JOURNAL OF BACTERIOLOGY, May 1986, p. 591–597
0021-9193/86/050591-07$02.00/0

Vol. 166, No. 2

Phenotypic Characterization of 10 Methanol Oxidation Mutant Classes in *Methylobacterium* sp. Strain AM1

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Received 2 December 1985/Accepted 18 February 1986

Twenty-five methanol oxidation mutants of the facultative methylotroph *Methylobacterium* sp. strain AM1 have been characterized by complementation analysis and assigned to 10 complementation groups, Mox A1, A2, A3, and B through H (D. N. Nunn and M. E. Lidstrom, J. Bacteriol. 166:582–591, 1986). In this study we have characterized each of the mutants belonging to the 10 Mox complementation groups for the following criteria: (i) phenazine methosulfate-dichlorophenolindophenol dye-linked methanol dehydrogenase activity; (ii) methanol-dependent whole-cell oxygen consumption; (iii) the presence or absence of methanol dehydrogenase protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting; (iv) the absorption spectra of purified mutant methanol dehydrogenase proteins; and (v) the presence or absence of the soluble cytochrome *c* proteins of *Methylobacterium* sp. strain AM1, as determined by reduced-oxidized difference spectra and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. With this information, we have proposed functions for each of the genes deficient in the mutants of the 10 Mox complementation groups. These proposed gene functions include two linked genes that encode the methanol dehydrogenase structural protein and the soluble cytochrome c_L, a gene encoding a secretion function essential for the synthesis and export of methanol dehydrogenase and cytochrome c_L, three gene functions responsible for the proper association of the pyrrolo-quinoline quinone prosthetic group with the methanol dehydrogenase apoprotein, and four positive regulatory gene functions controlling the expression of the ability to oxidize methanol.

We have previously isolated mutants of *Methylobacterium* sp. strain AM1 that are no longer able to utilize methanol as a growth substrate because of their inability to oxidize this compound. These mutants are able to grow on methylamine and formate and therefore are impaired only in gene functions specific to methanol oxidation. Twenty-five methanol oxidation (Mox) mutants were characterized by complementation analysis and found to fall into 10 distinct classes, designated Mox A1, A2, A3, and B through H (19).

To determine the functions deficient in each of the Mox complementation class mutants, it is important to survey the characteristics of methanol dehydrogenase (MDH) and methanol oxidation in *Methylobacterium* sp. strain AM1 and methylotrophs in general.

It has been noted in our laboratory and others that the synthesis of MDH in wild-type *Methylobacterium* sp. strain AM1 is inducible, i.e., can be induced by methanol in the presence of alternative substrates such as succinate and methylamine (9, 17; D. N. Nunn and M. E. Lidstrom, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H50, p. 116). When induced, *Methylobacterium* sp. strain AM1 MDH can represent 10 to 20% of total cell protein and can be easily visualized on sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Native MDH of *Methylobacterium* sp. strain AM1 is composed of two identical 60,000-molecular-weight (MW) subunits (the holoenzyme is 120 kilodaltons) (13). Each subunit is associated with a single pyrrolo-quinoline quinone (PQQ) prosthetic group (8). PQQ is also thought to be the prosthetic group of methylamine dehydrogenase (7), an enzyme necessary for the oxidation of and growth on methylamine. The PQQ group can be readily dissociated from the MDH and does not appear to be covalently attached. A characteristic peak of absorption at 345 nm can be observed in MDH properly associated with the PQQ prosthetic group (4, 8). Attempts to reconstitute purified PQQ with MDH apoprotein have had mixed success. No reassociation could be detected by Patel et al. (21), as determined by reconstitution of dye-linked MDH activity. Davidson et al. (6) have reported the reassociation of PQQ and MDH apoprotein of Bacterium W3A1, as determined by the absorption spectrum of the reconstituted versus native MDHs. Although the characteristic spectrum was observed after incubation of purified apo-MDH and PQQ, no report of enzyme activity was given.

MDH activity can be detected in a dye-linked phenazine methosulfate-dichlorophenolindophenol assay, and purified preparations of MDH or crude, cell-free extracts of the organism may be assayed for activity (3). For whole cells of *Methylobacterium* sp. strain AM1 to oxidize methanol, the two soluble *c*-type cytochromes of this organism (cytochrome c_H, high isoelectric point, 11,000 MW; cytochrome c_L, low isoelectric point, 20,900 MW) are required (2, 5). One or both may accept electrons directly from the MDH in vivo. In vitro experiments strongly suggest that cytochrome c_L is the direct electron acceptor of MDH and that cytochrome c_H acts to mediate electron transfer from cytochrome c_L to the terminal oxidase (5). Cytochrome c_H also participates in electron transfer during the oxidation of methylamine by methylamine dehydrogenase.

