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PROGRESS REPORT, JULY 1, 1987 - JUNE 30, 1989

SUMMARY

In this project we are developing genetic techniques for methylotrophic bacteria and using these to study genes involved in methanol oxidation. We have defined the genetic organization of these Mox genes and have started to define their regulatory regions. It is clear that some of these genes encode transcriptional regulators of unknown function.

PROGRESS

In the past two years we have studied methanol oxidation in the facultative autotroph, *Xanthobacter* H4-14, the facultative serine cycle methylotroph, *Methylobacterium* sp. AM1, and two methanotrophic bacteria. Our studies suggest that in all cases the methanol oxidation system requires a large number of genes that are loosely clustered. In the case of the *Xanthobacter*, mutants were isolated that produced methanol dehydrogenase constitutively. These mutants were extremely sensitive to methanol because they produced toxic levels of formaldehyde. Apparently the formaldehyde consumption systems were repressed during growth on succinate, and when methanol was added to a methanol dehydrogenase constitutive mutant, it produced formaldehyde and killed the cells.

We have cloned the *moxF* gene, encoding the large subunit of methanol dehydrogenase, from three different methylotrophs, and hybridization studies suggest it is highly conserved. We have shown that in the *Methylobacterium* species, the region encoding MoxF also encodes three other polypeptides, which we have designated MoxJ, MoxG, and MoxI. Our evidence suggests that MoxG is the cytochrome *c* that is the electron acceptor for methanol dehydrogenase, and surprisingly, MoxI was shown to be a subunit of methanol dehydrogenase.

We have also spent some time on development of genetic techniques, including the development of a relatively rapid method for isolating plasmid DNA from the *Methylobacterium* species.

This work has resulted in 4 publications.

PUBLICATIONS

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2. Stephens, R. L., M. G. Haygood and M. E. Lidstrom. 1988. Identification of putative methanol dehydrogenase (moxF) structural genes in methylotrophs and cloning of moxF genes from Methylococcus capsulatus Bath and Methylomonas albus BG8. J. Bacteriol. 170:2063-2069.

3. Anderson, D. A. and M. E. Lidstrom. 1988. The "moxFG" region encodes four polypeptides in the methanol-oxidizing bacterium, Methylobacterium sp. strain AM1. J. Bacteriol. 170:2254-2262.

4. Kim, Y. M. and M. E. Lidstrom. 1989. Plasmid analysis in pink facultative methylotrophic bacteria using a modified acetone-alkaline hydrolysis method. FEMS Microbiol. Lett. in press.

PROGRESS REPORT

July 1, 1989 - June 30, 1992

SUMMARY

This project focuses on transcription and transcriptional regulation of genes involved in methylotrophic growth in both methane and methanol utilizing bacteria. A large number of genes are involved in methylotrophy, and their expression must be carefully coordinated, in order to balance formaldehyde producing and consuming activities so that formaldehyde does not build to toxic levels. In addition, in the facultative methylotrophs methylotrophy is an inducible metabolism, which requires an additional level of regulation by carbon source.

The work we have carried out in the past three years has involved defining genetic organization of key methylotrophic genes, studying upstream regions involved in transcription, and defining key regulatory genes for these systems. This work has suggested that different methylotrophic metabolic segments are intercoordinated, and while it is likely that multiple regulatory signals play a role in this, formaldehyde may be the key that links them together. We are just beginning to define the DNA sequences and regulatory proteins involved in this sensing system, but it seems clear that a complex interconnected network exists that helps the cell maintain the appropriate balance between formaldehyde production and consumption, and between dissimilatory and assimilatory metabolism.

Previous Goals: The previous goals of this project were to clone and characterize promoters of methylotrophy genes, and to characterize transcripts and transcriptional regulation. Although we have made progress in promoter characterization, because of budget constraints and technical difficulties with the promoter and transcript work, we have concentrated more recent work on analyzing regulatory genes and the regulatory network for the methanol oxidation system. This has resulted in two published papers, and the data are now complete for a total of five others, which are in various stages of preparation for submission within the next few months.

Progress:

The work we have carried out for the past three years has focused on three areas, defining the genetic organization of the systems under study, characterizing the promoter regions of key genes in those systems, and isolating and characterizing regulatory genes for these systems. Most of this work has involved the methanol oxidation (Mox) system, but we have also carried out some studies on genes involved with the serine pathway for formaldehyde assimilation.

1. Genetic Organization

a. Methanol oxidation genes. In order to carry out comparative studies of the transcription of methanol oxidation (*mox*) genes, we have continued to isolate and characterize these genes and their upstream regions, from both methanol and methane utilizing bacteria, especially those encoding the structural polypeptides of methanol dehydrogenase (MeDH), *moxFJGI*. We have published the sequence of *moxF* and *moxJ* from the facultative methanol

utilizer *M. extorquens* AM1, including the upstream region of *moxF* and the transcription start site mapping of this gene (see reprint, Anderson et al., 1990). We have also finished characterizing the *moxFJGI* region of a marine Type I methanotroph, *Methylomonas* sp. A45, including upstream sequence data and T7 expression analysis of the encoded polypeptides, and a manuscript describing this work is in preparation. The order of the genes was found to be the same as in the methanol utilizers (*moxFJGI*) and the expressed polypeptides were similar in size to those found in *M. extorquens* AM1 (Fig. 1). Analysis of the promoter region is described below.

We have also studied the *moxAKL* region, which encodes functions involved in maturation of the MeDH. Expression data for the *moxAKL* region of a Type I methanotroph, *Methylomonas albus* BG8 show that four polypeptides can be detected in *E. coli* using a T7 expression system, of approximately 38, 36, 26 and 19 kDa. (The cloning and characterization of this region is currently being written up for publication). Expression of the corresponding region from *M. extorquens* AM1 shows only two polypeptides, of 34 and 19 kDa. We have sequenced this region from *M. extorquens* AM1, and four open reading frames have been detected, all transcribed in the same direction and encoding polypeptides of 34, 23, 37 and 19 kDa (Fig. 2). The last two polypeptides are predicted to be periplasmic, and the mature sizes would be 34 and 17 kDa, respectively. It is likely that the first and third polypeptides (mature form) would comigrate on gels, but it is not clear why the 23 kDa polypeptide was not observed. It may be poorly expressed, for some reason. The last three open reading frames appear to be translationally coupled, with the last codon of each upstream open reading frame overlapping with the first codon of the downstream open reading frame. These data coupled to previous mutant complementation results suggest that this region contains four genes, *moxA* (34 kDa), *moxK* (23 kDa) and *moxL* (37 kDa, 34 kDa mature), and a new gene, tentatively designated *moxW* (19 kDa, 17 kDa mature). It is likely that we do not have any mutants of *moxW* in our collection. In order to confirm these genes and their products, we are now constructing clones with specific deletions and insertions, which will be used for mutant complementation. In addition, we will construct specific chromosomal mutants by using homologous recombination with the insertion clones, to determine the phenotype of each. These will be constructed in such a way that they do not exert a polar effect on downstream genes. The sequence data have not revealed any potential promoter sequences. It is possible that these genes are cotranscribed with the upstream *moxFJGI* region.

b. Serine pathway genes. Under NIH support, we have characterized a chromosomal region of *M. extorquens* AM1 that contains at least four genes of the serine pathway for formaldehyde assimilation, encoding hydroxypyruvate reductase (*hprA*), C-1 specific PEP carboxylase (*ppcAB*) and malyl CoA lyase (*mclA*) (Fig. 3). As part of the DOE project, we are now examining transcription of this region, to compare this system involved in carbon assimilation to the Mox system, involved in energy metabolism. We have shown that these genes are clustered and transcribed in the same direction (*hprA-ppcAB-mclA*), and we have defined the region encoding them using a combination of mutant complementation and T7 expression studies. The expression studies have shown that the malyl CoA lyase subunit is 35 kDa, while the complementation studies suggest the PEP carboxylase region consists of two genes. No detectable polypeptides were expressed from the PEP carboxylase region in the T7 experiments. A manuscript is in preparation describing these results. The *hprA* gene has been sequenced,

including the regions upstream and downstream of it. A second open reading frame (orf-1 in Fig.3) has been identified downstream of *hprA*, transcribed in the same direction, but insertion mutants constructed by homologous recombination of three different insertion clones into the chromosome, grow normally on methanol. If this is a gene involved in methylotrophic growth, it is not obligatory. No open reading frames have been identified upstream of *hprA*, although a putative promoter is present (see below).

