INTRODUCTION

Methylobacterium extorquens AM1 is a pink-pigmented facultative methylo troph that can grow on single-carbon compounds such as methanol as sole carbon and energy source. Methanol is oxidized to formaldehyde by the periplasmic enzyme methanol dehydrogenase (MDH). The formaldehyde is consumed inside the cell, either assimilated into biomass through the serine cycle or oxidized to CO₂ with the generation of energy (Lidstrom, 1991; Anthony, 2000). MDH is an α₂β₂ tetramer with two active sites, each containing a pyrroloquinoline quinone (PQQ) prosthetic group and a calcium atom (Anthony, 2000).

Genetic analysis of M. extorquens AM1 has shown that at least 25 genes are involved in the oxidation of methanol to formaldehyde (Lidstrom, 1991). These genes have been mapped to five gene clusters on the M. extorquens AM1 chromosome: mxa, mxb, pqqABC/DE, pqqFG and mxc. The first of these loci contains a cluster of 14 genes all transcribed in the same direction: mxaFGIJRSAACKLDEHB with an additional upstream gene, mxaW, which is divergently transcribed (Anderson et al., 1990; Morris et al., 1995; Springer et al., 1995, 1998). mxaF and mxaI encode the α and β subunits of MDH, respectively. mxaG encodes the cytochrome c₁ structural polypeptide (Anderson & Lidstrom, 1988; Nunn & Lidstrom, 1986a, b; Nunn et al., 1989). mxaF, mxaR, mxaS, mxaD, mxaE and mxaH encode genes of unknown function, thought to be involved in MDH stability and/or assembly (Lidstrom, 1991). mxaACK and I are involved in inserting the calcium into the enzyme (Morris et al., 1995; Richardson & Anthony, 1992). mxaB is a transcriptional regulator of methanol oxidation genes (Springer et al., 1998). The function of mxaW is unknown. Although mutants in mxaW show no phenotype, a methanol-inducible promoter is present upstream of the gene (Springer et al., 1998). In addition, six genes, pqqABC/DE and pqqFG, are required for PQQ biosynthesis in M. extorquens AM1 (Morris et al., 1994; Springer et al., 1996; Toyama et al., 1997). In most PQQ-synthesizing bacteria, pqqC and pqqD are separate genes, but in M. extorquens AM1 we have shown that these genes are fused into a single polypeptide, which we have designated pqqCD (Toyama et al., 1997). Four more genes are involved in transcriptional regulation of the methanol oxidation system, mxbDM and mxcQE (Springer et al., 1995, 1997).

Although the genes involved in methanol oxidation are
identified and sequenced, little is known about their transcriptional organization. Using reporter gene fusions, methanol-inducible promoters have been detected upstream of \textit{mxaF}, \textit{mxaW}, \textit{mbxD} and \textit{pqqA} (Ramamoorthy & Lidstrom, 1995; Springer et al., 1997) and transcriptional start sites have been mapped for \textit{mxaF} and \textit{pqqA} (Anderson et al., 1990; Ramamoorthy & Lidstrom, 1995). In addition, two transcripts were detected by Northern blots in the \textit{pqqAB} region, a major one encoding \textit{pqqA} and a minor one encoding \textit{pqqAB} (Ramamoorthy & Lidstrom, 1995). In this study, we have focused on the promoters and transcriptional organization for the major gene clusters encoding structural genes involved in synthesis of active MDH and in PQQ synthesis.

**METHODS**

**Chemicals and enzymes.** All chemicals used were analytical grade and obtained from Baker Chemicals or Fisher Scientific. X-Gal was from ISC Bioexpress. Enzymes for molecular biology were purchased from Roche Molecular Biochemicals and New England Biolabs, and used according to the suppliers’ instructions. Taq DNA polymerase was obtained from Gibco-BRL.

**Media and growth conditions.** \textit{Methylobacterium} strains were grown at 30 °C on minimum medium described previously (Fulton et al., 1984), containing 0·5 % (v/v) methanol or 0·4 % (v/v) succinate. \textit{Escherichia coli} strains were grown on Luria–Bertani (LB) broth or solid media (Sambrook et al., 1989) by adding 1·5 % agar (Difco). Appropriate antibiotics, all of which were obtained from Sigma, were added to the following final concentration (mg l\(^{-1}\)): tetracycline, 12·5 (10 for \textit{M. extorquens} AM1); kanamycin, 50; ampicillin, 50; streptomycin, 25.

