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# Screening of microorganisms producing α-methylserine hydroxymethyltransferase, purification of the enzyme, gene cloning, and application to the enzymatic synthesis of α-methyl-L-serine

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ABSTRACT

Through the screening of microorganisms capable of utilizing α-methylserine, three representative strains belonging to the bacterial genera *Paracoccus*, *Aminobacter*, and *Ensifer* were selected as potent producers of α-methylserine hydroxymethyltransferase, an enzyme that catalyzes the interconversion between α-methyl-L-serine and D-alanine via tetrahydrofolate. Among these strains, *Paracoccus* sp. AJ110402 was selected as the strain exhibiting the highest α-methylserine hydroxymethyltransferase activity. The enzyme was purified to homogeneity from a cell-free extract of this strain. The native enzyme is a homodimer with apparent molecular mass of 85 kDa and contains 1 mol of pyridoxal-5′-phosphate per mol of the subunit. The $K_m$ for α-methyl-L-serine and tetrahydrofolate was 0.54 mM and 73 μM, respectively. The gene from *Paracoccus* sp. AJ110402 encoding α-methylserine hydroxymethyltransferase was cloned and expressed in *Escherichia coli*. Sequence analysis revealed an open reading frame of 1278 bp, encoding a polypeptide with a calculated molecular mass of 46.0 kDa. Using *E. coli* cells as whole-cell catalysts, 9.7 mmol of α-methyl-L-serine was stereoselectively obtained from 15 mmol of D-alanine and 13.2 mmol of formaldehyde.

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## 1. Introduction

α-Methylserine hydroxymethyltransferase (MSHMT) (EC 2.1.2.7) catalyzes the interconversion between α-methyl-L-serine and D-alanine with hydroxymethyl transfer by tetrahydrofolate (THF). It has been reported that MSHMT from *Pseudomonas* sp. can also act on α-ethyl-L-serine and hydroxymethylserine; however, no activity toward L-serine was detected [1]. The interconversion between α-methylserine and D-alanine has also been demonstrated to be catalyzed by serine hydroxymethyltranseferase (SHMT) (EC 2.1.2.1), which plays an important role in C-1 metabolism [2–11], although the activity of SHMT toward α-methyl-L-serine is considerably lower than that for L-serine [12,13].

Both the aforementioned enzymes require THF and pyridoxal-5′-phosphate (PLP) for their activity. However, since there have been no reports on the properties of purified MSHMT, its amino acid sequence, or the cloning of its gene, the difference between the two enzymes is still unclear.

In recent years, there has been a growing interest in α-alkyl-α-amino acids and peptides containing their residues for pharmaceutical use. The stereospecific hydroxymethyl transfer to α-amino acids appears to be one of the most effective methods for α-alkyl-L-serine production.

In order to obtain MSHMTs suitable for α-methyl-L-serine production, microorganisms capable of growing with α-methyl-DL-serine as the sole carbon source were isolated. This paper describes the purification and characterization of MSHMT, and the cloning of its gene. This is the first report of the cloning of the gene encoding MSMHT, thus offering the potential of an enzymatic process for the production of α-methyl-L-serine.

## 2. Materials and methods

### 2.1. Chemicals

α-Methyl-L-serine and α-methyl-D-serine were purchased from Acros Organics Inc. (Geel, Belgium). α-Methyl-DL-serine,

*Abbreviations:* SHMT, serine hydroxymethyltransferase; MSHMT, α-methylserine hydroxymethyltransferase, D-alanine 2-hydroxymethyltransferase; PLP, pyridoxal-5′-phosphate; DTT, dithiothreitol; EDTA, *N,N*′-ethylenediaminetetraacetic acid; THF, tetrahydrofolate or tetrahydropteroylglutamate; NADP, nicotinamide adenine dinucleotide phosphate.

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5,10-methylenetetrahydrofolate dehydrogenase and tetrahydrofolate were obtained from Sigma Chemical Co., Ltd. (MO, USA). Pyridoxal-5′-phosphate and formaldehyde solution were obtained from Nacalai Tesque (Kyoto, Japan). Casamino acids, nutrient broth, and yeast nitrogen base without amino acids and ammonium sulfate were obtained from BD Biosciences (CA, USA). A Resource Q column, a HiLoad 16/10 Phenyl Sepharose HP column, and a HiLoad 16/60 Superdex 200 pg column with LMW and HMW Gel Filtration calibration Kits were purchased from Amersham Bioscience Corp. (NJ, USA).

