JOURNAL OF BACTERIOLOGY, Jan. 1992, p. 71–77
0021-9193/92/010071-07$02.00/0

Vol. 174, No. 1

Cloning, Mutagenesis, and Physiological Effect of a Hydroxypyruvate Reductase Gene from *Methylobacterium extorquens* AM1

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Received 2 August 1991/Accepted 25 October 1991

The gene encoding the serine cycle hydroxypyruvate reductase of *Methylobacterium extorquens* AM1 was isolated by using a synthetic oligonucleotide with a sequence based on a known N-terminal amino acid sequence. The cloned gene was inactivated by insertion of a kanamycin resistance gene, and recombination of this insertion derivative with the wild-type gene produced a serine cycle hydroxypyruvate reductase null mutant. This mutant had lost its ability to grow on C-1 compounds but retained the ability to grow on C-2 compounds, showing that the hydroxypyruvate reductase operating in the serine cycle is not involved in the conversion of acetyl coenzyme A to glycine as previously proposed. A second hydroxypyruvate-reducing enzyme with a low level of activity was found in *M. extorquens* AM1; this enzyme was able to interconvert glyoxylate and glycollate. The gene encoding hydroxypyruvate reductase was shown to be located about 3 kb upstream of two other serine cycle genes encoding phosphoenolpyruvate carboxylase and malyl coenzyme A lyase.

Methylobacterium extorquens AM1 is a pink-pigmented facultative methylotroph that utilizes the serine cycle for C-1 assimilation (23). In the serine cycle, a C-1 unit is condensed with glycine to form serine, which is converted via hydroxypyruvate and D-glycerate into 3-phosphoglycerate (16). The net assimilation of C-1 compounds into cell constituents by this pathway requires the regeneration of one molecule of glycine for each molecule of 3-phosphoglycerate formed. The mechanism of synthesis of glycine from C-1 compounds remains unknown, but it is known that acetyl coenzyme A (acetyl-CoA) is a precursor (30). The use of labeled compounds and mutant studies have shown that the unknown route involved in the synthesis of glycine from acetyl-CoA is also essential for growth on ethanol, pyruvate, lactate, malonate, and hydroxybutyrate (6–8, 26, 27, 30). The *M. extorquens* AM1 methanol mutant 20BL was generated by nitrosoguanidine mutagenesis and lacks hydroxypyruvate reductase (HPR) (12). It does not grow on ethanol, but it grows normally on hydroxybutyrate (8, 30). These data suggested that this key enzyme of the serine cycle might play a second role in the route for conversion of acetyl-CoA into glycine during growth on C-2 compounds (8) but not during growth on hydroxybutyrate (30). However, the genetic lesion in 20BL was not determined, and so it was not clear whether the phenotype observed was due to a single mutation or whether the structural gene for the serine cycle HPR was affected.

To clarify this situation, it was necessary to construct a null serine cycle HPR mutant from the cloned structural gene and to determine its phenotype. In a recent paper, we described purification of the serine cycle HPR from *M. extorquens* AM1 and presented its N-terminal amino acid sequence (3). The present work was devoted to cloning of the gene encoding the serine cycle HPR and constructing an insertion HPR mutant of *M. extorquens* AM1 by gene replacement.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown in LB medium in the presence of appropriate antibiotics as described by Maniatis et al. (19). Isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were added at 40 μg/ml. *M. extorquens* AM1 was grown in the minimal medium described previously (9). Succinate (20 mM), methanol (100 mM), methylamine (20 mM), ethanol (20 mM), and ethylamine (20 mM) were used as growth substrates. The following antibiotic concentrations were used for *M. extorquens* AM1: tetracycline, 20 μg/ml; kanamycin, 150 μg/ml; rifamycin, 50 μg/ml. The growth responses of mutants were tested on plates containing the indicated substrates.

DNA-DNA hybridizations. DNA-DNA hybridizations were carried out with dried agarose gels as described by Meinkoth and Wahl (21) or with nitrocellulose filters as described by Maniatis et al. (19). Hybridizations with an oligonucleotide probe end labeled as described by Maniatis et al. (19) were carried out, and the filters were washed at 45°C. Hybridization with plasmid DNA labeled by the random primed method as described by the manufacturer (Boehringer GmbH, Mannheim, Germany) was carried out, and the filters were washed at 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.

