JOURNAL OF BACTERIOLOGY, Nov. 1984, p. 718–723
0021-9193/84/110718-06$02.00/0

Vol. 160, No. 2

Molecular Cloning of a Malyl Coenzyme A Lyase Gene from *Pseudomonas* sp. Strain AM1, a Facultative Methylotroph

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Received 20 June 1984/Accepted 3 August 1984

A genomic library containing *Hin*dIII partial digests of *Pseudomonas* sp. strain AM1 DNA was constructed in the broad-host-range cosmid pVK100. PCT57, a *Pseudomonas* sp. strain AM1 methanol mutant deficient in malyl coenzyme A lyase activity, was complemented to a methanol-positive phenotype by mobilization of the pVK100 library into PCT57 recipients with the ColE1/RK2 mobilizing plasmid pRK2013. Six different complemented isolates all contained a recombinant plasmid carrying the same 19.6-kilobase-pair *Pseudomonas* sp. strain AM1 DNA insert. Subcloning and complementation analysis demonstrated that the gene deficient in PCT57 (*mcl-1*) was located in a 1.6-kilobase-pair region within a 7.4-kilobase-pair *Eco*RI-*Hin*dIII fragment.

Pseudomonas sp. strain AM1 is a facultative methylotroph that assimilates C_1 compounds via the serine pathway for formaldehyde fixation (Fig. 1) (1, 23). Six enzymes of the serine pathway, hydroxypyruvate reductase, serine-glyoxylate aminotransferase, glycerate kinase, malyl coenzyme A (CoA) lyase, glyoxylate-activated serine transhydroxymethylase, and the C_1-specific phosphoenol pyruvate (PEP) carboxylase, as well as the methanol oxidation enzyme methanol dehydrogenase, have been shown to be inducible by growth on C_1 compounds (6, 19). The first four of these activities are also repressed by the presence of multicarbon compounds such as succinate or glucose, and results of induction studies have suggested that these activities might constitute a genetic regulon (19).

Until recently, the genetic analysis of C_1 functions in *Pseudomonas* sp. strain AM1 has not been possible, since no classical genetic systems exist. Attempts to transform both linear and plasmid DNA by a variety of techniques have been unsuccessful (unpublished data), no transducing phage are available, and no natural conjugation systems are present. However, the broad-host-range conjugative plasmid R68.45 has now been used to determine linkage relationships among a variety of mutants in *Pseudomonas* sp. strain AM1 (29, 30). Linkage was observed between drug resistance markers and methanol mutants, suggesting that some genes required for growth on methanol might be linked to each other. However, physical distances have not been assigned, and in some cases the genetic defects of these mutants are not clear.

We are interested in the transcriptional regulation of C_1-specific genes in methylotrophs. To obtain fine-structure genetic maps of C_1-specific genes in *Pseudomonas* sp. strain AM1, we used an approach involving molecular cloning techniques and mutant complementation. The *Pseudomonas* sp. strain AM1 methanol mutant PCT57 seemed to be a good candidate for these initial studies since it lacks an inducible serine pathway activity, malyl CoA lyase (6, 25), and appears to be a point mutant (25). We report here the construction of a *Pseudomonas* sp. strain AM1 gene library in a cosmid cloning vector and the use of PCT57 to clone a C_1-specific gene necessary for malyl CoA lyase activity.

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MATERIALS AND METHODS

Strains, media, and growth conditions. The strains and plasmids used in this study are listed in Table 1. PCT57 was provided by J. R. Quayle, University of Bath. Rifamycin-resistant strains of *Pseudomonas* sp. strain AM1 and PCT57 were isolated by plating ca. 10^8 cells onto nutrient agar plates containing rifamycin (20 μg/ml).

Pseudomonas sp. strain AM1 strains were grown at 30°C on either the ammonia mineral salts medium described by Harder et al. (9) or nutrient broth (Difco Laboratories, Detroit, Mich.), and *Escherichia coli* strains were grown at 37°C on L broth (20). Agar (Difco) was added to 1.5% (wt/vol) for plates. Supplements were sterilized separately and added to sterile medium at the following final concentrations: methanol, 0.5% (vol/vol); succinate, 0.2% (wt/vol); rifamycin, 20 μg/ml; tetracycline, 10 μg/ml; kanamycin, 50 μg/ml.