### 2.2. Screening of microorganisms and culture conditions

A small amount of various soil samples was added to 3 ml of a selection medium consisting of 0.4% (w/v) α-methyl-DL-serine and 0.17% yeast nitrogen base without amino acids and ammonium sulfate (pH 7.0 adjusted with NaOH), and cultivation was performed at 30 °C. After 2–3 days, 0.15 ml of culture broth was inoculated into new selection medium, and then repeated twice more. The colonies were isolated on a selection medium plate by spreading the final broth obtained. Isolates were purified and stored on nutrient broth plates. Each strain was cultivated using this selection method, and then the enzyme activities were measured.

### 2.3. Enzyme assay

MSHMT activity was measured using a modification of Wilson's method [1,14]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4), 10 mM α-methyl-DL-serine, 0.5 mM tetrahydrofolate, 10 mM 2-mercaptoethanol, 10 μM PLP, 10 mM sodium ascorbate, 0.4 mM NADP, 1 U ml$^{-1}$ 5,10-methylenetetrahydrofolate dehydrogenase, and the appropriate amount of enzyme in a total volume of 0.1 ml. After incubation at 30 °C for 10 min, the reaction was stopped by the addition of 0.15 ml of 6 N HCl. The activity was calculated from the absorbance change at 350 nm due to the formation of 5,10-methenyltetrahydrofolate using a value of $\varepsilon_{350}$ = 29,400 M cm$^{-1}$. One unit of activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product per minute under the above conditions.

### 2.4. Purification of MSHMT from Paracoccus sp. AJ110402

*Paracoccus* sp. AJ110402 was grown in the selection medium at 30 °C, and cells from 2 l cultures were harvested by centrifugation (8000 × *g*, 10 min, 4 °C) and washed twice with 25 mM potassium phosphate buffer containing 20 μM PLP (pH 7.4) (buffer A). The cell suspension in buffer A was sonicated using an Insonator 201 ultrasonic oscillator (Kubota, Tokyo, Japan), and centrifuged at 12,000 × *g* for 20 min at 4 °C for the removal of cell debris. In order to remove the insoluble fraction, the supernatant was ultracentrifuged at 200,000 × *g* for 30 min at 4 °C, and the resultant supernatant was used as the cell-free extract.

All procedures were carried out at 4 °C or on ice. The cell-free extract was applied to a Resource Q column (1.6 cm × 3.0 cm) equilibrated with buffer A. The enzyme was eluted with a linear gradient of 0 to 1 M NaCl.

The active fractions were collected and mixed with buffer A containing 2 M ammonium sulfate, and applied to a HiLoad 16/10 Phenyl Sepharose HP column equilibrated with buffer A containing 1 M ammonium sulfate, and then eluted with a linear gradient of 1 to 0 M ammonium sulfate.

The active fractions were pooled and dialyzed against 2.5 mM potassium phosphate buffer containing 20 μM PLP (pH 6.8). The dialysate was loaded onto a Cellulofine HAp column (1.6 cm × 10 cm) (Seikagaku Corp., Tokyo, Japan) equilibrated with

**Table 1**
Identification of MSHMT producers from natural sources

| Isolated strains | Specific activity (U mg protein$^{-1}$) | Similarity with 500 bp of 16S rDNA | |
|---|---|---|---|
| AJ110402 | 0.110 | *Paracoccus alcaliphilus* ATCC 51199$^{T}$ | 96.60% |
| AJ110403 | 0.105 | *Aminobactor aminovorans* DSM6450$^{T}$ | 100% |
| AJ110404 | 0.054 | *Ensifer meliloti* LMG6133$^{T}$ | 99.80% |

**Table 2**
Effects of the medium composition on MSHMT production in *Paracoccus* sp. AJ110402

| Medium | α-Me-DL-ser[a] (%) | Additional carbon/nitrogen source[b] | Specific activity (U mg protein$^{-1}$) |
|---|---|---|---|
| Selection medium | 0.2 | – | 0.113 |
| | 0.2 | 0.1% D-glucose | 0.077 |
| | 0.2 | 0.1% glycerol | 0.084 |
| | 0.2 | 0.1% $Na_2$ fumarate | 0.063 |
| | 0.2 | 0.1% casamino acids | 0.048 |
| Nutrient broth | None | – | ND |
| | 0.1 | – | 0.037 |
| | 0.2 | – | 0.028 |
| | 0.4 | – | 0.020 |

The cultivation was performed at 30 °C for 24 h, and then the enzyme activity was measured using the cell-free extract.
[a] α-Me-DL-ser, α-methyl-DL-serine.
[b] –, no addition.

**Table 3**
Purification of MSHMT from *Paracoccus* sp. AJ110402

| Fraction | Protein (mg) | Specific activity (U mg protein$^{-1}$) | Total activity (U) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Cell-free | 136 | 0.096 | 13.0 | 1.0 | 100 |
| Resource Q | 31.2 | 0.523 | 16.3 | 5.5 | 125 |
| Phenyl Sepharose HP | 2.7 | 1.21 | 3.3 | 12.6 | 25.0 |
| Cellulofine HAp | 1.1 | 3.51 | 4.0 | 36.6 | 30.7 |

the dialysis buffer, and then eluted with a linear gradient of 2.5–250 mM potassium phosphate buffer (pH 6.8).

