Reduced Folate Supply as a Key to Enhanced L-Serine Production by Corynebacterium glutamicum

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The amino acid L-serine is required for pharmaceutical purposes, and the availability of a sugar-based microbial process for its production is desirable. However, a number of intracellular utilization routes prevent overproduction of L-serine, with the essential serine hydroxymethyltransferase (SHMT) (glyA) probably occupying a key position. We found that constructs of Corynebacterium glutamicum strains where chromosomal glyA expression is dependent on P_{lac} and lacI^P are unstable, acquiring mutations in lacI^P, for instance. To overcome the inconvenient glyA expression control, we instead considered controlling SHMT activity by the availability of 5,6,7,8-tetrahydrofolate (THF). The pabAB and pabC genes of THF synthesis were identified and deleted in C. glutamicum, and the resulting strains were shown to require folate or 4-aminobenzoate for growth. Whereas the C. glutamicum ΔsdaA strain (pserACB) accumulates only traces of L-serine, with the C. glutamicum ΔpabBCΔsdaA strain (pserACB), L-serine accumulation and growth responded in a dose-dependent manner to an external folate supply. At 0.1 mM folate, 81 mM L-serine accumulated. In a 20-liter controlled fed-batch culture, a 345 mM L-serine accumulation was achieved. Thus, an efficient and highly competitive process for microbial L-serine production is available.

1L-serine is a nonessential amino acid but plays an important role in stabilizing the blood sugar concentration in the liver (16). It relates, furthermore, to many other substances, including sphingosine and the phosphatides, which are part of the myelin covering of the nerves, as well as the formation of activated C_1 units used for a number of anabolic processes (20). Therefore, L-serine is present in selected infusion solutions and also has other applications. For instance, it is an ingredient of skin lotions to ensure a proper hydration status. The total annual demand for L-serine is estimated to be 300 tons (5).

The production processes currently used still rely on the extraction of L-serine from protein hydrolysates or from molasses, as well as on the enzymological conversion of glycine plus a C_3 compound, like methanol, to L-serine. The latter uses the reverse reaction of the serine hydroxymethyltransferase (SHMT) (6). Thus, an enzymatic system has been designed to convert glycine plus formaldehyde to L-serine (4). The cellular systems assayed employed, among other things, resting cells of methanol-utilizing bacteria, such as Hyphomicrobium methylotrorum, where L-serine formation from glycine plus methanol was achieved (6). In such a system, up to 45 g liter^{-1} L-serine accumulation was possible, but only at a glycerol yield of 50%, thus making the system less attractive. Also, alginate-entrapped cells of Corynebacterium glycophilum were used for L-serine formation from glycine (21). It is self-evident that it would be most profitable to directly convert cheap sugar into L-serine. Although microbial processes for amino acid production are in general advancing quickly, attempts to develop L-serine producers have as yet yielded merely strains that form traces of this amino acid (7, 25).

We are engaged in exploring the production capabilities of Corynebacterium glutamicum, including flux directions, flux quantifications, and metabolite export, with the focus so far on L-lysine, L-isoleucine, L-valine, L-threonine, and D-pantothenate (2). Due to the apparent lack of a convincing strain for L-serine formation, we recently also explored the metabolism of this amino acid in C. glutamicum. We studied in detail the 3-phosphoglycerate dehydrogenase, SerA, catalyzing the initial reaction of the three-step pathway of L-serine biosynthesis (13). As a result of deletion of 197 amino acyl residues of the carboxy-terminal end of the SerA polypeptide, the 3-phosphoglycerate dehydrogenase activity is no longer inhibited by L-serine. Furthermore, we identified a high capacity of C. glutamicum to degrade L-serine, which is strongly reduced upon deletion of the sdaA-encoded L-serine dehydratase (11). Degradation is apparently a key issue in microbial L-serine formation, certainly with respect to the central role of this amino acid in metabolism. This agrees with the observation that overexpression of engineered serA together with serB and serC in C. glutamicum yielded only traces of L-serine (14). However, when the L-serine dehydratase gene was additionally deleted, a transient accumulation of up to 16 mM was observed. A further and substantial increase of up to 86 mM occurs when the SHMT activity is reduced, apparently by reducing L-serine degradation to glycine plus 5,10-methylene-tetrahydrofolate. Since the glyA-encoded SHMT is essential (14), a reduction of SHMT activity was required by a controllable promoter integrated in the chromosome. However, this strain is inconvenient, since it requires the control of iso-

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propyl-thio-β-D-galactopyranoside for production and tends to be unstable. We here describe our successful attempts to further improve the strain and to control the essential SHMT activity by a novel physiological approach.