Both the MDH and the soluble cytochromes are found predominantly, if not exclusively, in the periplasmic space (1, 14, 15). The results of Davidson et al. (6) suggest that the MDH of Bacterium W3A1 contains a signal peptide of approximately 1,500 MW, as would be expected for a

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TABLE 1. *Methylobacterium* sp. strain AM1 strains used in this study

Strains	Relevant traits	Source or reference
Wild type		J. R. Quayle (22)
AM1rif	Wild type, rifamycin resistant	(19)
M15A	Mox A3 class mutant	J. R. Quayle (11)
M15Arif	Rifamycin resistant	(19)
PG1	Mox A1 class mutant	P. Goodwin (23)
PG1rif	Rifamycin resistant	(19)
UV21	Mox A2 class mutant	(19)
UV4, UV25	Mox B class mutants	(19)
AA18, AA40, UV40, UV41, UV42, UV44, UV45, UV47, UV50, UV52	Mox C class mutants	(19)
UV46, UV9, UV27	Mox D class mutants	(19)
AA31, AA32	Mox E class mutants	(19)
UV26	Mox F class mutant (MDH structural gene mutant)	(19)
UV10, UV19, UV24	Mox G class mutants	(19)
UV48	Mox H class mutant	(19)

gram-negative periplasmic protein. They have also shown that no high-MW precursor form of MDH could be detected by pulse-chase labeling. It was concluded that synthesis of MDH in Bacterium W3A1 occurs exclusively by cotranslation.

In this study we describe the characterization of 25 methanol oxidation mutants that have been previously determined to belong to 10 distinct genetic complementation groups. We have determined the phenotypes of each of the mutant classes for a number of important characteristics and used this information to tentatively identify functions deficient in each of the 10 Mox complementation mutant classes.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1. *Methylobacterium* sp. strain AM1 mutant M15A was provided by J. R. Quayle, University of Bath, Bath, England; mutant PG1 was provided by P. Goodwin, Northeast Surrey College of Technology, Ewell, England.

Media and growth conditions. Media and growth conditions have been previously described (19). Unless otherwise noted, methanol (0.5%) and methylamine (0.2%) minimal media with 0.1% Casamino Acids added were used for growing all *Methylobacterium* sp. strain AM1 strains. Strains were maintained on nutrient agar with rifamycin (40 μg/ml).

Whole-cell methanol oxidation measurements. Freshly harvested methanol- and methylamine-grown cells were washed in 20 mM Tris hydrochloride (pH 8.0). Washed cells were immediately assayed for methanol-dependent oxygen consumption with an oxygen electrode (Rank Bros., Ltd., Bottisham, England).

Preparation of strain AM1 cell extracts. *Methylobacterium* sp. strain AM1 strains were grown to late-log phase in methanol plus methylamine liquid cultures (400 ml in 2,000-ml flasks) at 32°C. Cells were harvested and washed with 20 mM Tris hydrochloride (pH 8.0) and suspended to 0.25 g (wet weight) of cells per ml in the same buffer. Cell suspensions were broken by three passes through a French pressure cell at 20,000 lb/in^2, and the lysed cells were spun at 30,000 × *g* for 30 min. The supernatant was frozen as crude cell extract or used for further fractionations.

Protein determinations. Proteins were assayed using the Lowry method of protein analysis (16).

Purification of MDH. The purification of MDH from *Methylobacterium* sp. strain AM1 has been previously described (19). Preparation of apo-MDH (minus PQQ) was performed as described by Patel et al. (21).

Enzyme assays. MDH activity of crude and purified preparations was assayed with phenazine methosulfate and dichlorophenol-indophenol as electron acceptors, as described by Anthony and Zatman (3) with modifications as described by Weaver and Lidstrom (24).

Absorption spectra of purified MDHs. The absorption spectra of purified MDHs were measured in a Gilford Response double-beam spectrophotometer at 25°C. The slit width was 2 mm, and 300-μl cuvettes were used. The reference cuvette contained 150 mM potassium phosphate (pH 7.0). Spectra were scanned at 1 nm/s.

SDS-PAGE. Purified proteins, whole cells and crude cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 8, 10, or 15% polyacrylamide gels. Proteins were stained with Coomassie blue R or were electrophoretically transferred to nitrocellulose filters and probed with anti-MDH antibody, as previously described (19).

