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# Genetics of the Serine Cycle in *Methylobacterium extorquens* AM1: Cloning, Sequence, Mutation, and Physiological Effect of *glyA*, the Gene for Serine Hydroxymethyltransferase

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**The gene (*glyA*) of *Methylobacterium extorquens* AM1 encoding serine hydroxymethyltransferase (SHMT), one of the key enzymes of the serine cycle for $C_1$ assimilation, was isolated by using a synthetic oligonucleotide with a sequence based on amino acid sequence conserved in SHMTs from different sources. The amino acid sequence deduced from the gene revealed high similarity to those of known SHMTs. The cloned gene was inactivated by insertion of a kanamycin resistance gene, and recombination of this insertion derivative with the wild-type gene produced an SHMT null mutant. Surprisingly, this mutant had lost its ability to grow on $C_1$ as well as on $C_2$ compounds but was still able to grow on succinate. The DNA fragment containing *glyA* was shown not to be linked with fragments carrying serine cycle genes identified earlier, making it the fourth chromosomal region of *M. extorquens* AM1 to be indicated as being involved in $C_1$ assimilation.**

*Methylobacterium extorquens* AM1 is a pink-pigmented serine cycle methylotroph able to grow on methanol and methylamine as well as on a variety of multicarbon substrates (26, 34). This organism has been successfully used as a model for genetic studies of methanol and methylamine oxidation (14, 17, 18). Recently, progress has also been achieved in studying the genetics of the serine cycle in this strain, with three regions coding for serine cycle enzymes having been identified. One of these regions has been shown to encode genes for serine glyoxylate aminotransferase (*sgaA*), hydroxypyruvate reductase (*hprA*), methylenetetrahydrofolate dehydrogenase (*mtdA* [7]), malate thiokinase (*mtkA* and *mtkB* [8]), phosphoenolpyruvate carboxylase (*ppcA* [2]), and malyl coenzyme (CoA)-lyase (*mclA* [12]). Mutations in all of these genes have been obtained to confirm that they are required for the operation of the serine cycle (2, 6–8). Two other regions of the *M. extorquens* AM1 chromosome that contain serine cycle genes have been less extensively studied. One of these complements glycerate kinase mutants, and another complements mutants with lesion(s) in the unknown acetyl-CoA oxidation pathway portion of the serine cycle (33). Neither of these fragments overlaps the region containing *sgaA*, *hprA*, *mtdA*, *mtkAB*, *ppcA*, and *mclA* (7). The gene (*glyA*) for another serine cycle enzyme, serine hydroxymethyltransferase (SHMT), has been recently cloned and sequenced from an obligate methylotroph, *Hyphomicrobium methylovorum* GM2 (24); however, its location relative to other methylotrophy genes in that bacterium is unknown.

We were interested in cloning *glyA* from *M. extorquens* AM1 in order to clarify its role in this organism. It has been suggested that during growth of *M. extorquens* AM1 on succinate and other multicarbon compounds, the serine necessary for cell biosynthesis is produced by the phosphorylated pathway (15). During growth on $C_1$ compounds, the phosphorylated pathway plays a minor role, since the serine cycle is the major source of serine under these growth conditions (Fig. 1) (1, 13). A role was ascribed to SHMT in the production of glycine from serine during growth on succinate, and a glycine auxotroph mutant, G82, was isolated which was not able to synthesize SHMT during growth on succinate. However, this mutant was able to grow on methanol and expressed normal levels of SHMT on this substrate (13). This finding led to the suggestion that *M. extorquens* AM1 might contain two isoenzymes of SHMT, one expressed during growth on multicarbon compounds and another induced during growth on $C_1$ substrates (13). However, mutants in the methylotrophic SHMT have not been isolated by chemical mutagenesis. The two isoenzymes were purified from a related organism, *Methylobacterium organophilum* XX (25); however, means for molecular analysis of the corresponding genes were not available at that point.

