JOURNAL OF BACTERIOLOGY, Jan. 1995, p. 206–211 Vol. 177, No. 1
0021-9193/95/$04.00+0

Transcriptional Analysis of *pqqD* and Study of the Regulation of Pyrroloquinoline Quinone Biosynthesis in *Methylobacterium extorquens* AM1

ROOPA RAMAMOORTHI AND MARY E. LIDSTROM*

W. M. Keck Laboratories, California Institute of Technology, Pasadena, California 91125

Received 14 July 1994/Accepted 28 October 1994

Methanol dehydrogenase, the enzyme that oxidizes methanol to formaldehyde in gram-negative methylotrophs, contains the prosthetic group pyrroloquinoline quinone (PQQ). To begin to analyze how the synthesis of PQQ is coordinated with the production of other methanol dehydrogenase components, the transcription of one of the key PQQ synthesis genes has been studied. This gene (*pqqD*) encodes a 29-amino-acid peptide that is thought to be the precursor for PQQ biosynthesis. A unique transcription start site was mapped to a guanidine nucleotide 95 bp upstream of the *pqqD* initiator codon. RNA blot analysis identified two transcripts, a major one of 240 bases encoding *pqqD* and a minor one of 1,300 bases encoding *pqqD* and the gene immediately downstream, *pqqG*. Both transcripts are present at similar levels in cells grown on methanol and on succinate, but the levels of PQQ are about fivefold higher in cells grown on methanol than in cells grown on succinate. These results suggest that PQQ production is regulated at a level different from the transcription of *pqqD*. The genes *mxbM*, *mxbD*, *mxcQ*, *mxcE*, and *mxaB* are required for transcription of the genes encoding the methanol dehydrogenase subunits and were assessed for their role in PQQ production. PQQ levels were measured in mutants defective in each of these regulatory genes and compared with levels of *pqqD* transcription, measured with a transcriptional fusion between the *pqqD* promoter and *xylE*. The results showed that only a subset of these regulatory genes (*mxbM*, *mxbD*, and *mxaB*) is required for transcription of *pqqD*, and only *mxbM* and *mxbD* mutants affected the final levels of PQQ significantly.

Methylobacterium extorquens AM1 is a facultative methylotrophic bacterium capable of growing on single-carbon compounds such as methanol and methylamine as well as multicarbon compounds such as succinate (13). Methanol is oxidized to formaldehyde by the periplasmic enzyme methanol dehydrogenase, and then the formaldehyde is either assimilated into the cell or is oxidized to CO_2, with the generation of energy. The methanol dehydrogenase contains pyrroloquinoline quinone (PQQ) as the prosthetic group (2).

A complex array of genes is involved in methanol oxidation (14, 15) in *M. extorquens* AM1, and functions have been determined for a number of them. *mxaF* encodes the large subunit of methanol dehydrogenase, *mxaI* encodes the methanol dehydrogenase small subunit, and *mxaG* encodes the cytochrome c_L structural polypeptide (1, 22, 23). *mxaA*, *mxaK*, and *mxaL* are involved in the insertion of calcium into the active site of methanol dehydrogenase (22–24). *mxbM*, *mxbD*, *mxcQ*, *mxcE*, and *mxaB* are genes required for transcription of methanol oxidation genes (14, 20, 22, 23, 29).

The transcriptional regulation of the *mxaF* promoter in *M. extorquens* AM1 regulatory mutants has been studied, and in the wild-type strain, a sixfold increase in *mxaF* transcription was found in cells grown on methanol compared with cells grown on succinate. In strains defective in *mxcQ*, *mxcE*, *mxbM*, *mxbD*, or *mxaB*, the transcription from the *mxaF* promoter was negligible in cells grown both on succinate and on medium containing methanol and methylamine (20, 21). The function of these gene products is not yet known, but some evidence suggests that the transcription of *mxcQ*, *mxcE*, and *mxaB* is dependent on *mxbM* and *mxbD* (29). It is not yet clear how the production of the components for this complex methanol oxidation system is coordinated.

Seven genes, called *pqq* genes, are required for PQQ biosynthesis in *M. extorquens* AM1, but their functions are unknown (19, 22, 23). The *pqqD* gene encodes a small polypeptide of 29 amino acids containing conserved tyrosine and glutamate residues separated by three amino acids (19). Tyrosine and glutamate have been shown to be the precursors of PQQ biosynthesis, and it has been proposed that the peptide might serve as the substrate for PQQ biosynthesis (7, 10, 17).

We are interested in determining how the production of the different components of the methanol oxidation system is coordinated at the transcriptional level in *M. extorquens* AM1. One component that must be produced to obtain active methanol dehydrogenase is PQQ, but nothing is known concerning how the synthesis of PQQ is regulated in this strain. Since the PqqD peptide appears to be a key element in PQQ biosynthesis, we have studied the transcription of *pqqD* by carrying out RNA blot analysis, by mapping the transcriptional start site upstream of *pqqD*, and by assessing both transcription of *pqqD* and PQQ production in wild-type strains and in mutant strains defective in transcription of methanol dehydrogenase genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Plasmid pHX200 was provided by R. S. Hanson, University of Minnesota.