Fractionation of soluble *c*-type cytochromes. The cytochrome c_H and MDH proteins of *Methylobacterium* sp. strain AM1 have a high pI and do not bind to the anion-exchange resin DEAE-cellulose (20). Crude cell extracts, prepared as described above, were loaded on DEAE-cellulose minicolumns (1 by 4 cm) and eluted with 20 mM Tris hydrochloride (pH 8.0). The void volume was collected and labeled as the "cytochrome c_H fraction." The cytochrome c_L remained bound to the column and was eluted with a minimum volume of 250 mM Tris hydrochloride (pH 8.0)–100 mM potassium chloride. This fraction was labeled the "cytochrome c_L fraction." Each fraction, as prepared from wild-type *Methylobacterium* sp. strain AM1, was analyzed by SDS-PAGE, heme stain, and reduced-oxidized difference spectra (procedures described below). The observed MW and difference spectra agreed with those reported for the two cytochromes by O'Keeffe and Anthony (20).

Reduced-oxidized difference spectroscopy of cytochrome *c* fractions. Cytochrome c_H and c_L fractions were adjusted to 10 mg of total protein per ml with 20 mM Tris hydrochloride (pH 8.0) or 250 mM Tris hydrochloride (pH 8.0)–100 mM potassium chloride, respectively. Dithionite-reduced–ferricyanide-oxidized difference spectra were performed as described by O'Keeffe and Anthony (20).

Heme stain of cytochrome c_L fraction. Samples (400 μg) of protein from wild-type and Mox D mutant cytochrome c_L fractions were separated on a 15% SDS-polyacrylamide gel. Proteins were fixed in 10% trichloroacetic acid and stained with *o*-anisidine as described by Francis and Becker (10). After a 2-h incubation the stained gel was photographed and then further stained with Coomassie blue R to visualize total proteins.

TABLE 2. Summary of the phenotypes of the ten Mox mutant classes

Mox class	MDH protein (size in kilodaltons)	MDH activity	MDH absorption spectrum (peaks [nm])	Cytochrome c_L protein (size in kilodaltons)	Cytochrome c_L difference spectrum (peaks [nm])
Mox A1 (PG1)	+++ (60)	−	None	+++ (20.9)	520, 549
Mox A2	+++ (60)	−	329.5	+++ (20.9)	520, 549
Mox A3 (M15A)	+++ (60)	−	348.5	+++ (20.9)	520, 549
Mox B	−	−	—[a]	−	—[b]
Mox C	+ (60)	−	329.5	+ (20.9)	520, 549
Mox D	−	−	—[a]	+++ (23)	522.5, 552
Mox E	−	−	—[a]	+/− (23?)	522.5, 552
Mox F	−	−	—[a]	+ (20.9)	520, 549
Mox G	+++ (60)	+++	345	−	—[b]
Mox H	+ (60)	−	329.5	+ (20.9)	520, 549

[a] —, No MDH protein present.
[b] —, No cytochrome c_L protein present.

RESULTS

Growth conditions for methanol oxidation (Mox) mutants. Twenty-five methanol oxidation mutants were previously found to belong to 10 distinct complementation groups, Mox A1, A2, A3, and B through H (19). All mutants reverted to growth on methanol at a frequency of 10^{-9} or less. In addition, all mutants used in this study grew on methylamine minimal medium (0.1% Casamino Acids added) with a generation time not significantly different from that of the wild type (3.5 to 4.0 h). We have previously observed that cells grown on methylamine alone or methylamine plus methanol have fully induced levels of MDH activity (Nunn and Lidstrom, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H50, p. 116). For this reason, all wild-type and Mox mutant strains of *Methylobacterium* sp. strain AM1 were grown on a mixture of methanol and methylamine to fully induce the methanol-inducible Mox gene functions.

Characterization of wild-type *Methylobacterium* sp. strain AM1 and Mox mutants. Methanol oxidation mutants were characterized by a number of criteria and compared with wild-type *Methylobacterium* sp. strain AM1. These criteria include: (i) whole-cell methanol-dependent oxygen consumption; (ii) phenazine methosulfate-dichlorophenolindophenol dye-linked MDH activity; (iii) presence or absence of MDH protein by SDS-PAGE and Western blotting; (iv) absorption spectra of mutant and wild-type MDHs; (v) analysis of soluble cytochrome *c* fractions by reduced-oxidized difference spectra and SDS-PAGE gel electrophoresis.

The results of the characterizations for members of each Mox complementation class are described below and summarized in Table 2. While the various Mox mutant classes were observed to have different levels of the MDH and cytochrome c_L proteins, all Mox mutants characterized contained wild-type levels of the cytochrome c_H (data not shown).

(i) Mox A. The Mox A complementation class can be subdivided into three distinct subclasses, Mox A1, Mox A2, and Mox A3 (19). All three Mox A complementation classes are similar in that no methanol-dependent whole-cell oxidation (less than 1.0% of wild-type activity) or dye-linked MDH activity (less than 0.5% of wild-type activity) can be detected.

