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Identification and Nucleotide Sequences of *mxaA*, *mxaC*, *mxaK*, *mxaL*, and *mxaD* Genes from *Methylobacterium extorquens* AM1

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The DNA sequence for a 4.4-kb *Hin*dIII-*Xho*I *Methylobacterium extorquens* AM1 DNA fragment that is known to contain three genes (*mxaAKL*) involved in incorporation of calcium into methanol dehydrogenase (I. W. Richardson and C. Anthony, Biochem. J. 287:709–7115, 1992) was determined. Five complete open reading frames and two partial open reading frames were found, suggesting that this region contains previously unidentified genes. A combination of sequence analysis, mutant complementation data, and gene expression studies showed that these genes correspond to *mxaSACKLDorf1*. Of the three previously unidentified genes (*mxaC*, *mxaD*, and *orf1*), mutant complementation studies showed that *mxaC* is required for methanol oxidation, while the function of the other two genes is still unknown.

The oxidation of methanol to formaldehyde in the gram-negative, pink-pigmented, facultative methylotroph *Methylobacterium extorquens* AM1 is catalyzed by the periplasmic quinoprotein methanol dehydrogenase (MDH) (4, 24). Its prosthetic group is pyrroloquinoline quinone (PQQ), which is noncovalently bound and is present at 2 molecules per MDH dimer (5, 6). MDH requires the specific electron acceptor cytochrome c_L (12), and calcium ion (Ca^{2+}) is also essential for MDH activity (1, 35). Recent structural data show that each PQQ site contains a calcium ion, which apparently plays a role in maintaining PQQ in the correct configuration (6, 46). The methanol oxidation (Mox) system of *M. extorquens* AM1 has proved to be complex, and to date 24 genes (*mox* genes) have been shown to play a role in MDH synthesis, assembly, or regulation or PQQ synthesis in this microorganism (25).

The Mox system has also been studied in a number of other gram-negative methylotrophs, including *Methylobacterium organophilum* XX (8, 26, 27, 42), *Methylobacterium organophilum* DSM 760 (25), and *Paracoccus denitrificans* (17, 19, 20, 42). Overlap in gene designations has caused confusion, and so a new unified nomenclature for the methanol oxidation genes has been introduced (25). This paper will utilize these new gene designations.

The *mox* genes in *M. extorquens* AM1 are clustered in different loci (25). The largest of these, the *mxa* locus, contains several *mox* genes arranged in three clusters, *mxaFJGIR*, *mxaAKL*, and *mxaB* (25). *mxaF* and *mxaI* encode the large (α) MDH subunit of 60 kDa and small (β) MDH subunit of 8 kDa, respectively, which form the $\alpha_2\beta_2$ heterodimer (2, 33, 34). *mxaG* encodes the cytochrome c_L electron acceptor (19 kDa) (32), but the functions of *mxaJ* (30 kDa) and *mxaR* remain unknown (2, 3, 42). However, in *Acetobacter methanolicus*, a 32-kDa polypeptide with similarity to MxaJ has been isolated in association with MDH at a single molecule per tetramer ($\alpha_2\beta_2\gamma$) (29).

Therefore, it was proposed that MxaJ might play a role in vivo in electron transfer to the cytochrome c_L, in enabling correct structural conformation of MDH, or in correct assembly of the PQQ, Ca^{2+}, and MDH (27). Phenotypic characterization of a *mxaJ* deletion mutant constructed in *P. denitrificans* also suggested a chaperonin-like role for MxaJ (42). Van Spanning et al. (42) constructed a *P. denitrificans mxaR* insertion mutant and proposed that the cytoplasmic MxaR has a role in the regulation of formation of active MDH. In *M. extorquens* AM1, *mxaB* has been shown to be required for transcription of *mxaF* (31). Finally, *mxaA*, *mxaK*, and *mxaL* have been shown to encode polypeptides essential for correct incorporation of calcium ion into MDH (34, 35, 37). MDH isolated from strains defective in these genes is inactive, lacks Ca^{2+} ion, and has PQQ in a fully oxidized form rather than the normal semiquinone form (37). The absorption spectra of these inactive MDHs suggest that PQQ is bound differently in the absence of Ca^{2+} (37). However, incubation with high levels of calcium salts (0.5 to 10 mM) in vitro restores MDH activity (37). It was proposed that MxaA, MxaK, and MxaL either maintain a high level of calcium in the periplasm, binding Ca^{2+} and inserting it into the MDH, or stabilize a configuration of MDH that permits incorporation of Ca^{2+} at low concentrations (37).