2. Promoter Characterization

The gene organization experiments described above have defined genes and their transcriptional orientation, so that promoter studies can be carried out. In addition, the upstream sequence data obtained have been compared to sequences upstream of other methylotrophy genes, to identify conserved regions.

a. *moxF promoters*. We had previously mapped the transcriptional start site for the *M. extorquens* AM1 *moxF* region to 170 bp upstream of the translational start site and shown that a single start was detected in RNA from both methanol and succinate grown cells (see reprint Anderson et al., 1991). Approximately 10-fold more RNA was detected in the start site mapping in preparations from methanol-grown cells than from succinate-grown cells. By comparing the -10 and -35 regions with those of other *moxF* genes, we proposed a putative consensus promoter sequence for the *moxF* promoters (AAAGACA - 16-18bp - TAGAAA - 5-7bp - +1; ref.1). We initiated our studies of this promoter region using a 1.55 kb *Xho*I-*Sal*I fragment that covers this region and includes a portion of the *moxF* open reading frame (see reprint, Morris and Lidstrom, 1992; Fig. 4). However, study of this promoter in *M. extorquens* AM1 has been hampered by what appears to be a cloning artifact. Using the promoter cloning vector pGD500 (IncPI, low copy, *lacZ* reporter gene), the *Xho*I-*Sal*I fragment has been shown to exhibit orientation-specific β-galactosidase activity in *M. extorquens* AM1. However, we expected approximately 6-10 fold higher levels in methanol grown cells than succinate grown cells. Instead, we observed high constitutive levels in cells grown on both substrates. The same cells regulate the chromosomal MeDH activity and structural polypeptides normally (see reprint, Morris and Lidstrom, 1992). When this construction was inserted into the chromosome by homologous recombination, both MeDH and *lacZ* were regulated normally. These insertions result in fusion of *lacZ* to the complete upstream region and fusion of *moxF* to the *Xho*I-*Sal*I fragment, with regeneration of a complete *moxF* (see reprint). Therefore, the promoter activity of the fragment is reflected in the level of MoxF, measured by antibody assays, and the normal promoter activity is measured by β-galactosidase activity, as an internal positive control. These data show that the 1.55 kb *Xho*I-*Sal*I fragment contains all of the cis-acting sequences necessary for transcription and for regulation by methanol.

We have observed similar constitutive expression with constructions in a new promoter cloning vector we have generated, that has an IncPI replicon, but carries *catA* as a reporter gene and is an entirely different construction. This problem has also been observed with both vectors using promoters from other methylotropy genes (see below). We assume that this problem is due to some artifact of the promoter being in a plasmid. The result is that all promoter studies must be carried out with chromosomal constructions. This greatly adds to the work and time involved. Due to the difficulties involved in genetic manipulations of *M. extorquens* AM1 and its slow

growth rates, these constructions take 2-3 weeks to generate, another 2 weeks to confirm, and the analysis of expression takes another 2-3 weeks. In addition, efficient recombination requires a cloned fragment of greater than 0.5 kb, which restricts the kinds of tests that can be carried out. Therefore, we have not yet carried out detailed mutational analysis of these promoters. However, we have cloned two smaller fragments into pGD500 for insertion into the chromosome and testing in cells grown on different substrates (Fig. 4). These both cover the putative promoter region, but the larger one includes an upstream region that contains a number of inverted repeats and which has been implicated in *moxF* function, while the smaller one omits this region. This smaller fragment is only 262 bp, and therefore the frequency of recombination into the chromosome will be low. We hope to isolate the appropriate insertions by screening a large number of cells. When we have inserted these into the chromosome and tested their activities, the data should tell us whether that upstream repeat region is required for regulation by methanol.

We have also studied the moxF promoters in two Type I methanotrophs, *Methylomonas albus* BG8 and *Methylomonas* A4. In both cases, upstream sequence data had identified putative promoter sequences similar to those observed for the *Methylobacterium moxF* genes (Fig. 5). Our initial attempts to map the transcriptional start sites for these two strains using procedures that had been successful in *M. extorquens* AM1 failed, and Northern blots suggested the difficulty was with the RNA preparations. After a great deal of effort testing a variety of RNA procedures and modifications to these, we had success with a modified hot phenol extraction procedure (2). Recent experiments using RNA prepared in this manner and carrying out primer extension using two different primers for each have mapped the transcriptional start site of *M. albus* BG8 to the sequence containing the consensus promoter, but in *Methylomonas* A45, the start site has been mapped to a sequence about 50 bp downstream of the consensus (44 bp upstream of the translational start site; Fig. 5). In the latter case, this sequence does not resemble consensus sequences for sigma-54 or sigma-32 dependent promoters, nor consensus sequences for *Pseudomonas* promoters. We are continuing this work, to confirm the mapping and we will carry out Northern blot analysis in attempts to define the transcripts. However, these data suggest that the *moxF* promoter is not universally conserved. It is possible that the consensus sequence noted above reflects a regulatory sequence, rather than the promoter. Future work will address this question (see Research Plan).

We have also cloned into pGD500 a 1.5 kb fragment of *M. albus* BG8 DNA containing the putative *moxF* promoter, and have observed orientation-specific β-galactosidase activity (Table 1). We are now in the process of generating smaller subclones of this fragment, as well as a series of subclones containing the putative promoter region of *Methylomonas* A45, to test for promoter activity. When this work is completed, a manuscript will be written up for publication describing these two promoter regions.

b. *hprA* promoter. The sequence data from the region upstream of *hprA* and of the ORF immediately downstream of *hprA* revealed a sequence similar to that observed for the *Methylobacterium moxF* promoters (Fig. 5). Therefore, we have also attempted to define this promoter region. We have cloned a 350 bp DNA fragment containing the putative promoter sequence into pGD500, and this construction shows orientation-specific β-galactosidase activity. However, it is only a few-fold over background (Table 1). Purified hydroxypyruvate reductase has a high specific activity, and comparisons to the activity in cell extracts suggest that it is a

minor protein in methanol grown cells. Therefore, it is possible that this gene is transcribed at a low rate in methanol-grown cells. As with the *moxF* promoter, this expression is constitutive, and we are currently attempting to insert this construction into the chromosome to test its regulation. This promoter is also constitutively expressed in the new *catA* promoter cloning vector we have constructed, described above. We are now attempting to map the transcriptional start site for this gene, although if the transcript is present at low levels, this may be difficult.

