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Genetics of Serine Pathway Enzymes in *Methylobacterium extorquens* AM1: Phosphoenolpyruvate Carboxylase and Malyl Coenzyme A Lyase

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***Methylobacterium extorquens* AM1 is a facultative methylotrophic bacterium that uses the serine pathway for formaldehyde incorporation as its assimilation pathway during growth on one-carbon compounds. A DNA region from *M. extorquens* AM1 previously shown to contain genes for the serine pathway enzymes malyl coenzyme A (CoA) lyase and hydroxypyruvate reductase has been characterized in more detail. Insertion mutagenesis revealed an additional region required for growth on one-carbon compounds, and all of the insertion mutants in this region lacked activity for another serine pathway enzyme, the acetyl-CoA-independent phosphoenolpyruvate (PEP) carboxylase. Expression analysis with *Escherichia coli* of DNA fragments that included the malyl-CoA lyase and PEP carboxylase regions identified five polypeptides, all transcribed in the same direction. Three of these polypeptides were expressed from the region necessary for the acetyl-CoA-independent PEP carboxylase, one was expressed from the region containing the malyl-CoA lyase gene, and the fifth was expressed from a region immediately downstream from the gene encoding hydroxypyruvate reductase. All six genes are transcribed in the same direction, but the transposon insertion data suggest that they are not all cotranscribed.**

Methylobacterium extorquens AM1 (formerly *Pseudomonas* sp. strain AM1) is a facultative methylotroph of the α-2 subdivision of the *Proteobacteria* (37). In this strain, methanol and methylamine are oxidized to formaldehyde by their respective dehydrogenases, and the formaldehyde produced is partitioned between two pathways that either oxidize it to CO_2 or assimilate it into cell carbon (2). *M. extorquens* AM1 uses the serine pathway for formaldehyde incorporation (Fig. 1) as its assimilatory pathway during methylotrophic growth, and the enzymes of this pathway are induced during growth on one-carbon compounds (2). However, little is known concerning the regulatory mechanisms involved in controlling serine pathway enzymes or how this formaldehyde-consuming pathway is coordinated with formaldehyde production.

In order to study these regulatory mechanisms in detail, it is necessary to clone and characterize the genes for serine pathway enzymes. In *M. extorquens* AM1, four serine pathway genes have been cloned, one for malyl coenzyme A (CoA) lyase (10), one for glycerate kinase (32), one for the unknown pathway that converts acetyl-CoA to glyoxylate (32), and one for hydroxypyruvate reductase (7) (Fig. 1). The gene for malyl-CoA lyase was cloned by complementation of a malyl-CoA lyase mutant and is located within a 2-kb region on a 19.6-kb chromosomal DNA fragment (10). It was later shown that this gene was located approximately 10 kb from a cluster of eight genes involved in the methanol oxidation system (22). The gene for hydroxypyruvate reductase (*hprA*) was cloned with an oligonucleotide probe based on an N-terminal amino acid sequence of purified hydroxypyruvate reductase (7), and it was localized within a 1-kb region of a 3-kb chromosomal DNA fragment. This DNA fragment was shown to be adjacent to that containing the gene for malyl-CoA lyase, with the two genes separated by approximately 7 kb (7). The genes for glycerate kinase and the unknown acetyl-CoA oxidation pathway were cloned by mutant complementation but were not precisely located on the large DNA fragments obtained (32). Neither of these clones shows overlap with the clones described above (7), and so at least three separate chromosomal regions encode genes of the serine pathway in *M. extorquens* AM1. We have now analyzed the region between the genes for hydroxypyruvate reductase and malyl-CoA lyase in *M. extorquens* AM1 and have shown that it contains additional genes involved in methylotrophy, including at least one required for synthesizing another serine pathway enzyme, the acetyl-CoA-independent phosphoenolpyruvate (PEP) carboxylase. All of these clustered genes are transcribed in the same direction, and we have also shown that the malyl-CoA lyase gene encodes a polypeptide of approximately 39 kDa.

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 at 37°C in Luria-Bertani medium in the presence of appropriate antibiotics. *M. extorquens* AM1 was grown at 30°C in a minimal medium described previously (10) or in nutrient broth (Difco Laboratories, Detroit, Mich.). Supplements were filter sterilized separately and were added to sterile medium at the following final concentrations: methanol, 0.5% (vol/vol); succinate, 0.2% (wt/vol); methylamine, 0.2% (wt/vol); kanamycin (Km), 50 μg/ml; tetracycline (Tc), 10 μg/ml; rifamycin (Rf), 30 μg/ml; and ampicillin (Ap), 100 μg/ml.

