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The *moxFG* Region Encodes Four Polypeptides in the Methanol-Oxidizing Bacterium *Methylobacterium* sp. Strain AM1

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The polypeptides encoded by a putative methanol oxidation (*mox*) operon of *Methylobacterium* sp. strain AM1 were expressed in *Escherichia coli*, using a coupled in vivo T7 RNA polymerase/promoter gene expression system. Two *mox* genes had been previously mapped to this region: *moxF*, the gene encoding the methanol dehydrogenase (MeDH) polypeptide; and *moxG*, a gene believed to encode a soluble type *c* cytochrome, cytochrome c_L. In this study, four polypeptides of M_r 60,000, 30,000, 20,000, and 12,000 were found to be encoded by the *moxFG* region and were tentatively designated *moxF*, *-J*, *-G*, and *-I*, respectively. The arrangement of the genes (5′ to 3′) was found to be *moxFJGI*. The identities of three of the four polypeptides were determined by protein immunoblot analysis. The product of *moxF*, the M_r-60,000 polypeptide, was confirmed to be the MeDH polypeptide. The product of *moxG*, the M_r-20,000 polypeptide, was identified as mature cytochrome c_L, and the product of *moxI*, the M_r-12,000 polypeptide, was identified as a MeDH-associated polypeptide that copurifies with the holoenzyme. The identity of the M_r-30,000 polypeptide (the *moxJ* gene product) could not be determined. The function of the M_r-12,000 MeDH-associated polypeptide is not yet clear. However, it is not present in mutants that lack the M_r-60,000 MeDH subunit, and it appears that the stability of the MeDH-associated polypeptide is dependent on the presence of the M_r-60,000 MeDH polypeptide. Our data suggest that both the M_r-30,000 and -12,000 polypeptides are involved in methanol oxidation, which would bring to 12 the number of *mox* genes in *Methylobacterium* sp. strain AM1.

Methylotrophic bacteria are able to grow by using reduced one-carbon compounds as a sole source of carbon and energy. *Methylobacterium* sp. strain AM1 (formerly *Pseudomonas* sp. strain AM1) is a facultative methylotroph that can utilize single-carbon compounds such as methanol and methylamine as well as numerous multicarbon substrates (2, 14). When growing on methanol, *Methylobacterium* sp. strain AM1 oxidizes the substrate via a series of two electron transfer reactions (2). The initial step in the methanol oxidation pathway is catalyzed by a periplasmic methanol dehydrogenase (MeDH; EC. 1.1.99.8) (1, 20), which oxidizes methanol to formaldehyde. The formaldehyde can then be further oxidized or assimilated into cell carbon via the serine pathway (2).

The MeDH from *Methylobacterium* sp. strain AM1 has been well characterized biochemically. The native form has been reported to consist of two identical M_r-60,000 subunits, each associated noncovalently with a pyrroloquinoline quinone prosthetic group (2). Most MeDHs purified from methanol-oxidizing methylotrophs have been found to have a similar dimer structure with subunit size ranging from M_r 60,000 to 76,000 (2).

A low-molecular-weight polypeptide (M_r 12,000) of unknown function has been found to copurify with the MeDH (D. Nunn and M. Lidstrom, unpublished data; C. Anthony, personal communication). Traditionally, it has been assumed that this small polypeptide represents an artifact of the purification procedure. However, proteins of similar size (M_r 10,000) have been reported to copurify with the alcohol (methanol) dehydrogenase of *Xanthobacter autotrophicus* (18) and the MeDH of *Methylomonas* sp. strain J (26). Whether the MeDH-associated protein from either methylotroph was functionally important could not be determined. Therefore, the subunit structure of MeDH is still somewhat uncertain.

Methanol oxidation in *Methylobacterium* sp. strain AM1 also requires specific electron acceptors. Electrons from the oxidation of methanol are transferred to the pyrroloquinoline quinone cofactor of MeDH (11) and then to two soluble (periplasmic) type *c* cytochromes, cytochrome c_L and cytochrome c_H (4, 27). The direct electron acceptor in vivo is not known; however, in vitro evidence suggests that electrons are transferred from MeDH to cytochrome c_L, to cytochrome c_H, and finally to the terminal cytochrome oxidase (2, 14). Cytochrome c_H also appears to be the direct electron acceptor for methylamine oxidation by methylamine dehydrogenase (2). Therefore, only cytochrome c_L appears to be specifically involved in methanol oxidation.

Previous studies in this laboratory have shown the genetics of methanol oxidation in *Methylobacterium* sp. strain AM1 to be quite complex (24, 25). A group of *Methylobacterium* sp. strain AM1 mutants specifically defective in their ability to oxidize methanol were isolated and classified by complementation analysis. Those mutants were found to comprise 10 distinct complementation groups (24). The 10 identified *mox* (methanol oxidation) genes were found to be located on five separate *Hin*dIII fragments, and on the basis of mutant phenotype studies, functions were proposed for the products encoded by these genes (25). Products of *moxB*, *moxC*, *moxE*, and *moxH* may be involved in regulation or stability. The *moxA1*, *moxA2*, and *moxA3* gene products seem to play a role in the proper association of the pyrroloquinoline quinone cofactor with the MeDH apoprotein. The product encoded by *moxD* appears to affect the transport of both MeDH and cytochrome c_L. The MeDH M_r-60,000 polypeptide was determined to be the *moxF* gene product, and cytochrome c_L was believed to be the *moxG* gene product (24, 25).

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moxF and *moxG* were found to map to the same 8.6-kilobase (kb) *Hin*dIII fragment, HINDIII-FG (Fig. 1). The MeDH structural gene was localized to a 3.58-kb *Xho*I fragment and was found to span the *Sal*I site, with transcription proceeding from left to right as drawn (24). Complementation analysis with various HINDIII-FG subclones suggested that *moxG* is located downstream from *moxF*. Additional data compiled from complementation experiments with Tn*5*-mutagenized HINDIII-FG clones suggested further that *moxF* and *moxG* comprise a single transcriptional unit. The mutagenesis data also indicated that the 5′ boundary of the putative operon lay approximately 2 kb from the left end of the HINDIII-FG fragment and that the 3′ boundary extended at least to the *Bam*HI site (24, 25).

To study the putative *moxFG* operon in greater detail, we have utilized a dual plasmid bacteriophage T7 RNA polymerase/promoter gene expression system to express in *Escherichia coli* the polypeptides encoded by this operon. A DNA fragment containing *moxFG* was subcloned into the T7 expression vector, and the polypeptides encoded by the various subclones were subsequently expressed and labeled. We have been able to determine the location and order of the genes encoding the four expressed polypeptides and, via protein immunoblot analysis, have conclusively identified three of the four as MeDH, the copurifying MeDH-associated polypeptide, and cytochrome c_L. We thus propose an expanded and more detailed map of the region previously designated the *moxFG* operon.

