APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2005, p. 7139–7144 Vol. 71, No. 11
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.11.7139–7144.2005

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

Petra Peters-Wendisch,* Michael Stolz, Helga Etterich, Nicole Kennerknecht, Hermann Sahm, and Lothar Eggeling

Institut für Biotechnologie 1, Forschungszentrum Jülich, D-52425 Jülich, Germany

Received 12 April 2005/Accepted 16 June 2005

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, $serA^{\Delta 197}$, 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 $serA^{\Delta 197}$, *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* Δ*sdaA*::pK18mob*glyA′* (pEC-T18mob2$serA^{\Delta 197}CB$), accumulated up to 86 mM L-serine with a maximal specific productivity of 1.2 mmol h^{-1} g (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 C_1 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, resting 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 glycinophilum* 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.

* Corresponding author. Mailing address: Institut für Biotechnologie 1, Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany. Phone: 49 2461 61 5430. Fax: 49 2461 61 2710. E-mail: p.wendisch@fz-juelich.de.

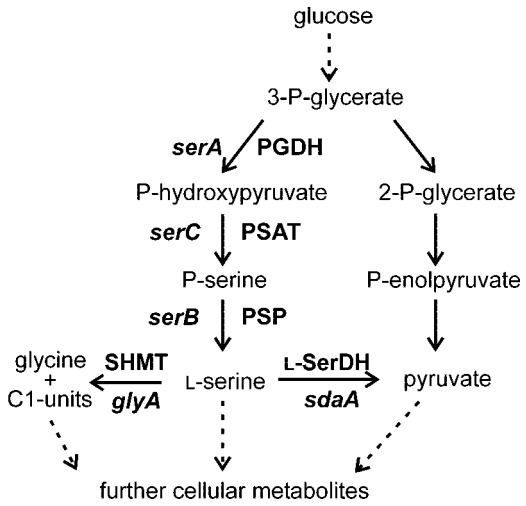

FIG. 1. Scheme of L-serine biosynthesis and its metabolism in *C. glutamicum* during growth on glucose. Dotted arrows represent pathways consisting of more than one reaction. Genes are given in italics.

MATERIALS AND METHODS

Bacteria and plasmids. Bacterial strains and plasmids used in this work are listed in Table 1.

Growth conditions. Luria-Bertani medium (LB) was used as the standard medium for *Escherichia coli*, while brain heart infusion medium (BHI; Difco) was used as complex medium for *C. glutamicum*. As minimal medium, CGXII was used (17), but including 30 mg protocatechuic acid l^{-1} and 220 mM glucose as a carbon source. When appropriate, *E. coli* strains were cultured with carbenicillin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}), or tetracycline (5 μg ml^{-1}) and *C. glutamicum* strains with kanamycin (25 μg ml^{-1}) or tetracycline (5 μg ml^{-1}). A reduced concentration of kanamycin (15 μg ml^{-1}) or tetracycline (4 μg ml^{-1}), respectively, was used to obtain transformants of *C. glutamicum*. *E. coli* was grown at 37°C and *C. glutamicum* at 30°C in 50 ml medium in 500 ml baffled shake flasks and 120 rpm agitation. Strains harboring the chromosomally integrated plasmid pK18mob*glyA′* were cultivated in the presence of 100 μM isopropyl-β-thiogalactoside (IPTG). For L-serine production experiments, these strains were cultivated without IPTG.

Isolation of serine-auxotrophic mutants and localization of transposon insertion sites. The transposon (Tn) delivery vector pCGL0040 was isolated from *E. coli* GM2929 grown in the presence of 50 μg of kanamycin and 20 μg of chloramphenicol ml^{-1} (1). The plasmid was used to transform *C. glutamicum* ATCC 14752 to kanamycin resistance by using LBHIS plates (20) with 15 μg of kanamycin ml^{-1}. The resulting Tn mutants were transferred to CGXII plates containing 25 μg of kanamycin ml^{-1} and either no peptide or a 3 mM concentration of the dipeptide Ser-Ala. Four clones that exhibited only growth in the presence of Ser-Ala were isolated. These clones were retrieved from the LBHIS master plate and tested on CGXII plates containing 25 μg of kanamycin ml^{-1}, 2 mM Ser-Ala, 1 mM L-serine, 1 mM L-alanine, or no supplement. Three mutants required Ser-Ala or L-serine for growth. The cloning and sequencing of the Tn insertion site in these mutants was performed as described previously (32).