DNA manipulations. Plasmid isolation, *E. coli* transformation, restriction enzyme digestion, ligation, blunting ends with T4 DNA polymerase, and polynucleotide kinase reaction were carried out as described by Maniatis et al. (19). The chromosomal DNA of *M. extorquens* AM1 was isolated by the procedure of Saito and Miura (25).

Oligonucleotide probe synthesis. The oligonucleotide probe 5′-AAIAAGGTNGTNTTCCTNGAYCG-3′ was synthesized based on known amino acid sequence of HPR (3) by the California Institute of Technology Microchemical Facility.

DNA sequencing. DNA sequencing was carried out with an Applied Biosystems automated sequencer by the University

TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
DH5α	F⁻ *recA1* Δ(*lacZYA-argF*) U169 *hsdR17 thi-1 gyrA66 supE44 endA1 relA1* Δ80d*lac* Δ(*lacZ*) M15	Bethesda Research Laboratories
HB101	*F⁻ hsdS20*(r⁻ m⁻)*recA13 ara-14 proA2 lacY1 galK2 rpsL20*(Sm^r)*xyl-5 mtl-1 supE44* λ⁻	1a
S17-1	F⁻ *pro recA1* r⁻ m⁺ RP4-2, integrated (Tc::Mu) (Km::Tn*7* [Sm^r Tp^r])	28
M. extorquens AM1		
AM1rif	Wild type, Rif^r	22
20BL	*hpr-1*, Rif^r	12
HPR38	*hprA*(*hpr*::Km^r) Rif^r	This study
HPR42	*hprA*(*hpr*::Km^r) Rif^r	This study
Plasmids		
pAYC61	Ap^r Tc^r, *mob*⁺, Inc(ColE1)	A. Chistoserdov
pAYC139	Tc^r, IncP1, *mob*⁺ *mauBEDAC*	2
pRK310	Tc^r, *lacZ' mob*⁺, IncP1	4
pRK2013	Km^r, *tra*⁺, Inc(ColE1)	4
pUC4K	Ap^r Km^r	32
pUC19	Ap^r, *lacZ'*	Pharmacia
pVK100	Tc^r Km^r Cos, IncP1	14
pLC3	Ap^r, *hpr*⁺	This study
pLC3.2	Ap^r, Δ*hpr*	This study
pLC13	Ap^r Tc^r, *hpr*⁺ *mob*⁺, IncP1	This study
pLC21	Ap^r Tc^r, *hpr*⁺ *mob*⁺, IncP1	This study
pLC311	Ap^r Tc^r, *hpr*::Km^r	This study
pLC613	Ap^r Tc^r, *hpr*::Km^r *mob*⁺, Inc(ColE1)	This study
pDN24	Tc^r, IncP1, *mob*⁺ *moxAKLB*	22
pDN202	Tc^r, IncP1, *mob*⁺ *moxEQ*	22
pM2	Tc^r, IncP1, *mob*⁺ *ppc mcl*	9
p8-25	Tc^r, IncP1, *mob*⁺ *moxRS*	18
pDN411	Tc^r, IncP1, *mob*⁺ *moxFJGI*	22
p48C-46	Tc^r, IncP1, *mob*⁺ *moxH*	22
p1130-BH-310	Tc^r, IncP1, *mob*⁺ *moxCPOMNDTV*	22
pSS13-1	Tc^r, IncP1, *mob*⁺ *mmf-2*	29
pSS48-1	Tc^r, IncP1, *mob*⁺ *mmf-1*	29
pBE7.21	Tc^r Ap^r, Inc(ColE1)	1

[a] *mau*, methylamine utilization; *mox*, methanol oxidation; *mcl*, malyl-CoA lyase; *mmf-1*, acetyl-CoA conversion to glycine; *mmf-2*, glycerate kinase; *ppc*, PEP carboxylase.

of California, Los Angeles, Sequencing Facility. The sequences were also read manually and were determined from both strands.

