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Cometabolism of a Nongrowth Substrate: L-Serine Utilization by *Corynebacterium glutamicum*

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Despite its key position in central metabolism, L-serine does not support the growth of *Corynebacterium glutamicum*. Nevertheless, during growth on glucose, L-serine is consumed at rates up to 19.4 ± 4.0 nmol $\min^{-1}$ (mg [dry weight]$)^{-1}$, resulting in the complete consumption of 100 mM L-serine in the presence of 100 mM glucose and an increased growth yield of about 20%. Use of ^{13}C-labeled L-serine and analysis of cellularly derived metabolites by nuclear magnetic resonance spectroscopy revealed that the carbon skeleton of L-serine is mainly converted to pyruvate-derived metabolites such as L-alanine. The *sdaA* gene was identified in the genome of *C. glutamicum*, and overexpression of *sdaA* resulted in (i) functional L-serine dehydratase (L-SerDH) activity, and therefore conversion of L-serine to pyruvate, and (ii) growth of the recombinant strain on L-serine as the single substrate. In contrast, deletion of *sdaA* decreased the L-serine cometabolism rate with glucose by 47% but still resulted in degradation of L-serine to pyruvate. Cystathionine β-lyase was additionally found to convert L-serine to pyruvate, and the respective *metC* gene was induced 2.4-fold under high internal L-serine concentrations. Upon *sdaA* overexpression, the growth rate on glucose is reduced 36% from that of the wild type, illustrating that even with glucose as a single substrate, intracellular L-serine conversion to pyruvate might occur, although probably the weak affinity of L-SerDH (apparent K_m, 11 mM) prevents substantial L-serine degradation.

Cometabolism is often observed for xenobiotic compounds which do not enable growth as a single carbon- and energy source (21). This is due, for example, to long degradation pathways and unnatural structures. On the other hand, microorganisms with a restricted metabolism, such as *Lactococcus lactis*, are dependent on cometabolism of essential natural compounds, e.g., amino acids (35). The amino acid L-serine is characterized by the fact that several organisms have the ability to introduce it into the central metabolism via pyruvate (35, 2, 16). Another distinguishing feature is its high cellular demand, exceeding the simple provision of L-serine for protein synthesis, since L-serine is additionally required for glycine, cysteine, tryptophan, and phospholipid synthesis as well as for 1-carbon-unit generation (52). Glycine, in turn, is a precursor for purines and heme. In *Corynebacterium glutamicum* about 7.5% of the total carbon flux toward L-serine is utilized for these purposes (28). Prior estimates for *Escherichia coli* determined that as much as 15% of the carbon assimilated from glucose involves L-serine (42). Due to the high demand and its key position in the precursor supply, L-serine has to be regarded as an intermediate of the central metabolism (52).

In spite of the presence of L-serine dehydratase (L-SerDH) (47) and the key position of L-serine, growth of *E. coli* on L-serine as a carbon source is very poor, allowing doubling times of about 60 h only (63). When the organism is additionally exposed to low concentrations of glycine, isoleucine, and threonine, growth is enhanced (36). Interestingly, during growth on tryptone broth, where a number of amino acids are present, L-serine is utilized immediately and earlier than any other amino acid (43). Taken together, L-serine is clearly a poor growth substrate for *E. coli* and is preferably cometabolized. *Klebsiella aerogenes* and *Salmonella enterica* serovar Typhimurium can also grow only slowly on L-serine as a carbon source (30), and modest growth of *K. aerogenes* is supported with L-serine as a nitrogen source (58). Utilization of L-serine has also been reported for *Helicobacter pylori* (31). This bacterium exhibits a strict respiratory form of metabolism and is unable to utilize glucose but prefers amino acids such as L-serine and L- or D-alanine, which are oxidized, thereby serving as an important energy source (32).

C. glutamicum is able to grow on a variety of mixed carbon sources (3, 44, 61), which, with the exception of the sequential consumption of glucose and glutamate (25), are metabolized in parallel. It has been shown that *C. glutamicum* is also able to degrade a xenobiotic compound in cometabolism with readily metabolizable carbon sources (10).

Our interest was to study L-serine utilization by *C. glutamicum*. This bacterium is used for industrial production of L-glutamate and L-lysine (5); the latter amino acid accumulates to as much as 170 g liter^{-1} in the medium with mutant strains (41). One reason for the exceptional L-lysine-synthesizing property of this bacterium is its inability to degrade this amino acid. In exploring the ability to produce L-serine with *C. glutamicum* (38), it is therefore obviously necessary to assay for utilization of this amino acid also. Here we report studies which revealed that L-serine is a cometabolized substrate with very high utilization rates, enabling its channeling into the central metabolism.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. The wild-type *C. glutamicum* strain ATCC 13032 was used for construction of recombinant strains. For plasmid construction, *E. coli* DH5αMCR (13) was used. Plasmids used were pGEM-T (Promega, Madison, Wisc.) for subcloning of PCR fragments in *E. coli*,

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pK19mob*sacB* (48) for construction of the L-SerDH-negative mutant, and pXMJ19 (19) for construction of the *sdaA*-overexpressing strain of *C. glutamicum*. For overexpression of *metC* (*aecD*), plasmid pSL173 (24) was used, and for *metC* deletion, plasmid pCR007d (46) was used.