**Bacterial matings.** Triparental matings were performed as described previously (Chistoserdov et al., 1994).

**Construction of plasmids for promoter studies.** The PCR products that included the upstream region of the gene to be studied were first cloned into the pCR2.1 TOPO vector. These fragments were then cut and inserted into the appropriate promoter probe vector using the multiple cloning sites in front of the reporter gene.

**RT-PCR.** The RT-PCR kit was obtained from Gibco-BRL or Roche Molecular Biochemicals and the experiment was performed according to the suppliers’ instructions. The primers used for RT-PCR were designed across the intergenic region of the two genes to be studied, to generate a PCR product of approximately 100–500 bp.

**RNA isolation.** Total bacterial RNA was isolated from \textit{M. extorquens} AM1 cells grown to mid-exponential phase on succinate or methanol, using the Epicentre Technologies RNA purification kit. The concentration and quality of total RNA was analysed using an Agilent Bioanalyzer 2100 and an Agilent separations chip by the Center for Expression Arrays (University of Washington, Seattle, WA).

**β-Galactosidase assays.** Quantitative analyses of lacZ expression were performed in cell extracts according to Miller (1972). Cell extracts of \textit{M. extorquens} AM1 were obtained by passing concentrated cell suspension through a French pressure cell at 37 kPa (Amino) as described by Chistoserdova & Lidstrom (1991).

**Catechol-2,3-dioxygenase activity (XylE assays).** Catechol-2,3-dioxygenase was assayed in cell extracts as described by Zukowski et al. (1983).

**Transcriptional start site mapping.** The \textit{mxaW} transcriptional start site was mapped by means of primer extension using the ThermoScript (Gibco-BRL) cDNA synthesis protocol or 1st Strand AMV synthesis kit (Roche) using 8–10 μg total RNA. Primers were labelled with \([\gamma\text{-}32\text{P}]}\text{ATP}\) (6000 Ci mmol\(^{-1}\) (222 TBq mmol\(^{-1}\)); NEN), using T4 polynucleotide kinase (Roche). In each case, the primers for the reverse transcription reaction were 18–25 mers and were located at different sites with relation to the start codon.

**RESULTS**

**RT-PCR of \textit{mxa} and \textit{pqq} gene clusters**

The clustering of genes involved in methanol oxidation in the \textit{mxa} gene cluster (Fig. 1) and of genes involved in PQQ synthesis in the two \textit{pqq} gene clusters (Fig. 2) suggested the possibility that the genes in each case might be co-transcribed. However, attempts to obtain clear and reproducible bands with Northern blots have not been successful (data not shown). Therefore, we used RT-PCR across each pair of genes in each cluster to assess the possibility of a contiguous transcript. The first set of genes tested was in the \textit{mxa} cluster (Fig. 1). In this case, RT-PCR products of the correct size and sequence were obtained across each pair of genes in the entire 14-gene set (\textit{mxaFJGIRSACKLDEHB}), which covers 12·6 kb. No products were obtained between \textit{mxaF} and the upstream region, or between \textit{mxaB} and the downstream region. In each case, controls for DNA contamination of the RNA preparations using direct PCR without the RT step were negative (data not shown). These data suggest that this 14-gene cluster may be transcribed as a single operon.

Similar experiments were not carried out for \textit{mxaW}, because it is flanked by genes transcribed in the opposite orientation and must be a single-gene transcript. However, the two \textit{pqq} gene clusters involved in PQQ synthesis have been analysed. For \textit{pqqABCDE}, it had previously been shown that \textit{pqqA} and \textit{pqqB} were co-transcribed (Ramamoorthy & Lidstrom, 1995). Positive bands of the correct size were obtained for the other two intergenic regions of this cluster, but not for the upstream and downstream regions, suggesting that these genes constitute an operon transcribed by the single promoter upstream of \textit{pqqA} (Fig. 2). Little is known about the other PQQ cluster containing \textit{pqqFG}. The genome sequence showed a group of six genes in this region, all transcribed in the same direction, including \textit{pqqFG} (Fig. 2). The first of these is predicted to encode an isoleucyl tRNA synthetase and the sixth a dioxygenase, while the other two (\textit{orf181} and \textit{orf219}) are ORFs of unknown function. Positive products for the \textit{orf181–orf219–pqqF–pqqG}–dioxygenase intergenic regions were also obtained but not for the region between the gene predicted to encode isoleucyl tRNA synthetase and \textit{orf181}, suggesting that the last five genes might constitute an operon (Fig. 2).
Promoter analysis for *mxa* and *pqq* intergenic regions

