High yield production of L-serine through a novel identified exporter combined with synthetic pathway in Corynebacterium glutamicum

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Research

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Abstract

Background L-Serine has wide and expanding applications in industry with a fast-growing market demand. Strategies for achieving and improving L-serine production in C. glutamicum have focused on inhibiting its degradation and enhancing its biosynthetic pathway, however, L-serine yield remains relatively low. Exporters play an essential role in the fermentative production of amino acids. Improvements to L-serine yield should consider the improvement of L-serine export from the cell. In C. glutamicum , ThrE, which can export L-threonine and L-serine, is the only identified L-serine exporter so far.

Results In this study, a novel L-serine exporter NCgl0580 was identified and characterized in C. glutamicum ΔSSAAI (SSAAI), and named as SerE (encoded by serE). Deletion of serE in SSAAI led to a 56.5% decrease in L-serine titer, whereas overexpression of serE compensated for the lack of serE with respect to L-serine titer. A fusion protein with SerE and enhanced green fluorescent protein (EGFP) was constructed to confirm that SerE localized at the plasma membrane. The function of SerE was studied by peptide feeding approaches, and the results showed that SerE is a novel exporter for L-serine and L-threonine in C. glutamicum. Subsequently, the interaction of a known L-serine exporter ThrE and SerE was studied, and the results suggested that SerE is more important than ThrE in L-serine export in SSAAI. In addition, probe plasmid and electrophoretic mobility shift assays (EMSA) revealed NCgl0581 as the transcriptional regulator of SerE. Comparative transcriptomics between SSAAI and the NCgl0581 deletion strain showed that NCgl0581 is a positive regulator of NCgl0580. Finally, by overexpressing the novel exporter SerE combined with L-serine synthetic pathway key enzyme serA Δ197, serC and serB , the resulting strain lead to the L-serine titer of 43.9 g/L with yield of 0.44 g/g sucrose, which was the highest yield reported so far for any organism.

Conclusions This study provides a novel target for L-serine and L-threonine export engineering as well as a novel global transcriptional regulator NCgl0581 in C. glutamicum .

Background

L-serine has been identified as one of the top 30 most interesting building blocks for a range of chemicals and materials, and is used in cosmetic, pharmaceutical, and food industries [1, 2]. Metabolic engineering of Corynebacterium glutamicum (C.glutamicum) for L-serine production has been focused on its terminal synthesis pathways and degradation pathways, and proven to be very useful for improving L-serine production in this organism [3–6]; however, the L-serine productivity is still low for large-scale L-serine production, the highest reported L-serine titer reached 42.62 g/L and 50 g/L with the yield to be 0.21 g/g sucrose and 0.26 g/g glucose in C. glutamicum and E.coli respectively, however, L-serine has the potential to be made from sugar by fermentation with a very high theoretical yield (1.17 g/g glucose,1.22 g/g sucrose) [2].

Improvements to L-serine production should consider the improvement of L-serine export from the cell. Export plays an essential role in metabolic engineering strategies for production of amino acids [7], as it reduces intracellular amino acid concentrations, and thereby alleviates feedback inhibition and circumvents toxicity problems [1, 8–10]. In recent decades, a number of export systems have been identified for excreting amino acids, such as L-lysine, L-cysteine, L-glutamate, L-threonine, L-arginine, L-methionine, and branched-chain amino acids, in C. glutamicum and E.coli [11–17]. However, to the best of our knowledge, except for ThrE (L-threonine and L-serine exporter) [15, 18], no other L-serine exporters have been reported in C. glutamicum so far. In E. coli, Mundhada et al. found that intracellular L-serine accumulation was toxic to the engineered strain modified to produce L-serine, and that following overexpression of eamA (which encodes L-cysteine exporter in E. coli), the engineered strain exhibited increased tolerance toward L-serine with higher L-serine productivity [2]. Therefore, L-serine exporter in C. glutamicum could be a potential target for strain optimization to further improve L-serine production.

It has been reported that homologs similar to the exporters in E. coli might fulfil a comparable function in C. glutamicum [17, 19, 20]. Accordingly, we hypothesized that the homolog to eamA (L-serine exporter in E. coli) might be involved in L-serine export in C. glutamicum. In the present study, three homologs to eamA, namely, NCgl2050, NCgl2065, and NCgl0580, were determined, and their functions were identified by targeted gene deletion, respectively. The results showed that one of the genes, NCgl0580, was involved in L-serine export. Subsequently, localization and function of NCgl0580 were investigated, and the interaction of a known L-serine exporter ThrE (encoded by thrE) and the novel exporter NCgl0580 was studied. Furthermore, the transcriptional regulator of NCgl0580 was identified and studied. Finally, the effect of overexpression of L-serine exporter in combination with L-serine synthetic pathway enzyme to L-serine production were evaluated.

Results

Exploring putative L-serine exporters in *C. glutamicum*

In past studies, homologs of *E. coli* exporters have been shown to have similar functions in *C. glutamicum* [17, 19, 20]. Therefore, we hypothesized that the *C. glutamicum* homolog to *eamA* (L-serine exporter in *E. coli*) [2] might be involved in L-serine export in this organism. According to the NCBI database, EamA belongs to the RhaT superfamily, and 15 records of related proteins associated with RhaT superfamily in *C. glutamicum* ATCC13032 were obtained. After eliminating duplicate records, three related genes, NCgl2050, NCgl2065, and NCgl0580, were obtained, which might be involved in L-serine export in *C. glutamicum*.

To verify the function of these putative proteins in *C. glutamicum* SSAAI (SSAAI), NCgl2050, NCgl2065, and NCgl0580 were respectively deleted in this strain. The results showed that the deletion of NCgl2050 and NCgl2065 did not produce any changes in cell growth and L-serine titer (Fig. 1A and 1B). Strikingly, deletion of NCgl0580 significantly reduced the L-serine titer in SSAAI, but did not affect the growth of the strain (Fig. 1C). SSAAI ΔNCgl0580 produced 11.31 g/L L-serine, which was 56.5% lower than that noted

in SSAAI (Fig. 1C). However, plasmid-borne overexpression of NCgl0580 compensated for the lack of NCgl0580 with respect to L-serine titer, resulting in 26.76 g/L L-serine titer, similar to that generated by the parent strain SSAAI (Fig. 1D). As shown in Fig. 1D, when compared with SSAAI, the strain harboring the plasmid grew slowly to some extent in the logarithmic growth phase, finally reaching similar cell growth to SSAAI. This finding suggested that NCgl0580 might act as the L-serine exporter in *C. glutamicum*, and was named as SerE and its function was further investigated.

Localization and function of *SerE*

According to the NCBI, SerE was presumed to be a hypothetical membrane protein of 301 amino acids, similar to permease of the drug/metabolite transporter (DMT) superfamily. The transmembrane helices of SerE were predicted by TMHMM Server v. 2.0, and SerE exhibited ten transmembrane-spanning helices with both amino- and carboxy-terminal ends in the cytoplasm.

To confirm the localization of SerE, SerE-EGFP fusion protein was expressed in SSAAI. Confocal microscopic observations of SSAAI-*egfp* and SSAAI-*serE-egfp* confirmed that EGFP and SerE-EGFP fusion proteins were successfully expressed, respectively (Fig. S1). To further verify the localization of SerE, membrane and cytoplasmic proteins from these two strains were extracted by ultrasonication, and the fluorescence of these proteins was determined using a fluorescence spectrophotometer. The fluorescence of the cytoplasmic proteins of SSAAI-*egfp* and membrane proteins of SSAAI-*serE-egfp* (Fig. 2A) affirmed that SerE was localized at the plasma membrane in SSAAI.

