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Identification of Putative Methanol Dehydrogenase (*moxF*) Structural Genes in Methylotrophs and Cloning of *moxF* Genes from *Methylococcus capsulatus* Bath and *Methylomonas albus* BG8

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An open-reading-frame fragment of a *Methylobacterium* sp. strain AM1 gene (*moxF*) encoding a portion of the methanol dehydrogenase structural protein has been used as a hybridization probe to detect similar sequences in a variety of methylotrophic bacteria. This hybridization was used to isolate clones containing putative *moxF* genes from two obligate methanotrophic bacteria, *Methylococcus capsulatus* Bath and *Methylomonas albus* BG8. The identity of these genes was confirmed in two ways. A T7 expression vector was used to produce methanol dehydrogenase protein in *Escherichia coli* from the cloned genes, and in each case the protein was identified by immunoblotting with antiserum against the *Methylomonas albus* methanol dehydrogenase. In addition, a *moxF* mutant of *Methylobacterium* strain AM1 was complemented to a methanol-positive phenotype that partially restored methanol dehydrogenase activity, using broad-host-range plasmids containing the *moxF* genes from each methanotroph. The partial complementation of a *moxF* mutant in a facultative serine pathway methanol utilizer by *moxF* genes from type I and type X obligate methane utilizers suggests broad functional conservation of the methanol oxidation system among gram-negative methylotrophs.

Methanotrophic bacteria have recently received a great deal of attention due to their unique ability to utilize methane as a sole carbon and energy source. The enzyme systems involved in methanotrophic metabolism have interest from a commercial point of view (1, 4), and the role of methanotrophic bacteria in the global methane cycle has also become a significant topic (35). To understand and exploit the activities of methanotrophs, it is important to develop capabilities for genetic manipulation and to study gene organization and expression in these bacteria.

One system of particular interest in methanotrophs is that of methanol dehydrogenase (MeDH), which oxidizes methanol to formaldehyde (1). This enzyme carries out a key step in one-carbon metabolism, since it produces formaldehyde, the C_1 intermediate used for both assimilative and dissimilative metabolism. MeDH is universally found in gram-negative bacteria that grow aerobically on methane or methanol and is known to be conserved at the biochemical level (7) and, in some cases, at the immunological level as well (29, 30, 38). In most strains, MeDH appears to be a dimer of a subunit that ranges from 56 to 76 kilodaltons (kDa) in size and contains the cofactor pyrrolo-quinoline quinone (1, 7). The enzyme appears to be part of a periplasmic system (7) that is coupled to at least one soluble cytochrome *c* (7, 28). In some cases, a monomeric form of the enzyme is functional in vitro, but the in vivo significance of this protein is unknown (7).

We have reported a genetic analysis of functions necessary for methanol oxidation (Mox functions) in the facultative serine pathway methanol utilizer *Methylobacterium* sp. strain AM1, which suggests that at least 10 different gene products are involved (26). The 10 *mox* genes apparently include 2 encoding the MeDH and cytochrome *c* and 8 involved in assembly and regulation (27). A more recent study of the Mox system in a related methanotroph, *Methylobacterium organophilum* XX, has suggested that at least 11 genes are necessary for methanol oxidation in this organism (19). Therefore, it appears that the Mox system is quite complex in the facultative serine pathway methanol utilizers, but it is not known whether it is similarly complex in the obligate methanotrophs. Genetic studies of methanol metabolism in obligate methanotrophs are more difficult than in facultative methanol utilizers. The obligate methanotrophs are not capable of growth on compounds containing carbon-carbon bonds (37), and methanol oxidation is a required function for growth on all known substrates. Therefore, Mox mutations will be lethal in obligate methanotrophs. *Methylobacterium* strain AM1 provides an alternative host system for addressing questions of methanotrophic Mox functions. However, this approach is dependent upon biochemical and genetic conservation of Mox functions between distantly related methylotrophic bacteria. This paper reports the utilization of a portion of the *Methylobacterium* strain AM1 MeDH structural gene (*moxF*) as a hybridization probe to identify putative *moxF* genes in a variety of methylotrophs and to isolate and characterize the *moxF* genes from two obligate methanotrophs.

(A preliminary report of this work has been presented [R. L. Stephens and M. E. Lidstrom, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, I-114, p. 281].)

