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Enhancing Production of L-Serine by Increasing the *glyA* Gene Expression in *Methylobacterium* sp. MB200

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Abstract Microbial fermentation using methylotrophic bacteria is one of the most promising methods for L-serine production. Here we describe the metabolic engineering of a *Methylobacterium* strain to increase the production of L-serine. The *glyA* gene, encoding serine hydroxymethyltransferase (SHMT), was isolated from the genomic DNA of *Methylobacterium* sp. MB200, using a DNA fragment encoding *Methylobacterium extorquens* AM1 SHMT as a probe, and inserted into the vector pLAFR3. The resulting construct was transformed into *Methylobacterium* sp. MB200 using triparental mating. The genetic-engineered strain, designated as *Methylobacterium* sp. MB202, was shown to produce 11.4±0.6 mg/ml serine in resting cell reactions from 30 mg/ml wet cells, 20 mg/ml glycine, and 70 mg/ml methanol in 2 days, representing a 4.4-fold increase from that of the wild strain. The results demonstrated the potential for improving L-serine production by manipulating the *glyA* in bacteria and should facilitate the production of L-serine using *Methylobacterium* sp. strains.

Keywords *Methylobacterium* sp. · *glyA* · Serine hydroxymethyltransferase (SHMT) · L-Serine · Genetic engineering

Introduction

Methanol, a compound easily synthesized and widely used as an industrial chemical, is an attractive raw material for biotechnology industry because of its abundance, high purity, and low cost. Therefore, methanol-utilizing bacterial strains are not only of ecological relevance with respect to the global carbon cycle, but also interesting candidate organisms for the production of amino acids such as L-serine, vitamins, glyoxylate, cytochromes, coenzymes, polyhydroxyalkanoates, and proteins [1–3]. Methanol

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has been widely exploited for use in amino acid production, especially in the production of L-serine. L-Serine is used as a component of amino acid infusion, an additive of cosmetics, and a starting material for the enzymatic production of tryptophan. It is also used in the biosynthesis of purines, pyrimidines, and other amino acids. Therefore, it is of great economic interest to develop a cost-effective method for L-serine production as the demand for it continues to grow.

A number of microorganisms, such as *Methylobacterium* sp. [4], *Hyphomicrobium* sp.[5–7], *Pseudomonas* sp.[8], *Sarcina albida* [9], and *Nocardia butanica* [10], utilize methanol as the carbon source for the biosynthesis of L-serine. Of them, methylotrophs are the most important producers because of their high rate of bioconversion [4]. Many methylotrophic bacteria possess serine pathway to assimilate C1 compounds [11]. In this cycle, serine hydroxymethyltransferase (SHMT), encoded by *glyA* gene, plays an important role as the first enzyme in the transfer of formaldehyde to glycine, yielding L-serine as the main intermediate in the pathway (Scheme 1). It was also shown that the recombinant SHMT itself is sufficient to synthesize L-serine from formaldehyde and glycine.

In this study, we report that increasing the SHMT expression level by increasing the copy numbers of *glyA* gene in *Methylobacterium* sp. through genetic engineering could dramatically improve the yield of L-serine production compared with the wild strain.

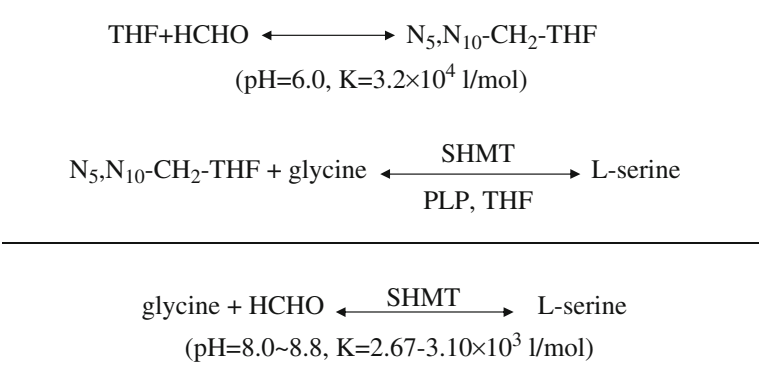
Materials and Methods

Materials

Restriction enzymes, pGEM T-easy cloning vector, T4 DNA ligase, and DNA polymerase were purchased from Promega (CA, USA). Kits for DNA extraction, plasmid extraction, and DNA purification were obtained from Qiagen (Valencia, CA, USA). All chemicals including L-serine and pyridoxal-5-phosphate were obtained from commercial sources and were of the highest grade.