Construction of plasmids and strains. Plasmids were constructed in *E. coli* DH5αMCR from PCR-generated fragments (Expand High Fidelity PCR kit; Roche Diagnostics) by using *C. glutamicum* ATCC 13032 DNA as a template prepared according to a method described elsewhere (10). *E. coli* was transformed by the $RbCl_2$ method (12) and *C. glutamicum* via electroporation (36). All transformants were analyzed by plasmid analysis and/or PCR with appropriate primers, respectively.

In order to construct pEC-T18mob2*serCB*, *serC* and *serB* were amplified by

TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli strains		
DH5αMCR	*endA1 supE44 recA1 gyrA96 relA1 deoR U169* ϕ80*dlacZ*Δ*M15 mcrA* Δ(*mrr-hsdRMS-mcrBC*)	11
GM2929	*dam-13*::Tn*9 dcm-6 hsdR2 recF143 mcrA mcrB*	27
C. glutamicum strains		
WT	Wild type, ATCC 13032	ATCC[b]
ATCC 14752	Requires biotin	ATCC
WTΔ*sdaA*	WT carrying a deletion in the *sdaA* gene	24
WT::pK18mob*glyA′*	WT with *glyA* under control of *tac* promoter	32
WTΔ*sdaA*::pK18mob*glyA′*	WTΔ*sdaA* with *glyA* under control of *tac* promoter	This work
Plasmids		
pCGL0040	Donor of Tn*5531* (IS*1207*, Km^r), $Ap^r ori V_{Ec}$	U53587[c]
pUC18	Cloning vector; Ap^r	26
pUC18*serC*	pUC18 with 1.8-kb PCR product containing *serC*	This work
pUC18*serB*	pUC18 with 1.8-kb PCR product containing *serB*	This work
pUC18*serCB*	Ligation of 3.5-kb NotI-ScaI fragment from pUC18*serC* with 2.8-kb NotI-ScaI fragment from pUC18*serB*	This work
pUC18*serA*$^{\Delta 197}$	pUC18 with 1.25-kb PCR fragment containing *serA* with a deletion of 197 aa at the C terminus	30
pZ1*serA*	pZ1 with 1.9-kb EcoRI-BamHI fragment from pUC18*serA* containing *serA*	30
pZ1*serA*$^{\Delta 197}$	pZ1 with 1.3-kb EcoRI-BamHI fragment from pUC18*serA*$^{\Delta 197}$ containing *serA*$^{\Delta 197}$	30
pEC-T18mob2	*E. coli-C. glutamicum* shuttle vector, Tet^r	35
pEC-T18mob2*serCB*	pEC-T18mob2 with 3.6-kb EcoRI-XbaI fragment containing *serC* and *serB* from pUC18*serCB*	This work
pEC-T18mob2*serA*$^{\Delta 197}$*CB*	pEC-T18mob2*serCB* with 3.6-kb EcoRI-XbaI fragment containing *serC* and *serB* from pUC18*serCB*	This work
pK18mob*glyA′*	Mobilizable vector, nonreplicative in *C. glutamicum*, Km^r, containing *lacI*^{q} and P*tac* fused to 5′-terminal fragment of *glyA*	32

[a] Km^r, kanamycin resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.
[b] ATCC, American Type Culture Collection.
[c] GenBank accession number.