In this study, the region of DNA in *M. extorquens* AM1 known to contain *mxaA*, *mxaK*, and *mxaL* has been investigated further. Five genes were identified and designated *mxaACKLD*. Four genes (*mxaA*, *mxaC*, *mxaK*, and *mxaL*) correspond to the identified complementation groups; however, none of the mutants screened were defective in *mxaD*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. In addition, of 214 new *mox* mutants isolated as described by Morris et al. (30), 14 were complemented by pDN24, classifying them as *mxaAKLB* mutants. They were mutants 18, 35, 61, 7-1, 7-3, 7-4, 7-21, 7-28, 7-37, 7-39, 7-43, 8-19, 9-32, and 9-42.

Media and growth conditions. *M. extorquens* AM1 strains were grown at 30°C on the ammonium-mineral salts medium described by Harder et al. (16) supplemented with a vitamin solution (39). Succinate was added to 0.2% (wt/vol), and methanol was added to 0.5% (vol/vol). For growth on methylamine, medium was supplemented with both methylamine at 0.2% (wt/vol), and methanol at 0.2% (vol/vol). Mox-negative mutants were occasionally grown in the presence of allyl alcohol at 0.05% (vol/vol) to prevent reversion to wild type. *Escherichia coli* strains were grown at 37 or 30°C in Luria broth (28). Antibiotics were added to sterile medium in the following concentrations: rifamycin, 20 μg/ml; tetracycline, 10 μg/ml; kanamycin, 40 μg/ml; and ampicillin, 100 μg/ml. When kanamycin and ampicillin were used together, the concentrations were 40 μg/ml each.

Bacterial matings. Triparental matings with *M. extorquens* AM1 were performed as described previously (15). Mating mixtures were plated on both suc-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant trait	Source or reference
E. coli		
DH5α	r^- m^+ *recA1 lacZYA* ϕ80d*lac*Δ(*lacZ*)M15	BRL, Inc.
HB101	r^- m^- *recA13*	11
MM294	*recA*$^+$	7
HMS174	r^- m^+ *recA1* Rif^r	Stan Tabor
M. extorquens AM1 strains		
AM1rif	Rif^r derivative	34
PG1rif	*mxaA* mutant; Rif^r	34
UV21rif	*mxaK* mutant; Rif^r	34
M15a	*mxaL* mutant	21
UV4rif	*mxaB* mutant; Rif^r	34
SM29	*Methylobacterium organophilum* XX mutant	8
Plasmids		
pRK2013	Km^r, mobilizing "helper" plasmid	14
pVK100	Tc^r Km^r; IncP cosmid	22
pRK310	Tc^r *lacPOZ′*; IncP plasmid	13
pUC19	Ap^r *lacZ′*; multiple cloning site	4
pBR322	Ap^r Tc^r; ColE1 replicon	9
M. extorquens AM1 clones		
pDN24	Tc^r; HINDIII-AB in pVK100; complements *mxaAKLB*	34
pDN9	Tc^r; 4.4-kb *Hin*dIII-*Xho*I subclone from pDN24 in pVK100; complements *mxaAKL*	34
pDN30	Tc^r; 9.4-kb *Eco*RI-*Eco*RI subclone from pDN24 in pVK100; complements *mxaLB*	34
PstA-322	Tc^r; 7-kb *Pst*I-*Pst*I subclone from pDN24 in pBR322	D. Nunn
EcoA-322	Ap^r; 9.4-kb *Eco*RI-*Eco*RI subclone from pDN24 in pBR322	D. Nunn
pCM91	Ap^r; 4.4-kb *Hin*dIII-*Xho*I subclone from pDN24 in pBR322	This study
pT7-3A130	Ap^r; 0.9-kb *Pst*I-*Bam*HI subclone from pCM91 in pT7-3	This study
pT7-5A36	Ap^r; 0.9-kb *Pst*I-*Bam*HI subclone from pCM91 in pT7-5	This study
pT7-5B23	Ap^r; 1.5-kb *Bgl*II-*Bgl*II subclone from pCM91 in pT7-5. Plasmids contain fragment in	This study
pT7-5B24	opposite orientations.	
pT7-3C11	Ap^r; 1.6-kb *Bgl*II-*Xho*I subclone from PstA-322 in pT7-3	This study
pT7-5C31	Ap^r; 1.6-kb *Bgl*II-*Xho*I subclone from PstA-322 in pT7-5	This study
pT7-53	Ap^r; 4.4-kb *Hin*dIII-*Xho*I subclone from pDN9 in pT7-5	This study
pUC19-A_2BS	Ap^r; 1.7-kb *Bam*HI-*Sal*I subclone from pDN9 in pUC19	This study
pUC19-A_3S_1	Ap^r; 1.9-kb *Sal*I-*Sal*I subclone from EcoA-322 in pUC19. Plasmids contain fragment	This study
pUC19-A_3S_2	in opposite orientations.	
pRK310A_2BSPB	Tc^r; 1.7-kb *Pst*I-*Bam*HI subclone from pUC19-A_2BS in pRK310	This study
pRK310A_3S_1	Tc^r; 1.9-kb *Pst*I-*Bam*HI subclone from pUC19-A_3S_1 in pRK310	This study
pRK310A_3S_2	Tc^r; 1.9-kb *Pst*I-*Bam*HI subclone from pUC19-A_3S_2 in pRK310	This study
pCMPN5	Ap^r; 2.1-kb *Pst*I-*Nru*I subclone from pT7-53 in pUC19	This study
pCMB3	Ap^r; 0.8-kb *Bgl*I-*Bgl*I subclone from pT7-53 in pUC19	This study
pCMEB1	Ap^r; 1.6-kb *Eco*RI-*Bsa*AI subclone from pT7-53 in pUC19. Plasmids contain	This study
pCMEB41	fragment in opposite orientations.	
pCMBS8	Ap^r; 1.7-kb *Bam*HI-*Sal*I subclone from pUC19A_2BS in pUC19 at *Hin*cII site. Plasmid contains fragment in opposite orientation to pUC19-A_2BS.	This study
pCMHB1	Tc^r; 1.2-kb *Hin*dIII-*Bam*HI subclone from pCM91 in pRK310	This study
pCMHB18	Tc^r; 1.6-kb *Hin*dIII-*Bam*HI subclone from pCMEB41 in pRK310	This study
pCM34	Tc^r; 0.8-kb *Hin*dIII-*Bam*HI (partial) subclone from pCMB3 in pRK310	This study
pCM59	Tc^r; 2.1-kb *Hin*dIII-*Bam*HI (partial) subclone from pCMPN5 in pRK310	This study
pCMBS81	Tc^r; 1.7-kb *Hin*dIII-*Bam*HI subclone from pCMBS8 in pRK310	This study
pCMEB14	Tc^r; 1.6-kb *Hin*dIII-*Bam*HI subclone from pCMEB1 in pRK310	This study

