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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Engineering of High Yield Production of L-Serine in *Escherichia Coli*

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ABSTRACT: L-serine is a widely used amino acid that has been proposed as a potential building block biochemical. The high theoretical yield from glucose makes a fermentation based production attractive. In order to achieve this goal, serine degradation to pyruvate and glycine in *E. coli* MG1655 was prevented by deletion of three L-serine deaminases *sdaA*, *sdaB*, and *tdcG*, as well as serine hydroxyl methyl transferase (SHMT) encoded by *glyA*. Upon overexpression of the serine production pathway, consisting of a feedback resistant version of *serA* along with *serB* and *serC*, this quadruple deletion strain showed a very high serine production yield (0.45 g/g glucose) during small-scale batch fermentation in minimal medium. Serine, however, was found to be highly toxic even at low concentrations to this strain, which lead to slow growth and production during fed batch fermentation, resulting in a serine production of 8.3 g/L. The production strain was therefore evolved by random mutagenesis to achieve increased tolerance towards serine. Additionally, overexpression of *eamA*, a cysteine/homoserine transporter was demonstrated to increase serine tolerance from 1.6 g/L to 25 g/L. During fed batch fermentation, the resulting strain lead to the serine production titer of 11.7 g/L with yield of 0.43 g/g glucose, which is the highest yield reported so far for any organism.

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KEYWORDS: amino acids; E. coli; fermentation; L-serine production; L-serine toxicity; metabolic engineering

Introduction

L-serine is an amino acid currently used in the cosmetics, pharmaceutical, and medical industry. The estimated annual production of serine is between 300–1,000 tons (Leuchtenberger et al., 2005). Additionally, the compound has been identified as one of the top 30 most interesting building block biochemical, because of its potential industrial uses (Werpy et al., 2004). If the production cost can be reduced, a significantly larger market may therefore open up. The current production is based on conversion of glycine and methanol using resting cells (Hagishita et al., 1996), where methylotrophs convert methanol to formaldehyde and transfer the CH2-OH unit of the molecule to glycine using serine hydroxymethyltransferase (*glyA*). In this process, around 90% of glycine supplied is converted to serine, taking 5 days to complete. Glycine is significantly more expensive than glucose leading to a high production cost of L-serine.

Serine has the potential to be made from glucose by fermentation with a very high theoretical yield (23/10 mmol of glucose) (Burgard and Maranas, 2001). However, several challenges need to be addressed in order to increase the titer and yield, the most crucial ones being degradation of serine in the production organism. Serine has two main degradation pathways to either glycine or pyruvate. Conversion of serine to pyruvate is in *E. coli* encoded by three L-serine deaminases, *sdaA*, *sdaB*, and *tdcG*, while only *sdaA* is found in *C. glutamicum*. In both organisms, the conversion of serine to glycine through serine hydroxymethyl transferase (SHMT) is encoded by *glyA*. Production of serine by deleting only L-serine deaminases has been attempted in *E. coli* (Li et al., 2012) and *C. glutamicum* (Peters-Wendisch et al., 2005). In *E. coli,* transient accumulation of 3.8 mg/L from 1 g/L glucose was observed when only one of the serine pathway genes (*serA*) was overexpressed. In *C. glutamicum,* deletion of the L-serine deaminase *sdaA* leads to marginal and transient increase of the serine titer of 0.05 to 0.06 g/L from 40 g/L glucose (Peters-Wendisch et al., 2005). Serine hydroxymethyl transferase (SHMT) converts serine to glycine while transferring one carbon unit to tetrahydrofolate (THF), which is an important cofactor required for C1-metabolism. Down regulation of *glyA* (Peters-Wendisch et al., 2005) in *C. glutamicum* resulted in the production of 9.07 g/L serine from 40 g/L glucose, however, also leading to an unstable strain prone to acquire mutations in the *glyA* promoter region, which in turn upregulated the expression of the enzyme. Removal of the folic acid pathway and supplementation of folate was demonstrated to be a successful method to tune SHMT activity and lead to stable serine production in *C. glutamicum* (Stolz et al., 2007). The strain resulted in the production of 36 g/L serine in a 20 L fermenter, however, with a

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relatively low yield. Recently, a *C. glutamicum* strain (SYPS-062) isolated from soil was engineered to eliminate feedback inhibition and reduced synthesis of other amino acids produced as a byproduct in the strain (L-alanine and L-valine). This strain could produce 46.2 g/L of L-serine with yield of 0.21 g/g of sucrose, which is the highest so far reported in any strain (Zhu et al., 2015).

Recently, *E. coli* was engineered to increase the flux towards the serine precursor, 3-phosphoglycerate, by perturbing both the TCA cycle and the glyoxylate shunt. The final strain produced 8.45 g/L serine from 75 g/L glucose (Gu et al., 2014). The relatively low yield was most likely due to presence of other serine catabolic enzymes and parallel channeling of flux towards other byproducts. The removal of both serine degradation pathways should lead to stable production strain producing at high yield. The gene *glyA* has been shown to be essential in *C. glutamicum*, but it can be deleted in *E. coli* (Vidal et al., 2008). Combinatorial deletion of *glyA* together with the three L-serine deaminases has however not been demonstrated so far.

Serine plays a pivotal role in central carbon metabolism, where it supplies hydroxymethyl groups to cellular anabolic reactions through conversion to glycine, and it also serves as a precursor for other amino acids and phospholipids (Aboulwafa et al., 2004). However, in high concentrations, serine is toxic to cells as it interferes with branched chain amino acid biosynthesis (Hama et al., 1990) and it may be converted into reactive byproducts such as hydroxypyruvate and acrylates (de Lorenzo et al., 2015). Therefore, despite being a relatively poor growth substrate (Newman et al., 1982), *E. coli* harbors three deaminases and a strong feedback regulation of the serine biosynthesis pathway, in order to regulate serine concentration. When all three L-serine deaminases are deleted, the resulting strain shows only marginal growth in concentrations as low as 1.5 g/L serine (Zhang and Newman, 2008).

In this study, we have successfully removed the two major serine degradation pathways from *E. coli* MG1655 by deleting the three L-serine deaminases (*sdA*, *sdaB*, and *tdcG*) together with *glyA*, resulting in increased serine production. The strain, however, was very sensitive to even low concentrations of serine. Increased tolerance towards L-serine was achieved by random mutagenesis and by over expression of a cysteine and acetyl serine exporter encoded by *eamA*. The resulting strain showed increased production during fed batch fermentation, resulting in the highest yield reported so far (0.43 g/g glucose consumed).