Enzyme assays. Enzyme activities were determined in *M. extorquens* AM1 and *E. coli* crude extracts prepared as described earlier (3). All measurements were done at room temperature in a total volume of 1 ml. HPR was assayed as described by Chistoserdova and Lidstrom (3). The reaction mixture for the malate thiokinase–malyl coenzyme A (malyl-CoA) lyase coupled assay contained 50 mM Tris-HCl (pH 7.6), 2 mM ATP, 5 mM $MgCl_2$, 1 mM CoA, 1.5 mM phenylhydrazine, 10 mM L-malate, and the cell extract. The rate of increase of optical density at 324 nm caused by formation of glyoxylic acid phenylhydrazone from glyoxylate and phenylhydrazine (5) after L-malate addition was determined. Phosphoenolpyruvate (PEP) carboxylase (acetyl-CoA independent) and serine glyoxylate aminotransferase were assayed as described by Goodwin (10). For measuring the latter activity in HPR-deficient mutants, exogeneous HPR purified from *M. extorquens* AM1 was added. Serine transhydroxymethylase activity was measured in a discontinuous assay described by Lidstrom (17). Spectrophotometric methods (13, 33) were used for protein determination.

Isoelectrofocusing. Isoelectrofocusing was performed with PhastGel IEF gels of the appropriate pH range and the PhastSystem in accordance with the recommendations of the manufacturer (Pharmacia LKB). HPR was visualized by specific staining as described by Chistoserdova and Lidstrom (3) with D-glycerate or glycollate as a substrate. The reaction mixture for activity staining of serine transhydroxymethylase contained 100 mM Tris-HCl buffer (pH 7.6), 1 mM NADP, 0.1 mM pyridoxal phosphate, 0.25 mM tetrahydrofolate, 20 mM L-serine, 0.5 mM phenazine methosulfate, 1 mM nitrotetrazolium blue, and 1 U of methylenetetrahydrofolate dehydrogenase per 20 ml.

Matings. Triparental and biparental matings between *E. coli* and *M. extorquens* AM1 were performed overnight on filters on succinate-minimal agar. Cells were washed with sterile medium and plated on selective medium at appropriate dilutions. Rifamycin was used for *E. coli* counterselection.

Nucleotide sequence accession number. The sequence of the 200-bp *Kpn*I-*Eco*RI fragment described here has been deposited with Gen Bank under accession number M81443.

RESULTS

Cloning and analysis of the serine cycle HPR gene. On the basis of the N-terminal amino acid sequence of the HPR of

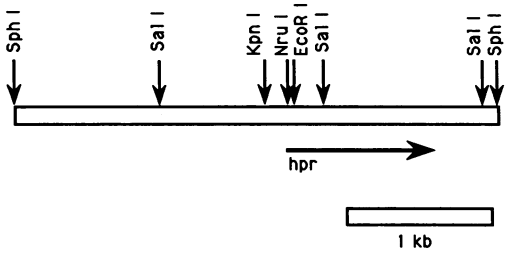

FIG. 1. Restriction map of the 3-kb *Sph*I fragment containing the HPR coding region. The arrow shows the direction of transcription and the size of the gene.

M. extorquens AM1, an oligonucleotide of 23 bases was synthesized with a redundancy of 128. It was deduced from amino acid residues 2 through 9 (3). A 3-kb *Sph*I fragment of the *M. extorquens* AM1 chromosome that hybridized with the probe was isolated from an agarose gel by electroelution and cloned into pUC19, and the resulting plasmid was transformed into *E. coli* DH5α. A few positive clones were selected, and restriction analysis revealed that these carried the plasmid with the cloned 3-kb fragment in two different orientations. A restriction map of the cloned fragment carried in a plasmid designated pLC3 is shown in Fig. 1. A more precise location of the hybridizing region of the insert was determined by Southern hybridization of restriction fragments with the same probe, which narrowed the region to a 200-bp *Kpn*I-*Eco*RI fragment. This fragment was isolated from an agarose gel by electroelution and cloned into pUC19, and the resulting plasmid (pLC3.2) was transformed into *E. coli* DH5α. The cloned fragment was sequenced on both strands and was found to contain the beginning of the serine cycle HPR (*hprA*) gene. The resulting nucleotide and deduced amino acid sequences are presented in Fig. 2. The amino acid sequence of the N-terminal part of HPR is in complete agreement with that of purified HPR (3). The nucleotide sequence, however, revealed the presence of methionine and threonine at the N terminus instead of one unidentified amino acid in the protein sequence. Possibly, the start methionine is removed posttranslationally. The sequence also revealed the presence of a putative ribosome-binding site (AGG) 8 bp upstream of the start codon at the 5′ end of the gene. In accordance with the known subunit molecular mass of HPR (37 kDa [3]), the 3-kb fragment is sufficient for encoding the entire *hprA*.

