TCT→ACT)           | 0.58±0.005    |
| 149      | A→T             | Q50L(CAG→CTG)           | 0.24±0.02     |
| 301      | G→T             | A101S(GCT→TCT)          | 0.22±0.016    |
| 483      | A→T             | Q161H(CAA→CAT)          | 0.66±0.018    |
| 532      | T→C             | S178P(TCC→CCC)          | 0.36±0.028    |
| 572      | A→C             | D191A(GAC→GCC)          | 0.6±0.019     |
| 685/686  | AA→GG           | K229G(AAA→GGA)          | 0.13±0.005    |
| 743      | T→G             | L248R(CTG→CGG)          | 0.56±0.014    |
| 902      | T→G             | V301G(GTG→GGG)          | 0.57±0.017    |
| 1073     | T→C             | V358A(GTG→GCG)          | 0.54±0.01     |
| 1163     | A→T             | D388V(GAC→GTC)          | 0.57±0.008    |
| 1195     | A→T             | I399F(ATC→TTC)          | 0.62±0.032    |
| 1225     | A→C             | I409L(ATC→CTC)          | 0.44±0.004    |
| 1238     | A→C             | Y413S(TAC→TCC)          | 0.59±0.012    |

The data are presented as the means ± SDs from three measurements.

*pT-glyA was used as the positive control.

Figures
Figure 1

L-Serine biosynthesis and its regulation in E. coli. The blue arrows indicate overexpression of the relevant genes using the SP vector. The red "X" indicates deletion of relevant genes. The red dashed line indicates feedback inhibition.
Figure 2

Optimization of the synthetic serAfrCB operon with pgk and screening for high-level L-serine production (A) PGDHmut (serAf, open squares), PGDHwt (serA, open circles). (B) The structure of different plasmids. (C) L-serine production by shake flask fermentation with different plasmids. (D) L-serine production by SSW-02/SP-08 by fed-batch fermentation. Cell growth (filled squares), L-serine concentration (filled circles), glucose consumption (upward-pointing filled triangles), specific growth rate (open squares), and L-serine productivity (open circles). The arrow indicates the starting point of induction. The data are presented as the means ± SDs from three measurements.
Figure 3

SSW-03/SP-08 L-serine production during fed-batch fermentation. (A) Structural modelling of SHMT mutant K229G (light gray) and comparison with the wild-type (light orange, PDB ID, 1DFO) in cartoon format. The cofactor PLP and THFA are sticks in green. The L-serine degradation products glycine and formyl group are sticks in yellow. K229 of the wild-type is stick in cyan and G229 of mutant is stick in magenta. The O is in red and the N is in blue. (B) SSW-03/SP-08 L-serine production by fed-batch fermentation. Cell growth (filled squares), L-serine concentration (filled circles), glucose consumption (upward-pointing filled triangles), specific growth rate (open squares), and L-serine productivity (open circles). The arrow indicates the starting point of induction. The data are presented as the means ± SDs from three measurements.

Figure 4

Effect of sdaB, llvA, tdcB or tdcG deletion on L-serine production and biomass. (A) Genotypes of the strains. The genes marked in red indicate targets for knockout. (B) L-serine production by and growth of SSW-05/SP-08 cells. (C) Cell density achieved by SSW-06/SP-08. (D) L-serine production by and growth of SSW-07/SP-08 cells. (E) L-serine production and cell density achieved by SSW-08/SP-08. Cell growth (filled squares), L-serine concentration (filled circles), glucose consumption (upward-pointing filled triangles), specific growth rate (open squares), and L-serine productivity (open circles). The arrow indicates the starting point of induction. The data are presented as the means ± SDs from three measurements.
Figure 5

L-serine production by SSW-10/SP-08 and SSW-10/SP-09 during fed-batch fermentation. (A) Genotype of strain SSW-10 and structures of different SP-09 plasmids. The genes marked in red indicate targets for knockout. (B) L-serine production by and growth of SSW-011/SP-08 cells. (C) L-serine production and cell density achieved by SSW-011/SP-09. Cell growth (filled squares), L-serine concentration (filled circles), glucose consumption (upward-pointing filled triangles), specific growth rate (open squares), and L-serine productivity (open circles). The arrow indicates the starting point of induction. The data are presented as the means ± SDs from three measurements.
Gene expression levels related to the glycolysis, TCA cycle and amino acid pathways related to L-Ser synthesis. (A) Glycolysis, (B) TCA cycle, (C) L-serine, (D) L-glycine and (E) L-threonine biosynthesis of SSW-10/SP-09. Significant changes of expression ratio to transcript levels are represented by color (p-value < 0.05, and fold changes > 2.0), three independent replicate. The red colour indicates higher expression level of SSW-10/SP-09; blue colour indicates lower expression level of SSW-10/SP-09; gray colour indicates no changes.

Figure 7
PCA score and intracellular metabolites of SSW-10/SP-09 and E. coli W3110. (A) 17 intracellular metabolites of SSW-10/SP-09 and E. coli W3110 (three independent replicates). Significant changes in cofactors are represented by *(p<0.05) and **(p<0.01). (B) PCA score.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.docx
- Additionalfile2.docx
- Additionalfile3.docx
- Additionalfile4.docx