PCR using the upstream primers serC-upper (5′-GACCACCCACAGCCACCG TAATC-3′; the nucleotide (nt) corresponding to nt 877628 of NC003450 is underlined) and serB-upper (5′-**GCGGCCGC**GTTGATGATCCTTGGGGTTA CG-3′; the nucleotide corresponding to nt 2671294 of NC003450 is underlined), respectively, and the respective reverse primers serC-lower (5′-**GCGGCCGC**TT TCCCGCATGTTGACTCCTTCTA-3′; the nucleotide corresponding to nt 875874 of NC003450 is underlined) and serB-lower (5′-GAAGGATCCTCGCT ATGTGG-3′; the nucleotide corresponding to nt 2669483 of NC003450 is underlined). Boldfaced nucleotides correspond to the introduction of a NotI restriction site. The PCR fragments were blunted and cloned into the SmaI site of pUC18. The obtained plasmids, pUC18*serC* and pUC18*serB*, were digested with ScaI and NotI, and the inserts containing *serC* and *serB* were isolated and ligated together, resulting in plasmid pUC18*serCB*. 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-T18mob2*serCB*.

To construct pEC-T18mob2*serA*$^{\Delta 197}$*CB*, plasmid pUC18*serA*$^{\Delta 197}$ (30) was digested with EcoRI and BamHI and the *serA*$^{\Delta 197}$-containing insert obtained was blunted and ligated in EcoRI-linearized and blunted plasmid pEC-T18*serCB*.

In order to place the *glyA* gene in the chromosome of *C. glutamicum* under the control of the IPTG-inducible *tac* promoter, the respective strains were transformed via electroporation with the nonreplicative plasmid pK18mob*glyA*′ to kanamycin resistance. Selection for kanamycin resistance was performed in the presence of 100 μM IPTG. The correct integration into the chromosome via homologous *glyA* sequences was verified by PCR with appropriate primer pairs and controls. The resulting mutants carried one intact copy of *glyA* under the control of the inducible *tac* promoter and one incomplete copy under its own promoter.

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 $MgCl_2$, 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 chromatrography as previously described (33).

3-Phosphoglycerate dehydrogenase activity was determined spectrophotometrically by the formation of NADH as described elsewhere (30).

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 Tn*5531* to screen for L-serine auxotrophs (18). Three clones unable to grow on minimal medium CGXII unless supplemented with 1 mM L-serine were identified. Sequencing of the transposon flanking regions revealed that in two mutants, the insertions were at different sites within the open reading frame NCgl2436, whereas in the remaining mutant, the insertion was apparently in the promoter region of NCgl0794, 35 nucleotides upstream of the deduced open reading frame. The latter open reading frame encodes a polypeptide of 376 amino acids which exhibits identities of 20% over its entire length to the phosphoserine aminotransferase (PSAT; *serC*) of *E. coli* (14). NCgl2436 encodes a deduced polypeptide of 433 amino acids and its C-terminal half shares 41% identical amino acids with the phosphoserine phosphatase (PSP, *serB*) from *E. coli* (25). Interestingly, the N-terminal half of the *C. glutamicum* polypeptide, which exhibits an ACT-like domain (2), is absent from the *E. coli* PSP, which is 93 amino acids shorter. The sequence similarities to *E. coli* and the serine auxotrophy of the transposon mutants identified the genes as *serC* and *serB* from *C. glutamicum*. Additionally, we constructed plasmid pEC-T18mob2*serCB* 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-T18mob2*serCB*). Furthermore, we demonstrated that this plasmid complemented the *serC* and *serB* transposon mutants as expected (not shown).

TABLE 2. L-Serine accumulation of various *C. glutamicum* strains

Strain	L-Serine concn (mM)[a] at:	
	28 h	54 h
WT	<0.01	<0.01
WT(pZ1)(pEC-T18mob2)	<0.01	<0.01
WT(pZ1*serA*)(pEC-T18mob2)	<0.01	0.12 ± 0.10
WT(pZ1*serA*$^{\Delta 197}$)(pEC-T18mob2)	<0.01	0.08 ± 0.05
WT(pZ1)(pEC-T18mob2*serCB*)	<0.01	0.04 ± 0.03
WT(pZ1*serA*)(pEC-T18mob2*serCB*)	<0.01	0.11 ± 0.03
WT(pZ1*serA*$^{\Delta 197}$)(pEC-T18mob2*serCB*)	<0.01	0.05 ± 0.02
WT Δ*sdaA*(pZ1)(pEC-T18mob2)	0.08 ± 0.03	0.05 ± 0.01
WT Δ*sdaA*(pZ1*serA*)(pEC-T18mob2*serCB*)	0.09 ± 0.01	0.15 ± 0.01
WT Δ*sdaA*(pZ1*serA*$^{\Delta 197}$)(pEC-T18mob2*serCB*)	0.44 ± 0.28	0.14 ± 0.01

[a] L-Serine accumulation was determined on minimal medium CGXII with 220 mM glucose in two or three independent experiments.

