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 C₃ 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 C₃ as well as on C₁ 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 C₃ 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*) (14), malate thiokinase (14) and malyl coenzyme (CoA)-lyase (14). 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, *Hyphomicrobiun methyllovorum* 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 C₁ 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 C₁ 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 ethyamine (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 rifampicin, 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 Maniatis et al. (19). The chromosomal DNA of *M. extorquens* AM1 was isolated by the procedure described by Saito and Miura (29).
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 RNA-derived polypeptide sequences were performed using PCGene (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 3 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. Rifampycin 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 (GGHILTHG; 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 GCCGCCACCT(C/G)AC (C/G)CACGMC, 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 0.6 kb with HindIII inserts 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.

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. methylotrophicus* (12) (24), *Brachyzoium 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 (GGHILTHG) 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 *BamHI* 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 EcoRI-*Sal*I fragment containing the middle portion of glyA was cloned into pUC19, and the *Sal*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, pLC181IES, contained the unique *Sal*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 *Pst*I and treated with T4 DNA polymerase, and then the *Hind*III fragment from pUC4K (37), containing the Km′ gene, was inserted. A plasmid, pLC181Sp2, in which the Km′ gene was inserted 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.
coli S17-1, which was used as a donor in biparental matings with *M. extorquens* AM1. Km\(^\text{r}\) transconjugants were selected on rich medium with low frequency. Those that were simultaneously Tc\(^\text{r}\) 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 BamHI fragment from pLC181a, the 1.4-kb HincII fragment from pUC4K carrying the Km\(^\text{r}\) 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.

TABLE 1. Activities of some enzymes participating in C\(_1\) metabolism in mutant and wild-type *M. extorquens* AM1

<table>
<thead>
<tr>
<th>Strain and growth condition*</th>
<th>Enzyme activity (nmol/min/mg of protein)(b)</th>
<th>HPR</th>
<th>SHMT</th>
<th>MTHFDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM1 Succ</td>
<td>330</td>
<td>5</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>MeOH</td>
<td>1,200</td>
<td>30</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>Gly1 Succ</td>
<td>340</td>
<td>0</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>MeOH(f)</td>
<td>1,300</td>
<td>0</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>Gly16 Succ</td>
<td>345</td>
<td>0</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>MeOH(f)</td>
<td>1,300</td>
<td>0</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>Gly16(pLC310.181a) Succ</td>
<td>320</td>
<td>60</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>MeOH</td>
<td>1,400</td>
<td>75</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>Gly6(pLC310.181b) Succ</td>
<td>380</td>
<td>90</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>MeOH</td>
<td>1,350</td>
<td>120</td>
<td>280</td>
<td></td>
</tr>
</tbody>
</table>

* Succ, cells were grown on succinate; MeOH, cells were grown on methanol except as indicated otherwise.

\(f\) Data are averages of two to four independent measurements. Values agreed ± 15%.

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

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 pl of about 6, which is the calculated pl 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\(_2\) compounds supplemented with glycine or serine, underscoring the central role of SHMT in carbon assimilation during methylo trophic 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.
exorquens 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 C2 compounds. This is surprising, since current proposals for growth on C2 compounds do not include SHMT (1, 11). However, a part of the serine cycle, involving the conversion of acetyl-CoA to glycine, is known to be common for both C1 and C2 metabolism. The biochemical details of this pathway are unknown, but glycine will restore growth of mutants defective in this pathway on both C1 and C2 compounds (11). The glyA mutants are unusual in this respect, since glyoxylate restores growth on C2 compounds but not on C1 compounds. This suggests a dual role for SHMT during methylo trophic growth: the expected central role in the incorporation of C1 units into the serine cycle, and an unexpected secondary role in the conversion of acetyl-CoA to glycine. This secondary role would also be involved in growth on C2 compounds and would explain the growth phenotype of the glyA mutants.

Expression of glyA in M. exorquens AM1. The 6-kb PstI 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 pLC310.181, 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. To confirm their ability to grow on methanol, the fragment was able to restore a C1-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 C2 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 levels 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. exorquens AM1 chromosome involved in C1 assimilation. The HindIII insert into pLC2 identified in this work did not reveal any common restriction patterns with the HindIII fragment cloned in pM2, which was identified earlier (12) and shown to contain a cluster of serine cycle genes. Two other HindIII 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 glycine 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. exorquens AM1 chromosome that have been shown so far to contain genes involved in oxidation or assimilation of C1 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. exorquens AM1 known to be involved in C1 assimilation. Its location with respect to other chromosomal regions involved in C1 metabolism is not known.

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REFERENCES