c. *pqq* promoters. Several of the genes originally identified as *mox* genes are required for the biosynthesis of the cofactor, PQQ, and will be renamed *pqq* genes. It has become clear that the methanol oxidation system is quite complex, and the *pqq* genes may be regulated separately from the other *mox* genes. Therefore, we are beginning to study the transcription of these genes. This work has just been initiated, but we have isolated Tn5*lac* insertions into cloned *pqq* genes and have started characterizing them. These insertions create transcriptional fusions to *lacZ*, and our preliminary screens show that they do exhibit orientation-specific β-galactosidase activity. It is possible that these may prove to be a better alternative for studying transcription than direct constructions in promoter cloning vectors. This work has suggested that transcription occurs in the direction, *moxOVTCP* (see Table 1, main proposal). We have started to sequence the region upstream of the *moxO* (*pqqD*) gene, since in analogy to other *pqq* genes that have been sequenced from non-methylotrophic bacteria, this should be the first gene in a 5-gene transcriptional unit (3,4). These preliminary sequence data have confirmed the presence of the first three *pqq* genes, and their correspondence to known *pqq* genes in *Acinetobacter aceti* (3) and *Klebsiella pneumoniae* (4), as well as the direction of transcription. Further sequencing is necessary to define the upstream promoter region. Once this is finished, we will generate subclones in promoter cloning vectors, and test their activity in both plasmid and chromosomal contructions.

d. RNA polymerase. Nothing is known about RNA polymerase in any methylotrophs, and yet the transcriptional start site mapping data from *Methylobacterium* strains suggests the presence of a novel promoter sequence, and therefore the presence of a novel sigma subunit for RNA polymerase (sigma M?). I currently have a visiting fellow in my laboratory, Dr. Juan Davagnino, who purified and characterized RNA polymerase from *Thermus aquaticus* for his thesis research. He will spend 6 months in the laboratory, purifying RNA polymerase from methanol grown cells of *M. extorquens* AM1 and developing an in vitro transcription assay for methylotrophic promoters. Transcription specificity will be compared to a promoter we have cloned from *M. extorquens* AM1 that has a classical σ-70 consensus sequence and to a promoter for methylamine dehydrogenase, which has an unrelated sequence. The *moxF* promoter fragment is being used as a template for transcription assays, but once a purified enzyme is available, the *pqqD* and *hprA* promoters will also be tested. If Dr. Davagnino is successful, we will also purify RNA polymerase from succinate-grown cells, using the σ-70 type promoter for transcription assays. That will allow us to compare the sigma subunit present in RNA polymerase that specifically transcribes methylotrophic promoters to that present in the enzyme that transcribes the σ-70 type promoter (presumably the latter will contain the *M. extorquens* AM1 σ-70 subunit). This system will be extremely useful for later footprinting and in vitro transcription experiments. In addition, the sequencing of *mox* regulatory genes proposed in the Research Plan may identify candidates for a sigma factor, and it will then be important to have isolated "sigma M" on hand.

3. Regulation and Regulatory Genes

Because of the technical difficulties involved with studying the promoter structure of methylotrophy genes, and the difficulties we have had with mRNA preparations, we have focused our more recent work on defining regulatory genes and the regulatory network in *M. extorquens* AM1. Our approach so far has involved both physiological studies and also experiments using our promoter fusions to test transcription of *moxF* in six *mox* mutants that have pleiotropic phenotypes. This work shows great promise for understanding growth on C-1 compounds and how the numerous functions for methylotrophy are coordinated in the cell.

a. Physiological regulation. These experiments have focused on the inducing signal for methylotrophic functions. We have examined the levels of MeDH and Serine pathway (SP) enzymes in *M. extorquens* AM1 cells grown on methylated glycines (methylglycine, or sarcosine; dimethylglycine; and trimethylglycine, or betaine). *M. extorquens* AM1 grows on all three compounds, apparently by removing methyl groups sequentially and using these methylotrophically, although the oxidative enzymes have not yet been identified. Both MeDH and SP enzymes are induced during growth of *M. extorquens* AM1 on these compounds, and as expected, mutants in SP enzymes do not grow on these compounds. However, all tested *mox* and *pqq* mutants grow normally on all three methylated glycines, suggesting that these oxidative pathways do not share common functions. Specifically, these results show that PQQ is not required for growth on methylated glycines, and none of the putative *mox* regulatory genes affect growth on these compounds.

The ability of five different methylated substrates (methanol, methylamine and the three methylated glycines) to induce MeDH activity suggests the possibility that the common intermediate formaldehyde, rather than methanol is the inducing signal for both the Mox and Pqq systems. It has been hypothesized that formaldehyde is the regulatory signal in *P. denitrificans*, and in that strain regulation is thought to occur by derepression (5). However, we have shown that a MoxK mutant of *M. extorquens* AM1, which has no MeDH activity but contains normal levels of the structural polypeptides, induces these polypeptides to a significant level when exposed to methanol overnight (the standard methanol- mutant induction protocol). These data suggest that methanol itself can be an inducing signal for the *moxFJGI* operon. We do not yet know whether PQQ synthesis is also induced under these conditions. It is possible that a number of methylated compounds can activate the regulatory system for Mox, due to a lack of binding specificity. This does not rule out a role for formaldehyde in the regulation of this system, but simply shows that formaldehyde is not the sole signal.

b. *moxB*. Mutants in *moxB* are highly pleiotropic, showing only trace amounts of the MeDH structural polypeptides (6). Therefore, *moxB* is a good candidate for encoding a positive transcription factor. We have tested the *moxF*-promoter-*lacZ* fusion discussed above in *M. extorquens* AM1 *moxB* mutants and have shown that no β-galactosidase activity above the vector background is observed in these constructions (see reprint, Morris and Lidstrom). Surprisingly, this was true for both the vector and chromosomal constructions, suggesting that both constitutive and inducible transcription were deficient in these mutants. This seemed to indicate that *moxB* might be defective in a sigma factor, since the loss of an activator protein should only affect the inducible portion of the transcription. However, we have now observed the same phenotype in

mutants that are apparently deficient in a positive regulatory system (see below) and it appears that multiple components of the system are required for transcription.

c. *moxQ* and *moxE*. We have examined MoxQ and E mutants in detail, and have discovered that they are also highly pleiotropic, showing low levels of MeDH structural polypeptides. These genes are known to correspond to *mox* genes present in *Paracoccus denitrificans*, which have been named *moxX* and *moxY* (N. Harms, personal communication). Nellie Harms' group in the Netherlands has sequenced *moxX* and *moxY* from *P. denitrificans*, and have found substantial homology to one class of regulatory/sensor pairs. MoxX has homology to the regulator class containing ComA, NarL, UhpA and DegU, while MoxY has homology to the sensor class containing ComP, NarX, UhpB and DegS. These regulators are all DNA-binding proteins, and so it is assumed that *moxX* and *moxY* encode a two-component regulatory system that senses a methylotrophy signal and regulates transcription via a phosphorylated activator protein. A third gene is present downstream of *moxY*, called *moxZ*, and mutants constructed in this gene are unable to grow on methanol. However, they contain normal MeDH activity. Therefore, Nellie Harms has proposed that this gene functions to coordinate methanol oxidation with formaldehyde consumption. If this coordination were disrupted, the cells could be killed on methanol due to formaldehyde toxicity, and would show a methanol-negative growth phenotype in the presence of normal MeDH acitivity. It is possible that in analogy to other two-component regulatory systems, this third gene affects relative phosphorylation levels of a signal transducer or it may be a completely separate component.