Materials and Methods

Strains, Media, and Materials

Tolerance studies, growth experiments, and engineering of serine production was carried out using *E. coli* MG1655 as a parental strain. Further strains constructed in this study are shown in Table I. Minimal M9 media supplemented with antibiotics (if required) containing 2 g/L glucose, 0.1 mM $CaCl_2$, 2.0 mM $MgSO_4$, 1× trace element solution, and 1× M9 salts. The 1× trace element stock solution consisted of 6.75 mg/L $FeCl_3{}^*6H_2O$, 0.4 mg/L $ZnCl_2{}^*4H_2O$, 0.5 mg/L $CoCl_2{}^*6H_2O$, 0.5 mg/L $NaMoO_4{}^*2H_2O$, 0.25 mg/L $CaCl_2{}^*H_2O$, 0.325 mg/L $CuCl_2{}^*6H_2O$, 0.125 mg/L H_3BO_3, and concentrated HCl dissolved in double distilled water and sterile filtered. The 1× M9 salts stock solution consisted of 6.8 g/L Na_2HPO_4 anhydrous, 3 g/L KH_2PO_4, 5 g/L NaCl, and 1 g/L NH_4Cl dissolved in double distilled water and autoclaved. Precultures were done in 2× YT media containing 16 g/L bacto tryptone, 10 g/L yeast extract, 5 g/L NaCl adjusted to pH 7 and autoclaved. Glucose (0.1%) and respective antibiotics were added to precultures used for production. 1 mM of threonine was supplemented to batch and fed batch fermentation media for serine production studies, as it has been shown to partly alleviate serine inhibition of branched amino acid biosynthesis (Hama et al., 1990).

All chemicals were ordered from Sigma–Aldrich (Taufkirchen, Germany). Restriction enzymes and polymerase were bought from Thermoscientific (Waltham, MA). DNA purifications and plasmid preps were done by using Machery Nagel kits (Duren, German). DNA samples were measured by Nanodrop (Thermoscientific, Wilmington, DE). Growth profiles of strains were studied in Biolector (M2P labs, Baeswieler, Germany) or microtiter plate reader (Biotek, Winooski VT). DNA oligonucleotides were ordered from Integrated DNA Technologies (Leuven, Belgium) and sequences are given in Table II. Strains and plasmids constructed are shown in Table I.

Construction of Deletion Strains

Deletion of deaminases was done sequentially by using Lambda red system (Datsenko and Wanner, 2000). Primers used for amplification of the kanamycin cassette from plasmid pKD4 are shown in Table II. The PCR product was column purified and subjected to overnight DpnI digestion. Cells transformed with pKD46 were grown in 2YT-amp media at 30° and 250 rpm. The expression of exo, beta, and gamma proteins were induced by addition of 20 mM arabinose at mid log phase and harvesting the cells after additional 1 h incubation. Cells were washed twice with ice cold 10% glycerol and 200 ng of purified PCR product was transformed by electroporation. Transformants were plated on LB plates containing kanamycin. The kanamycin cassette was subsequently removed using plasmid pCP20 encoding flippase gene. Serine hydroxymethyl transferase encoded by *glyA* was deleted using a previously described P1 phage system (Thomason et al., 2007).

Overexpression of Serine Biosynthesis Pathway for Production of Serine

The genes, *serA*, *serB*, and *serC*, were PCR amplified from *E. coli* MG1655 using primers shown in Table II. After column purification, the gene products and plasmids were digested using Fast digest enzymes (Thermoscientific, Waltham, MA). *serA* was subjected to double digestion with NcoI and NotI while *serC* was digested with NdeI and PacI. pCDFDuet-1 was first digested with NcoI and NotI for cloning of *serA* and this plasmid was later used for cloning of *serC*. The gene encoding *serB* was cloned in a pACYCDuet-1 vector at the NcoI and PacI site leading to pACTCDuet-1-*serB*. Subsequently, the *eamA* gene was included in the pACYC vector backbone along with *serB* by cloning *serB* at first MCS by NcoI and HindIII double digestion followed by ligation. The resulting vector (pACYCDuet-1-*serB*) was then subjected to double digestion by NdeI and PacI for

Table I. Strains and plasmids used in the study.

Strains and plasmids	Genotype	Reference
Strains		
MG1655	*F- λ- ilvG- rfb-50 rph-1*	Lab stock
S1	MG1655 *ΔsdaA λ(DE3)*	This study
T1	MG1655 *ΔsdaAΔsdaBΔtdcG λ(DE3)*	This study
Q1	MG1655 *ΔsdaAΔsdaBΔtdcGΔglyA λ(DE3)*	This study
Q3	MG1655*ΔsdaAΔsdaBΔtdcGΔglyA [rhtA] [mntS] λ(DE3)*	This study
Q1 *ΔrhtA*	MG1655 *ΔsdaAΔsdaBΔtdcGΔglyAΔrhtA::kan λ(DE3)*	This study
Q1 *ΔompX*	MG1655*ΔsdaAΔsdaBΔtdcGΔglyAΔompX::kan λ(DE3)*	This study
Q1 *ΔopgE*	MG1655 *ΔsdaAΔsdaBΔtdcGΔglyAΔopgE::kan λ(DE3)*	This study
Q1 *ΔrybA*	MG1655 *ΔsdaAΔsdaBΔtdcGΔglyAΔrybA::kan λ(DE3)*	This study
Q1 *ΔsstT*	MG1655 *ΔsdaAΔsdaBΔtdcGΔglyAΔsstT::kan λ(DE3)*	This study
Plasmids		
pCDF-Duet-*serAmut-serC*	$Spec^R$, containing *serA*mut and *serC*	This study
pACTCDuet-1-serB	*cat*, containing *serB*	This study
pACYCDuet-1-*serB-eamA*-c-His	*cat*, containing *serB* and *eamA*-c-His	This study
pCDF-1b-*eamA*-c-His	$Spec^R$, containing *eamA*-c-His	This study
pKD46	*bla*, helper plasmid for recombination	(Datsenko and Wanner, 2000)
pCP20	*bla* and *cat*, encoding flippase to loop out *kan* casette	(Datsenko and Wanner, 2000)
pKD4	*bla*, FRT-*kan*-FRT	(Datsenko and Wanner, 2000)

cloning of eamA-c-His to form pACYCDuet-1-*serB-eamA*-c-His. Feedback inhibition of *serA* was prevented by mutating three residues H344, N346, and N364 to alanine (Al-Rabiee et al., 1996) by site directed mutagenesis using the two step protocol (Edelheit et al., 2009) and primers shown in Table II.