To substantiate the function of SerE, a peptide feeding approach was employed by incubating SSAAI and SerE deletion strain, SSAAI Δ*serE,* with 2 mM of the dipeptide Ser-Ser, respectively, and measuring the concentration of extracellular L-serine. As shown in Fig. 2B, a higher L-serine concentration was detected in SSAAI, when compared with that in SSAAI Δ*serE*, confirming that SerE is a novel exporter of L-serine in *C. glutamicum*.

It is known that L-cysteine accept system in *E. coli* (encoded by *eamA*) also catalyzes L-serine accept [2], and that L-threonine exporter in *C. glutamicum* (encoded by *thrE*) also transports L-serine [15]. We therefore analyzed whether the novel exporter SerE could accept L-cysteine and L-threonine. The export experiments with dipeptides (Thr-Thr, Cys-Cys) were performed using SSAAI and SSAAI Δ*serE*. The dipeptides were added at a concentration of 2 mM to the medium, and the extracellular amino acid concentrations at different time intervals were determined by HPLC. The results revealed that the concentration of L-cysteine was comparable in both strains and did not significantly change (data not shown), indicating that SerE does not export L-cysteine. Interestingly, the concentrations of L-threonine in SSAAI Δ*serE* were lower than those in SSAAI (Fig.2C), indicating that SerE is also an exporter of L-threonine in *C. glutamicum*.

Interaction of a known exporter ThrE and a novel exporter SerE

It is well known that *thrE* encodes ThrE that can export L-threonine and L-serine in *C. glutamicum* ATCC13032 [15]. To understand the interaction of ThrE and SerE on L-serine accept, *thrE* was deleted in SSAAI (SSAAI Δ*thrE),* and no significant change was observed in L-serine excretion for the deletion mutant (Fig. 3A and 3B). In contrast, deletion of SerE significantly reduced the L-serine titer in SSAAI, and resulted in little change in cell growth (Fig. 1C). The SSAAI Δ*serE* produced 11.31 g/L L-serine, which was 56.5% lower than that produced by SSAAI (Fig. 1C). Subsequently, *thrE* and *serE* double deletion mutant was constructed, which exhibited cell growth comparable to that of SSAAI (Fig. 3B), and produced 10.34 g/L L-serine, which was 60% lower than that observed in SSAAI (Fig. 3A and. 3B).

Furthermore, *thrE* and *serE* were overexpressed alone or in combination in SSAAI, then SSAAI-*thrE,* SSAAI-*serE* and SSAAI-*thrE-serE* were obtained. While L-serine accumulation in SSAAI-*thrE* was similar to that in SSAAI (Fig. 3A and 3C), the production of L-serine in SSAAI-*serE* reached 28.67 g/L, which was 10.5% higher than that noted in SSAAI (Fig. 3A and 3C). However, a decrease in cell growth was observed in SSAAI-*serE* before 72 h of fermentation, when compared with that found in SSAAI (Fig. 3C). And no significant difference of L-serine titer was found in the time courses of both SSAAI-*serE* and SSAAI-*thrE-serE* (Fig. 3C), and SSAAI-*thrE-serE* exhibited a lower cell growth than SSAAI-*thrE* before 96 h of fermentation. These observations might be due to the fact that over-efflux L-serine inhibited the cell growth. And the results suggested that SerE is more important than ThrE in L-serine export in SSAAI.

Transcriptional regulator of the novel exporter SerE

The gene NCgl0581, located upstream of *serE* and divergently transcribed from *serE* (Fig. S2), and its product (consisting of 303 amino acids) was found to be a member of the LysR-type transcriptional regulators (LTTRs) family. It must be noted that LTTRs were initially described as regulators of divergently transcribed genes [21]. In a previous study on *C. glutamicum,* LysG*,* located upstream of L-lysine exporter gene *lysE,* was observed to encode a LysR-type transcriptional regulator, confirming that LysG is a positive transcriptional regulator of *lysE* [22]. Accordingly, we speculated that NCgl0581 might be involved in the control of *serE* transcription.

To determine the function of NCgl0581, a mutant strain with NCgl0581 deletion was constructed. As shown in Fig. 4A, the growth of SSAAI ΔNCgl0581 was similar to that of the parent strain SSAAI. However, the L-serine titer of SSAAI ΔNCgl0581 was 11.08 g/L, which was 57.4% lower than that of the parent strain, indicating that NCgl0581 played an important role in L-serine production. Subsequently, the effect of NCgl0581 on *serE* expression was further investigated by using the probe plasmid pDXW-11. Two recombinant strains, SSAAI ΔNCgl0581-1 (harboring the plasmid pDXW-11-1, Fig. S3A) and SSAAI ΔNCgl0581-0 (harboring the plasmid pDXW-11-0, Fig. S3B) were constructed, and their fluorescence during fermentation was measured. The fluorescence of SSAAI ΔNCgl0581-1 was stronger than that of SSAAI ΔNCgl0581-0 during the fermentation process (Fig. S3C), revealing that NCgl0581 functioned as a positive regulator of *serE* expression. To verify whether the regulatory protein NCgl0581 binds to the upstream region of SerE, electrophoretic mobility shift assay (EMSA) was performed by using the DNA probe labeled with biotin, and the result clearly indicated that NCgl0581 binds to this region (Fig. S4).

To confirm whether NCgl0581 is a specific regulator of SerE, transcriptome sequencing was performed using SSAAI and NCgl0581 deletion strain. The findings showed that the transcription levels of 115 genes were altered, including 56 genes upregulated and 59 genes downregulated, in response to NCgl0581 deletion, indicating that NCgl0581 is a global transcriptional regulator in *C. glutamicum.* The genes with significant transcriptional change (≥4-fold) are shown in Tables 1 and 2.

TABLE 1 The genes significantly up regulated by NCgl0581 deletion

Gene id	SSAAI Δ0581	SSAAI	Fold change	Protein function
NCgl2897	701.56	71.07	9.87	Starvation-inducible DNA-binding protein
NCgl0546	17.78	2.75	6.45	Hypothetical protein
NCgl1405	15.94	2.71	5.88	ABC transporter periplasmic component
NCgl1302	10.05	1.96	5.13	Aldo/keto reductase
NCgl1344	286.87	55.96	5.12	Ornithine carbamoyltransferase
NCgl1343	280.65	57.24	4.9	Acetylornithine aminotransferase
NCgl0746	43.30	9.04	4.7	Hypothetical protein
NCgl1342	134.70	29.07	4.63	Acetylglutamate kinase
NCgl2946	672.93	155.87	4.31	Hypothetical protein
NCgl1022	89.53	21.28	4.20	Cysteine sulfinate desulfinase
NCgl1023	368.88	88.67	4.15	Nicotinate-nucleotide pyrophosphorylase
NCgl1341	108.49	27.09	4.00	Bifunctional ornithine acetyltransferase/N-acetylglutamate synthase