Media and growth conditions. *M. extorquens* AM1 strains were grown at 30°C on the ammonium-mineral salts medium described by Harder et al. (9), supplemented with a vitamin solution (27). Succinate was added to 0.27% (wt/vol), and methanol was added to 0.5% (vol/vol). For growth of the *M. extorquens* AM1 mutants and wild-type strain on methanol-plus-methylamine medium, methanol was added to 0.2% (vol/vol) and methylamine was added to 0.2% (wt/vol). *Escherichia coli* strains were grown at 37°C in Luria broth (16). *Pseudomonas testosteroni* was grown on the same mineral salts medium used for *M. extorquens* AM1, and 0.3% (vol/vol) ethanol was added as the carbon source. The following

* Corresponding author. Mailing address: W. M. Keck Laboratories, 138-78, California Institute of Technology, Pasadena, CA 91125. Phone: (818) 395-2132. Fax: (818) 395-2940.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli		
DH5α	r^- m^+ *recA1 lacZYA* ϕ80d*lac*Δ(*lacZ*)*M15*	BRL
HB101	*recA* St^r	3
P. testosteroni	ATCC 15667 (wild type)	8
M. extorquens		
AM1		
AM1 rif	Rif^r derivative	22
UV25 rif	*mxaB* mutant of AM1 rif	22
UV9 rif	*mxbD* mutant of AM1 rif	22
EMS7-20 rif	*mxbM* mutant of AM1 rif	12
EMS7.10	*mxcQ* mutant of AM1 rif	12
AA31	*mxcE* mutant of AM1 rif	22
Plasmids		
pRK2073	Sm^r mobilizing helper	5
pUC18/19	Ap^r, *lacZ*, multiple cloning site	30
pBluescript	Ap^r, *lacZ*, large multiple cloning site	Pharmacia
pRK310	Tc^r IncP1	4
pHX200	Tc^r IncP1, promoterless *xylE* transcriptional fusion vector	29
pCM187	Ap^r, 2.1-kb *Hin*dIII-*Eco*RI subclone from p1130D-HBg2.1 in pUC18	19
pRR7	0.5-kb *Bgl*II-*Eco*RI fragment from pCM187 cloned between the *Bam*HI and *Eco*RI sites of pBluescript	This study
pRR8	0.5-kb *Hin*dIII-*Xba*I fragment from pRR7 cloned into pHX200 (correct orientation with respect to *xylE*)	This study
pRR9	0.5-kb *Xba*I-*Kpn*I fragment cloned from pRR7 into pHX200 (opposite orientation with respect to *xylE*)	This study
p1130D-HBg2.1	pRK310 containing a 2.1-kb *Hin*dII-*Bgl*II fragment (*pqqG*) inserted between the *Hin*dIII and *Bam*HI sites of pRK310	19
p1130D::Tn*5-19*	Tc^r Km^r Tn*5lac* insertion in p1130D, no complementation of Pqq^- mutants of *pqqG* complementation group	19
p1130D::Tn*5-136*	Tc^r Km^r Tn*5lac* insertion in p1130D, no complementation of Pqq^- mutants of *pqqG* complementation group	19

antibiotics were added to sterile medium in the concentrations indicated (in micrograms per milliliter): rifamycin, 20; tetracycline, 10; ampicillin, 100; kanamycin, 30 (*M. extorquens* AM1) or 50 (*E. coli*); and streptomycin, 10.

DNA manipulations. Restriction enzyme digestions, ligations, plasmid isolations, and transformations of DNA into *E. coli* DH5α were carried out as described in Maniatis et al. (16). DNA sequencing was done according to the dideoxy chain termination method of Sanger et al. (25), with Sequenase (U.S. Biochemical Corp., Cleveland, Ohio).

Bacterial matings. Triparental matings were performed as described previously (6). *M. extorquens* AM1 (the recipient), the *E. coli* strain containing pRR8, pRR9, p1130D::Tn*5-136*, or p1130D::Tn*5-19* (the donor), and *E. coli* HB101 containing the mobilizer plasmid pRK2073 were spotted on nutrient agar (Difco, Detroit, Mich.) plates with no antibiotic, in the ratio of about 5:1 of *M. extorquens* AM1 to *E. coli*. The conjugation was allowed to proceed overnight, and then the mated mixture was plated on succinate minimal medium with the appropriate antibiotics. Rifamycin was used to select for *M. extorquens* AM1 strains, tetracycline was used to select for pRR8 or pRR9, and kanamycin was used to select for p1130D::Tn*5-136* or p1130D::Tn*5-19*.

RNA isolation. Total bacterial RNA was isolated from *M. extorquens* AM1 cells grown to mid-exponential phase on methanol or succinate. A total of 50 ml of cell culture was quickly chilled on dry ice and centrifuged (10,000 × *g*) at 4°C, and the pellet of cells was resuspended in 15 ml of diethylpyrocarbonate-treated water. The remaining steps in the RNA isolation procedure were done as described by Waechter-Brulla et al. (28).