Luria-Bertani medium was used as the standard medium for cultivation of *E. coli*, while all *C. glutamicum* strains were precultured on brain heart infusion medium (Difco). The CgXII minimal medium used for growth of *C. glutamicum* has been described previously (23); it contained 30 μg of protocatechuic acid ml^{-1} and was supplemented with the respective carbon source. When appropriate, *E. coli* strains received carbenicillin or kanamycin (each at 50 μg ml^{-1}) or chloramphenicol (30 μg ml^{-1}). *C. glutamicum* strains received kanamycin (50 μg ml^{-1}) or chloramphenicol (10 μg ml^{-1}). After transformation, *C. glutamicum* received reduced kanamycin or chloramphenicol concentrations of 15 or 4 μg ml^{-1}, respectively. In *sdaA* overexpression experiments using pXMJ19 as a vector, 1 mM isopropyl-β-D-thiogalactopyranoside was added for induction of P$_{tac}$. All *C. glutamicum* cultures (60 ml in 500-ml baffled Erlenmeyer flasks) were inoculated to give an optical density at 600 nm (OD$_{600}$) of about 1 and then incubated aerobically at 30°C on a rotary shaker at 120 rpm. For determination of L-serine utilization, *C. glutamicum* was precultured in CgXII medium containing 200 mM glucose as a carbon source. The cells were harvested in the early-stationary phase and used to inoculate fresh CgXII medium with glucose or glucose plus L-serine at the concentrations described in Results. The substrate consumption rate for growing cells is given as growth rate (μ) × (substrate concentration at t_1 − substrate concentration at t_0)/(dry weight at t_1 − dry weight at t_0), where t_1 is the time of complete glucose depletion. For nongrowing cells in phase 2, L-serine consumption rates were determined by the following formula: (serine concentration at t_2 − serine concentration at t_1)/(mean dry weight at phase 2) × ($t_2 - t_1$), where t_2 is the time of complete serine depletion. The relation established previously (an OD$_{600}$ of 1 is equivalent to 0.3 mg [dry weight] ml^{-1}) was used to calculate the dry weight of the cultures.

NMR spectroscopy. For the ^{13}C-labeling experiments, L-[U-^{13}C]serine (98% enrichment) from Cambridge Isotope Laboratories, Andover, Mass., was used. Wild-type *C. glutamicum* and the Δ*sdaA* mutant were harvested after growth for 20 h, cells lyophilized, and hydrolyzed, and the hydrolysate was used to record a heteronuclear single-quantum correlated two-dimensional nuclear magnetic resonance (NMR) spectrum as described previously (37). Isotopomer distributions of proteinogenic amino acids and glycerol were determined from this two-dimensional spectrum. NMR measurements were performed on an AMX-400 WB spectrometer system (Bruker, Karlsruhe, Germany).

Gene overexpression. Plasmids were constructed in *E. coli* DH5αMCR from PCR-generated fragments (Expand High Fidelity PCR kit; Roche Diagnostics) by using *C. glutamicum* DNA, prepared as previously described (6), as a template. *E. coli* was transformed by the RbCl$_2$ method (14). *C. glutamicum* was transformed via electroporation (56). All transformants were analyzed by plasmid analysis and/or PCR with appropriate primers.

In order to construct pXMJ19*sdaA*, the *sdaA* gene from *C. glutamicum* was amplified by PCR using the upstream primer 5′-GC**TCTAGA**AAGGAGATATAGAT[r]ATGGCTATCAGTGTTGTTGAT-3′ (nucleotide 1744884 of NC003450 is underlined) and the reverse primer 5′-GC**GAATTC**GCCAAGCAAGACAAAATCCAAGCC-3′ (nucleotide 1746274 of NC003450 is underlined). Boldfaced nucleotides correspond to an XbaI and an EcoRI restriction site, respectively, and italicized nucleotides correspond to the ribosome binding site of T7 gene 10. The PCR product was subcloned into the pGEM-T vector by using the pGEM-T vector system (Promega). The resultant plasmid, pGEM-T*sdaA*, was digested with EcoRI and XbaI, and the *sdaA*-containing insert obtained was ligated with EcoRI- and XbaI-treated pXMJ19, resulting in plasmid pXMJ19*sdaA*.

Gene inactivation. The *sdaA* gene was inactivated by modified gene replacement methods as described previously (27, 48). According to the sequence of the *sdaA* gene, four primers (primers Δ*sdaA*_1 [5′-TCGTGCAACTTCAGACTC-3′], Δ*sdaA*_2 [5′-CCCATCCACTAAACTTAAACACGTCATAATGAACCCACC-3′], Δ*sdaA*_3 [5′-TGTTTAAGTTTAGTGGATGGGCCGACTAATGGTGCTGCG-3′], and Δ*sdaA*_4 [5′-CGGGAAGCCCAAGGTGGT-3′]) were designed, with primers Δ*sdaA*_2 and Δ*sdaA*_3 containing homologous extensions of 21 bp (underlined) at the 5′ end used as a linker sequence in order to allow crossover PCR. The primer pair Δ*sdaA*_1 and Δ*sdaA*_2 was used to amplify a 504-bp fragment of the 5′ end, and primer pair Δ*sdaA*_3 and Δ*sdaA*_4 was used to amplify a 509-bp fragment of the 3′ end, of the *sdaA* gene by PCR from denatured cells from *C. glutamicum*. The resulting PCR fragments were used as templates for PCR with primer pair Δ*sdaA*_1 and Δ*sdaA*_4 to amplify the *sdaA* gene harboring a 415-bp deletion plus the 21-bp linker sequence. The resulting 1,034-bp fragment was ligated into the SmaI restriction site of the mobilizable *E. coli* vector pK19mob*sacB*, which is nonreplicative in *C. glutamicum*, leading to pK19mob*sacB*Δ*sdaA*. By use of a method described previously (39), the resulting vector pK19mob*sacB*Δ*sdaA* was used to replace the intact chromosomal *sdaA* gene in *C. glutamicum* ATCC 13032 with the truncated *sdaA* gene. PCR with primers located upstream and downstream of the truncated gene was performed to verify the replacement at the chromosomal *sdaA* locus (data not shown). The *sdaA* mutant was designated 13032Δ*sdaA*. Site-specific deletion of the *metC* gene of strain 13032Δ*sdaA* was performed by using plasmid pCR007d (46). The desired deletion was also verified by PCR (data not shown), and the resulting mutant was designated 13032Δ*sdaA*Δ*metC*.

