Metabolic Engineering of *Corynebacterium glutamicum* for L-Serine Production

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Although L-serine proceeds in just three steps from the glycolytic intermediate 3-phosphoglycerate, and as much as 8% of the carbon assimilated from glucose is directed via L-serine formation, previous attempts to obtain a strain producing L-serine from glucose have not been successful. We functionally identified the genes *serC* and *serB* from *Corynebacterium glutamicum*, coding for phosphoserine aminotransferase and phosphoserine phosphatase, respectively. The overexpression of these genes, together with the third biosynthetic *serA* gene, *serA*197, encoding an L-serine-insensitive 3-phosphoglycerate dehydrogenase, yielded only traces of L-serine, as did the overexpression of these genes in a strain with the L-serine dehydratase gene *sdaA* deleted. However, reduced expression of the serine hydroxymethyltransferase gene *glyA*, in combination with the overexpression of *serA*197, *serC*, and *serB*, resulted in a transient accumulation of up to 16 mM L-serine in the culture medium. When *sdaA* was also deleted, the resulting strain, *C. glutamicum* ΔsdaA::pK18mobglyA*” (pec-T18mob2serA197CB), accumulated up to 86 mM L-serine with a maximal specific productivity of 1.2 nmol h⁻¹ g⁻¹ (dry weight)⁻¹. This illustrates a high rate of L-serine formation and also utilization in the *C. glutamicum* wild type. Therefore, metabolic engineering of L-serine production from glucose can be achieved only by addressing the apparent key position of this amino acid in the central metabolism.

The demand of L-serine is about 300 tons per year, and this amino acid is required for the pharmaceutical and the cosmetic industries, in addition to being a building block for chemical and biochemical purposes (6). The current production relies mainly on its enzymatic or cellular conversion from the precursor glycine plus a C1 compound. Utilizing the condensing activity of serine hydroxymethyltransferase, an enzymatic system has been elaborated to convert glycine plus formaldehyde to L-serine (15). The cellular systems employed, among others, restoing cells of methanol-utilizing bacteria such as *Hyphomicrobium methylovorum* where L-serine accumulation from glycine plus methanol was achieved (16). Also, a fermentative production of L-serine from glycine alone by *Corynebacterium glycophilum* was described (19). However, there is not much information on the direct fermentative production of L-serine from glucose.

Attempts to isolate L-serine-producing strains using different bacteria by applying undirected mutagenesis yielded mutants accumulating only traces of L-serine (38). Apparently, the direct conversion of glucose is a demanding challenge, probably due to the role of L-serine as a central intermediate for a number of cellular reactions (Fig. 1).

We are interested in the amino acid-synthesizing capabilities of *Corynebacterium glutamicum*, which is traditionally used for the large-scale production of L-glutamate and L-lysine (9). In general, the efforts to engineer producing strains were focused on the enzymes of the biosynthesis pathways. For instance, considerable formation of L-lysine resulted in the deregulation of the key enzyme aspartate kinase (4). Using similar approaches, *C. glutamicum* strains were developed by overproducing L-isoleucine, L-valine, L-threonine, or D-pantothenate (8, 13). Besides the supply of precursors (29, 31) or reducing power (23), export of amino acids was also found to be relevant (7). Another focus of strain development was degradation. For instance, production of L-threonine with *C. glutamicum* could be increased by decreasing its intracellular degradation (33) and in fact, one of the reasons for the success of L-lysine formation with *C. glutamicum* was its inability to degrade the product L-lysine. Thus, for the production of specific amino acids, a number of cellular reactions have to be considered in the ensemble and not just the biosynthesis pathway alone.

Since there is not yet a convincing strain for L-serine production from glucose, we met the challenge to engineer *C. glutamicum* for this purpose. In *C. glutamicum* as in other bacteria, L-serine is synthesized via phosphorylated intermediates starting with the glycolytic intermediate 3-phosphoglycerate, which is oxidized to phosphohydroxypyruvate. Subsequent transamination leads to the formation of phosphoserine, which is dephosphorylated to yield L-serine (Fig. 1). We have previously studied 3-phosphoglycerate dehydrogenase (PGDH; *serA*) from *C. glutamicum* catalyzing the initial reaction of the three-step pathway of L-serine biosynthesis (30). As a result of deleting the 197 carboxy-terminal amino acids of the SerA polypeptide, PGDH activity is no longer inhibited by L-serine (30). *C. glutamicum* possesses a high capacity to degrade L-serine in the presence of glucose, and we could demonstrate that *sdaA*-encoded L-serine dehydratase is involved in L-serine degradation (24). Based on these studies, we describe here the construction of an L-serine-producing strain from *C. glutamicum* by metabolic engineering.