MATERIALS AND METHODS

Bacterial strains and plasmid vectors. The bacterial strains and plasmid vectors used in this study are listed in Table 1. Plasmid pTZ19TT was kindly provided by S. Lory and M. Strom, University of Washington, Seattle. Plasmid pGP1-2 was provided by S. Tabor, Harvard Medical School, Boston, Mass.

Media and growth conditions. *E. coli* strains were grown at 30 or 37°C in Luria broth (22) or at 30°C in M9 medium (22) supplemented with 0.2% glycerol, 20 μg of thiamine per ml, and 0.01% each of 18 amino acids (minus cysteine and methionine). Agar was added to 1.5% (wt/vol) for plates. *Methylobacterium* sp. strain AM1 isolates were grown at 30°C on the ammonium-mineral salts medium described by Harder et al. (15) supplemented with 1.0% (vol/vol) Staley's vitamin solution (29). Isolates were maintained on nutrient agar with rifampin to 40 μg/ml. When appropriate, filter-sterilized supplements were added to sterile media as follows: 0.5% (vol/vol) methanol, 0.2% (wt/vol) methylamine, 100 μg of ampicillin per ml, 50 μg of kanamycin per ml, 40 μg of rifampin per ml, and 10 μg of tetracycline per ml.

DNA manipulations. Plasmid DNA was isolated from *E. coli* by the rapid screening method of Holmes and Quigley (16). Large-scale plasmid isolation was carried out by the method of Ish-Horowicz and Burke (17), with two cycles of cesium chloride-ethidium bromide density gradient centrifugation. All restriction enzymes were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and were used as suggested by the manufacturer. T4 DNA ligase was also purchased from the same company, and ligations were performed as described by Maniatis et al. (22), except that incubations were done at 20°C for 3 to 5 h.

Agarose gels of 0.8 to 1.2% (wt/vol) were run with 1× Tris-borate buffer (22) in a horizontal electrophoresis apparatus. DNA fragments were purified by the procedure described by Maniatis et al. (22).

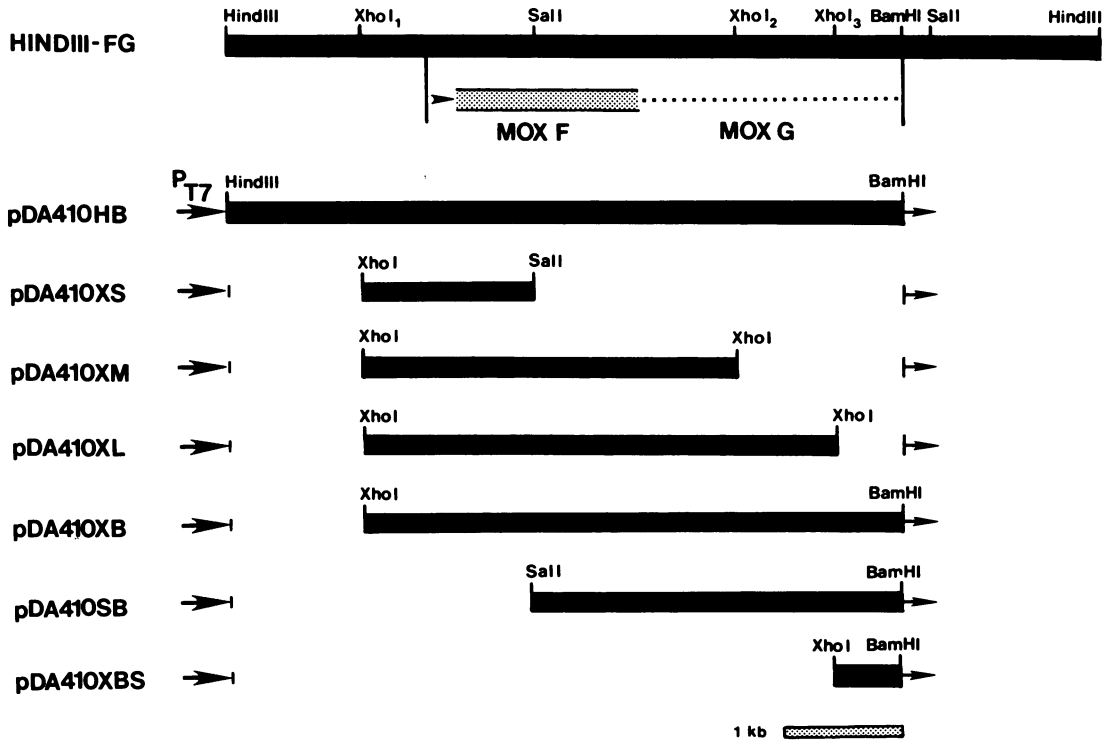

FIG. 1. Restriction maps and characteristics of HINDIII-FG and T7 expression HINDIII-FG subclones. P_{T7}, Promoter of bacteriophage T7. Vector sequences are represented by arrows.

TABLE 1. Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Relevant traits	Source or reference
E. coli		
DH5α	$r^- m^+$ *recA1 lacZYA* φ80d*lacZM15*	BRL, Inc.
HB101	*recA* Str^r	6
MM294	*recA*$^+$	3
Methylobacterium sp. strain AM1		
AM1*rif*	Rif^r derivative	24
AM1 UV26	*moxF* mutant of AM1 *rif*	24
AM1 UV10	*moxG* mutant of AM1 *rif*	24
AM1 UV19	*moxG* mutant of AM1 *rif*	24
AM1 UV24	*moxG* mutant of AM1 *rif*	24
Plasmid		
pTZ19R	T7 promoter gene expression, MCS, Ap^r	U.S. Biochemical Corp.
pTZ19TT	Derivative of pTZ19R containing translation terminators	This study M. Strom
pKK232-8	Translation terminator, promoterless Cm^r gene	7
pGP1-2	T7 RNA polymerase gene, *c*I857, Km^r	30
pDN410	*Methylobacterium* sp. strain AM1 *HIN*DIII-FG; IncP1 cosmid Tc^r	24
pDA410HB	*HIN*DIII-FG 6.6-kb HB/pTZ19TT, Ap^r	This study
pDA410XS	*HIN*DIII-FG 1.53-kb XS/pTZ19TT, Ap^r	This study
pDA410XM	*HIN*DIII-FG 3.58-kb X/pTZ19TT, Ap^r	This study
pDA410XL	*HIN*DIII-FG 4.55-kb X/pTZ19TT, Ap^r	This study
pDA410XB	*HIN*DIII-FG 5.35-kb XB/pTZ19TT, Ap^r	This study
pDA410SB	*HIN*DIII-FG 3.8-kb SB/pTZ19TT, Ap^r	This study
pDA410XBS	*HIN*DIII-FG 0.8-kb XB/pTZ19TT, Ap^r	This study
pRK310	IncP1, *lacPOZ'*, Tc^r	10
pRK2013	Mobilizing plasmid, Tc^r	12
pDA3410XM	*HIN*DIII-FG 3.58-kb X/pRK310	This study
pDA3410XL	*HIN*DIII-FG 4.55-kb X/pRK310	This study
pDA3410XB	*HIN*DIII-FG 5.35-kb XB/pRK310	This study

[a] BRL, Inc., Bethesda Research Laboratories, Inc.; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Rif^r, rifampin resistance; Str^r, streptomycin resistance; Tc^r, tetracycline resistance; MCS, multiple cloning site; B, *Bam*HI; H, *Hin*dIII; S, *Sal*I; X, *Xho*I.