Enzyme assays. Cells were grown to mid-log phase in

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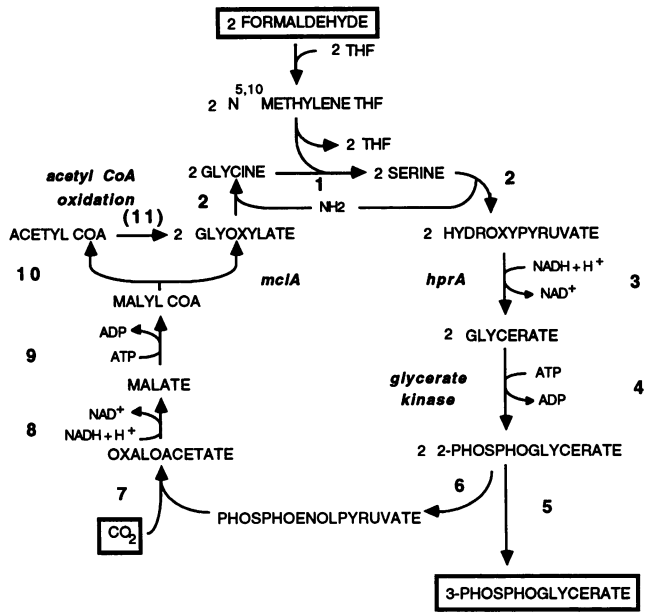

FIG. 1. Serine pathway for formaldehyde assimilation in *M. extorquens* AM1 (2). Enzymes (in boldface): 1, serine hydroxymethyltransferase; 2, serine-glyoxylate aminotransferase; 3, hydroxypyruvate reductase; 4, glycerate kinase; 5, phosphoglycerate mutase; 6, enolase; 7, PEP carboxylase; 8, malate dehydrogenase; 9, malate thiokinase; 10, malyl-CoA lyase; 11, the pathway by which acetyl-CoA is converted to glyoxylate (the biochemical steps are unknown) (2). The genes that have been cloned are marked in boldface italics (*mclA*, malyl-CoA lyase; *hprA*, hydroxypyruvate reductase).

succinate minimal medium, washed with sterile minimal medium, and resuspended to the original culture volume with sterile minimal medium containing methanol. These cultures were incubated for 20 h to allow for the induction of methylotrophy enzymes. For those strains with Tn*5* and Km cassette insertions, Km was also added to all media to prevent the growth of strains that had lost the Km marker. Cells were harvested, washed with potassium phosphate buffer (20 mM, pH 7.0), and broken by two passes through a French pressure cell at 137 mPa. Cell debris was removed by centrifugation at 10,000 × *g* for 15 min, and the supernatant was used for enzyme assays. For glycerate kinase assays, an aliquot of the supernatant was centrifuged at 100,000 × *g* for 1 h to remove NADH oxidase activity, and the supernatant was used for enzyme assays.

The following enzymes were assayed by previously published procedures: hydroxypyruvate reductase (D-glycerate: NAD^+ oxidoreductase, EC 1.1.1.29) (20), serine-glyoxylate aminotransferase (L-serine:glyoxylate aminotransferase, EC 2.6.1.45) (4), methanol dehydrogenase (EC 1.1.99.8) (29), malyl-CoA lyase (EC 4.1.3.24) (30), acetyl-CoA-independent PEP carboxylase (orthophosphate:oxaloacetate carboxylyase [phosphorylating], EC 4.1.1.31) (11), glyoxylate-activated serine hydroxymethyltransferase (5,10-methylenetetrahydrofolate:glycine hydroxymethyltransferase, EC 2.1.2.1) (23), glycerate kinase (EC 2.7.1.31) (11), and formate dehydrogenase (EC 1.2.1.2) (16). Malyl-CoA for the malyl-CoA lyase assays was a gift from J. R. Quayle, University of Bath. Protein was determined by the method of Lowry et al. (24).

DNA manipulations. Rapid small-scale isolation of plasmid DNA was performed by the method of Holmes and Quigley (14) or that of Birnboim and Doly (3) with chloramphenicol amplification (25). Plasmid DNA for cloning and restriction enzyme analysis was prepared by the large-scale method of Ish-Horowicz and Burke (15) and was purified by centrifugation on two CsCl density gradients. Chromosomal DNA of *M. extorquens* AM1 was isolated as described previously (10).

Restriction enzyme digests of DNA were carried out as specified by the suppliers (Bethesda Research Laboratories, Rockville, Md., and New England Biolabs, Beverly, Mass.). Restriction fragments were isolated from agarose gels by electrophoresis by using an Elutrap (Schleicher & Schuell) electroelution system or as described in reference 26. Ligations of restriction fragments were with T4 DNA ligase (Bethesda Research Laboratories) as recommended by the supplier. *E. coli* cells were transformed by the $CaCl_2$ procedure of Mandel and Higa (25).