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TABLE 1. Strains and plasmids used

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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</thead>
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<td>DH5α MCR</td>
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<tr>
<td>GM2929</td>
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<td><em>C. glutamicum</em> strains</td>
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<tr>
<td>ATCC 14752</td>
<td>Requires biotin</td>
<td>ATCC 8739</td>
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<td>WT carrying a deletion in the <em>sdaA</em> gene</td>
<td>This work</td>
</tr>
<tr>
<td>WT:pK18mobglyA’</td>
<td>WT with <em>glyA</em> under control of tac promoter</td>
<td>This work</td>
</tr>
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<td>Plasmids</td>
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<td>Donor of Tn5351 (IS1207, Km’), Ap’/rV’</td>
<td>U53587’</td>
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<td>Cloning vector; Ap’</td>
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<td>pUC18serB</td>
<td>pUC18 with 1.8-kb PCR product containing <em>serB</em></td>
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<td>pUC18serCB</td>
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<td><em>E. coli</em>-C. glutamicum shuttle vector, Tet’</td>
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<td>pEC-T18mob2serCB</td>
<td>pEC-T18mob2 with 3.6-kb EcoRI-XbaI fragment containing <em>serC</em> and <em>serB</em> from pUC18serCB</td>
<td>This work</td>
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<td>pEC-T18mob2 with 3.6-kb EcoRI-XbaI fragment containing <em>serA</em> and <em>serB</em> from pUC18serCB</td>
<td>This work</td>
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<tr>
<td>pK18mobglyA’</td>
<td>Mobilizable vector, nonreplcative in <em>C. glutamicum</em>, Km’, containing lacI8 and P<em>tc</em> fused to 5’-terminal fragment of <em>glyA</em></td>
<td>32</td>
</tr>
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*a* Km’, kanamycin resistance; Ap’, ampicillin resistance; Cm’, chloramphenicol resistance.

*b* ATCC, American Type Culture Collection.

*GenBank accession number.*
PCR using the upstream primers serC-upper (5′-GACCAACACGCCAACCAG 3′; the nucleotide (nt) corresponding to nt 877628 of NC003450 is underlined) and serB-lower (5′-ACCCGATCCGCTCCTTGGGTATG 3′; the nucleotide corresponding to nt 877628 of NC003450 is underlined), respectively, and the respective reverse primers serC-lower (5′-GAGGCTCCTTGCTATGGGTTA 3′) and serB-upper (5′-AACCGATCCGCTCCTTGGGTATG 3′; the nucleotide corresponding to nt 877628 of NC003450 is underlined). Boldfaced nucleotides correspond to the introduction of a NotI restriction site. The PCR fragments were blunted and cloned into the Smal site of pUC18. The obtained plasmids, pUC18serC and pUC18serB, were digested with SacI and NotI, and the inserts containing serC and serB were isolated and ligated together, resulting in plasmid pUC18serCB. The plasmid was digested with EcoRI and XbaI, and the serCB-containing insert obtained was ligated into EcoRI- and XbaI-treated pEC-T18mob2, resulting in plasmid pEC-T18mob2serCB.

To construct pEC-T18mob2serAA197mob2 plasmid pUC18serA4197 (30) was digested with EcoRI and BamHI and the serA4197-containing insert obtained was blunted and ligated in EcoRI-linearized and blunted plasmid pEC-T18serCB.

In order to place the glyA gene in the chromosome of C. glutamicum under the control of the IPTG-inducible sac promoter, the respective strands were transformed via electroporation with the nonreplicative plasmid pK18mobglyA (17) and the respective strains were transduced with the respective glyA-containing plasmid pK18mobglyA to kanamycin resistance. Selection for kanamycin resistance was performed in the presence of 100 μg/mL kanamycin.