The purified enzyme was dialyzed against 25 mM potassium phosphate buffer containing 1 mM EDTA, 1 mM DTT, and 20 μM PLP (pH 7.5).

### 2.5. Protein analysis

Protein concentrations were determined using the Bradford method [15] with bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 10–20% polyacrylamide gel (Daiichi Pure Chemicals, Tokyo, Japan) with Precision Protein Marker (Bio-Rad Laboratories, CA, USA) used as marker proteins. The native relative molecular mass was determined using a HiLoad 16/60 Superdex 200 pg column with LMW and HML Gel Filtration Calibration Kits. N-terminal amino acid sequence analysis was carried out using an automated protein sequencer (PPSQ-21A; Shimadzu, Kyoto, Japan).

### 2.6. PLP content

After the purified enzyme was extensively dialyzed against 25 mM potassium phosphate buffer containing 1 mM DTT, 1 mM EDTA, and 20 μM PLP (pH 7.5), the concentration of PLP inside and outside the dialysis bag was determined using phenylhydrazine [16].

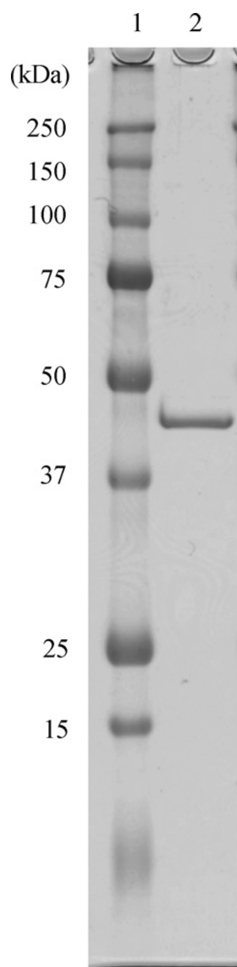


**Fig. 1.** SDS-PAGE of purified MSHMT. Lane 1, standard proteins; lane 2, purified MSHMT (0.2 μg).

### 2.7. Cloning of the gene (mshmt) encoding MSHMT

A probe for the gene encoding MSHMT was obtained using a TaKaRa LA PCR in vitro Cloning Kit (TaKaRa Biomedicals, Kyoto, Japan) as follows. The oligonucleotide primers S1 (5′-AAY GAR CTS ACS CGS ACS TTY TTY AA-3′) and S2 (5′-GTS CAY GAY ACS GAY CCS CTS ATH GC-3′) were synthesized, and degenerate PCR was performed according to the manufacturers instruction manual. The 0.7-kb-length fragment was amplified with the ligation product of the genomic DNA from AJ110402 digested with *Pst*I and a *Pst*I cassette linker as the template, and then inserted into a pGEM-T Easy vector (Promega Corp., WI, USA). The 0.7-kb fragment obtained by *Eco*RI/*Pst*I digestion of the plasmid was used as the probe. The genomic DNA from AJ110402 was digested with *Bgl*II/*Nru*I, and then 3.5-kb-length fragments were collected and inserted into *Bam*HI/*Sma*I-digested pUC19. This construct was used to transform *Escherichia coli* JM109, and the resulting library was screened by colony hybridization with the probe described above. The positive clones carrying the plasmid pHMT01 were used for further analysis.

In order to construct a plasmid for expression, the *mshmt* gene was amplified with pHMT01 using the primer pair PHMT_SD_Eco (5′-CGG AAT TCC GGA GAG ACC GCC ATG AAC GAA TT-3′) and PHMT_ter_Hind (5′-CCC CAA GCT TCA GTG GGC GTA GAC CGG GAA GGC C-3′), digested with *Eco*RI/*Hin*dIII, and inserted into pUC18 digested with *Eco*RI/*Hin*dIII, resulting in pUCPHMT01. The DNA sequence of the PCR product was determined in order to confirm that no errors had occurred during the amplification by the DNA sequencer (ABI-3100, Applied Biosystems, USA).