Each of these three classes produces wild-type levels of the 60,000 MW MDH protein when crude cell extracts are analyzed by SDS-PAGE. Western blots of whole-cell samples were probed with anti-MDH antibody and showed a significant amount of MDH protein as well as a number of other bands (Fig. 1). A band of approximately 58 kilodaltons in size could be seen in all mutant and wild-type strains and

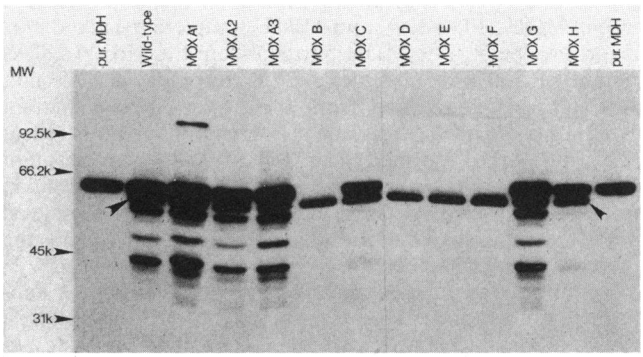

FIG. 1. Western blot of wild-type *Methylobacterium* sp. strain AM1 and Mox mutant proteins. Whole cells (~500 μg, wet weight) were boiled in Laemmli buffer with 1% SDS and 20 mM beta-mercaptoethanol added for each sample. Samples were separated on a 12% SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose filter. The filter was incubated with antiserum prepared against strain AM1 MDH, washed, and incubated with ^{125}I-conjugated staphylococcal protein A. The arrow denotes a protein recognized by the antiserum preparation but not cross-reactive with MDH (common antigen protein, discussed in Results). The high-MW band seen in the Mox A1 lane may represent either an MDH dimer protein undissociated due to inadequate denaturation during sample preparation or a truly nondenaturable MDH dimer form.

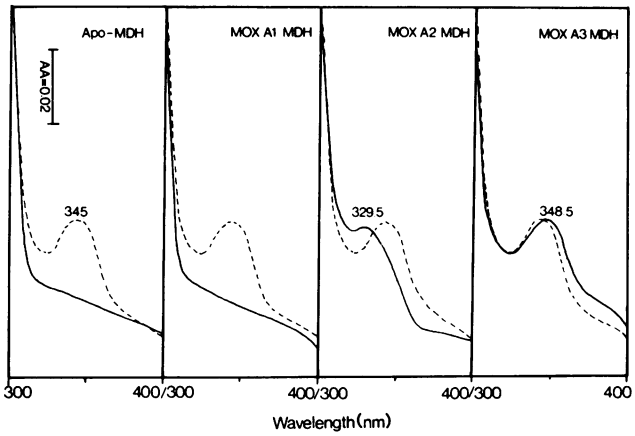

FIG. 2. Absorption spectra of purified MDHs (500 μg/ml in 150 mM potassium phosphate, pH 7.0) from wild-type *Methylobacterium* sp. strain AM1 (broken line), apo-MDH (minus PQQ) prepared from the wild-type enzyme, and the Mox A1 through A3 mutants. No differences in the recorded spectra were seen after further reduction of the same samples with sodium borohydride (data not shown).

might represent the gram-negative "common antigen" described by Jensen et al. (12) and previously observed in this laboratory (19). A number of smaller MW, cross-reactive proteins were also seen; these probably represent specific MDH degradation products, as their presence was always correlated with that of the full-size MDH protein.

MDH protein was purified from each of the three Mox A complementation class mutants. No dye-linked MDH activity could be detected with any of the three MDHs. Each of the mutant MDH proteins was examined for its absorption spectrum. The scans of the wild-type MDH, the prepared PQQ-less apoprotein, and the mutant MDHs are shown in Fig. 2. The peak of absorption at 345 nm is a characteristic of the PQQ prosthetic group in proper association with the native MDH. PQQ-less apo-MDH completely lacked this absorption peak. The MDH purified from the Mox A1 class mutant (PG1) was also devoid of the peak at 345 nm. Similarly, MDH purified from the Mox A2 class mutant (UV21) lacked this peak, but a new shoulder appeared at 329.5 nm. MDH purified from the Mox A3 class mutant (M15A) appeared to have a peak at 348.5 nm instead of 345 nm. The analyses of the Mox A MDHs were repeated at least twice on different preparations of the proteins, and the altered absorption spectra were reproducibly observed.

Membrane-free cell extracts of each of the Mox A subclasses of mutants were separated on DEAE-cellulose minicolumns into a cytochrome c_H fraction and a cytochrome c_L fraction as described in Materials and Methods. The difference spectra of the cytochrome c_L fraction showed that all of the Mox A mutant subgroups also appeared to have normal levels of this cytochrome (data not shown).