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. The media and growth conditions for *Methylobacterium* sp. strain AM1 and *Escherichia coli* strains have been previously described (8). Succinate was added to 0.2% (wt/vol) and methanol was

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant trait(s)[a]	Source or reference
E. coli		
HB101	*recA* Str^r	3
DH5α	*recA1*	15
CSR603	*recA uvrA6*, host for pRK2013	33
Methanol utilizers		
Methylobacterium sp. strain AM1		
AM1rif	Rif^r derivative of AM1; SP, 64% GC	26
UV26	*moxF* mutant of AM1rif	26
Hyphomicrobium sp. strain X	SP, 62% GC	2
Methylobacterium sp. strain 3A2	SP, 65% GC	5
Methylobacterium organophilium XX	SP, 65% GC	9, 31
Methane utilizers		
Methylococcus capsulatus Bath	Type X, SP, RMP, 65% GC	36, 37
Methylomonas albus BG8	Type I, RMP, 53% GC	37
Methylomonas sp. strain A1	Type I, marine, SP, RMP, 54% GC	Our laboratory
Methylosinus trichosporium OB3b	Type II, SP, 62% GC	37
Methylosinus sporium 5	Type II, SP, 62% GC	
Plasmids		
pVK100	Tc^r Km^r, IncP1 cosmid	16
pRK310	Tc^r *lacPOZ'*, IncP1	6
pRK2013	Km^r (mobilizing plasmid)	6
pHC79	Tc^r Am^r, ColE1 cosmid	11
pTZ18R	Am^r *lacZ'*, T7 promoter	U.S. Biochemical Corp.
pGP1-2	*c*I857 Km^r, T7 RNA polymerase	34

[a] Abbreviations: Str^r, streptomycin resistance; Rif^r, rifamycin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Am^r, ampicillin resistance; SP, serine pathway; RMP, ribulose monophosphate pathway; % GC, percent GC ratio.

added to 0.5% (vol/vol) where required. Antibiotics were added to the sterile medium in the following concentrations: tetracycline, 10 μg/ml; rifamycin, 10 μg/ml; kanamycin, 50 μg/ml; and ampicillin, 100 μg/ml. When kanamycin and ampicillin were used together, the concentrations were 40 μg/ml each.

Methylococcus capsulatus Bath was grown at 42°C on nitrate-mineral salts medium (36) to which a sterile vitamin solution (18) was added. The plates contained 1.5% (wt/vol) agar (Difco Laboratories, Detroit, Mich.). *Methylomonas albus* BG8 was grown at 30°C on nitrate-mineral salts medium as described above, but the plates contained 1.5% agarose (Difco or Sea-Chem). Both methanotrophs were maintained on plates in gas canisters under a methane-air (1:1) atmosphere and were grown in liquid culture under an atmosphere of the same composition.

Preparation of extracts and enzyme assays. Mid-log-phase *Methylobacterium* strain AM1 cells (100-ml culture) grown on minimal medium (26) with 0.5% methanol (vol/vol) were harvested by centrifugation at 10,000 × *g* for 10 min, washed once in 300 mM Tris hydrochloride buffer (pH 9.0), and suspended in 1 ml of the same buffer. The cells were broken by three passes through a French pressure cell at 20,000 lb/in^2, and the supernatant remaining after centrifugation at 15,000 × *g* for 15 min was used for enzyme assays. MeDH activity was measured in an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) as described previously (18). Whole-cell methanol oxidation was measured as methanol-dependent O_2 uptake in the oxygen electrode by cultures washed with minimal medium as described above and concentrated 10× in the same medium.

Cosmid clone bank constructions. (i) *Methylomonas albus* BG8. The BG8 clone bank of a *Hin*dIII partial digest in pVK100 was prepared by D. Nunn by a procedure previously described (8) and was maintained in *E. coli* HB101.

(ii) *Methylococcus capsulatus* Bath. The Bath clone bank was prepared as described above, except that the cosmid cloning vector was pHC79 and the inserts were from a *Pst*I partial digest which had been size fractionated to between 35 and 45 kilobases (kb) by electroelution from agarose gels.

Matings for complementation. Plasmids (pRK310 containing appropriate inserts) were mobilized into *Methylobacterium* sp. strain AM1 in three-way matings between an *E. coli* DH5α donor, an *E. coli* CSR603(pRK2013) mobilizer, and a *Methylobacterium* sp. strain AM1 Rif^r recipient. Mid-log-phase cultures of donor, mobilizer, and recipient were washed of antibiotic, mixed in a 1:1:10 ratio, and spotted onto a 0.45-μm-pore-size nitrocellulose membrane filter. This filter was incubated at 30°C on nutrient agar medium (Difco) for 12 h. The cells were then washed from the filter and plated on selective medium containing rifamycin, tetracycline, and methanol. Mobilization of plasmids from *Methylobacterium* sp. strain AM1 into *E. coli* was carried out in a similar manner, but matings were on nutrient agar and platings were on L agar (23) containing tetracycline. *Methylobacterium* strain AM1 will not grow on L agar. Single colonies of putative transconjugants were streaked onto selective medium to purify them of any residual donor cells.