### 2.8. Enzymatic synthesis of α-methyl-L-serine by whole-cell catalysis

An overnight culture of *E. coli* JM109/pUCPHMT grown in Lauria-Bertani (LB) medium containing 100 μg $ml^{-1}$ ampicillin Na was diluted into the same medium (1/20 dilution). After 1 h shaking at 37 °C, isopropyl-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and then the cells were cultivated for 4 h. The cells were washed twice with 25 mM potassium phosphate buffer containing 20 μM PLP (pH 7.4). The standard reaction mixture contained 50 mM potassium phosphate buffer, 150 mM D-alanine, 20 mM formaldehyde, 0.3 mM THF, 0.1 mM PLP, 10 mM 2-mercaptoethanol, and 10–90 mg (as dry weight) of cells (pH 8.0)

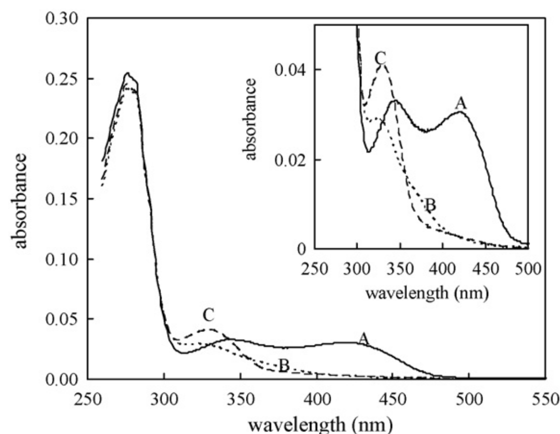


**Fig. 2.** Absorption spectra of the purified MSHMT. Absorption spectra were recorded using a Beckman DU800 spectrophotometer. The purified enzyme (0.25 mg $ml^{-1}$) in 25 mM potassium phosphate buffer containing 1 mM EDTA and 1 mM DTT (pH 7.5) was used. Curve A, native enzyme; curve B, hydroxylamine-treated enzyme; curve C, sodium borohydrate-reduced enzyme.

in a total volume of 100 ml. When a larger amount of formaldehyde was added, 600 mM formaldehyde was fed at a rate of 2.5 ml $h^{-1}$. The mixture was incubated at 30 °C with moderate stirring.

### 2.9. HPLC analysis

α-Methyl-L-serine, α-methyl-D-serine, L-alanine, and D-alanine were determined using a high-performance liquid chromatograph packed with Sumichiral OA-6100 (4.6 mm × 150 mm) (Sumika Chemical Analysis Service, Ltd., Osaka, Japan). The detection was performed at 210 nm at 30 °C, and 0.5 mM $CuSO_4$ was used as the mobile phase.

## 3. Results and discussion

### 3.1. Screening and identification

Through the screening, 14 types of microorganism capable of growing with α-methyl-DL-serine as a sole carbon and nitrogen source were obtained from natural sources. Among these, three strains exhibiting high activity (more than 0.05 mU mg $protein^{-1}$) in the cell-free extracts (Table 1) were selected. From sequence analysis of 500 bp of 16S rDNA, these strains were identified as *Paracoccus* sp., *Aminobacter* sp., and *Ensifer* sp. [17]. The *Paracoccus* sp., which we designated AJ110402, was observed to be the best producer of MSHMT, and was consequently selected for further experiments.

### 3.2. Cultivation conditions

Optimal culture conditions for enzyme production were investigated. The selection medium, described in Section 2, was supplemented with D-glucose, glycerol, disodium fumarate or casamino acids (0.1%, w/v in each case) as a carbon and/or nitrogen source. In each case, the L-form of α-methyl-serine in the medium was completely degraded; however, most of the D-form remained after 24 h of culture. Enzyme activity was detected in the cell-free extract prepared from each medium containing α-methyl-DL-serine. Among these, the selection medium gave the highest activity (Table 2). Activity was also detected using nutrient broth containing 0.1–0.4% (w/v) α-methyl-DL-serine, although no activity was detected unless α-methyl-DL-serine was added to the nutrient broth (Table 2). The enzyme appears to be induced by α-methyl-DL-serine, suggesting that the enzyme plays an important role in the

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Paracoccus 110402   1:-------MNE LTRTFFNSSV HDTDPLIAQA LDDERARQKN QIELIASENI VSQAVLDALG 53
M. extorquens       1:MSAGTATDTT DLDSFFSAHL AETDPEIAKA ISQELGRQQH EIELIASENI VSRAVLEAQG 60
S. tokodaii         1:---------- -----MSQIP KELEKVIELT REQNRWRRTE VINLIASENV MSPLAETVYM 45
E. coli             1:---------- --MLKREMNI ADYDAELWQA MEQEKVRQEE HIELIASENY TSPRVMQAQG 48
B. subtilis         1:---------- ------MKHL PAQDEQVFNA IKNERERQQT KIELIASENF VSEAVMEAQG 44
                                                              *    * ******   *