The goal of the present study was to determine how many *glyA* genes are present in the *M. extorquens* AM1 chromosome and to study the phenotypes of insertion mutants in *glyA*.

The *Escherichia coli* strains DH5α (Bethesda Research Laboratories, Inc.) and S17-1 (32) were grown in Luria-Bertani (LB) medium in the presence of appropriate antibiotics as described by Maniatis et al. (19). *M. extorquens* AM1 was grown in the minimal medium described previously (12). Succinate (20 mM), methanol (100 mM), methylamine (20 mM), ethanol (40 mM), or ethylamine (20 mM) was used as substrate. Methanol induction of mutants was carried out as described by Dunstan et al. (10). The following antibiotic concentrations were used for *M. extorquens* AM1: tetracycline, 10 μg/ml; kanamycin, 100 μg/ml; and rifamycin, 50 μg/ml. The growth responses of mutants were tested on plates containing the substrates listed above in the presence or absence of supplements of serine or glycine (1 and 5 mM), glyoxylate (1, 2, or 10 mM), or glycolate (20 mM). DNA-DNA hybridizations were carried out with dried agarose gels as described by Meinkoth and Wahl (23) at 42 or 68°C. For hybridizations, 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) was used, and 0.5× SSC was used for washes. Plasmid isolation, *E. coli* transformation, restriction enzyme digestion, ligation, blunting ends with T4 DNA polymerase, and filling in ends with Klenow enzyme were carried out as described by Maniatis et al. (19). The chromosomal DNA of *M. extorquens* AM1 was isolated by the procedure described by Saito and Miura (29).

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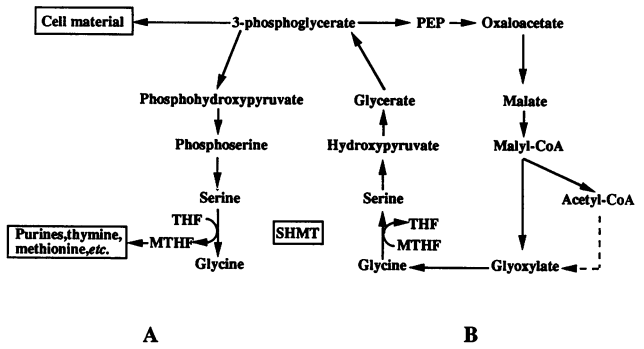


FIG. 1. Proposed dual role of SHMT in the serine cycle (B) and in generation of glycine and $C_1$ units (A) during growth on multicarbon compounds. THF, tetrahydrofolate; MTHF, 5,10-methylene tetrahydrofolate.

DNA sequencing was carried out with an Applied Biosystems automated sequencer by the Sequencing Facility at the University of California, Los Angeles. Translation and analyses of DNA and DNA-derived polypeptide sequences were carried out using PC/Gene (Genofit SA., Geneva, Switzerland) and DNA-Master (California Institute of Technology, Pasadena, Calif.). Enzyme activities were determined for *M. extorquens* AM1 crude extracts prepared as described earlier (5). All measurements were done at room temperature in a total volume of 1 ml. Activity of hydroxypyruvate reductase was measured as described elsewhere (5). The activity of methylene tetrahydrofolate dehydrogenase (MTHFDH) was determined as described elsewhere (31). The activity of SHMT was measured by a continuous or discontinuous assay (30) using MTHFDH as a coupling enzyme. Spectrophotometric methods (16, 38) were used for protein determinations. Isoelectrofocusing was performed with PhastGel isoelectric focusing gels (pH range, 3 to 9 or 5 to 8) and the PhastSystem in accordance with the recommendations of the manufacturer (Pharmacia LKB.) SHMT was visualized by specific staining as described earlier (6), except that the commercial preparation of MTHFDH was replaced by cell extract of *M. extorquens* AM1 carrying pLC410a (7) and overproducing MTHFDH. Triparental or biparental matings between *E. coli* and *M. extorquens* AM1 were performed overnight on nutrient agar. Cells were then washed with sterile medium and plated on selective medium at appropriate dilutions. In triparental matings, pRK2013 (9) was used as a helper plasmid. Rifamycin was used for *E. coli* counterselection.