(ii) Mox B. Two Mox B mutants were analyzed and showed no detectable methanol-dependent whole-cell oxygen consumption or dye-linked MDH activity. Cell extracts of these mutants contained no MDH protein (or degradation products) as determined by SDS-polyacrylamide gels and Western blots probed with anti-MDH antibody (Fig. 1).

Difference spectra of the Mox B cytochrome fractions showed that no appreciable amount of cytochrome c_L was made by these mutants (Fig. 3). In addition, no 20,900 MW cytochrome c_L protein could be seen on Coomassie-stained 15% polyacrylamide gels (Fig. 4).

(iii) Mox C. Ten Mox C mutants were characterized and were all found to be devoid of whole-cell or dye-linked MDH activity. Cell extracts of the mutants did contain detectable levels of the 60,000 MW MDH protein but significantly less protein was present than in wild-type cells, as seen on Western blots (Fig. 1). The decreased amount of MDH protein was seen in all Mox C mutants examined.

MDH protein was purified from a Mox C class mutant and showed no detectable dye-linked MDH activity. The absorption spectrum appeared to be similar to that seen in the Mox A2 class MDH, i.e., no peak absorbing at 345 nm but a new shoulder at 329.5 nm (data not shown).

Difference spectra of Mox C cytochrome fractions showed that less cytochrome c_L was present in these mutants than in wild-type *Methylobacterium* sp. strain AM1 (Fig. 3 and 4).

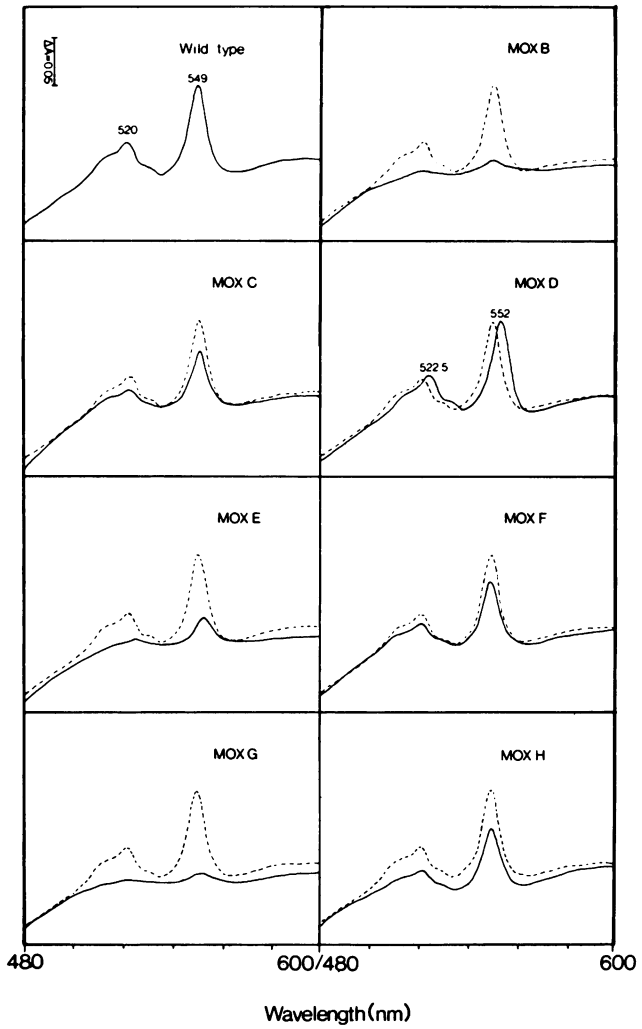

FIG. 3. Reduced-oxidized difference spectra of the cytochrome c_L fractions of wild-type *Methylobacterium* sp. strain AM1 and Mox mutants (data not shown for Mox A1 through A3 mutants). Samples (10 mg of total protein per ml in 250 mM Tris hydrochloride pH 8.0, 100 mM potassium chloride) were reduced with a few crystals of sodium dithionite or oxidized with a few crystals of potassium ferricyanide. The wild-type strain AM1 spectrum is shown as a broken line in the Mox mutant panels for comparison.