TABLE 2 The genes significantly down regulated by NCgl0581 deletion

Gene id	SSAAI Δ0581	SSAAI	Fold change	Protein function
NCgl0580	18.40	5152.54	280.02	Hypothetical protein
NCgl0638	1.71	20.97	12.22	ABC transporter permease
NCgl0639	11.00	82.47	7.49	ABC transporter periplasmic component
NCgl2943	207.03	1355.55	6.54	Hypothetical protein
NCgl0943	16.19	103.52	6.39	AraC family transcriptional regulator
NCgl0484	2.32	14.57	6.28	ABC transporter permease
NCgl2942	283.52	1776.15	6.26	NADH: flavin oxidoreductase
NCgl0166	13.41	79.70	5.94	Hypothetical protein
NCgl0324	2.11	11.87	5.61	Zn-dependent alcohol dehydrogenase
NCgl0282	5.19	28.25	5.44	4-Hydroxyphenyl-beta-ketoacyl-CoA hydrolase
NCgl1975	102.94	503.75	4.89	Hypothetical protein
NCgl2893	1.25	6.08	4.84	Efflux system protein
NCgl0155	9.11	43.69	4.79	5-Dehydro-2-deoxygluconokinase
NCgl0014	10.02	47.76	4.76	Hypothetical protein
NCgl2953	7.68	35.80	4.66	Sugar permease
NCgl2744	12.26	55.19	4.50	Hypothetical protein
NCgl2970	15.22	67.51	4.43	ABC transporter periplasmic component
NCgl0608	23.06	100.35	4.35	ABC transporter permease
NCgl0258	4.51	19.50	4.32	Arsenite efflux pump ACR3
NCgl0281	16.83	67.69	4.02	Dehydrogenase

The transcriptional level of *serE* was significantly decreased by 280-fold following NCgl0581 deletion, revealing that NCgl0581 is a positive regulator of *serE*. Furthermore, NCgl0581 deletion downregulated the two ABC transporter permeases (NCgl0638 and NCgl0484) and ABC transporter periplasmic component (NCgl0639) by 12-, 6.3-, and 7.5-fold, respectively, and upregulated ABC transporter periplasmic component (NCgl1405) by 5.88-fold, suggesting that NCgl0581 is involved in the synthesis of substances transported through ABC transporter.

Overexpression of the novel L-serine exporter system regarding to SerE and NCgl0581

Considering that NCgl0581 could activate the expression of SerE in strain SSAAI, the overexpression of NCgl0581, *serE* or their co-expression was studied, and strains SSAAI-NCgl0581 and SSAAI-NCgl0581-*serE* were constructed respectively. As shown in Fig. 4B, a decrease in cell growth was observed in SSAAI-

NCgl0581-*ser*E and SSAAI-NCgl0581 before 96 h of fermentation, and SSAAI-NCgl0581-*ser*E showed the lowest growth rate, the time courses for L-serine production were similar in all, furthermore, the yield of L-serine to biomass (Yp/x) increased in both SSAAI-NCgl0581-*serE* and SSAAI-NCgl0581(Fig. 4C and 4D), which suggested that overexpression of a novel exporter SerE and it's transcriptional regulator NCgl0581 was beneficial for L-serine efflux, but not for the cell growth, with regard to Yp/x, SSAAI-NCgl0581-*serE* and SSAAI-NCgl0581 exhibited 9.67% and 19.17% higher value in 96 h respectively, when compared with SSAAI. The similar decrease in cell growth was observed in SSAAI-*serE* (Fig.3C), the titer of L-serine in SSAAI-*serE* reached 28.67 g/L, which was 10.5% higher than that noted in SSAAI. A decrease in cell growth was observed in recombinant strain, we inferred that L-serine was transported out of the cell, when L-serine was synthesized, and the intracellular L-serine was not enough for the cell growth, next we would replenish L-serine by overexpressing L-serine synthetic pathway key enzyme.

Improve L-serine yield and production

To direct more flux to L-serine synthesis, L-serine exporter SerE and L-serine synthetic pathway key enzyme (containing a feedback insensitive *serA*Δ197, *serB* and *serC* encoding the deregulated 3-phosphoglycerate dehydrogenase, phosphoserine phosphatase, and phosphoserine aminotransferase) were co-overexpressed in SSAAI, SSAAI-*serA*Δ197-*serB-serC-serE* had been constructed, the recombinant strain shared similar typical growth curves as the parent strain SSAAI, and reached a final L-serine titer of 32.8 g/L, which was 22.1% higher than that noted in SSAAI. Then L-serine exporter *serE*, *serA*Δ197, *serB* and *serC* were overexpressed in strain A36, and A36-*serA*Δ197-*serB-serC-serE* had been constructed, A36 was stemmed from SSAAI by using ARTP mutation, and it could produce 34.78 g/L L-serine [23], As shown in Fig. 5, the tandem expression strain A36-*serA*Δ197-*serB-serC-serE* shared similar typical growth curves as the parent strain A36 in the overall process. Sucrose level decreased with time in a similar pattern for the both strains. Interestingly, when the process time in the batch cultivations exceeded 72 h, the cell growth and L-serine titer of A36-*serA*Δ197-*serB-serC-serE* increased higher than the parent strain A36. After 120 h cultivation, A36-*serA*Δ197-*serB-serC-serE* consumed all of the sucrose, and reached a final L-serine titer of 43.9 g/L, with the conversion rate of 0.44 g/g. These results demonstrate that overexpression of L-serine exporter in combination with L-serine synthetic pathway could facilitate L-serine production in *C.glutamicum.*

Discussion

Transport engineering is becoming an attractive strategy for strain improvement [11, 16, 17]. However, only a relatively limited number of exporters of amino acids have been identified in C. glutamicum (Table S1) [8, 12, 14–17, 24–27]. In this study, SerE was identified as a novel L-serine exporter in C. glutamicum. Further analysis showed that SerE could also accept L-threonine (Fig. 2C), but not L-cysteine, similar to ThrE, which could export both L-serine and L-threonine in C. glutamicum [15]. It was assumed that the presence of -OH in both L-serine and L-threonine might be the reason for these exporters to transport the two substrates. Based on homology search, SerE was found to be similar to a member of the DMT superfamily. Although DMT superfamily proteins were involved in the transport of a wide range of

substrates, there were only a few reports available on their structures and mechanisms of substrate transport. Christian et al. performed structural and functional analyses of YddG, a DMT protein, and provided insight into the common transport mechanism shared among the DMT superfamily members [28]. It has been reported that analyses of the crystal structure data of exporters could help to elucidate the elusive transport mechanism [29], and in the future, we will plan to investigate further the SerE structures and mechanisms of substrate transport.

To explore the interaction between the known L-serine exporter ThrE and the novel exporter SerE on L-serine accept, ThrE and SerE single and double mutants were constructed. The results showed that serE and thrE double deletion mutant could still accumulate 10.34 g/L L-serine (Fig. 3B), suggesting that C. glutamicum might also possess other L-serine exporter systems. The evolution of multiple exporter systems for a single substrate is beneficial for the survival of bacteria in variable environment [7, 30]. It must be noted that overexpression of serE in SSAAI resulted in 10.5% increase in L-serine titer, but a decrease in cell growth. This could be due to the use of constitutive-type promoter to overexpress SerE, which resulted in higher L-serine efflux. As sufficient L-serine content is important to maintain cell growth, a decrease in cell growth was noted as a stress response to serE overexpression. In future studies, better tuning of the serE expression will be performed in SSAAI by testing different promoters and RBS. When thrE and serE were co-overexpressed in SSAAI, SSAAI-thrE-serE exhibited a lower cell growth than SSAAI, and similar L-serine titer with SSAAI-serE was observed (Fig. 3C). A severe decline in cell growth was observed in all exporter overexpression strains, it was likely that this may be caused by accumulation of L-serine in the media as well as the additional burden on the cell of overexpressing the exporters, the similar result was observed by Mundhada et,al[31].