Serine Production During Small Scale Batch Fermentation

In order to use pET vectors as expression system, a DE3 cassette containing T7 polymerase was integrated into the genome of each deletion mutant using a Lambda DE3 lysogenization kit (Millipore, Damstadt Germany). Subsequently, Q1 (DE3) strain was transformed with pCDFDuet-1-*serA*mut-*serC* and pACYCDuet-1-*serB*. The resulting strains were grown overnight in 2YT medium containing 0.1% glucose and supplemented with required antibiotics. Overnight cultures were inoculated in duplicates into flasks containing M9 medium supplemented with 0.2% glucose, 1 mM threonine, 2 mM glycine, and required antibiotics. Flasks were incubated at 37°C at 250 rpm, and serine production was induced at different IPTG concentrations after the cultures reached an optical density of 0.55 to 0.65. Effects of gene deletions on serine production was also studied in the similar set up involving strains carrying single (*ΔsdaA*) S1, triple (*ΔsdaA*, *ΔtdcG*, *ΔsdaB*) T1, and quadruple deletions (*ΔsdaA*, *ΔtdcG*, *ΔsdaB*, and *ΔglyA*) Q1, transfomed with above vectors containing the serine pathway. Overnight cultures were inoculated in triplicates in flasks containing M9 medium supplemented with 0.2% glucose, 1 mM threonine, required antibiotics (for strain Q1, 2 mM glycine was also supplemented). Serine production was induced with 40 μM IPTG after the cultures reached an optical density of 0.55 to 0.65.

Toxicity Studies With Over Expression of *eamA*

A transporter encoded by *eamA*, was cloned in pCDF-1b at NcoI and PacI site by amplification with primers (Table II) and the above mentioned protocol for digestion and ligation. The use of NcoI leads to mutation of serine to alanine at second position. Q1 was transformed with the pCDF-1b empty vector and the pCDF-*eamA*-c-His plasmid. Overnight cultures of these transformants were inoculated in M9 media supplemented with 2 mM glycine and spectinomycin. Cultures were incubated at 37°C and 250 rpm until reaching an optical density of 0.4 to 0.5. The expression of *eamA* was induced with 100 m M IPTG for 1 h, after which 800 μL was added to a 48-well biolector plate (M2P labs, Baeswieler, Germany) containing 800 μL M9 media with varying concentrations of serine (6.25, 12.5, 25, and 50 g/L) and 2 mM glycine. The growth was then monitored using a Biolector instrument at 37°C and 70% humidity with continuous shaking.

Random Mutagenesis for Increasing Serine Tolerance

The Q1 strain was grown in M9 media supplemented with 2 mM glycine and 3 g/L serine overnight. 1 mL of culture was transferred to a 6-well petri plate and exposed to UV irradiation (CBS Scientific, San Diego, CA) for 30 min. The culture was then added to 5 mL M9 media supplemented with 2 mM glycine and 50 g/L serine for enrichment of the tolerant mutants. The culture was incubated at 37°C and 250 rpm for 3 days and then plated on M9 plate supplemented with 50 g/L serine for the selection of tolerant variants.

Genome Sequencing

Genomic DNA was extracted from 1.5 mL of overnight cultures of *E. coli* strains using QIAamp DNA Mini Kit (QIAGEN, Germany). Genomic libraries were generated using the TruSeq ® Nano DNA LT Sample Preparation Kit (Illumina Inc., San Diego CA). The breseq pipeline version 0.23 (Deatherage and Barrick, 2014) with bowtie2 (Langmead and Salzberg, 2012) was used to map sequencing reads and identify sequence variants relative to the *E. coli* K12 MG1655 reference genome (NCBI accession number NC_000913.2). Gene

Table II. (A) Primers used for constructing gene deletions. (B) Primers used for amplification and cloning of given vectors. (C) Primers used for site directed mutagenesis to remove feedback inhibition by serine. Target genes are mentioned in primer name while 'F' or 'R' reflects forward or reverse primer respectively.

Primer name	Sequence	Plasmids constructed
A. Gene deletion primers		
sdaA_F	5′GCGCTGTTATTAGTTCGTTACTGGAAGTCCAGTCACCTTGTCAGGAGTATTATCGTGGTGTAGGCTGGAGCTGCTTCG3′	
sdaA_R	5′CGCCCATCCGTTGCAGATGGGCGAGTAAGAAGTATTAGTCACACTGGACCATATGAATATCCTCCTTAGTTCC3′	
sdaB_F	5′CGCTTTCGGGCGGCGCTTCCTCCGTTTTAACGCGATGTATTTCCTATGGTGTAGGCTGGAGCTGCTTCG3′	
sdaB_R	5′GGCCTCGCAAAACGAGGCCTTTGGAGAGCGATTAATCGCAGGCAACCATATGAATATCCTCCTTAGTTCC3′	
tdcG_F	5′CGTTCCGCTCCACTTCACTGAACGGCAATCCGAGGGTGTGGATATGGTGTAGGCTGGAGCTGCTTCG3′	
tdcG_R	5′GTGCACCCAAGGATGAAAGCTGACAGCAATGTCAGCCGCAGACCACCATATGAATATCCTCCTTAGTTCC3′	
ompX_F	5′GATATATTTAAAACTTAGGACTTATTTGAATCACATTTGAGGTGGTTATGGTGTAGGCTGGAGCTGCTTCG3′	
ompX_R	5′GAGGCGGATTTTTTATATCACCAAAGTGATTAGAAGCGGTAACCAACACCATATGAATATCCTCCTTAGTTCC3′	
opgE_F	5′CTTGACGCCAGGGTAAGCACATGGCGTTTGTTACGATAGTGGCATATGGTGTAGGCTGGAGCTGCTTCG3′	
opgE_R	5′GATATAAAAAATCCGCCTCTCGGGGCGGATTTTTGTTTTTAAGGTTTCGCATATGAATATCCTCCTTAGTTCC3′	
rhtA_F	5′AGTACACTCCACGCTTTACTTAAGTCTAGATATTTGTGGGAGAAAGGATGGTGTAGGCTGGAGCTGCTTCG3′	
rhtA_R	5′TTAATTAATGTCTAATTCTTTTATTTTGCTCTCTTTGCGTACTGTCAGCGCATATGAATATCCTCCTTAGTTCC3′	
rybA_F	5′CGTTTTAGCAATAGCTATATAATATAGCCTGTGCTATATCTGTATGTAATGGTGTAGGCTGGAGCTGCTTCG3′	
rybA_R	5′ATCCGCGAGCCGCAATATACGCAGGGCTGCAAGAAGATAGAGCGAGCCCATATGAATATCCTCCTTAGTTCC3′	
sstT_F	5′CGCACCAGGGATGTGCGACAACACAATGAAAGGATCGAAAAATGGTGTAGGCTGGAGCTGCTTCG3′	
sstT_R	5′GTTGAGAAAACCCCTTCCGCCGTAGACGAAAGGGGTTAAACCATATGAATATCCTCCTTAGTTCC3′	
B. Cloning primers		
serA_NcoI_F	5′GGCCCATGGCAAAGGTATCGCTGGAG3′	pCDFDuet-1-serA-serC
serA_NotI_R	5′ATTGCGGCCGCTTAGTACAGCAGACGGGCGCGA3′	pCDFDuet-1-serA-serC
serC_NdeI_F	5′GGCCATATGATGGCTCAAATCTTCAATTTTAGTTCTGG3′	pCDFDuet-1-serA-serC
serC_PacI_R	5′GCCTTAATTAATCATTAACCGTGACGGCGTTCGAAC3′	pCDFDuet-1-serA-serC
serB_NcoI_F	5′GGCCCATGGCTAACATTACCTGGTGCG3′	pACTCDuet-1-serB
serB_PacI_R	5′GCCTTAATTAATTATTACTTCTGATTCAGGCTGCC3′	pACTCDuet-1-serB
serB_HindIII_R	5′GCCAAGCTTTTATTACTTCTGATTCAGGCTGCC3′	pACYCDuet-1-serB-eamA-c-His
eamA_NdeI_F	5′CCGCATATGTCGCGAAAAGATGGGGTG3′	pACYCDuet-1-serB-eamA-c-His
eamA_PacI_R	5′GCCTTAATTAATGATGATGATGATGATGACTTCCCACCTTTACCGCTTTACGCC3′	pACYCDuet-1-serB-eamA-c-His and pCDF-1b-eamA-c-His
eamA_NcoI_F	5′CCGCCATGGCGCGAAAAGATGGGGTG3′	pCDF-1b-eamA-c-His
C. Site directed mutagenesis primers used for mutation of serA to serAmut		
N364A_F	5′CGAGCAGGGCGTCGCTATCGCCGCGCAATA3′	
N364A_R	5′TATTGCGCGGCGATAGCGACGCCCTGCTCG3′	
H344AN346A_F	5′CTGAT CACATCGCTGAAGCT CGTCCGGGCGTGC3′	
H344AN346A_R	5′GCACGCCCGGACGAGCTTCAGCGATGTGCATCAG3′	