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 *serA*$^{\Delta 197}$ allele) provided a 3-phosphoglycerate dehydrogenase devoid of feedback inhibition by L-serine (30). Here, we studied whether overexpression of *serA* or *serA*$^{\Delta 197}$ is sufficient to increase L-serine accumulation in the WT. Therefore, the strains WT(pZ1*serA*)(pEC-T18mob2) and WT(pZ1*serA*$^{\Delta 197}$)(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 *serA*$^{\Delta 197}$ nor the WT allele yielded significant L-serine concentrations. Enzyme assays confirmed, for WT(pZ1*serA*) (pEC-T18mob2), a specific PGDH activity of 700 nmol (min mg)$^{-1}$ and for WT(pZ1*serA*$^{\Delta 197}$) (pEC-T18mob2), a specific activity of 690 nmol (min mg)$^{-1}$ 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-T18mob2*serCB*), WT(pZ1*serA*)(pEC-T18mob2*serCB*), and WT(pZ1*serA*$^{\Delta 197}$) (pEC-T18mob2*serCB*) 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 (WTΔ*sdaA*) to assay for the influence of the overexpression of the L-serine biosynthetic genes *serA*,

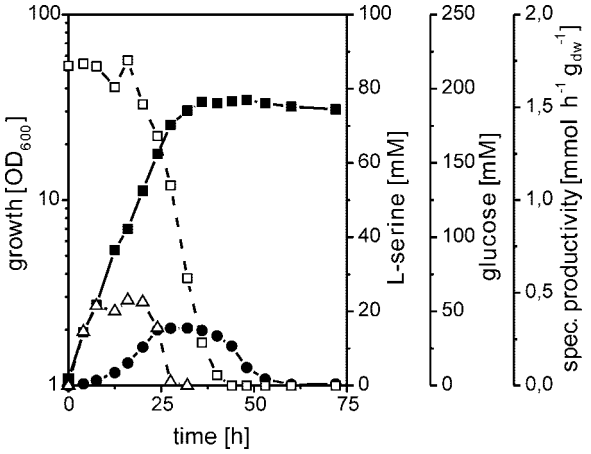

FIG. 2. Growth (■), glucose (□) and L-serine (●) concentrations in the medium, and specific L-serine productivity (△) of strain WT::pK18mob*glyA′*(pEC-T18mob2*serA*$^{\Delta 197}$*CB*) on minimal medium with 220 mM glucose. OD_{600}, optical density at 600 nm; dw, dry weight.

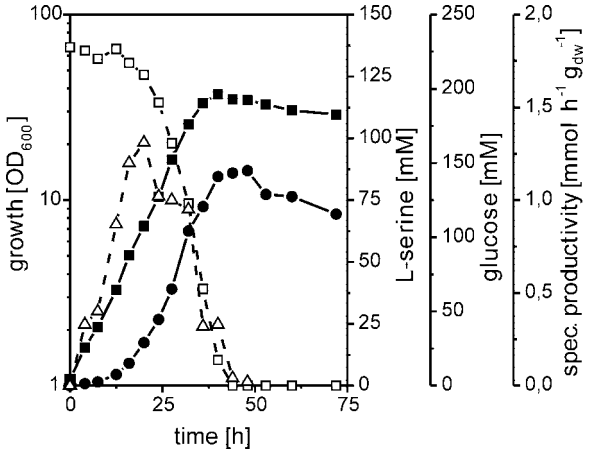

FIG. 3. Growth (■), glucose (□) and L-serine (●) concentrations in the medium, and specific L-serine productivity (△) of strain WTΔ*sdaA*::pK18mob*glyA′*(pEC-T18mob2*serA*$^{\Delta 197}$*CB*) on minimal medium with 220 mM glucose. OD_{600}, optical density at 600 nm; dw, dry weight.