Primer extension analysis. Two oligonucleotides, 5′-GACTTTATGGAACGCCGGAACCGCGCG-3′ (R1) and 5′-ACTTCATGGTGTCCTCTCCTCGACTTTATGG-3′ (R3), complementary to nucleotides −14 to −40 and 6 to −23, respectively, with respect to the translation start site of *pqqD*, were synthesized by the Caltech Microchemical Facility. Superscript reverse transcriptase (GIBCO-BRL) was used for primer extension according to the manufacturer's instructions, except that each reaction mixture contained 7.5 μg of RNA and a pulse-chase label was used. Initially, 1 μl of mixed deoxynucleoside triphosphate stock (10 mM [each] dATP, dGTP, and dTTP) and 1 μl of [α-^{32}P]dCTP were added, and the reaction mixture was incubated for 10 min. For the chase phase, 1 μl of 10 mM dCTP was then added, and the incubation was continued for an additional 40 min. The products of transcription were prepared as described previously (28) and subjected to electrophoresis on 6% (wt/vol) polyacrylamide gels simultaneously with a sequencing ladder generated with the same primer, with DNA from plasmid pCM187 as the template.

RNA blot analysis. *M. extorquens* AM1 RNA (10 μg) was subjected to electrophoresis in 1% (vol/vol) formaldehyde-agarose gels with 1× buffer (20 mM MOPS [morpholinepropanesulfonic acid, pH 7.0], 8 mM sodium acetate, 1 mM EDTA [pH 8.0]) as described by Maniatis et al. (16). Transfer to a Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, Calif.) was accomplished by using a dry electroblot apparatus with 0.17× buffer (conductivity, 320 mΩ), and the transfer was allowed to continue for 4 h at 50 mA. Hybridization was done as described by Maniatis et al. (16), with two [γ-^{32}P]ATP 5′-end-labeled oligonucleotides, 5′-CAGATCTCGGAAACGATGGGGGCAGCCCACTTC-3′ (R2), complementary to nucleotides 2 to 34 with respect to the translation start site of *pqqD*, and 5′-TATCGCCAATTCCGGCCCCGCCTGCAAGAGCC-3′ (R4), complementary to nucleotides −8 to −40 with respect to the transcription start site.

Preparation of cell extracts. *M. extorquens* AM1 strains were grown in liquid culture with the appropriate additions of succinate, methanol, methylamine, vitamins, and antibiotics. Cells (150 ml of culture at mid to late exponential phase) were harvested, washed once with the ammonium-mineral salts medium, recentrifuged, and resuspended in 2 ml of the same medium. Cells in the suspension were then broken by three passes through a French pressure cell at 20,000 lb/in^2. Cell suspensions were kept on ice, and the French pressure cell was chilled to 4°C. The cell extracts were centrifuged at 30,000 × *g* for 30 min, and the supernatants were decanted and stored at −20°C (20). For the catechol dioxygenase assay, a small amount of the lysate (about 200 μl) was immediately spun at 14,000 rpm for 4 min in an Eppendorf microcentrifuge, and the supernatant was assayed immediately for activity, as this enzyme was unstable.

Methanol dehydrogenase assay. Methanol dehydrogenase activity was assayed by the phenazine methosulfate-dichlorophenol indophenol dye-linked method described previously (20).

Catechol 2,3-dioxygenase activity (XylE assay). Catechol 2,3-dioxygenase (expressed from *xylE*) transforms catechol into a yellow product, 2-hydroxymuconic semialdehyde. The enzyme reaction was conducted in a cuvette in a total volume of 1 ml containing 960 μl of 50 mM phosphate buffer (pH 7.5), 20 μl of cell-free protein lysate or dilutions, and 20 μl of catechol (10 mg/ml). A kinetic assay was conducted at 376 nm (29) by using a Hewlett Packard model 8452A diode array UV-vis spectrophotometer.

Protein determination. Protein was assayed by using the Bio-Rad protein assay. Stock solutions of bovine serum albumin were used as standards.

PQQ assay. The *P. testosteroni* alcohol dehydrogenase apoenzyme was partially purified as described by Groen et al. (8), except that the cells were grown on ethanol and a Tris-acryl column was used rather than a DEAE-Sephacel column. No further purification of the apoenzyme was carried out. PQQ was measured in the following assay. Apoenzyme (specific activity, 0.7) was diluted 1 to 5 in 0.1 M potassium phosphate buffer (pH 7.0). Diluted apoenzyme (20 μl) was then mixed with 170 μl of 100 mM Tris-OH (pH 7.0)–3 mM $CaCl_2$ containing pure PQQ (1 to 5 pmol) or various volumes of culture supernatant. The culture supernatant was obtained when the cells were harvested by centrifugation (as described above for "Preparation of cell extracts"). KCN (10 μl) was added to a final concentration of 5 mM. The mixture was incubated at room temperature for 5 min. To this volume, 800 μl of a mixture containing 0.2 mM butanol, 55 mM Tris-OH (pH 7.0), 1.66 mM $CaCl_2$, 1.65 mM phenazine methosulfate, and 0.1 mM dichlorophenol indophenol was added, and the kinetics were monitored for 2 min at 610 nm. PQQ amounts were determined by comparison with a PQQ calibration curve and expressed as nanomoles of PQQ produced by a given number of cells, normalized to 1 mg of cell protein total.

β-Galactosidase assay. The β-galactosidase activity was assayed in cell extracts as described previously by Miller (18).