Paracoccus 110402  54:HEMTNKTLEG YPGNRFHGGG QFVDVVEQAA IDRAKQLFNC GYANVQPHSG TQANLAVFFL 113
M. extorquens      61:SVLTNKYAEG YPGRRYYGGC QFVDIAEELA IDRAKRLFGC GFANVQPNSG SQANQGVFMA 120
S. tokodaii        46:SDFMSRYAEG KPYKRYYQGT KYVDEVETLA MQLMNEITNT KFCDLRATSG TIANAAVFRV 105
E. coli            49:SQLTNKYAEG YPGKRYYGGC EYVDIVEQLA IDRAKELFGA DYANVQPHSG SQANFAVYTA 108
B. subtilis        45:SVLTNKYAEG YPGKRYYGGC EHVDVVEDIA RDRAKEIFGA EHVNVQPHSG AQANMAVYFT 104
                              **  *  *   *    **  *  *                    **   **  *

Paracoccus 110402 114:LVKPGDRILS LDLAAGGHLS HGMKGNLSGR WFEAHNYNVD PQNEVINYDE MERIAEEVKP 173
M. extorquens     121:LMQPGDTFLG LDLAAGGHLT HGAPPNVSGK WFKPVSYTVR REDQRIDMEQ VERLAQEHKP 180
S. tokodaii       106:LANPGEKALI APVQAGAHVS HTKFGTLGAL GIEHIELPYD ADKMNVDVDK AIKMIEQIKP 165
E. coli           109:LLEPGDTVLG MNLAHGGHLT HGSPVNFSGK LYNIVPYGID ATGH-IDYAD LEKQAKEHKP 167
B. subtilis       105:ILEQGDTVLG MNLSHGGHLT HGSPVNFSGV QYNFVEYGVD KETQYIDYDD VREKALAHKP 164
                          *   *       * *   *                                        **

Paracoccus 110402 174:KLLITGGSAY PRELDFARMA QIAKKVGAFF MVDMAHIAGL VAGGAHPSPF PH-ADIVTCT 232
M. extorquens     181:KVIIAGGSGY PRHWDFAKFR EIADSVGAYF FVDMAHFAGL VAAGLHPSPF PH-AHVATTT 239
S. tokodaii       166:KFIVMGGSLY LFPHPVKELA PHAHAVGAKV VYDAAHVYGL ITGKAWHNPL EEGADIMTSS 225
E. coli           168:KMIIGGFSAY SGVVDWAKMR EIADSIGAYL FVDMAHVAGL VAAGVYPNPV PH-AHVVTTT 226
B. subtilis       165:KLIVAGASAY PRTIDFKKFR EIADEVGAYF MVDMAHIAGL VAAGLHPNPV PY-ADFVTTT 223
                      *    * * *              *   **     * **  **         *     *   *

Paracoccus 110402 233:TTKTLRGPRG GLILTNN--E EWYKKLQTAV FPGVQGSLHS NVLAAKAICL GEALRPEFRD 290
M. extorquens     240:THKTLRGPRG GMILTND--E ALAKKFNSAI FPGLQGGPLM HVIAAKAAAF GEALKPEFKI 297
S. tokodaii       226:THKTFPGPQG GAVFSNE--E EIFKKVADTI FPWFVSNHHL HRLPATAVTA LE-MKYFGED 282
E. coli           227:THKTLAGPRG GLILAKGGSE ELYKKLNSAV FPGGQGGPLM HVIAGKAVAL KEAMEPEFKT 286
B. subtilis       224:THKTLRGPRG GMILCRE--E -FGKKIDKSI FPGIQGGPLM HVIAAKAVSF GEVLQDDFKT 280
                      * **  ** * *        *    **      **               *     *

Paracoccus 110402 291:YVAQVVKNAK VLAETLTSRG IRIVS---GG TDTHIVLLDL SSKGLNGKQA EDALARANIT 347
M. extorquens     298:YAKQVIDNAR ALADTIISGG YDITS---GG TDNHLMLVDL QKKGLTGKAA EAALSRADIT 354
S. tokodaii       283:YAKQITKNAK AFAEALAAEG FKVIGEHLGY TQSHQVVLDV RNLGGGAKIA K-LFEDANII 341
E. coli           287:YQQQVAKNAK AMVEVFLERG YKVVS---GG TDNHLFLVDL VDKNLTGKEA DAALGRANIT 343
B. subtilis       281:YAQNVISNAK RLAEALTKEG IQLVS---GG TDNHLILVDL RSLGLTGKVA EHVLDEIGIT 337
                      *      **           *         *  *  *    *         * *         *

Paracoccus 110402 348:SNKNPIPNDS PRPA-EWVGM RLGVSAATTR GMKEDEFRKL GNVVADLL-- EAESAGNGPE 404
M. extorquens     355:CNKNGVPFDP QKPT-ITSGI RLGTPASTTR GFGVAEFKQV GSLIVQVLDG IAEKGDGGDA 413
S. tokodaii       342:TNKNLLPYDP PSAVKDPSGI RLGVQEMTRF GMKEEEMREI AKLMREVAID GKDPNEVKKK 401
E. coli           344:VNKNSVPNDP KSPF-VTSGI RVGTPAITRR GFKEAEAKEL AGWMCDVL-- ---DSINDEA 397
B. subtilis       338:SNKNAIPYDP EKPF-VTSGI RLGTAAVTSR GFDGDALEEV GAIIALAL-- ---KNHEDEG 391
                       ***  * *          *  * *    *   *