DNA-DNA hybridizations. Preparation of nitrocellulose filters and DNA probes for DNA-DNA hybridizations was carried out as described previously (10). Hybridizations were carried out as described by Toukdarian and Lidstrom (36), with the conditions 50% formamide at 37°C and washing at 65°C.

Tn*5* and Km cassette mutagenesis. Plasmid DNA (pBE7.21 and pB11.30) was mutagenized in *E. coli* HB101 with bacteriophage lambda::Tn*5* (38). An insertion mutation designated 5PK was also constructed by ligating a DNA fragment containing the Km resistance cassette of pUC4K (Pharmacia) into a *Bgl*II site in the 11.3-kb *Hin*dIII fragment of pB11.30. Plasmids containing Tn*5* or the Km cassette insertion were transferred to *M. extorquens* AM1 by three-way matings as described previously (10), selecting for Km resistance in the presence of Rf as counterselection against *E. coli*. Km-resistant colonies were screened on Tc, since Ap resistance from pBR322 is not expressed in *M. extorquens* AM1 (9a). The majority of colonies were Tc resistant. The small percentage of colonies that were Tc sensitive were screened for growth on methanol and methylamine.

Protein expression. Protein expression from cloned genes was carried out with *E. coli* DH5α with the T7 promoter vectors pT7-5, pT7-6, and pT7-3 and plasmid pGP1-2 containing the T7 polymerase gene by the procedure of Tabor (33). Cells containing the expression plasmid plus pGP1-2 were grown in Luria-Bertani medium plus Ap (40 μg/ml) and Km (40 μg/ml). After heat induction at 42°C for 15 min, Rf was added to 200 μg/ml and the sample was incubated at 42°C for 10 to 15 min. The sample was transferred to 30°C for 20 to 30 min, and then 10 μCi of [^{35}S]methionine was added for 5 to 10 min. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (19). Following electrophoresis, the gels were shaken at room temperature in 0.5 M sodium salicylic acid plus 1% (vol/vol) glycerol for 30 min before being dried (5). Dried gels were exposed to X-ray film at −70°C for several days. Protein molecular mass standards were either from Bio-Rad (phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.3 kDa) or from Amersham (same as above plus myosin, 200 kDa).

TABLE 1. Bacterial strains, plasmids, and phage used in this study

Strain, plasmid, or phage	Relevant trait(s)	Source or reference
Strains and/or characteristics		
E. coli		
DH5α	r⁻ m⁺ *recA1* Δ(*lacZYA-argF*)U169 *hsdR thi-1 gyrA supE endA1 relA1* 80δ*lac*Δ(*lacZ*) M15	BRL, Inc.[a]
HB101	r⁻ m⁻ *hsdS recA ara-14 supE lacY mcrB galK proA xyl-5 mtl-1 rpsL*	26
M. extorquens AM1		
AM1 Rf^r	Rf^r	10
PCT-57 Rf^r	Rf^r *mcl-1*	
b	AM1 Rf^r (near *mclA*)::Tn*5*(Km^r) (methanol$^+$ and methylamine$^+$)[b]	10
m	AM1 Rf^r (near *mclA*)::Tn*5*(Km^r) (methanol$^+$ and methylamine$^+$)	10
Plasmids		
pVK100	Tc^r Km^r IncP1 *rlx* cosmid Mob$^+$	18
pRK2013	Km^r ColE1 replicon Mob$^+$ with RK2 *tra* genes	9
pBR322	Tc^r Ap^r ColE1 replicon Bom$^+$	26
pM2	pVK100(19.6-kb AM1 *Hin*dIII); *mclA moxPC*	10
pV11.88	pVK100(11.3 kb AM1 *Hin*dIII); *mclA*	10
pB11.30	pBR322(11.3 kb AM1 *Hin*dIII); *mclA*	10
pBE7.21	pBR322(7.2 kb AM1 *Eco*RI); *mclA*	This study
pGP1-2	Km^r p15A *ori* t7 gene 1 (RNA polymerase) inducible P_{lac} promoter λ*c*I85	34
pT7-3	Ap^r T7 promoter	S. Tabor
pT7-5	Ap^r, same as pT7-3 except that *bla* gene is reversed	33
pT7-6	Ap^r, same as pT7-3 except that polylinker sequence is reversed	33
Phage λ::Tn*5*	Km^r defective λ*rex*::Tn*5*	38

[a] BRL, Inc., Bethesda Research Laboratories, Inc.
[b] Methanol$^+$ and methylamine$^+$, able to grow on methanol and methylamine.