Construction of an insertion mutant in *hprA* of *M. extorquens* AM1. The strategy for introduction of a specific mutation in the chromosomal gene encoding HPR was based on the exchange of DNA via homologous recombination in vivo between the wild-type gene and a gene that had been inactivated by an insertion mutation in vitro (24, 31). The suicide vector pAYC61 used in this study was constructed and provided by A. Chistoserdov (1b) (Table 1).

```
KpnI     10         20         30
GGTACCTCATGAAACTCCCGGTCACCGACCGTTCTCG

 40         50         60         70
CTCCGGCGATGACGGGGCGGCAGGGGTTTTGTGAGCG

    80         90        100        110
ACACGATTGCCCGCCGGTCGATCCGGAAGGGGTCCAGA

        120        130             NruI
ATG ACA AAG AAA GTC GTC TTC CTC GAT CGC
Met Thr Lys Lys Val Val Phe Leu Asp Arg

        150        160          EcoRI
GAG TCG CTC GAT GCG ACC GTG CGC GAA TTC
Glu Ser Leu Asp Ala Thr Val Arg Glu Phe
```

FIG. 2. Nucleotide sequence of the 5′ region of *hprA* and predicted amino acid sequence. A putative Shine-Dalgarno sequence is underlined.

The sequenced 5′ part of *hprA* was located in the middle of the cloned fragment in plasmid pLC3 and had adjacent DNA fragments large enough to allow recombination events on both sides of an *Nru*I site within the gene (Fig. 1 and 2). *hprA* was mutated in vitro by digestion of the sequence at the *Nru*I site and subsequent ligation of a 1.3-kb *Hin*cII fragment of pUC4K containing the kanamycin resistance gene from transposon Tn*503*. The resulting plasmid, pLC311, was digested with *Sph*I, and a 4.3-kb fragment containing the interrupted gene was ligated, after the single-stranded ends were removed, into the single *Sma*I site of pAYC61. This construct, pLC613, was then used for transformation of the mobilizing strain, *E. coli* S17-1.

E. coli S17-1 harboring pLC613 was used as the donor strain in a biparental mating with *M. extorquens* AM1, and kanamycin-resistant colonies were isolated. About 20% of these colonies were tetracycline sensitive, which should be the result of a double recombination event leading to a complete gene replacement.

To confirm the presence of the in vitro-mutated *hprA* at the proper location in the chromosome of *M. extorquens* AM1, chromosomal DNA of the parental and mutant strains was isolated and digested with *Sph*I for Southern analysis. An *Sph*I digest of pLC3 was used as a probe (data not shown). The probe hybridized with a 3-kb fragment of the wild-type chromosomal DNA and, as expected, with a 4.3-kb DNA fragment of the mutant strains.

Analysis of the *hprA* insertion mutants. Growth responses and the presence of HPR activity were determined in seven insertion mutants. All of the mutants had lost their ability to grow on C-1 compounds (methanol and methylamine) but retained the ability to grow on C-2 compounds (ethanol and ethylamine). All of the mutants possessed very low HPR activity in comparison with that of the wild-type strain when grown on succinate plus methanol. Two mutants, HPR38 and HPR42, were used for further enzymological analysis.

Five serine cycle enzyme activities were determined in the mutants and the wild-type *M. extorquens* AM1. Because the serine cycle enzymes are inducible by C-1 compounds but repressed by the presence of multicarbon compounds (16), the activities were determined after methanol induction as described by Dunstan et al. (7) (Table 2). In general, all of the activities tested in the mutants except HPR activity were comparable with those in the wild-type strain. Since it is known that some serine cycle methylotrophs contain two separate serine transhydroxymethylases (11, 17, 20), the presence of both enzymes was tested by isoelectrofocusing of crude extracts followed by activity staining. Isoelectrofocusing revealed the presence of one form of the enzyme (with a pI of about 6.0) in succinate-grown cultures and two isoforms (with pIs of about 6.0 and 6.5) in methanol-induced cultures of both the wild-type and mutant strains (data not shown).