MATERIALS AND METHODS

Bacteria, plasmids, and growth. The bacterial strains and plasmids used in this work are listed in Table 1. Luria-Bertani medium (9) was used as the standard medium for Escherichia coli, while brain heart infusion medium (Difco) was used as complex medium for C. glutamicum. As minimal medium, CGXII was used to generate 13032 C. glutamicum ATCC 13032 with the truncated pabAB genes, resulting in strain 13032 pabAB. Alternatively, pabAB DEL C was made in a similar manner. In the first PCR, primers pabAB-del-A and pabAB-del-D were used to amplify a 563-bp fragment of the 5′ end of pabAB and primers pabAB-del-A and pabAB-del-D were used to amplify a 528-bp fragment of the 3′ end of pabAB. The resulting PCR fragments were used in a second PCR with pabAB-del-A and pabAB-del-D as primers. The resulting 1,122-bp fragment was ligated into the BamHI restriction site of the mobilizable E. coli vector pK19mobSacB, leading to pK19mobSacB-pabAB. This was used to replace the intact chromosomal pabAB genes in C. glutamicum ATCC 13032 with the truncated pabAB genes, resulting in strain 13032 ΔpabAB.

Similarly, pK19mobSacB-pabAB was made for pabC deletion. In the first PCR, primers pabC-del-A and pabC-del-D were used with pabC-del-C and pabC-del-D, respectively. The resulting DNA was used in the second PCR with primers pabC-del-A and pabC-del-D, and the resulting 1,178-bp fragment was ligated into the BamHI site of pK19mobSacB to generate pK19mobSacB-pabC. This was used to generate 13032 ΔpabC.

Plasmid pK19mobSacB-pabC was made in a similar manner. In the first PCR, primers pabC-del-A and pabC-del-D were used with pabC-del-C and pabC-del-D, respectively. The resulting DNA was used in the second PCR with primers pabC-del-A and pabC-del-D, and the resulting 1,178-bp fragment was ligated into the BamHI site of pK19mobSacB to generate pK19mobSacB-pabC. This was used to construct strain 13032 ΔpabAB.

Product formation. For fed-batch fermentations, a 20-liter stirred-tank reactor (Bioengineering, Wald, Switzerland) was used. Cells were pregrown in shake...
RESULTS

Stability of reduced glyA expression. We previously found that for high l-serine accumulation, among other aspects, a reduced SHMT activity is necessary (14). This was achieved by placing in the chromosome the SHMT-encoding glyA gene under the control of P_{lac}, thus greatly reducing the SHMT activity if no isopropyl-thio-β-D-galactopyranoside is present. This leads to high l-serine formation while simultaneously reducing growth of the 13032 glyA::pK18mob strain. As a further means of characterizing the engineered strain, from another 10 single colonies, primer pairs amplifying the glyA locus as present in the wild type. We nevertheless found that 17 fermentations on the 20-liter scale, only 4 displayed the expected high l-serine formation. In order to identify the reason for this apparent instability, the strain was cultivated in brain heart infusion medium on a 50-ml scale without isopropyl-thio-β-D-galactopyranoside and inoculated six times in series in the same medium, with each cultivation lasting 8 to 15 h. From the final culture, single colonies were derived and 10 of them analyzed by PCR with primer pairs amplifying the glyA gene and parts of the repressor LacI. In three clones, the identical transition of C to T was detected, resulting in the exchange of Ala in position 13 of the LacI repressor for Thr. In one further clone, T was mutated to C in sequences upstream of lacI. For a further confirmation in two clones with mutated LacI, the SHMT activity was determined. It was 48 and 40 nmol min^{-1} mg (protein)^{-1}, respectively, instead of 8 nmol min^{-1} mg (protein)^{-1} determined for the control. Altogether this shows that the strain with SHMT activity controlled by P_{lac} is explicitly prone to chromosomal mutations and rearrangements, thus making the strain unsuitable for large-scale fermentations.