We have analyzed the *M. extorquens* AM1 *moxF*-promoter-*lacZ* fusion described above in *moxQ* and *moxE* mutants of *M. extorquens* AM1 and have shown that the β-galactosidase activity is similar to that of the vector background, in both the plasmid or chromosomal constructions. However, when the promoter fusion is inserted into *moxA* or *moxK* mutants, a transcription pattern like the wild-type is observed, so this is not an artifact found in all Mox mutants. As pointed out above, this is somewhat surprising. If the constitutive expression of the *moxF* promoter found in the vector constructions were due to transcription from a fortuitous promoter, or transcription from the methanol-inducible promoter independent of the inducing system, then the loss of the methanol-dependent activator system should only affect transcription of the chromosomal construction. However, our data suggest either that the constitutive expression in the vector constructions requires the activator system, but it somehow operates in the absence of the inducer in this case, or that multiple components of the transcription system are required even for basal transcription in the absence of inducer. The fact that the transcription of *moxF* from the chromosomal construction was reduced to vector background rather than the higher level observed in succinate-grown cells supports the latter hypothesis. A simple model to explain these data would be that the regulator (non-phosphorylated) is required for transcription in the absence of the inducing signal, and this basal transcription is increased when the regulator is phosphorylated in response to the inducing signal. If this system is interconnected, then loss of any single component could result in loss of expression of the regulator, leading to loss of *moxF* transcription. It should be quite interesting to test expression of other methylotrophy genes (*pqqD* and *hprA*) in these *moxQ* and *moxE* mutants.

d. *moxM, moxD* and *moxN*. These three genes are located adjacent to the *pqq* cluster, but preliminary Tn5*lac* analysis suggests they are transcribed in the opposite orientation (see

Table 1, main proposal). We have characterized *moxM*, *moxN* and *moxD* mutants, and find that they synthesize low amounts of the MeDH structural polypeptides, but have no MeDH activity. They do not grow on methanol in the presence of PQQ. It is possible that these genes are involved in regulation of both *mox* and *pqq* genes. This hypothesis is supported by evidence from studies in *P. denitrificans* (7). These investigators have identified a gene for a previously unknown cytochrome *c*, and have shown that it is adjacent to a gene having substantial similarity to *moxF*, the MeDH large subunit, especially in the region that is conserved in all known PQQ-containing dehydrogenases. They have hypothesized that these genes encode an unknown PQQ-containing dehydrogenase and its electron acceptor (7). Mutants in these genes grow normally on all tested methylotrophic and non-methylotrophic substrates. A similar cytochrome is present at very high levels in *M. extorquens* AM1 *moxD* mutants (8), and amino acid sequence data suggest this is identical to the new cytochrome in *P. denitrificans* (D. Nunn, personal communication). If *moxD* encodes a function involved in coordinating PQQ synthesis, mutants deficient in this function might be derepressed for alternate PQQ-using systems. Since these mutants contain decreased levels of MeDH polypeptides and do not grow on methanol + PQQ, they must affect *mox* genes in addition to those that synthesize PQQ.

We do not yet know whether any of the pleiotropic mutants discussed so far (*moxB*, *moxQ*, *moxE*, *moxM*, *moxD*, *moxN*) synthesize PQQ, but we are testing all of them. The bioassay for PQQ uses an ethanol dehydrogenase apoprotein from a *Pseudomonas testosteroni* strain (9) and we are in the process of purifying the apoprotein for the assays now. In addition, we will test transcription of the *moxF* promoter construction and the *moxO* (*pqqD*) Tn*5lac* fusions in the *moxM*, *moxD* and *moxN* mutants to determine whether these mutants are involved in transcription of either set of genes.

A manuscript describing the isolation, mapping and preliminary characterization of the *moxQ, E, M, N* and *D* genes is currently in preparation.

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Research Papers:

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3. Arps, P.J., B. Speer, Y.M. Kim and M.E. Lidstrom. The *moxAKL* genes of *Methylomonas albus* BG8. in preparation for J.Bacteriol.

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5. Arps, P.J., B. Speer and M.E. Lidstrom. Characterization of the genes for phosphoenolpyruvate carboxylase and malyl CoA lyase from *Methylobacterium extorquens* AM1. in preparation for J.Bacteriol.

6. Lee, E., W.-H. Fan and M.E. Lidstrom. Isolation and characterization of new *mox* genes from *Methylobacterium extorquens* AM1. in preparation for J.Bacteriol.

7. Chistoserdova, L. and M.E. Lidstrom. Sequencing of the *hpr* gene from *Methylobacterium extorquens* and identification of the promoter region. in preparation for J.Bacteriol.

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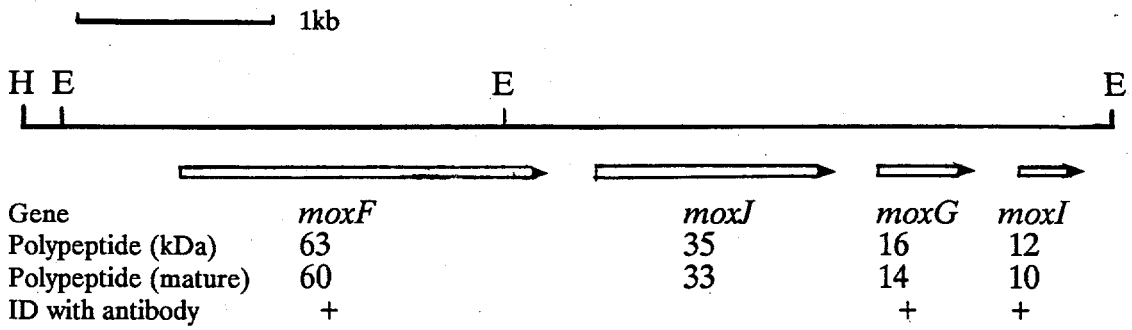
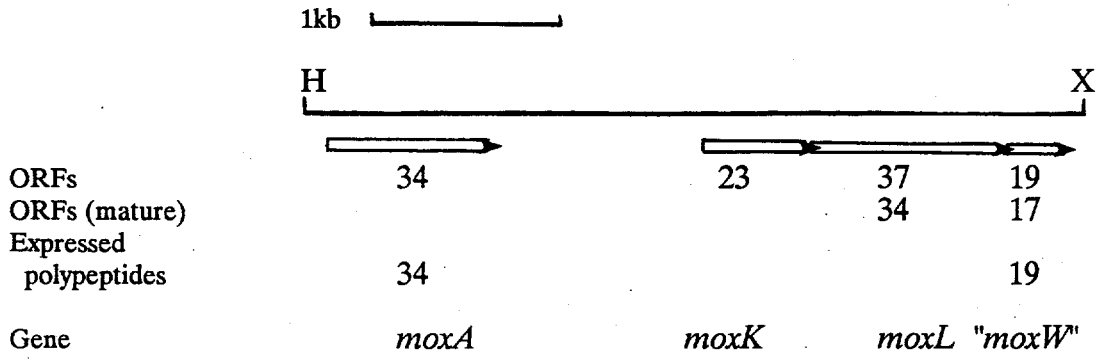


Figure 1. *moxFJGI* region of *Methylomonas* sp. A45. H, *Hin*dIII; E, *Eco*RI.