DNA manipulations. Plasmid DNA from *E. coli* was isolated by one of two methods. Rapid screenings were carried out by the method of Holmes and Quigley (12), and large-scale plasmid preparations were carried out by the method of Ish-Horowicz and Burke (13). Plasmid DNA from the large-scale preparation was further purified by two rounds of banding in CsCl-ethidium bromide density gradients.

Chromosomal DNA preparations were by the method of Marmur (21).

Restriction enzymes were obtained from Bethesda Research Laboratories, Rockville, Md.; New England Bio-

Labs, Inc., Beverly, Mass.; or Molecular Biology Resources, Inc., Milwaukee, Wis. They were used according to the instructions of the manufacturers.

Agarose gel electrophoresis purification of DNA fragments from agarose gels and DNA ligations and transformations were performed as described by Maniatis et al. (20).

Radiolabeling of probe DNA was performed by the procedure of Rigby et al. (32).

DNA-DNA hybridizations. Hybridizations were performed with dried agarose gels as described by Meinkoth and Wahl (22) with the following modifications. Gels were denatured and neutralized after the gel had been dried. The prehybridization and hybridization solutions contained 6.6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% (wt/vol) sodium dodecyl sulfate, 0.5% (wt/vol) nonfat dried milk, and 0.01 M EDTA (pH 8.0). Gels were incubated with 1×10^6 to 5×10^6 cpm of ^{32}P-labeled probe DNA overnight at 58°C and washed four times. The first two washes were in 2X SSC at room temperature for 5 min each, and the last two were in 2X SSC at 58°C for 15 min each.

Colony hybridizations were performed by the procedure described by Maniatis et al. (20) with 1×10^6 to 5×10^6 cpm of ^{32}P-labeled probe DNA.

Protein electrophoresis and immunoblotting. Proteins were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (17). Proteins were blotted onto nitrocellulose membranes by using the Trans-Blot (Bio-Rad Laboratories, Richmond, Calif.) system according to the protocol of the manufacturer. Tris hydrochloride-buffered saline with 10% nonfat dried milk was used as the blocking solution. Filter-bound antibodies were detected with either horseradish peroxidase or alkaline phosphatase assays performed according to the instructions of the manufacturer (Bio-Rad).

Protein expression. Protein expression from cloned genes was accomplished with the GeneScribe-Z vector pTZ18R (U.S. Biochemical Corp., Cleveland, Ohio) and plasmid pGP1-2 containing the T7 polymerase gene. The procedure followed was that of Tabor and Richardson (34).

***Methylomonas albus* BG8 MeDH purification.** All purification steps were performed at 0 to 4°C. Cells were harvested at an optical density (at 600 nm) of 0.7, washed with and suspended in 150 mM Tris hydrochloride (pH 8.0), and then sonicated (four 30-s bursts at maximum power; sonicator from Branson Sonic Power Co., Danbury, Conn.) with cooling. Cellular debris was pelleted at 10,000 × *g* for 30 min. The supernatant was fractionated by ammonium sulfate precipitation. The fraction precipitating at 50 to 70% of saturation was centrifuged at 10,000 × *g* for 10 min, and the pellet was suspended in 20 mM Tris hydrochloride (pH 8.0) and dialyzed with three changes against buffer of the same composition. The fractionated extract was loaded onto a DEAE-cellulose column (2.5 by 30 cm) equilibrated with 20 mM Tris hydrochloride (pH 8.0) and then washed with the column buffer; this was followed by washes with 50 mM Tris hydrochloride (pH 8.0) and then 50 mM Tris hydrochloride (pH 8.0)–50 mM KCl. A small fraction of the sample was eluted with 50 mM Tris hydrochloride (pH 8.0)–100 mM KCl. The remainder was eluted with 50 mM Tris hydrochloride (pH 8.0)–150 mM KCl. Fractions containing activity were pooled and dialyzed against 10 mM Tris hydrochloride (pH 8.0). The column was washed with column buffer and then with 50 mM Tris hydrochloride (pH 8.0)–50 mM KCl, and it was finally eluted with 50 mM Tris hydrochloride (pH 8.0)–75 mM KCl. The eluted proteins were concentrated with a stirred cell (YM30 membrane filter; Diaflow Corp.) and dialyzed against 10 mM Tris hydrochloride (pH 8.0). The proteins were then resolved on a preparative isoelectric focusing bed (10 by 20 cm; LKB, Bromma, Sweden). The MeDH protein banded at pH 4.70 to 5.10 and was eluted from the Ultrodex resin with 10 mM Tris hydrochloride (pH 8.0) and dialyzed against 10 mM KPO_4 buffer (pH 7.0). The protein solution was loaded into a hydroxyapatite column (1.0 by 20 cm) equilibrated with 10 mM KPO_4 buffer (pH 7.0), washed with the column buffer and then with 50 mM KPO_4 buffer (pH 7.0), and finally eluted with 100 mM KPO_4 buffer (pH 7.0). The eluted protein was then concentrated with a stirred cell (YM30 filter) and dialyzed against 10 mM Tris hydrochloride (pH 8.0). Sodium dodecyl sulfate-polyacrylamide gels revealed only two protein bands of approximately equal intensity, with apparent molecular masses of 60 and 10 kDa.