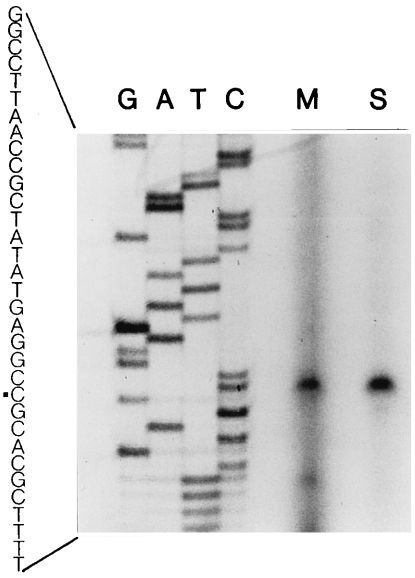

FIG. 1. Transcription start site analysis. α-^{35}S-dATP-labeled sequencing reaction mixtures, synthesized from primer R1 and subjected to electrophoresis through a 6% polyacrylamide sequencing gel, are labeled G, A, T, or C. Adjacent lanes indicate extension products synthesized from primer R1 and labeled with [α-^{32}P]dCTP, with 7.5 μg of total RNA isolated from methanol-grown (M) or succinate-grown (S) cells as the template. A 1 to 10 dilution of the extension reaction mixture was loaded per lane. The nucleotide sequence including the transcription start site (■) is indicated.

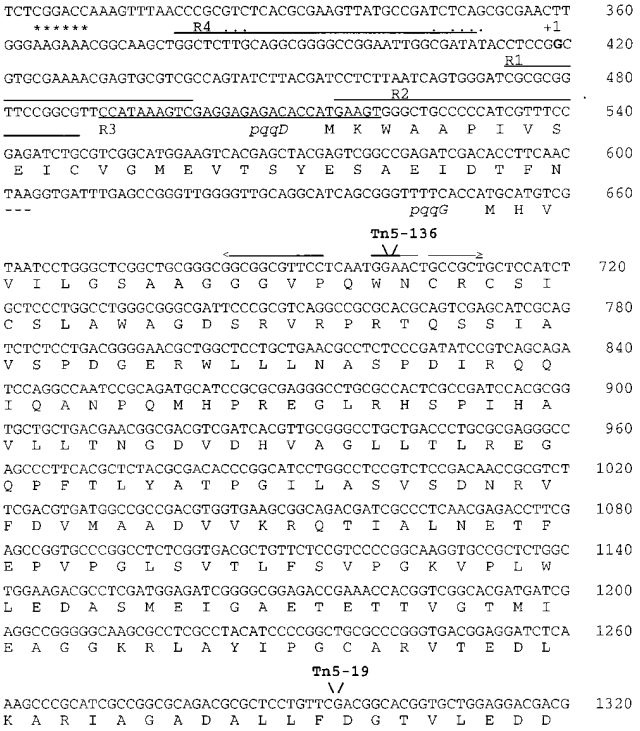

FIG. 2. Nucleotide sequence of *M. extorquens* AM1 *pqqD*, a portion of *pqqG*, and the region upstream of *pqqD* (19). The relevant deduced amino acid residues are indicated below the nucleotide sequence. Termination codons are indicated by dashed lines. The sequences complementary to the primers used to determine the transcription start site and for the RNA blots are indicated by lines labeled R1, R2, and R4 above the bases or labeled R3 below the bases. The transcription start site is indicated in bold type and labeled +1. Bases in common with the *K. pneumoniae* −10 and −35 sequences are indicated by dots above the nucleotides. Asterisks mark the AAGAAA sequence similar to the proposed methylotrophic consensus promoter (15). Sites of insertion for Tn*5-136* and Tn*5-19* are indicated. Arrows above the nucleotide sequence identify inverted repeats that could form a 10-bp stem–5-base loop structure in the mRNA.

RESULTS

Transcription start site analysis. Two oligonucleotide primers complementary to regions within *pqqD* were used to map the *M. extorquens* AM1 *pqqD* transcription start site. Figure 1 shows a major extension product produced with primer R1 (see Materials and Methods), indicating that the 5′ end of the *pqqD* mRNA lies 95 bp upstream of the *pqqD* translation start site and is initiated at a guanidine nucleotide at position 419 in the sequence (Fig. 2). The primer extension product produced with a second primer, R3, confirmed this nucleotide as the transcription start site (data not shown). Some minor extension products were also seen, but they varied from experiment to experiment and were not confirmed by both primers. The levels of transcription observed in cells grown on succinate were equivalent to those seen in cells grown on methanol and were initiated at the same *pqqD* transcription start site (Fig. 1).

RNA blot analysis. RNA blot analysis with *M. extorquens* AM1 RNA from methanol-grown cells was carried out with an oligonucleotide probe complementary to the beginning of *pqqD* (R2) (Fig. 2). Two transcripts were detected (Fig. 3), a major transcription product of approximately 240 bases and a second, less abundant transcript of approximately 1,300 bases. The same pattern and relative amounts of transcription products were also observed for RNA isolated from *M. extorquens* AM1 cells grown on succinate. Neither of these transcripts was detected when oligonucleotide R4 was used in RNA blots, showing that both were initiated approximately between nucleotides −8 and 97 with respect to the identified transcription start site (Fig. 2). Since only one transcription start site was identified in this region, it seems likely that both transcripts were initiated at 1 (Fig. 2). If so, the smaller one would terminate near the beginning of *pqqG* (Fig. 4) just upstream of a region containing a potential stem-loop structure (Fig. 2). Within *pqqG* at this region (nucleotides 685 to 694 and 700 to 710), two 10-bp inverted repeats exist with a single mismatched base (T-705). These could potentially form a 10-bp stem topped by a 5-base loop with a free energy value of $\Delta G^{o'}$ of 22.7 kcal (~95.0 kJ). The second transcript is predicted to terminate near the start of *pqqC* (Fig. 4).