RESULTS

Isolation and cloning of an *Eco*RI fragment that overlaps pM2. In a previous study, a 19.6-kb DNA fragment that contained a gene for the serine pathway enzyme malyl-CoA lyase was cloned into the vector pVK100 to generate plasmid pM2 (10). This insert contained two *Hin*dIII fragments of 11.3 and 8.3 kb which were shown to be adjacent on the chromosome (10). A chromosomal fragment that contains genes for the methanol oxidation system was later identified and shown to be adjacent to the 8.3-kb *Hin*dIII fragment in pM2 (22, 27, 28). In order to analyze the region adjacent to the other side of the insert in pM2 (adjacent to the 11.3-kb *Hin*dIII fragment), an overlapping *Eco*RI fragment was isolated. It had been previously shown by chromosomal probing that two *Eco*RI fragments of approximately 7 and 10 kb overlapped pM2 (10). The 10-kb fragment was shown to overlap pM2 within the 8.3-kb *Hin*dIII fragment (27, 28), and so the 7-kb fragment was assumed to overlap the 11.3-kb *Hin*dIII fragment on the other side. Chromosomal DNA was digested with *Eco*RI and separated on an agarose gel. Fragments of approximately 7 kb were excised from the gel and electroeluted, and the resulting DNA was ligated to the vector pBR322. Transformants were screened, and a plasmid (pBE7.21) that contained a 7.2-kb fragment was identified. The identity of this overlapping fragment was confirmed by restriction enzyme analysis and probing with the 11.3-kb *Hin*dIII fragment. A map of this fragment and its overlap with the 11.3-kb *Hin*dIII fragment is presented in Fig. 2.

Isolation and characterization of insertion mutants. Fifty Tn*5* insertions were generated in pBE7.21 and pB11.30. These were mapped by restriction enzyme analysis with established restriction sites within the transposon (17, 39). About half of these were mapped to sites within the insert, and of these, 13 that were well separated were chosen for further study (Fig. 2). In addition, two Tn*5* insertions (insertions b and m) that had been previously reported (10) and a Km resistance insertion constructed in a *Bgl*II site of the 11.3-kb *Hin*dIII insert (5PK) were also studied further (Fig. 2).

Each of the insertion clones noted above was transferred to *M. extorquens* AM1 via three-way matings, and transconjugants were selected on Km and Rf. Since none of these plasmids is capable of replicating in *M. extorquens* AM1 (13), Km-resistant colonies should be the result of recombination of the plasmid into the chromosome. Km-resistant colonies that are also Tc sensitive should result from a double-crossover recombinational event, and these were chosen for further testing. The correct position of each transposon in the chromosome was confirmed by isolating chromosomal DNA from a Km-resistant, Tc-sensitive isolate for each insertion and probing chromosomal digests with the insert in pBE7.21 or pB11.30, as appropriate, and with either Tn*5* or the Km cassette, as required (data not shown). Chromosomal DNA from all mutant strains was also tested for hybridization to pBR322 to confirm that vector sequences were not present in the insertion mutants (data not shown).

All Km-resistant, Tc-sensitive colonies identified for each insertion were tested for growth on methanol and methylamine. This analysis identified a new region in the chromosome necessary for growth on both substrates (defined by insertions 201, 55, 53, g, n, and 5PK), adjacent to the region previously shown to contain a gene necessary for malyl-CoA lyase activity (10) (includes insertion t). One insertion mutant (insertion b) between these two regions grew normally on methanol and methylamine (Fig. 2).

Enzyme activities in insertion mutants. Several enzymes involved in methylotrophic metabolism were assayed for seven of the insertion mutants (Table 2). The results were compared with those for the wild type and the methylotro-

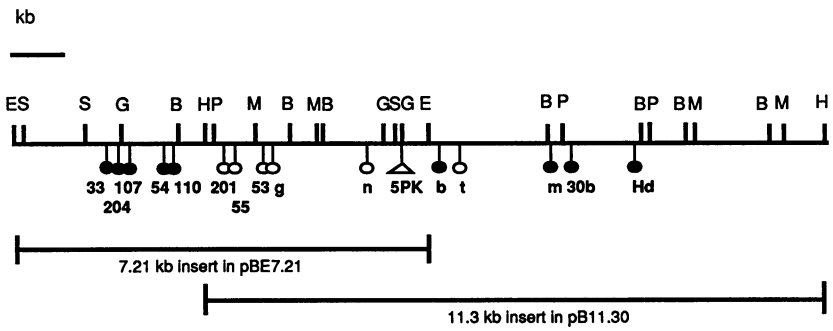

FIG. 2. Location of the Tn*5* and Km^r cassette insertions used in this study. Insertions 33, 204, 107, 54, 110, 201, 55, and 53 were generated in pBE7.21. Insertions g, n, 5PK, b, t, m, 30b, and Hd were generated in pB11.30. Each of these was used to generate chromosomal insertion mutants. Those mutants that grow normally on methanol and methylamine are denoted by solid circles, and those showing no growth on methanol or methylamine are designated by open circles. The Km^r cassette insertion (5PK) is designated by an open triangle. The chromosomal region shown covers a 14.6-kb section, including all of the insert DNA in pBE7.21 and pB11.30. E, *Eco*RI; S, *Sal*I; G, *Bgl*I; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; M, *Sma*I. *Bgl*II sites are shown for the insert in pBE7.21 only. kb, 1 kb.