deletions were verified manually based on missing coverage regions in the genome. Common variants found in MG1655 stock cultures (Freddolino et al., 2012) were excluded from further analysis. All sequencing samples had an average mapped coverage of at least 25×.

Growth Rate Analysis for Serine Tolerance

Serine tolerance was studied using a 96 well microtiter plate reader (Biotek, Winooski VT). Single colonies were precultured in 2× YT media for overnight and were inoculated in 150 μL of M9 media supplemented with 0.2% glucose, 2 mM glycine and either 0, 12.5, 25, or 50 g/L serine. Growth was monitored in triplicates in a microtiter plate reader at 37°C with continuous shaking, and the log phase was used to estimate growth rate.

Serine Production by Fed-Batch Fermentation

Serine production was demonstrated during fed batch fermentation in 1 L fermenters (Sartorius, Gottingen, Germany). The media contained 2 g/L $MgSO_4{}^*7H_2O$, 2 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 7.5 g/L glucose, 2 g/L yeast extract, 0.6 g/L glycine, 0.12 g/L threonine, 4X trace elements, 50 mg/L spectinomycin and 25 mg/L chloramphenicol. For the *E. coli* Q1 strain, the initial glucose concentration was 10 g/L in order to enhance the growth before induction. Two hundred and fifty milliliters of feed contained 140 g/L glucose$^*H_2O$, 24 g/L $(NH_4)_2SO_4$, 2 g/L glycine, 2.5 g/L each of $MgSO_4{}^*7H_2O$ and KH_2PO_4, 1X trace elements, 40 μM IPTG, 50 mg/L spectinomycin, and 25 mg/L chloramphenicol.

A log phase culture was used to inoculate 500 mL media by 1:50 inoculum ratio. The cultures were allowed to grow over night in the fermenter at 37°C, 1,000 rpm stirrer speed under aerobic conditions assured by keeping DO concentrations above 30% by mixing pure oxygen to the gassing stream if required. The pH was controlled at 7.0 by addition of 2 M ammonia. Feed at the rate of 8 g/h was started after glucose concentration decreased below 250 mg/L. Production was induced at OD_{600nm} 7.0–8.5 by the addition of 40 μM IPTG, and the feed rate was reduced to 6 g/h. Samples were taken at regular intervals and were subjected to HPLC and LCMS analysis. The correlation between optical density (measured at 600 nM) to

cell dry weight (CDW) was determined to be 0.374 and was applied for CDW estimation.

Analytical Methods

Samples were filtered and subjected to HPLC for glucose and byproduct analysis using a method described previously (Kildegaard et al., 2014) with the only exception that the column temperature was kept at 30°C. Serine concentrations were determined using LCMS. The LC-MS/MS system consisted of a CTC autosampler module, a high pressure mixing pump and a column module (Advance, Bruker, Fremont, CA). The injection volume was 1 μl. The chromatography was performed on a ZIC-cHILIC column, 150 × 2.1 mm, 3 μm pore size, (SeQuant, Merck Millipore). In front of the separation column was a 0.5 μm depth filter (KrudKatcher Classic, phenomenex) and guard column ZIC-cHILIC, 20 × 2.1 mm(SeQuant, Merck). Eluent A contained 20 mM ammonium acetate pH adjusted to 3.5 with formic acid in milliQ water while Eluent B consisted of acetonitrile. The total flow rate of eluent A and B was 0.4 mL/min. The isocratic elution 35%, and the total run time was 5 min. Retention time was 2.8 min for serine. The MS–MS detection was performed on an EVOQ triple quadrupole instrument (Bruker, CA) equipped with an atmospheric pressure ionization (API) interface. The mass spectrometer was operated with electrospray in the positive ion mode (ESI+). The spray voltage was set to 4,500 V. The cone gas flow was 20 L/hr, and the cone temperature was set at 350°C. The heated probe gas flow was set at 50 L/hr with a temperature of 350°C. Nebulizer flow was set at 50 L/hr, and the exhaust gas was turned on. Argon was used as collision gas at a pressure of 1.5 mTorr. Detection was performed in multiple reacting monitoring (MRM) mode. The quantitative transition was 106 → 60, and the qualitative transition was 106 → 70. The collision energy was optimized to 7eV for both transitions. Calibration standards of serine were prepared in media used for serine production and diluted 50:50 with 0.2% formic acid in acetonitrile.