Transformation of *E. coli* with plasmid DNA was accomplished by the procedures described by Bergsman et al. (5).

Expression vector modification. Modification of pTZ19R, creating pTZ19TT, was done by Mark Strom, University of Washington, Seattle. Plasmid pKK232-8 (7) was digested with *Hin*dIII. The *Hin*dIII ends were filled in as described by Maniatis et al. (22), using the large fragment of DNA polymerase I (Bethesda Research Laboratories). The plasmid was ligated and transformed into *E. coli* DH5α. Clones were screened for the presence of the modified plasmid pKK232-8HF, which was then purified and digested with *Sma*I and *Eco*RI. A 0.3-kb *Sma*I-*Eco*RI fragment from pKK232-8 (7) containing translational terminators in all three reading frames along with the 5′ end of a promoterless chloramphenicol acetyltransferase gene was isolated, purified, and ligated into pTZ19R digested with *Sma*I and *Eco*RI. *E. coli* DH5α was transformed with a sample of the ligation reaction.

Expression vector subcloning of HINDIII-FG. In each of the following cases, pDA410HB, pDA410XS, pDA410XM, pDA410SB, and pDA410XBS, the indicated fragments (Fig. 1) were purified and ligated in the presence of pTZ19TT cut with the appropriate restriction nucleases. For pDA410XL, the 6.6-kb *Hin*dIII-*Bam*HI fragment of HINDIII-FG was purified from pDA410HB and partially digested with *Xho*I. The pool of *Xho*I partial digestion products, which included the desired 4.55-kb *Xho*I fragment, was ligated into *Sal*I-digested pTZ19TT. For pDA410XB, plasmid pDA410HB was digested with *Sal*I, and the *Sal*I fragment containing the 3.8-kb *Sal*I-*Bam*HI fragment of HINDIII-FG was isolated and purified. The *Sal*I fragment was ligated into *Sal*I-digested pDA410XS, and the resulting plasmid contained a reconstructed 5.35-kb *Xho*I-*Bam*HI HINDIII-FG fragment in pTZ19TT.

Expression of HINDIII-FG subclone polypeptides. A coupled bacteriophage T7 RNA polymerase/promoter gene expression system was utilized to express the polypeptides encoded by the subcloned HINDIII-FG fragments. Transcription of insert DNA was driven by the T7 promoter present on the modified expression vector pTZ19TT. T7 RNA polymerase was supplied in *trans* by pGP1-2 (30). The procedures described by Tabor and Richardson (30) were used for maximum expression of plasmid-encoded polypeptides (2.5-h induction) and for [^{35}S]methionine labeling of plasmid-encoded polypeptides (20-min induction). [^{35}S]methionine was purchased from New England Nuclear Research Products, Boston, Mass.

Broad-host-range vector subcloning of *Methylobacterium* sp. strain AM1 HINDIII-FG. For pDA3410XM, the 3.58-kb *Xho*I ("Xho M") fragment of HINDIII-FG was isolated from pDA410XM as a *Pst*I cassette and ligated into *Pst*I-digested pRK310. For pDA3410XL, the 4.55-kb *Xho*I ("Xho L") fragment of HINDIII-FG was isolated from pDA410XL as a *Pst*I cassette and ligated into *Pst*I-digested pRK310. For pDA3410XB, the 5.35-kb *Xho*I-*Bam*HI fragment of HINDIII-FG was isolated from pDA410XB as a *Pst*I cassette and ligated into *Pst*I-digested pRK310. A sample of each ligation reaction was transformed into *E. coli* DH5α, and the appropriate constructs were mobilized into the *Methylobacterium* sp. strain AM1 MoxF mutant UV26, using a previously described three-way conjugation procedure (13).

MeDH purification. MeDH was purified from *Methylobacterium* sp. strain AM1 by Lorie Buchholz according to published procedures (24).

Antibody preparation. Antiserum to the purified *Methylobacterium* sp. strain AM1 MeDH was generated by Cocalico Biologicals, Inc., Reamstown, Pa. Female New Zealand White rabbits were immunized with 0.5 mg of MeDH in Freund complete adjuvant and received 0.1-mg boosts every 2 weeks. The antisera were purified by the method of Nowotny (23). Antibody to purified *Methylobacterium* sp. strain AM1 cytochrome c_L was a gift of David Nunn, The University of Southampton, Southampton, England.

Preparation of crude cell extracts. *Methylobacterium* sp. strain AM1 cells were grown to mid-log phase (optical density at 600 nm = 0.4 to 0.6) in liquid culture containing

methanol, methylamine, vitamins, and antibiotics when appropriate. The cultures were centrifuged, washed with cold 20 mM Tris hydrochloride buffer, pH 8.0, recentrifuged, and suspended in the same buffer at a concentration of 0.25 g (wet weight) of cells per ml. Cells in the suspensions were broken either by three passes through a French pressure cell at 18,000 lb/in^2 or by four 5-s bursts, using the microtip of the Sonifier cell disruptor (model 1400; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) set at the tip limit. Cell suspensions were kept on ice throughout both procedures. The cell extracts were spun at 35,000 × *g* for 30 min. The supernatants were decanted, frozen in liquid nitrogen, and stored at −70°C.

Protein determination. Proteins were assayed by using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.). Stock solutions of lysozyme were used as standards.

SDS-polyacrylamide gel electrophoresis. Whole-cell extracts from the expression experiments, crude cell extracts, and purified proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, as described by Laemmli (21), on 7.5, 12.5, and 15% polyacrylamide gels. Gels containing [^{35}S]methionine-labeled expression system polypeptides were stained with Coomassie blue R. Following destaining, the gels were soaked for 30 min in 0.5 M salicylate–1.5% glycerol (8). The gels were dried and exposed to Kodak X-Omat AR film at −70°C.