Bacterial Strains and Plasmids

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



Scheme 1 The reaction for synthesizing L-serine from glycine and formaldehyde

Table 1 Plasmids and strains used in this study.

Strain/plasmid	Relevant characteristics	Source
Bacteria strains		
Methylobacterium.sp MB200	Wild-type strain, Nm^r	Laboratory stock
Methylobacterium.sp MB202	MB200 containing pLAFRg	This study
E. coli DH5α	SupE44,Δ*lacU*169 (ψ80*lacZ*ΔM15) hsdR17 RecA1 endA1 gyrA96 thi-1 relA1	[12]
E. coli Tuner (DE3)	Expression strain, $Carb^r$, Amp^r	Novagen
Plasmids		
pGEM–T easy	T-A cloning vector, Amp^r	Promega
pET-blue	Expression vector, Cm^r	Novagen
pRK2013	Helper plasmid, Mob^+, ColE1, Km^r, Tra^+	[13]
pLAFR3	Broad-host range cloning vector, Mob^+, Tra^-, cosmid, Tc^r	[14]
pLAFRg	pLAFR3 containing *glyA* gene	This study

Media and Growth Conditions

Methylobacterium sp. MB200 was grown at 32 °C in an ammonium mineral salt medium (MM medium) supplemented with 1.2% methanol as the sole carbon source [15]. *Escherichia coli* strains DH5α and Tuner (DE3) were cultivated in Luria–Bertani (LB) medium at 32 °C and 37 °C, respectively. Antibiotics were added where appropriate to the medium at the following final concentrations: ampicillin (50 μg/ml), kanamycin (25 μg/ml), carbencillin (200 μg/ml), choramphenicol (50 μg/ml), nalidixic acid (50 μg/ml), and tetracycline (75 μg/ml).

DNA Manipulation

Isolation of genomic DNA and plasmid, DNA ligation and digestion were performed according to the suppliers' instructions for the kits or reagents. Other DNA manipulations were performed according to standard procedures [16].

Sequencing of *glyA* Gene

The DNA probe was prepared by polymerase chain reaction (PCR) using the genomic DNA of *Methylobacterium* sp. MB200 as the template and primers P1(5′-CGACTCCTTCTTCTCGGCTC-3′) and P2 (5′-CGGCGAAGTGGGCCATGTCG-3′), designed according to the *glyA* gene sequence of *Methylobacteria extorquens* AM1 (GenBank accession number L33463), under the following conditions: one cycle at 95 °C, 5 min; 30 cycles of each at 94 °C, 40 s, 55 °C, 30 s, and 72 °C, 30 s; and one cycle at 72 °C, 3 min. The resulting PCR product, 650 bp (designated as pg), was sequenced using an ABI 377 sequencer (AME Bioscience, Toroed, Norway) and used as the probe for Southern blot hybridization. The genomic DNA of *Methylobacterium* sp. MB200 was digested separately with *Bam*HI, *Pst*I, *Sac*I, *Sal*I, and *Hin*dIII. The digests were fractionated on an agarose gel and then transferred to a nylon membrane. The DNA probe was labeled with P^{32}-dCTP by Klenow random primer extension. The hybridization was performed overnight at 65 °C. The nylon membranes were washed under high-stringency conditions and exposed to an erased

storage phosphor screen, which was scanned with a Typhoon 9410 Scanner (Amersham Biosciences, USA). Based on the result of Southern blot hybridization, *Bam*HI was chosen for the construction of genomic library because it only produced one single fragment of 3–4 kb; it also demonstrated that there is only one copy of *glyA* gene in wild-type strain MB200. After digestion of the genomic DNA with *Bam*HI, the 3–4 kb DNA fragment was gel-purified and ligated to pGEM-3Zf (+) to prepare the genomic library. Approximately 3,000 clones were screened by colony hybridization in situ using above procedure, and one of the positive clones was analyzed by sequencing.