Enzyme assays. L-Serine dehydratase activity was measured by the formation of pyruvate from L-serine via high-performance liquid chromatography (HPLC) after derivatization with 4,5-dimethoxy-1,2-diaminobenzene (DDB) (22). Crude extracts were acquired by ultrasonication in 50 mM HEPES (pH 8.0)–10% glycerol–3 mM FeSO$_4$–10 mM dithiothreitol. Assays were performed in mixtures (1.5 ml) containing 50 mM HEPES (pH 8.0), 1% glycerol, 10 mM dithiotreitol, 0 to 500 mM L-serine, and different amounts of crude extract (10 to 100 μg of protein ml^{-1}). D-Serine, L-threonine, D-threonine, L-*allo*-threonine, or D-*allo*-threonine (50 mM each) was included in the mixture instead of L-serine, respectively, for determination of the substrate specificity. The reaction was stopped after 10 min by adding 150 μl of DDB solution (80 μg of DDB ml^{-1}, 0.5 M HCl, 0.21 M β-mercaptoethanol) to a 150-μl reaction mixture. Derivatization was performed by 2 h of incubation at 102°C.

Cystathionine β-lyase activity was determined by a method analogous to that described for L-serine dehydratase by measuring pyruvate formation via HPLC. Crude extracts were prepared by resuspension of the cell pellets in 1 ml of 100 mM Tris-HCl, pH 8.5 (24), and disruption by ultrasonic treatment. Enzyme activity was determined immediately in the cell-free supernatant. The assay mixture contained 100 mM Tris-HCl (pH 8.5), 200 μM pyridoxal-5′-phosphate, 5 mM cystathionine, 5 mM L-cystine, 50 mM cysteine or 50 mM L-serine, respectively, and an appropriate amount of crude extract. Pyruvate formation was analyzed as described above.

Gene expression analysis. For the DNA microarray analysis (60), wild-type *C. glutamicum* was grown after precultivation in CgXII medium in two parallel cultures with 100 mM glucose as the carbon source. After 4 h of growth, from an OD$_{600}$ of 0.8 to an OD$_{600}$ of 3.5, 1 mM seryl-tripeptide was added to one of the cultures and the cells were further incubated to reach an OD$_{600}$ of 7 before harvest. Intracellular quantification of the amino acid pools (51) confirmed that addition of 1 mM seryl-tripeptide to the exponentially growing culture resulted in an intracellular L-serine concentration of 95 mM, whereas the concentration in the control culture without peptide was 1 mM. The harvesting of 25-ml aliquots of the cultures and the entire procedure of RNA preparation, cDNA synthesis, DNA microarray hybridization, washing, data normalization, and gene expression analysis have been described previously (18, 26).

Analytical methods. Amino acid concentrations in the culture supernatant were determined by reversed-phase liquid chromatography (HPLC) after derivatization with *ortho*-phthaldialdehyde as described previously (49). Glucose concentrations were determined with the D-glucose determination test kit (R-Biopharm, Darmstadt, Germany).

RESULTS

Utilization of L-serine by *C. glutamicum*. Initial growth experiments showed that wild-type *C. glutamicum* was not able to utilize L-serine either as a sole carbon source or as a sole nitrogen source (data not shown), although L-serine was utilized in the presence of glucose. We therefore analyzed co-utilization of L-serine in detail. For this purpose, *C. glutamicum* was grown on minimal medium with different glucose concentrations in the absence (Fig. 1A) or presence (Fig. 1B) of 100 mM L-serine. As can be seen, with all glucose concentrations, an increase in the final biomass was attained due to addition of L-serine. After 30 h of cultivation, the increase in the OD$_{600}$ was 18 to 22%. Addition of L-serine reduced the growth rate slightly, i.e., from 0.37 h^{-1} at 60 mM glucose to 0.30 h^{-1}. Cometabolism of L-serine with glucose is therefore different from that for *E. coli*, where 1 mM L-serine abolishes growth on glucose or lactate (36).

For all cultures grown on the glucose–L-serine mixtures (Fig.

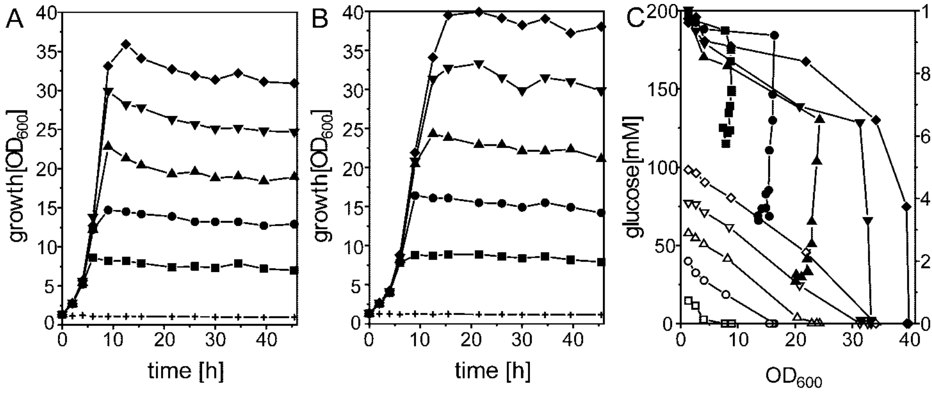

FIG. 1. Growth (A, B) and substrate consumption (C) of wild-type *C. glutamicum* in minimal medium containing different glucose concentrations (+, 0 mM; ■, 20 mM; ●, 40 mM; ▲, 60 mM; ▼, 80 mM; ♦, 100 mM) in the absence (A) or presence (B) of L-serine. (C) Consumption of glucose (open symbols) and serine (closed symbols) as a function of OD_{600}.