Tn*5lac* insertions. Two Tn*5lac* insertions (Tn*5-136* and Tn*5-19*) in p1130D that abolish complementation of *pqqG* mutants have been reported (19). Tn*5lac* is constructed in such a way that in the correct orientation, a transcriptional fusion to *lacZ* is generated (11). The sites of insertion of these two transposons have previously been determined by sequencing to lie within *pqqG* between nucleotides 701 and 702 and 1295 and 1296, respectively (Fig. 2) (19), and their orientations were found to be that in which their internal β-galactosidase genes would both be transcribed in the same direction as *pqqDGC*. The insertion site of Tn*5-136* disrupts the possible stem-loop structure described above, and Tn*5-19* is inserted further downstream, near the end of *pqqG*. Therefore, these transposon insertions were used as indicators of the transcription levels from the *pqqD* promoter. β-Galactosidase activities were determined in cell extracts of methanol-grown *M. extorquens* AM1 containing plasmids that carried the two different transposon insertions. Extracts of methanol-grown cells containing p1130D::Tn*5-136* had 10-fold-greater β-galactosidase activity (192 nmol min^{-1} mg of protein $^{-1}$) than extracts of cells containing p1130D::Tn*5-19* (19 nmol min^{-1} mg of $protein^{-1}$). β-Galactosidase activities were similar in extracts of succinate-

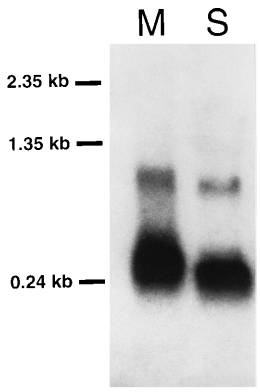

FIG. 3. RNA blot analysis. Total RNA (10 μg) isolated from methanol-grown (lane M) and succinate-grown (lane S) cells was subjected to electrophoresis through a 1% agarose-formaldehyde gel. Hybridization was done with [γ-^{32}P]ATP-labeled oligonucleotide R2. The sizes indicated on the left were determined by using an 0.24- to 9.5-kb RNA ladder (BRL).

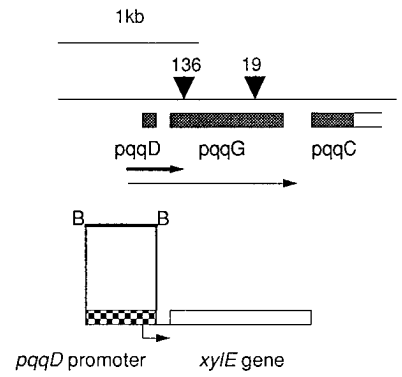

FIG. 4. Physical map of *pqqDGC* region showing the apparent locations of the two transcripts detected and expanded map of the transcriptional fusion in pRR8 between the *pqqD* promoter and *xylE*. The arrows show the direction of transcription. B, *Bgl*II.

grown cells (p1130D::Tn*5-136*, 146 nmol min^{-1} mg of protein^{-1}; p1130D::Tn*5-19*, 23 nmol min^{-1}mg of protein^{-1}). These β-galactosidase activity levels correlate with the levels of the two transcripts observed (Fig. 3), suggesting that the insertion site of p1130D::Tn*5-136* lies within the part of this region encoding the major (smaller) transcript while p1130D::Tn*5-19* is located within the minor (larger) transcript (Fig. 2 and 4).

Construction of a *pqqD-xylE* transcriptional fusion. A 0.5-kb *Bgl*II-*Eco*RI fragment that should contain the *pqqD* promoter and contains part of the *pqqD* peptide-coding region was linked to a promoterless *xylE* reporter gene (Fig. 4) to create a transcriptional fusion in pHX200, a low-copy-number plasmid with broad host range. This plasmid was called pRR8. The 0.5-kb fragment should contain the entire *pqqD* promoter, as *mxbM* is located just upstream of *pqqD*, is transcribed in the same direction as *pqqD*, and terminates in the 0.5-kb fragment (26).

Expression of *xylE* from *pqqD* promoter. Plasmid pRR8 was conjugated into *M. extorquens* AM1 and *mxbM*, *mxbD*, *mxcQ*, *mxcE*, and *mxaB* mutant strains by using triparental matings as described in Materials and Methods. Transconjugants were isolated and grown in liquid culture with either succinate, methanol (wild type only), or methanol plus methylamine as the carbon sources. The latter condition is optimal for induction of methanol oxidation functions in methanol oxidation mutants, which are unable to grow on methanol. Cells were harvested in exponential phase, and cell extracts were prepared. Catechol 2,3-dioxygenase activity was then measured to provide an indication of the level of transcription from the *pqqD* promoter in the wild-type and mutant strains (Table 2). Methanol dehydrogenase activity was also measured to confirm that the mutants had not reverted during growth and was always below detectable levels. In the wild type, the catechol dioxygenase activity was similar in methanol- and succinate-grown cells but was approximately twofold higher in cells grown on methanol plus methylamine. In the *mxcQ* mutant, the activity levels in cells grown on succinate and on methanol plus methylamine were similar to those in the wild type. Activities were lower in all of the other mutants, although like the *mxcQ* mutant, the *mxcE* mutant showed a normal induction pattern. The other mutants showed no significant differences in the activity levels in cells grown on succinate or on methanol plus methylamine. When a plasmid containing the *pqqD* promoter in the opposite direction from that of the *xylE* gene (pRR9) was conjugated into the wild type, the catechol dioxygenase activity was found to be negligible (less than 0.1 μmol min^{-1} mg of protein^{-1}) both on methanol and on succinate.