A. *M. extorquens* AM1



B. *M. albus* BG8

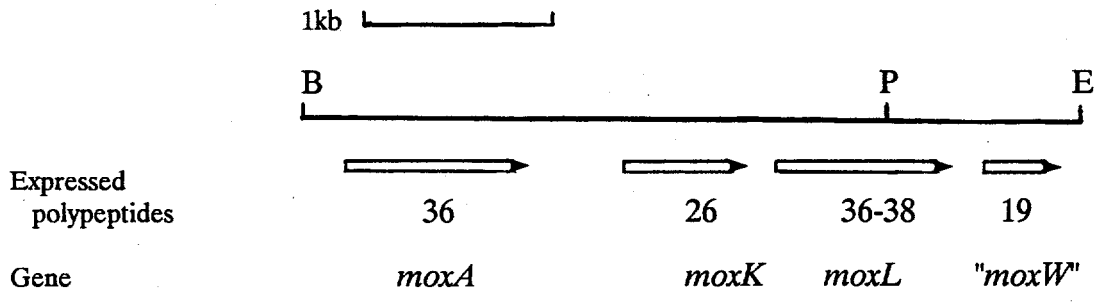


Figure 2. The *moxAKL* regions of *M. extorquens* AM1 and *M. albus* BG8. H, *Hin*dIII; X, *Xho*I; B, *Bam*HI; P, *Pst*I.

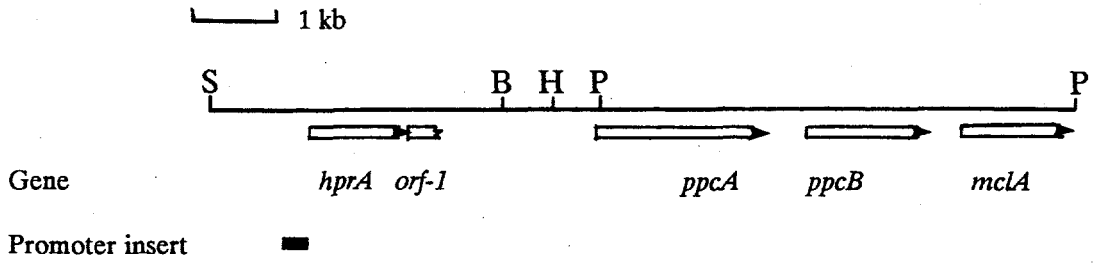


Figure 3. The *M. extorquens* AM1 chromosomal region containing serine pathway genes. *hprA*, hydroxypyruvate reductase; *orf-1*, partial open reading frame identified from sequencing; *ppcAB*, two genes necessary for the C-1 specific PEP carboxylase; *mclA*, malyl CoA lyase. S, *Sph*I; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I.

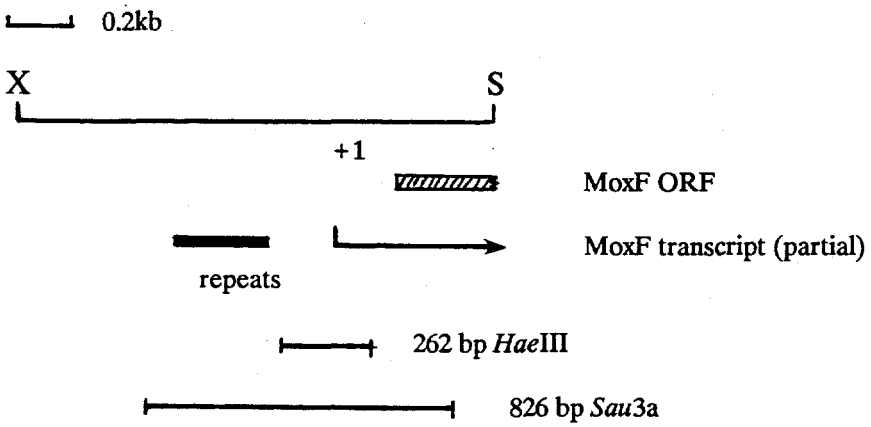


Figure 4. Two subclones of the *moxF* region of *M. extorquens* AM1 for promoter testing.

Figure 5. COMPARISON OF UPSTREAM SEQUENCES FOR *MOX F* and *HPR A* GENES

	-35		-10	+1
E. COLI	TTGACA	XXXXXXXXXXXXXXXXX	TATAAT	XXXXX**G**

MOX F

AM1 CT **AAAGACA** TCGCGTCCAATCAAAGCC **TAGAAA** ATATA**G**---170bp--- first Met

XX GT **AAAGACA** TCTCCTTCAATCAACGCC **TAGAAA** CGATA ---171bp--- first Met

Pd GATCGGAC GG**GAAA** AACCC---157bp--- first Met

BG8 AA **AAAG**GA**A** CTTTTCCCGACTCACA CG**GAAA** AGCCATA ---212bp--- first Met

A45 AA **AAA**TC**CA** AGGCATACGTTTTGATC A**AGAAA** AGCTGAT-
TGCTGCAAGTTGACACAAATAAACACATCGGCGCGTTAAGC ---43bp--- first Met

HPR A (AM1)

GG **AAA**CGGG GCCGGCGTCGGTACCTC AT**GAAA** CTCCCGG---91bp--- first Met

ORF-1 (AM1)

AG **AAA**CG**CA** CCGGCCTCGGAGTGGGGCCGCGT AC**GAAA** AGAC---99bp--- first Met

AM1 = *Methylobacterium extorquens* AM1 (serine pathway methanol-utilizer)
XX = *Methylobacterium organophilum* XX (serine pathway methanol-utilizer)
Pd = *Paracoccus denitrificans* (autotrophic methanol-utilizer)
BG8 = *Methylomonas albus* BG8 (RuMP pathway methane-utilizer)
A45 = *Methylomonas* sp. A45 (marine RuMP pathway methane-utilizer)

Transcriptional start sites for *moxF* in AM1, XX, BG8 and A45 are marked in bold-face.

Table 1. Beta-galactosidase activities in promoter-*lacZ* fusions in pGD500.

Construct	Organism	beta-galactosidase units		Insert
pGD500 alone	BG8	<1		
pGD500::$p_{moxF-BG8}$ (correct orientation)	BG8	400		1.5kb *Hin*dIII-*Bam*HI
pGD500::$p_{moxF-BG8}$ (incorrect orientation)	BG8	20		1.5kb *Bam*HI-*Hin*dIII
		methanol	succinate	
pGD500 alone	AM1	55	50	
pGD500::p_{hprA} (correct orientation)	AM1	130	145	0.35 kb *Kpn*I-*Eco*RI
pGD500::p_{hprA} (incorrect orientation)	AM1	40	45	0.35 kb *Eco*RI-*Kpn*I

BG8, *Methylomonas albus* BG8 (Type I methanotroph); grown on methane
AM1, *Methylobacterium extorquens* AM1 (facultative serine pathway methanol-utilizer

PROGRESS REPORT

July 1, 1992 - June 30, 1995

SUMMARY

The long-term thrust of this DOE project has been to identify and characterize *mox* genes and other methylotrophy genes in both methane- and methanol-utilizing bacteria, and study expression of those genes. In the last three years of support, the project has focused on identifying methylotrophy genes and the regions involved in their expression for comparative purposes, and has begun the process of analyzing the genes involved in transcriptional regulation of the Mox system in the strain for which we have the most information, *M. extorquens* AM1.