Expression of *glyA* in *E. coli*

A 1,305-bp DNA fragment containing the coding region of *Methylobacterium* sp. MB200 *glyA* was amplified by PCR using the 3–4 kb DNA fragment encoding SHMT obtained above as the template and primers P3 (5′-CTA *GGATCC* CAT GAG CGC CGG AAC TG-3′) and P4 (5′-ACG *AAGCTT* GTT GTA GAT CGG GAA GC-3′) (*Hin*dIII and *Bam*HI sites are in italics). The PCR conditions were as follows: one cycle at 95 °C, 5 min; 30 cycles each at 94 °C, 30 s, 54 °C, 30 s, and 72 °C, 1 min; and one cycle at 72 °C, 3 min. The PCR product was confirmed by sequencing, digested with *Hin*dIII and *Bam*HI, and cloned into the expression vector pET-blue. The resulting plasmid (pET:og) was transformed into *E. coli* Tuner (DE3) to construct the expression clone Tuner (pET:og). Tuner (pET:og) was cultured to optical density (OD) 0.5–1.0, induced by 0.8 mM isopropyl β-D-1-thiogalactopyranoside for 4 h. Cells were harvested by centrifugation and lysed by sonication using a JY92-II ultrasonic cell disrupter (Ningbo Kesheng Instrument Factory, Ningbo, China) operating at a power level of 300–400 W, in 30 cycles of 10-s bursts separated by 10-s intervals. After centrifugation at 14,000×*g*, the cell-free supernatant was recovered and stored at 4 °C until analyzed. SHMT was purified using a Ni-NTA column (Novagen, Madison, WI, USA) according to the manufacturer's instructions, and the purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or stored at 4 °C.

Methylobacterium sp. MB202 Strain Construction

The *glyA* gene in the pET:og was digested by *Bam*HI and *Hin*dIII and inserted into the pLAFR3 vector to obtain a plasmid designated as pLAFRg, which was transformed into *E. coli* DH5α. *Methylobacterium* sp. MB200 was cultured in MM medium with nalidixic acid at 32 °C for 48 h. *E. coli* strain HB101 containing plasmid pRK2013 and DH5α containing pLAFRg were cultivated in LB with kanamycin (for HB101) and tetracycline (for DH5α) at 37 °C overnight. Aliquots of these cultures (200 μl of *Methylobacterium* sp. MB200, 50 μl of HB101 and DH5α, respectively) were pooled and plated onto a MM plus 20% LB plate, which was incubated at 32 °C for 2 days. Representative colonies were spread onto tetracycline- and nalidixic acid-amended MM for selecting the recombinant strains after incubation for 3–4 days at 32 °C. pLAFRg was conjugated from *E. coli* DH5α into *Methylobacterium* sp. MB200 to generate the recombinant strain designated as *Methylobacterium* sp. MB202.

SHMT Activity Assay

SHMT activity was determined according to the method reported by Zuo et al. [17] with slight modifications. Briefly, 1 ml assay mixture containing 0.05 mM DL-threo-β-

phenylserine, 0.05 μM pyridoxal-5-phosphate, 0.03% cetyl trimethyl ammonium bromide, 0.6 mg cells or proper amount of purified protein, and 50 mM sodium phosphate buffer (pH 8.0) was incubated at 37 °C. The absorbance at 279 nm was read to measure the synthesis of benzaldehyde. One unit of enzyme activity was defined as the amount of the enzyme required to produce 1.0 μmol benzaldehyde per minute at 37 °C.

Resting Cell Reaction

One loopful of the plate culture was inoculated into 10 ml of MM medium and incubated at 32 °C for 3 days with reciprocal shaking (200 strokes/min). An aliquot of the resulting culture suspension was inoculated into 100 ml of the fresh medium and incubated under the same condition. Cells were harvested by centrifugation at 8,000×*g*, 4 °C for 10 min, when the OD at 600 nm of the culture reached 0.8–1.2, and washed twice with 0.9% saline. The resting cell reactions for L-serine production were carried out as reported by Qian Ou in volumes of 1 ml reaction containing 50 μM Tris–HCl (pH 8.8), 20 mg glycine, 70 mg methanol, and 30 mg wet cells [18]. The reactions were incubated for 48 h at 37 °C.