Results and Discussion

Deletion of *glyA* Enhances Serine Production

As a first step towards production of serine, the three L-serine deaminases (*sdaA*, *sdaB*, and *tdcG*) were deleted sequentially. Deletion of *sdaA* created a single deletion strain referred to as S1. A strain carrying deletions of all three deaminases was referred to as T1. Subsequent deletion of *glyA*, encoding serine hydroxymethyl transferase (SHMT), resulting in strain Q1, which has *sdaA*, *sdaB*, *tdcG*, and *glyA* deleted (Fig. 1).

Serine is synthesized by three enzymes: 3-phosphoglycerate dehydrogenase, encoded by *serA*, catalyzes the first step followed by catalysis of phoserine aminotransferase, encoded by *serC*, and finally dephosphorylation of phosphoserine by phosphatase, encoded by *serB*. *SerA* is known to be feedback regulated by serine in *E. coli*. The feedback inhibition was overcome by mutating three residues (344, 346, and 364) to alanine, as previously described (Al-Rabiee et al., 1996), resulting in the gene named *serA*mut. The three genes were overexpressed using a pET-based vectors (pCDFDuet-1-*serA*mut-*serC* and pACTCDuet-1-serB) as shown in Figure 1. The effect of IPTG induction was investigated for strain Q1 (DE3) transformed with the serine biosynthesis vectors. The highest production per cell dry weight (CDW) was achieved at an induction level of 40 and 60 μM (data not shown). An induction level of 40 μM was chosen for subsequent experiments since higher concentrations resulted in reduced growth.

In order to investigate the effect of deletion of the serine catabolic pathways on serine production, the single deletion strain (S1), triple deletion strain (T1), and quadruple deletion strain (Q1), each carrying the vectors required for serine biosynthesis, were studied in batch fermentations. The S1 strain showed the lowest serine production (0.49 from 2 g/L glucose), which was expected because these cells can still degrade serine. Strain T1 accumulated 0.53 g/L of serine in 24 h (Fig. 2A). The marginal difference in serine titer between the S1 and T1 strains indicates that deletion of the L-serine deaminases is not sufficient to prevent serine degradation. For the T1 strain, significantly reduced growth was observed after induction of the serine production pathway (Fig. 2B), which may be due to the toxic effect of serine (Zhang and Newman, 2008). The Q1 strain accumulated 0.89 g/L of serine, which is 1.8 and 1.7-fold higher than S1 and T1 respectively (Fig. 2A). The T1 strain had a higher glucose consumption rate (Fig. 2A) in spite of lower cell growth when compared to the Q1 strain (Fig. 2B). The specific production of the Q1 strain was 2.73 g/L/CDW, which is 3.4-fold higher when compared to strain S1 (0.73 g/L/CDW) and 1.3-fold higher when compared to strain T1 (1.94 g/L/CDW). Down regulation of *glyA* was also found to be important for enhancing serine production in *C. glutamicum* (Peters-Wendisch et al., 2005; Stolz et al., 2007). In the present experiments, the sensitivity towards serine would, however, render the strain unsuitable for fed batch fermentations. In order to overcome this, two approaches were followed to increase serine tolerance as described below.

Overexpression of Potential Serine Exporter Enhances Serine Tolerance

Efflux pumps are known to increase tolerance towards small molecules by exporting compounds to the external medium (Dunlop et al., 2011). Furthermore, overexpression of amino acid exporters has previously been shown to increase amino acid production (Burkovski and Kramer, 2002). *C. glutamicum* has a dedicated serine exporter, ThrE (Simic et al., 2001). When overexpressed in *E. coli*, it did not lead to increased tolerance, potentially due to lack of functional incorporation into the cell membrane as observed by western blotting using His-tag antibodies (data not shown). In *E. coli* no well-characterized serine exporters have so far been described. Since serine is structurally similar to cysteine, we hypothesized that cysteine transporters could have specificity towards serine. Ten different exporters in *E. coli* have previously been shown to have potential cysteine export function (Yamada et al., 2006). Since EamA is one of the best characterized cysteine exporters (Dassler et al., 2000), this transporter was first chosen for overexpression using the pCDF-1b vector. Growth of the Q1 strain with and without overexpression of the exporter was monitored at different serine concentrations. Overexpression of *eamA* resulted in tolerance of the Q1 strain towards 25 g/L of serine, whereas the empty vector control did not grow in the presence of 6.25 g/L of serine in the media