Analysis of the glyA locus. Requiring a more stable and convenient strain, we searched for an alternative for controlling SHMT activity. Since SHMT activity requires pyridoxal 5'-phosphate as well as 5,6,7,8-tetrahydrofolate (Fig. 1) to catalyze l-serine conversion to 5,10-methylene tetrahydrofolate and glycine, we considered controlling SHMT activity with a limited supply of cells with 5,6,7,8-tetrahydrofolate. A genome analysis revealed two open reading frames at the 3' end of glyA (NCgl0954) and transcribed in the same direction, putatively involved in tetrahydrofolate synthesis (Fig. 1). The N-terminal part of NCgl0955 shows strong sequence similarities (43% identity) to the para-aminobenzoate synthase component I (PabA) of E. coli, whereas its C-terminal part resembles the para-aminobenzoate synthase component II (PabB; 38% identity). Apparently, in C. glutamicum both polypeptides involved in the synthesis of para-aminobenzoate are fused, which is also the case for Corynebacterium efficiens and Corynebacterium diphtheriae (not shown) but not in the related species Mycobacterium tuberculosis and Mycobacterium bovis. The product of the PabAB activity is 4-amino-4-deoxychorismate, and NCgl0956 might encode the lyase, PabC, subsequently converting this product within the tetrahydrofolate pathway into para-aminobenzoate and pyruvate (3).

Construction and analysis of folate auxotrophs. Using the appropriate allelic-exchange vectors, the genes pabAB, pabC, and pabABC, respectively, were deleted from the chromosome of 13032ΔxdaA (see Materials and Methods). The resulting strains were streaked on minimal medium CGXII without further additions to assay for their folate auxotrophy. However, there was no visible difference in growth from that of wild-type cells. After a subsequent transfer onto a further minimal medium plate, colonies were somewhat smaller than the control, but only after a third transfer was no growth of strain 13032ΔxdaAΔpabABC and 13032ΔxdaAΔpabAB apparent, whereas there was still limited growth of 13032ΔxdaAΔpabC (Fig. 2). The growth of all three mutants could be fully restored by supplementing either 1 mM folate or 4-aminobenzoate. The partial growth of

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**FIG. 1.** Synthesis of tetrahydrofolate and its linkage to serine hydroxymethyltransferase. At the top is shown the genomic region of *C. glutamicum* encompassing nucleotides 1051865 to 1056075 of NC_006958, including glyA, pabABC, and pabC. Below are shown the corresponding enzymatic steps of tetrahydrofolate synthesis (simplified) and methylene tetrahydrofolate synthesis.
the ΔpabC mutant could indicate that the substrate of the pabC-encoded enzyme is also nonenzymatically converted in C. glutamicum, which is in accord with results reported by Tewari et al. (23) showing that the intermediate 4-aminobenzoate is labile and decomposes spontaneously to 4-aminobenzoate.

**Growth and L-serine accumulation by 13032ΔsdaAΔpabABC (psrACB).** Based on the observation that the pabABC deletion was more favorable than that of pabC, C. glutamicum 13032ΔsdaAΔpabABC was transformed to tetracycline resistance with psrACB to determine its L-serine production capabilities. The resulting strain was cultivated overnight in complex brain heart infusion medium and subsequently transferred to minimal medium CGXII without any addition of folate. After growing for 10 h, cells of this culture were used to inoculate the main culture (CGXII) with different folate concentrations. The resulting growth curves are shown in Fig. 3 (top). Without the addition of folate and with the lowest folate concentration of 0.01 mM, growth of strain 13032ΔsdaAΔpabABC (psrACB) was severely impaired and the growth rate did not exceed 0.1 h⁻¹. The weak growth without the addition of folate was due to traces of folate still present in the inoculum, since cells taken at the end of the cultivation to inoculate a new culture did not grow. The addition of 1 mM folate fully restored growth of the auxotrophic strain so that it was almost identical to that of its ancestor strain, 13032ΔsdaA (psrACB), and the intermediate concentrations of 0.1 and 0.25 mM enabled partial growth with respect to both rate and final cellular optical density reached.

Whereas with the control strain 13032ΔsdaA (psrACB), the L-serine concentration was in the micromolar range, with 13032ΔsdaAΔpabABC (psrACB) and 1 mM folate, up to 1.8 mM L-serine accumulated (Fig. 3, bottom). Lowering the folate concentration to 0.25 mM drastically increased the L-serine accumulation to concentrations of up to 60 mM. Even further-increased L-serine concentrations, up to 94 mM, were obtained upon reducing the folate concentration to 0.1 mM. High final L-serine titers were also obtained at a concentration of 0.01 mM and without folate addition, although this required extended production times.