Identification of L-Serine

The rest cell reaction mixture was centrifuged for 20 min at 14,000×*g*, 4 °C, and the supernatant was analyzed for L-serine using a L-8800 AAA System Manager amino acid analysis system with 26223C column (Hitachi, Japan). Qualitative examination was performed as reported by Qian Ou using thin-layer chromatography (TLC) [18].

Results

Cloning and Sequencing of *glyA*

A 650-bp DNA fragment was amplified by PCR with a pair of primers, designed based on the *glyA* gene sequence, from chromsomal DNA of *Methylobacterium* sp. MB200. The sequencing of the PCR product revealed 95% identity to the *glyA* region of *M. extorquens* AM1, suggesting that this fragment was likely part of the *glyA* gene. Using the 650-bp fragment as a probe, we were able to select *Bam*HI to digest the genomic DNA for the construction of the genomic library because there was only one single band with a reasonable size upon probing the *Bam*HI digest of the genomic DNA with the this probe. After digestion with *Bam*HI, the genomic DNA was gel fractionated, and the DNA fragments about 3–4 kb was excised and cloned into pGEM-3Zf (+) to construct a genomic library. About 3,000 clones were generated, and several positive clones were screened by colony hybridization with the above probe (Fig. 1). The plasmid DNA was extracted from one of the positive clones and characterized by restriction enzyme digestion and direct sequencing. The plasmid contained a 1,305-bp open reading frame (ORF) with 95% identity to the *glyA* of *M. extorquens* AM1 at the nucleotide level. The phylogenetic neighbor-joining tree clearly depicts a relationship of *glyA* gene between *Methylobacterium* sp. MB200 and other organisms (Fig 2). Its gene product consisted of 435 amino acids with a calculated molecular mass of 48.23 kDa. The amino acid sequence showed 94% identity to the sequence of the SHMT from *M. extorquens* AM1 and 90% identity to that from *Methylobacterium* sp. 4-46. All these observations suggested we have sequenced and cloned the correct gene.

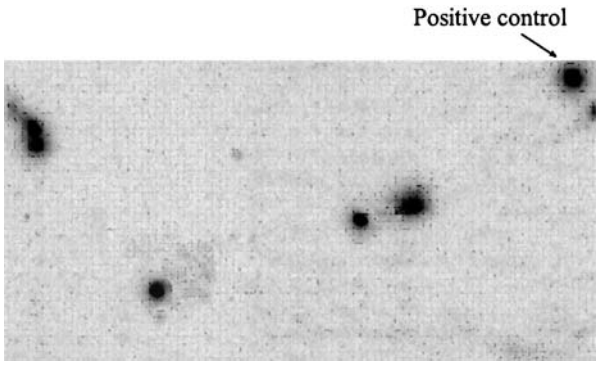


Fig. 1 Colony hybridization of the partial genomic library of *Methylobacterium* sp. MB200. The *arrow* indicates the positive control, the *black dot* represents positive clone

Expression and Purification of SHMT

To further confirm we had isolated the right gene, the gene was cloned into pET-blue and expressed in Tuner (DE3). The expressed protein was purified and characterized to determine if the product of *Methylobacterium* sp. MB200 *glyA* was a SHMT. The ORF containing *glyA* was cloned into the vector pET-blue to generate the expression vector pET:og. The recombinant plasmid pET:og was transformed into expression host strain Tuner (DE3) to generate the expression strain Tuner (pET:og). The His6-tagged