Paracoccus 110402 405:AAEKA-KVTV RELTEAFPVY AH-------- --                               425
M. extorquens     414:AVEAAVKEKV HALTDRFPIY A--------- --                               434
S. tokodaii       402:VIEFRKNYLE VKYTFSVDLS KYSNGKMLPL LI                               433
E. coli           398:VIERI-KGKV LDICARYPVY A--------- --                               417
B. subtilis       392:KLEEA-RQRV AALTDKFPLY KELDY----- --                               415
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**Fig. 3.** Amino acid sequence alignment of MSHMT from *Paracoccus* sp. AJ110402 to SHMTs. The N-terminal amino acid sequence found in the purified enzyme is underlined. Presumed PLP-binding lysine residue is indicated with box, and the conserved residues are with asterisks. Paracoccus 110402, MSHMT from *Paracoccus* sp. AJ110402 (GenBank accession number, AB426468); *M. extorquens*, SHMT from *Methylobacterium exorquens* (AAA64456); *S. tokodaii*, SHMT from *Sulfolobus tokodaii* (BAB66416); *E. coli*, SHMT from *Escherichia coli* (AAA23912); *B. subtilis*, SHMT from *Bacillus subtilis* (CAA86110).

degradation of α-methyl-L-serine. It has also been reported that MSHMT from *Pseudomonas* MS [1,18] and several isolates utilizing α-methyl-DL-serine [19] exhibit similar properties. Based on these results, the selection medium was used for the subsequent enzyme purification experiments.

### 3.3. Purification of MSHMT

MSHMT from *Paracoccus* sp. AJ110402 was purified chromatographically with a 30.7% yield (Table 3). The purified enzyme appeared homogeneous on SDS-PAGE with a molecular mass of 45 kDa (Fig. 1). The native molecular mass was determined to be 85 kDa by gel filtration, suggesting this enzyme consists of a homodimer. The N-terminal amino acid sequence was determined to be N-E-L-R-T-F-F-N-S-S-V-H-D-T-D-P-L-I-A-Q-A-L-D-D-E-D/R-A-D/R-Q.

### 3.4. Enzyme properties

The $V_{max}$ value of MSHMT from *Paracoccus* sp. AJ110402 was 8.15 μmol min$^{-1}$ mg$^{-1}$ toward α-methyl-L-serine, and the $K_m$ for α-methyl-L-serine and THF was 0.54 mM and 73 μM, respectively. No activity, however, was found toward α-methyl-D-serine, L-serine, D-serine, α-*iso*-butyl-DL-serine, α-*iso*-propyl-DL-serine, and α-benzyl-DL-serine. MSHMT from *Pseudomonas* MS is also unable to act on L-serine, although it does act on α-methyl-L-serine, α-ethyl-L-serine, and hydroxymethylserine [1,18].

The optimal pH was observed at pH 7.4–8.0, and more than 95% of the activity remained at pH 6.0–7.5 after 16 h at 4 °C. Further, the enzyme activity was not decreased after 30 min treatment at 40 °C in 25 mM potassium phosphate buffer containing 1 mM EDTA, 1 mM DTT, and 20 μM PLP (pH 7.5), whereas 86 and 13% of the remaining activity was detected after treatment at 50 and 60 °C, respectively.

The enzyme exhibited absorption maxima at 280, 340, and 420 nm at pH 7.5 with an $A_{280}/A_{420}$ ratio of approximately 5.9 (Fig. 2). Treatment with 1 mM hydroxylamine resulted in the loss of activity with the complete disappearance at 420 nm, suggesting the existence of the linkage between PLP and a lysine residue of the enzyme (Fig. 2). However, 75% of the activity was restored after the re-dialysis against the buffer without hydroxylamine. The addition of sodium borohydride [20] also caused the immediate loss of activity, with the disappearance of the absorption peak at 420 nm and the appearance of a new peak at 328 nm (Fig. 2). The PLP content of the enzyme was calculated to be 0.8–0.9 mol/mol of subunit. The spectral changes induced by treatment with sodium borohydride or hydroxylamine suggest that PLP is bound to the ε-amino group of a lysine, as has previously been reported for other PLP-bound enzymes [21–23].