**Cloning of *glyA*.** To isolate *glyA* from *M. extorquens* AM1, an oligonucleotide probe was developed on the basis of an amino acid sequence (GGHLTHG; amino acid residues 124 to 130 in *E. coli* SHMT [27]) which is known to be conserved in SHMTs (24). The known preference of *M. extorquens* AM1 for G or C in the third triplet position was taken into consideration, resulting in the oligonucleotide GGCGGCCACCT(C/G)AC (C/G)CACGGC, with only a fourfold degeneracy. DNA-DNA blot analysis using this oligonucleotide probe showed that it hybridized to the chromosome of *M. extorquens* AM1 digested with different restriction enzymes, producing one strong band and a few minor bands in each case (data not shown). The oligonucleotide probe was used for screening an existing clone bank of *M. extorquens* AM1 (12), and a positive clone containing pVK100 with a *Hin*dIII insertion of about 25 kb (pLC2) was isolated. Within this insert, a 6-kb *Pst*I fragment was identified that hybridized with the probe, corresponding to a 6-kb fragment detected as a major band in the hybridization experiment with the *Pst*I digest of the *M. extorquens* AM1 chromosome. This fragment was subcloned into pUC19 (37) to generate pLC181a, and its physical map is shown in Fig. 2.

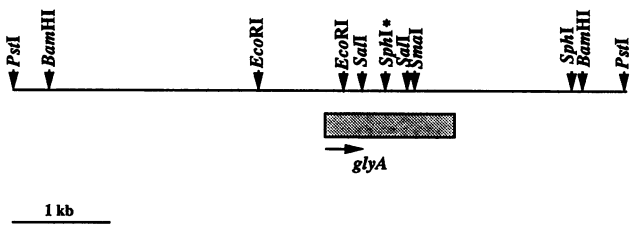


FIG. 2. Physical map of the *M. extorquens* AM1 chromosomal region containing *glyA*. Arrow, direction of transcription; asterisk, site of the insertion mutation.

The part of this fragment that hybridized to the oligonucleotide probe was identified as the 0.3-kb *Sph*I-*Sal*I fragment (Fig. 2), and the sequence of this fragment was determined, revealing the presence of an open reading frame with similarity to *glyA* genes from *H. methylovorum* GM2 (24), *Bradyrhizobium japonicum* (28), *Salmonella typhimurium* (36), *E. coli* (27), *Campylobacter jejuni* (3), *Neurospora crassa* (22), and rabbits (20, 21). The complete sequence of *glyA* from *M. extorquens* AM1 was obtained (data not shown), consisting of 1,305 nucleotides, including the stop codon, which were able to encode a polypeptide with a molecular mass of 46,305 Da, and it was found to contain the conserved sequence (GGHLTHG) used to design the oligonucleotide probe. The identities of the deduced amino acid sequence with those from the *glyA* genes noted above varied between 43 and 74%.

The 5-kb *Bam*HI fragment containing the entire *glyA* gene and its flanking regions was used as a probe for DNA-DNA hybridization analysis of the *M. extorquens* AM1 chromosome, and only one positive band was identified for different restriction enzyme digestions under both low- and high-stringency conditions (data not shown), suggesting that only one copy of *glyA* was present in the chromosome of *M. extorquens* AM1. A few chromosomal fragments were isolated that showed slight hybridization with the oligonucleotide probe for *glyA* at low stringency. The probe-binding regions of these fragments, ranging in size from 0.3 to 1 kb, have been sequenced; however, none of them contained an open reading frame coding for *glyA* or a gene similar to *glyA*, although all of the DNA fragments contained regions potentially able to hybridize to the probe.

