Identification of an upstream regulatory sequence that mediates the transcription of mox genes in *Methylobacterium extorquens* AM1

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A multiple A-tract sequence has been identified in the promoter regions for the *mxaF*, *pqqA*, *mxbD* and *mxcQ* genes involved in methanol oxidation in *Methylobacterium extorquens* AM1, a facultative methylotroph. Site-directed mutagenesis was exploited to delete or change this conserved sequence. Promoter-xyrE transcriptional fusions were used to assess promoter activity in these mutants. A fiftyfold drop in the XylE activity was observed for the *mxaF* and *pqqA* promoters without this sequence, and a five- to sixfold drop in the XylE activity was observed for the *mxbD* and *mxcQ* promoters without this sequence. Mutants were generated in the chromosomal copies in which this sequence was either deleted or altered, and these mutants were unable to grow on methanol. When one of these sequences was added to Plac of *Escherichia coli*, which is a weak constitutive promoter in *M. extorquens* AM1, the activity increased two- to threefold. These results suggest that this sequence is essential for normal expression of these genes in *M. extorquens* AM1, and may serve as a general enhancer element for genetic constructs in this bacterium.

At least 25 genes have been identified to be involved in the methanol oxidation reaction in *M. extorquens* AM1 (Lidstrom, 1991; Zhang & Lidstrom, 2003). These Mox genes are distributed between three different loci: *mxa*, *mxb* and *mxc*. Fourteen genes (*mxaFGJKLMNRSACKLDEHB*) transcribed in the same direction, together with an additional gene (*mxaW*) transcribed in the opposite direction, are located on the *mxa* gene cluster (Anderson et al., 1990; Morris et al., 1995; Springer et al., 1998, 1995). The large and small subunits of methanol dehydrogenase are encoded by *mxaF* and *mxaL*, respectively (Anderson & Lidstrom, 1988; Nunn & Lidstrom, 1986a, b; Nunn et al., 1989). The gene *mxaG* encodes cytochrome *c* (Anderson & Lidstrom, 1988; Nunn et al., 1989). The gene *mxaD* encodes the 17 kDa periplasmic protein that directly or indirectly stimulates the interaction between methanol dehydrogenase and cytochrome *c* (Toyama et al., 2003), and the other genes in this region are involved in either assembly or regulatory functions (Anthony, 2000; Lidstrom & Tsygankov, 1991). Two other regulatory genes (*mxbDM*) (Springer et al., 1997) and genes required for synthesis of the PQQ prosthetic group, *pqqABC/DE* (Morris et al., 1994; Toyama et al., 1997), are found at the *mxb* locus. The other two known *pqq* genes (*pqqFG*) reside within another cluster in the genome (Springer et al., 1996). Two additional regulatory genes, *mxcQE*, are located in the *mxc* gene cluster. Of the five Mox regulatory genes identified (*mxB*, *mxbDM*, and *mxcQE*), four are predicted to encode two sets of sensor-kinase/
response regulator pairs (MxbDM and MxcQE) and the fifth is predicted to encode an additional response regulator (MxA) (Nunn & Lidstrom, 1986a, b; Springer et al., 1997, 1998, 1995).

Each of the Mox gene clusters has been shown to constitute a single transcriptional unit, and promoter regions have been identified for all of the Mox gene clusters except the mxcQE pair (Zhang & Lidstrom, 2003). Each of these Mox promoters has been shown to be expressed at higher levels during growth on methanol than on succinate (Zhang & Lidstrom, 2003). Transcriptional start sites have been determined for all of the promoters except the mxbDM and mxcQE pair (Anderson et al., 1990; Ramamoorthi & Lidstrom, 1995; Zhang & Lidstrom, 2003). Although no consensus promoter sequence could be identified from this small set, these promoters all shared similarity with the Escherichia coli $d^{70}$ promoter consensus sequence (Zhang & Lidstrom, 2003).

The regulation of expression of these Mox promoters by carbon source as well as the presence of putative response regulators required for transcription of the Mox promoters suggested that a regulatory region might be present upstream of the promoters involved either in the increased expression during growth on methanol, or in the basal expression that disappears in the Mox regulatory mutants. A putative regulatory sequence was identified, a multiple A-tract sequence in the promoter region of most genes involved in methanol oxidation (Zhang & Lidstrom, 2003). Little is known concerning the role of this consensus sequence in M. extorquens AM1. In other organisms, multiple A- or T-tracts function as upstream recognition elements, increasing promoter activity (Cheema et al., 1999) or facilitating DNA curvature for the binding of regulatory proteins (Aiyar et al., 1998). In this study we have investigated the function of this sequence upstream of Mox promoters by site-directed mutagenesis.