NCgl0581 was identified as a novel L-serine exporter SerE's transcriptional regulator, and EMSA was performed to confirm the binding sites of NCgl0581 with the promoter of SerE. A previous study reported that the first member of the protein-gene pairs, ArgP-argO in E. coli and LysG-lysE in C. glutamicum, is a LysR-type transcriptional regulator, while the second member is its target gene encoding an amino acid exporter [22, 32, 33]. Similarly, NCgl0581-serE might also be a protein-gene pair sharing the same regulation mechanism. A serine-biosensor based NCgl0581 was reported by Binder et al.[34], and based on this study, we constructed a biosensor for L-serine and found that NCgl0581 activated NCgl0580 (SerE) expression in the presence of L-serine, the expression of SerE enhancing with the L-serine titer increasing [35]. However, NCgl0581 did not activate the expression of SerE in the presence of L-alanine and L-valine. To further confirm whether SerE could accept L-alanine and L-valine, peptide feeding assays were employed using dipeptides (Ala-Ala, Val-Val) with SSAAI and SSAAI ΔserE. The results revealed that SerE could neither export L-alanine nor L-valine (data not shown). Moreover, transcriptome sequencing showed that NCgl0581 regulated 115 genes in C. glutamicum, suggesting that NCgl0581 was a novel global transcriptional regulator in C. glutamicum. Transcriptional regulators and their roles in expression control of target genes are important for metabolic engineering of C. glutamicum for industrial applications [36], and this study provided a new member of transcriptional regulator family.

When L-serine exporter SerE was overexpressed alone, which resulted in increase of 10.8% of L-serine titer, at same time, a decrease in cell growth was observed in recombinant strain, we inferred that L-serine was transported out of the cell, when L-serine was synthesized, and the intracellular L-serine was not enough for the cell growth, to replenish L-serine by overexpressing L-serine synthetic pathway key enzyme, the cell growth was restored, and L-serine titer was increased to 43.9 g/L, and L-serine yield was 0.44 g/g sucrose, which were the highest titer and yield reported so far for any organism. The results indicated that serAΔ197, serC, serB overexpression ensured sufficient L-serine supply avoided the cell growth inhibition. In Mundhada's study, 37 g/L of L-serine was produced with a yield of 0.24 g/g glucose in E. coli [2], and in their previous study, L-serine titer was 11.7 g/L with highest yield of 0.43 g/g glucose [31]. Interestingly, we found that L-serine titer increased significantly by overexpressing serB in A36, with L-serine titer of 37.9 g/L, which was 24% higher than that of A36, serB encoded phosphoserine phosphatase (PSP, EC 3.1.3.3), catalyzed the last step of L-serine biosynthesis, however, L-serine titer had not significantly change when serAΔ197, serC was overexpressed in A36 respectively (with L-serine titer of 31.1 g/L and 32.78 g/L) (Fig.S5), and these results were in consistent with the previous report completely, in which overexpression of the mutant allele serAΔ197 in C. glutamicum13032 either alone or in combination with overexpression of serC and serB did not result in significant L-serine accumulation [3],which might be due to the poor accumulation and intracellular conversion of L-serine in C. glutamicum13032. In a recent study, 50 g/L of L-serine was produced with glucose as carbon source in E. coli, with a yield of 0.26–0.30 g/g glucose [37], in which 50 g/L is the highest reported so far for L-serine production, however, the yield was also lower than our present study (0.44 g/g sucrose). It is possible that fine controlling the three enzymes of the L-serine biosynthesis pathway to enhance L-serine production further.

Conclusion

In the present work, a novel exporter SerE and its positive regulator NCgl0581 were identified in C. glutamicum, with SerE also exhibiting the ability to accept L-threonine and NCgl0581 acting as a novel global transcriptional regulator in C. glutamicum, and by overexpressing novel exporter combined with L-serine synthetic pathway enzyme, increased L-serine yield significantly. These results enrich our understanding of amino acid transport and can provide additional targets for exporter engineering in C. glutamicum.

Materials And Methods

Strains, plasmids, and growth conditions

The strains and plasmids used in this study are listed in Table 3. *E. coli* JM109 was used as the cloning host, and was grown in lysogeny broth (LB) medium (containing 5.0 g/L yeast extract, 10.0 g/L tryptone, and 10.0 g/L NaCl) at 37^{o}C and 220 rpm. The engineered SSAAI (CGMCC No.15170) was constructed in our laboratory by knocking out 591 bp of the C-terminal domain of *serA*, deleting *sdaA*, *avtA*, and *alaT*, as well as attenuating *ilvBN* in the genome of *C. glutamicum* SYPS-062-33a (CGMCC No. 8667). The seed

and fermentation media for *C. glutamicum* were prepared as described previously [5]. The *C. glutamicum* strains were pre-incubated in the seed medium overnight to an optical density (OD_{562}) of about 25, and then inoculated at an initial concentration of OD_{562}=1 into a 250 mL flask containing 25 mL of the fermentation medium at 30^{o}C and 120 rpm. The antibiotic kanamycin (50 mg/L) was added when necessary. Samples were withdrawn periodically for the measurement of residual sugar, amino acids, and OD_{562} as described previously [5].

TABLE 3 Strains and plasmids used in this study.

Strain/Plasmid	Description	Sources or reference
E. coli		
JM109	*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, *supE*44, *relA*1	Laboratory strain
C. glutamicum		
SSAAI	*C. glutamicum* SYPS-33a with deletion of the 591 bp in the C-terminus of *serA*, deletion of *sdaA*,*alaT*,*avta* and attenuation of*ilvBN*	[5]
A36	SSAAI mutant strain	[35]
SSAAI-*thrE*	SSAAI harboring plasmid pDXW-10-*thrE*	This study
SSAAIΔ*thrE*	SSAAI with deletion of *thrE*	This study
SSAAIΔNCgl2050	SSAAI with deletion of NCgl2050	This study
SSAAIΔNCgl2065	SSAAI with deletion of NCgl2065	This study
SSAAIΔNCgl0580	SSAAI with deletion of NCgl0580	This study
SSAAI-10	SSAAI harboring plasmid pDXW-10	This study
SSAAI-*egfp*	SSAAI harboring plasmid pDXW-10-*egfp*	This study
SSAAI-*serE-egfp*	SSAAI harboring plasmid pDXW-10-*serE-egfp*	This study
SSAAI-NCgl0581	SSAAI harboring plasmid pDXW-10- NCgl0581	This study
SSAAI-NCgl0581-*serE*	SSAAI harboring plasmid pDXW-10- NCgl0581-*serE*	This study
SSAAIΔNCgl0581	SSAAI with deletion of NCgl0581	This study
SSAAIΔNCgl0581-1	SSAAIΔNCgl0581 harboring pDXW-11-1	This study
SSAAIΔNCgl0581-0	SSAAIΔNCgl0581 harboring pDXW-11-0	This study
SSAAIΔNCgl0580-*serE*	SSAAIΔ*serE* harboring plasmid pDXW-10-*serE* (NCgl0580)	This study
SSAAI-*serE*	SSAAI harboring plasmid pDXW-10-*serE* (NCgl0580)	This study
ATCC13032	Wild type	Laboratory strain
ATCC13032Δ*serE*	ATCC13032 with deletion of *serE* (NCgl0580)	This study
pK18mob*sacB*	Integration vector, *oriV*, *oriT*, *mob*, *sacB*, Km^r	[38]
pK18mob*sacB*Δ*thrE*	pK18mob*sacB* carrying the up- and downstream homologous fragments of *thrE* gene for *thrE* deletion	This study