cinate minimal medium and methanol minimal medium for complementation analysis, with appropriate antibiotics.

DNA manipulations. Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, and Klenow enzyme were purchased from New England Biolabs, Inc. (Beverly, Mass.); Promega Corp. (Madison, Wis.); Bethesda Research Laboratories, Inc. (Rockville, Md.); or Boehringer Mannheim GmbH (Mannheim, Germany) and used according to the manufacturer's instructions. Agarose gel electrophoresis, plasmid isolations, and transformations of DNA into *E. coli* DH5α or HB101 were carried out as described by Maniatis et al. (28). DNA sequencing was done by the dideoxy chain-termination method of Sanger et al. (38), with Sequenase from U.S. Biochemical Corp. (Cleveland, Ohio), or by the University of California–Los Angeles Sequencing Facility with an Applied Biosystems model 373A automated sequencer. Primers used were either purchased from U.S. Biochemical Corp. or synthesized by the Caltech Microchemical Facility.

DNA and DNA-derived polypeptide analysis. Translation and analyses of DNA and DNA-derived polypeptide sequences were carried out with the PC/Gene (Genofit SA., Geneva, Switzerland), Genepro version 4.0 (Riverside Scientific Enterprises, Seattle, Wash.), DNA-Master (California Institute of Technology, Pasadena), and Genetics Computer Group (GCG) (Madison, Wis.) programs. The GenBank international protein and DNA data banks were searched for homologous sequences with the GCG FASTA program. An attempt was also made to determine whether the sequences of the various polypeptides were compatible with a known chain fold, as described before (10), emphasizing calcium-binding proteins. More than 50 different folds were tried for each of the *mxa* gene products.

Protein expression and electrophoresis. Protein expression from genes cloned in T7 promoter vectors pT7-3, pT7-4, pT7-5, and pT7-6 was done in *E. coli* DH5α containing pGP1-2 as described by Tabor (40) and Tabor and Richardson (41). [^{35}S]methionine from New England Nuclear, Inc. (Wilmington, Del.) was used for labeling polypeptides as described by Waechter-Brulla et al. (45). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the procedure of Laemmli (23) through 15% (wt/vol) polyacrylamide gels. The protein molecular mass standards (Bio-Rad, Richmond, Calif.) used were as follows (in daltons): phosphorylase *b*, 97,400; bovine serum albumin, 66,200; ovalbumin, 42,699; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400.

Nucleotide sequence accession number. The nucleotide sequence of the *mxaACKLD* region reported here has been assigned GenBank accession number L41608.