Antiserum production and isolation of immunoglobulin G fraction. Antiserum to purified MeDH from *Methylomonas albus* BG8 was generated by Cocalico Biologicals, Inc., Reamstown, Pa. by injection of female New Zealand White rabbits. The injection protocol involved primary immunization with 0.5 mg of MeDH in Freund complete adjuvant followed by three 0.1-mg boosts at 2-week intervals. The antisera resulting from each individual rabbit were pooled and purified as described by Nowotny (25).

RESULTS

Screening of genomic DNA from methanotrophs for hybridization to *moxF*. An open-reading-frame (ORF) fragment (ORF9) internal to the *moxF* gene from *Methylobacterium* strain AM1 (Fig. 1) (26) was used as a probe to screen *Pst*I genomic digests of DNA from a variety of methylotrophs, including type I, II, and X methane oxidizers and serine pathway restricted and facultative methanol utilizers. Hybridization was detected in all strains tested, using a stringency allowing approximately 30% base pair mismatch, although the strength of hybridization varied (Fig. 2). No hybridization was detected in controls under these conditions with DNA from the vector pVK100 or lambda bacteriophage, and only light bands were observed with DNA from *Pseudomonas aeruginosa* and *E. coli* (data not shown).

Cloning of putative *moxF* genes. Two obligate methane utilizers, *Methylococcus capsulatus* Bath and *Methylomonas albus* BG8, were used for cloning studies. These were chosen for several reasons: they have been well studied, they grow relatively well on agar plates, they represent two groups of methanotrophs (type X and type I, respectively), and their genomic DNAs showed strong hybridization bands with the ORF9 probe. Additional probing confirmed hybridization to a 6.25-kb *Pst*I fragment from *Methylomonas albus* BG8 (data not shown). Cosmid clone banks were con-

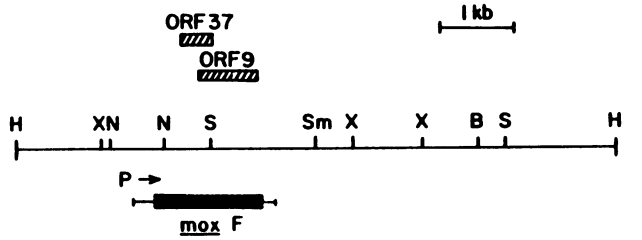

FIG. 1. *moxF* region of *Methylobacterium* strain AM1. The ORF fragments used for probes (ORF9 and ORF37) are noted. Abbreviations: P, promoter; H, *Hin*dIII; X, *Xho*I; N, *Nru*I; S, *Sal*I; Sm, *Sma*I; B, *Bam*HI.

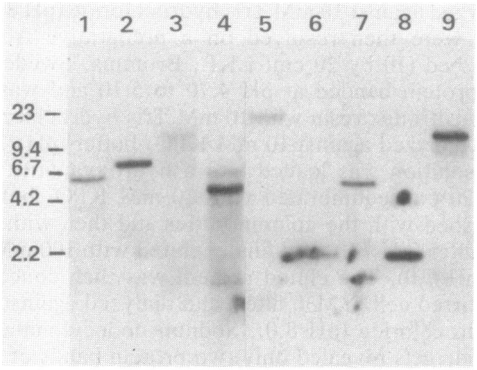

FIG. 2. DNA blots of genomic DNA from methanotrophs, using the ORF9 probe. Lanes: 1 through 4, genomic DNA cut with *Pst*I; 5 through 9, genomic DNA cut with *Eco*RI; 1, *Methylococcus capsulatus* Bath; 2, *Methylosinus trichosporium* OB3b; 3, *Methylobacterium* strain 3A2; 4, *Methylobacterium organophilum* XX; 5, *Hyphomicrobium* strain X; 6, *Methylomonas* strain A1; 7, *Methylomonas albus* BG8; 8, *Methylosinus sporium* 5; 9, *Methylobacterium* strain AM1. Molecular weight standards are shown on the left, in kilobases.