**METHODS**

**Bacterial strains and plasmids.** These are listed in Table 1.

**Chemicals and enzymes.** All chemicals used were analytical grade and obtained from Baker Chemical or Fisher Scientific. Enzymes for molecular biology were purchased from New England Biolabs and used according to the suppliers’ instructions. Taq DNA polymerases were obtained from Invitrogen.

**Media and growth conditions.** E. coli strains were grown on Luria–Bertani (LB) (Sambrook et al., 1989) broth or solid medium made by addition of 1-5% agar (Difco). M. extorquens AM1 was grown aerobically at 30°C either in liquid or on agar plates using either 0.5% (v/v) methanol or 0.4% (w/v) succinate. Where necessary, media were supplemented with appropriate antibiotics, all of which were obtained from Sigma. Ampicillin (Ap) and kanamycin (Km) were used at 50 μg ml$^{-1}$ for both E. coli and M. extorquens AM1. Rifamycin (Rif) (50 μg ml$^{-1}$) was routinely added to plates for growth of M. extorquens AM1. Tetracycline (Tc) was added at final concentration of 12.5 μg ml$^{-1}$ for E. coli and 10 μg ml$^{-1}$ for M. extorquens AM1. Transformations of E. coli were performed using commercially available cells (JM109 and TOP10, from Promega and Invitrogen, respectively).

**Generation of directed mutations.** Data from the M. extorquens AM1 genome project (http://www.integratedgenomics.com/ genomereleases.html#list6) were used to design PCR primers to amplify the putative promoter of mxcQ. Other promoters containing the multiple A-tract region were amplified by PCR using chromosomal DNA of wild-type M. extorquens AM1 as a template and were cloned into pCR2.1 TOPO. Site-directed mutagenesis was performed.

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**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasmids</strong></td>
<td>tac promoter cloned into PCR2.1TOPO</td>
<td>C. J. Marx, unpublished</td>
</tr>
<tr>
<td>pCM42</td>
<td>Helper plasmid, Sm$^r$</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pRK2073</td>
<td>Promoter probe vector, Tc$^r$</td>
<td>Marx &amp; Lidstrom (2001)</td>
</tr>
<tr>
<td>pCM184</td>
<td>tac promoter fragment in pCM130</td>
<td>This study</td>
</tr>
<tr>
<td>pKF01</td>
<td>61 bp promoter fragment with AAGAAA in pCM130</td>
<td>This study</td>
</tr>
<tr>
<td>pKF03</td>
<td>pTSGex containing AAGAAA upstream of P tac</td>
<td>This study</td>
</tr>
<tr>
<td>pKF113</td>
<td></td>
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</tbody>
</table>

| **Mutant strains** | | |
| MZ116K.1 | AAGAAA sequence of mxaF promoter deleted, Km$^r$ | This study |
| MZ141K.1 | AAGAAA sequence of mxaF promoter changed to GGGGGG, Km$^r$ | This study |
| MZ135K.1 | GAAAA of mxbD promoter deleted, Km$^r$ | This study |
| MZ139K.1 | AAGAAAA of mxcQ promoter deleted, Km$^r$ | This study |
| MZ157K.1 | AAGAAA of pqqA promoter deleted, Km$^r$ | This study |
using the Quick Change site-directed mutagenesis kit (Stratagene) according to the supplier’s instructions. The mutations were verified by sequencing (Department of Biochemistry, University of Washington) and were subcloned into pCM130 as either EcoRI–EcoRI or BamHI–HindIII fragments in the correct orientation such that xyle was expressed from the cloned promoter. The pCM130 constructions were transferred into wild-type M. extorquens AM1 by triparental matings (Chistoserdov et al., 1994a) with the helper plasmid pRK2073. For chromosomal insertion constructions, the respective fragments downstream of genes of interest were cloned into pCM184 using SacI–SacII or ApaI–SacII sites and the fragments upstream of the genes of interest were cloned at the NadI–BglII or NadI–KpnI sites. The resulting plasmid DNA was electroporated into E. coli S17-1. These strains were used as donors in biparental matings with wild-type M. extorquens AM1, and KmR-Th progeny were identified on minimal medium agar plates containing succinate. In all cases, the identity of the double-crossover mutants was confirmed by diagnostic PCR using chromosomal DNA as template.

**Construction of altered Ptac-xyle vector.** pKF03 was constructed by annealing the overlapping oligos KpaptaAf (5’-CTAGTAA-GAAATCTGAAATGAGCTGTTGACAATTAATCATCGGCTCGTATA-3’) and KpaptaAr (5’-CTAGTATTAATCATCGGCTCGTATA-ATGTGTGGAT-3’). The resulting plasmid DNA was electroporated into E. coli S17-1. These strains were used as donors in biparental matings with wild-type M. extorquens AM1, and KmR-Th progeny were identified on minimal medium agar plates containing succinate as described previously (Chistoserdov et al., 1994b). In all cases, the identity of the double-crossover mutants was confirmed by diagnostic PCR using chromosomal DNA as template.