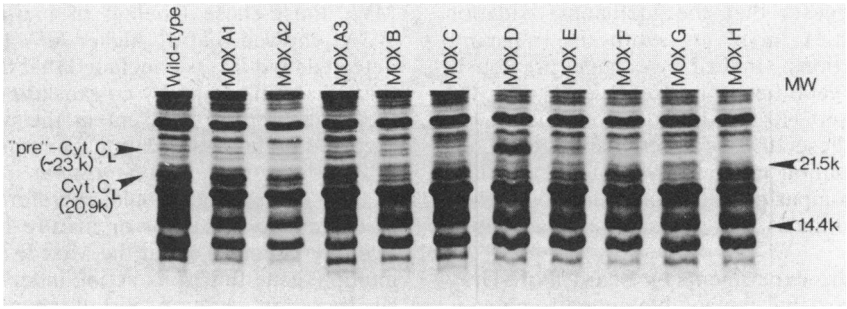

FIG. 4. Coomassie blue R-stained 15% SDS-polyacrylamide gel of *Methylobacterium* sp. strain AM1 and Mox mutant cytochrome c_L fractions (250 μg of total cell protein per lane). The size of the mature cytochrome c_L protein is 20,900 MW (20). The proteins indicated by the arrows were confirmed to be cytochrome c_L proteins by heme stains (data not shown).

We have repeated these analyses a number of times and have consistently seen less cytochrome c_L in the Mox C mutants than in the wild type.

(iv) Mox D. Three Mox D mutants were characterized and exhibited no methanol-dependent whole-cell oxygen consumption or dye-linked MDH activity. Western blots of Mox D whole-cell extracts showed that no MDH protein was detectable in whole cells or crude extracts (Fig. 1).

The Mox D mutants appeared to have normal levels of cytochrome c_L. The absorption maxima of the cytochrome c_L fraction, however, showed a dramatic and reproducible red-shift of about 3 nm from that of the wild-type cytochrome c_L (Fig. 3). When this fraction was analyzed on 15% polyacrylamide gels, the normal 20,900 MW cytochrome c_L protein was absent and a new 23,000 MW protein was seen (Fig. 4). Cytochrome stains of Mox D and wild-type fractions (Fig. 5) showed that this higher MW protein is a *c*-type cytochrome and may represent the untransported or uncleaved (or both) cytochrome c_L precursor protein with a signal peptide still attached.

(v) Mox E. Two Mox E mutants were characterized and found to exhibit no whole-cell or dye-linked MDH activity, and no MDH protein could be detected on Western blots (Fig. 1).

Only a small amount of cytochrome c_L was detectable in the Mox E mutants. The difference spectrum of this fraction showed a red-shift similar to that of the Mox D-type cytochrome c_L fraction (Fig. 3). SDS-PAGE gels showed the absence of the 20,500 MW mature cytochrome c_L protein, but no appreciable amount of 23,000 MW precursor form could be detected (Fig. 4).

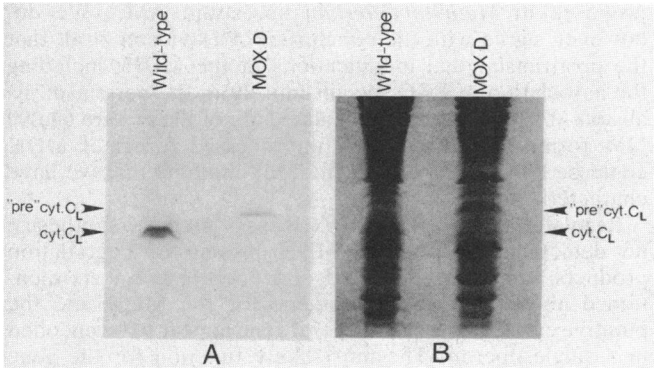

FIG. 5. (A) Cytochrome stain of a 15% SDS-polyacrylamide gel of wild-type *Methylobacterium* sp. strain AM1 and Mox D cytochrome c_L fractions (400 μg of total cell protein per ml) showing the mature wild-type cytochrome c_L (20,000 MW) and the high-MW Mox D cytochrome c_L (~23,000 MW). (B) Same gel as in panel A but stained further with Coomassie blue R to show total proteins.

(vi) Mox F. We have previously shown that the DNA sequences that complement the Mox F mutant encode the MDH structural gene and appear to be cotranscribed with downstream sequences that complement the Mox G mutants (19). As would be expected, the Mox F mutant class exhibited no whole-cell or dye-linked MDH activity. In addition, no MDH protein could be detected on Western blots (Fig. 1).

A less than normal amount of cytochrome c_L protein appeared to be present in the Mox F mutant. The absorption spectrum of the cytochrome c_L fraction appeared similar to that of wild-type *Methylobacterium* sp. strain AM1 cytochrome c_L (Fig. 3 and 4).

(vii) Mox G. Three Mox G mutants were characterized and found to exhibit no methanol-dependent whole-cell oxygen consumption. Crude cell extracts did, however, show wild-type levels of dye-linked MDH activity. SDS-PAGE and Western blots showed normal levels of the 60,000 MW MDH protein (Fig. 1). MDH was purified from the mutants and found to have a specific activity and absorption spectrum similar to those of the wild-type MDH (data not shown).