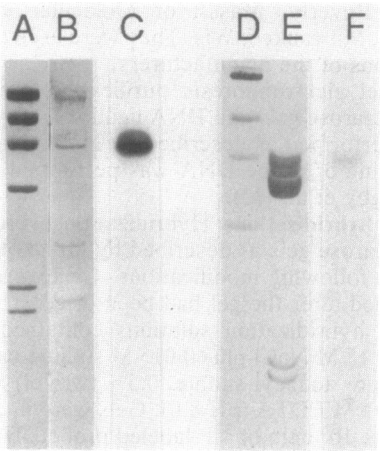

FIG. 3. Ethidium bromide-stained agarose gels (negatives) and DNA blots (ORF9 probe) of *Pst*I-cut plasmids containing insert DNA from *Methylomonas albus* BG8 or *Methylococcus capsulatus* Bath. Lanes: A and D, *Hin*dIII-cut lambda phage DNA as a standard; B, pRS8600 (containing an insert of strain BG8 DNA), agarose gel; C, pRS8600, DNA blot; E, pRS2100 (containing an insert of strain Bath DNA), agarose gel; F, pRS2100, DNA blot.

structed with DNA from each methanotroph. For *Methylococcus capsulatus* Bath, the vector was pHC79 and the construction utilized a *Pst*I partial digest; for strain BG8, the vector was pVK100 and involved a *Hin*dIII partial digest. The host in each case was *E. coli* HB101. Colony blots were hybridized with the ORF9 probe, and in each case a few positive colonies were identified. The background hybridization to *E. coli* DNA observed in the genomic blots did not interfere with detection of positive clones in the colony blots. Plasmids were purified from the positive colonies and used for further study. The plasmid containing insert DNA from strain BG8 was designated pRS8600, and the one containing insert DNA from strain Bath was designated pRS2100.

*Pst*I digests of the putative *moxF*-containing clones yielded fragments identical in size to those showing hybridization to the ORF9 probe in the genomic *Pst*I digests. These putative *moxF*-containing *Pst*I fragments hybridized to the ORF9 probe, while other *Pst*I fragments in the clones did not (Fig. 3). The appropriate *Pst*I fragments were subcloned into pRK310, and these plasmids were designated pRS2117 (for *Methylococcus capsulatus* Bath) and pRS8604 (for *Methylomonas albus* BG8). Probing with the isolated ORF9 fragment confirmed the presence of hybridizing DNA in each.

Mapping and subcloning. Restriction mapping of these two DNA segments was performed with the inserts in the pRK310 vector. The agarose gels from these restriction digests were dried and used for DNA-DNA hybridizations with two different probes, ORF9 and ORF37 (Fig. 1). The data obtained from these experiments identified the general location of the MeDH genes within each clone (Fig. 4 and 5). In the *Methylobacterium* strain AM1 *moxF* gene, ORF9 and ORF37 overlap by only 180 base pairs, with ORF37 representing the more 5′ portion of *moxF* and ORF9 representing the more 3′ portion (26). Therefore, it is possible to use these two probes to determine orientation, if appropriate restriction sites are available. The data were sufficient to indicate the orientation of the gene for *Methylococcus capsulatus* Bath but not for *Methylomonas albus* BG8.

Protein expression. The subclones shown in Figs. 4 and 5 were chosen for expression in *E. coli* with the T7 expression vector pTZ18R. These subclones contained a 3.1-kb *Hin*dIII fragment for *Methylomonas albus* BG8 and a 2.4-kb *Eco*RI-*Kpn*I fragment for *Methylococcus capsulatus* Bath. Plasmid constructs were used that contained inserts in both orientations, which were named pRS2.417 (orientation as shown in Fig. 4) and pRS2.414 (opposite orientation to that shown in Fig. 4) for the pRS2117 clones and pRS3.139 (orientation as shown in Fig. 5) and pRS3.104 (opposite orientation to that shown in Fig. 5) for the pRS8604 clones. Expression of each of these constructions was heat induced, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell extracts of protein expression was performed, and the polyacrylamide gels were blotted to nitrocellulose membranes. These protein blots were then subjected to enzyme immu-