1B), consumption of both substrates was monitored and the respective concentrations determined were plotted against the OD_{600} reached at the time when the sample was taken (Fig. 1C). Interestingly, we observed different phases of L-serine utilization. For instance, on 60 mM glucose plus 100 mM L-serine, glucose was utilized completely by the cells, whereas at the same time only about 40% of the L-serine was consumed. However, after exhaustion of glucose (at an OD_{600} of 24), the culture still continued to consume L-serine without additional growth to a concentration of approximately 15 mM, where utilization stopped. This result shows that distinct phases of cometabolism exist: in phase 1, growth occurs during coutilization of glucose with L-serine; in phase 2, after growth has stopped, L-serine is still utilized; in phase 3, L-serine is not utilized at all. As can be seen from Fig. 1C, the largest part of L-serine is consumed during phase 2, and this depends on the amount of biomass present. The average consumption rate of glucose for all cultures was 43.8 ± 4.9 nmol min^{-1} (mg [dry weight])$^{-1}$. For L-serine it was 19.4 ± 4.0 nmol min^{-1} (mg [dry weight])$^{-1}$ in phase 1 and 8.2 ± 2.6 nmol min^{-1} (mg [dry weight])$^{-1}$ in phase 2.

Intracellular fate of L-serine. In order to obtain direct information on the fate of L-serine, a tracer analysis was performed. For this purpose *C. glutamicum* was grown on 100 mM glucose with 90 mM L-[U-^{13}C]serine. Cells were harvested after 20 h, when all glucose and 80% of the L-serine had been consumed. The cell pellet was hydrolyzed, and a sample was analyzed by two-dimensional (^{1}H, ^{13}C) HSQC NMR (54) to quantify ^{13}C fractional enrichments and isotopomer distributions of glycine, alanine, aspartate, phenylalanine, glycerol, and histidine in the biomass hydrolysate (37). Table 1 shows that 92.6% of the L-serine was labeled uniformly, giving rise to a doublet of doublet (dd) signals in the NMR ^{13}C spectrum. The fact that almost all labeled cellular L-serine was labeled uniformly corresponded to the situation expected when the L-[U-^{13}C]serine is taken up and integrated into the cellular protein. More interesting was the analysis of the proteinogenic L-alanine, because this gives direct information about the labeling in pyruvate (29). The fine structure of the ^{13}C NMR signal from C-2 of L-alanine is shown in Fig. 2. It is apparent that the dominant signal was again a doublet of doublets resulting from the isotopomer in which all three carbons were labeled. Also the singlet signal(s) from the isotopomer in which only C-2 of L-alanine is labeled was visible, as well as signals due to the two isotopomers in which two carbon atoms were labeled. The results of the quantitative analysis of selected NMR signals are given in Table 1. It shows that 85.5% ± 2.0% of the labeled L-alanine was uniformly labeled, which was further confirmed by the result for C-3 of L-alanine, showing 86.8% ± 0.5% coupling with C-2. This result verified that the carbon skeleton of L-serine was converted as an entity to pyruvate and used for different purposes such as transamination to L-alanine. Glycine was almost uniformly labeled in both C atoms (96.3%), indicating that it was also derived directly from L-serine. Moreover,

TABLE 1. Isotopomer labeling patterns of central metabolites from wild-type *C. glutamicum*

Central metabolite and carbon atom position	Measured from biomass compound	Fine structure of ^{13}C multiplet signals (%)				
		s	d_{-1}	$d_{-1} + d_{+1}$[a]	d_{+1}	dd
Serine C-2	Serine C-2	2.0 ± 1.0	3.4 ± 0.5		2.0 ± 1.0	92.6 ± 2.0
3-Phosphoglycerate C-2	Glycine C-2	3.7 ± 0.5	96.3 ± 0.5			
Pyruvate C-2	Alanine C-2	5.0 ± 2.0	5.9 ± 1.0		3.6 ± 2.0	85.5 ± 2.0
Pyruvate C-3	Alanine C-3	13.2 ± 0.5	86.8 ± 0.5			
Phosphoenolpyruvate C-2	Phenylalanine C-2	21.0 ± 5.0	15.0 ± 1.0		5.0 ± 2.0	59.0 ± 4.0
Triose phosphates C-1/C-3	Glycerol C-1/C-3	40.0 ± 3.0	60.0 ± 2.0			
Triose phosphates C-2	Glycerol C-2	38.0 ± 3.0		19.0 ± 2.0	43.0 ± 4.0	
Pentose phosphates C-3	Histidine C-3	39.0 ± 2.0	61.0 ± 2.0			
Oxaloacetate C-2	Aspartate C-2	14.7 ± 0.5	25.4 ± 0.5		12.9 ± 1.0	47.0 ± 1.0

[a] Since glycerol is a symmetrical molecule, only the sum of d_{-1} and d_{+1} is determined.