The presence of low HPR activity in the mutants and its increased activity under conditions of methanol induction was rather surprising, because the in vitro mutation should lead to complete absence of the enzyme. To determine whether some alternative enzyme is able to catalyze the reaction, crude extracts of the mutants and the wild-type

TABLE 2. Serine cycle enzyme activities in *hprA* insertion mutants and the wild-type strain of *M. extorquens* AM1

Strain	Growth conditions	Enzyme activity (nmol min^{-1} mg of $protein^{-1}$)[a]				
		Serine transhydroxy-methylase	Serine glyoxylate aminotransferase	HPR	Malate thiokinase–malyl-CoA lyase	PEP carboxylase
AM1	Succinate[b]	35		265		
	Methanol induced[c]	60	203	1,200	220	30
HPR38	Succinate	14		1		
	Methanol induced	36	152	23	220	59
HPR42	Succinate	10		2		
	Methanol induced	33	109	29	172	40

[a] The results are averages of two to three independent experiments, which agreed within ±15%.
[b] Cells were grown on succinate (20 mM).
[c] Cells were grown on 20 mM succinate, collected, washed with sterile medium, and incubated for 18 to 20 h with 100 mM methanol.

strain were isoelectrofocused and specifically stained by using the reverse reaction with D-glycerate as a substrate. An intense, methanol-inducible activity band corresponding to HPR with a pI of 4.75 (3) was present in the wild-type strain extract but not in extracts of the insertion mutants or in 20BL (Fig. 3). A second, less intense methanol-inducible band (pI 6.5) was present in all extracts (Fig. 3), indicating the presence of a second enzyme. Staining of the gel by using the reverse reaction with glycollate as a substrate has shown that the second enzyme gave a positive response but not HPR, indicating that the second hydroxypyruvate-reducing enzyme is able to convert glycollate into glyoxylate (data not shown). Purified HPR does not oxidize glycollate (3). The activity of the second hydroxpyruvate-reducing enzyme was absent in extracts of ethanol- or ethylamine-grown cells of the insertion mutants, and the pI 6.5 activity band did not appear on gels of the same extracts after activity staining, showing that the enzyme is not present in cells grown on C-2 compounds.

The second hydroxypyruvate-reducing enzyme of *M. extorquens* AM1. The pI 6.5 hydroxypyruvate-reducing enzyme was briefly characterized by using crude extracts of HPR38 (Table 3) to determine whether its properties differed from those of the HPR described earlier (3). The pI 6.5 enzyme catalyzed the reduction of glyoxylate and the reduction of hydroxypyruvate equally effectively, and catalyzed the reverse reaction with D-glycerate almost as effectively as it catalyzed the direct reaction. Although we did observe the reverse reaction with glycollate in gels (see above), we were not able to measure this activity in crude extracts, possibly because the equilibrium of the reaction lies toward the formation of glycollate.

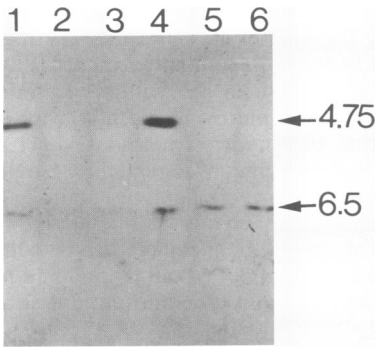

FIG. 3. Specific staining for HPR activity after isoelectrofocusing (pH 3.0 to 9.0) of crude extracts of the wild-type and *hpr* mutants of *M. extorquens* AM1. Lanes: 1 through 3, AM1, HPR38, and 20BL, respectively, grown on succinate; 4 through 6, AM1, HPR38, and 20BL, respectively, methanol-induced cultures (see footnote *c* of Table 2). Each lane contained 10 μg of protein.

Expression of *hprA* in *M. extorquens* AM1 and *E. coli*. Plasmid pLC3 was cloned into the *Hin*dIII site of pRK310 (4) to study the expression of the cloned gene. Both orientations of the 3-kb fragment were obtained, resulting in plasmids pLC13 (*hprA* is downstream of the *lac* promoter for pUC19) and pLC21 (*hprA* is in the opposite orientation with respect to the *lac* promoter), which were transformed into *E. coli* DH5α. The resulting strains of *E. coli* DH5α were used as the donors in triparental matings with mutants 20BL, HPR38, and HPR42. HPR activity was determined in the donor strains carrying pLC13 and pLC21 and in transconjugants of *M. extorquens* AM1 obtained in the matings (Table 4).