Strain/Plasmid	Description	Sources or reference
pK18mob*sacB*ΔNCgl2050	pK18mob*sacB* carrying the up- and downstream homologous fragments of NCgl2050 gene for NCgl2050 deletion	This study
pK18mob*sacB*ΔNCgl2065	pK18mob*sacB* carrying the up- and downstream homologous fragments of NCgl2065 gene for NCgl2065 deletion	This study
pK18mob*sacB*ΔNCgl0580	pK18mob*sacB* carrying the up- and downstream homologous fragments of NCgl0580 gene for NCgl0580 deletion	This study
pK18mob*sacB*ΔNCgl0581	pK18mob*sacB* carrying the up- and downstream homologous fragments of NCgl0581 gene for NCgl0581 deletion	This study
pDXW-10	*E. coli-C. glutamicum* shuttle vector, *tacM* promoter, Km^r	[39]
pDXW-10-*thrE*	pDXW-10 carrying the gene of *thrE*	This study
pDXW-10-*serE*	pDXW-10 carrying the gene of *serE*	This study
pDXW-10-*egfp*	pDXW-10 carrying the gene of *egfp*	This study
pDXW-10-*egfp-serE*	pDXW-10 carrying the gene of *egfp and serE* for the expression of fusion protein EGFP-SerE	This study
pDXW-10- NCgl0581	pDXW-10 carrying the gene of NCgl0581	This study
pDXW-10- NCgl0581-*serE*	pDXW-10 carrying the gene of NCgl0581 and *serE*	This study
pDXW-11	*E. coli-C. glutamicum* shuttle vector, probe plasmid, Km^r	[40]
pDXW-11-1	pDXW-11 carrying the fragments of NCgl0581, the intergenic region between NCgl0581 and NCgl0580, and *egfp*	This study
pDXW-11-0	pDXW-11 carrying the fragments of the intergenic region between NCgl0581 and NCgl0580, and *egfp*	This study

Km^r, kanamycin resistance.

Construction of plasmids and strains

The primers used in this study for gene expression/deletion are listed in Table S2. Gene deletion was performed using the nonreplicable deletion vector pK18mob*sacB*, as reported previously [38]. For example, to achieve *thrE* deletion, the homologous-arm fragments for *thrE* deletion were amplified from SSAAI chromosome using the primer pairs *thrE*1/2 for the upstream fragment and *thrE*3/4 for the downstream fragment. Then, with the two fragments as templates, a crossover PCR was performed using

the primer pair *thrE*1/4. The truncated product of *thrE* was digested with *Xba*Ⅰ and *Hind*Ⅲ and ligated to the vector pK18mob*sacB* that was similarly treated. The recombinant plasmid pK18mob*sacB*Δ*thrE* was transformed into SSAAI competent cells by electroporation, and chromosomal deletion was performed by selecting cells that were kanamycin resistant and sucrose nonresistant, and verified by PCR.

The pDXW-10 and pDXW-11 plasmids were used to overexpress genes in *C. glutamicum* [39, 40]. The recombinant plasmids were constructed as follows: the genes *thrE* and *serE* were amplified, digested, and ligated to the pDXW-10 plasmid that was digested with *Hind*Ⅲ/*Bgl*Ⅱ. The plasmid harboring the fusion protein, SerE-EGFP (enhanced green fluorescent protein) was constructed by using the method reported in a previous study [19]. To confirm the role of NCgl0581 on NCgl0580 expression, the fragment consisting of intergenic region of NCgl0581 and NCgl0580 and EGFP with or without NCgl0581 was ligated to the plasmid pDXW-11 by Clon Express MultiS One Step Cloning Kit (Vazyme, Nanjing, China). The strains were constructed by electroporation with the corresponding plasmids.

The genes, *serA*Δ197, *serB*, and *serC*, were PCR amplified from SSAAI using primers shown in Table S2. To construct plasmid pDXW-10-*serA*Δ197*,* the resultant fragment of *serA*Δ197 was digested with *EcoR*I and *Not*I and cloned into pDXW-10. To construct plasmid pDXW-10-*serA*Δ197-*ser*B-*ser*C, PCR fragments of *serB*, and *serC* were digested with the appropriate restriction enzymes and successively cloned into the corresponding plasmids to form plasmid pDXW-10-*serA*Δ197-*serB-serC.* The resulting plasmid (pDXW-10-*serA*Δ197-*serB-serC*) was then subjected to double digestion by *Nde*I and *Pac*I for cloning of NCgl0580 to form pDXW-10-*serA*Δ197-*serB-serC*-NCgl0580.

Confocal microscopic observation

The strains SSAAI-10 (SSAAI harboring plasmid pDXW-10), SSAAI-*egfp*, and SSAAI-*serE-egfp* were grown in the seed medium and harvested during the exponential phase. The cells were washed twice and maintained in PBS (pH 7.4), mounted on a microscope slide, and observed under a Leica laser scanning confocal microscope (Leica, TCS SP8; Leica, Wetzlar, Germany) equipped with a HC PL Apo 63x/1.40 Oil CS2 oil-Immersion objective, with excitation filter at 488 nm and emission filter at 510-550 nm. The digital images were acquired and analyzed with Lecia Application Suite X 2.0.

Membrane and cytoplasmic protein extraction and fluorescence measurements

The strains SSAAI-10, SSAAI-*egfp*, and SSAAI-*serE-egfp* were used for extracting membrane and cytoplasmic proteins to determine SerE localization. The extraction was performed using Membrane and a Cytoplasmic Protein Extraction Kit according to the manufacturer's protocol (Beyotime, Nanjing, China). The cells were washed twice with PBS (pH 7.4) and disrupted by ultrasonication on ice (pulse, 4 s; interval, 6 s; total duration, 30 min) (Sonics Vibra-Cell™, Sonics, Newtown, CT, USA). The supernatant containing cytoplasmic proteins was collected by centrifugation (700 × *g*, 4 ^{o}C for 10 min), and the precipitate was used for extracting membrane proteins. The protein concentration was determined with a Modified BCA Protein Assay Kit (Sangon, China). After extraction, the fluorescence intensity (excitation at

488 nm, emission at 517 nm) of the membrane and cytoplasmic proteins was determined using fluorescence spectrophotometer (Synergy H4; BioTek, Winooski, VT, USA).

Amino acid export assay

For ascertaining the function of *serE*, a dipeptide Ser-Ser addition assay was conducted [15]. In brief, pre-incubated cells (in seed medium) were washed once with CGX minimal medium [41], inoculated into CGX minimal medium with 2 mM Ser-Ser (other dipeptide), and incubated for 2 h at 30^{o}C. Then, the cells were harvested, washed once with cold CGX minimal medium, and resuspended in CGX minimal medium. Amino acid excretion was initiated by adding 2 mM Ser-Ser (another dipeptide). HPLC was used to determine the concentrations of amino acids [19].

Analytical procedures

Cell density (OD_{562}) was measured using an AOE UV-1200S UV/vis spectrophotometer (AOE Instruments Co. Inc., Shanghai, China). Glucose concentration was determined using SBA-40E glucose analyzer (Biology Institute of Shandong Academy of Sciences, China). For measurement of extracellular L-serine concentration in shake-flask fermentation, 500 μL of the culture were centrifuged at 700 ×*g* for 5 min, and the supernatant was used for detection after appropriate dilution. To ascertain intracellular L-serine concentration, 300 μL of the culture were centrifuged at 700 ×*g* and 4^{o}C for 10 min, and 300 μL of water were added to the cells. The cells were disrupted by FastPrep-24 5G instrument (5 m/s, 120 s, MP Biomedicals, Shanghai, China). The cytoplasmic volume was assumed to be 2 μL/mg dry cell weight [27]. The titers of intracellular and extracellular L-serine and other amino acids were analyzed by HPLC using phenyl isothiocyanate as a precolumn derivatization agent, according to our previously study [8].