**L-serine accumulation on an increased scale.** As is evident, reduced folate availability is promising for assaying for L-serine formation on a larger scale. In order to also investigate the properties of the strain constructed under such conditions and in a less-defined medium probably more relevant for industrial conditions, the performance of strain 13032ΔsdaAΔpabABC (psrACB) was evaluated by using a 20-liter reactor based on corn steep liquor medium. The medium contained 35 g liter⁻¹ solid corn steep liquor plus initially 15 g liter⁻¹ fructose. The minimum dissolved oxygen concentration was set to 50% saturation to ensure no oxygen limitation. As can be seen in Fig. 4, inoculation of the reactor with cells derived from the preculture CGXII enabled rapid growth, up to a maximum specific growth rate of 0.25 h⁻¹. L-Serine formation occurred from the beginning up to a final concentration of 345 mM, suggesting a suitable folate supply in the culture due to corn steep liquor use, which can be assumed to contain at least traces of folate. The maximum oxygen uptake rate was about 110 mol liter⁻¹ h⁻¹, which was present at the end of the logarithmic growth of the culture. The maximal specific productivity was 1.45 mmol g⁻¹ h⁻¹, and the volumetric productivity was about 1.4 g liter⁻¹ h⁻¹.

![FIG. 2. Growth of mutants of C. glutamicum deleted of genes of folate biosynthesis. Growth of the corresponding mutants compared to that of the control (WT) without vitamin addition (w/o), plus 1 mM 4-aminobenzoate (+pAB), or plus 1 mM folate (+Fol). All strains were isogenic with respect to sdaA deletion.](http://aem.asm.org/)

![FIG. 3. Growth (top) and L-serine production (bottom) of C. glutamicum 13032ΔsdaAΔpabABC (psrACB) in minimal medium containing different folate concentrations (●, 0 mM; ○, 0.01 mM; ▲, 0.1 mM; ●, 0.25 mM; ●, 1 mM). The control strain, 13032ΔsdaA (psrACB), did not receive folate (■). OD, optical density.](http://aem.asm.org/)
DISCUSSION

The SHMT is essential in _C. glutamicum_, as is also the case for other organisms (14). Besides glycine, the enzyme activity generates the activated one-carbon units required for a number of cellular processes, for instance, for the synthesis of formylated methionine bound to the initiator tRNA^Met^, which is necessary for translation initiation. This cellular demand cannot be bypassed by external metabolite addition. Furthermore, SHMT activity is involved in the generation of a number of metabolites and reactions, and it is therefore not surprising that the total carbon flux towards L-serine on minimal medium with glucose as the substrate amounts to 7.5%, as estimated for _C. glutamicum_ (10). Earlier estimates for _E. coli_ determined that as much as 15% of the carbon assimilated from glucose involves L-serine (15). Due the high demand and its key position in cellular physiology, L-serine has to be regarded as an intermediate of the central metabolism (20). These aspects altogether might explain the strong selective pressure against its cellular reduction is the key to achieving L-serine accumulation. As our previous studies have shown, SHMT activity clearly has a major impact (11, 13, 14). The reduction of glyA expression alone resulted in an approximately 1 mM accumulation of L-serine (13), which was not the case upon deletion of the serine dehydratase gene _sdaA_. As the present work has shown, limitation of folate is an ideal tool for limiting L-serine conversion and directing its flux towards extracellular L-serine. Similarly, control of d-pantothenate availability is known to influence the formation of selected amino acids. The basis is that d-pantothenate is a constituent of coenzyme A, and a reduced coenzyme A availability results in reduced activity of the pyruvate dehydrogenase, thus limiting pyruvate decarboxylation. This has been exhaustively used in developing a _C. glutamicum_ strain producing L-valine, which is made up of two pyruvate molecules (17). It should be noted that vitamin limitations in strain constructions are entirely different from the well-established “pathway tailoring” by removing competing reactions or removing bottlenecks (1). The reason is that at a fixed low vitamin concentration, the cell as a catalyst is still active but its proliferation reduced, which might affect in many ways the physiology of the cell. For instance, in _E. coli_ a YgfZ protein is present, which may be a folate-dependent regulatory protein involved in C-1 metabolism (12), and a similar protein is present in _C. glutamicum_ (NCgl2492).

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REFERENCES