RESULTS AND DISCUSSION

Sequencing of the *mxaAKL* region. A 4,404-bp *Hin*dIII-*Xho*I fragment of *M. extorquens* AM1 DNA known to complement *mxaA*, *mxaK*, and *mxaL* mutant strains was sequenced (Fig. 1). The sequenced fragment encodes five complete open reading frames (ORFs) and two partial ORFs (Fig. 2), suggesting that three of these ORFs must correspond to *mxaAKL* and the others must represent previously unidentified genes.

The first full ORF encodes a predicted polypeptide of 307 amino acids (aa). The N terminus has features of a signal sequence (48). A molecular mass of 33.7 kDa was calculated for the polypeptide prior to cleavage of the signal sequence, and the mature polypeptide would be 31.8 kDa. Immediately upstream of this ORF lies the C terminus of an ORF encoding 32 aa, showing 34% identity with the C terminus of *mxaS* of *P. denitrificans* (17, 18). Immediately downstream of the first ORF is a second ORF, encoding a predicted polypeptide of 355 aa with a molecular mass of 37.5 kDa. A larger ORF of 563 aa is also present in this region (Fig. 2). However, this ORF does not contain the expected codon preference for *M. extorquens* AM1 genes and is not thought to be a bona fide gene. The termination codon of the second ORF overlaps the initiation codon of the third full ORF by 1 bp. The third ORF encodes a predicted polypeptide of 208 aa with a calculated molecular mass of 23.1 kDa. The fourth full ORF encodes a predicted polypeptide of 336 aa, the first 27 aa of which have features of a signal sequence. A molecular mass of 35.8 kDa was calculated for the polypeptide before signal sequence cleavage, and a molecular mass of 32.6 kDa was calculated for the mature polypeptide. The termination and initiation codons of the third and fourth ORFs, respectively, are linked in the sequence CCATGAAT, with a 4-bp overlap. The fifth full ORF overlaps the termination codon of the fourth ORF by 1 bp and encodes a predicted polypeptide of 176 aa with a calculated molecular mass of 18.5 kDa. The first 19 aa could encode a signal sequence, resulting in a mature polypeptide with a calculated molecular mass of 16.6 kDa. The final ORF contained in the *Hin*dIII-*Xho*I fragment is truncated by the *Xho*I site. Only 39 aa of the N terminus are known, but it does not appear to contain a signal sequence. It was designated *orf1*. The identity of the genes corresponding to the five full ORFs was determined by complementation and gene expression analyses.

Complementation of *mxaAKLB* mutants. Three clones had been shown previously to complement *M. extorquens* AM1 *mox* mutants that were classified as *mxaA*, *mxaK*, *mxaL*, and *mxaB* mutants (34). These are the 19.4-kb *Hin*dIII fragment in pDN24 (called HINDIII-AB [34]), which was able to complement all of the mutants; the 4.4-kb *Hin*dIII-*Xho*I fragment in pDN9, which was able to complement the *mxaA*, *mxaK*, and *mxaL* mutants; and the 9.4-kb *Eco*RI-*Eco*RI fragment in pDN30, which was able to complement *mxaL* and *mxaB* mutants (34). Subclones of this region were constructed in pRK310 and used to determine which ORFs were responsible for the complementation of which mutants and consequently to identify the genes (Fig. 2). The *M. extorquens* AM1 mutants used in this study included the published mutant strains PG1 (*mxaA*), UV21 (*mxaK*), M15a (*mxaL*), and UV4 (*mxaB*) (34) and an additional 14 new mutant strains complemented by pDN24 (see Materials and Methods). The *M. organophilum* XX *mxaA* mutant strain SM29 (8) was also included for complementation analysis. Transconjugants from complementation tests capable of growth on methanol-supplemented plates were scored for either complementation or recombinational rescue by comparison of the colony frequency on methanol with that grown on succinate. Complementation resulted in a similar number of colonies on both substrates and presumably reflected the presence of the complete gene on the clone being tested. Recombinational rescue gave lower numbers (10- to 100-fold) on methanol plates than on succinate plates but still significantly higher than the numbers on methanol plates from control matings carried out with the vector, pRK310, and presumably reflected the presence of a partial gene on the clone, so that a methanol$^+$ phenotype could only be achieved after recombination occurred. In general, these assumptions are borne out by a comparison of sequence and complementation data (Fig. 2 and Table 2).