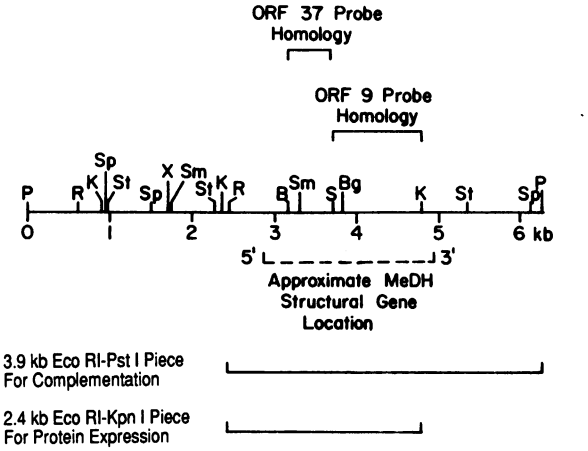

FIG. 4. Restriction map of the insert in pRS2117 (*Methylococcus capsulatus* Bath). The approximate location of the MeDH structural gene was defined by the fragments of DNA showing homology to the ORF9 and ORF37 probes. Fragments indicated were used for protein expression and complementation. (Note that the full 6.25-kb segment was also used for complementation experiments.) Restriction enzyme abbreviations: P, *Pst*I; R, *Eco*RI; K, *Kpn*I; Sp, *Sph*I, St, *Sst*I; X, *Xho*I, Sm, *Sma*I; B, *Bam*HI; S, *Sal*I; Bg, *Bgl*II.

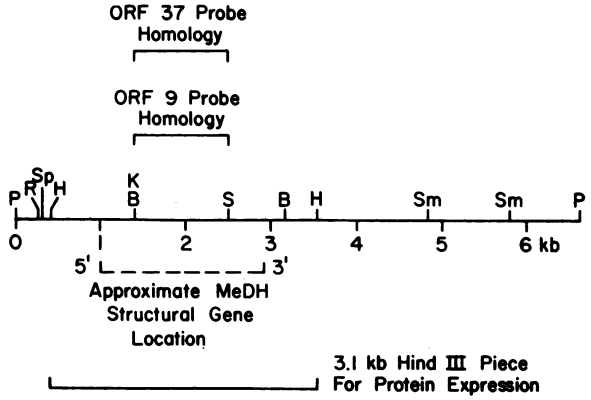

FIG. 5. Restriction map of the insert in pRS8604 (Methylomonas albus BG8). The approximate location of the MeDH structural gene was defined by the segments of DNA showing homology to the ORF9 and ORF37 probes. The DNA segment indicated was used for protein expression, and the entire 6.5-kb segment was used for complementation experiments. Restriction enzyme abbreviations: P, *Pst*I; R, *Eco*RI; Sp, *Sph*I; H, *Hin*dIII; K, *Kpn*II; B, *Bam*HI; S, *Sal*I; Sm, *Sma*I.

noassays, using the goat anti-rabbit horseradish peroxidase system for the blots of the pRS8604 clones and the goat anti-rabbit alkaline phosphatase system for the blots of the pRS2117 clones. The alkaline phosphatase system was used in the latter case because of its greater sensitivity in our hands.

Figure 6 shows the strong cross-reaction of the *Methylomonas albus* BG8 MeDH antiserum with a single band of approximately 60 kDa in the nitrocellulose membrane blot in the lane containing extract from pRS3.139 (lane D) but no interaction with protein bands in extracts from pRS3.104 (lane B) or from the pTZ18R vector without an insert (lane C). This cross-reacting band corresponded in size to the large band of the purified MeDH from *Methylomonas albus* BG8. The results were similar but cross-reaction was much weaker in the reaction of the strain BG8 MeDH antiserum with a band of approximately 57 kDa in the nitrocellulose membrane blot of the expressed *Methylococcus capsulatus* Bath MeDH protein from plasmid pRS2.417 (data not shown). No cross-reaction was observed with pRS2.414. In whole-cell extracts from strain Bath, the MeDH protein (57 kDa) cross-reacts with antiserum against the BG8 enzyme, but the reaction is weak (L. Buchholz and M. Lidstrom, unpublished data).

The purified MeDH from *Methylomonas albus* BG8 used for the antiserum preparation contained a small polypeptide of approximately 10 kDa in addition to the 60-kDa MeDH polypeptide (Fig. 6, lane A), and the antiserum contained antibody that cross-reacted with this small polypeptide (Fig. 6b, lane A). A small polypeptide of this size is present in cell-free extracts and copurifies with MeDH preparations from a variety of methanotrophs (14; C. Anthony, personal communication; L. Buchholz, A. DiSpirito, and M. Lidstrom, unpublished data), but its function, if any, is unknown. It was not detected in any of the whole-cell extracts from the protein expression system.