Enzyme assays. Wild-type *Pseudomonas* sp. strain AM1 was grown on methanol, and PCT57 was grown to mid-log phase on succinate, washed with sterile medium containing methanol, and incubated for 20 h. 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.

Enzymes were assayed by the following published procedures: hydroxypyruvate reductase (D-glycerate:NAD^+ oxidoreductase, EC 1.1.29) (15), serine-glyoxylate aminotransferase (L-serine:glyoxylate aminotransferase, EC 2.6.1.45) (4), methanol dehydrogenase (EC 1.1.99.8) (22), malyl CoA lyase (24), acetyl CoA-independent PEP carboxylase (orthophosphate:oxaloacetate carboxy-lyase [phosphorylating], EC 4.1.1.31) (5), glyoxylate-activated serine transhydroxymethylase (5,10-methylene-tetrahydrofolate:glycine hydroxymethyl transferase, EC 2.1.2.1) (21), and formate dehydrogenase (EC 1.2.1.2) (13). A small amount of malyl CoA was provided by J. R. Quayle, University of Bath. Protein was determined by the method of Lowry.

DNA isolation. For isolation of total cellular DNA from *Pseudomonas* sp. strain AM1 strains, 20 ml of cells was harvested in the late log phase and suspended in 0.5 ml of TE buffer (25 mM Tris, 10 mM EDTA [pH 8.01]) with 50 mM glucose and 1 mg of lysozyme per ml. Suspended cells were then frozen in a dry ice-ethanol bath. Sodium dodecyl sulfate

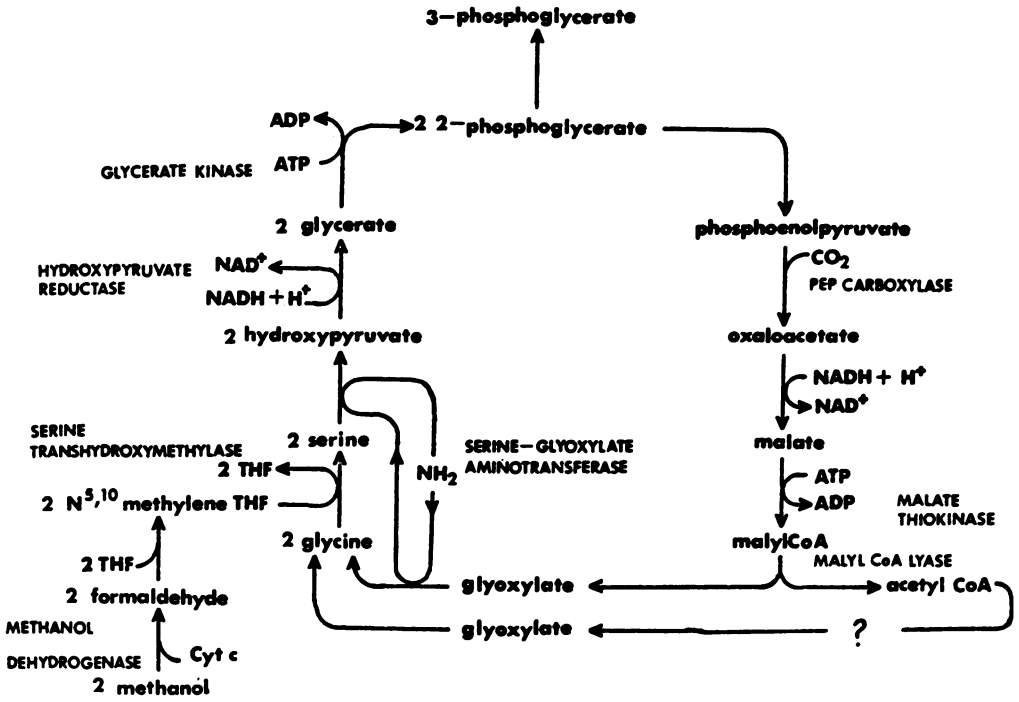

FIG. 1. The isocitrate lyase-negative serine pathway for the incorporation of formaldehyde (adapted from reference 9). The pathway for conversion of acetyl CoA to glyoxylate is unknown.