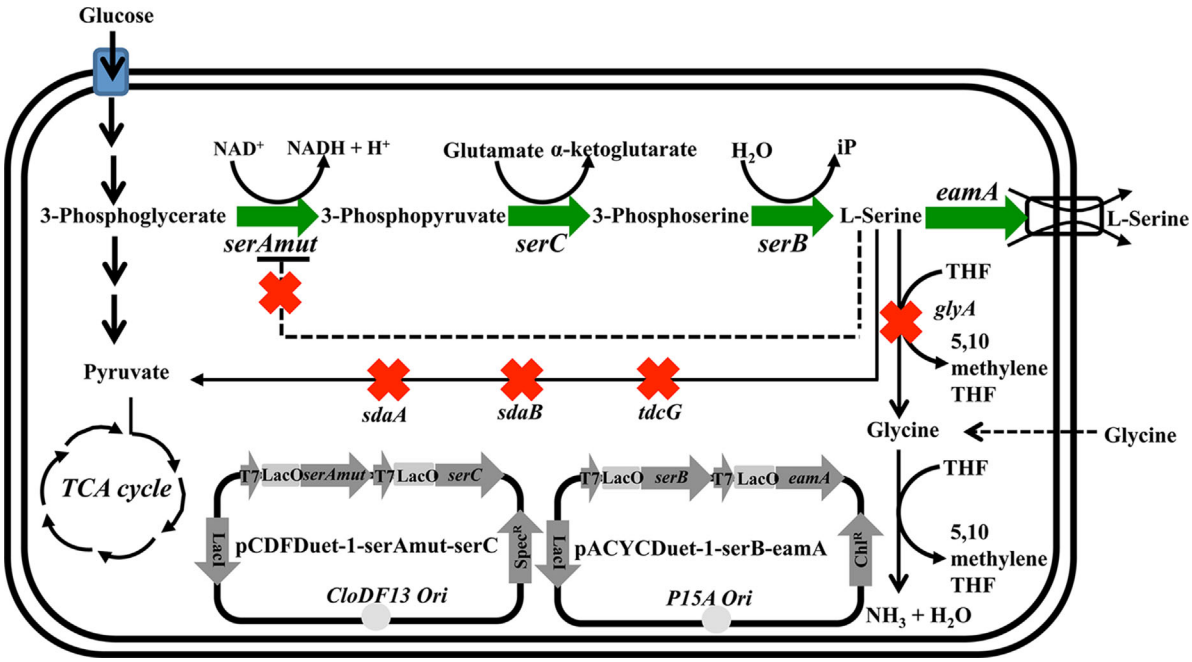

Figure 1. Schematic representation of metabolic pathways involved in the construction of a serine producing *E. coli* strain. SerA was rendered feed back resistant by mutating three amino acid at position 344, 346, and 364 to alanine, resulting in a variant is named serAmut. Potential serine exporter *eamA* demonstrated to exhibit tolerance when over expressed. Serine production was therefore carried out with and without over expression of *eamA*. Arrows highlighted in green indicate pathways genes upregulated (*serAmut, serB, serC,* and *eamA*) by overexpression using pET—Duet vectors. Red crosses on solid lines indicate genes that were deleted, while the cross on the dotted line indicates removal of feed back inhibition.

(Fig. 3A). This suggests that *eamA* may act as potential serine exporter in *E. coli*.

Evolution of Serine Tolerance Through Random Mutagenesis

In order to further increase the tolerance towards serine, the Q1 strain was evolved by random mutagenesis as described in the materials and methods section. The strain that exhibited the highest tolerance, termed Q3, was selected for further studies. The Q1 and Q3 strains were grown in M9 media with varying concentrations of L-serine (0, 12.5, and 50 g/L). As shown in Figure 3B, the Q1 strain showed negligible growth at 12.5 g/L of L-serine. The Q3 strain, on the other hand, showed significant and similar growth profile and growth rate (Fig. 3C) of around 0.12 h^{-1} at both 12.5 g/L and 25 g/L, and it showed negligible growth at concentrations of 50 g/L of serine. This demonstrates that the evolved strain Q3 had acquired significant tolerance when compared to the parental strain Q1. The wild type MG1655 showed a 1.8-fold higher growth rate when compared to the Q3 strain at 12.5 and 25 g/L serine (Fig. 3C). The tolerance is due to the efficient serine degradation pathways present in the wild type strain.

Genome sequencing revealed that the Q3 strain had only acquired a deletion of 2,855 bp comprising the first five bp of *rhtA*, a complete deletion of *ompX* and *opgE,* as well as deletion of a major part of the bifunctional RNA encoding for sRNA *rybA* and the regulatory protein *mntS* (Fig. 3D). The deletion resulted in the truncation of around 239 bp of *rybA* and 77 bp of *mntS*. There were no other point mutations observed in this strain (two different clones were genome sequenced), likely because of the short selection time used in the experiment. To study the effects of the observed mutations, single deletions of each gene (*ΔrhtA*, *ΔompX*, *ΔopgE*, *ΔrybA)* were constructed in the Q1 strain and their effect on serine tolerance was studied (Fig. 3C) as described below.

Deletion of *ompX* has previously been shown to increase osmotic tolerance of *E. coli* (Otto and Hermansson, 2004), and it is therefore speculated that this mutation could also result in increased tolerance towards serine. Studies of the *ompX* deletion confirmed this hypothesis, as the serine growth rate was found to be increased by more than 5-fold compared to Q1 at 12.5 g/L serine. At 25 g/L of serine, this mutation almost (0.7-fold) restored the growth rate observed for the Q3 strain.

Surprisingly, deletion of *rhtA* resulted in a 6.8-fold increased growth rate at 12.5 g/L serine. It is not clear why deletion of this exporter increases tolerance. Since *rhtA* is a threonine exporter (Livshits et al., 2003), the deletion of this gene could potentially help maintain a higher intracellular concentration of threonine. It is known that high concentrations of serine results in the inhibition of branched amino acid biosynthesis in *E. coli*, which can be partly alleviated by the addition of threonine to the media (Hama et al., 1990). The other deleted gene, *opgE*, is reported to have phosphoethanolamine transferase activity on osmoregulated periplasmic glucans (Bontemps-Gallo et al., 2013), and deletion of this gene may potentially enhance osmotolerance of the cell. The *opgE* deletion strain showed around 3.7-fold higher growth rate than Q1 at 12.5 g/L serine. Furthermore it grew even at 25 g/L serine.

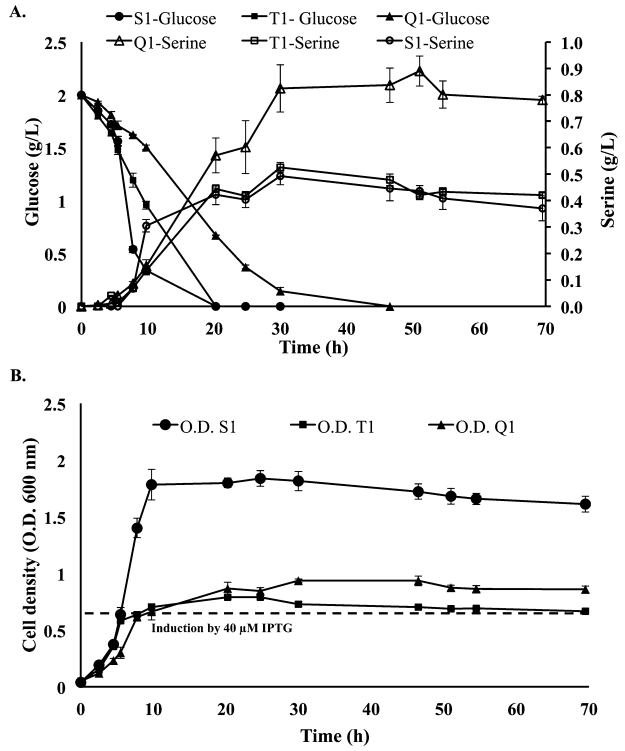

Figure 2. Comparison of serine production in S1, Q1, and T1 in batch fermentations (**A**) Profiles of serine production and glucose consumption for strains S1, T1, and Q1. (**B**) Profiles of cell density for the strains S1, T1, and Q1. Error bars represent deviations from triplicate experiments.