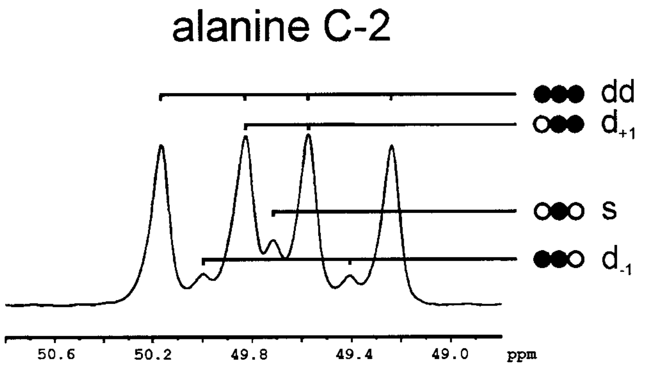

FIG. 2. ^{13}C NMR spectra of the C-2 of alanine and illustration of the signal fine structure composition. s, singlet peak of [2-^{13}C]alanine (no neighboring labels); d_{-1}, ^{13}C in the preceding position ([1,2-$^{13}C_2$]alanine) produces a doublet peak, split by scalar coupling; d_{+1}, ^{13}C in the following position ([2,3-$^{13}C_2$]alanine) yields another doublet split with a different coupling constant; dd, "doublet of doublet" signal of [$^{13}C_3$]alanine.

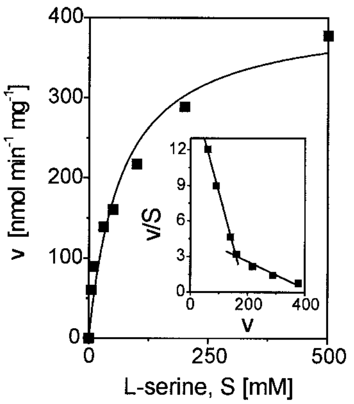

FIG. 3. Specific activity (v) of L-SerDH in crude extracts of *C. glutamicum* 13032(pXMJ19*sdaA*) as a function of the L-serine concentration (S). Inset represents the respective Eadie-Hofstee plot.

significant ^{13}C label was detected in further cellular compounds (Table 1) such as L-phenylalanine or L-aspartate, from which it can be concluded that label derived from L-serine was further transferred to cellular metabolites via pyruvate.

Identification, deletion, and overexpression of *sdaA*. Based on the tracer experiment, we searched the genome of *C. glutamicum* for an L-SerDH which could be a candidate enzyme for generating pyruvate from L-serine. By using the *E. coli* SdaA, SdaB, or TdcG sequence invariably, one single protein sequence was identified in *C. glutamicum* (encoded by NCgl1583) exhibiting high identities (about 40%) to all three *E. coli* polypeptides. The protein encoded by *C. glutamicum* NCgl1583 exhibits three conserved cysteine residues which could serve for the coordination of a [4Fe-4S] cluster as postulated for the L-SerDH of the anaerobic bacterium *Peptoniphilus asaccharolyticus* (formerly known as *Peptostreptococcus asaccharolyticus*) (17). In order to investigate whether the gene identified encodes a functional L-SerDH, the corresponding gene, termed *sdaA*, was amplified and cloned into the *E. coli-C. glutamicum* shuttle vector pXMJ19 (19). The resulting plasmid was used to transform wild-type *C. glutamicum* to yield the recombinant strain 13032(pXMJ19*sdaA*). In addition, a deletion plasmid was constructed which was used in two rounds of positive selection (48) to enable deletion of *sdaA* in the genome of *C. glutamicum*. The deletion in the resulting 13032Δ*sdaA* strain was confirmed by PCR (data not shown).

Since known L-SerDHs often exhibit only weak activities (7), we used a highly sensitive assay based on the direct quantification of pyruvate via HPLC (see Materials and Methods). Cells were harvested from a glucose-plus-L-serine culture, and crude extracts prepared were immediately used in the L-SerDH assay. From the linear increase in pyruvate formation, the specific activities were calculated. With plasmid-encoded *sdaA* a high specific activity of 210.7 ± 8.7 nmol min^{-1} (mg of protein)$^{-1}$ was obtained, and the activity was dependent on reducing conditions and iron (data not shown), as has been described for *E. coli* (53). This result clearly showed that *sdaA* encodes a functional L-SerDH. As expected, the mutant 13032Δ*sdaA*(pXMJ19) strain, with *sdaA* deleted, did not give any activity (specific activity, 0.7 ± 0.9 nmol min^{-1} [mg of protein]$^{-1}$). Surprisingly, with the wild type carrying the empty plasmid also, no significant activity was observed (specific activity, 1.0 ± 0.6 nmol min^{-1} [mg of protein]$^{-1}$), suggesting that the enzyme is not active under the chosen conditions.

In order to determine the substrate constant for L-serine, the activity of the L-SerDH was quantified with an extract of the 13032(pXMJ19*sdaA*) strain at different substrate concentrations. As shown in Fig. 3, substrate conversion does not follow classical Michaelis-Menten type kinetics. Instead, the Eadie-Hofstee plot (Fig. 3 inset) revealed a clear biphasic dependence of the activity on the L-serine concentration. Whereas at the lower substrate concentrations (up to 50 mM) an apparent K_m of 11 mM was determined, at the higher concentrations (up to 500 mM) the apparent K_m determined was 90 mM. The possibility that L-serine itself influences the assay at exceptionally high concentrations cannot be excluded. Nevertheless, the L-SerDH of *C. glutamicum* exhibited a relatively low affinity for its substrate L-serine, as was shown for the *E. coli* SdaA enzyme with its estimated K_m of 42 mM (34). When D-serine, L-threonine, D-threonine, L-*allo*-threonine, and D-*allo*-threonine were assayed as possible substrates, in no case did the activity exceed 1.6% of the activity obtained with L-serine.