phy mutant PCT-57. PCT-57 was originally isolated after nitrosoguanidine mutagenesis; it is unable to grow on either methanol or methylamine (8) and is defective in malyl-CoA lyase activity (30). An Rf-resistant mutant of PCT-57 was subsequently isolated (10) and was used throughout this study.

A number of enzyme activities tested for the mutants unable to grow on one-carbon compounds were at lower levels than those in the strains capable of growing on one-carbon compounds. However, this phenomenon is commonly observed in *M. extorquens* AM1 serine pathway mutants, and it is apparently due to the induction protocol (10, 32). For mutant strains unable to grow on one-carbon compounds, induction of serine pathway functions is accomplished by exposing washed succinate-grown cells to methanol for 20 h. Under these conditions, the serine pathway mutants are in a nongrowing state, and induction is apparently incomplete. Therefore, the low enzyme activity levels observed for the mutants probably do not reflect defects in the genes responsible for those enzymes. However, activity for one enzyme of the serine pathway, the acetyl-CoA-independent PEP carboxylase, was not detectable in insertion mutants 201, 53, g, n, and 5PK, suggesting that these mutants were altered in a function necessary for this enzyme. The PEP carboxylase activity was at low but detectable levels in insertion mutant t. Insertion t is located in the region previously shown to complement mutant PCT-57 (10), and the mutant t is assumed to be missing malyl-CoA lyase. This assumption could not be verified directly, as at the time this mutant was tested we were unable to obtain additional malyl-CoA to carry out the assay. This substrate is currently not available commercially.

It is possible that one or more enzymes involved in biochemical steps for which there is no assay are also missing from these insertion mutants. For instance, growth on both methanol and methylamine requires the oxidation of formaldehyde to formate. However, *M. extorquens* AM1 has multiple formaldehyde dehydrogenase activities, and it is not known whether any of these are required for growth on one-carbon compounds (2). It is possible that the real formaldehyde oxidation activity is coupled to transport from the periplasm into the cytoplasm and has never been identi-

TABLE 2. Enzyme activities in wild-type and insertion strains in the *hpr-mcl* region of *M. extorquens* AM1[a]

Enzyme	Activity (nmol/min/mg of protein)								
	Wild type[b]	b[b]	PCT-57[c]	201	53	g	n	5PK	t
Methanol dehydrogenase	64	62	38	NT[d]	NT	40	40	NT	NT
Formate dehydrogenase	84	75	19	36	22	32	73	43	58
Hydroxypyruvate reductase	343	375	396	350	144	184	247	304	180
Serine-glyoxylate aminotransferase	165	175	33	132	78	27	62	34	79
Serine hydroxymethyltransferase	37	23	34	47	55	55	21	29	60
Glycerate kinase	11	24	3	NT	4	8	6	NT	10
PEP carboxylase	16	36	20	0[e]	0	0	0	0	1.2
Malyl-CoA lyase	150	270	0	NT	NT	51	34	NT	NT

[a] Cultures were grown to mid-log phase on succinate, washed, resuspended in medium containing methanol, and incubated for 20 h to allow induction.
[b] Grows normally on methanol.
[c] Malyl-CoA lyase mutant (30).
[d] NT, not tested.
[e] 0, not detectable.

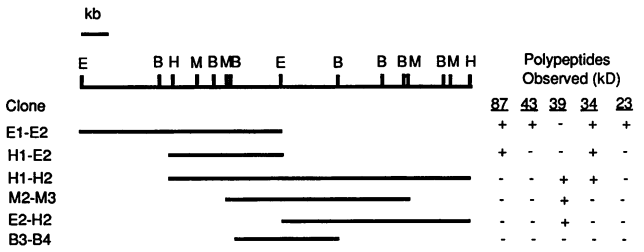

FIG. 3. Map of the clones used in the T7 expression experiments and the polypeptides observed from each. In all cases, the polypeptides were only observed when the indicated fragments were cloned into the appropriate expression vector such that the orientation with respect to the T7 promoter was left to right, as shown. Restriction sites are as described in the legend to Fig. 2. kb, 1 kb.

fied. Therefore, it is presently not possible to assess levels of the key methylotrophic formaldehyde oxidation activity. In addition, it has been inferred from pulse-labelling experiments that a section of the serine pathway must convert acetyl-CoA to glyoxylate, but the biochemical steps are unknown, and they cannot be assayed either individually or as a pathway (2).