In order to carry out comparative studies of the transcription of methylotrophy genes, we have cloned and characterized genes involved in methanol oxidation (*mox* genes) from two Type I methanotrophs, *Methylobacter marinus* A45 (formerly *Methylomonas* sp. A45) and *Methylobacter albus* BG8 (formerly *Methylomonas albus* BG8). In both cases, the organization of the genes was found to be identical, and the transcriptional start sites upstream of the *mxaF* genes were mapped. Other methylotrophy genes have been cloned and characterized from these methanotrophs, including *mxaAKL* and *fdh*.

The rest of this project has focused on the regulatory network for the *mox* system in *M. extorquens* AM1 . We have sequenced two *mox* regulatory genes, *mxbD* and *mxbM* and they show identity with a specific group of sensor-kinase/response regulator pair systems. We have shown that these genes are required for expression of *mox* genes and of *pqq* genes, and also for repression of a PQQ-linked dehydrogenase of unknown function. A second set of *mox* regulatory genes, *mxcQE* also have similarity to sensor-kinase/response regulator pairs, but to a different subfamily than *mxbDM*. We have studied the regulation of *pqq* genes in more detail, and have shown that the major regulatory step is post-transcriptional. Finally, we have shown that an RNA polymerase preparation purified from methanol-grown *M. extorquens* AM1 contains two polypeptides with similarity to known sigma factors, and this preparation initiates transcription at the *mxaF* transcription start site.

Progress

Each of the publications resulting from this project period are cited by author names in the text below, and are listed at the end of this Progress Report. The numbered references are found in the list at the end of the main proposal.

1. Promoter regions of methylotrophic genes

a. Methanotrophs. At the beginning of this project period, we continued a series of studies to characterize promoter regions of genes involved in methylotrophic growth in both methane- and methanol-utilizing bacteria, for comparative purposes. We had previously cloned the region containing *mxaF* from two methanotrophs, *Methylobacter albus* BG8 and *Methylobacter marinus* A45. We first determined the order of the *mxa* genes and found they were the same as in *M. extorquens* AM1 (*mxaFJGIR*). Next, we mapped the transcriptional start sites for the two *mxaF* genes. The regions immediately upstream of both transcriptional start sites did not show substantial similarity to each other or to the conserved -10, -35 sequence for *mxaF* from *Methylobacterium* strains, nor to any recognizable promoters. Transcriptional fusions to a *lacZ* reporter gene confirmed the presence of promoter activity in the case of *M. albus* BG8. This work has been published (Waechter-Brulla et al., 1993; Chistoserdova et al., 1994). Another gene involved in methylotrophy encoding a formaldehyde dehydrogenase (*fdh*) was cloned and sequenced from *M. marinus* A45 (Speer et al., 1994) and a gene cluster involved in the Mox system (*mxaAKL*) was also cloned from *M.albus* BG8 and partially sequenced (Arps et al., 1995). An analysis of the sequence upstream of these genes did not show resemblance to the *Methylobacterium mxaF* promoter, to the regions upstream of the methanotroph *mxaF* genes, nor to any of the known promoters noted above. This effort has resulted in the identification of a number of methylotrophic genes from methanotrophs, and has shown that the promoter sequences for methylotrophic genes in methanotrophs and methylotrophs must be different. Since promoter mutation studies are difficult to carry out in methanotrophs, we have focused our efforts for promoter characterization and genetic regulation on genes from *M. extorquens* AM1, a strain more amenable to genetic analysis.

b. *M. extorquens* AM1. We had previously mapped the transcription start site for *mxaF* from *M. extorquens* AM1 (11). We cloned and characterized a number of genes involved in the serine cycle (assimilatory metabolism) from this organism (Arps et al., 1993) and generated *xylE* transcriptional fusions to two of these (*hprA*, encoding hydroxypyruvate reductase, and *sgaA*, encoding serine-glyoxylate aminotransferase). These enzymes are induced about 3-fold during growth on C_1 compounds (30). In accordance with these data, the fusions were also expressed at a three-fold higher level in cells grown on methanol than in cells grown on succinate. However, the expression levels were very low, only about 1% that of *mxaF*. This is in keeping with the high specific activity of the purified enzymes. We attempted to map the transcriptional start sites, but we were unable to obtain sufficient sensitivity to detect them, presumably because of the low level of transcription. An analysis of upstream sequences did not reveal any recognizable promoter sequences.

The next system we studied was that for PQQ synthesis. We focused on the first gene of a five-gene cluster (*pqqDGCBA* ; see Fig. 3, main proposal), which turned out to be highly transcribed. We mapped the transcriptional start site and detected two transcripts that initiated at that site, one encoding only *pqqD* and a second encoding *pqqDG* (Ramamoorthi and Lidstrom, 1995). We were unable to detect a transcript for the entire 5-gene cluster, but it may have been present at too low a level for us to detect. Once again, the -10,-35 region of *pqqD* did not show similarity to these regions from *mxaF*, but it did show significant similarity to the -10, -35 sequences of the gene equivalent to *pqqD* from *Klebsiella pneumoniae* (Ramamoorthi and Lidstrom, 1995). *K. pneumoniae* does not grow on methanol, but it has a PQQ-linked glucose dehydrogenase and therefore, has *pqq* genes (21). We have also sequenced two other *pqq* genes (*pqqEF*), unlinked to the first gene cluster, and have shown that *pqqE* is equivalent to *pqqF* of *K. pnuemoniae* (Springer et al., in prep.; see Fig. 3, main proposal). Both of these genes appear to encode a protease, but its role in PQQ synthesis is still unknown.

2. *M. extorquens* AM1 RNA polymerase.

The data we have obtained so far suggest that methylotrophic genes in *M. extorquens* AM1 may be transcribed using a novel sigma factor, since we have not identified promoter sequences indicative of known promoters. In order to assess this question, we have purified RNA polymerase from *M. extorquens* AM1 cells grown on succinate or on methanol, and in both cases, have obtained a preparation that has 6 polypeptides. Three of these are the correct size to be α, ß, and ß', and in the case of the putative α polypeptide, N-terminal sequencing of the polypeptide after blotting to a nylon membrane has revealed high identity to *E. coli* α subunit of RNA polymerase. The putative ß and ß' subunits have not been sequenced. The other three polypeptides are 97, 40 and 24 kDa in mass. These have been blotted to nylon membranes and N-terminal sequences have been determined. The sequence from the 24 kDa polypeptide has 50% identity to the N-terminal sequence of the 24 kDa ω polypeptide found in RNA polymerase preparations of *E. coli* (Fig. A). The sequence from the 97 kDa polypeptide has 58% identity to the N-terminal sequence of the 77 kDa vegetative sigma factor from *Agrobacterium tumefaciens* (Fig. A), which keys into the large class of RpoD/SigA sigma factors (31). The sequence of the 40 kDa polypeptide has 33% identity to the Group 2 sigma factor from *Streptomyces coelicolor*, HrdD (Fig. A), including 10 residues that are highly conserved in this group (31). Neither of the putative sigma factors was detected in immunoblots using antibody against *E. coli* σ^{70}.