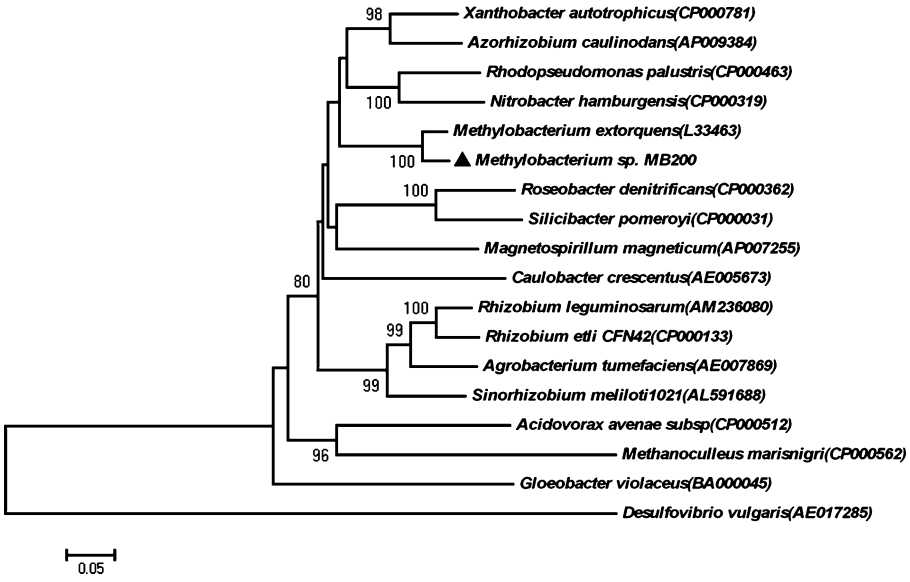


Fig. 2 Phylogenetic relationship of the *glyA* gene. Phylogenetic tree was constructed based on 100-fold bootstrap analyses using the neighbor-joining with the MEGA program. *Filled and open circles* at the nodes represent bootstrap values >80% and >50%, respectively

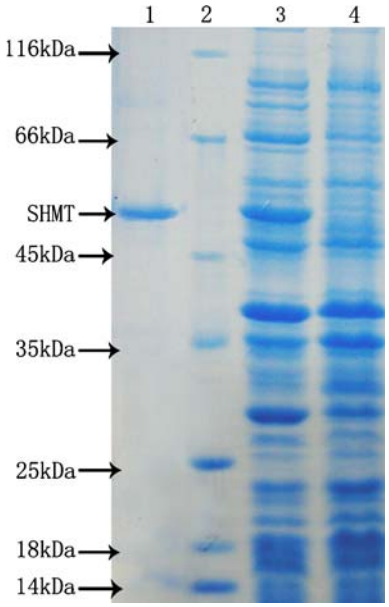


Fig. 3 SDS-PAGE analysis of the expression and purification of the recombinant SHMT. *Lane 1* purified protein; *lane 2* protein molecular weight marker (116, 66, 45, 35, 22, 18, and 14.4 kDa); *lane 3* total protein in the induced lysate; and *lane 4*, total protein in the uninduced lysate

protein was about 48 kDa as shown by SDS-PAGE (Fig. 3), consistent with its calculated size of 48.23 kDa. Enzyme assay showed that the purified protein from *E. coli* Tuner (pET:og) had SHMT activity.

Overexpression of the *glyA* Gene in *Methylobacterium* sp. MB202

The *glyA* gene was cloned into pLAFR3 to generate pLAFRg, which was transformed into *E. coli* DH5α to generate a donor strain for the triparental mating with *M.* sp MB200. The triparental mating process was carried out by the co-cultivation of *Methylobacterium* sp. MB202 with the donor strain and HB101 containing plasmid pRK2013 under double antibiotics selection condition. Tc^r transconjugant MB202 was selected for further characterization and study of SHMT activity. SHMT activities of the wild-type strain MB200 and the recombinant strain MB202 cultivated with methanol as the sole carbon source were determined. The results demonstrated that the SHMT activity in cell-free extracts of MB200 was about 33 U/mg wet cells, while in cell-free extracts of MB202, it was about 115 U/mg wet cells. The SHMT activity of MB202 strain represented an approximately 3.5-fold increase over the activity displayed by MB200. The time courses of SHMT activity produced in the wild-type MB200 strain and the recombinant MB202 strain were also investigated. The activity peaked at 36 h in both strains (Fig. 4).

Production of L-Serine by MB202 and MB200 Using Resting Cell Reactions

After cultivation for 36 h, the MB202 and MB200 cells were collected and used for resting cell reaction in the presence of 30 mg/ml of cells (wet matter), 20 mg/ml glycine, and 70 mg/ml methanol, respectively. TLC analysis showed that there was L-serine produced from the reaction systems of both strains (Fig. 5); however, there was