The 19 *mxaAKLB* mutants, all complemented by pDN24 and not pRK310, were further divided into five distinct complementation groups. One group comprises mutants complemented by only pDN24 and pDN30 and not any of the other plasmids, and these were classified as *mxaB* mutants. The complementation data show that *mxaB* is not present on the sequenced 4.4-kb *Hin*dIII-*Xho*I fragment (34), and therefore, none of the ORFs identified corresponds to *mxaB*. A second complementation group contains mutants complemented by pDN24, pDN30, and pDN9 and includes a mutant previously designated as a *mxaL* mutant. These mutants were also complemented by pCMEB14, which contains only one complete ORF, the fourth one in this region. These results identified this ORF as *mxaL*. pCMHB18 also encodes *mxaL* but does not complement the *mxaL* mutant M15a and only rescues another *mxaL* mutant (9-32) by recombination. Since in pCMHB18 *mxaL* is present in the opposite orientation with respect to the *lacZ* promoter of pRK310, it seems likely that the lack of complementation is due to a lack of gene expression in this construction. Recombinational rescue was also seen for *mxaL* mutant 9-32 with plasmids pRK310A_3S_1 and pRK310A_3S_2, both of which encode a major portion of *mxaL*.

Another group of mutants, including previously identified *mxaA* mutants, were all complemented by pDN24, pDN9, and pCMHB1. The only common ORF among these plasmids is the first complete ORF in this region, which must correspond to *mxaA* (Fig. 2). *M. extorquens* AM1 *mxaA* mutants also showed recombinational rescue with pCM59, which encodes a major portion of *mxaA*. No recombinational rescue was observed for the *M. organophilum* XX *mxaA* mutant SM29, as expected with heterologous strains.

A fourth group of mutants, which included a previously identified *mxaK* mutant, were all complemented by pDN24, pDN9, and pCMBS81. These plasmids have only the third ORF in common, and therefore this ORF must be *mxaK*. As was the case for *mxaL* expression, plasmids with *mxaK* cloned in the opposite orientation with respect to the *lacZ* promoter of the pRK310 vector did not complement *mxaK* mutants.

The remaining mutants were only complemented by pDN24 and pDN9. However, recombinational rescue was observed with at least one other plasmid (pCM59, pCM34, pCMHB1, pCMBS81, and pRK310A_2BSPB), all of which contain DNA