In vitro runoff transcription assays were carried out to determine whether this RNA polymerase preparation initiates transcription from *mxaF* at the same start site measured using bulk RNA. The template used was a 392 bp fragment generated by PCR that contains the *mxaF* transcription start site within it, and is predicted to produce an RNA of 133 nucleotides. A major RNA species was observed of approximately this size (between 123 and 147 nucleotides), and this species was not produced with a control DNA fragment from upstream of *mxaF* that lacked the *mxaF* promoter. These data suggest that this RNA polymerase preparation does initiate transcription at the correct site. This work is currently being written up for submission to J. Bacteriol. (Davagnino et al., in prep.) The sequence comparisons suggest that the 97 kDa polypeptide may be the σ^{70} equivalent, and the 40 kDa polypeptide may be the sigma factor responsible for transcribing *mxaF*, but further work will be necessary to define the role of each.

```
Mex 97        ATKATERDDADAAQDQPTDGPLLDLTDAAVK
Atu SigA     MATKVKENEEAENERDGATDGPLLDLSDDAVK

                           **  **   ***     *  **
Mex 40        AGALAVLANEGGLSRYLDEIRKFPMLEPAEEFTLAK
Ace HrdD      RANGGELADRDLVGMYLDEIARTPLLDAAKEVELSQ

Mex 24        ARVTVEDSIEKVENRFELVLLAAHRARLLAAG
ω             ARVTVQDA-EKIGNRFDLVLAARARQMQVGGK
```

Fig. A. N-terminal amino acid sequences determined from three polypeptides found in the *M. extorquens* AM1 RNA polymerase purification, and comparison to the sequences in the protein database that were most similar. The identical residues are shown in bold-face, the asterisks denote residues conserved in Group 2 sigma factors (in those cases in which these are not identical, they are conserved as either one of the pair). Atu SigA is the 77 kDa vegetative sigma factor from *Agrobacterium tumefaciens* (31), Sce HrdD is a Group 2 sigma factor from *Streptomyces coelicolor* (31) and ω is the 24 kDa omega polypeptide from *E. coli* (32). Mex 97 is the *M. extorquens* AM1 97 kDa polypeptide, Mex 40 is the *M. extorquens* AM1 40 kDa polypeptide and Mex 24 is the *M. extorquens* AM1 24 kDa polypeptide.

3. *M. extorquens* AM1 Mox regulatory genes.

a. Mapping and sequence. We had previously identified 3 loci containing genes that appeared to be involved in regulation of Mox functions (see Fig. 3, main proposal), and one of these, *mxaB*, had been shown to be defective in transcription of a *mxaF-lacZ* fusion (11). We mapped the other two loci to 5 complementation groups, *mxbDM* and *mxcBQE*(Springer et al., 1995). We have sequenced the *mxbDM* region, and have shown that the gene product of *mxbM* has homology to a group of response regulators, while the gene product of *mxbD* has homology to a corresponding group of sensor kinases. The highest identity at the amino acid level for MxbM is with *E. coli* PhoB (38%), and for MxbD (in the C-terminal 280 amino acids) it is with *E. coli* CpxA (30%) although it also shows substantial identity with *E. coli* PhoM (26%) (Springer et al., 1995). We have partial sequence of the *mxcE* region, suggesting that *mxcE* encodes a response regulator, although in this case the homology is with a separate subfamily, with the highest homology being to *mxaX* of *Paracoccus denitrificans* (17). We have not completed the sequence of *mxcQE*, since the laboratory of R.S. Hanson has now sequenced the equivalent genes from a related *Methylobacterium* strain, and have shown that they have homology with *mxaYX* of *P. denitrificans* (R.S. Hanson, personal communication). We are however completing the sequence of *mxcB*. A gene of similar size (*mxaZ*) is present adjacent to *mxaYX* of *P. denitrificans* in the same transcriptional orientation (17) and *mxcB* may be the *mxaZ* equivalent. The function of *mxaZ* is not known. A strain with a mutation in *mxaZ* still grows on methanol, although with reduced growth yield (17). We are also beginning to sequence in the region of the sixth regulatory gene, *mxaB*.

b. Expression using gene fusions. Our next set of experiments has been to assess expression of specific *mox* genes using reporter gene fusions. This work has involved two promoter fusion vectors described earlier, pGD500 (*lacZ* reporter) and pHX200 (*xylE* reporter). With pGD500, *mxaF-lacZ* fusions were generated using a 1.55 kb *M. extorquens* AM1 fragment, which contained the transcriptional start site for *mxaF*, about 1 kb of upstream DNA, and a small portion of *mxaF* (Fig. B). The fusions (both orientations) were studied in wild-type and in the *mxaB* mutant. This work was supported by a previous DOE funding period and has been published (11), but it is summarized here for completeness. ß-galactosidase activity was at a high constitutive level in wild-type, and not detectable in the mutant. A chromosomal construction, pCM301 that regenerates a complete *mxaF* in the chromosome (see Fig. B), was generated, and in this case, the regulation of ß-galactosidase activity correlated well with the regulation of the MxaF, MxaG and MxaI polypeptides and more generally with MeDH activity (11).

We have subsequently used the same 1.55 kb promoter-containing fragment to generate a *mxaF-xylE* fusion in pHX200, and in this case, the expected regulation was observed in the plasmid construction (Fig. B), as was reported for *M. organophilum* XX promoter fusions using this vector (12). The background activity for pHX200 alone in *M. extorquens* AM1 is very low, often not detectable. A low level methanol-inducible activity was observed when the fusion was in the incorrect

orientation (pCMSau4), suggesting the possibility of a divergently transcribed low-level promoter (Springer et al., submitted), which is probably analogous to the *mxaW* promoter studied in *M. organophilum* XX (12). An analysis of this region in *M. extorquens* AM1has revealed that it contains a potential partial open reading frame of 220 amino acids, transcribed in the direction opposite to *mxaF* that shows good correlation with the expected codon usage of *M. extorquens* AM1. The N-terminal 34 amino acid overlap with the putative *mxaW* from *M. organophilum* XX shows 52% identity, but neither sequence shows substantial similarity to any entries in GenBank. We have not yet constructed a mutant in this putative gene, and so its potential function is unknown.

For the *mxaF-xylE* fusion, the increase in catechol dioxygenase activity measured in cells grown on methanol and succinate (10-14 fold) is similar to that observed for MxaF levels, as measured by immunoblots, suggesting that this system faithfully reports transcription from this promoter (Springer et al., submitted). We have used this fusion to assess transcription from the *M. extorquens* AM1 *mxaF* promoter in each of the 6 regulatory mutant classes noted above. In all cases, no detectable catechol dioxygenase activity (above the very low vector background) was found, regardless of growth substrate (succinate, methylamine, or methanol + methylamine) (Springer et al., submitted). These results confirm with a *M. extorquens* AM1 promoter fusion the phenotype of the MxaB, MxbD, and MxcE mutant classes as measured by Xu et al. using *M. organophilum* XX promoter fusions and cells grown on C_1 substrates (12). This also extends the information to the MxbM, MxcQ and MxcB mutant classes and a broader range of growth substrates. We have not yet tested the expression of the putative *mxaW* promoter in these mutant classes. However, the *mxaW* promoter of *M. organophilum* XX was not expressed in the *M. extorquens* AM1 MxbD mutant class, although it was expressed in the MxaB and MxcE mutant classes (12).