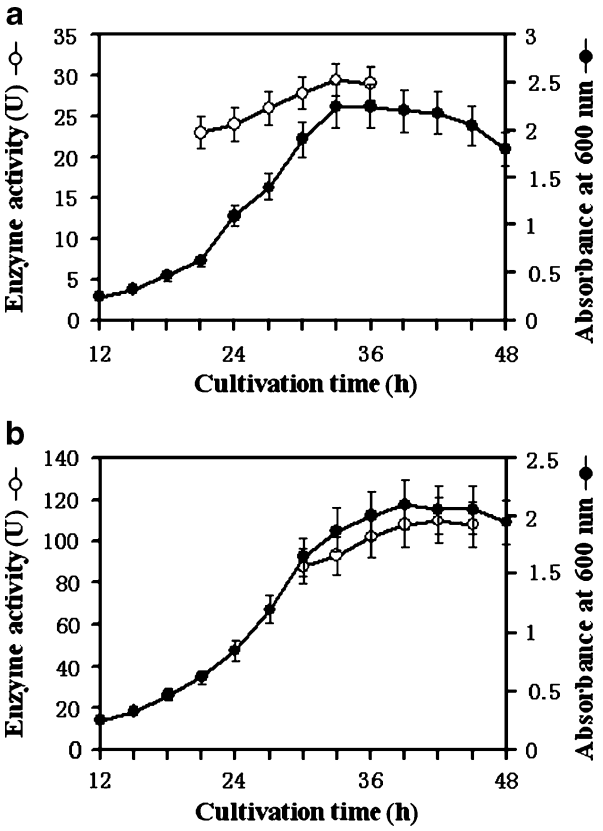


Fig. 4 Time course dependence of SHMT activity in *Methylobacterium* sp. cultures. **a** The wild-type strain MB200; **b** the genetically engineered strain MB202

much more L-serine produced from MB202 resting cell system than that produced from MB200 system according to the sizes of L-serine spots.

Quantitative detection found that a yield of 2.6±0.4 mg/ml L-serine was obtained in 2 days using the strain MB200, while 11.4±0.6 mg/ml L-serine was obtained using improved strain MB202, representing a 4.4-fold increase from that produced by the wild-type strain MB200 (Fig. 6).

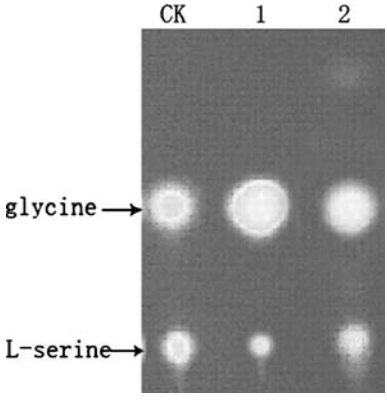


Fig. 5 TLC analysis of L-serine synthesis by MB200 and MB202. *CK* indicates the standard of L-serine and glycine, *lane1* and *lane 2* indicate L-serine and glycine from the reaction systems of MB200 and MB202