Immunoblot analysis. Following electrophoresis, expression polypeptides were transferred to nitrocellulose (Schleicher & Schuell, Keene, N.H.), using a Transphor TE series electrophoresis unit (Hoeffer Scientific Instruments, San Francisco, Calif.), by the method of Towbin and co-workers (31). Detection of antibody-polypeptide complexes was accomplished by using the Bio-Rad Immun-Blot assay with slight modification. Tris hydrochloride-buffered saline with 10% nonfat dry milk was used as a blocking solution, and Tris hydrochloride-buffered saline with 0.05% Tween 20 and 5% nonfat dry milk was used as antibody buffer. Goat anti-rabbit alkaline phosphatase conjugate antibody was used according to the instructions of the manufacturer.

RESULTS

Analysis of polypeptides encoded by the putative *moxFG* operon. To study the putative *moxFG* operon in greater detail, it would be helpful to express these methanol oxidation genes in *E. coli*. Previous attempts to express *Methylobacterium* sp. strain AM1 *mox* genes in alternate hosts have been unsuccessful. The reason for this is unknown; however, preliminary evidence suggests that nonrecognition of *Methylobacterium* sp. strain AM1 *mox* promoter sequences and premature termination of transcription within the cloned *Methylobacterium* sp. strain AM1 sequences may be contributing factors (Nunn and Lidstrom, unpublished data).

In an attempt to circumvent these obstacles, we have utilized a bacteriophage T7 RNA polymerase/promoter gene expression system that has been successfully used to express *Pseudomonas aeruginosa* exotoxin A in *E. coli* (21a). The expression vector used in the present study, pTZ19TT, is a derivative of GeneScribe-Z vector pTZ19R (U.S. Biochemical Corp., Cleveland, Ohio). A 0.3-kb *Sma*I-*Eco*RI fragment from pKK232-8 (7), containing translation termination signals in all three reading frames and approximately 470 base pairs of a promoterless chloramphenicol acetyltransferase gene, was cloned into the multiple cloning site of pTZ19R (Fig. 2).

Various regions of the 8.6-kb HINDIII-FG fragment, previously identified by its ability to complement *Methylobacterium* sp. strain AM1 MoxF and MoxG mutant classes (24), were subcloned into pTZ19TT (Fig. 1). Each subclone was then isolated and transformed into *E. coli* DH5α containing pGP1-2. The selected transformants were subsequently pulse-labeled with [^{35}S]methionine under conditions in which polypeptides encoded by the particular pDA410 construct would be preferentially labeled (30). A fluorogram of whole-cell extracts after electrophoresis through a 15% SDS-polyacrylamide gel shows the pattern of the labeled polypeptides (Fig. 3).

Four HINDIII-FG insert-encoded polypeptides with molecular weights of approximately 60,000, 30,000, 20,000, and 12,000 could be detected in the extract of the strain containing the largest subclone, pDA410HB (Fig. 3, lane 4). Other protein bands visible in this and other expression subclone lanes appeared to be background host- or vector-encoded polypeptides or both, as determined by overexposure of control lanes 1 to 3 (data not shown). The dark band at M_r 29,000 represents β-lactamase, which is encoded by expression vector sequences and was used as an internal control.

The nested set of pDA410 subclones allowed us to deduce the approximate location and order of the genes encoding these four polypeptides. None of the protein bands could be detected upon induction of the subclone containing the 1.53-kb *Xho*I-*Sal*I fragment pDA410XS (Fig. 3, lane 5). A larger subclone (pDA410XM), which contains the overlapping *Xho*I fragment, was found to encode the M_r-60,000 polypeptide (Fig. 3, lane 6). This result suggested that the detected polypeptide might represent the MeDH subunit since the MeDH structural gene had been previously mapped to this region (24). A still larger overlapping subclone (pDA410XL), which contains a 4.55-kb insert, was found to encode both the M_r-60,000 and M_r-30,000 polypeptides (Fig. 3, lane 7). This indicated that the gene encoding the M_r-30,000 polypeptide (*moxJ*) was located downstream of the gene encoding the M_r-60,000 polypeptide but upstream of the *Xho*I$_3$ site. The larger subclone (pDA41XB), containing the 5.35-kb *Xho*I-*Bam*HI fragment, was found to encode all four polypeptides (Fig. 3, lane 8), placing the genes encoding the M_r-20,000 and the M_r-12,000 polypeptides downstream of those encoding the two larger polypeptides. Gene expression of subclone pDA410SB, which con-

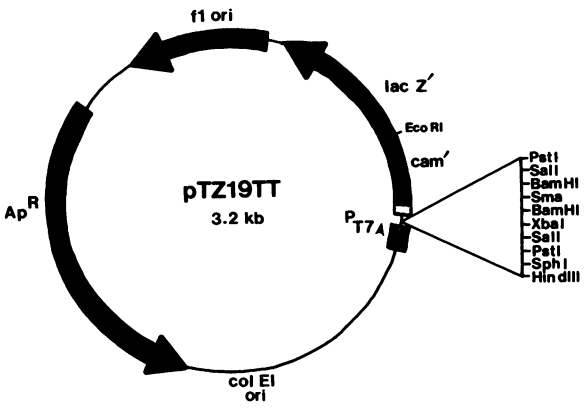

FIG. 2. Modified T7 promoter gene expression vector pTZ19TT used for HINDIII-FG subclone protein expression in *E. coli*. P_{T7}, Promoter of bacteriophage T7; ColE1 ori, origin of replication from plasmid ColE1; Ap^R, ampicillin resistance; f1 ori, origin of replication from bacteriophage f1; cam', portion of the chloramphenicol acetyltransferase gene. Hatched area, T7 promoter; open area, translation termination codons.