serA$^{\Delta 197}$, *serC*, and *serB* on L-serine accumulation in this background (Table 2). The control strain WTΔ*sdaA*(pZ1)(pEC-T18mob2) accumulated 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* WTΔ*sdaA*(pZ1*serA*)(pEC-T18mob2*serCB*) 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 WTΔ*sdaA* (pZ1*serA*$^{\Delta 197}$)(pEC-T18mob2*serCB*), 0.44 mM (28 h) and 0.14 mM (54 h) L-serine concentrations were determined. This comparison shows an advantage of the *serA*$^{\Delta 197}$ allele over *serA* 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 pK18mob*glyA′* was employed to reduce the SHMT activity by replacing the native *glyA* promoter with the IPTG-inducible *tac* promoter (33). We used strain WT::pK18mob*glyA′* 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 *serA*$^{\Delta 197}$ together with *serC* and *serB* in the kanamycin-resistant strain WT::pK18mob*glyA′*, all three genes were cloned into vector pEC-T18mob2 (35) to generate pEC-T18*serA*$^{\Delta 197}$*CB* (see Materials and Methods). Using this tetracycline resistance-conferring plasmid, strain WT::pK18mob*glyA′*(pEC-T18*serA*$^{\Delta 197}$*CB*) 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 pK18mob*glyA′*. 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 mmol 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 L-SerDH (*sdaA*) was deleted (24).

Influence of combining the deletion of *sdaA* with reduced SHMT activity on growth and L-serine accumulation. We used plasmid pK18mob*glyA′* to exchange the native *glyA* promoter with the *tac* promoter in strain WTΔ*sdaA* as well as plasmid pEC-T18mob2*serA*$^{\Delta 197}$*CB* to overexpress the L-serine biosynthesis genes. The resulting strain, WTΔ*sdaA*::pK18mob*glyA′* (pEC-T18mob2*serA*$^{\Delta 197}$*CB*), 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 ($Y_{P/S}$) 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 WTΔ*sdaA*::pK18mob *glyA′*(pEC-T18mob2*serA*$^{\Delta 197}$*CB*), 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*$^{\Delta 197}$, *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-

TABLE 3. Comparison of L-serine fluxes[a] in recombinant *C. glutamicum* strains

Characteristics	WT	WT (p*serA*)(p*serCB*)[b]	WTΔ*sdaA* (p*serA*)(p*serCB*)[b]	WT::pK18mob*glyA′* (p*serACB*)[c]	WTΔ*sdaA*::pK18mob*glyA′* (p*serACB*)[c]
μ_{max}[d] (h^{-1})	0.38	0.26	0.21	0.11	0.11
Serine flux for cellular demand	9.50	6.5	5.25	2.75	2.75
Serine excretion flux	0	0	0	10	21
Sum of serine fluxes	9.5	6.5	5.25	12.75	23.75

[a] Fluxes are the maximal fluxes observed in nmol min^{-1} mg (dry weight)$^{-1}$.
[b] (p*serA*)(p*serCB*) stands for (pZ1*serA*$^{\Delta 197}$)(pEC-T18mob2*serCB*).
[c] p*serACB* stands for (pEC-T18mob2*serA*$^{\Delta 197}$*CB*).
[d] μ_{max}, maximum growth rate.

served as a consequence of reduced *glyA* expression. The strains WT::pK18mob*glyA′*(pEC-T18mob2*serA*$^{\Delta 197}$*CB*) and WTΔ*sdaA*::pK18mob*glyA′*(pEC-T18mob2*serA*$^{\Delta 197}$*CB*) 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*, *Methanococcus 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*, *Mycobacterium tuberculosis*, 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 present only in the *E. coli* protein and which is involved in pyridoxal-5′-phosphate synthesis. These differences are also apparent from the fact that only the N-terminal part of the *C. glutamicum* PSAT protein, but the entire *E. coli* PSAT protein, is classified as an aminotransferase class V (pfam00266).