No cytochrome c_L could be detected by difference spectra or SDS-PAGE of the Mox G cytochrome c_L fraction (Fig. 3 and 4).

(viii) Mox H. One Mox H mutant was characterized and found to have no detectable whole-cell or dye-linked MDH activity. However, small amounts of MDH protein could be detected on SDS-PAGE and Western blots (Fig. 1). MDH purified from this mutant showed no dye-linked activity, and the absorption spectrum of the protein looked similar to that of the Mox A2 and Mox C proteins (data not shown).

Reproducibly less cytochrome c_L was detected in the Mox H mutant than was seen in the wild-type *Methylobacterium* sp. strain AM1 cytochrome c_L fraction (Fig. 3 and 4).

SDS-PAGE analysis of purified wild-type and mutant MDH. MDH was purified from each of the Mox classes that contained detectable amounts of this protein and was separated on an 8% polyacrylamide gel and stained with Coomassie blue R. All MDHs were found to be of the mature 60,000 MW MDH subunit size, and no higher MW protein could be detected in any of the Mox mutants (data not shown).

DISCUSSION

MDH plays a vital role in methylotrophic bacteria as the first enzyme in the oxidation and utilization of methanol as a growth substrate. This key role suggests that the synthesis and utilization of an active MDH might be well regulated. In addition, the presence of MDH and the cytochrome c_L in the

periplasmic space suggests that the methanol oxidation system might be complex, involving synthesis, transport, and modification functions. Indeed, we have previously shown that at least 10 gene functions are necessary for the ability to oxidize methanol (19). In this study our goal was to determine the role of these 10 gene functions by characterizing 25 methanol oxidation mutants by a number of biochemical criteria and comparing this information with existing data about the synthesis and utilization of an active MDH.

Cytochrome c_L. In vitro experiments by Beardsmore-Gray et al. (5) strongly suggest that the soluble cytochrome c_L is the physiological electron acceptor for the MDH of *Methylobacterium* sp. strain AM1. No in vivo evidence, however, exists that proves this is indeed the case. Strain AM1 mutants have been previously isolated that are missing both the soluble cytochrome c_H and c_L and do not grow on methanol or methylamine (2). We have isolated a Mox mutant class (Mox G) that produces wild-type amounts of active MDH and cytochrome c_H but no detectable amounts of cytochrome c_L. These mutants are unable to grow on methanol and exhibit no whole-cell methanol oxidation, yet contain in vitro MDH activity and retain the ability to oxidize and utilize methylamine as a growth substrate. The DNA sequences that complement the Mox G mutants may, therefore, encode the cytochrome c_L structural protein. However, alternative explanations are possible. For instance, Mox G mutants may instead be deficient in a regulatory gene necessary for the expression of this cytochrome or may perhaps be deficient in a gene function required for the stability of the cytochrome c_L protein. These possibilities remain to be tested. In any case, the absence of cytochrome c_L in this mutant class strongly suggests that cytochrome c_L is essential only for the oxidation of methanol and is not required for oxidation of or growth on methylamine.

We have previously shown that the sequences that complement the Mox G mutants appear to reside downstream, in the same operon, from the gene encoding the MDH (deficient in the Mox F mutant) (19). We have characterized the Mox F class mutant and found that no MDH protein could be detected, although soluble cytochrome c_L was present. The level of the cytochrome c_L protein, however, was less than that seen in the wild-type cytochrome c_L fraction and may represent some degree of translational polarity, though incomplete. If the MDH and the putative cytochrome c_L gene reside in the same operon, we would expect that the two proteins would be coregulated. In support of this, a number of Mox mutants (discussed below) did show the concurrent loss of both the MDH and cytochrome c_L proteins.

Synthesis and secretion of MDH and cytochrome c_L. Both the cytochrome c_L and the MDH have been shown to be located in the periplasmic space of methylotrophic bacteria (1, 14, 15). All gram-negative periplasmic proteins so far characterized possess a small 1,000 to 3,000 MW signal peptide that directs the transport of the protein to the periplasm (18). The Mox D mutants were observed to synthesize a 23,000 MW cytochrome c_L protein which is 1,100 MW larger than the mature 20,900 MW form. This suggests that the gene function deficient in the Mox D mutants is involved in the secretion and transport of this cytochrome to the periplasm. Interestingly, the Mox D mutants do not appear to make detectable levels of the MDH protein. Davidson et al. have shown that the MDH of Bacterium W3A1, a ribulose monophosphate pathway methylotroph, has a signal peptide of approximately 1,500 MW. Pulse-chase labeling of methanol-grown Bacterium W3A1 showed that no higher MW form of MDH could be detected, and it was concluded that the transport of MDH in this organism occurs by cotranslation (6). It is possible that the gene product deficient in the Mox D mutants is also involved in the cotranslational synthesis, transport, or both, of the MDH of *Methylobacterium* sp. strain AM1. The lack of this gene product would, therefore, result in the absence of detectable precursor or mature MDH protein. Alternatively, it is possible that the Mox D mutants are deficient in multiple gene functions acting independently in the synthesis, secretion, and transport of the cytochrome c_L and MDH proteins.