The RT-PCR results suggested that the three gene clusters analysed might each be transcribed as single transcripts. However, a positive RT-PCR product could be obtained between two transcripts if the transcripts overlap. Therefore, we screened the larger intergenic regions for promoter activity, using *xylE* as a reporter, first with the promoter probe vector pCM76 and later with a low background vector (pCM130). The regions screened were those upstream of *mxaJ*, *mxaG*, *mxaI*, *mxaR*, *mxaS*, *mxaE*, *mxaH*, *mxaB*, *pqqF*, *pqqG* and *orf219*. However, no significant activity above background was found for any of these constructs, suggesting that no promoter was present in these intergenic regions (data not shown).

Analysis of *mxaF* and *mxaW* promoter regions

It had previously been shown that a 0.4 kb region between *mxaF* and *mxaW* has full activity compared to a 1.5 kb region that had been analysed previously (Marx & Lidstrom, 2001) and the transcriptional start site had been previously mapped to a position 168 bp upstream of the translational start site (Anderson et al., 1990). To more precisely define the promoter region, a number of smaller fragments were tested for promoter activity, using the *xylE* reporter in both pCM76 and pCM130, and the results are shown in Fig. 3. A fragment covering 100 bp upstream of the transcriptional start site showed full activity, a fragment covering 89 bp upstream showed intermediate activity, and a fragment covering 61 bp upstream showed activity at the vector background level. Therefore, the full promoter activity appeared to require a region approximately 90–100 bp upstream of the transcriptional start site and no activity could be detected when the −10, −35 region alone was present.

The 0.4 kb region between *mxaF* and *mxaW* has methanol-inducible *xylE* activity when cloned in the orientation opposite to *mxaF* (Marx & Lidstrom, 2001; Springer et al., 1998), suggesting the promoter of *mxaW* is also within this 0.4 kb region but divergently transcribed compared to the...
14-gene mxa cluster (Fig. 3). Since the transcriptional start site of mxaW was not known, we mapped it and found it to be 52 bp upstream of the mxaW translational start site (Fig. 4). The region encompassing the mxaW promoter was further defined by subcloning into a vector that targets low activity promoters (pCM132 using lacZ as a reporter), and

![Image of RT-PCR for the intergenic regions of pqq genes. Asterisks indicate positive controls using chromosomal DNA as template; other lanes used cDNA as template; multiple lanes show replicates with the amounts of cDNA on top of each lane. The arrows under the pqq gene clusters show the putative transcripts. All RT-PCR reactions were repeated with the same results.](image)

**Fig. 2.** RT-PCR for the intergenic regions of pqq genes. Asterisks indicate positive controls using chromosomal DNA as template; other lanes used cDNA as template; multiple lanes show replicates with the amounts of cDNA on top of each lane. The arrows under the pqq gene clusters show the putative transcripts. All RT-PCR reactions were repeated with the same results.

14-gene mxa cluster (Fig. 3). Since the transcriptional start site of mxaW was not known, we mapped it and found it to be 52 bp upstream of the mxaW translational start site (Fig. 4). The region encompassing the mxaW promoter was further defined by subcloning into a vector that targets low activity promoters (pCM132 using lacZ as a reporter), and

![Image of the M. extorquens AM1 mxa promoter region showing the DNA fragments tested for promoter activity, the catechol dioxygenase and β-galactosidase activities from those fragments, and the transcriptional start sites. The transcriptional start site for mxaF was published previously (Anderson et al., 1990). The data on each line show the bp upstream of the transcription start site of mxaF or mxaW.](image)

**Fig. 3.** The *M. extorquens* AM1 mxa promoter region showing the DNA fragments tested for promoter activity, the catechol dioxygenase and β-galactosidase activities from those fragments, and the transcriptional start sites. The transcriptional start site for mxaF was published previously (Anderson et al., 1990). The data on each line show the bp upstream of the transcription start site of mxaF or mxaW.
found to be between 92 and 146 bp upstream of the transcriptional start site (Fig. 3). Since the distance between the two transcriptional start sites is 148 bp, the −10, −35 regions of the two promoters do not overlap. However, the regions shown to be essential for both promoters do overlap (Fig. 3).