Physiological consequences of altered *sdaA* expression. We performed growth experiments on minimal medium containing 100 mM glucose and/or 100 mM L-serine with the recombinant strains constructed. Most interestingly, the 13032(pXMJ19*sdaA*) strain was able to grow on L-serine as a sole carbon source, albeit at a low growth rate of 0.07 h^{-1} (Fig. 4) and not entirely consuming the substrate (data not shown). This indicates that *sdaA* expression enables utilization of L-serine, but that at the same time an unknown regulatory mechanism might prevent its entire consumption. Furthermore, growth of the 13032(pXMJ19*sdaA*) strain on glucose was significantly affected, since its growth rate was reduced from 0.36 h^{-1} for the 13032(pXMJ19) strain as a control to 0.23 h^{-1} (Fig. 4). These effects were confirmed with separate clones and in separate growth experiments, and they illustrate the in vivo activity of L-SerDH in the overexpressing strain.

The consequences of altered *sdaA* expression on L-serine coutilization were derived from the cultures grown on glucose

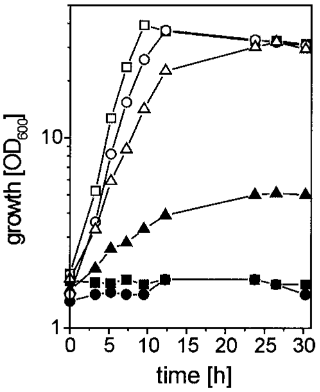

FIG. 4. Growth of different *C. glutamicum* strains in minimal medium containing 100 mM glucose (open symbols) or 100 mM L-serine (closed symbols). Triangles, 13032(pXMJ19*sdaA*); circles, 13032(pXMJ19); squares, 13032Δ*sdaA*(pXMJ19).

plus L-serine. Upon overexpression of *sdaA*, the L-serine consumption rate was increased about twofold to 30.0 nmol min^{-1} (mg [dry weight])$^{-1}$, compared to 16.4 nmol min^{-1} (mg [dry weight])$^{-1}$ obtained with the control strain 13032(pXMJ19). Moreover, L-serine utilization by the *sdaA* deletion mutant was reduced to 8.7 nmol min^{-1} (mg [dry weight])$^{-1}$. This shows that L-SerDH contributes significantly to L-serine utilization. However, due to the clearly observable residual L-serine consumption by the *sdaA* deletion mutant, we analyzed the fate of L-[U-^{13}C]serine in the same manner as that described for the wild type. Indeed, the ^{13}C enrichments in cellular metabolites determined by two-dimensional (^{1}H, ^{13}C) HSQC NMR were comparable to that for the wild type. For instance, for the multiplet signals obtained for C-2 of alanine, $s = 6\% \pm 2\%$, $d_{-1} = 6\% \pm 2\%$, $d_{+1} = 7\% \pm 3\%$, and dd = 81% ± 4%. This demonstrates that L-serine is still converted via pyruvate to L-alanine in the deletion mutant. Furthermore, other multiplet signals also were almost identical. The dd obtained for C-2 of aspartate was 43% ± 1% in the mutant compared to 47% ± 1% in the wild type. This indicates that the pyruvate resulting from L-serine is used to synthesize oxaloacetate by the anaplerotic pyruvate carboxylase (39, 40), which is further converted to L-aspartate. These data confirm that besides L-serine dehydratase, further L-serine-converting reactions yielding pyruvate are operating.

Global gene expression analysis. To get access to such reactions, we used DNA microarrays to probe for altered mRNA levels as a further approach to identify L-serine-degrading reactions (26). To enable a high intracellular L-serine concentration, we added the tripeptide Ser-Ser-Ser, which is hydrolyzed upon uptake and thus ensures a high intracellular L-serine concentration (51). Total RNA was isolated 70 min after peptide feeding, and relative RNA levels were determined by hybridization to DNA microarrays representing 95.5% of the open reading frames of *C. glutamicum* (NCBI NC003450). In total only a relatively small number of genes (69 genes) exhibited an mRNA level exceeding a twofold alteration, with 62 of them showing increased expression due to peptide addition. Table 2 lists expression changes of eight open reading frames (ORFs) related to amino acid metabolism. NCgl2241 encodes the putative ATP-binding protein of an oligopeptide transporter. Its increased mRNA level could thus be a direct consequence of the peptide present to enable its efficient uptake. With the others, a correlation to L-serine is probably less apparent (see Discussion). Most interestingly, the cystathionine β-lyase mRNA level (*metC*) is increased 2.4-fold, and the respective enzyme of L-methionine synthesis (24), catalyzing a β-elimination reaction, has been reported to have a broad substrate specificity in *C. glutamicum* and in *E. coli*, also reacting with L-cysteine, which is structurally related to L-serine (1, 45, 59).