The Mox D mutants are capable of growing as well as wild type on methylamine and other substrates, which suggests that this gene function(s) is specific to the transport and secretion of the cytochrome c_L (and possibly MDH) to the periplasm. We have not, however, rigorously examined the profile of other *Methylobacterium* sp. strain AM1 periplasmic proteins to determine whether Mox D mutants are impaired in the ability to transport proteins other than the MDH and cytochrome c_L.

PQQ prosthetic group. A number of researchers have reported little success in reconstituting MDH activity by incubating purified MDH apoprotein (minus the PQQ prosthetic group) with purified PQQ from a variety of methylotrophs (8, 21). It has been suggested that the separation of the prosthetic group from the native MDH results in the irreversible denaturation of the MDH protein. An alternative explanation might be that the proper association of the prosthetic group with the apo-MDH requires the functions of other proteins and cannot be carried out using only purified apo-MDH and PQQ. We have characterized three independent groups of Mox mutants, Mox A1, A2, and A3, whose phenotypes suggest that the latter explanation is the more likely. Each of the three classes synthesizes an inactive MDH that appears not to be in proper association with the PQQ prosthetic group. Davidson et al. (6) have shown that the MDH from Bacterium W3A1 can be reconstituted with purified PQQ to yield an MDH having an absorption spectrum similar to that of the wild-type enzyme, which might, therefore, suggest that the *Methylobacterium* sp. strain AM1 and Bacterium W3A1 enzymes are different in this regard. They did not, however, report the reconstitution of MDH activity. Thus, this activity might similarly require the functions of at least one of the three gene functions we have proposed in *Methylobacterium* sp. strain AM1. We do, however, agree with the conclusion of Davidson et al. that the posttranslational modifications of the MDH, including the association of PQQ, occur entirely in the periplasm, as all apo-MDHs that we have isolated are of the mature 60,000 MW form. No higher MW, unprocessed forms of MDH could be detected in any of the Mox mutants that we have characterized.

Regulatory factors. The Mox B class mutants synthesize no detectable amounts of MDH protein or degradation products and are also devoid of cytochrome c_L. As mentioned above, the structural gene for the MDH and the putative cytochrome c_L structural gene appear to be encoded in a single operon. The most likely function for the gene deficient in the Mox B mutants is as a positive regulatory factor necessary for the induction of the putative MDH-cytochrome c_L operon.

The Mox E class of mutants has a similar phenotype to the Mox B mutants and has no detectable MDH protein or degradation products. These mutants do, however, appear

to make a small amount of cytochrome c_L protein that shows the same red-shift as seen in the Mox G-type cytochrome c_L. We propose that the gene(s) deficient in the Mox E mutants encodes a positive regulatory factor controlling expression of the MDH-cytochrome c_L regulon but also acts to regulate the Mox D gene product. The absence of the Mox D gene product would explain both the red-shift in the difference spectrum of the cytochrome c_L that is made by the Mox E mutants and the lack of detectable MDH in these mutants. This, is at best, a tentative working hypothesis, and it will be necessary to carry out RNA and transcriptional analyses to rigorously define the function of the gene products deficient in the Mox E and Mox B mutants. However, their pleotropic nature suggests these products have a regulatory function.

The phenotypes of the Mox C and Mox H mutants appear similar in a number of respects. Low but detectable amounts of the MDH and cytochrome c_L proteins are present in both classes of mutants. No dye-linked MDH activity could be detected, however, and further examination of the MDH from each of the classes showed an absorption spectrum similar to that of the Mox A2 mutant MDH. The difference spectra of each of the cytochrome c_L fractions appeared normal and showed no apparent red-shift. The mature 20,900 MW cytochrome protein was detected on SDS-PAGE gels. The genes deficient in the Mox C and H mutants most likely function as positive regulatory factors that regulate the expression of the MDH and cytochrome c_L but also regulate the expression of the Mox A2 (and possibly Mox A3) gene products. The Mox D gene product(s) appears to function in the Mox C and H mutants, as MDH protein could be detected and the cytochrome c_L that was seen was of the mature 20,900 MW form. As in the case of the Mox B and E mutants, these mutants must be further characterized by RNA and transcriptional fusion experiments.

ACKNOWLEDGMENT

This research was supported by a grant from the U.S. Department of Energy (DE-AT06-80ER10680).

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