Protein expression analysis. The regions defined by the transposon insertions were analyzed with *E. coli* for the production of encoded polypeptides by a T7 RNA polymerase-T7 promoter expression system (33). We have used this system successfully in the past to study methylotrophic genes of *M. extorquens* AM1 (1, 6). A series of subclones in both orientations with respect to the T7 promoter were constructed in the expression vectors pT7-5, pT7-6, and pT7-3 (33) and were used for expression experiments with *E. coli* (Fig. 3 and 4). Five polypeptides of approximately 87, 43, 39, 34, and 23 kDa were observed from these expression clones that were not present in controls with vector alone or with the clones in the opposite orientation (Fig. 3 and 4). All five were transcribed in the same direction, from left to right as shown in Fig. 3. No polypeptides from clones in the opposite orientation other than those observed with controls containing vector alone were observed (data not shown).

The 43- and 23-kDa polypeptides were only observed in clone E_1-E_2, containing the leftmost *Eco*RI fragment (Fig. 4, lane 5). The 87-kDa polypeptide was poorly expressed and was observed in two clones, E_1-E_2 (Fig. 4, lane 5) and H_1-E_2 (Fig. 4, lane 2), which share a common internal *Hin*dIII-*Eco*RI fragment. However, this fragment is also found in the H_1-H_2 clone, and the 87-kDa polypeptide was not observed in cells containing that clone (Fig. 4, lane 1). The 34-kDa polypeptide was observed in all three clones that have this interior *Hin*dIII-*Eco*RI fragment, E_1-E_2 (Fig. 4, lane 5), H_1-E_2 (Fig. 4, lane 2), and H_1-H_2 (Fig. 4, lane 1). The 39-kDa polypeptide was highly expressed compared with the other polypeptides, and it was observed in three clones, H_1-H_2 (Fig. 4, lane 1), M_2-M_3 (Fig. 4, lane 4), and E_2-H_2 (Fig. 4, lane 3), which have in common an internal *Eco*RI-*Sma*I fragment. One clone designated B_3-B_4 containing a *Bam*HI fragment internal to M_2-M_3 did not produce any of the five polypeptides (Fig. 4, lane 6). Additional expression experiments were carried out with different incubation times and different exposure times, but no other polypeptides above vector background were distinguishable in any of these experiments.

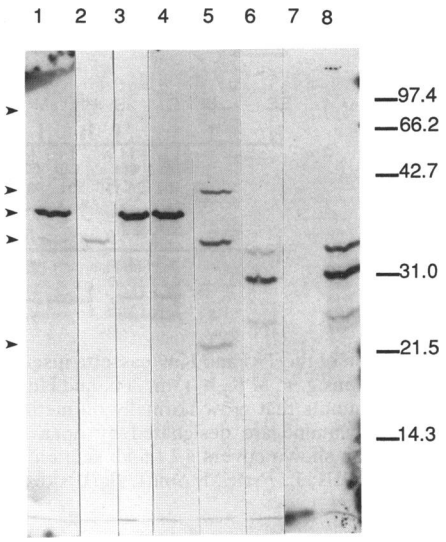

FIG. 4. Autoradiogram of polypeptides expressed by the T7 expression system in *E. coli* strains containing H_1-H_2 (lane 1), H_1-E_2 (lane 2), E_2-H_2 (lane 3), M_2-M_3 (lane 4), E_1-E_2 (lane 5), B_3-B_4 (lane 6), pT7-6 (lane 7), and pT7-3 (lane 8). See Fig. 3 for maps of the inserts. All inserts are in pT7-6 except for B_3-B_4, which is in pT7-3. Arrows denote polypeptides listed in Fig. 3 (from the top down, 87, 43, 39, 34, and 23 kDa), and molecular weight standards (in thousands) are on the right.

DISCUSSION

The results presented in this paper show that, in *M. extorquens* AM1, a cluster of genes involved in the serine pathway for formaldehyde incorporation is located in a chromosomal DNA segment of approximately 10 kb. Two serine pathway genes had previously been shown to be present in this region, *hprA*, the structural gene for the 37-kDa hydroxypyruvate reductase subunit (7), and a second gene encoding a function required for malyl-CoA lyase (10), which we now propose to call *mclA*. The locations of *hprA* and *mclA* are shown in Fig. 5A.