Activity of cystathionine β-lyase. Based on these observations, the possibility that cystathionine β-lyase also uses L-serine as a substrate was tested. We therefore used plasmid pSL173, encoding *metC* (24), to overexpress *metC* in *C. glutamicum* 13032Δ*sdaA*. The resulting 13032Δ*sdaA*(pSL173) strain and the control 13032Δ*sdaA*(pZ1) strain were grown in minimal medium with 200 mM glucose as the carbon source. Enzyme activities were determined with crude extracts of both strains with the natural substrate cystathionine (5 mM) as well as with L-cystine (5 mM), L-cysteine (50 mM), and L-serine (50 mM), and pyruvate formation was quantified. With cystathionine and L-cystine, the control exhibited comparable specific activities of 0.13 and 0.11 μmol min^{-1} mg^{-1}, respectively. With *metC* overexpressed, the specific activities were increased to

TABLE 2. ORFs showing altered relative mRNA levels in response to L-serine in wild-type *C. glutamicum*

ORF	NCBI no.[a]	Function or annotation[b]	Gene[c]	Increase in mRNA level in response to L-serine (fold)
2793	NCgl1222	Acetolactate synthase large chain	*ilvB*	4.6
3236	NCgl2133	Glutamine synthetase I	*glnA*	2.4
3118	NCgl2227	Cystathionine β-lyase	*metC*	2.4
844	NCgl0371	Strong similarity to *purU (Shigella flexneri)*, encoding formyltetrahydrofolate deformylase		2.3
1500	NCgl0895	Similarity to *livM (E. coli)*, encoding a leucine transport protein		2.2
3696	NCgl2139	Threonine synthase	*thrC*	2.1
1396	NCgl0811	Inositol monophosphatase family protein; similarity to *cysQ (E. coli)*		2.0
3102	NCgl2241	Strong similarity to *oppD (L. lactis)*, encoding an oligopeptide transport ATP-binding protein		1.9

[a] Number for the corresponding ORF in the revised *C. glutamicum* genome in the National Center for Biotechnology Information (NCBI) database.
[b] Most similar gene in public databases.
[c] Only characterized *C. glutamicum* genes are given.

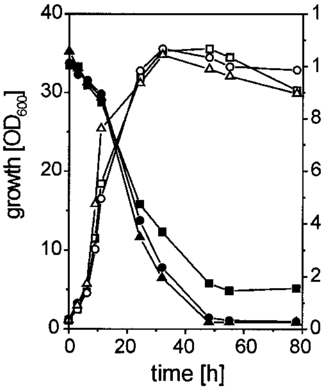

FIG. 5. Growth (open symbols) and L-serine consumption (closed symbols) of different *C. glutamicum* strains in minimal medium containing 100 mM glucose plus 100 mM L-serine. Triangles, 13032Δ*sdaA*(pSL173); circles, 13032Δ*sdaA*(pZ1); squares, 13032Δ*sdaA*Δ*metC*.

1.05 and 1.34 μmol min^{-1} mg^{-1} for the respective substrate. With L-cysteine as a substrate and an extract of the overexpressing strain, the specific activity was 0.35 ± 0.07 μmol min^{-1} mg^{-1}, corroborating the finding that cystathionine β-lyase has L-cysteine desulfhydration activity (59). Also with L-serine a significant activity of 0.04 ± 0.01 μmol min^{-1} mg^{-1} was determined. This result showed that cystathionine β-lyase of *C. glutamicum* is capable of deaminating L-serine to pyruvate in vitro, as was shown for *E. coli* (2) and *Neurospora crassa* (8).

To test the influence of cystathionine β-lyase on L-serine degradation, a Δ*sdaA* Δ*metC* double mutant was constructed. The 13032Δ*sdaA*Δ*metC* strain was cultivated on minimal medium containing 100 mM glucose plus 100 mM L-serine, and growth and L-serine utilization were monitored (Fig. 5). The *metC*-overexpressing 13032Δ*sdaA*pSL173 strain and the 13032Δ*sdaA*pZ1 strain were analyzed as controls. After 55 h of cultivation, the remaining concentration of L-serine in the medium was 15 mM for the double-mutant 13032Δ*sdaA*Δ*metC* strain, whereas for the 13032Δ*sdaA*(pSL137) and 13032Δ*sdaA*(pZ1) strains, the remaining concentration for both cultures was below 3 mM. This result shows that chromosomally encoded *metC* has in fact a significant influence on L-serine degradation.

DISCUSSION

Cometabolism of L-serine by *C. glutamicum* can be divided into distinct phases and therefore clearly differs from the strict parallel metabolism of glucose and acetate (61) as well as from the sequential metabolism of glucose and glutamate (25). In phase 1 at a growth rate of 0.30 h^{-1}, the demand for L-serine for cell material formation, including the many metabolites derived from this amino acid, is 7.5 nmol min^{-1} (mg [dry weight]$)^{-1}$ (28). Thus, about half of the L-serine consumed could be directly incorporated into cell material in this phase, resulting in an increased growth yield. This is consistent with the finding that a large amount of L-serine is metabolized via pyruvate. Integration of L-serine into biomass occurs even though *C. glutamicum* is not able to grow on L-serine as a sole carbon source. A similar situation is observed with *Campylobacter jejeuni*, which utilizes this amino acid in the presence of pyruvate, resulting in an increased growth yield of 46% (57). Also, it has been reported for *E. coli* that L-serine as an auxiliary carbon source results in a greatly increased growth yield (33). In phase 2, L-serine utilization was decoupled from growth, and thus, other reasons for L-serine utilization must exist, as, for instance, its degradation to derive energy for maintenance. Indeed, an *E. coli* mutant is known which has a growth advantage in the stationary phase and which at the same time has an increased capability to grow with L-serine, enabling generation times of 6.7 h instead of 61.2 h (63). Another discernible consequence with *C. glutamicum* is that after consumption of glucose, the cultures which received L-serine appear to maintain a higher OD_{600} for a longer time than cultures without L-serine (compare Fig. 1A and B). Considering that after depletion of glucose, L-serine is still consumed, its metabolism might serve to increase the maintenance of the culture under starvation conditions. Although wild-type *E. coli* is not capable of effective L-serine utilization (47), mutants with increased utilization are known, where mutations increase the fitness in starved cultures (58).