Analysis of pqq cluster promoter regions

The RT-PCR and promoter cloning data suggested that the two pqq gene clusters were each transcribed from a single promoter upstream of pqqA (for the pqqABC/DE cluster) and orf181 (for the orf181–orf219–pqqFG–dioxygenase cluster). The transcriptional start site upstream of pqqA had previously been determined to be 94 bp upstream of the translational start site (Ramamoorthi & Lidstrom, 1995) (Fig. 5). We also mapped the transcriptional start site for orf181 and found it to be 101 bp upstream of the translational start site of orf181 (Figs 4 and 5). Subclones were used to narrow down the promoter region for the pqqA and orf181 clusters, which were found to lie within 46 bp and 225 bp upstream of the transcriptional start site, respectively (Fig. 5).

Fig. 4. Transcriptional start site mapping for mxaW and orf181. G, A, T and C are sequencing lanes. PE, primer-extension reaction. The nucleotides labelled with * are the transcriptional start sites.

Fig. 5. The M. extorquens AM1 pqq regions, showing the DNA fragments tested for promoter activity, the catechol dioxygenase activities from those fragments, and the transcriptional start sites for both clusters. The transcriptional start site for pqqA was published previously (Ramamoorthi & Lidstrom, 1995). The data on each line show the number of bp upstream of the transcriptional start site for pqqA or the translation start site for orf181.
DISCUSSION

Twenty-five genes are known to be involved in methanol oxidation to formaldehyde in *M. extorquens* AM1, found in five gene clusters. In this study, we have analysed transcripts and promoter regions for the three gene clusters encoding structural genes involved in the synthesis of active MDH and in PQ synthesis, involving 21 of the known methanol oxidation genes. The *mxa* cluster contains 15 genes, most of which are required for growth on methanol and for active methanol oxidation. In this study, we have shown that the 15 genes are transcribed by two divergent methanol-inducible promoters, one for *mxaW* and the other for *mxaFJGIRSACKLDEHB*. The genes immediately downstream of *mxaW* and *mxaB* are transcribed in the opposite orientation and are not involved in methanol oxidation (Springer et al., 1998). Our results from this study suggest that the *mxa* region is expressed as two transcripts, one for *mxaW* and one for the other 14 genes. The two regions important for transcription apparently overlap.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong> JM109</td>
<td>F’traD36 lacZ Δ(lacZ)M15 proA+ B+ /el14’ (McrA-) Δ (lac-proAB) thi gyrA96 (Nalr) endA1 hsdR17 (r+) mcr (lac-)proAB D80 lacZ M15 lacX74 recA1</td>
<td>Promega</td>
</tr>
<tr>
<td><strong>TOP 10</strong></td>
<td>F’ mcrA Δ(mrr–hsdRMS–mcrBC) Δ80 lacZ Δ155 ΔlacX74 recA1 deoR araD139 Δ(ara–leu)7697 galU galK rpsL (Strr) endA11 nupG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>M. extorquens AM1</strong></td>
<td>Wild-type strain</td>
<td>Peel &amp; Quayle (1961)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRK2073</td>
<td>Sm’ mobilizing helper</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pCR2.1-TOPO</td>
<td>Cloning vector for PCR products; Ap’ Km’</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pCM66lacZ</td>
<td>IncP cloning vector, Km’, lacZ as reporter gene</td>
<td>Marx &amp; Lidstrom (2001)</td>
</tr>
<tr>
<td>pCM130</td>
<td>pCM76 containing transcriptional terminator upstream of multiple cloning sites</td>
<td>Marx &amp; Lidstrom (2001)</td>
</tr>
<tr>
<td>pCM132</td>
<td>pCM66lacZ containing transcriptional terminator upstream of multiple cloning sites</td>
<td>Marx &amp; Lidstrom (2001)</td>
</tr>
<tr>
<td>pMZ2</td>
<td>453 bp mxaF fragment in pCM76, Tc’</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ3</td>
<td>414 bp mxaF fragment in pCM76, Tc’</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ10</td>
<td>0.8 kb pqqA fragment in pCM76, Tc’</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ11</td>
<td>0.6 kb pqqA fragment in pCM76, Tc’</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ12</td>
<td>484 bp pqqA fragment in pCM76, Tc’</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ13</td>
<td>454 bp pqqA fragment in pCM76, Tc’</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ14</td>
<td>0.4 kb pqqA fragment in pCM76, Tc’</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ22</td>
<td>442 bp mxaF fragment in pCM76, Tc’</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ26</td>
<td>365 bp mxaW fragment in pCM76, Tc’</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ27</td>
<td>311 bp mxaW fragment in pCM76, Tc’</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ23</td>
<td>Like pMZ2, but in pCM130, Tc’</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ24</td>
<td>Like pMZ3, but in pCM130, Tc’</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ106</td>
<td>1.3 kb fragment covering orf181 and 3’ region of upstream gene in pCM130</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ107</td>
<td>1.1 kb fragment covering orf181 and 3’ region of upstream gene in pCM130</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ108</td>
<td>0.8 kb fragment covering orf181 and 3’ region of upstream gene in pCM130</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ109</td>
<td>0.6 kb fragment covering orf181 and 3’ region of upstream gene in pCM130</td>
<td>This study</td>
</tr>
<tr>
<td>pMZ110</td>
<td>0.4 kb fragment covering orf181 and 3’ region of upstream gene in pCM130</td>
<td>This study</td>
</tr>
</tbody>
</table>
Likewise, we have shown that the two gene clusters involved in PQQ synthesis are also each transcribed as a single transcript and each contains a single upstream promoter. *pqqFG* appears to be co-transcribed with three other genes of unknown function; *orf181, orf219* and a gene predicted to encode a dioxygenase. Genes with identity to *orf181, orf219* and the putative dioxygenase are found in the genomes of four bacteria known to synthesize PQQ and containing the other known PQQ genes, *Sinorhizobium meliloti, Mesorhizobium loti, Pseudomonas aeruginosa* and *Rhodopseudomonas palustris* with identities, respectively, of 23 %, 23 %, 24 %, 28 % to *orf181; 23 %, 21 %, 40 %, 30 % to *orf219; and 60 %, 63 %, 29 %, 37 % to the gene encoding the dioxygenase. Although the role of these genes in PQQ synthesis is unknown, their co-transcription with *pqqFG* in *M. extorquens* AM1 suggests they may be involved.