The wild-type *serA* derivative and the *serA*$^{\Delta 197}$ allele were overexpressed either alone or in combination with the genes *serB* and *serC*, but L-serine accumulated only in traces not exceeding 0.1 mM. This distinguishes L-serine accumulation from, for instance, L-lysine formation by *C. glutamicum*, where deregulation of the initial enzyme of the pathway already results in substantial L-lysine accumulation of around 40 mM (4), indicating that intracellular L-serine turnover precludes its production. This is corroborated by our findings that L-serine production was high only when *glyA* expression was reduced and L-serine dehydratase activity absent. The corresponding strain WTΔ*sdaA*::pK18mob*glyA′*(pEC-T18mob2*serA*$^{\Delta 197}$*CB*) accumulated L-serine up to 86 mM, which is in the same range as that observed for L-threonine accumulation with a respective threonine-producing *C. glutamicum* strain (33). Moreover, this strain produced a yield of 0.64 mol L-serine per mol glucose, which is about two- to sixfold higher than that obtained with processes where glycine or glycine plus methanol were used as substrates (16, 19). However, the reduced *glyA* expression in the L-serine-producing strain led to a slower growth rate and a lower final optical density compared to a strain with native *glyA* expression likely due to perturbation of the C-1 metabolism. Moreover, since the deletion of *sdaA* alone or in combination with overexpression of the L-serine biosynthetic genes did not result in an appreciable L-serine accumulation, it is inconclusive whether a significant intracellular flux increase due to the overexpression of the *serA* alleles together with *serB* and *serC* is present. These results denote that the L-serine pathway is a rather unusual amino acid biosynthesis pathway. This idea is supported 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 and C_1 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 WTD*sdaA* (pEC-T18mob2 *serA*$^{\Delta 197}$*CB*) illustrates that reducing the L-serine degradation to glycine and C_1 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 C_1 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 WTD*sdaA*::pK18mob*glyA′* (pEC-T18mob2*serA*$^{\Delta 197}$*CB*) 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.

ACKNOWLEDGMENTS

We thank A. Schmitz and S. Knebel for technical assistance and V. F. Wendisch for fruitful discussions and critical reading of the manuscript.

This work was supported in part by the Deutsche Bundesstiftung Umwelt (DBU AZ13037 and AZ13089).