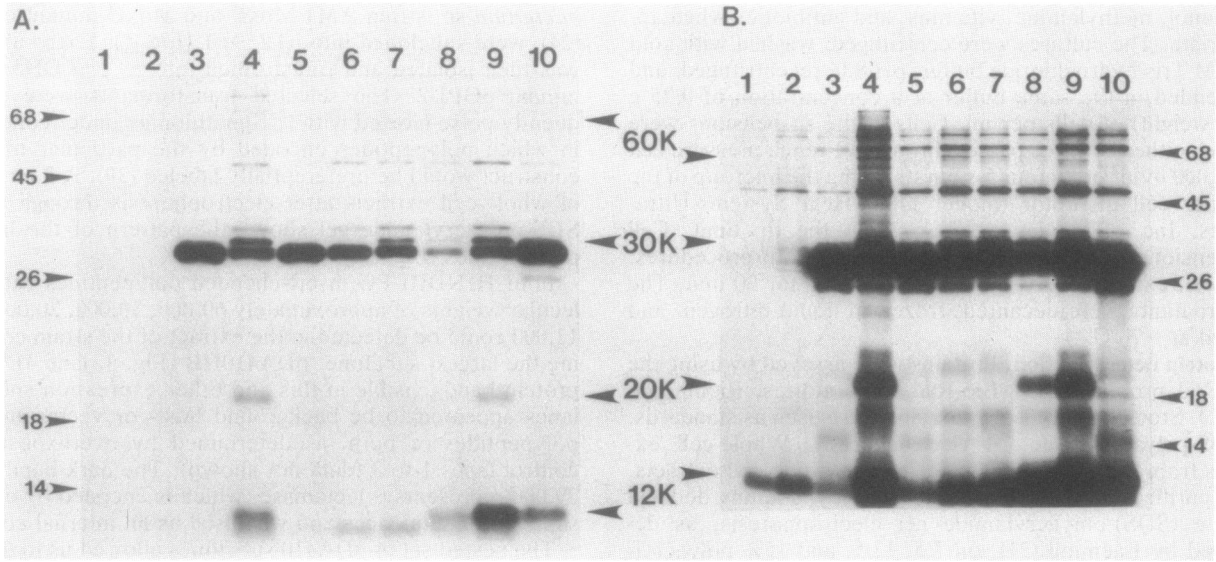

FIG. 3. Fluorogram of a 15% SDS-polyacrylamide gel of T7 expression whole-cell extracts labeled with [^{35}S]methionine. *E. coli* DH5α is the host strain for all constructs, and cells were induced for 20 min. (A) Lightly exposed fluorogram. (B) Overexposed fluorogram to highlight the M_r-60,000 polypeptides. Lanes: 1, no plasmid; 2, pGP1-2; 3, pTZ19TT; 4, pDA410HB; 5, pDA410XS; 6, pDA410XM; 7, pDA410XL; 8, pDA410XB; 9, pDA410SB; 10, pDA410XBS. Strains containing pTZ19TT or a T7 expression HINDIII-FG subclone also contained pGP1-2. Positions of the HINDIII-FG-encoded polypeptides (in kilodaltons) are indicated in the center, and those of the protein standards in kilodaltons are shown on either side.

tains a 3′-overlapping 3.82-kb insert, confirmed that the M_r-60,000 polypeptide is encoded 5′ to the other polypeptides, as only the M_r-30,000, -20,000, and -12,000 polypeptides could be detected (Fig. 3, lane 9). To determine the order of the genes encoding the M_r-20,000 and M_r-12,000 polypeptides, the small 0.8-kb *Xho*I-*Bam*HI fragment was also subcloned into the expression vector. This insert was found to encode the M_r-12,000 polypeptide (Fig. 3, lane 10), indicating that the gene encoding this polypeptide (*moxI*) is located downstream of the gene encoding the M_r-20,000 polypeptide.

Identification of the M_r-60,000, -20,000, and -12,000 polypeptides by protein immunoblot analysis. The identities of three of the polypeptides detected in the expression experiments are suggested by the sizes observed. The M_r-60,000 polypeptide is similar to the size of the MeDH subunit, the size of the M_r-20,000 polypeptide is near that of the mature cytochrome c_L, and the M_r-12,000 polypeptide is similar to the size of the small polypeptide known to copurify with the MeDH subunit from *Methylobacterium* sp. strain AM1. Protein immunoblot (Western blot) analysis of the induced subclone extracts was performed to determine the identity of these three polypeptides.

An M_r-12,000 MeDH-associated polypeptide routinely copurifies with the M_r-60,000 subunit of MeDH and for the purpose of discussion will be called an MeDH-associated polypeptide. We had previously obtained antisera to the M_r-60,000 subunit alone by excising this protein band from SDS-polyacrylamide gels and using it as an antigen (25). For the current study antiserum was generated with an MeDH preparation that contained both polypeptides in approximately equal proportions, but no other detectable polypeptides as determined by SDS-polyacrylamide gel electrophoresis (data not shown).

Antibody raised against the sample containing both the MeDH polypeptide and the MeDH-associated polypeptide was reacted with induced T7 expression subclone extracts analogous to those used in the ^{35}S-labeling experiments (Fig. 4). Several host- or vector-encoded protein bands or both cross-react with the anti-MeDH/anti-MeDH-associated antibody preparation (control lanes 1 to 3). The identities of these cross-reactive protein bands are unknown; however, the dark band at M_r 58,000 may represent the so-called common antigen of gram-negative bacteria observed first by Jensen et al. (19). Only a few protein bands in addition to the MeDH protein and the MeDH-associated protein bands can be detected in the *Methylobacterium* sp. strain AM1 crude cell extract blots reacted with this antibody preparation (see Fig. 6). However, these bands apparently represent specific MeDH protein degradation products and are discussed below. The M_r-60,000 polypeptide was found to react with the anti-MeDH subunit antibody (Fig. 4, lanes 4, 6, 7, and 8), suggesting that this polypeptide is the MeDH subunit, the product of *moxF*. No larger polypeptide that might represent an unprocessed MeDH protein precursor was detected, even in blots from gels that were run for longer time periods to separate the higher-molecular-weight bands.

In addition to the M_r-60,000 polypeptide, we found that antibody also reacted with the M_r-12,000 polypeptide encoded by three of the expression subclones, pDA410HB, pDA410XB, and pDA410SB (Fig. 4, lanes 4, 7, and 8, respectively), suggesting that the low-molecular-weight protein observed in the ^{35}S-labeling experiments is the copurifying MeDH-associated polypeptide. We propose to designate the gene encoding this polypeptide as *moxI*. These data support our earlier finding that *moxI* is located downstream of *moxF*. Surprisingly, the M_r-12,000 polypeptide could not be detected in the T7 expression extract containing pDA410XBS (Fig. 4, lane 10) despite the fact that the polypeptide had been observed in the [^{35}S]methionine-labeled extract. The reason for this is not clear. The two procedures do, however, differ in that the cells used for protein immunoblots were incubated for >2 h longer after induction than were the ^{35}S-labeled cells.