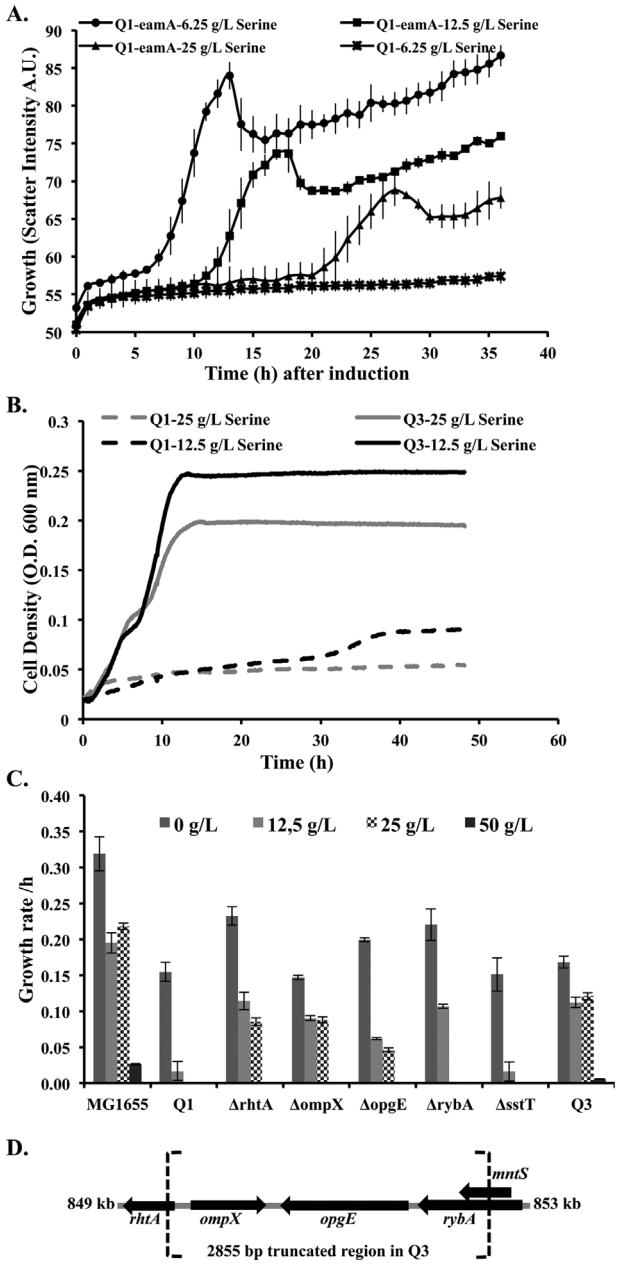

Figure 3. Improvement of serine tolerance by random mutagenesis and expression of exporters. (**A**) Growth profile of Q1 with or without overexpression of *eamA* in M9 medium supplemented with 0.2% glucose containing different concentration of serine. (**B**) Growth profile of Q1 and Q3 in M9 media containing 0.2% glucose, 2 mM glycine and supplemented with 12.5 or 25 g/L serine. (**C**) Growth rate of *E. coli* MG1655, Q1, Q3, and single deletions of genes observed in M9 medium containing 0.2% glucose and 2 mM glycine (except MG1655 wt) and different concentrations of serine. Error bars denote deviations observed in triplicates. (**D**) Overview of the truncated region identified by genome sequencing of the Q3 strain.

Downstream of *opgE* is a bi-functional RNA encoding a sRNA, *rybA*, and a 42 amino acid small regulatory protein, *mntS* (Wassarman et al., 2001; Waters et al., 2011). The growth rate of the *rybA* deletion mutant was found to be 6.37-fold higher than Q1 at 12.5 g/L. The sRNA, *rybA*, negatively regulates aromatic amino acid production under stress conditions (Gerstle et al., 2012). Apart from branched chain amino acid synthesis, serine has also been reported to inhibit aromatic amino acid biosynthesis (Tazuya-Murayama et al., 2006) by an unknown mechanism. If *rybA* is involved in this inhibition, then the deletion of this gene could potentially lead to increased tolerance towards serine.

In addition to the previously mentioned deletions, a serine importer encoded by *sstT* (Kim et al., 2002) was also deleted in order to investigate if blocking or diminishing of serine import could enhance tolerance. As shown in the Figure 3C, the deletion of *sstT* was found not to have any effect on serine tolerance when compared to the parental strain Q1.

None of the above single deletions could match the tolerance of the Q3, which shows 6.7-fold higher growth rate than Q1 at 12.5 g/L serine and around 1.4-fold higher growth rate than *ΔrhtA* at 25 g/L serine. Furthermore the Q3 strain shows marginal growth even at 50 g/L serine. It is possible that the deletion of the above mentioned genes may show positive epistasis towards serine tolerance, since the potential tolerance mechanisms are different. The Q3 strain was therefore used for further studies of serine production during fed batch fermentation. Surprisingly, the mutations were not related to factors that have previously been shown to be involved in serine toxicity. These include the inhibition of branched amino acid biosynthesis resulting from inhibition of the aspartate kinase/homoserine dehydrogenase (*thrA*) (Hama et al., 1990).

Serine Tolerance Results in Increased Serine Production During Fed Batch Fermentation

Based on the experiments described above, we decided to test the production of serine during fed batch fermentation using the Q1 and Q3 strains. In order to test the effect of the potential serine exporter, *eamA*, on serine production during fed batch fermentation, the *eamA* gene was cloned into the second site of pACYCDuet-1 vector, resulting in the overexpression of *serAmut*, *serB*, *serC*, and *eamA*. The plasmids, pACYCDuet-1-*serB* or pACYCDuet-1-*serB*-*eamA*-c-His, were transformed into the Q1 and Q3 strains containing the pCDFDuet-1-*serA*mut-*serC*, and at least two independent fermentations were carried out for each strain.

For the Q1 strain, upregulation of *eamA* increased the productivity in the initial phase of fermentation (Fig. 4A), but later during the fermentation a severe decline in cell dry weight was observed, which lead to decreased serine production after 25 h induction, reaching 6.69 g/L. It is likely that this may be caused by accumulation of serine in the media as well as the additional burden on the cell of overexpressing the membrane protein *eamA*. Overexpression of membrane proteins has previously been shown to result in increased stress response (Wagner et al., 2006). Q1 without overexpression of *eamA* showed lower decline in cell density during the fermentation and continued to produce serine for a longer period of time, reaching a final average titer of 8.3 g/L (Fig. 4B and Table III). The yield of Q1 without overexpression of *eamA* was 0.44 g/g glucose consumed (as observed in batch fermentations) compared to 0.26 g/g for Q1 overexpressing *eamA*, indicating that overexpression of *eamA* in the Q1 background leads to a higher maintenance energy.