was added to 1% (wt/vol) on top of the frozen cells, and the mixture was transferred to a 60°C water bath for 10 min, when lysis was complete. Proteins were removed by extraction with phenol-chloroform, and DNA was precipitated with isopropanol as described by Maniatis and co-workers (17). Purified DNA was dissolved in a minimal amount of TE buffer.

Plasmid DNA from *E. coli* was isolated by the methods described by Holmes and Quigley for rapid screening (11). Large-scale plasmid DNA preparations were done by the method of Ish-Horowicz and Burke (12) or Birnboim and Doly (3), and the DNA was purified by banding in a cesium chloride-ethidium bromide equilibrium gradient.

Digestion of DNA with restriction endonucleases and dephosphorylation of DNA. Restriction enzymes were obtained from Bethesda Research Laboratories, Rockville, Md. The enzymes were used as described by Maniatis and co-workers (17).

For digestion of total cellular DNA, enzyme was added at 5 to 10 U of enzyme per μg of DNA. Reactions were typically incubated at 37°C overnight. For digestion of plasmid DNA, enzymes were used at 1 to 2 U per μg of DNA, and incubations were carried out at 37°C for 2 to 3 h.

Bacterial alkaline phosphatase was purchased from Bethesda Research Laboratories and was used according to the suggestions of Maniatis et al. (17).

Ligation and transformation. T4 DNA ligase was purchased from Bethesda Research Laboratories. Ligations were incubated either for 4 h at 22°C or for 16 h at 15°C with the buffer recommended by the vendor. Transformation of *E. coli* was performed as described by Mandel and Higa (16).

Clone bank construction. Total genomic DNA was partially digested with *Hin*dIII and combined with a complete *Hin*dIII digest of genomic DNA to ensure complete representation of all *Hin*dIII fragments. This DNA was then size-fractionated by electroelution from agarose gels (17) to yield fragments of greater than 15 kilobase pairs (kb). pVK100 DNA was digested with *Hin*dIII and dephosphorylated with bacterial alkaline phosphatase. The fractionated *Pseudomonas* sp. strain AM1 DNA was ligated to the dephosphorylated pVK100 DNA at a ratio of 3:1 (vector to insert), with a total DNA concentration of 300 μg/ml, and the resulting DNA was packaged in vitro into lambda procapsids (28). This preparation was used to infect *E. coli* HB101, selecting for tetracycline resistance (Tc^r). A pool of ca.

TABLE 1. Characteristics of the strains, plasmids, and phage used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Pseudomonas sp. strain AM1		
Wild type	Facultative methylotroph	23
Rf^r	Rifamycin resistant	This study
PCT57	Methanol (*mcl-1*)	6
PCT57 Rf^r	Rifamycin resistant PCT57	This study
E. coli		
HB101	*recA*	17
MM294	*recA*$^+$	2
CSR603	*recA uvrA6*	26
Plasmids and phage		
pVK100	Tc^r Km^r IncP1 *rlx*	14
pRK310	Tc^r IncP1 *rlx*, PUC9 linker	G. Ditta
pRK2013	Km^r ColE1 with RK2 *tra*	7
pBR322	ColE1 Tc^r Ap^r	17
λ::Tn*5*	Defectiveλ *rex*::Tn*5*	33

[a] Gene abbreviations: *mcl*, malyl CoA lyase; *recA*, recombination deficient; *uvrA6*, UV repair deficient; *rlx*, mobilization positive.