### 3.5. Gene cloning and expression

Sequence analysis of the plasmid pHMT01 identified one complete open reading frame (ORF) comprising 1278 bp with an initial ATG codon (GenBank accession number, AB426468). A probable ribosome binding site, AGAGA, is six bases upstream of the putative initial codon. The ORF encodes a protein of 425 amino acid residues with a predicted molecular mass of 46 kDa, which is in agreement with the value determined by SDS-PAGE. Further, the amino acid sequence from the second codon coincided with the results of N-terminal analysis of the purified enzyme, and the two unidentified residues by the protein sequencer were both arginine (28th and 30th residue) (Fig. 3).

Sequence homology analysis revealed that the predicted amino acid sequence had a 55.5% similarity with the SHMT of *Methylobacterium extorquens* AM1 [4]. By alignment of the amino acid sequence with SHMTs, the Lys 235 of MSHMT from *Paracoccus* sp. was identified as an expected PLP-binding residue that forms a Schiff base. Since the His 228 of SHMT from *E. coli*, which lies next to the Lys binding to PLP, plays an important role in substrate specificity [13,24], the Thr 234 of MSHMT from *Paracoccus* sp., the corresponding residue to His 228 of SHMT from *E. coli*, also appears to be an interesting residue from the viewpoint of substrate specificities (Fig. 3).

The plasmid for gene expression, pUCPHMT01, was introduced into *E. coli* JM109. An MSHMT activity of 2.54 U mg$^{-1}$ was detected in the cell-free extract prepared from the cells cultivated in LB medium containing 100 μg ml$^{-1}$ ampicillin Na under the induction of IPTG (1 mM), although no activity was detected from *E. coli* JM109 carrying pUC18.

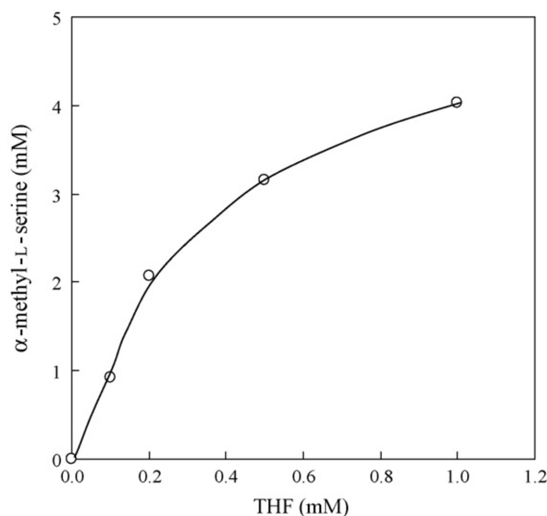


**Fig. 4.** Effect of tetrahydrofolate (THF) on α-methyl-L-serine synthesis activity. The reaction mixture contained 50 mM potassium phosphate buffer, 100 mM D-alanine, 20 mM formaldehyde, 0–1 mM THF, 0.1 mM PLP (pH 8.0), 10 mM 2-mercaptoethanol and the cells of *E. coli* JM109/pUCPHMT (2.5 mg as dry cells per liter). The reaction was performed at 30 °C for 2 h.

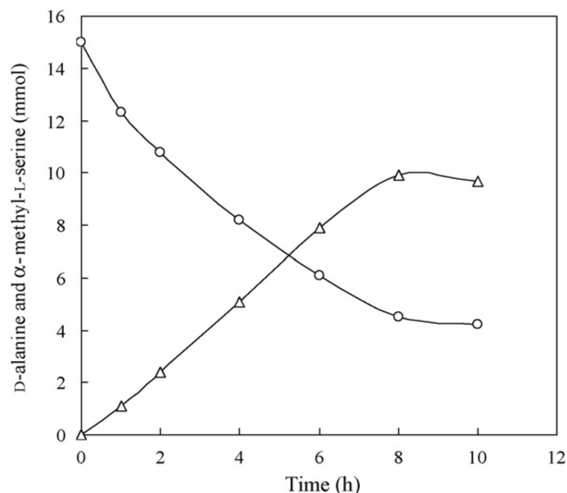


**Fig. 5.** Enzymatic synthesis of α-methyl-L-serine by the whole cells of *E. coli* JM109/pUCPHMT. The reaction was carried out with the feeding of formaldehyde as described in Section 2. Cells (90 mg as dry cells) were added to 100 ml of the reaction mixture. Circles, D-alanine; triangles, α-methyl-L-serine.

### *3.6. Enzymatic synthesis of α-methyl-L-serine by whole-cell catalysis*

To produce α-methyl-L-serine with *E. coli* cells expressed the gene encoding MSHMT, the optimal condition was investigated.