EMSA

To identify the binding site of NCgl0581 in the NCgl0580 promoter region, EMSA was conducted by using Non-Radioactive EMSA Kits with Biotin-Probes User's Manual VER. 5.11 (Viagene Biotech Inc, Changzhou, China), according to the manufacturer's instruction. The consensus oligonucleotides were BIO-JNZXM-TP (5'-AAACAGCCAA CTATAGTTAAGTAATA-3') and BIO-JNZXM-BM (5'-TATTACTTAACTATAGTTGGCTGTTT-3').

Supplementary Information

Supplementary information accompanies this paper.

Abbreviations

C.glutamicum: *Corynebacterium glutamicum*SSAAI : *C. glutamicum* ΔSSAAI EGFPEnhanced green fluorescent proteinEMSAElectrophoretic mobility shift assays DMTThe drug/metabolite transporter superfamilyThr-Thr: L-Threonine dipeptidesCys-Cys: L-cysteine dipeptidesAla-AlaL-alanine

dipeptides，Val-Val，L-valine dipeptides，LTTRs，LysR-type transcriptional regulators family，Yp/x ，The yield of L-serine to biomass，PSP，Phosphoserine phosphatase ，LB: Lysogeny broth .

Declarations

Authors' contributions

XM Z, YJ G and ZW C conceived and designed the experiments. XM Z led the performance of the experiments, YJ G and ZW C performed the experiments. XM Z, GQ X and XJ Z analyzed the data. XM Z, YJ G and ZW C wrote the manuscript, JS S, MK and ZH X gave some suggestions for the experiments and revised the manuscript. All authors read and approved the final manuscript

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and analyzed during this study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Figures

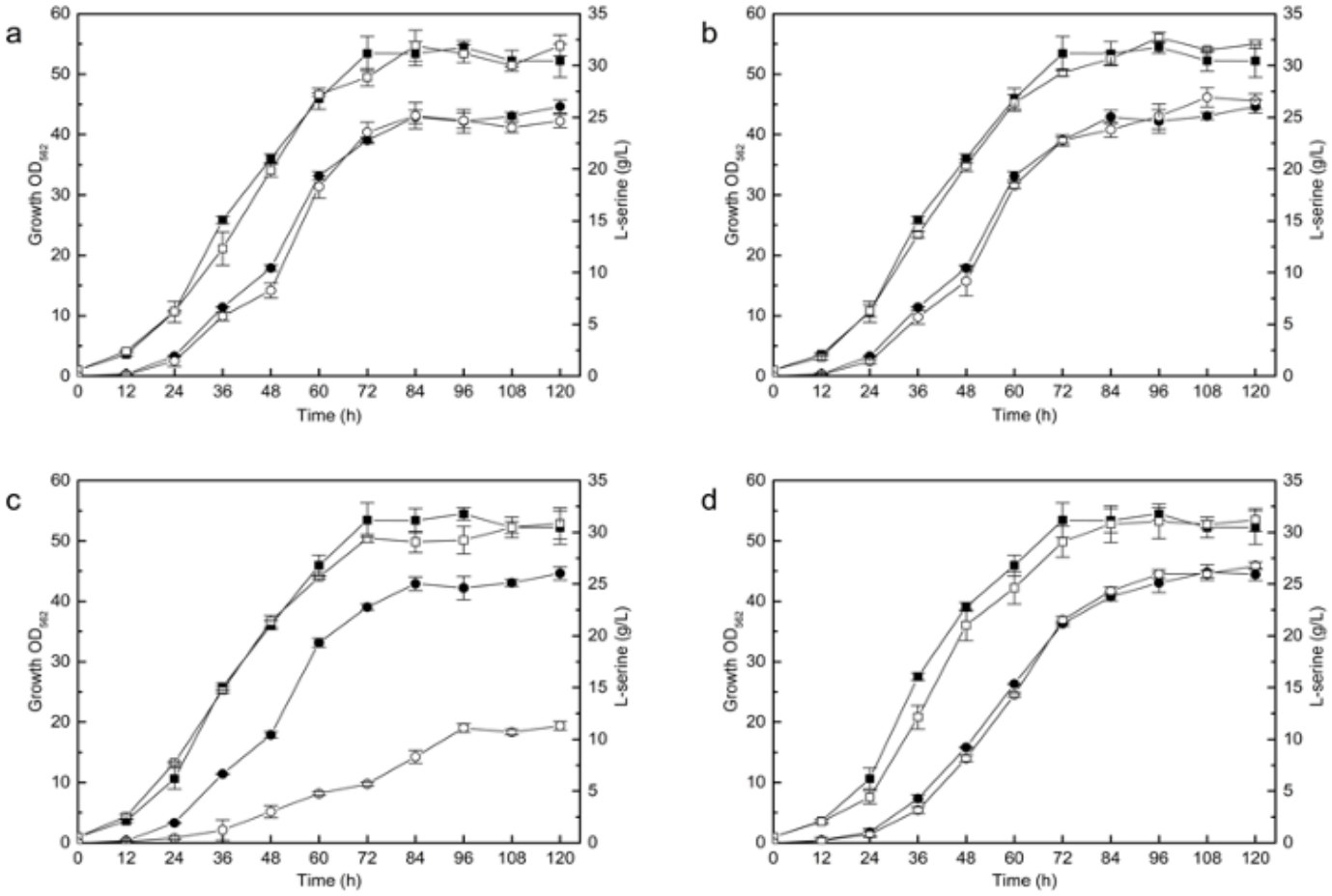

Figure 1

Effect of NCgl2050, NCgl2065, NCgl0580 deletion and plasmid compensating strain on SSAAI. (a) NCgl2050 deletion strain SSAAIΔNCgl2050 (open symbols), SSAAI (solid symbols). (b) NCgl2065 deletion strain SSAAIΔNCgl2065 (open symbols), SSAAI (solid symbols). (c) NCgl0580 deletion strain SSAAIΔNCgl0580 (open symbols), SSAAI (solid symbols). (d) The plasmid compensating strain SSAAIΔN0580-NCgl0580 (open symbols). SSAAI (solid symbols). Squares and circles indicate the cell growth OD562 and L-serine titer, respectively.