An alignment of these four methanol-inducible promoters (Fig. 6) does not show an obvious consensus sequence in the −10, −35 regions or upstream within the defined promoter regions. The −35 regions show similarity to the *E. coli* σ^70−35 consensus (Fig. 6), but the −10 regions are more divergent. The promoter region of *mxaF* in a closely related strain, *Methylobacterium organophilum* XX, was investigated previously (Xu et al., 1993). A region of dyad symmetry was found between 30 and 50 bp upstream of the transcriptional start site in this strain. However, this structure is not present in *M. extorquens* AM1, even though the overall sequences in this region between *mxaF* and *mxaW* are very similar in both strains. The only obvious conserved region upstream of the −35 region within the defined *M. extorquens* AM1 promoter regions is a hexanucleotide, AAGAAA. A similar hexanucleotide has been previously suggested as a potential regulatory site in *M. organophilum* XX, based on its presence upstream of *mxaF* in that organism (Xu et al., 1993). Site-directed mutagenesis will be required to address the sequences important both in the −10, −35 regions and upstream regions.

Five regulatory genes are known that are all required for detectable expression of the *mxaF* promoter, *mxbDM, mxxQE* and *mxaB* (Springer et al., 1995, 1997). *mxbD* and *mxxQ* are predicted to encode sensor kinases, while *mxaB, mxbM* and *mxxE* are all predicted to encode response regulators. Of these, only *mxbDM* are required for normal expression of the *mxaW* and *pqqA* promoters, while *mxxQE* are required for elevated expression of *mxbDM* (Ramamoorthi & Lidstrom, 1995; Springer et al., 1997). The precise regions required for this regulatory cascade are not yet known, but must reside within the promoter regions identified in this study.

**ACKNOWLEDGEMENTS**

This work was supported by a grant from the DOE (DEFG03-96ER20226).

**REFERENCES**