The reaction was stopped after 5 and 10 min by adding 10 μL of 0.2 M EDTA and placing it on ice. The amount of Pi released was determined with an EnzChek phosphate assay kit (Molecular Probes) as described previously. 3-Phosphoglycerate dehydrogenase activity was determined spectrophotometrically as previously described (32). The reaction was performed in 20 mM Tris-HCl (pH 7.5), 1 mM MgCl2, and 5 mM phosphoserine.

Enzyme assays. Phosphoserine phosphatase activity was analyzed as previously described (3) by the determination of inorganic phosphate (P_i) released from phosphoserine. Assays were performed discontinuously in mixtures (100 μL) containing 20 mM Tris-HCl (pH 7.5), 1 mM MgCl2, and 5 mM phosphoserine. The reaction was stopped after 5 and 10 min by adding 10 μL of 0.2 M EDTA and placing it on ice. The amount of P_i released was determined with an EnzChek phosphate assay kit (Molecular Probes) as described previously.

Serine hydroxymethyltransferase was assayed discontinuously by the quantification of glycine formed from serine and 5,10-methylene tetrahydrofolate via high-performance liquid chromatography as previously described (33).

RESULTS

Identification of the genes coding for phosphoserine aminotransferase and phosphoserine phosphatase. While the serA gene was known (30), the aim was to identify the complete L-serine biosynthetic pathway in C. glutamicum. Therefore, we used a recently established transposon mutant bank of C. glutamicum ATCC 14752 with Tn5531 to screen for L-serine auxotrophs (18). Three clones unable to grow on minimal medium with 220 mM glucose as the carbon source and the L-serine auxotrophy of the transposon mutants identified the genes as serC and serB from C. glutamicum. Additionally, we constructed plasmid pEC-T18mob2serCB carrying both genes. With this moderate-copy-number plasmid, the PSP activity was increased threefold, from 110 nmol min⁻¹ mg (protein)⁻¹ in the wild type (WT) to 320 nmol min⁻¹ mg (protein)⁻¹ in strain WT (pEC-T18mob2serCB). Furthermore, we demonstrated that this plasmid complemented the serC and serB transposon mutants as expected (not shown).

Influence of the overexpression of the L-serine biosynthesis genes on L-serine accumulation. We previously showed that truncation of serA from C. glutamicum by 197 amino acids at its C terminus (encoded by the serA4197 allele) provided a 3-phosphoglycerate dehydrogenase devoid of feedback inhibition by L-serine (30). Here, we studied whether overexpression of serA or serA4197 is sufficient to increase L-serine accumulation in the WT. Therefore, the strains WT(pZ1serA) (pEC-T18mob2) and WT(pZ1serA4197) (pEC-T18mob2) were grown in minimal medium with 220 mM glucose as the carbon source and the L-serine concentration in the culture medium was determined (Table 2). However, neither the overexpression of mutant serA4197 nor the WT allele yielded significant L-serine concentrations. Enzyme assays confirmed, for WT(pZ1serA) (pEC-T18mob2), a specific PGDH activity of 700 nmol (min mg⁻¹)⁻¹ and for WT(pZ1serA4197) (pEC-T18mob2), a specific activity of 690 nmol (min mg⁻¹)⁻¹ equivalent to an 8- to 10-fold overexpression compared to the WT (30).

In order to test whether the additional expression of serC and serB or even their expression alone resulted in L-serine accumulation, the respective strains WT(pZ1) (pEC-T18mob2serCB), WT(pZ1serA) (pEC-T18mob2serCB), and WT(pZ1serA4197) (pEC-T18mob2serCB) were constructed. Surprisingly, also with these strains, no substantial L-serine accumulation occurred (Table 2).

Influence of deletion of sdaA on L-serine accumulation. Based on the result that the overexpression of the serine biosynthetic genes is not sufficient for L-serine production and our previous observation of a significant contribution of sdaA-encoded L-serine dehydratase (L-SerDH) to L-serine degradation in C. glutamicum (24), we used the wild-type derivative containing the sdaA deletion (WTΔsdaA) to assay for the influence of the overexpression of the L-serine biosynthetic genes serA, serB, and serC.
three genes were cloned into vector pEC-T18mob2 (35) to
accumulate 0.08 mM L-serine after 28 h of cultivation and
<0.05 mM after 54 h, showing that at the early time point, sdaA deletion alone resulted in traces of L-serine. C. glutamicum WT::pZ1serA(pEC-T18mob2serA) accumulated 0.09 mM L-serine after 24 h and up to 0.15 mM after 54 h, showing a slight increase in L-serine accumulation compared to the control. However, with strain WT::sdaA(pZ1serA) (pEC-T18mob2serCB), 0.44 mM (28 h) and 0.14 mM (54 h) L-serine concentrations were determined. This comparison shows an advantage of the sdaA gene deletion over sdaA but that, despite the deletion of sdaA, degradation of L-serine is still occurring.