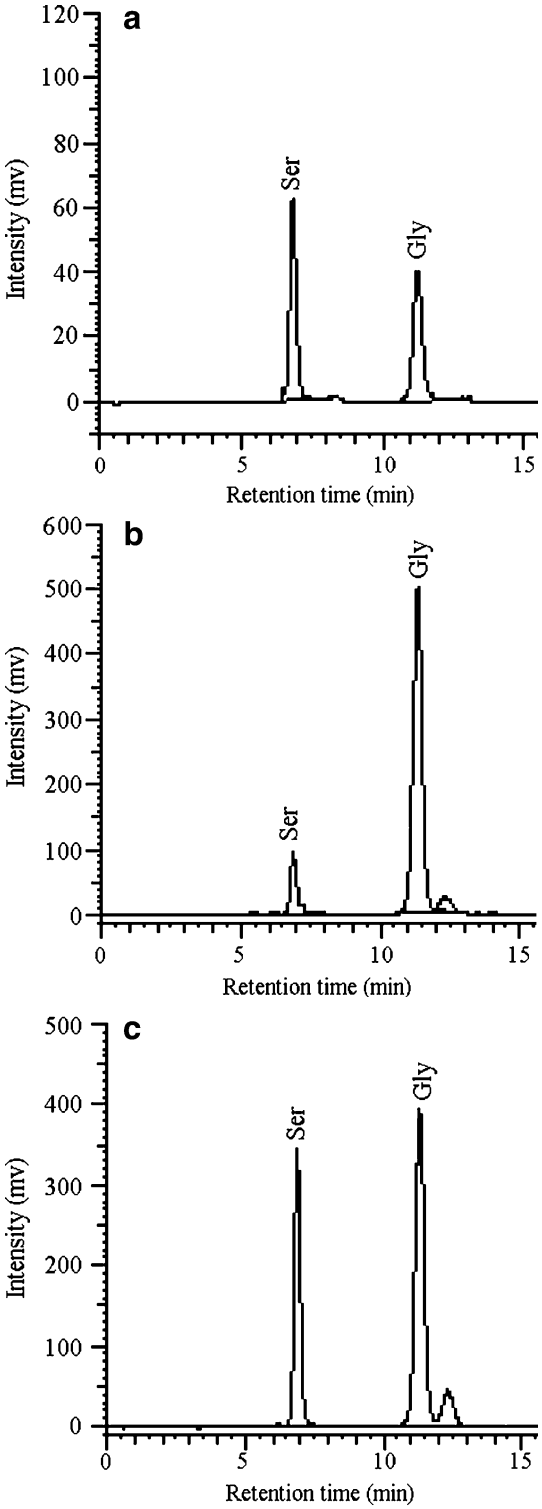


Fig. 6 Detection of L-serine synthesis by MB200 (**b**) and MB202 (**c**) on L-8800 AAA System Manager amino acid determination system with 26223C column. **a** Glycine and L-serine standards

Discussion

Among the many methods for L-serine production [19], microbial production is considered one of the most effective methods and has been widely studied [20]. In particular, serine production by methylotrophic bacteria exploiting the serine pathway that converts glycine and formaldehyde to L-serine using resting cell system has been investigated extensively. For example, the production of L-serine level reached 45 mg/ml in 3 days using the resting cell system of *Hyphomicrobium* sp. NCIB10099 consisting of 30 mg of cells (as dry matter), 100 mg glycine, and 88 mg methanol in a final volume of 1 ml [6]. In studies on L-serine production using *Methylobacterium* sp. MN43, 65 mg/ml L-serine was produced from 50 mg glycine, 104 mg methanol, and 40 mg of cells (as dry matter) in 5 days [4]. It is also considered that methylotrophs have tremendous potential in the biotransformation of one-carbon compounds into L-serine. However, little is reported on the enhancement of serine production by altering the expression level of SHMT in the production strain. In this report, we showed that the productivity of L-serine by methylotrophic bacteria *Methylobacterium* sp. MB200 was dramatically improved by elevating the SHMT activity in the cells.

The *glyA*, a gene which encodes serine hydroxymethyltransferase, has been identified and characterized in microorganisms, plants, and mammals [21–24]. SHMT catalyzes the conversion of serine to glycine, which serves as the major source of one-carbon units for the biosynthesis of purine, thymidylate, and methionine. Thus, it is very difficult to accumulate L-serine extracellularly or intracellularly. But in some methylotrophic bacteria that possess serine cycle to assimilate C1 compounds, SHMT plays an important role as the first enzyme in the assimilation of C1 compounds through the transfer of formaldehyde to glycine, producing the main intermediate, L-serine, in the process. L-Serine is an important intermediate and involved in the biosynthesis of a number of compounds such as amino acids (glycine, methionine, cysteine, and tryptophan), thymine, purine, and phosphatide. The rate of the metabolism of L-serine is high, and it is difficult to obtain L-serine by traditional fermentation. Enzymatic means of L-serine production has also been investigated; the shortcoming is that the cost is too high because the SHMT enzyme needs pyridoxal-5′-phosphate and tetrahydrofolic acid as cofactors.

In the present study, resting cell reaction system was applied for L-serine production to decrease L-serine degradation. A yield of 11.4±0.6 mg/ml L-serine was produced from 30 mg/ml wet cells, 20 mg/ml glycine, and 70 mg/ml methanol in 2 days by *Methylobacterium* sp. MB202, about 4.4-fold increase compared with that produced by the wild-type strain *Methylobacterium* sp. MB200. This result demonstrated the potential of L-serine production using *Methylobacterium* sp strains from methanol and warranted further study to optimize the reaction conditions and investigate other ways to suppress L-serine degradation.

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