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PROGRESS REPORT

DEPARTMENT OF ENERGY, BASIC ENERGY SCIENCES

Principal Investigator: Mary E. Lidstrom

Grant # DE-FG03-87ER13753

Genetics in Methylotrophic Bacteria

I. Promoters in *Methylobacterium extorquens* AM1

We have concentrated our effort on studying promoters in *M. extorquens* AM1. First, we have continued our work on the *moxF* promoter. We have constructed chromosomal *lacZ* fusions of this promoter to avoid the regulation problems of plasmid-borne fragments, and have shown that this is regulated normally in the chromosome. This construction has been used to study transcription in two *moxB* mutants, which have a strongly pleotropic phenotype and may be involved in transcriptional regulation. Both showed only background, vector level beta-galactosidase activity, suggesting that *moxB* encodes a function required for transcription of *moxF*. This work is described in the enclosed preprint, which has been submitted to J. Bacteriol. We have now carried out similar work with *moxQ* and *moxE* mutants, which also have pleotropic phenotypes, and these show only background beta-galactosidase activity as well. We are now in the process of generating smaller subclones to further define the promoter region. We are also constructing chromosomal fusions in other pleiotropic *mox* mutants to determine beta-galactosidase activities.

We have constructed *lacZ* fusions to some of the *mox* genes involved in the synthesis of the cofactor, PQQ, in order to carry out a similar analysis of transcription of PQQ genes (to be renamed *pqq* genes). It is likely that they are regulated separately from the other *mox* genes. However, current data suggest that expression of *pqq* genes is loosely coordinated with expression of *mox* genes.

The serine pathway enzymes are regulated differently from the *mox* and *pqq* functions, being subject to catabolite repression and showing different induction kinetics. However, they are involved in the consumption of formaldehyde, while the other two are involved in its production, and it seems clear that some coordination of expression occurs. We have now cloned the structural gene for hydroxypyruvate reductase (a key serine pathway enzyme) and are sequencing the upstream region. Preliminary *lacZ* fusion experiments suggest that this region contains a weak promoter, and we are currently defining this region.

The data available so far suggest that the *mox* and *pqq* systems are at the heart of a complex regulatory network which includes the serine pathway genes, that senses and responds to formaldehyde levels. We have developed a working model for this regulatory network that involves both two-component sensor-regulator systems as well as "coordinators" that cross-talk between the systems. Although

much of this model is hypothetical at this stage, the goal of continued research on this project will be to test and improve this working model.

II. Promoters in Methanotrophic Bacteria

We have continued to isolate *mox* genes in methanotrophs, for the purpose of studying their promoters and transcriptional regulation. We now have the *moxFJGI* operon cloned and characterized from *Methylomonas* A45, and have sequenced the upstream region. A putative "Mox" promoter sequence has been identified, and we are planning to define it in more detail. In addition, we have analyzed the *moxAKL* region from *M. albus* BG8 and will study this promoter as well.

Progress Report

1. Dates since last competitive review: July 1, 1986 - July 1, 1989

(Note: this grant was terminated at the University of Wisconsin-Milwaukee June 30, 1987 and reinitiated at the California Institute of Technology July 1, 1987 due to a move by the PI.)

2. Previous aims

The previous aims of this project were to study the organization and regulation of genes involved in methanol metabolism in methylotrophs. The specific aims were:

a) To develop basic genetic techniques and isolate new strains.
b) To clone and characterize C-1 genes.
c) To clone and begin characterizing promoter regions from these genes.

We have accomplished most of the work for the first two goals and are currently working on the third.

3. Progress

The initial intent of the previous proposal was to concentrate genetic studies on three methanotrophic strains. One of these (*Methylosinus trichosporium* OB3b), was not studied, as information became available that other groups were working on this system and we did not feel it was necessary to duplicate that effort. Instead, we have continued to study the facultative methanol-utilizer *Methylobacterium* AM1. This has proven extremely useful, as information gained from these studies has facilitated work with the methanotrophs.

Although the move to Caltech in August 1987 was of clear benefit, it did necessitate a complete change of personnel on this project. As a result, progress was slowed, and papers from these investigators are just beginning to be prepared and submitted for publication.