**DNA manipulations.** Plasmid DNA isolation, restriction enzyme digestion, ligation, and E. coli transformation were carried out by standard protocols (Sambrook et al., 1989). The chromosomal DNA of M. extorquens AM1 was isolated as described by Saito & Miura (1963).

**Catechol 2,3-dioxygenase assays.** The activity of Xyle was determined in M. extorquens AM1 in crude extracts as described previously (Zhang & Lidstrom, 2003; Zukowski et al., 1983).

**RESULTS**

**Sequence comparison of regions upstream of mxa promoters**

A region upstream of the −35 sequence of mxaF, pqqA and mxaW (Anderson et al., 1990; Ramamoorthi & Lidstrom, 1995; Zhang & Lidstrom, 2003) was analysed and found to contain a multiple A sequence. A similar sequence was found upstream of the putative promoter of mxbD (Springer et al., 1997) (Fig. 1). For both mxaF and pqqA, a sequence of AGAAAG was present 14 bp upstream of the −35 sequence. For mxaW, a sequence of AAAAG was present 67 bp upstream of the transcription start site, while for mxbD, AAAAG was present 83 bp upstream from the translation start site. The mxcQ gene (http://www.integratedgenomics.com/genomereleases.html) also showed a similar sequence (AAGAAAAA) 227 bp upstream of the translation start site.

**Promoter activity in mutants in which the multiple A-track sequence was deleted or altered**

In order to investigate the function of the multiple A-track sequence, site-directed mutagenesis was performed to either delete this sequence in each promoter or alter all As in the sequence to Gs. Each altered promoter region was generated as a transcriptional fusion to a xyle reporter in a plasmid vector. Xyle activity was measured in wild-type M. extorquens AM1 (Table 2). For the mxaF promoter without AAAAG, the Xyle activity decreased to 7–4 mU and 4–4 mU in the cells grown on methanol and succinate, respectively, only slightly above the vector background. The Xyle activity of the pqqA, mxbD and mxcQ promoters without the conserved sequence decreased to the background level in the cells grown on methanol and succinate. However, the Xyle activity of the mxaW promoter increased in the deletion strain, twofold on succinate and threefold on methanol. We also tested the Xyle activity for altered versions of all five promoters, in which all of the As in each conserved sequence were converted to Gs. As shown in Table 2, for the mxaF, mxaW, mxbD and mxcQ promoters, the Xyle activity of the deletion and A→G constructs was similar on both methanol and succinate. However, the Xyle activity of the altered pqqA promoter was similar to that of the wild-type promoter on succinate, in cells grown on either succinate or methanol.

**Phenotype of the double-crossover mutants**

To analyse phenotypic characteristics, mutants were constructed in the chromosomal genes, in which the conserved sequence was deleted. MZ116K.1, a strain containing the

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**Fig. 1.** The sequence upstream of Mox genes, with the multiple A-track designated in bold. Transcription start sites (where known) are labelled with an asterisk. ATG is the translation start site.
Multiple A-tract sequences act as enhancer elements. In support of a more general role, addition of this sequence to all Gs resulted in activities similar to those of the wild-type grown on succinate, in cells grown on either succinate or methanol. These results suggest that the loss of activity in these pqqA promoter deletion constructs had more to do with the spacing upstream of the promoter than the actual sequence. Previous work has defined the regions responsible for full promoter activity for each of these promoters to within 20–200 bp upstream of the −35 sequence, depending on the promoter (Zhang & Lidstrom, 2003), but no other consensus sequence or hairpin structure is conserved in that region of these promoters.

Multiple A-tract sequences act as enhancer elements for promoters in many bacterial systems, including the very highly expressed rrr promoters as well as others (Aiyar et al., 1998; Cheema et al., 1999). In some cases these have been shown to bind the z subunit of RNA polymerase (Aiyar et al., 1998), and act as positive transcriptional elements via their DNA-curvature characteristics. Therefore, it is possible that the sequence we have identified is analogous to one of these elements. In support of a more general role, addition of this sequence to an E. coli promoter that is poorly transcribed in M. extorquens AM1 (Plac) did increase promoter activity two- to threefold in M. extorquens AM1. In other bacteria, these elements are present in tracts of multiple sets of A-rich or T-rich sequences and in our case these promoters all contain only one upstream A-rich sequence. In one case in which a single A-tract was studied, for the malT promoter of E. coli, deletion of the sequence had little effect on transcription but moving the spacing enhanced transcription (Tagami & Aiba, 1999). The fact that the addition of this sequence alone did not generate promoter activity at the same high level as the mxaf promoter suggests that other elements in the mxaf promoter region contribute to the high activity. Since no other conserved elements exist upstream of mxaf promoters in other bacteria (Zhang & Lidstrom, 2003), it is likely that the high activity is due to