α-Methyl-L-serine was produced from D-alanine and formaldehyde with THF by the whole cells of *E. coli* JM109/pUCPHMT at pH 6.2–8.2; however, no product was detected when L-alanine was used as the substrate instead of D-alanine under the same conditions. The optimal pH was approximately 7.4–8.0. No α-methyl-L-serine synthesis activity was detected under conditions without THF (Fig. 4). Further, the activity was almost constant by the washed cells in the presence of more than 5 μM of PLP, although the activity was reduced to 1.3% unless PLP was supplemented.

In the reaction with the feeding of formaldehyde, 9.7 mmol of α-methyl-L-serine was obtained after 10 h from 15 mmol of D-alanine and 13.2 mmol of formaldehyde (Fig. 5). After the reaction, 1.1 mmol of L-alanine was produced; however, no α-methyl-D-serine was detected. In L-serine production from glycine and formaldehyde catalyzed by SHMT, it was found important to control pH and the concentration of formaldehyde [25], and the formation of 5,10-methylenetetrahydrofolate [26,27]. These factors might help to achieve an α-methyl-L-serine production with a higher yield. The racemization of alanine was detected during the reaction, suggesting that MSHMT is capable of catalyzing racemization via the PLP–alanine quinonoid complex, as are SHMTs [28].

## 4. Conclusion

*Paracoccus* sp. AJ110402 was isolated and selected as the potent producer of MSHMT and this enzyme was purified. The gene encoding MSHMT was firstly cloned in this study, and we succeeded in constructing a useful system of whole-cell catalysis for optically pure α-methyl-L-serine.

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## References

[1] E.M. Wilson, E.E. Snell, J. Biol. Chem. 237 (1962) 3171–3179.
[2] S. Angelaccio, R. Chiaraluce, V. Consalvi, B. Buchenau, L. Giangiacomo, F. Bossa, R. Contestabile, J. Biol. Chem. 278 (2003) 41789–41797.
[3] S. Chaturvedi, V. Bhakuni, J. Biol. Chem. 278 (2003) 40793–40805.
[4] L.V. Chistoserdova, M.E. Lidstrom, J. Bacteriol. 176 (1994) 6759–6763.
[5] S.D. Flatte, R.H. White, B. Maras, F. Bossa, V. Shirch, J. Bacteriol. 179 (1997) 7456–7461.
[6] V.R. Jara, V. Prakash, N.A. Rao, H.S. Savithri, J. Biosci. 27 (2002) 233–242.
[7] S.S. Miyazaki, S. Toki, Y. Izumi, H. Yamada, Eur. J. Biochem. 162 (1987) 533–540.
[8] L. Schirch, D. Peterson, J. Biol. Chem. 255 (1980) 7801–7806.
[9] L. Schirch, M. Mason, J. Biol. Chem. 237 (1962) 2578–2581.
[10] V. Schirch, S. Hopkins, E. Villar, S. Angelaccio, J. Bacteriol. 163 (1985) 1–7.
[11] R.J. Ulevitch, R.G. Kallen, Biochemistry 16 (1977) 5324–5350.
[12] L. Schirch, M. Mason, J. Biol. Chem. 238 (1963) 1032–1037.
[13] P. Stover, M. Zamora, K. Shostak, M. Gautam-Basak, V. Schirch, J. Biol. Chem. 267 (1992) 17679–17687.
[14] E.M. Wilson, Methods in Enzymology, vol. 17, part B, Academic Press, New York and London, 1971, pp. 341–346.
[15] M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[16] H. Wada, E.E. Snell, J. Biol. Chem. 236 (1961) 2089–2095.
[17] JP Patent, JP2006320294A1.
[18] E.M. Wilson, E.E. Snell, J. Biol. Chem. 237 (1962) 3180–3184.
[19] G.T. Sperl, Curr. Microbiol. 19 (1989) 135–138.
[20] Y. Matsuo, D.M. Greenberg, J. Biol. Chem. 234 (1959) 507–515.
[21] R. Contestabile, A. Paiardini, S. Pascarella, M.L. di Salvo, S. D'Aguanno, F. Bossa, Eur. J. Biochem. 268 (2001) 6508–6525.
[22] A.C. Eliot, J.F. Kirsch, Annu. Rev. Biochem. 73 (2004) 383–415.
[23] M.D. Toney, Arch. Biochem. Biophys. 433 (2005) 279–287.
[24] J.N. Scarsdale, S. Radaev, G. Kazanina, V. Schirch, H.T. Wright, J. Mol. Biol. 296 (2000) 155–168.
[25] H.Y. Hsiao, T. Wei, Biotechnol. Bioeng. 28 (1986) 1510–1518.
[26] R.G. Kallen, W.P. Jencks, J. Biol. Chem. 241 (1966) 5851–5863.
[27] R.G. Kallen, W.P. Jencks, J. Biol. Chem. 241 (1966) 5864–5878.
[28] K. Shostak, V. Schirch, Biochemistry 27 (1988) 8007–8014.