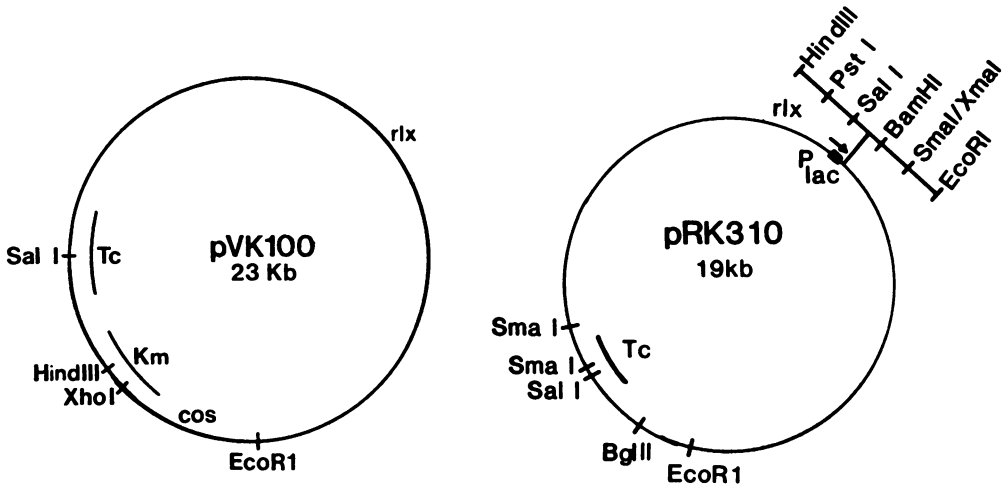

FIG. 2. The broad-host-range cosmid cloning vector pVK100 (14) and a related plasmid used for subcloning; pRK310; *rlx*, region necessary for mobilization; Tc, tetracycline resistance; Km, kanamycin resistance; *cos*, cohesive ends of lambda phage.

10,000 transductants was obtained by washing resultant colonies from plates into a minimum volume of LB broth. The pool was stored in 50% glycerol at −70°C.

Matings. Plasmids were mobilized into *Pseudomonas* sp. strain AM1 in three-way crosses between an *E. coli* HB101 donor, an *E. coli* CSR603(pRK2013) mobilizer, and a *Pseudomonas* sp. strain AM1 Rif^r recipient. Mid-log phase cultures of donor, mobilizer, and recipient were mixed in a 1:1:5 ratio and filtered onto a 0.45-μm membrane filter. The filter was incubated at 30°C on nutrient agar medium for 18 to 24 h, and the cells were stripped from the filter and plated onto selective medium (ammonia minimal salts agar, 10 μg of rifamycin per ml, 20 μg of tetracycline per ml, and either succinate or methanol). For complementation analysis of pM2 and its subclones, the cells were plated onto succinate, and then individual colonies were tested for growth on methanol plates.

Mobilization of plasmids from *Pseudomonas* sp. strain AM1 into *E. coli* was carried out in a similar manner, except that the selective medium was L agar plus 20 μg of tetracycline per ml.

All putative transconjugants were purified by a second single-colony transfer to selective medium to ensure the absence of donor cells.

Tn*5* mutagenesis. The recombinant plasmid pB11.30 (the 11.3-kb *Hin*dIII *Pseudomonas* sp. strain AM1 chromosomal fragment cloned into pBR322) was mutagenized in *E. coli* HB101 with Tn*5*, using lambda Tn*5* as described previously (33). Plasmids containing Tn*5* insertions were screened on *E. coli* MM294 (2).

DNA filter hybridization. Approximately 1 μg of DNA was restricted, electrophoresed on agarose gels, and transferred onto nitrocellulose paper by the method of Southern (27). DNA probes were radiolabeled by nick-translation with [^{32}P]dCTP and [^{32}P]dATP (New England Nuclear Corp., Boston, Mass.) to ca. 10^7 to 10^8 cpm/μg of DNA (18) and then hybridized to the DNA on the filters as described previously (31). Hybridization conditions were 50% formamide at 37°C, with washing at 65°C.