**DISCUSSION**

A multiple A-tract sequence upstream of five promoters for methanol oxidation genes has been shown to be essential for normal expression of those promoters regardless of the presence or absence of inducing conditions. In four of the five promoters (mxaf, pqqA, mxbd and mxcq) the sequence exerted a positive effect, in that expression dropped to near the vector background in constructs in which the A-tract sequence had been deleted. In the fifth case, the mxaw promoter, the sequence exerted a negative effect, as expression increased in the deletion construct. The function of mxaw is not known, since mutants defective in mxaw show no detectable phenotype, including no change in **mxaf** promoter activity (Springer et al., 1998). However, the promoter is weakly methanol-inducible, so the role of the A-tract sequence in mxaw expression does not appear to involve methanol induction.

In all cases except the pqqA promoter, the effect was the same whether the sequence was deleted or altered to all Gs. However, in the case of the pqqA promoter, altering the sequence to all Gs resulted in activities similar to those of the wild-type grown on succinate, in cells grown on either succinate or methanol. These results suggest that the loss of activity in these pqqA promoter deletion constructs had more to do with the spacing upstream of the promoter than the actual sequence. Previous work has defined the regions responsible for full promoter activity for each of these promoters to within 20–200 bp upstream of the −35 sequence, depending on the promoter (Zhang & Lidstrom, 2003), but no other consensus sequence or hairpin structure is conserved in that region of these promoters.

**Table 2. XyIE activity of wild-type and mutant promoters [nmol (mg protein)−1min−1]**

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Wild-type</th>
<th>Deletion</th>
<th>Altered (As→Gs)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Methanol</td>
<td>Succinate</td>
<td>Methanol</td>
</tr>
<tr>
<td>Pmxaf</td>
<td>34.8 ± 1.0</td>
<td>72 ± 5.6</td>
<td>7.4 ± 1.1</td>
</tr>
<tr>
<td>PqqA</td>
<td>16.4 ± 0.8</td>
<td>59 ± 5.8</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>Pmxaw</td>
<td>25 ± 4.7</td>
<td>14 ± 0.6</td>
<td>73.1 ± 6.4</td>
</tr>
<tr>
<td>Pmxbd</td>
<td>19 ± 2.9</td>
<td>10 ± 0.6</td>
<td>3.2 ± 1.8</td>
</tr>
<tr>
<td>PmxcQ</td>
<td>9.4 ± 1.9</td>
<td>6.1 ± 0.8</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Vector</td>
<td>1 ± 5.0</td>
<td>2 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

mxaf promoter without AAGAAA, and MZ141K.1, the strain containing the mxaf promoter with the altered sequence (GGGGGG), did not grow on methanol. However, other mutants such as MZ135K.1 (sequence deleted for mxbd promoter), MZ139K1 (sequence deleted for mxcq promoter) and MZ157K1 (sequence deleted for pqqA promoter), for which the plasmid constructs showed either background or higher XyIE activity when the conserved sequence had been deleted, grew normally on methanol.

**Use of the A-tract sequence as an enhancer element**

The importance of the A-tract sequence in transcription of mx promoters suggested that it might serve as an enhancer element for other promoters in M. extorquens AM1. In order to test this hypothesis, the mxaf A-tract sequence, AAGAAA, was inserted 17 bp upstream of the E. coli lac promoter in the promoter-probe vector pCM130. The lac promoter had been previously shown to be a weak promoter in M. extorquens AM1 (Marx & Lidstrom, 2001). The cloned lac promoter had an activity of 56–60 mU, while the construct containing the AAGAAA showed an increase to 128–180 mU. For comparison, the mxaf promoter showed an activity of 350 mU under these conditions. These results suggest that this sequence can be used to significantly increase promoter activity of non-C1 promoters in this bacterium, but supports the hypothesis that the high activity of the mxaf promoter is only partly due to the A-tract sequence.
spacing of the A-rich sequence and/or sequences within the −10/−35 region of the promoter.

Alternatively, it is possible that this sequence represents a binding site for a transcriptional regulator. However, so far all attempts to demonstrate binding of the three predicted DNA-binding proteins in the Mox system (MxA, MxBM and MxCE) to these promoters have been unsuccessful (M. Franke, A. Stover & M. E. Lidstrom, unpublished). Therefore, it is possible that this sequence is either not a site for protein binding, or is a site for an as-yet-unidentified regulator.

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REFERENCES