**Nucleotide sequence accession number.** The sequence of 1,500 bp has been deposited with GenBank under accession number L33463.

**Construction of an insertion mutation into *glyA*.** The 0.6-kb *Eco*RI-*Sma*I fragment containing the middle portion of *glyA* was cloned into pUC19, and the *Sph*I site in the pUC19 linker was removed by cutting it with *Pst*I, blunting the ends with T4 DNA polymerase, and ligating. The resulting plasmid, pLC181ES, contained the unique *Sph*I site approximately in the middle of the 0.6-kb insert. This site was used for generation of an insertion mutation. The plasmid was digested with *Sph*I and treated with T4 DNA polymerase, and then the *Hin*cII fragment from pUC4K (37), containing the $Km^r$ gene, was inserted. A plasmid, pLC181Sp2, in which the $Km^r$ gene was transcribed in the same direction as *glyA*, was selected. pLC181Sp2 was ligated with the suicide vector pAYC61 (4), and the resulting plasmid, pLC181gly, was transformed into *E.*

TABLE 1. Activities of some enzymes participating in $C_1$ metabolism in mutant and wild-type *M. extorquens* AM1

| Strain and growth condition[a] | Enzyme activity (nmol/min/mg of protein)[b] | | |
|---|---|---|---|
| | HPR | SHMT | MTHFDH |
| AM1 | | | |
| Succ | 330 | 5 | 70 |
| MeOH | 1,200 | 30 | 210 |
| Gly1 | | | |
| Succ | 340 | 0 | 80 |
| MeOH[c] | 1,300 | 0 | 240 |
| Gly16 | | | |
| Succ | 345 | 0 | 75 |
| MeOH[c] | 1,300 | 0 | 240 |
| Gly16(pLC310.181a) | | | |
| Succ | 320 | 60 | 80 |
| MeOH | 1,400 | 75 | 250 |
| Gly6(pLC310.181b) | | | |
| Succ | 380 | 90 | 85 |
| MeOH | 1,350 | 120 | 280 |

[a] Succ, cells were grown on succinate; MeOH, cells were grown on methanol except as indicated otherwise.

[b] Data are averages of two to four independent measurements. Values agreed ± 15%.

[c] Cells were grown on succinate, washed, and incubated with methanol for at least 24 h to allow for induction.

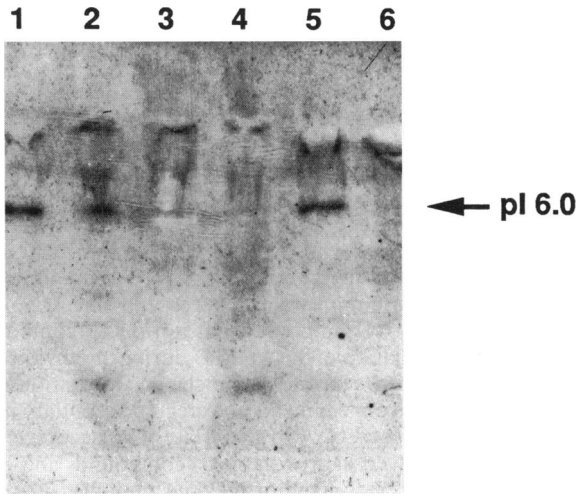


FIG. 3. Specific staining for the SHMT activity after isoelectrofocusing of cell extracts, pH 3 to 9. Lanes: 1 and 2, GlyA(pLC181b) grown on methanol and succinate, respectively; 3 and 4, GlyA grown on succinate and then induced with methanol and grown on succinate, respectively; 5 and 6, wild-type AM1 grown on methanol and succinate, respectively.