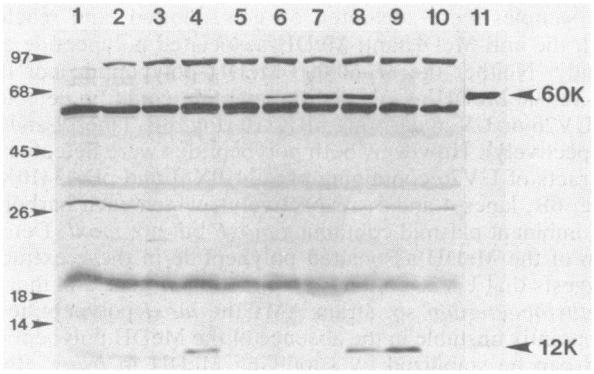

FIG. 4. Protein blot of T7 expression HINDIII-FG subclone whole-cell extracts after reaction with *Methylobacterium* sp. strain AM1 anti-MeDH/anti-MeDH-associated polypeptide antibody. All strains were grown under conditions of maximal plasmid-encoded protein expression, involving induction for 2.5 h. Whole-cell extracts were separated on a 12.5% SDS–polyacrylamide gel and electrophoretically transferred to nitrocellulose. The protein blot was incubated with *Methylobacterium* sp. strain AM1 anti-MeDH/anti-MeDH-associated polypeptide antibody, washed, and then incubated with goat anti-rabbit alkaline phosphatase conjugate antibody. Lanes: 1, no plasmid; 2, pGP1-2; 3, pTZ19TT; 4, pDA410HB; 5, pDA410XS; 6, pDA410XM; 7, pDA410XL; 8, pDA410XB; 9, pDA410SB; 10, pDA410XBS; 11, purified MeDH/MeDH-associated polypeptide. Strains containing pTZ19TT or a T7 expression HINDIII-FG subclone also contained pGP1-2. The positions of the two HINDIII-FG-encoded polypeptides (in kilodaltons) recognized by the anti-MeDH/anti-MeDH-associated polypeptide antibody (M_r 60,000 and 12,000) are indicated on the right, and those of the protein standards (in kilodaltons) are shown on the left.

Protein blots of induced T7 expression HINDIII-FG subclone extracts were also reacted with antibody raised to purified cytochrome c_L in an attempt to verify the identification of the M_r-20,000 polypeptide as cytochrome c_L. Several background host- and vector-encoded protein bands can be seen to cross-react with the cytochrome c_L antibody (Fig. 5, lanes 1 to 3); their identities are unknown. A few cross-reactive protein bands are also detected in the wild-type *Methylobacterium* sp. strain AM1 crude cell extract (Fig. 5, lane 11). The M_r-20,000 polypeptide previously observed to be encoded by pDA410HB, pDA410XB, and pDA410SB was found to react with anti-cytochrome c_L antibody (Fig. 5, lanes 4, 8, and 9, respectively). This result suggests not only that the labeled M_r-20,000 polypeptide is cytochrome c_L, but also that the cytochrome c_L structural gene (*moxG*) is indeed located, as previously hypothesized, in the *moxFG* region (25). A faint protein band representing a polypeptide of approximately M_r 23,000 was also detected in all expression extracts in which cytochrome c_L was observed (Fig. 5, lanes 4, 8, and 9). A polypeptide of similar molecular weight has been previously identified as a putative cytochrome c_L precursor (25).

A smaller polypeptide of approximately M_r 14,500 encoded by pDA410XL (Fig. 5, lane 7) was also found to react with the anti-cytochrome c_L antibody. This smaller protein band probably represents a truncated form of the cytochrome c_L polypeptide. The decreased intensity of this protein band compared with bands representing the full-length mature cytochrome c_L polypeptide may be the result of lessened stability of the truncated cytochrome c_L message or polypeptide or decreased anti-cytochrome c_L antibody recognition.

Characterization of the MeDH-associated polypeptide: protein stability. To further characterize the M_r-12,000 MeDH-associated polypeptide, additional protein immunoblot analysis was carried out. First, four *Methylobacterium* sp. strain AM1 Mox mutants, whose defects mapped to the region encoding both the MeDH polypeptide and the MeDH-associated polypeptide (24), were tested. *Methylobacterium* sp. strain AM1 mutant UV26 belongs to the MoxF complementation class, with its mutation mapping to the MeDH structural gene. Mutants UV10, UV19, and UV24 are of the MoxG complementation class, with their mutations mapping to the cytochrome c_L structural gene region. The MoxF class mutant does not contain detectable MeDH subunit, but does contain normal cytochrome c_L, albeit at reduced levels (25). All three of the MoxG class mutants contain the MeDH protein, but no detectable cytochrome c_L (25).

In light of the results presented in this study, we were interested in determining whether the M_r-12,000 MeDH-associated polypeptide was present in these MoxF and MoxG class mutants. A protein blot of crude extracts from *Methylobacterium* sp. strain AM1 wild-type and MoxF and -G mutants grown under inducing conditions (0.2% methanol–0.2% methylamine) (25) was reacted with anti-MeDH/anti-MeDH-associated polypeptide antibody. The M_r-60,000 MeDH subunit and the M_r-12,000 MeDH-associated polypeptide could both be detected in the *Methylobacterium* sp. strain AM1 wild-type sample (Fig. 6A, lane 1) and also in all of the MoxG class mutant samples (lanes 3, 4, and 5). No MeDH protein was observed in the MoxF class mutant sample, as expected (Fig. 6A, lane 2). Interestingly, no MeDH-associated protein could be detected either. Absence of the MeDH-associated polypeptide in the MoxF class mutant cannot be attributed to premature transcriptional or translational termination of the putative *moxFG* operon message within *moxF*, since mature cytochrome c_L, whose structural gene lies between *moxF* and *moxI*, can be

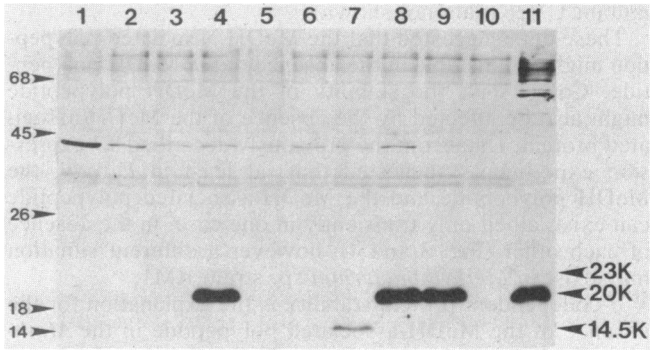

FIG. 5. Protein blot of T7 expression HINDIII-FG subclone whole-cell extracts after reaction with anti-cytochrome c_L antibody. Strains were grown under conditions of maximal plasmid-encoded protein expression as in the legend to Fig. 4. Whole-cell extracts were separated on a 12.5% SDS–polyacrylamide gel and electrophoretically transferred to nitrocellulose. The protein blot was incubated with anti-cytochrome c_L antibody, washed, and then incubated with goat anti-rabbit alkaline phosphatase conjugate antibody. Lanes: 1, no plasmid; 2, pGP1-2; 3, pTZ19TT; 4, pDA410HB; 5, pDA410XS; 6, pDA410XM; 7, pDA410XL; 8, pDA410XB; 9, pDA 410SB; 10, pDA410XBS; 11, *Methylobacterium* sp. strain AM1 wild-type crude cell extract (20 μg of total cell protein). Strains containing pTZ19TT or a T7 expression HINDIII-FG subclone also contained pGP1-2. The positions of the HINDIII-FG-encoded polypeptides recognized by anti-cytochrome c_L antibody (M_r 23,000, 20,000, and 14,500) are indicated on the right and those of the protein standards (in kilodaltons) are shown on the left.