The insertion mutant analysis presented here has identified an additional region between *hprA* and *mclA* that is required for methylotrophic growth. This region is approximately 4.3 kb and extends at maximum from 0.3 kb 5′ to the first *Hin*dIII site shown in Fig. 5A to the second *Eco*RI site (see Fig. 2). The insertion mutants in this region contain detectable activities of the methanol and formate dehydrogenases and all assayable serine pathway enzymes, except for the acetyl-CoA-independent PEP carboxylase. Therefore, one or more functions required for activity of the acetyl-CoA-independent PEP carboxylase are encoded in this region. Since this is a 4.3-kb segment of DNA, it probably contains more than one gene, and the expression data confirm this assumption (see below). It is possible that not all of the genes in this region encode functions required for PEP carboxylase activity. For instance, upstream insertions might exert a polar effect on a downstream gene required for PEP carboxylase activity. If so, these mutants in upstream genes would be missing PEP carboxylase activity even though they were deficient in a gene encoding a different function. One or more of these upstream genes might code for enzymes involved in unknown pathways that could not be assessed, including formaldehyde oxidation and the conversion of acetyl-CoA to glyoxylate (2).

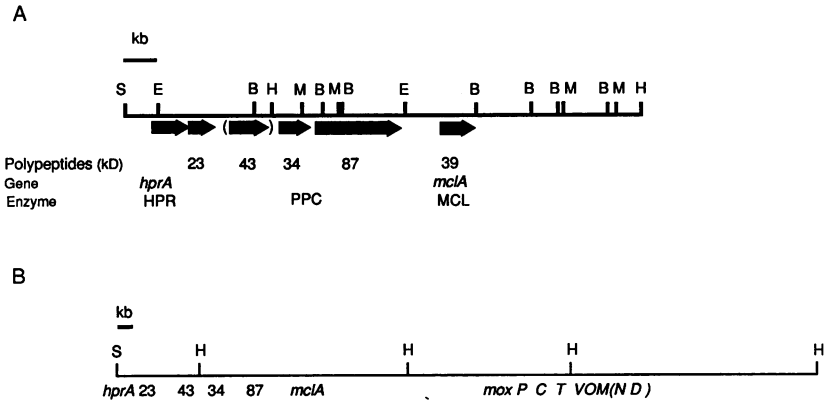

FIG. 5. Proposed map of the *M. extorquens* AM1 methylotrophy gene cluster studied. (A) Genes in a 15.6-kb region of *M. extorquens* AM1 chromosomal DNA containing *hprA* and *mclA*. Data for *hprA* are from reference 7, and the placement of *mclA* relies partly on data from reference 10. Parentheses denote uncertainty with regard to the location of the gene encoding the 43-kDa polypeptide (see text). (B) A 38-kb *M. extorquens* AM1 DNA region showing the location of the genes in panel A with respect to a cluster of eight *mox* genes. Data for *mox* genes are from reference 22. Restriction enzymes are as described in the legend to Fig. 2. *hprA*, structural gene for hydroxypyruvate reductase (HPR); *mclA*, gene required for malyl-CoA lyase (MCL) activity; PPC, acetyl-CoA-independent PEP carboxylase; *moxPCTVOMND*, genes required for methanol oxidation. Parentheses denote uncertain gene order.

The expression studies carried out in this work show that five polypeptides could be detected from the 14.6-kb DNA region analyzed. One of these is approximately 39 kDa and is encoded by the region between the second *Eco*RI site and the second *Sma*I site shown in Fig. 5. This region had been previously shown to contain *mclA*, which was located between Tn*5* insertions b and m (10) (Fig. 2). The 39-kDa polypeptide was not observed in expression experiments with the *Bam*HI fragment (B_3-B_4) (Fig. 4, lane 6) which overlaps part of this region. This is consistent with the fact that this fragment does not complement the malyl-CoA lyase mutant PCT-57 (10) and that *mclA* apparently extends just beyond this fourth *Bam*HI site. Therefore, these results suggest that *mclA* encodes the 39-kDa polypeptide. Malyl-CoA lyase has been previously purified, and it was found by sedimentation equilibrium to be an enzyme of 190 kDa (12). However, the subunit structure is unknown. A tetramer of a 39-kDa subunit would be 156 kDa, which is smaller than the mass reported for the purified holoenzyme. Therefore, it is not known whether *mclA* is a structural gene for malyl-CoA lyase or it encodes a regulatory or accessory function. It is not possible to assay expression extracts for malyl-CoA lyase activity, since the substrate for this enzyme (malyl-CoA) is not commercially available.