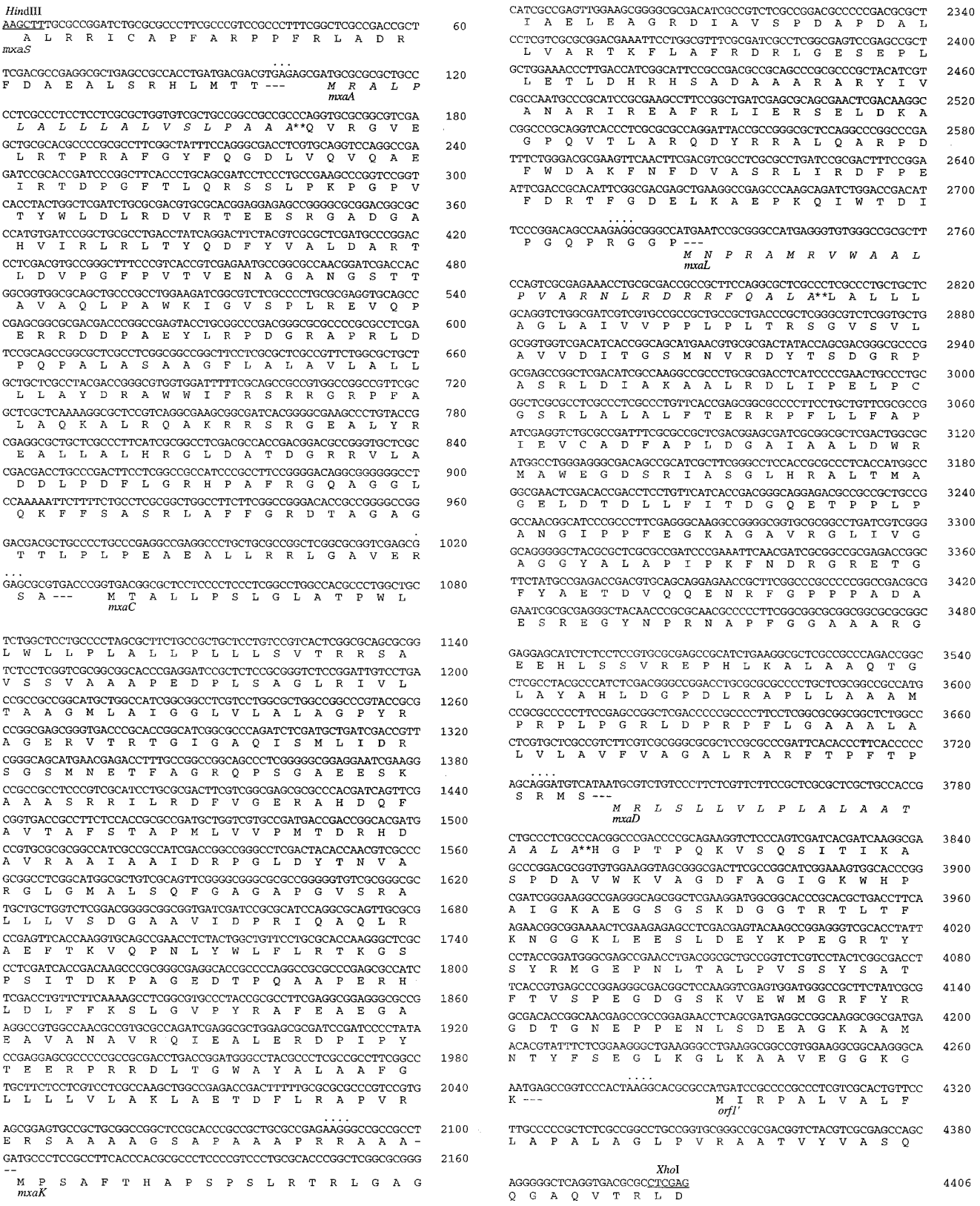

FIG. 1. Nucleotide sequence of *M. extorquens* AM1 *mxaACKLD*. The 4,404-bp *Hin*dIII-*Xho*I fragment encodes the C terminus of *mxaS*; *mxaA*, *mxaC*, *mxaK*, *mxaL*, and *mxaD*; and the N terminus of *orf1'*. The relevant deduced amino acid residues are indicated below the nucleotide sequence, and termination codons are indicated (– – –). Proposed signal sequences are indicated in italics, and probable cleavage sites are marked (**). Shine-Dalgarno sequences are indicated by dots (·) above the nucleotides.