As shown by incorporation of ^{13}C-labeled L-serine in protein, the advantage of coutilization consists in part in a reduced requirement for the synthesis of L-serine from glucose. However, as revealed by the high uptake rate, together with the labeling information, the Δ*sdaA* Δ*metC* mutant studies, and the enzyme analysis, the largest part of externally added L-serine is converted to pyruvate. This was also shown for *L. lactis*, where carbon flux analysis with ^{14}C-labeled L-serine revealed that 72% of the initial L-serine flux is found in metabolites originating from pyruvate (35). While *L. lactis* has a restricted metabolism and is therefore necessarily dependent on the cometabolism of L-serine with glucose, an advantage for *C. glutamicum* in cometabolism could be utilization of the generated pyruvate for biosynthetic purposes as well as its oxidation to produce energy for maintenance. Although the current label information alone cannot give an answer to whether substantial quantities of L-serine are oxidized via pyruvate up to carbon dioxide, thus contributing to energy generation, it is clear that a large part of pyruvate contributes to synthesis of cell material, since the ^{13}C label from L-serine-derived pyruvate is present in phosphoglycerate, triose phosphates, and even pentose phosphates (Table 1). Therefore, it can be concluded that the pyruvate skeleton is converted to glyceraldehyde-3-phosphate in vivo in sufficiently high concentrations to enable reverse metabolic flux from pyruvate up to fructose-1,6-bisphosphate.

As found for other L-SerDHs (12, 47, 57), the enzyme from *C. glutamicum* has high substrate specificity and does not accept substrates other than L-serine. Unlike the pyridoxal-5-phosphate-containing D-serine and L-threonine dehydratases, it is most likely that all highly specific bacterial L-SerDHs contain an [4Fe–4S]-cluster as the prosthetic group (11). The *C. glutamicum* L-SerDH polypeptide also exhibits three of the four possible conserved cysteine residues (9), and the activity is dependent on reducing conditions. Accordingly, the L-SerDH is oxygen sensitive, as demonstrated for the enzymes of the strictly anaerobic organisms *P. asaccharolyticus* (12) and *Clostridium propionicum* (16) as well as for those of the microaerophilic bacterium *C. jejeuni* (57). Although *C. glutamicum* possesses a functional L-serine dehydratase, no activity was observed in crude extracts of the wild type under the chosen conditions, but since deletion of the enzyme resulted in 30% decreased L-serine degradation, it must be concluded that the enzyme is active in vivo in the wild type. This is similar to the situation for *E. coli*, where activity in crude extracts was detectable only after treatment with iron and reducing agents (53, 34). Even though this dependence was confirmed for the overexpressed *C. glutamicum* L-SerDH, the possibility that other, yet unknown factors are necessary to stabilize the activity cannot be ruled out.

The respective L-SerDHs of *P. asaccharolyticus* (12), *C. propionicum* (16), and *C. jejeuni* (57) were isolated from cells grown on complex media containing L-serine, implying that coutilization of this amino acid might occur. Whereas these organisms have a restricted metabolism, it is astonishing that *E. coli* is not able to grow on L-serine, although it has three L-SerDHs, encoded by *sdaA* (53), *sdaB* (50), and *tdcG* (15), which are subject to complex regulation by a number of different effectors (for a review, see reference 47). With the *sdaA*-overexpressing strain of *C. glutamicum*, we found growth on L-serine, albeit at a lower growth rate and final biomass yield than on pyruvate (data not shown). This indicates that although the specific activity of L-SerDH in this strain would be sufficient for growth on L-serine comparable to growth on pyruvate, there are either regulatory phenomena or L-SerDH-specific biochemical features that prevent the entire conversion of L-serine. Since the conversion of L-serine to pyruvate liberates ammonium, a high internal ammonium concentration might be toxic for the cell as well.

This assumption is further substantiated by the result of the DNA-Chip experiment, where the *glnA* transcript of glutamine synthetase I, the only ammonia-fixing enzyme in *C. glutamicum* (20), is increased 2.4-fold at an elevated intracellular L-serine concentration. The notably increased level of *ilvB* (acetolactate synthase) could be an indirect consequence of an increased pyruvate pool, since the synthase utilizes two pyruvate molecules. Interestingly, the *metC* transcript level is also increased, presumably as an indirect consequence of increased L-serine concentrations. This gene encodes the cystathionine β-lyase of L-methionine synthesis (24), but we could clearly demonstrate that it is also able to deaminate L-serine to pyruvate in vitro. This result shows that the enzyme not only degrades sulfur-containing amino acids (59), but is also able to degrade L-serine in a β-elimination reaction, as has been found for cystathionine β-lyases from *N. crassa* (8) and *E. coli* (2). Deletion of the *metC* gene in the Δ*sdaA* background resulted in a strain that degraded significantly less L-serine than the Δ*sdaA* single mutant, demonstrating that L-serine is also converted by this enzyme in vivo. Nevertheless, L-serine degradation still occurs in that strain, indicating that at least one additional activity is present. For instance, threonine dehydratase (55) or the β-subunit of L-tryptophan synthase from *E. coli* and *Salmonella* serovar Typhimurium (4, 62) is known to deaminate L-serine to pyruvate in vitro. Whether these enzymes are involved in L-serine degradation in *C. glutamicum* remains to be elucidated.

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