In *E. coli* DH5α carrying pLC13, the activity was doubled in comparison with that of the plasmid-free strain or DH5α carrying pLC21 when cells were grown without induction by IPTG and about five times increased in the induced cells. It is known that *E. coli* contains its own HPR (glycerate dehydrogenase) (15). To check whether the increase in HPR activity in DH5α(pLC13) was caused by *M. extorquens* AM1 *hprA* expression or was an activity variation, crude extracts of *E. coli* strains were isoelectrofocused and specifically stained for HPR activity (Fig. 4). A purified preparation of HPR from *M. extorquens* AM1 was used as a control. Activity staining of the gel revealed that plasmid-free DH5α and DH5α(pLC21) extracts contained an HPR band with a pI of about 4.5. DH5α carrying pLC13 contained the pI 4.5 band and an additional band with pI 4.75, corresponding to the purified HPR band. This result showed that *hprA* of *M. extorquens* AM1 is expressed in *E. coli* when placed in the direction of the *lac* promoter but not when placed in the opposite orientation. The low level of induction observed may be due to transcriptional and/or translational inefficiencies.

High HPR activity was measured in transconjugants of *M. extorquens* AM1 mutants carrying pLC13 or pLC21, indicating that the cloned fragment contains not only the structural gene for HPR but probably also its own promoter. The activity in succinate-grown transconjugants was higher than that in the wild type (Table 2); this might be due to either increased gene copies (5 to 7 copies) or titration of a

TABLE 3. Properties of two hydroxypyruvate-reducing enzymes from *M. extorquens* AM1

pI of HPR	% of total activity[a]	Induction of activity by methanol (fold)	Activity with glyoxylate (% of activity with hydroxypyruvate)	Velocity of reverse reaction with D-glycerate (% of direct reaction)	Activity with glycollate	pH optimum
4.75[b]	98	2.5–3	15	1.5	–	4.5
6.5[c]	2	5–10	100	99	+	6.6

[a] Methanol-induced cells. Activity was measured at pH 6.6.
[b] Data from Chistoserdova and Lidstrom (3).
[c] The enzyme was studied in crude extracts of an HPR (pI 4.75)-deficient mutant (HPR38).

repressor. A moderate induction by methanol was observed. The activity was higher in strains containing the pLC13 construct, suggesting that the *lac* promoter functions effectively in *M. extorquens* AM1. Isoelectrofocusing of crude extracts of transconjugants showed the presence of both the pI 4.75 and pI 6.5 activity bands (data not shown). As expected, insertion mutants HPR38 and HPR42 containing pLC13 or pLC21 regained the ability to grow on methanol and methylamine.

20BL mutant phenotype. As described above, the absence of HPR in insertion mutants of *M. extorquens* AM1 did not affect their ability to grow on C-2 compounds. However, mutant 20BL, also lacking HPR activity, is unable to grow on methanol or ethanol (8). This suggests that 20BL may contain a second mutation in a C-2 metabolic pathway or may contain a single mutation that affects both the HPR and a C-2 pathway. Revertants of 20BL have been described that contained wild-type levels of HPR and had regained their ability to grow on methanol (12). However, it was not specified whether they grew at the same rate as the wild-type strain or whether they were able to grow on ethanol. We have found that transconjugants of 20BL carrying *hprA* and possessing high HPR activity (Table 4) do grow on C-1 compounds but with much-reduced rates in comparison with that of the wild-type strain of *M. extorquens* AM1. The transconjugants, however, are still defective in the ability to grow on C-2 compounds. We have been unable to complement 20BL for growth on methanol with a broad-host-range cosmid clone bank (8a) that has been used successfully to isolate many other C-1 genes (2, 9, 18, 22, 29). These data further support the hypothesis that 20BL could contain at least two separate mutations.

TABLE 4. Activity of HPR in *M. extorquens* AM1 *hpr* mutants and *E. coli* DH5α carrying *hprA*

Strain	Activity (nmol min^{-1} mg of $protein^{-1}$)[a]	
	Noninduced	Induced
E. coli		
DH5α	53[b]	42[c]
DH5α(pLC13)	100[b]	250[c]
DH5α(pLC21)	41[b]	44[c]
M. extorquens AM1		
20BL	3[d]	31[e]
20BL(pLC13)	2,240[d]	3,015[e]
20BL(pLC21)	1,020[d]	1,400[e]
HPR38(pLC13)	2,000[d]	2,780[f]
HPR38(pLC21)	800[d]	1,100[f]

[a] The results are averages of two to three independent experiments, which agreed within ±15%.
[b] Cells were grown on LB medium.
[c] Cells were grown on LB medium in the presence of IPTG.
[d] Cells were grown on succinate.
[e] Cells were grown on succinate and then induced by methanol.
[f] Cells were grown on methanol.