Upregulation of *eamA* showed a positive effect on serine production in the Q3 background as opposed to what was observed for the Q1 strain background. Q3 with upregulated *eamA* showed significantly higher productivity during the initial phases of fermentation when compared to Q3 without *eamA* upregulation (Fig. 4B). However the burden of overexpression of *eamA* was also observed in Q3 after 20 h of induction as the cell density and serine production decreased with time. The Q3 strain without overexpression of *eamA*, on the other hand, showed increasing cell density with time. The serine production yield of Q3 overexpressing *eamA* was 0.43 g/g, and the maximum serine titer observed was 12.6 g/L compared to 8.3 g/L of Q1 strain. Q3 without overexpression of *eamA* reached maximum titer of 10.1 g/L but consumed more glucose, thereby resulting in a yield of 0.33 g/g glucose. It is likely that the increased growth of the more tolerant Q3 strain results in a lower overall production yield. Deviations in serine production were only observed in the last stage of the fermentations, where serine production was found to be either increased or marginally reduced. Careful balancing of the growth is therefore important in order to reach the highest possible yield. The achieved yield is significantly higher than what has previously been obtained, including a recently isolated and engineered *C. glutamicum* variant, which showed 21% mass yield from sucrose (Zhu et al., 2015).

Using the selected feeding profile, only negligible amounts of acetate, succinate, α-ketoglutarate, and pyruvate accumulated during the fermentation. This is opposite from other recent studies (Gu et al., 2014), and it may contribute to the higher serine yield obtained in the present experiments. Toxicity of serine has previously been attributed to the by-production of hydroxypyruvate through the serine pathway. The hydroxypyruvate levels were found to be in the range of 0.3–0.4 g/L regardless of serine concentration during the fermentation. The observed hydroxypyruvate may originate from the phosphatase activity of YeaB that converts phosphohydroxypyruvate (the product of SerA) to hydroxypyruvate, which is then channeled to pyridoxal phosphate synthesis by one of the serendipitous PLP pathways (Kim et al., 2010). We therefore decided to delete *yeaB* from the Q3 strain, however, this mutant showed significantly reduced growth rate and serine production in batch fermentation studies (data not shown). An additional byproduct that accumulates to significant amounts (4–6 g/L) was α-hydroxyglutarate. We speculate that this could be the result of a promiscuous activity of *serA*, which has previously been reported to result in the reduction of α-ketoglutarate to α-hydroxyglutarate (Zhao and Winkler, 1996). The toxicity of α-hydroxyglutarate has not been studied, however, it does drain the glutamate precursor (α-ketoglutarate) from the cell. Since

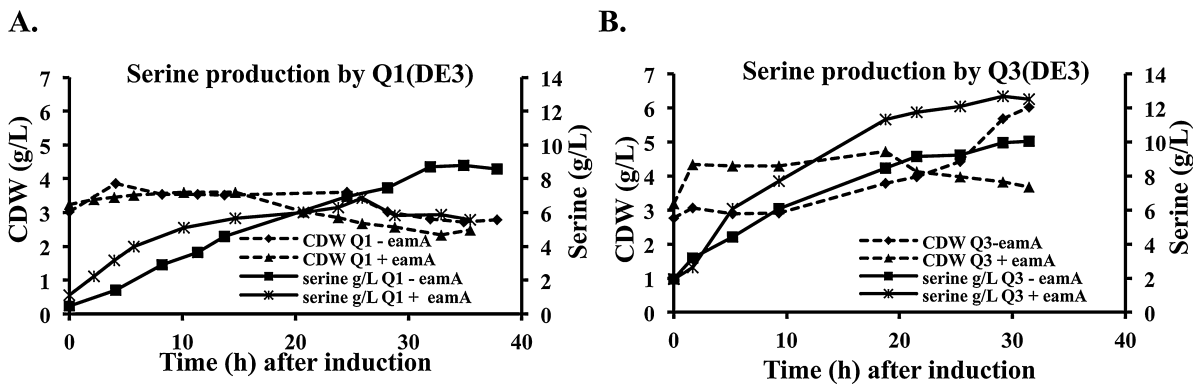

Figure 4. Serine production by fed batch fermentation. Serine production of the strains Q1 and Q3 was investigated during fed batch fermentation with or without parallel upregulation of the exporter encoded by *eamA*. Serine production and growth are shown for the strains Q1 (**A**) and Q3 (**B**).

Table III. Titer and yields of serine with respect to glucose consumed in gram (Yg/g), mole (Ymol/mol), and Cmol (YCmol/Cmol) along with cell density measured as cell dry weight per gram of glucose (Yx/s g/g). Serine productivity is given in g/L/h. The data was obtained from fed batch fermentations of Q3 and Q1 by upregulating the serine synthesis pathway with or without overexpression of potential serine exporter *eamA*.

	Q1 pCDF-Duet-*serA*mut-*serC* pACYCDuet-1-*serB*-		Q3 pCDF-Duet-*serA*mut-*serC* pACYCDuet-1-*serB*-	
Parameters	+ *eamA*	-*eamA*	+ *eamA*	-*eamA*
$Y_{g/g}$	0.26 ± 0.01	0.44 ± 0.01	0.43 ± 0.05	0.33 ± 0.01
$Y_{mol/mol}$	0.45 ± 0.01	0.75 ± 0.02	0.74 ± 0.08	0.56 ± 0.02
$Y_{Cmol/Cmol}$	0.22 ± 0.01	0.38 ± 0.01	0.37 ± 0.04	0.28 ± 0.01
$Yx/s_{g/g}$	0.1 ± 0	0.12 ± 0.03	0.15 ± 0.05	0.18 ± 0.02
Serine in g/L	6.69 ± 0.23	8.3 ± 0.71	11.73 ± 1.32	9.08 ± 1.31
Prod. (g/L/h)	0.13 ± 0	0.18 ± 0.01	0.24 ± 0.03	0.18 ± 0.01

glutamate is required as an amino group donor, it is possible that further balancing of the serine production pathway may be able to reduce this activity and enhance serine production.

Conclusion

Production of serine by direct fermentation of glucose has the potential to become an industrially attractive process. In this study we have shown that deletion of the genes required for conversion of serine to both glycine and pyruvate is highly important for increasing serine production and yield. This, however, renders the production strain sensitive to even low concentrations of serine. Tolerance towards serine was therefore achieved by adaptive evolution as well as by over-expression of an exporter, encoded by eamA. Genome sequencing revealed the deletion of a number of genes, *rhtA*, *ompX*, *opgE*, *rybA*, *mntS*, which each contributes to serine tolerance. Tolerance towards serine was particularly important for increasing serine titer and yield during fed batch fermentation, where a serine titer of 11.7 g/L with a mass yield from glucose of 43% was achieved, representing the highest yield so far demonstrated in any production organism. The described fermentation based process provides an important step towards industrial production of serine directly from glucose. Although the identified mutations significantly increase tolerance towards serine, it remains a challenge that needs to be addressed more systematically.

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