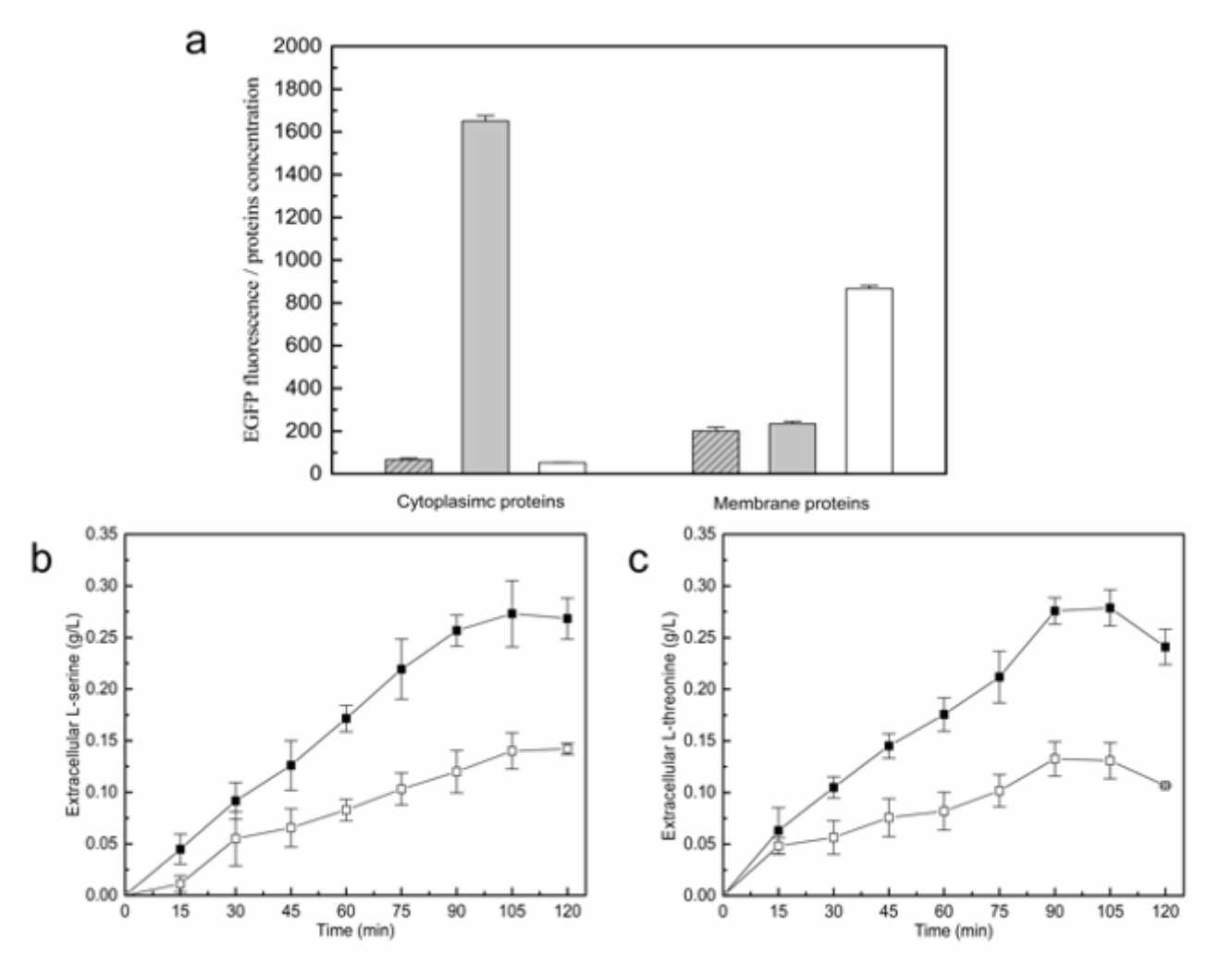

Figure 2

The fluorescence of cytoplasmic proteins and membrane proteins and the result of amino acid export of SerE by using peptide feeding approach in SSAAI. (a) The fluorescence of cytoplasmic proteins and membrane proteins of SSAAI-10 (SSAAI harboring plasmid pDXW-10 only, grey bar with slash), SSAAI-egfp (SSAAI overexpressing EGFP protein with pDXW-10, grey bar) and SSAAI-serE-egfp (SSAAI overexpressing SerE-EGFP fusion protein with pDXW-10, white bar). (b) Extracellular concentration of L-serine in SSAAI (solid symbols) and serE deletion strain SSAAI ΔserE (open symbols). (c) Extracellular concentration of L-threonine in SSAAI (squares) and serE deletion strain SSAAI ΔserE (circles).

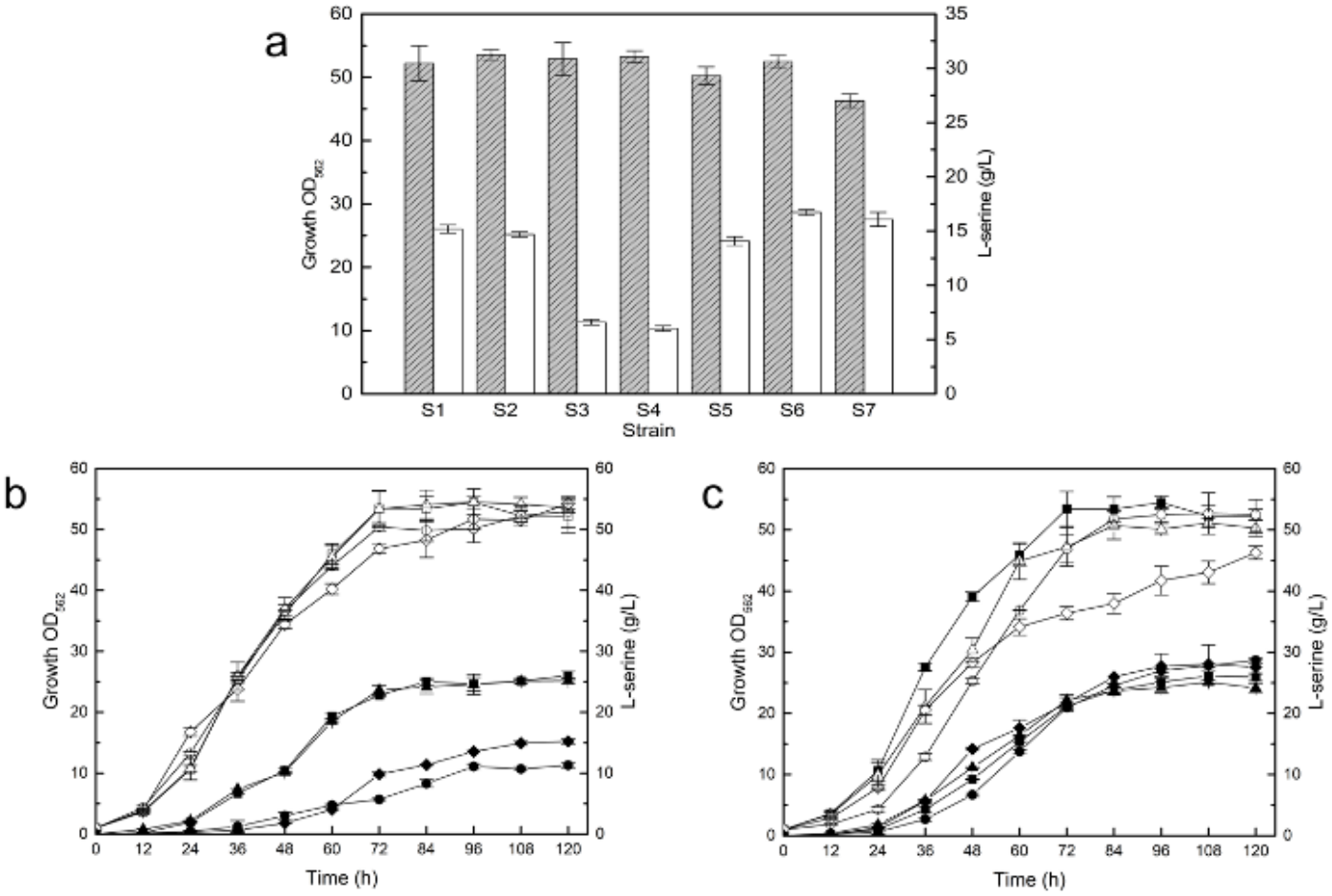

Figure 3

The effect of the exporter thrE, serE deletion or overexpression on strain SSAAI. (a)The cell growth (grey bar with slash), L-serine titer (white bar). SSAAI (S1), thrE deletion strain SSAAI ΔthrE (S2), serE deletion strain SSAAI ΔserE (S3), thrE and serE deletion strain SSAAI ΔserE ΔthrE (S4), thrE overexpression strain SSAAI-thrE (S5), serE overexpression strain SSAAI-serE (S6), thrE and serE double overexpression strain SSAAI-serE -thrE (S7). (b) The cell growth (open symbols) and L-serine titer (solid symbols) of stain SSAAI (squares), serE deletion strain SSAAI ΔserE (circles), thrE deletion strain SSAAI ΔthrE (triangles), thrE and serE deletion strain SSAAI ΔserE ΔthrE (rhombus). (c) The cell growth (open symbols) and L-serine titer (solid symbols) of stain SSAAI (squares), serE overexpression strain SSAAI-serE (circles), thrE overexpression strain SSAAI-thrE (triangles) and thrE and serE double overexpression strain SSAAI-serE -thrE (rhombus).