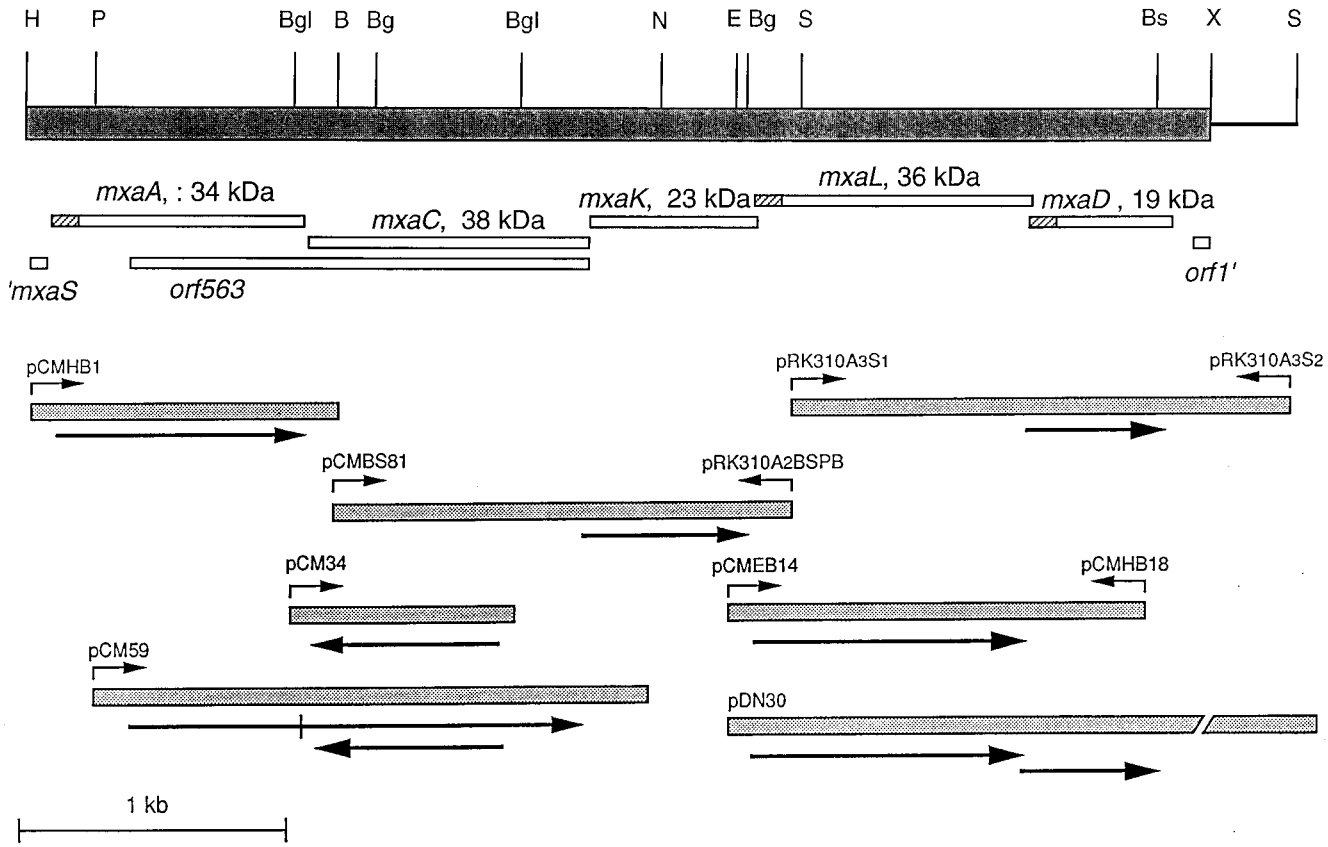

FIG. 2. Physical map of the *Hin*dIII-*Sal*I DNA fragment. The shaded box represents the sequenced 4.4-kb *Hin*dIII-*Xho*I region; an additional 200 bp to the *Sal*I site are indicated by the extended line. The open boxes represent possible ORFs deduced from the determined DNA sequence; proposed leader sequences are noted with hatching. All ORFs are transcribed left to right, as shown. Calculated molecular masses for the *mxa* polypeptides are indicated above the genes. Shaded boxes below indicate DNA fragments cloned into pRK310 for complementation of *M. extorquens* AM1 mutants. ORFs contained in the cloned DNA and their directions of transcription are indicated beneath the individual boxes. Small arrows at the ends of the boxes indicate the direction of transcription of the cloned DNA from the pRK310 *lacZ* promoter in the plasmids whose names are listed above those arrows. Plasmid pDN30 contains a 9.4-kb *Eco*RI-*Eco*RI fragment, and no *lacZ* promoter arrows are included, as it is in pVK100, not in pRK310. Restriction endonuclease sites: B, *Bam*HI; Bg, *Bgl*II; Bgl, *Bgl*I; Bs, *Bsa*AI; E, *Eco*RI; H, *Hin*dIII; N, *Nru*I; P, *Pst*I; S, *Sal*I; X, *Xho*I. Not all *Bgl*I, *Bsa*AI, and *Nru*I sites are indicated.

within the region between the first and third ORFs. Therefore, these mutants appear to have lesions in the gene corresponding to the second ORF, which was designated *mxaC*.

No mutants that corresponded to the fifth ORF in this region were identified.

Gene expression. T7 expression experiments were conducted to identify the polypeptide products of the genes corresponding to the five ORFs by using subclones of the 4.4-kb *Hin*dIII-*Xho*I fragment in the T7 expression plasmids pT7-3, pT7-4, pT7-5, and pT7-6 (40, 41). Recombinant plasmids containing the 4.4-kb *Hin*dIII-*Xho*I fragment (pT7-411, pT7-626, and pT7-66) or the 1.6-kb *Bgl*II-*Xho*I fragment (pT7-3C11 and pT7-5C31) expressed a single polypeptide of approximately 19 kDa, as observed in 15% (wt/vol) SDS-PAGE gels (Fig. 3). When the same DNA fragments were cloned in the opposite orientation with respect to the ϕ10 promoter, no new polypeptides were observed (data not shown). The size of the synthesized polypeptide and the cloned DNA fragments from which it was expressed indicate that this polypeptide is encoded by the fifth ORF, which was designated *mxaD*. Polypeptide products were not observed for any of the other genes with either [^{35}S]methionine- or ^{14}C-labeled amino acids used in the expression protocol.

These expression data suggest that the fifth ORF encodes a bona fide gene, *mxaD*. However, none of the mutants tested

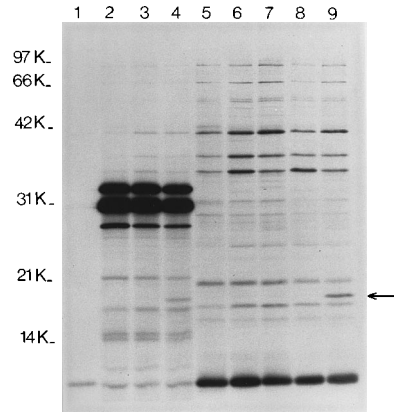

FIG. 3. Autoradiogram of polypeptides expressed by the T7 expression system from the *M. extorquens* AM1 *mxaACKLD* region. A representative gel, loaded with [^{35}S]methionine-labeled extracts of *E. coli* cells containing pGP1-2 and vector or recombinant plasmids, is shown. Lanes: 1, pGP1-2 alone; 2, pT7-3; 3, pT7-3A130; 4, pT7-3C11 (*mxaLD*); 5, pT7-5; 6, pT7-5A36; 7, pT7-5B23; 8, pT7-5B24; 9, pT7-5C31 (*mxaLD*). The 19-kDa polypeptide representing MxaD is indicated with an arrow. The positions of size standards are marked on the left (in kilodaltons).