*coli* S17-1, which was used as a donor in biparental matings with *M. extorquens* AM1. $Km^r$ transconjugants were selected on rich medium with low frequency. Those that were simultaneously $Tc^s$ should be a result of a double-crossover event, and a few of these were selected. DNA-DNA hybridization analysis was used to confirm the presence of the Km gene insertion in the appropriate location on the *M. extorquens* AM1 chromosome in the insertion mutants. The 5-kb *Bam*HI fragment from pLC181a, the 1.4-kb *Hin*cII fragment from pUC4K carrying the $Km^r$ gene, and the vector DNA were used as probes. The analysis has shown that the double-crossover mutants contained the expected insertion mutation inside the *Bam*HI fragment and did not carry any vector sequences (data not shown).

**Analysis of *glyA* insertion mutants.** Growth responses and the presence of SHMT activity were determined for representatives of insertion mutants in *glyA*. The mutants were able to grow normally on succinate but, surprisingly, did not grow on methanol, methylamine, ethanol, or ethylamine, showing that both $C_1$ and $C_2$ metabolic pathways were impaired.

Mutants of *E. coli*, *S. typhimurium*, and *B. japonicum* defective in *glyA* are known to be glycine auxotrophs (28, 35). A mutant of *M. extorquens* AM1, G82, which was induced by chemical mutagenesis that required glycine for growth on succinate and lacked SHMT activity when grown on this substrate, was described earlier (13). Neither glycine nor serine (1 or 5 mM each) was able to restore growth of the *glyA* mutant of *M. extorquens* AM1 on methanol or ethanol. No stimulation by serine or glycine was observed for growth on succinate. Since *glyA* mutants had a phenotype characteristic of mutants in the unknown pathway of glyoxylate biosynthesis from acetyl-CoA common for both $C_1$ and $C_2$ assimilative pathways (11), glyoxylate and glycolate were tested for the ability to complement the mutants. Glyoxylate (2 to 10 mM) was able to support growth of the mutants on ethanol or ethylamine, with the stimulating effect most pronounced at 10 mM glyoxylate. Glyoxylate in concentrations of up to 10 mM did not allow growth of the mutants on methanol and had no stimulating effect on *glyA* mutants during growth on succinate. Glycolate in concentrations of up to 20 mM did not stimulate growth of the *glyA* mutants on $C_1$ or $C_2$ compounds or on succinate.

The activity of SHMT was determined in cell extracts of two *glyA* mutants, Gly1 and Gly16, and the wild-type *M. extorquens* AM1 was grown on succinate and induced with methanol. The activity of hydroxypyruvate reductase (HPR) was measured to assess the level of methanol induction, and the activity of MTHFDH was measured to ensure that this coupling enzyme for the SHMT assay was present at sufficient levels. While normal levels of HPR and MTHFDH were found in mutant and wild-type cells (Table 1), SHMT activity was absent in *glyA* mutants, confirming that SHMT is the product of *glyA*. In addition, the SHMT-minus phenotype of the mutants was confirmed by specific staining in gels. The positive band corresponding to a pI of about 6, which is the calculated pI for SHMT, was present in the wild-type extract and was absent in Gly16 (Fig. 3).

Although the role of SHMT in assimilation of $C_1$ compounds in the serine cycle has been well known for some time, it has never been confirmed by the analysis of SHMT mutants. Our results confirm that SHMT is required for the serine cycle. *glyA* mutants lost the ability to grow on $C_1$ compounds, and they were unable to grow on $C_1$ compounds supplemented with glycine or serine, underscoring the central role of SHMT in carbon assimilation during methylotrophic growth. In addition, the *glyA* mutants grew normally on succinate, indicating that the SHMT encoded by *glyA* is serine cycle specific. However, *glyA* mutants did not contain detectable SHMT activity in extracts of cells grown on succinate; therefore, the enzyme that generates glycine during growth on succinate is not known. Activity gels of both wild-type and *glyA* mutant extracts revealed a few light bands that are constitutive. These may be artifacts or they may be SHMT activities that function to synthesize glycine during heterotrophic growth. Since it was shown that only a single copy of *glyA* was present in *M.*

*extorquens* AM1, if a second SHMT activity is present in this organism, it must be encoded by a gene not closely related to *glyA*.