Four other polypeptides (approximately 23, 43, 34, and 87 kDa) were expressed from the *Eco*RI fragment (E_1-E_2) (Fig. 4, lane 5) containing the 3′ region of *hprA* and approximately 6 kb of downstream DNA. The translation initiation site for *hprA* is not included in this fragment (7), and so none of these polypeptides should be encoded by *hprA*. Two of these polypeptides (23 and 43 kDa) were not expressed from a downstream *Hin*dIII-*Eco*RI fragment, suggesting that their translation is initiated within the *Eco*RI-*Hin*dIII region immediately downstream of *hprA* (Fig. 5A). Preliminary sequence data for the region downstream of *hprA* have revealed an open reading frame of at least 23 kDa (6a), confirming that a gene encoding a 23-kDa polypeptide could be present immediately downstream of *hprA*. Its function is currently unknown, but its position suggests that it may be involved in methylotrophic growth. The 43-kDa polypeptide observed in the expression experiments must be encoded further downstream. It is not yet clear whether the gene encoding this polypeptide lies within the region defined by the methylotrophy-positive insertions or that defined by the methylotrophy-negative insertions, since both are large enough to contain this gene.

Two of the polypeptides (34 and 87 kDa) expressed from the E_1-E_2 *Eco*RI fragment were also expressed from the leftmost *Hin*dIII-*Eco*RI fragment (H_1-E_2) (Fig. 4, lane 2) shown in Fig. 5A. This fragment is sufficiently large (3.95 kb) to encode both polypeptides. A discrepancy exists with regard to expression of the 87-kDa polypeptide. This polypeptide was poorly expressed in all cases, but it should have been observed in the H_1-H_2 clone containing the first *Hin*dIII fragment shown in Fig. 5A, and it was not. In other methylotrophs in which this T7 expression system has been used, it is sometimes found that a polypeptide is observed from smaller subclones but not from larger clones containing the same fragment internally (31). The reason for this is not known, but it presumably reflects problems of expression in heterologous systems. Since this polypeptide was observed from two different clones and was not detected when the clones were in the opposite orientation with respect to the T7 promoter, it seems likely that it is encoded by this region. The order of the 34- and 87-kDa polypeptides appears to be 34 and then 87 (Fig. 5A), since neither polypeptide was observed from the M_2-M_3 clone (Fig. 4, lane 4). If the 87-kDa polypeptide were encoded upstream of the 34-kDa polypeptide, the gene encoding the latter polypeptide would be located entirely within the M_2-M_3 fragment and should have been observed from the M_2-M_3 clone. Both of these polypeptides are encoded from the region shown to be necessary for methylotrophic growth, and therefore they appear to be the products of methylotrophy genes. Note that if the 43-kDa polypeptide is also required for methylotrophic growth, the gene encoding it must be present within the 4.3-kb methylotrophy-negative region defined by the insertion mutants. This region is just large enough to encode the 43-, 34-, and 87-kDa polypeptides. Determining the precise locations of these three genes will require sequencing of this region.

As noted above, the region from which the three polypeptides (43, 34, and 87 kDa) were expressed is required for PEP

carboxylase activity, and one or more of these may be structural polypeptides for this enzyme. Although this PEP carboxylase was purified from *M. extorquens* AM1 30 years ago (21), the size and subunit structure were not reported at that time. Therefore, the polypeptide sizes observed in the expression experiments cannot be correlated with known structural polypeptides. Most PEP carboxylase enzymes purified so far consist of a tetramer of subunits of approximately 90 to 100 kDa (35), and the 87-kDa polypeptide is an obvious candidate for the PEP carboxylase subunit. However, it was expressed at such low levels in *E. coli* that it has not been possible to confirm its identity by enzyme assay. Preliminary sequencing data from this region have identified a partial open reading frame that contains substantial similarity to PEP carboxylase of *E. coli* (6a), strongly suggesting that the 87-kDa polypeptide encodes the structural gene for PEP carboxylase and that it does occur in the position shown in Fig. 5A.

The results presented above suggest that there are at least six genes in the 15.6-kb fragment shown in Fig. 5A, all transcribed in the same direction. Four of these are required for growth on one-carbon compounds, and it seems likely that the other two are also involved in methylotrophic growth. Functions necessary for the following three serine pathway enzymes, hydroxypyruvate reductase, acetyl-CoA-independent PEP carboxylase, and malyl-CoA lyase, are encoded within this region. This set of genes is located approximately 10 kb from a previously described cluster of genes involved in the methanol oxidation system (Fig. 5B). These results show that a number of methylotrophy genes are clustered in this 38-kb region of the *M. extorquens* AM1 chromosome (Fig. 5B).

The identification of this gene cluster encoding serine pathway functions should now allow studies of the molecular details of regulation of serine pathway enzymes. Although these genes are all transcribed in the same direction, they are probably not cotranscribed, because the three regions identified as being required for methylotrophy were separated by insertion mutants which exhibited a methylotrophy-positive phenotype. It is unlikely that these methylotrophy-positive areas represent intergenic regions of an operon, because both methylotrophy-positive and methylotrophy-negative insertions analyzed were present in both orientations. Further studies with transcript mapping and gene fusions will be required in order to determine the promoter structure of this gene cluster.

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