1) Technique development

Because genetics in the methylotrophs is still a young and growing area, it is important to continually develop new techniques to make these studies easier and more productive. A few major breakthroughs in basic genetic capabilities for the methylotrophs have been made in this granting period, including an *E. coli* expression system, a transposon mutagenesis method, an electroporation method and ongoing work to construct a *rec*A-negative strain.

a. *E. coli* expression system. One major problem we faced previously in analyzing methylotrophic genes was the inability to obtain expression in alternate hosts. Transcription-test vectors suggested that in *E. coli*, the lack of expression was due to transcriptional termination occurring within the methylotrophic genes. This is not an uncommon problem with genes from organisms having a high GC ratio (*Methylobacterium* AM1 is 68% G+C). We have now been able to circumvent that difficulty using a dual T7 promoter/polymerase system for in *vivo* expression developed by Tabor and Richardson (33). Since the T7 polymerase does not recognize transcriptional terminators, good expression has been obtained with most methylotrophic genes we have tested, which has greatly aided our genetic analyses (see below).

b. Transposon mutagenesis. Another major problem with genetics in the methylotrophs is the lack of a good transposon mutagenesis technique. A Tn5 delivery system has been reported for *Methylobacterium* AM1 (19), but the frequency is low (10^{-7}) and very few mutants were isolated. We have screened a variety of mobilizable Tn5 delivery systems in five methylotrophic strains (Table 1). In most cases, no drug resistant cells were observed from the matings under a variety of conditions. However, high frequencies (10^{-3} - 10^{-4} per recipient) of Km^r colonies were observed for *Methylobacterium* AM1 and *M. parvus* OBBP using a 24 hr. mating time with the delivery vehicle pSUP5011 (35), which contains *mob* genes within the Tn5. We are currently developing and screening a bank of 5000 Km^r colonies for *Methylobacterium* AM1, for future mutant isolation (see Research Plan).

c. Electroporation. Another difficulty with these strains is our DNA transfer system. Our only method for transferring DNA into methylotrophs is by conjugation, using mobilizable plasmids, since many attempts by this and other laboratories to isolate transducing phage or to obtain high frequency transformation have failed. Mobilization is especially time-consuming due to the slow growth involved, and it runs the risk of alterations occurring in the transferred DNA. In addition, genetic manipulations that are facilitated by direct transformation such as gene replacement with linear DNA, are not possible in these strains. Electroporation is becoming the method of choice for transferring DNA into a variety of bacteria, including many with no direct transformation system. We have developed a successful electroporation protocol for *Methylobacterium* AM1 using a BioRad electroporation unit, and are in the process of testing several methanotrophic strains for plasmid transfer. Frequencies for *Methylobacterium* AM1 are $4 \times 10^3/\mu g$ DNA using the 23 kb vector, pVK100, which is about 2 orders of magnitude lower than *E. coli* with this plasmid. Plasmid screens have demonstrated that no gross alterations occurred as a result of transfer. Although the frequencies are somewhat low, they should still be sufficient for our purposes. We are currently attempting to electroporate linear DNA using a cloned *mox*A fragment and testing for complementation of a MoxA mutant.

d. Construction of a RecA strain. Many genetic manipulations are simpler when carried out in a strain deficient for recombination. We are in the process of generating a Rec-deficient strain using a procedure that has been successful for others (36). We have used our *Methylobacterium* AM1 cosmid clone bank to complement the RecA mutation in *E. coli* HB101. The complemented strains were identified by resistance to methylmethanesulfonate (MMS). We have shown that this clone imparts both MMS and UV resistance to HB101, and are in the process of confirming restoration of recombinational ability. The putative *rec*A gene will be mapped on the clone, and then a deletion derivative will be obtained by restriction with an appropriate enzyme and religation. If electroporation of linear fragments is successful, we will use this technique to transfer linear fragments containing the construction into *Methylobacterium* AM1, and survivors will be screened for MMS sensitivity. If the linear electroporation is not successful, we will clone the construction into a non-replicating vector and use that, either with electroporation or mobilization. Even though we will be screening for the replacement event instead of selecting for it, one of these approaches should be successful, if the frequency of recombination is sufficiently high. Although all of this screening sounds like a great deal of work, we have minimized the time involved with a rapid multiple screening system we have been using. This involves setting up 96-well microtiter plates as the "master plates" and using a 96-tine sterilizable transfer unit (CloneMaster). We have found that the CloneMaster conveniently transfers reproducible amounts of cells to selective plates for screening, and when conjugation is necessary, we can obtain high frequencies of mobilization in the microtiter wells. It is also possible

to use the CloneMaster to transfer sets from plate to plate, making the system very useful and flexible.