TABLE 2. Complementation of *mxaACKLB* mutants by *M. extorquens* AM1 clones and subclones

Plasmid	Complementation[a] of mutant by plasmid																		
	mxaA mutant						*mxaC* mutant					*mxaK* mutant				*mxaL* mutant		*mxaB* mutant	
	PG1	7-4	7-28	7-39	7-43	SM29	61	7-21	7-1	7-3	8-19	UV21	9-42	18	35	M15a	9-32	UV4	7-37
pRK310	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
pDN24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pDN9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
pDN30	−	−	−	−	−	−	−	−	−	−	−	−	−	r	r	+	+	+	+
pRK310A_2BSPB	−	−	−	−	−	−	−	−	−	r	r	r	r	r	r	−	−	−	−
pRK310A_3S_1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	r	−	−
pRK310A_3S_2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	r	−	−
pCMHB1	+	+	+	+	+	+	r	−	−	−	−	−	−	−	−	−	−	−	−
pCMHB18	−	−	−	−	−	−	−	−	−	−	−	−	−	r	r	−	r	−	−
pCM34	−	−	−	−	−	−	−	−	r	−	r	−	−	−	−	−	−	−	−
pCM59	r	r	r	r	r	−	r	r	r	r	r	−	−	−	−	−	−	−	−
pCMBS81	−	−	−	−	−	−	−	−	−	r	r	+	+	+	+	−	−	−	−
pCMEB14	−	−	−	−	−	−	−	−	−	−	−	−	−	r	r	+	+	−	−

[a] −, no complementation; +, complementation (similar numbers of colonies on methanol and succinate plates); r, recombinational rescue (10- to 100-fold fewer colonies on methanol plates than on succinate plates).

had a mutation that mapped to *mxaD*. Therefore, either this group of mutants was incomplete, containing no mutants defective in this gene, or a lesion in this gene does not result in a Mox^- phenotype. However, its location overlapping the *mxaL* termination codon and downstream of a cluster of four *mox* genes is suggestive that *mxaD* is involved in methanol oxidation in some way.

Sequence analysis. The *mxaACKLD* cluster is located immediately 3′ to the *mxaFJGIRS* cluster, and the data presented here show that these genes constitute a large cluster, *mxaFJG IRSACKLD*. In addition, the location of *orf1* between these genes and *mxaB* suggests that this *mxa* cluster contains at least two more genes.

Hydrophobicity analysis (39) of the products of *mxaACKLD* predicts them all to be soluble polypeptides except the *mxaL* gene product, which was predicted to be an integral membrane protein with two or three transmembrane segments. Since both the *mxaA* and *mxaD* gene products are predicted to have cleavable signal sequences, they are presumably periplasmic polypeptides.

All of the genes and gene products were compared with sequences in the DNA and protein databases, and no significant similarities were identified. However, the "threading" procedure, in which the sequence is placed onto a known structure and then interrogated for reasonableness (10), did show two significant scores for the *mxaA* gene product. Both involved the sequence from residues 210 to 260 threaded onto the structures of calcium-binding domains in calmodulin and the sarcoplasmic calcium-binding protein from sandworm. These domains have similar structures (43), and so it is possible that this region of the *mxaA* gene product, which is rich in aspartic acid residues at its C-terminal end, may have a calcium-binding function. Site-directed mutagenesis of the aspartates around residue 250 might provide a test for this hypothesis. The threading procedure did not produce any significant scores for the gene products of *mxaC*, *mxaK*, *mxaL*, or *mxaD*.

The sequence of *mxaACKLD* did not provide many clues to the functions for these gene products. However, since *mxaA*, *mxaK*, and *mxaL* are known to be involved in inserting Ca^{2+} into MDH, the other two genes may also have a role in this process. Since this process is thought to occur in the periplasm (37), it might be predicted that these gene products should be periplasmic polypeptides. Therefore, it is intriguing that the sequence data suggest a distribution of these gene products between the cytoplasm, membrane, and periplasm. It may be that MxaL (the predicted membrane polypeptide) serves to link the functions of the predicted cytoplasmic polypeptides (MxaC and MxaK) to those of the predicted periplasmic polypeptides (MxaA and MxaD), either directly or indirectly. MxaA was predicted to contain a Ca^{2+} binding site, consistent with a role for this polypeptide in directly providing Ca^{2+} to the MDH. Now that the gene products have been correlated with known mutant phenotypes, further work to determine the specific function of each of these gene products is possible.

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