PQQ assays. The catechol dioxygenase measurements provided an indication of how the transcription from the *pqqD* promoter was affected in the regulatory mutants tested. As seven gene products are involved in PQQ biosynthesis, the levels of PQQ were also measured in the mutant and wild-type strains grown on methanol (wild type only), on methanol plus methylamine, or on succinate to compare patterns of the final product made with those of *pqqD* transcription estimated from the gene fusion experiments. The cells were harvested in exponential phase, and PQQ was measured in both culture supernatants and cell extracts. All the detectable PQQ was excreted to the medium in both the mutant and wild-type strains, and a negligible amount of PQQ was accumulated within the cell as measured in cell extracts. This is in keeping with the hypothesis that PQQ is added to the methanol dehydrogenase apoprotein in the periplasm (2) and suggests that a PQQ export system must be present. Therefore, only the PQQ in the culture supernatant is reported (Table 3). In the wild type, the level of PQQ was maximal during growth on methanol, intermediate in methanol-plus-methylamine-grown cells, and lowest during growth on succinate. The levels of PQQ in the *mxcQ* and *mxcE* mutants grown on methanol plus methylamine were comparable to each other and to that of the wild type grown on methanol, intermediate in the *mxaB* mutant, and lower in the *mxbM* and *mxbD* mutants. In all the mutants the level of PQQ

TABLE 2. Results of catechol dioxygenase assays of cells containing plasmid pRR8

Strain	Defective gene	Catechol dioxygenase (μmol · min^{-1} mg of protein^{-1}) on[a]:		
		Succinate	Methanol + methylamine	Methanol
AM1 rif		2.9*	5.7	2.3
EMS7-10	*mxcQ*	2.3*	6.8	
AA31	*mxcE*	1.1	3.3	
EMS7-20 rif	*mxbM*	0.5	0.6	
UV9 rif	*mxbD*	0.3	0.3	
UV25 rif	*mxaB*	1.6	1.6	

[a] All determinations were carried out at least twice, and values agreed within ±20% except those indicated by an asterisk, which agreed within ±40%.

TABLE 3. PQQ assay results

Strain	Defective gene	PQQ (nmol mg of protein^{-1}) in cells grown on[a]:		
		Succinate	Methanol + methylamine	Methanol
AM1 rif		12	32	59
EMS7.10	*mxcQ*	11	64	
AA31	*mxcE*	13	49	
EMS7-20 rif	*mxbM*	2.5	19	
UV9 rif	*mxbD*	3.5	19	
UV4 rif	*mxaB*	7*	31*	

[a] All determinations were carried out at least twice, and values agreed within ±20% except those indicated by an asterisk, which agreed within ±40%.

on methanol plus methylamine was four- to sixfold higher than that on succinate (Table 3).

DISCUSSION

In gram-negative methylotrophs, the production of active methanol dehydrogenase requires the synthesis of both the methanol dehydrogenase subunits and the prosthetic group, PQQ, as well as gene products required for generating active enzyme (14, 15). It is not yet known how this complex system is coordinated at the transcriptional level. However, in *M. extorquens* AM1, the regulation of the genes encoding the methanol dehydrogenase subunits is complex and involves at least seven regulatory genes (14, 15). This study has focused on the production of PQQ in this strain. Seven genes are required specifically for PQQ synthesis (19). Five of these, *pqqDGCBA*, are clustered and transcribed in the same direction. To begin to understand how PQQ synthesis is regulated and how it coordinates with the synthesis of the methanol dehydrogenase subunits, we have studied transcription of the first gene of this cluster, *pqqD*, which encodes the peptide proposed to be the precursor for PQQ synthesis (19).

A unique transcription start site was identified 95 bp upstream of *pqqD*, and promoter activity was measured for the region including this site. Although the sequences required for promoter function are not yet known, some sequences that resemble sequences involved in transcription in related systems are present. The −10 and −35 sequences for *pqqD* (CGATAT and TTGCAG, respectively) have similarity to these sequences for the *Klebsiella pneumoniae pqq* operon (CAATAT and TT GATC, respectively) (17) (Fig. 2). In addition, Xu et al. (29) determined that the septanucleotide AGAAATG was associated with methanol-regulated promoters in *Methylobacterium organophilum* XX. Although this precise sequence is not found upstream of the *pqqD* transcription start site of *M. extorquens* AM1, the similar sequence AGAAACG is present at bases −54 to −48, with respect to the transcriptional start site (Fig. 2).