RESULTS

Construction of a *Pseudomonas* sp. strain AM1 clone bank. A clone bank of *Pseudomonas* sp. strain AM1 DNA was constructed in the broad-host-range cosmid pVK100 (Fig. 2). Total genomic DNA that was partially digested with *Hin*dIII and size-fractionated to greater than 15 kb was ligated to *Hin*dIII-digested pVK100 DNA that had been previously dephosphorylated with bacterial alkaline phosphatase. The ligated DNA was packaged in vitro into lambda procapsids (28) and used to infect *E. coli* HB101, resulting in a pool of ca. 10,000 transductants.

Random screening of individual clones for kanamycin sensitivity (Km^s) revealed that ca. 85% of the clones contained pVK100 with inserts, and plasmid screening showed that the average insert size was 20 kb.

Complementation of PCT57. The *Pseudomonas* sp. strain AM1 methanol mutant PCT57 lacks malyl CoA lyase activity (Mcl$^-$) and contains activity for methanol dehydrogenase, serine-glyoxylate aminotransferase, hydroxypyruvate reductase, and glycerate kinase (6, 25). To check our culture and to determine whether other C_1-specific genes were missing in this mutant, we tested growth substrates and assayed several

TABLE 2. Phenotypes and enzyme activities of *Pseudomonas* sp. strain AM1 wild type and PCT57

Growth substrate phenotype and enzyme activity	Strain	
	Wild type	PCT57
Growth substrates		
Methanol	+	−
Methylamine	+	−
Formate	+	−
Succinate	+	+
Ethanol	+	+
Enzyme activities (nmol/min/mg of protein)[a]		
Methanol dehydrogenase	78	19
Formate dehydrogenase	82	20
Hydroxypyruvate reductase	360	302
Serine-glyoxylate aminotransferase	176	63
PEP carboxylase (acetyl CoA independent)	22	12
Malyl CoA lyase	154	0
Serine transhydroxymethylase (glyoxylate activated)	29	31

[a] Values are the average of two or more determinations and agreed to within ± 10%.

TABLE 3. Complementation frequencies of PCT57

Donor	Recipient	Frequency Tc^r (per recipient)	Frequency methanol$^+$ (per Tc^r recipient)
E. coli HB101(pVK100)	*Pseudomonas* sp. strain AM1 PCT57 (Rf^r *mcl-1*)	7×10^{-2}	1.5×10^{-7}
E. coli HB101(pVK100:inserts)	*Pseudomonas* sp. strain AM1 PCT57 (Rf^r *mcl-1*)	9×10^{-2}	2.1×10^{-3}
E. coli HB101(pM2)[a]	*Pesudomonas* sp. strain AM1 PCT57 (Rf^r *mcl-1*)	(100%)	50 of 50

[a] pM2 is an *mcl*$^+$ plasmid.

C_1-specific enzymes in wild-type cultures grown on methanol and in PCT57 cultures grown on succinate and induced on methanol for 24 h (Table 2). Although some of the enzymes assayed were present at low levels compared with the wild type, all enzymes except malyl CoA lyase were detectable. The low levels of some C_1 enzymes may have been due to incomplete induction in the nongrowing cells.

pVK100 can be mobilized from *E. coli* strains into *Pseudomonas* sp. strain AM1 by the conjugative plasmid pRK2013 (7) at frequencies of 10^{-1} to 10^{-2} (Table 3). This allows the cloning of genes in this bacterium by direct mutant complementation. The *Pseudomonas* sp. strain AM1 (pVK100) clone bank was mated into PCT57 recipients, and methanol-positive clones were obtained at a frequency of 10^{-3} per Tc^r transconjugant. The hybrid plasmids in six complemented isolates (methanol$^+$, Tc^r) were mated into *E. coli* HB101 and then remated into PCT57. Of the Tc^r *Pseudomonas* sp. strain AM1 colonies in this second mating, 100% were able to grow on methanol, confirming the presence of a cloned DNA fragment that complemented the malyl CoA lyase mutation in *trans*.

Characterization of the plasmids complementing PCT57. The six complemented isolates tested were all found to contain plasmids with the same 19.6-kb insert. One of these, pM2, was chosen for further study, and the restriction map is shown in Fig. 3. Plasmid pM2 contained two *Hin*dIII fragments of 11.3 and 8.3 kb. The 11.3-kb *Hin*dIII fragment, in either orientation with respect to vector sequences, was sufficient for complementation of PCT57.