The orientation of the MeDH gene from *Methylococcus capsulatus* Bath was confirmed by these expression experiments, and the orientation of the gene from *Methylococcus albus* BG8 was determined to be left to right (Fig. 5).

It was not possible to assay for the MeDH in *E. coli*, because *E. coli* does not synthesize the MeDH cofactor, pyrrolo-quinoline quinone (24). This cofactor is required for MeDH activity and is an integral part of the MeDH complex (7). Although the cofactor does not appear to be covalently bound to the apoprotein, it has not yet proven possible to reconstitute active enzyme by mixing the apoprotein and the cofactor in vitro (1; D. Nunn, personal communication).

Complementation. Since it was not possible to confirm the presence of active MeDH in *E. coli* extracts, mutant complementation experiments were carried out. The *moxF* mutant of *Methylobacterium* strain AM1 (UV26) was mated with an *E. coli* donor carrying either pRS2117 (containing the 6.25-kb *Pst*I fragment from *Methylococcus capsulatus* Bath), pRS3.912 (containing the 3.9-kb *Eco*RI-*Pst*I fragment from strain Bath), or pRS8604 (containing the 6.5-kb *Pst*I fragment from *Methylomonas albus* BG8) (Fig. 4 and 5) and an *E. coli* strain containing the mobilizing plasmid pRK2013. Methanol-positive colonies of UV26 containing the plasmid were obtained at a high frequency with pRS2117 and pRS8604 but not with pRS3.912. Controls containing only the mobilizer and UV26 produced no methanol-positive colonies. In both cases of complementation, the whole cells oxidized methanol and contained in vitro MeDH activity (Table 2). However, the activities were lower than those of the wild-type, and the growth rates on methanol were less than those of the wild type. The mutant UV26 shows no growth on methanol and does not contain detectable MeDH activity or protein (26).

Plasmids from representative methanol-positive colonies from each mating were mated back into *E. coli*, and these recipients were shown by restriction digests to contain authentic pRS2117 or pRS8604, respectively (Fig. 7). These clones were remated into UV26 as before, and again, high frequencies of methanol-positive colonies were obtained, confirming complementation.

DISCUSSION

Methanol oxidation is a key step in C_1 metabolism by methanotrophs, and it is important to understand the components of the system, their biochemical functions, and how they are regulated by changes in growth conditions. This paper describes an approach to cloning Mox genes and

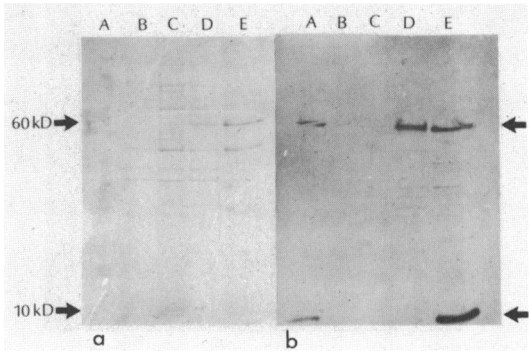

FIG. 6. Sodium dodecyl sulfate-polyacrylamide gel of whole-cell extracts of protein expression (Coomassie blue stain) (a) and protein immunoblot (b) of *Methylomonas albus* BG8 clones and controls. The antiserum used was generated against the BG8 MeDH. Lanes: A, purified BG8 methanol dehydrogenase; B through D, whole-cell extracts from *E. coli* containing the plasmids pRS3.104 (vector with insert in orientation shown in Fig. 5) (B), pTZ18R (vector with no insert) (C), and pRS3.139 (vector with insert in opposite orientation as B) (D); and E, whole-cell extract from BG8.

TABLE 2. Characteristics of strain UV26 (*moxF* mutant) complemented with methanotrophic DNA

Strain	Doubling time (h)[a]	MDH activity (nmol/min per mg of protein)	Whole-cell methanol oxidation (nmol/min per mg)
AM1 (wild type)	10	350	580
UV26(pRS2117) (*Methylococcus capsulatus* Bath insert)	34	30	56
UV26(pRS8604) (*Methylomonoas albus* BG8 insert)	28	63	76
UV26[b]	No growth	Not detectable	Not detectable

[a] Time for cultures grown in minimal medium plus methanol; average of two replicates (±15%).
[b] Data from reference 27.

studying Mox functions of obligate methanotrophs by using a facultative methanol utilizer as an alternate host.