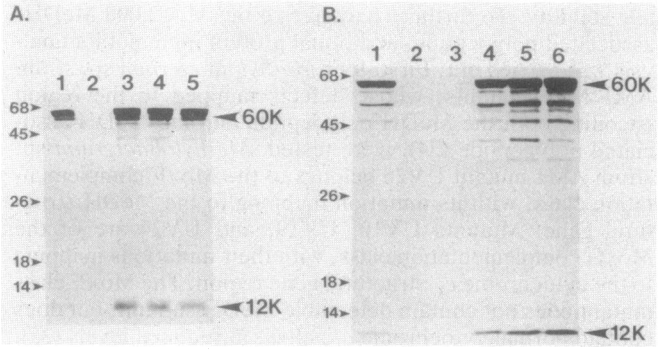

FIG. 6. Protein blots reacted with anti-MeDH/anti-MeDH-associated polypeptide antibody of *Methylobacterium* sp. strain AM1 wild type and (A) MoxF and MoxG class mutant crude cell extracts or (B) MoxF class mutant and MoxF class mutant containing cloned wild-type *moxF*. (A) Cells were grown under Mox-inducing conditions to mid-log phase, harvested, and broken with a French pressure cell. Cell extracts were centrifuged, and the supernatant was decanted and stored at −70°C. Crude cell extract samples (20 μg of total cell protein per lane) were separated on a 12.5% SDS–polyacrylamide gel and electrophoretically transferred to nitrocellulose. The protein blot was incubated with anti-MeDH/anti-MeDH-associated polypeptide antibody, washed, and then incubated with goat anti-rabbit alkaline phosphatase conjugate antibody. Lanes: 1, wild type; 2, MoxF UV26; 3, MoxG UV10; 4, MoxG UV19; 5, MoxG UV24. (B) Crude cell extracts were prepared and analyzed as described for panel A, except that the cells were lysed by sonication. Lanes: 1, wild type; 2, MoxF UV26; 3, MoxF UV26(pRK310); 4, MoxF UV26(pDA3410XM); 5, MoxF UV26 (pDA3410XL); 6, MoxF UV26(pDA3410XB). The positions of the polypeptides recognized by the anti-MeDH/anti-MeDH-associated polypeptide antibody (M_r 60,000 and 12,000) are indicated on the right, and those of the protein standards (in kilodaltons) are shown on the left.

detected via protein immunoblot analysis in the MoxF mutant UV26 (data not shown).

These data suggested that the MeDH-associated polypeptide might be unstable in the absence of the MeDH polypeptide. Conversely, the stability of the MeDH polypeptide might also be affected by the absence of the MeDH-associated protein. Data from the previously described T7 expression experiments suggested that, at least in *E. coli*, the MeDH polypeptide and the MeDH-associated polypeptide can exist, albeit only transiently in one case, in the absence of each other (Fig. 3 and 4); however a different situation may exist in *Methylobacterium* sp. strain AM1.

If codependent protein stability is the explanation for the absence of the MeDH-associated polypeptide in the *Methylobacterium* sp. strain AM1 MoxF class mutant, it should be possible to stabilize this polypeptide by supplying the M_r 60,000 MeDH polypeptide in *trans*. The 3.58-kb *Xho*I (XhoM) fragment, the larger 4.55-kb *Xho*I (XhoL) fragment, and the 5.35-kb *Xho*I-*Bam*HI fragment were cloned into broad-host-range cloning vector pRK310 (10). Each clone contained the MeDH structural gene, including upstream regulatory sequences such that transcription of the cloned *moxF* gene would be under the same control as the chromosomal genes of the *moxFG* region. Each construct was mobilized into UV26 (MoxF) in a three-way cross among the *E. coli* strain carrying the construct, *E. coli* carrying the mobilizing "helper" plasmid pRK2013 (12), and UV26. The selected *Methylobacterium* sp. strain AM1 MoxF class transconjugates were isolated and grown under Mox-inducing conditions. Crude cell extracts were prepared, and the samples were separated on gels, blotted, and reacted with the anti-MeDH/anti-MeDH-associated polypeptide antibody. Neither the M_r-60,000 MeDH polypeptide nor the M_r-12,000 MeDH-associated polypeptide could be detected in UV26 or UV26 carrying pRK310 (Fig. 6B, lanes 2 and 3, respectively). However, both polypeptides were detected in extracts of UV26 containing pDA3410XM and pDA3410XL (Fig. 6B, lanes 4 and 5, respectively), which each harbor a recombinant plasmid containing *moxF* but not *moxI*. Detection of the MeDH-associated polypeptide in these extracts suggests that UV26 contains an intact *moxI* gene and that in *Methylobacterium* sp. strain AM1 the *moxI* polypeptide is apparently unstable in the absence of the MeDH polypeptide and can be stabilized by supplying MeDH in *trans*. Both polypeptides were also detected in the control extract of UV26 containing pDA3410XB, in which the recombinant plasmid contained both *moxF* and *moxI* (Fig. 6B, lane 6).

In all *Methylobacterium* sp. strain AM1 extracts in which the M_r-60,000 MeDH polypeptide was detected (wild type, MoxG class mutants, and MoxF class mutants containing a pDA3410 recombinant plasmid), several protein bands reacted with the anti-MeDH/anti-MeDH-associated polypeptide antibody. These bands apparently represent discrete MeDH protein degradation products. A similar pattern of anti-MeDH-reactive protein bands has previously been detected in *Methylobacterium* sp. strain AM1 Mox mutant extracts in which the MeDH polypeptide was present (25).

We are at present unable to determine whether MeDH polypeptide stability is analogously dependent upon the MeDH-associated polypeptide in *Methylobacterium* sp. strain AM1, as no *Methylobacterium* sp. strain AM1 mutant has yet been isolated in which the mutation maps to *moxI*, specifically eliminating its synthesis.

DISCUSSION

To study the *Methylobacterium* sp. strain AM1 *moxFG* region in greater detail, we have utilized a coupled in vivo T7 RNA polymerase/promoter gene expression system to express in *E. coli* the polypeptides encoded by this region (Fig. 7). Using ^{35}S-labeling techniques, we have found that this region encodes not two but four polypeptides of approximate M_r 60,000, 30,000, 20,000, and 12,000. The order places the gene encoding the M_r-60,000 polypeptide most 5′, followed by the M_r 30,000, 20,000, and 12,000 polypeptide genes, respectively.