To determine whether the 11.3- and 8.3-kb *Hin*dIII fragments in pM2 were adjacent on the chromosome, chromosomal *Eco*RI digests were probed with pM2. By assuming that the two fragments were adjacent, pM2 would be expected to detect three *Eco*RI bands, the 14.2-kb internal fragment, and the two flanking fragments of unknown size but to be equal to or greater than 4.0 and 5.4 kb, respectively. When chromosomal *Eco*RI digests were probed with pM2, three fragments were detected, the 14.2-kb internal fragment plus two fragments of 9.6 and 7.4 kb. These data demonstrate, first, that the two *Hin*dIII fragments in pM2 were *Pseudomonas* sp. strain AM1 DNA and, second, that they were adjacent on the *Pseudomonas* sp. strain AM1 chromosome. *Pseudomonas* sp. strain AM1 contains three small plasmids, but the cloned *Hin*dIII fragments did not hybridize to them.

Further subcloning in pVK100 demonstrated that the 14.2-kb *Eco*RI fragment complemented PCT57 in either orientation with respect to vector sequences (Fig. 3). When the 3.6-kb *Bam*HI fragment was cloned in both orientations into another broad-host-range plasmid, pRK310 (Fig. 2), it was not capable of complementing PCT57, although other fragments (pVM6.2 and pVB7.2) cloned into this vector can complement PCT57 (see below). These data place the cloned Mcl gene within a 7.4-kb *Eco*RI-*Hin*dIII region (Fig. 3).

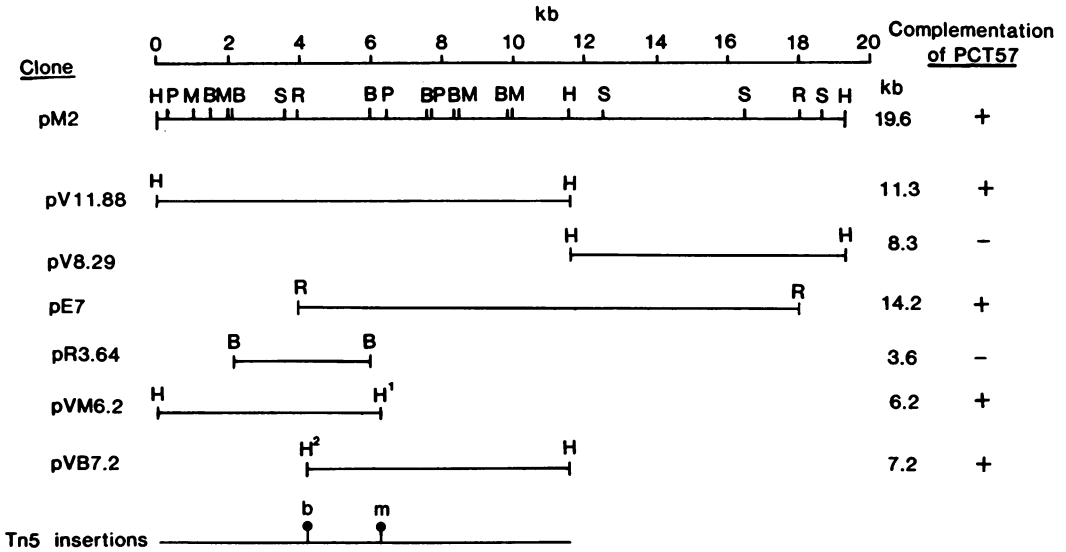

FIG. 3. Restriction map and characteristics of the clones and subclones used in this study. The sites of Tn5 insertions are indicated at the bottom of the figure. Abbreviations: H, *Hin*dIII; P, *Pst*I; M, *Sma*I; R, *Eco*RI; B, *Bam*HI; S, *Sal*I; [1], subcloned from insertion m; [2], subcloned from insertion b.