Influence of reduced serine hydroxymethyltransferase activity on L-serine accumulation. In growing C. glutamicum, only 16% of the L-serine synthesized is incorporated into protein (21) whereas the remainder is cleaved by serine hydroxymethyltransferase (SHMT; glyA) to provide 5,10-methylene tetrahydrofolate and glycine. A reduced activity of SHMT was already shown to be favorable for L-threonine production with C. glutamicum due to an L-threonine-degrading side activity of the enzyme. Since the glyA gene could not be deleted or disrupted in C. glutamicum, even when supplemented with glycine (33), plasmid pK18mobglyA’ was employed to reduce the SHMT activity by replacing the native glyA promoter with the IPTG-inducible tac promoter (33). We used strain WT::pK18mobglyA’ to analyze the influence of a reduced SHMT activity on L-serine production. In the first experiments, this strain already accumulated up to 1 mM L-serine (not shown), illustrating the principal importance of SHMT reduction for L-serine accumulation. In order to overexpress sdaA together with serC and serB in the kanamycin-resistant strain WT::pK18mobglyA’, all three genes were cloned into vector pEC-T18mob2 (35) to generate pEC-T18serA (see Materials and Methods). Using this tetracycline resistance-conferring plasmid, strain WT::pK18mobglyA’ (pEC-T18serA) was generated.

This strain was cultivated with or without 100 μM IPTG in minimal medium CGXII containing 220 mM glucose as the carbon source. Whereas in the presence of IPTG, the SHMT activity was 40 nmol min⁻¹ mg (protein)⁻¹, it was 10 nmol min⁻¹ mg (protein)⁻¹ without IPTG, confirming the successful application of pK18mobglyA’. With IPTG, L-serine accumulation was below 1 mM (not shown), but in the absence of the inducer, up to 16 mM L-serine accumulated (Fig. 2). Rate calculations showed that constant specific productivities of about 0.4 nmol h⁻¹ g (dry weight)⁻¹ occurred within 8 to 20 h of the cultivation. However, almost all L-serine was degraded again, which is consistent with our prior finding of the strong utilization of externally added L-serine by C. glutamicum unless sdaA (glyA) was deleted (24).

Influence of combining the deletion of sdaA with reduced SHMT activity on growth and L-serine accumulation. We used plasmid pK18mobglyA’ to exchange the native glyA promoter with the tac promoter in strain WT::sdaA as well as plasmid pEC-T18mob2serA to overexpress the L-serine biosynthesis genes. The resulting strain, WT::sdaA::pK18mobglyA’ (pEC-T18mob2serA), was cultivated on CGXII medium with 220 mM glucose as the carbon source. In order to reduce glyA expression, IPTG was omitted. Enzyme activity determinations confirmed the expected low SHMT activity (not shown). A typical cultivation profile is shown in Fig. 3. L-Serine accumulated up to about 86 mM in the culture medium, with a maximum specific productivity of 1.2 mmol h⁻¹ g (dry weight)⁻¹ at about 20 h and a molar yield (Y/S) of 0.64 mol/mol. This confirmed the positive effect of sdaA deletion, which was also visible, although at a drastically reduced level when just the L-serine biosynthesis genes were overexpressed, without reducing SHMT activity (see above). Despite the efficient L-serine production with strain WT::sdaA::pK18mob glyA’ (pEC-T18mob2serA), there was still significant degradation of L-serine at later time points (Fig. 3). A comparison of the growth rates of the different genetically modified strains with that of the wild type revealed that overexpression of the genes serA, serC, and serB alone or in combination with a deletion of the sdaA gene resulted in a decreased growth rate (Table 3). The largest reduction of growth rate was ob-

FIG. 2. Growth (■), glucose (□) and L-serine (●) concentrations in the medium, and specific L-serine productivity (△) of strain WT::pK18mobglyA’ (pEC-T18mob2serA) on minimal medium with 220 mM glucose. OD₆₀₀, optical density at 600 nm; dw, dry weight.