The data presented here suggest that the five genes, *pqqDGCBA*, may not be cotranscribed. Two transcripts that were apparently initiated at the *pqqD* transcription start site were detected with a probe to the 5′ portion of *pqqD*. The smaller, more abundant transcript (240 bases) would be expected to encode *pqqD* alone, while the larger product (1,300 bases) would encode both *pqqD* and *pqqG*. No larger transcripts were detected, although it is possible that they are present in the cell either in low abundance or as unstable structures. A potential stem-loop structure lies 265 bases downstream of the transcription start site within *pqqG* that is a candidate for a transcription terminator or a processing site. The β-galactosidase levels measured in extracts of cells containing the plasmids p1130D::Tn*5-19* and p1130D::Tn*5-136* support the hypothesis that the stem-loop structure is important for transcription levels. β-Galactosidase activity in extracts of cells containing p1130D::Tn*5-136*, in which Tn*5lac* is located within the putative stem-loop structure, was 10-fold greater than that in cells containing p1130D::Tn*5-19*, in which Tn*5lac* is located downstream of the putative stem-loop structure, within *pqqG*. Further studies are necessary to determine the precise role of this stem-loop structure in transcription of these genes.

The higher level of the smaller transcript compared with that of the larger one is consistent with the expectation that the small peptide, PqqD, would be required in greater amounts than the PQQ biosynthesis enzymes if it acts as a precursor of PQQ. In addition, the ribosome-binding sequence for *pqqD* is much stronger than those for *pqqG* and *pqqC* (20), and so it is possible that a further increase of the ratio of peptide to biosynthetic enzymes could be accomplished at the level of translation.

The catechol dioxygenase measurements in the various regulatory mutants containing the plasmid with a *pqqD-xylE* fusion (pRR8) show that *mxbM* and *mxbD* are involved in both the induction of *pqqD* by growth on methanol plus methylamine and the transcription of *pqqD*, apparently acting as positive regulators. In both *mxbM* and *mxbD* mutants, catechol dioxygenase activities were low and noninducible (Table 2). *mxaB* was required for induction of *pqqD*, but intermediate activity was observed in this mutant, suggesting that the *mxaB* mutation had only a moderate effect on transcription. *mxcE* and *mxcQ* did not appear to be required for transcription or induction, although the activity in the *mxcE* mutant was about half that in the *mxcQ* mutant. *mxcQ*, *mxcE*, *mxbM*, *mxbD*, and *mxaB* gene products are all required for transcription of the genes encoding the methanol dehydrogenase subunits (20, 21, 29), while the data presented here show that only a subset of these genes are involved in transcription and transcriptional regulation of *pqqD*. Therefore, there is partial overlap between the regulation of the methanol dehydrogenase subunit genes and *pqqD*, but part of the machinery involved in transcriptional regulation of the methanol dehydrogenase subunit genes is not involved in regulation of *pqqD*.

It had previously been shown in *M. organophilum* XX, a methylotroph closely related to *M. extorquens* AM1 (13), that *mxbM* and *mxbD* were required for transcription of two other methanol-inducible promoters in addition to the promoter for *mxaF* (the gene encoding the methanol dehydrogenase large subunit), while *mxcQ*, *mxcE*, and *mxaB* were specific to *mxaF* transcription (29). It is not yet known whether these gene products act together to simultaneously regulate the genes that they affect or whether they act in a sequential, linear, or branched pathway. However, our data support the hypothesis that *mxbM* and *mxbD* regulate a broader range of methylotrophy functions, including PQQ synthesis, while the other regulatory genes appear to affect specific subsets of methylotrophy genes.

When the data from the *pqqD-xylE* experiments were compared with PQQ levels in the same strains grown on the same substrates, the patterns were not the same. In the wild type, PQQ levels were fivefold higher in cells grown on methanol than in cells grown on succinate. This is similar to the relative change in the levels of the subunits of methanol dehydrogenase under similar conditions (20). However, catechol dioxygenase activity from the *pqqD-xylE* fusion was similar in cells grown on methanol and on succinate. This discrepancy did not appear to be due to expression artifacts involving the fusion nor to dif-

ferences in transcript stability under different growth conditions, since the results from the fusion correlated well with the RNA blot data and the transcriptional start site data. These results suggest that the regulation of PQQ production by growth on methanol must involve processes in addition to the transcription of *pqqD*, either via transcriptional regulation of other *pqq* genes or via posttranscriptional regulation of one or more *pqq* gene products. Some regulation of *pqqD* transcription must occur however, since in the wild-type catechol dioxygenase activity from the *pqqD-xylE* fusion was twofold higher in cells grown on methanol plus methylamine than in cells grown on succinate. As noted above, this regulation was disrupted in the *mxbM*, *mxbD*, and *mxaB* mutants. However, normal regulation of PQQ production was observed in these mutants, underscoring the fact that levels of regulation of PQQ production other than *pqqD* transcription must occur.

ACKNOWLEDGMENTS

We thank Ludmila Chistoserdova for advice with the RNA experiments, Juan Davagnino for help with the ethanol dehydrogenase apoenzyme purification, and Francis Biville for help with the PQQ assays.

This work was supported by grants from NIH (GM36296) and DOE (DEFG03-87ER13753).