To further define the malyl CoA lyase gene, a series of Tn*5* insertions were generated in the 11.3-kb *Hin*dIII fragment which had been cloned into pBR322. Since these insertions were carried by a plasmid that does not replicate in *Pseudomonas* sp. strain AM1, they could not be used for complementation. However, two insertions within the 7.4-kb *Eco*RI-*Hin*dIII region (insertions m and b) were useful for generating more subclones. The *Hin*dIII site in Tn*5* was used to construct two subclones in pRK310 (pVM6.2 and pVB7.2 [Fig. 3]). Both subclones complemented PCT57 in both orientations, demonstrating that the cloned Mcl gene is located in the 1.6-kb region between these two transposon insertions, and one end lies within the 0.2-kb region between the downstream *Bam*HI site in pR3.64 and insertion m.

Since no recombination-deficient strains of *Pseudomonas* sp. strain AM1 are available, it is possible that the observed complementation could be due to recombinational rescue rather than the presence of a complete gene on the complementing plasmid. This appears to be unlikely, since in other studies with *Pseudomonas* sp. strain AM1 genes, we have found that complementation of mutants with partial genes occurs at a frequency of only a few percent of the Tc^r recipients (unpublished data). All of the complementing clones reported here produced a methanol-positive phenotype in 100% of the Tc^r recipients, suggesting that each carried the entire region necessary for complementation of PCT57.

DISCUSSION

A gene necessary for malyl CoA lyase activity (*mcl-1*) was cloned from the methylotrophic bacterium *Pseudomonas* sp. strain AM1 by complementation of a malyl CoA lyase mutant with a *Pseudomonas* sp. strain AM1 cosmid clone bank. This gene is located within a 1.6-kb region of chromosomal DNA. Since complementing clones complement equally well in both orientations, it seems likely that the gene is expressed in *Pseudomonas* sp. strain AM1 from its native promoter.

Malyl CoA lyase is a soluble protein, and no evidence exists for a prosthetic group or processing function (8). Therefore, the defective gene in PCT57 should be either structural or regulatory. It seems unlikely that PCT57 is defective in a regulatory gene, since it is not pleiotropic and it shows no detectable malyl CoA lyase activity. Revertants regain normal malyl CoA lyase activity (25). If it is defective in a structural gene, the cloned region must have the correct coding capacity. The region defined is sufficient to encode a protein of 50,000 to 60,000 daltons. Malyl CoA lyase has been purified from *Pseudomonas* sp. strain AM1 and found to be a protein of 190,000 daltons (8). However, the subunit structure is not known, and so it is not possible to determine by size whether the cloned Mcl gene is a structural gene for this enzyme or, if so, whether it is the only structural gene for malyl CoA lyase.

A second test for a structural gene, expression in *E. coli*, was not possible due to the limited availability of the substrate for the assay (malyl CoA). In addition, attempts to express other *Pseudomonas* sp. strain AM1 genes in *E. coli* have not yet been successful (unpublished data). Until expression has been obtained, we cannot conclusively determine whether the cloned Mcl gene is structural.

Linkage analysis by Tatra and Goodwin (29, 30) with R68.45-mediated chromosome mobilization has suggested that the PCT57 mutation is linked to other C_1-specific mutations. These include a mutation in a gene necessary for methanol dehydrogenase activity (*mtd-1*) as well as two pleiotropic mutations. One of these affects glycerate kinase and PEP carboxylase (*mmf-2*), and a second affects methylamine dehydrogenase, cytochrome *c*, and methanol dehydrogenase (*mcc-1*). No linkage distances have been determined, but it is known that R68.45 can transfer up to 200 kb of DNA in *Pseudomonas aeruginosa* (10). We are now carrying out transposon mutagenesis of pM2 and marker exchange (32) into *Pseudomonas* sp. strain AM1 to determine whether other C_1-specific genes are present on this 19.6-kb DNA fragment. This approach will provide fine-structure mapping data to complement the R68.45 linkage analysis of Tatra and Goodwin.

ACKNOWLEDGMENTS

This work was supported by a grant from Genentech, Inc. D.N.N. was supported by predoctoral traineeship GM07270-09 from the National Institutes of Health.

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