Complementation analysis of the insertion mutants. Ten clones containing large DNA fragments of *M. extorquens* AM1 (2, 9, 18, 22, 29) carrying known genes associated with C-1 metabolism were introduced into the insertion mutants to determine whether they could complement the mutants. The clones tested were pAYC139 (*mauBEDAC*), pDN24 (*moxAKLB*), pDN202 (*moxEQ*), pM2 (*ppc mcl*), p8-25 (*moxRS*), pDN411 (*moxFJGI*), p48C-46 (*moxHU*), p1130BH-310 (*moxCPOMNDTV*), pSS13-1 (*mmf-2*), and pSS48-1 (*mmf-1*) (Table 1). These clones carry genes for methylamine utilization (*mau*) and methanol oxidation (*mox*) and also four genes responsible for synthesis of the serine cycle enzymes, malyl-CoA lyase, PEP carboxylase, glycerate kinase, and an enzyme involved in the synthesis of glycine from acetyl-CoA. Transconjugants obtained in triparental matings between *E. coli* HB101 carrying these cloned DNA fragments of *M. extorquens* AM1 DNA and mutants HPR38 and HPR42 were tested for their ability to grow on methanol. None of these DNA fragments was able to complement the mutations, and none of these fragments contained restriction enzyme cleavage patterns that matched those shown in Fig. 1.

Localization of *hprA*. A 7.21-kb *Eco*RI fragment of *M. extorquens* DNA was isolated in pBR322 (pBE7.21) (1). This fragment overlaps the end of the plasmid pM2, near the genes for the serine cycle enzymes PEP carboxylase (*ppc*)

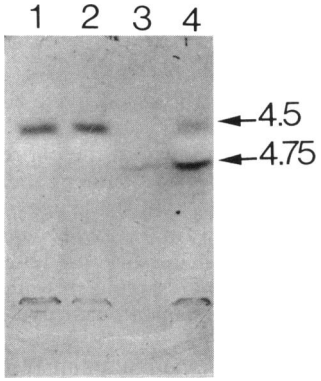

FIG. 4. Specific staining for HPR activity after isoelectrofocusing (pH 4.0 to 6.5) of crude extracts of *E. coli* strains harboring *hprA* from *M. extorquens* AM1. Lanes: 1, DH5α; 2, DH5α(pLC21); 3, purified HPR from *M. extorquens* AM1; 4, DH5α(pLC13).

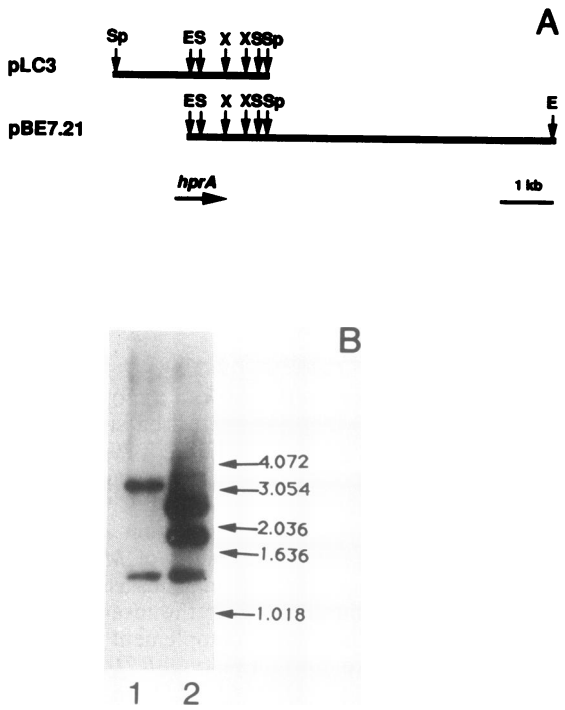

FIG. 5. Localization of *hprA*. (A) Restriction maps of pLC3 and pBE7.21 show an overlapping region. The arrow shows the location and direction of transcription of *hprA*. (B) Autoradiogram of *Eco*RI-*Sph*I-digested pBE7.21 (lane 1) and pLC3 (lane 2). An *Sph*I digest of pLC3 was used as a probe. The 3.7-kb fragment identified in lane 1 is the result of hybridization of the pBR322 replicon with the pUC19 replicon. The standards are fragments from a 1-kb ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Abbreviations: E, *Eco*RI; S, *Sal*I; Sp, *Sph*I; X, *Xho*I.