We have also studied regulation of PQQ synthesis and related this to transcription of *pqqD*, which encodes the peptide thought to be the precursor of PQQ. This work is described in a paper published in J. Bacteriol. (Ramamoorthi and Lidstrom, 1995). A single transcriptional start site for *pqqD* was mapped, and RNA blots identified two transcripts, a major one that encoded only *pqqD*, and a minor one that encoded both *pqqD* and the next gene, *pqqG* (Fig. C). PQQ levels were found to be about 5-fold higher in methanol-grown *M. extorquens* AM1 than in succinate-grown cells. However, both transcription of *pqqD* (as measured using a *pqqD-xylE* fusion; see Fig. C) and the steady-state levels of both *pqqD* transcripts were the same under both growth conditions. Therefore, the methanol regulation of PQQ synthesis must occur either at the level of transcription of different *pqq* genes, or at the post-transcriptional level. However, some regulation of *pqqD* does occur, since a 2-3 fold induction of *pqqD* transcription was observed in cells grown on methanol plus methylamine, but not in cells grown on single substrates (methylamine, methanol or succinate). This induction was abolished in the *mox* regulatory mutant classes MxbM, MxbD and MxaB, and in addition, activity from the *pqqD-xylE* fusion was decreased 5-10 fold in MxbM and MxbD mutants, but not affected significantly in any of the other regulatory mutants (Ramamoorthi and Lidstrom, 1995). These data are suggestive that MxbM and MxbD are involved either directly or indirectly in transcription of *pqqD*, and that *mxaB* mediates the induction of *pqqD* by C_1 compounds. However, MxcQE do not seem to be involved in transcription or transcriptional regulation by C_1 compounds of *pqqD*.

The Mox regulatory genes also affect a third system. We had previously shown that MxbD mutants contained very high levels of a cytochrome that is normally present in only trace amounts, which was later shown to be cytochrome *c*-553 (14). This cytochrome is now thought to be the electron acceptor for an unknown PQQ-linked dehydrogenase that has high identity with methanol dehydrogenase (23). We have now cloned and sequenced the genes for cytochrome *c*-553 and the dehydrogenase (*mxaF'J'G'*) from *M. extorquens* AM1 (see Fig. 3, main proposal), and have generated mutants defective in these genes by allelic exchange. As in *P. denitrificans* (23), mutants in these genes grow normally on methanol and all other tested substrates, and the role of this dehydrogenase is still unknown. However, we have shown by immunoblot that cytochrome *c*-553 is derepressed in all six of the regulatory mutant classes in cells grown on methylamine + methanol, but not in cells grown on succinate (Springer et al., submitted). Therefore, the derepression phenomenon is dependent upon the presence of methanol. Although we do not know the function of this dehydrogenase, it is possible that it is a detoxifying, periplasmic formaldehyde dehydrogenase, which might be expected to be regulated in the opposite manner to the formaldehyde-producing system (methanol dehydrogenase) in response to the presence of methanol. We are attempting to determine whether or not this is so.

In summary, the data available so far suggest that in *M. extorquens* AM1, *mxbD* and *mxbM* encode a sensor kinase/response regulator system that is involved in the expression of several sets of genes (Fig. D). These include positive regulation of several *mox* genes including at least one more sensor kinase/response regulator pair and a third linked gene (*mxcBQE*), as well as *pqqD* and *mxaW* *mxcBQE* in turn are involved in positive regulation of *mxaF* and negative regulation of the genes for a PQQ-linked dehydrogenase of unknown function, *mxaF'G'J'*. It is not yet known whether *mxaB* expression is dependent upon *mxbDM*, but current data suggest it is not dependent upon *mxcBQE*. *mxaB* apparently works with the *mxcBQE* system in the regulation of *mxaF* and *mxaF'J'G'*. One of the goals of this project is to determine which of the regulatory genes is required for expression of the others, in order to determine the hierarchy of expression in this system.

This regulatory system is a little unusual, in that the products of all six genes are required for any detectable expression of *mxaF*, regardless of the presence or absence of inducer. Therefore, at least one of them must encode a protein (for convenience, I will call it R_{mox}) that is absolutely required for transcription of *mxaF*. R_{mox} may be a DNA-binding activator protein, it may be a protein that binds to RNA polymerase and changes its activity (a sigma factor or other binding protein), it may be a protein that acts with other *mox* gene products in a complex to either bind DNA and/or bind RNA polymerase, or it may be an anti-sigma factor or a protein that inactivates a repressor protein. The other 5 regulatory genes may be involved in generating the active form of R_{mox}, either by transcription of required genes or direct activation of R_{mox}, and/or be required with R_{mox} to generate an active complex. In addition, one or more of these genes may be required for the induction phenomenon, or it is possible the induction may be mediated by a separate set of genes. Data from Xu et al., (12) show that transcription of the *mxaW* promoter from *M. organophilum* XX does not require *mxcE* or *mxaB* in *M. extorquens* AM1 and we have shown that transcription of the *pqqD* promoter does not require *mxcBQE* or *mxaB*. However, induction of the *pqqD* promoter by C_1 compounds does require *mxaB*. It is not yet known whether induction of the *mxaW* promoter requires *mxaB*. We also do not know whether intermediary regulators exist in the pathway between *mxbDM* and *pqqD* or *mxaW*, but they may exist, as indicated in Fig. D. It is also possible that another set of regulators exists in the pathway between *mxcBQE/mxaB* and the target genes, that has not yet been identified. One of the goals of this project is to obtain evidence as to whether other regulatory functions are operating in this system. What is the identity of R_{mox}? It could be the product of *mxcE*, which is predicted to encode a DNA-binding protein, the product of *mxaB*, or the product of an unknown gene. The experiments proposed in this project will provide clues as to the identity of R_{mox}, but definitive identification will probably require protein purification and in vitro studies.

Substantial MeDH activity (0.02 µmol/min/mg protein) and activity from the *mxaF-xylE* fusion (0.25 µmol/min/mg protein) are found in cells grown on succinate, in the absence of any external inducer. Since the products of all six regulatory genes are required for any detectable transcription of the *mxaF* promoter, these data indicate that these gene products must be present in an active state at some level in the absence of inducer. How might induction work in such a system? Two main possibilities exist, depending on whether the lower transcription in the absence of inducer is due to low levels of R_{mox} protein and/or other regulatory proteins, or whether it is due to presence of R_{mox} in a less active state. If the level of R_{mox} is limiting in the absence of the inducer, then the inducing signal might be sensed and transduced by one of the sensor kinase/response regulatory systems, with the result being increased levels of R_{mox}. Alternatively, if the steady-state levels of R_{mox} are sufficient for induction, the signal may be involved in generating a more active form of existing molecules of R_{mox}. This could occur through a phosphorylation cascade, in which case the signal would probably be sensed by one of the sensor kinase/response regulatory systems. Alternatively, R_{mox} itself might bind the inducer, creating a more active form. That scenario leaves open the question of what the sensor kinases sense, but it is possible that they are involved in more subtle regulation, such as coordination of formaldehyde-producing and formaldehyde-consuming systems. In that case they might sense a ratio of, for instance, formaldehyde/methylene THF or methylene THF/formate. It is also possible that a combination of these mechanisms (increased levels of R_{mox} plus activation of R_{mox}) is operating. A final possibility, noted above, is that another set of

unidentified regulatory genes is involved in induction. We do know however, that *mxaB* is involved in induction of *pqqD*. One of the goals of the new project period is to obtain information on the mechanism of the induction process and the inducing signal.

Publications from this project (starred papers are included in this Appendix):

Research Papers

1. Arps, P.J., E. Minnich, G. Fulton and M.E. Lidstrom. 1993. Genetics of serine pathway enzymes in *Methylobacterium extorquens* AM1: phosphoenolpyruvate carboxylase and malyl CoA lyase. J. Bacteriol. 175:3776-3783.

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