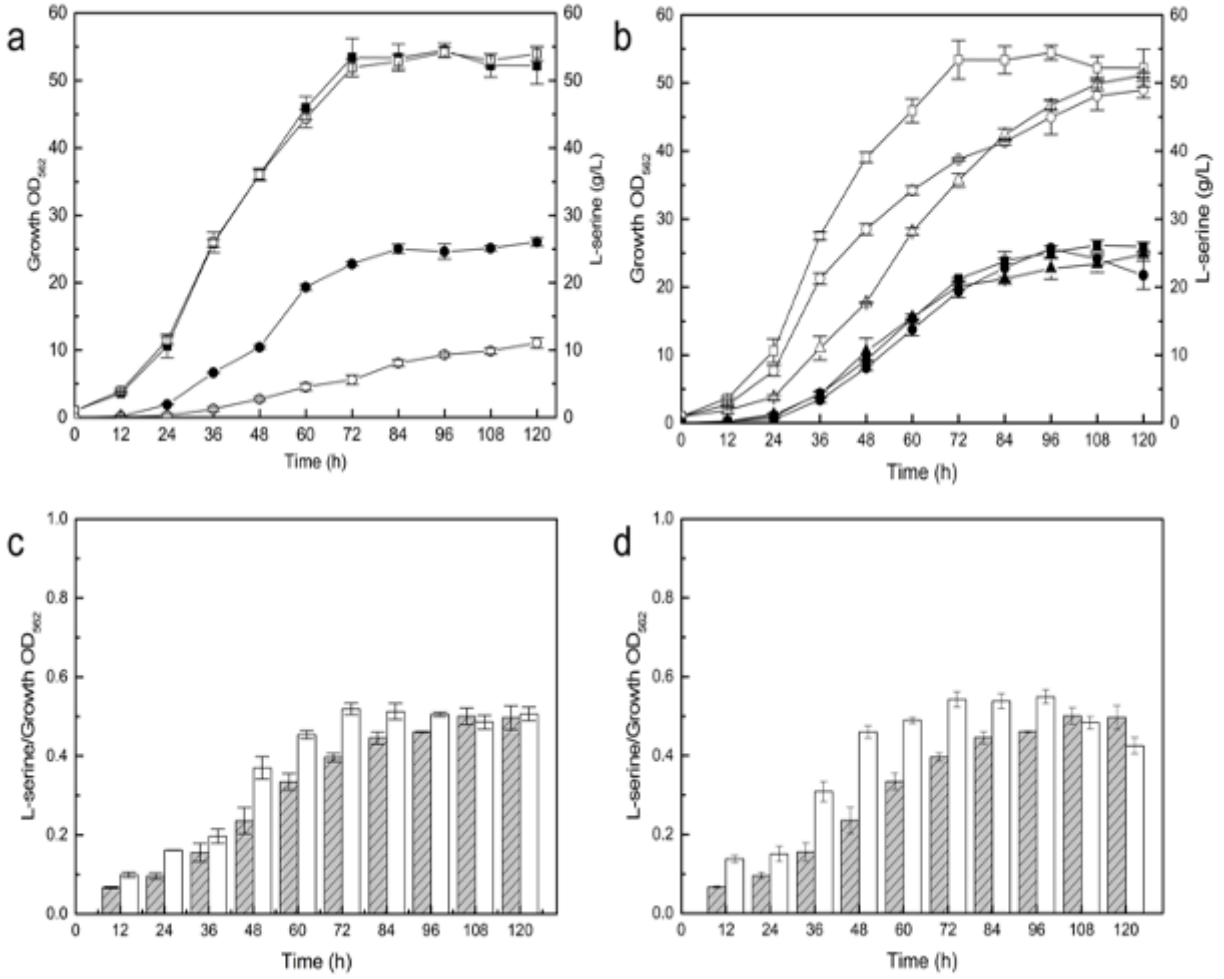

Figure 4

The effect of serE, NCgl0581 deletion or overexpression on SSAAI. (a) The cell growth (squares) and L-serine titer (circles) of SSAAI (solid symbols) and NCgl0581 deletion strain SSAAIΔNCgl0581 (open symbols), respectively. (b) The cell growth (open symbols) and L-serine titer (solid symbols) of stain SSAAI (squares), NCgl0581 overexpression strain SSAAI-NCgl0581 (circles), NCgl0581 and serE double overexpression strain SSAAI-serE-NCgl0581 (triangles). (c) The yield of L-serine to biomass (Yp/x) of SSAAI (grey bar with slash) and NCgl0581 overexpression strain SSAAI-NCgl0581 (white bar). (d) The yield of L-serine to biomass (Yp/x) of SSAAI (grey bar with slash) and NCgl0581 and serE double overexpression strain SSAAI-NCgl0581-serE (white bar).

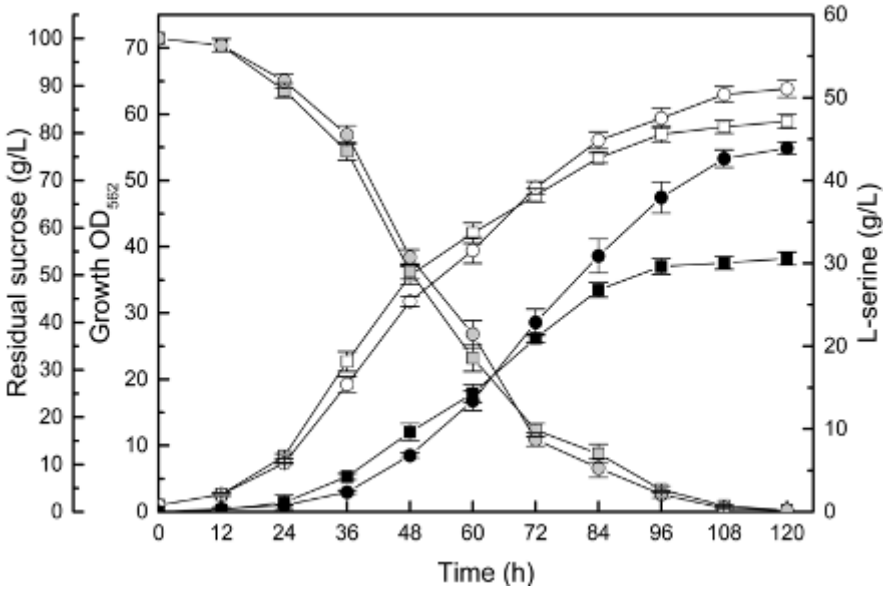

Figure 5

Fermentation process of strain A36 and strain A36-serAΔ197-serB-serC-serE.The cell growth (open symbols), L-serine titer (solid symbols) and residual sucrose (gray symbols) of stain A36 (squares) and A36-serAΔ197-serB-serC-serE (circles). Three parallel experiments were performed. Error bars indicate standard deviations of results from three parallel experiments.

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