and malyl-CoA lyase (*mcl*) (9, 18). This *Eco*RI fragment contains restriction enzyme cleavage patterns in common with those of the *Sph*I *hprA* fragment described here (Fig. 5A). To prove that the *Sph*I fragment carrying *hprA* overlaps with the *Eco*RI fragment, *Eco*RI-*Sph*I digests of pLC3 and pBE7.21 were hybridized with an *Sph*I digest of pLC3 labeled by the random priming method (Fig. 5B). Hybridization analysis revealed that a 1.4-kb *Eco*RI-*Sph*I fragment common to pLC3 and pBE7.21 hybridized with the 3-kb *Sph*I fragment carrying *hprA*. This places *hprA* about 3 kb from *ppc*, and the gene order is *hprA-ppc-mcl*.

DISCUSSION

The structural gene for the serine cycle HRP (*hprA*) of *M. extorquens* AM1 has been isolated. The expression and sequence data obtained show that the entire structural gene must be present on the cloned fragment and suggest that the promoter is also present on this fragment. Little is known about promoter sequences in *M. extorquens* AM1 (18), and further studies will be necessary to define the promoter region. Hybridization analysis has revealed that *hprA* is located about 3 kb from a pair of serine cycle genes, *ppc* and *mcl*.

Construction of a null *hprA* mutant in *M. extorquens* AM1 has shown that the absence of HPR does not affect growth on C-2 compounds. This is in contrast to the dual role of HPR in C-1 and C-2 metabolism in *M. extorquens* AM1 suggested by Dunstan et al. (8). Their proposal was based on the finding that a nitrosoguanidine-induced HPR mutant (20BL) was not able to grow on ethanol or ethanol plus glycollate but could grow on ethanol plus glyoxylate. These data suggested that the first role of HPR in *M. extorquens* AM1 metabolism would be the reduction of hydroxypyruvate in the serine cycle and that the second role would be oxidation of glycollate to glyoxylate in an unknown C-2 pathway for the biosynthesis of glycine from acetyl-CoA (8). However, other mutants shown to be defective in the glycine synthesis pathway differed phenotypically from 20BL. Their growth on both methanol and ethanol was restored in the presence of either glyoxylate or glycollate (6, 8, 26, 27). Since 20BL has been the only available HPR mutant in *M. extorquens* AM1, this phenotype has resulted in confusion concerning the assimilation of C-2 compounds (8, 30). Our data show that the product of *hprA*, which is clearly the major HPR in *M. extorquens* AM1, is not required for growth on C-2 compounds. We have also presented information suggesting that 20BL contains at least two separate lesions, one affecting HPR and one affecting C-2 metabolism. This putative second lesion, however, could not be the same as those in mutants PCT48 and C5, which have been shown to be defective in the glycine synthesis pathway (8, 27), since, unlike PCT48 and C5, 20BL can grow well on pyruvate (our results) and hydroxybutyrate (30). It is also possible that 20BL contains a single lesion that affects HPR as well as enzymes involved in glycine synthesis and that our inability to complement 20BL reflects a lack of the appropriate clone in our clone bank. Growth responses of 20BL transconjugants carrying *hprA* show that the second mutation in 20BL could affect an enzyme(s) that is essential for both C-1 and C-2 assimilation pathways. Further work will be required to analyze the defect(s) in this mutant.

Enzymological analysis of the *hprA* insertion mutant showed that *M. extorquens* AM1 contains a second hydroxypyruvate-reducing enzyme, with different properties, that is also inducible by methanol. Although we detected low activities of this enzyme, it is the first enzyme detected in *M. extorquens* AM1 with the ability to interconvert glycollate and glyoxylate. The function of this second HPR is not yet known, but it is a candidate for the proposed glycollate oxidation step in the pathway for glycine synthesis (6–8).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM 36296 from the National Institutes of Health.

We are grateful for the helpful discussions of Andrei Chistoserdov and his gift of pAYC61.

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