FIG. 3. Growth (■), glucose (□) and L-serine (●) concentrations in the medium, and specific L-serine productivity (△) of strain WT::sdaA::pK18mobglyA’ (pEC-T18mob2serA) on minimal medium with 220 mM glucose. OD₆₀₀, optical density at 600 nm; dw, dry weight.
served as a consequence of reduced glyA expression. The strains WT::pK18mobglyA' (pEC-T18mob2serA^197CB) and WTΔsdaA::pK18mobglyA' (pEC-T18mob2serA^197CB) exhibited three- to fourfold-decreased growth rates under production conditions compared to the wild type and a twofold rate compared to the respective strains without reduced glyA expression (Table 3). This corroborates our previous finding that a reduced SHMT activity correlates with a reduced growth rate (33).

**DISCUSSION**

Our functional studies identified the PSAT (serC) and PSP (serB) of *Corynebacterium glutamicum*. Although there are two further open reading frames (NCgl0400, NCgl0294) annotated as PSP in the genome of *C. glutamicum*, only NCgl2436 encodes a functional PSP. PSP of *C. glutamicum* contains 433 amino acyl residues, and its N terminus is extended by 93, 198, and 190 residues compared to the PSP proteins from *E. coli*, Methanothermus jannaschii, and humans, respectively (25, 28, 37). Notably, the additional N-terminal segment of *C. glutamicum* PSP includes a domain with similarity to an “ACT domain” that has been found in a number of proteins, including PGDH of *E. coli*, Mycoplasmatales, and *C. glutamicum* (2, 5, 30). This domain is proposed to represent a conserved regulatory ligand binding fold, but experimental evidence for PSP is absent. The N-terminal extension as characteristic for the *C. glutamicum* PSP is also present in that of *M. tuberculosis* (Rv3042c) and in PSPs of other *Actinomycetales*. On the other hand, PSAT of *C. glutamicum* has high identity (61%) to the protein of *M. tuberculosis* (Rv0884c) but only reduced identity (20%) to that of *E. coli* (14). The structural differences between PSAT from *C. glutamicum* and *E. coli* might be due to a second enzymatic function which is indicated by the atypical PGDH (serA) inhibition of *C. glutamicum* (30) and the facts that the equilibrium of the PGDH-catalyzed reaction is on the substrate side (34) and an ACT domain is present in PSP (serB).

Nevertheless, reduction of the glyA-encoded SHMT activity had clearly the major impact on L-serine accumulation. Already, the reduction of glyA expression alone resulted in an approximate 1 mM accumulation of L-serine (not shown), which was not the case upon sdaA deletion. The importance of reduced SHMT activity is also evident when comparing the maximal fluxes rates (Table 3). With SHMT reduction and overexpression of the biosynthesis pathway genes, the growth rate was 0.11 h^-1. The calculated flux over the pathway to satisfy the need for cellular synthesis, like phospholipid synthesis, of C1 generation under these conditions, is 2.75 nmol min^-1 mg (dry weight)^-1 (22). Taking a maximal L-serine excretion rate of 10 nmol min^-1 mg (dry weight)^-1 into account (Fig. 2), a total L-serine flux of 12.75 nmol min^-1 mg (dry weight)^-1 resulted (Table 3). Importantly, the comparison of this strain with the WT and WTDsdaA (pEC-T18mob2 serA^197CB) illustrates that reducing the L-serine degradation to glycine and C1 units favors an increased total L-serine flux, indicative of a stimulation of the L-serine synthesis probably due to a reduced availability of glycine and C1 units (Table 3). The strong increase in L-serine flux by 11.02 to 23.75 nmol min^-1 mg (dry weight)^-1 due to the additional sdaA deletion.
in WTDsdaA::pKl8mobg/h4’ (pPEC-T18mob2ser4 aktivCB) is largely in agreement with the difference in the l-serine degradation rates observed for the wild type and its sdaA deletion mutant with externally added l-serine where the sdaA deletion caused a decrease in l-serine degradation by 7.7 nmol min⁻¹ mg (dry weight)⁻¹ (24). This work demonstrates that engineering l-serine production from glucose requires considering the position of l-serine in metabolism instead of considering l-serine as an end product of a biosynthetic pathway.

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