REFERENCES

1. **Ankri, S., I. Serebrijski, O. Reyes, and G. Leblon.** 1996. Mutations in the *Corynebacterium glutamicum* proline biosynthetic pathway: a natural bypass of the *proA* step. J. Bacteriol. **178:**4412–4419.
2. **Chipman, D. M., and B. Shaanan.** 2001. The ACT domain family. Curr. Opin. Struct. Biol. **11:**694–700.
3. **Cho, H., W. Wang, R. Kim, H. Yokota, S. Damo, S. H. Kim, D. Wemmer, S. Kustu, and D. Yan.** 2001. BeF_3^- acts as a phosphate analog in proteins phosphorylated on aspartate: structure of a BeF_3^- complex with phosphoserine phosphatase. Proc. Natl. Acad. Sci. USA **98:**8525–8530.
4. **Cremer, J., L. Eggeling, and H. Sahm.** 1991. Control of the lysine biosynthesis sequence in *Corynebacterium glutamicum* as analyzed by overexpression of the individual corresponding genes. Appl. Environ. Microbiol. **57:**1746–1752.
5. **Dey, S., Z. Hu, X. L. Xu, J. C. Sacchettini, and G. A. Grant.** 2005. D-3-Phosphoglycerate dehydrogenase from *Mycobacterium tuberculosis* is a link between the *Escherichia coli* and mammalian enzymes. J. Biol. Chem. **280:**14892–14899.
6. **Drauz, K., B. Hoppe, A. Kleemann, H.-P. Krimmer, W. Leuchtenberger, and C. Weckbecker.** 2003. Amino acids. *In* M. Bohnet, C. J. Brinker, and B. Cornils (ed.), Ullmann's encyclopedia of industrial chemistry. Wiley-VCH Verlag GmbH, Weinheim, Germany.
7. **Eggeling, L., and H. Sahm.** 2003. New ubiquitous translocators: amino acid export by *Corynebacterium glutamicum* and *Escherichia coli*. Arch. Microbiol. **180:**155–160.
8. **Eggeling, L., S. Morbach, and H. Sahm.** 1997. The fruits of molecular physiology: engineering the L-isoleucine biosynthesis pathway in *Corynebacterium glutamicum*. J. Biotechnol. **56:**167–182.
9. **Eggeling, L., W. Pfefferle, and H. Sahm.** 2001. Amino acids, p. 281–303. *In* C. Ratledge and B. Kristiansen (ed.), Basic biotechnology. Cambridge University Press, Cambridge, England.
10. **Eikmanns, B. J., N. Thum-Schmitz, L. Eggeling, K. Lüdtke, and H. Sahm.** 1994. Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase. Microbiology **140:**1817–1828.
11. **Grant, S. G. N., J. Jessee, F. R. Bloom, and D. Hanahan.** 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA **87:**4645–4649.
12. **Hanahan, D.** 1985. Techniques for transformation of *Escherichia coli*, p. 109–136. *In* D. M. Glover (ed.), DNA cloning, vol. 1. IRL Press, Oxford, United Kingdom.
13. **Hermann, T.** 2003. Industrial production of amino acids by coryneform bacteria. J. Biotechnol. **104:**155–172.
14. **Hester, G., W. Stark, M. Moser, J. Kallen, Z. Markovic-Housley, and J. N. Jansonius.** 1999. Crystal structure of phosphoserine aminotransferase from *Escherichia coli* at 2.3 Å resolution: comparison of the unligated enzyme and a complex with alpha-methyl-L-glutamate. J. Mol. Biol. **286:**829–850.
15. **Hsiao, H.-Y., and T. Wei.** 1985. Enzymatic production of L-serine with a feedback control system for formaldehyde addition. Biotechnol. Bioeng. **28:**1510–1518.
16. **Izumi, Y., T. Yoshida, S. S. Miyazaki, T. Mitsunaga, T. Ohshiro, M. Shimao, A. Miyata, and T. Tanabe.** 1993. L-Serine production by a methylotroph and its related enzymes. Appl. Microbiol. Biotechnol. **39:**427–432.
17. **Keilhauer, C., L. Eggeling, and H. Sahm.** 1993. Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. J. Bacteriol. **175:**5595–5603.
18. **Kennerknecht, N., H. Sahm, M. R. Yen, M. Patek, M. H. Saier, Jr., and L. Eggeling.** 2002. Export of L-isoleucine from *Corynebacterium glutamicum*: a two-gene-encoded member of a new translocator family. J. Bacteriol. **184:**3947–3956.
19. **Kubota, K., and K. Yokozeki.** 1989. Production of L-serine from glycine by *Corynebacterium glycinophilum* and properties of serine hydroxymethyltransferase, a key enzyme in L-serine production. J. Ferment. Bioeng. **67:**387–390.
20. **Liebl, W., A. Bayerl, U. Stillner, and K. H. Schleifer.** 1989. High efficiency electroporation of intact *Corynebacterium glutamicum* cells. FEMS Microbiol. Lett. **65:**299–304.