Three of the four detected polypeptides have been identified by protein immunoblot analysis. The M_r-60,000 poly-

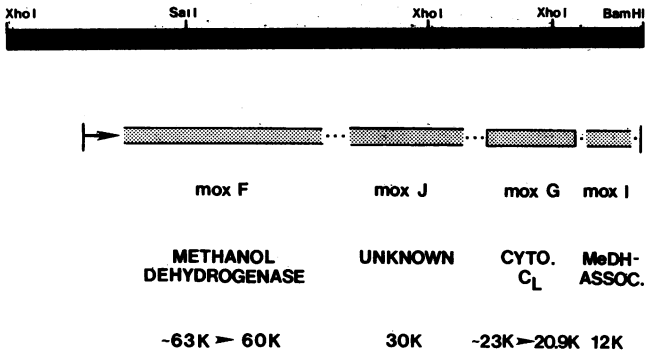

FIG. 7. Genes and gene products of the *moxFJGI* region. The sizes of precursors and mature polypeptides are noted at the bottom, in kilodaltons.

peptide, encoded by a region known to contain *moxF*, was identified as the MeDH polypeptide. The size of the detected *moxF* gene product suggests that this periplasmic *Methylobacterium* sp. strain AM1 protein is processed by and possibly transported in *E. coli*. Unprocessed MeDH has not been observed in methylotrophs, and synthesis of this periplasmic protein has been proposed to be cotranslational (9). That we were unable to detect any polypeptide slightly larger than M_r 60,000 suggests that synthesis of the MeDH subunit in *E. coli* is also cotranslational. Experiments are currently under way to determine whether a precursor form of the MeDH polypeptide can be isolated in *E. coli* or whether transport is obligately cotranslational.

The M_r-12,000 polypeptide was identified as the low-molecular-weight polypeptide that copurifies with the *Methylobacterium* sp. strain AM1 MeDH polypeptide. The gene that encodes this protein maps to the far 3′ end of the region, and we have designated it *moxI*. The function of this polypeptide is not known. A small polypeptide of M_r 10,000 to 12,000 copurifying with the MeDH has been observed in several methylotrophs (18, 26; unpublished data) in some cases in a 1:1 ratio, so it may be a subunit of the MeDH. Our data suggest that it may play a role in MeDH stability, since in *Methylobacterium* sp. strain AM1 both polypeptides were either present or absent as a pair. In addition, it was possible to restore the presence of the small polypeptide in a *moxF* mutant by providing *moxF* in *trans*. It is not clear whether the M_r-60,000 polypeptide is unstable in the absence of the M_r-12,000 polypeptide in *Methylobacterium* sp. strain AM1, since no *Methylobacterium* sp. strain AM1 mutant specifically defective for the MeDH-associated polypeptide has yet been isolated. In *E. coli* expression extracts, the larger polypeptide appeared to be stable alone, but the smaller polypeptide seemed less stable by itself. The MeDH-associated polypeptide was only transiently detected in extracts of the expression subclone containing *moxI* alone (pDA410 XBS). The protein was detected in ^{35}S-labeled extracts involving short-term incubations (Fig. 3, lane 10), but not in extracts prepared for protein immunoblot analysis, which are subjected to longer incubations (Fig. 4, lane 10). However, we do not at present understand why the MeDH-associated polypeptide was detected in both ^{35}S-labeled and protein immunoblot extracts of the expression subclone possessing pDA410SB, which contains *moxI* but only a partial, 5′-deleted *moxF*. It is possible that other *moxFJGI* sequences present in this subclone contribute to the ultimate stability of the MeDH-associated polypeptide, but it is unclear if or at what level this occurs. The difference in stability of this polypeptide in *E. coli* versus *Methylobacterium* sp. strain AM1 mutants suggests that the latter contain factors affecting stability of the MeDH which are not present in *E. coli*.

It was not possible to assess the role of the *moxI* gene product in MeDH activity because *E. coli* does not synthesize the required MeDH cofactor pyrroloquinoline quinone and it is not possible to reconstitute apoprotein and cofactor in vitro. Because we were unable to assay for MeDH activity in this bacterium, the effect of the small MeDH-associated polypeptide on MeDH activity in *E. coli* could not be determined. Likewise, we could not test for activity with our purified MeDH/MeDH-associated polypeptide preparation, as the only condition of several tested that allowed separation of the two polypeptides in our hands (0.1% SDS) resulted in their denaturation. Results from other researchers suggest that purified preparations lacking the small polypeptide do not have MeDH activity (D. Nunn and C. Anthony, personal communication). Definitive determination of the role of the MeDH-associated protein will require the isolation and characterization of *moxI* mutants. Regardless of whether the M_r-12,000 polypeptide plays a role in activity or stability, the data presented in this paper suggest that this small polypeptide probably is involved in methanol oxidation and may be a component of an MeDH enzyme complex.

The M_r-20,000 polypeptide was identified as the mature form of cytochrome c_L, the soluble periplasmic type *c* cytochrome believed to be the direct electron acceptor for MeDH in vivo. Earlier data from our laboratory suggested that the cytochrome c_L structural gene, *moxG*, was located downstream of *moxF*, the MeDH structural gene. This hypothesis has proven to be correct, and detection of what appears to be a truncated cytochrome c_L polypeptide in one of the expression subclone extracts has allowed a more accurate determination of the *moxG* location. *moxG* spans the *Xho*I$_3$ site (Fig. 1), with most of the gene being located upstream of this site. Cytochrome c_L appears to be synthesized in a precursor form of approximately M_r 23,000 (25), which becomes processed to the mature M_r-20,900 protein in *Methylobacterium* sp. strain AM1 and apparently in *E. coli* also. This suggests that the *moxG* sequences upstream of the *Xho*I site must in theory be able to encode a polypeptide of M_r 16,600, that being the predicted size of the truncated cytochrome c_L polypeptide with a signal sequence of about M_r 2,100 still attached. *moxG* thus appears to be located such that at least 450 base pairs of DNA coding sequence must lie upstream of the *Xho*I$_3$ site. It is not known whether cytochrome c_L or its putative precursor is located in the cytoplasm or periplasm in *E. coli*.

The identity of the M_r-30,000 polypeptide could not be determined nor is its function known. The gene encoding this polypeptide has been designated *moxJ*. A methanol-inducible polypeptide of similar molecular mass has previously been reported from this laboratory (32) during induction studies of *Methylobacterium* sp. strain AM1 and may correspond to the M_r-30,000 polypeptide observed in this study.

On the basis of these data, we propose an expanded map of the region previously described as the putative *moxFG* operon and have tentatively designated it the *moxFJGI* operon (Fig. 7). Earlier Tn*5* mutagenesis studies suggested that this entire region is one transcriptional unit; however, additional studies of transcripts will be necessary to verify this hypothesis.

Although we are presently unable to assign a function to either the unidentified M_r-30,000 polypeptide or the M_r-12,000 MeDH-associated polypeptide, it seems quite likely that they will prove to play a role in the oxidation of methanol. The discovery of two new putative *mox* genes brings the total to 12 genes that may be involved in methanol oxidation in *Methylobacterium* sp. strain AM1. Future studies of the putative *moxFJGI* operon and its protein products should provide a better understanding of this genetically complex step.

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