Our work has also shown that SHMT is required for growth on $C_2$ compounds. This is surprising, since current proposals for growth on $C_2$ compounds do not include SHMT (1, 11). However, a part of the serine cycle, involving the conversion of acetyl-CoA to glyoxylate, is known to be common for both $C_1$ and $C_2$ metabolism. The biochemical details of this pathway are unknown, but glyoxylate will restore growth of mutants defective in this pathway on both $C_1$ and $C_2$ compounds (11). The *glyA* mutants are unusual in this respect, since glyoxylate restores growth on $C_2$ compounds but not on $C_1$ compounds. This suggests a dual role for SHMT during methylotrophic growth: the expected central role in the incorporation of $C_1$ units into the serine cycle, and an unexpected secondary role in the conversion of acetyl-CoA to glyoxylate. This secondary role would also be involved in growth on $C_2$ compounds and would explain the growth phenotype of the *glyA* mutants.

**Expression of *glyA* in *M. extorquens* AM1.** The 6-kb *Pst*I fragment carrying *glyA* was ligated into pRK310 (9) for complementation experiments. Two different orientations of the fragment with respect to the *lac* promoter have been obtained. In plasmid pLC310.181b, *glyA* is under the control of the *lac* promoter, and in pLC310.181a, the fragment is in the opposite orientation. The plasmids were transferred to the *glyA* mutants Gly1 and Gly16 in triparental matings, $Tc^r$ transconjugants were obtained on succinate plates, and these were checked for their ability to grow on methanol. The fragment was able to restore a $C_1$-positive phenotype when it was cloned in either orientation with respect to the *lac* promoter, showing that a promoter is probably present upstream of *glyA*. The transconjugants also regained their ability to grow on $C_2$ compounds. The activity of SHMT was measured in transconjugants carrying pLC310.181a and pLC310.181b that were grown on succinate or methanol (Table 1). SHMT activity was present at high levels in constructs having *glyA* under the *lac* promoter in cells grown on succinate or methanol. For cells containing the construct with *glyA* in the opposite orientation, SHMT was present at lower levels in both succinate- and methanol-grown cells. Activity gels of Gly16 cells containing pLC310.181b confirmed the presence of the SHMT band at high levels on both methanol and succinate (Fig. 3).

**Identification of the fourth region on the *M. extorquens* AM1 chromosome involved in $C_1$ assimilation.** The *Hin*dIII insert into pLC2 identified in this work did not reveal any common restriction patterns with the *Hin*dIII fragment cloned in pM2, which was identified earlier (12) and shown to contain a cluster of serine cycle genes. Two other *Hin*dIII fragments that are not linked to the fragment from pM2 have been cloned; one is able to complement a glycerate kinase mutant, and another is able to complement mutants in the unknown pathway of synthesis of glyoxylate from acetyl-CoA (34). Since these last fragments are not well characterized, we tried to determine possible links between these fragments and the fragment from pLC2 by mutant complementation. pLC2 was transferred into mutants PG2 (glycerate kinase) and PT1005 (glyoxylate synthesis pathway) (33). None of the mutants was complemented. In addition, a digest of pLC2 was labelled and used as a probe in DNA-DNA hybridization with all of the fragments of the *M. extorquens* AM1 chromosome that have been shown so far to contain genes involved in oxidation or assimilation of $C_1$ compounds (2, 4, 18). None of the fragments hybridized to pLC2 (data not shown), indicating that the *glyA*-containing fragment identified in this work marks the fourth region on the chromosome of *M. extorquens* AM1 known to be involved in $C_1$ assimilation. Its location with respect to other chromosomal regions involved in $C_1$ metabolism is not known.

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