REFERENCES

1. **Anderson, D. J., C. J. Morris, D. N. Nunn, C. Anthony, and M. E. Lidstrom.** 1990. Nucleotide sequence of the *Methylobacterium extorquens* AM1 *moxF* and *moxJ* genes involved in methanol oxidation. Gene **90:**173–176.
2. **Anthony, C.** 1984. Bacterial oxidation of methane and methanol. Adv. Microb. Physiol. **27:**113–210.
3. **Boyer, H. W., and D. Roulland-Dussiox.** 1979. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
4. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X.-W. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski.** 1985. Plasmids related to the broad host range vector pRK290, useful for gene cloning and for monitoring gene expression. Plasmid **13:**149–153.
5. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
6. **Fulton, G. L., D. N. Nunn, and M. E. Lidstrom.** 1984. Molecular cloning of a malyl coenzyme A lyase gene from *Pseudomonas* sp. strain AM1, a facultative methylotroph. J. Bacteriol. **160:**718–723.
7. **Goosen, N., R. G. M. Huinen, and P. van de Putte.** 1992. A 24-amino-acid polypeptide is essential for the biosynthesis of the coenzyme pyrrolo-quinoline-quinone. J. Bacteriol. **174:**1426–1427.
8. **Groen, B. W., M. A. G. Van Kleef, and J. A. Duine.** 1986. Quinohaemoprotein alcohol dehydrogenase apoenzyme from *Pseudomonas testosteroni*. Biochem. J. **234:**611–615.
9. **Harder, W., M. Attwood, and J. R. Quayle.** 1973. Methanol assimilation by *Hyphomicrobium* spp. J. Gen. Microbiol. **78:**155–163.
10. **Houck, D. R., J. L. Hanners, and C. J. Unkefer.** 1991. Biosynthesis of pyrroloquinoline quinone. 2. Biosynthetic assembly from glutamate and tyrosine. J. Am. Chem. Soc. **113:**3162–3166.
11. **Kroos, L., and D. Kaiser.** 1984. Construction of Tn*5lac*, a transposon that fuses *lacZ* expression to exogenous promoters and its introduction into *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **89:**5816–5820.
12. **Lee, E., A. L. Springer, W.-H. Fan, and M. E. Lidstrom.** Unpublished data.
13. **Lidstrom, M. E.** 1991. The aerobic methylotrophic bacteria, p. 431–445. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes. Springer-Verlag, New York.
14. **Lidstrom, M. E., C. Anthony, F. Biville, F. Gasser, P. Goodwin, R. S. Hanson, and N. Harms.** 1994. New unified nomenclature for genes involved in the oxidation of methanol in gram negative bacteria. FEMS Microbiol. Lett. **117:**103–106.
15. **Lidstrom, M. E., and D. I. Stirling.** 1990. Methylotrophs: genetics and commercial applications. Annu. Rev. Microbiol. **44:**27–57.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Meulenberg, J. J. M., E. Sellink, N. H. Riegman, and P. W. Postma.** 1992. Nucleotide sequence and structure of the *Klebsiella pneumoniae pqq* operon. Mol. Gen. Genet. **232:**284–294.
18. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Morris, C. J., F. Biville, E. Turlin, E. Lee, K. Ellermann, W.-H. Fan, R. Ramamoorthi, A. L. Springer, and M. E. Lidstrom.** 1994. Isolation, phenotypic characterization, and complementation analysis of mutants in *Methylobacterium extorquens* AM1 unable to synthesize pyrroloquinoline quinone and sequence of *pqqD*, *pqqG*, and *pqqC*. J. Bacteriol. **176:**1744–1755.
20. **Morris, C. J., and M. E. Lidstrom.** 1992. Cloning of a methanol-inducible *moxF* promoter and its analysis in *moxB* mutants of *Methylobacterium extorquens* AM1. J. Bacteriol. **174:**4444–4449.
21. **Morris, C. J., and M. E. Lidstrom.** Unpublished data.
22. **Nunn, D. N., and M. E. Lidstrom.** 1986. Isolation and complementation analysis of 10 methanol oxidation mutant classes and identification of the methanol dehydrogenase structural gene of *Methylobacterium* sp. strain AM1. J. Bacteriol. **166:**581–590.
23. **Nunn, D. N., and M. E. Lidstrom.** 1986. Phenotypic characterization of 10 methanol oxidation mutant classes in *Methylobacterium* sp. strain AM1. J. Bacteriol. **166:**591–597.
24. **Richardson, I. W., and C. Anthony.** 1992. Characterization of mutant forms of the quinoprotein methanol dehydrogenase lacking an essential calcium ion. Biochem. J. **287:**709–715.
25. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
26. **Springer, A. L., and M. E. Lidstrom.** Unpublished data.
27. **Staley, J. T.** 1981. The genera *Prosthecomicrobium* and *Ancalamicrobium*, p. 456–460. *In* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), The procaryotes, vol. 1. Springer-Verlag, New York.
28. **Waechter-Brulla, D., A. A. DiSpirito, L. Chistoserdova, and M. E. Lidstrom.** 1993. Methanol oxidation genes in the marine methanotroph *Methylomonas* sp. strain A4. J. Bacteriol. **175:**3767–3775.
29. **Xu, H. H., M. Viebahn, and R. S. Hanson.** 1993. Identification of methanol-regulated promoter sequences from the facultative methylotrophic bacterium *Methylobacterium organophilum* XX. J. Gen. Microbiol. **139:**743–752.
30. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.