21. **Marx, A., A. A. deGraaf, W. Wiechert, L. Eggeling, and H. Sahm.** 1996. Determination of the fluxes in the central metabolism of *Corynebacterium glutamicum* by nuclear magnetic resonance spectroscopy combined with metabolite balancing. Biotechnol. Bioeng. **49:**111–129.
22. **Marx, A., K. Striegel, A. A. deGraaf, H. Sahm, and L. Eggeling.** 1997. Response of the central metabolism of *Corynebacterium glutamicum* to different flux burdens. Biotechnol. Bioeng. **56:**168–180.
23. **Marx, A., S. Hans, B. Möckel, B. Bathe, A. A. de Graaf, A. C. McCormack, C. Stapleton, K. Burke, M. O'Donohue, and L. K. Dunican.** 2003. Metabolic phenotype of phosphoglucose isomerase mutants of *Corynebacterium glutamicum*. J. Biotechnol. **104:**185–197.
24. **Netzer, R., P. Peters-Wendisch, L. Eggeling, and H. Sahm.** 2004. Cometabolism of a nongrowth substrate: L-serine utilization by *Corynebacterium glutamicum*. Appl. Environ. Microbiol. **70:**7148–7155.
25. **Neuwald, A. F., and G. V. Stauffer.** 1985. DNA sequence and characterization of the *Escherichia coli serB* gene. Nucleic Acids Res. **13:**7025–7039.
26. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26:**101–106.
27. **Palmer, B. R., and M. G. Marinus.** 1994. The *dam* and *dcm* strains of *Escherichia coli*: a review. Gene **143:**1–12.
28. **Peeraer, Y., A. Rabijns, C. Verboven, J. F. Collet, E. Van Schaftingen, and C. De Ranter.** 2003. High-resolution structure of human phosphoserine phosphatase in open conformation. Acta Crystallogr. Sect. D Biol. Crystallogr. **59:**971–977.
29. **Peters-Wendisch, P., B. Schiel, V. F. Wendisch, E. Katsoulidis, B. Möckel, H. Sahm, and B. J. Eikmanns.** 2001. Pyruvate carboxylase is a major bottleneck for glutamate and lysine production by *Corynebacterium glutamicum*. J. Mol. Microbiol. Biotechnol. **3:**295–300.
30. **Peters-Wendisch, P., R. Netzer, L. Eggeling, and H. Sahm.** 2002. 3-Phosphoglycerate dehydrogenase from *Corynebacterium glutamicum*: the C-terminal domain is not essential for activity but is required for inhibition by L-serine. Appl. Microbiol. Biotechnol. **60:**437–441.
31. **Riedel, C., D. Rittmann, P. Dangel, B. Möckel, S. Petersen, H. Sahm, and B. J. Eikmanns.** 2001. Characterization of the phosphoenolpyruvate carboxykinase gene from *Corynebacterium glutamicum* and significance of the enzyme for growth and amino acid production. J. Mol. Microbiol. Biotechnol. **3:**573–583.
32. **Simic, P., H. Sahm, and L. Eggeling.** 2001. L-Threonine export: use of peptides to identify a new translocator from *Corynebacterium glutamicum*. J. Bacteriol. **183:**5317–5324.
33. **Simic, P., J. Willuhn, H. Sahm, and L. Eggeling.** 2002. Identification of *glyA* (encoding serine hydroxymethyltransferase) and its use together with the exporter ThrE to increase L-threonine accumulation by *Corynebacterium glutamicum*. Appl. Environ. Microbiol. **68:**3321–3327.
34. **Sugimoto, E., and L. I. Pizer.** 1968. The mechanism of end product inhibition of serine biosynthesis. I. Purification and kinetics of phosphoglycerate dehydrogenase. J. Biol. Chem. **243:**2081–2089.
35. **Tauch, A., O. Kirchner, B. Löffler, S. Gotker, A. Pühler, and J. Kalinowski.** 2002. Efficient electrotransformation of *Corynebacterium diphtheriae* with a mini-replicon derived from the *Corynebacterium glutamicum* plasmid pGA1. Curr. Microbiol. **45:**362–367.
36. **van der Rest, M. E., C. Lange, and D. Molenaar.** 1999. A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Appl. Microbiol. Biotechnol. **52:**541–545.
37. **Wang, W., R. Kim, J. Jancarik, H. Yokota, and S. H. Kim.** 2001. Crystal structure of phosphoserine phosphatase from *Methanococcus jannaschii*, a hyperthermophile, at 1.8 A resolution. Structure **9:**65–71.
38. **Yoshida, H., and K. Nakayama.** 1974. Production of L-serine analog-resistant mutants from various bacteria and the effect of L-threonine and L-homoserine on the production of L-serine. J. Agric. Chem. Soc. Jpn. **48:**201–208.