The structural gene (*moxF*) for the MeDH subunit was chosen as a starting point for studying Mox systems. An internal fragment of the gene (*moxF*) from the facultative serine pathway methanol utilizer *Methylobacterium* strain AM1 was found to hybridize to putative *moxF* genes from a variety of methylotrophs: type I, type X, and type II methane utilizers and restricted and facultative serine pathway methanol utilizers. This probe showed little hybridization to DNA from nonmethylotrophs. Harms and coworkers have shown that the *moxF* gene from *Paracoccus denitrificans*, a facultative autotrophic methanol utilizer, hybridizes to the corresponding gene from *Methylobacterium* strain AM1 and *Methylobacterium organophilum* XX (10). Since the groups tested in these studies are thought to represent distantly related strains (9, 36), these results suggest that the *moxF* gene is broadly conserved among methylotrophs.

The similarity between diverse *moxF* genes has allowed us to isolate *moxF* genes from obligate methanotrophs without the necessity of isolating methanol oxidation mutants of these strains. The results of this study suggest that the intact *moxF* genes have been cloned from *Methylococcus capsulatus* Bath and *Methylomonas albus* BG8. The orientation-specific detection of the MeDH protein after expression in *E. coli* and the complementation of the *moxF* mutant of *Methylobacterium* AM1 are strong evidence for this conclusion.

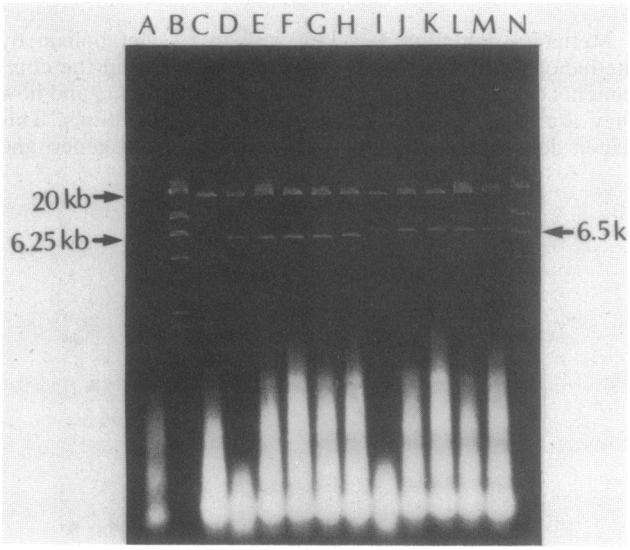

FIG. 7. Ethidium bromide-stained agarose gel of *Pst*I-cut plasmid from *E. coli* DH5α clones obtained by mating with a complemented *Methylobacterium* strain AM1 *moxF* mutant (UV26). Lanes: A, uncut pRK310; B and N, *Hin*dIII-cut lambda phage DNA; C, *Pst*I-cut pRK310; D, *Pst*I-cut pRS2117; E, F, G, and H, duplicates of two treatment groups each mated with UV26 complemented with the *Methylococcus capsulatus* Bath clone contained pRS2117; I, *Pst*I-cut pRS8604; J, K, L, and M, duplicates of two treatment groups each mated with UV26 complemented with the *Methylomonas albus* BG8 clone containing pRS8604.

Complementation only partially restored MeDH activity in both cases. However, the fact that some MeDH activity was detected shows that these heterologous genes were expressed to some degree. It is not known whether this expression was dependent upon vector or insert promoters, but we are currently addressing that question. It is more surprising that the heterologous MeDH proteins produced resulted in activity, since the MeDH apoprotein requires additional gene products to become active in *Methylobacterium* strain AM1. Current evidence suggests that the pro-apo-MeDH is secreted to the periplasm with cleavage of a signal peptide and addition of the cofactor pyrrolo-quinoline quinone (7, 27). This process requires at least three Mox-specific gene products in strain AM1 (27). In addition, growth on methanol requires effective coupling of active MeDH to a specific cytochrome *c*, the *moxG* product (27). The functional complementation of a *moxF* mutant of *Methylobacterium* strain AM1 strongly suggests that the Mox system must faithfully transport and assemble heterologous MeDH proteins and that the MeDH produced must couple to a *Methylobacterium* strain AM1 cytochrome. Likewise, these data suggest that at least some functions of the Mox system that have been identified for *Methylobacterium* strain AM1 are present in the Mox systems of type I and type X methanotrophs.

The results presented in this study show that it is possible to obtain information concerning Mox genes from obligate methanotrophs by using mutants and gene probes from facultative methylotrophs. Our data suggest that a similar approach should be useful for studying other Mox functions from methanotrophs. In addition, it is now possible to study transcriptional regulation of MeDH in the obligate methanotrophs by using broad-host-range promoter probe vehicles.

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