If it is not possible to identify a RecA strain by this method, we will use insertional inactivation of the RecA gene with a drug resistance cassette so that we can select for the recombinant. However, for future manipulations involving drug markers it would be better to isolate the strain without an inserted marker. Any putative RecA strains will be confirmed by assessing frequency of recombination by using one of our Mox clones containing a drug resistance cassette inserted at least 2 kb from either end. Isolation of a RecA strain of *Methylobacterium* AM1 will be extremely useful for future genetic manipulations involving screening of cloned fragments.

e. Isolation of new strains. We are continually looking for new strains of methanotrophs that may be more amenable to genetic analysis, especially of the methane oxidation system, as the classical strains we study are difficult. In the past granting period we have collaborated with other researchers to study two new strains, each of which was originally isolated according to its ability to degrade trichloroethylene (TCE). TCE is apparently epoxidized by the MMO of methanotrophs, after which it breaks down to products that are readily utilized by heterotrophic bacteria (41). This capability has aroused considerable interest from both governmental and industrial sectors, as TCE contamination of groundwater, terrestrial sites and toxic waste dumps is rapidly becoming a national crisis.

We have worked with a group from Oak Ridge National Laboratories to characterize and study a Type I methanotroph isolated from a site of long-term TCE contamination at ORNL (41; reprint appended), and also with a group from Stanford University to study another Type I methanotroph that has unusual properties concerning its MMO and TCE degradation (Henry et al., manuscript in preparation). In both cases, the organisms appear to be typical Type I *Methylomonas* strains, and the Oak Ridge strain has not been studied further. However, the Stanford strain has unusually high TCE epoxidation rates, and preliminary evidence suggests that the MMO from this strain has a high affinity for TCE. We have sought and have obtained funding from NIH to pursue work on the biochemistry of the MMO from this strain. However, we anticipate that the two projects will cross at some period in the future, when MMO genes are isolated and promoter regions become available for study.

2) Isolation of C-1 genes from methanotrophs

The second aim of the previous proposal was to isolate C-1 genes from methanotrophs, and this has been accomplished (37). We first attempted to isolate C-1 genes from methanotrophs by complementation of both methanol oxidation and assimilation mutants of *Methylobacterium* AM1 with clone banks constructed in the broad host-range cosmid vector pVK100, with no success. However, we were successful with DNA-DNA hybridization approaches.

a. Mox F. Using an internal fragment of the *mox*F gene from *Methylobacterium* AM1 (encoding the MeDH large subunit) as a probe, we have shown that specific DNA fragments hybridize in a variety of methylotrophs. We have used this hybridization to obtain clones containing *mox*F from genomic clone banks of two methanotrophs, the Type I strain *Methylomonas albus* BG8 and the Type X strain, *Methylococcus capsulatus* Bath. The regions of homology have been mapped to specific restriction fragments, and the genes have been located within 2 kb regions in each case (37).

The identity of the putative *mox*F genes was confirmed by two methods, expression in *E. coli*, and complementation of the *mox*F mutant of *Methylobacterium* AM1. The T7 system discussed above was used to obtain expression in *E. coli* for each clone. Antisera was generated to purified MeDH from *M. albus*, which was shown to cross-react with the *M. capsulatus* protein. This antisera was used in immunoblots of the expression extracts to identify the appropriate full-length polypeptide for each of the cloned *mox*F genes. Subclones were also constructed in the broad-host range vector pRK310 and used to complement a *mox*F mutant of *Methylobacterium* AM1. It is noteworthy that in each case, the original clone containing an approximately 20 kb *Hin*dIII fragment did not complement the mutant, explaining why we did not pick up complementation initially. Since the subclones that did complement placed the coding sequences much closer to vector promoters, it may be that the native promoters were not functional in *Methylobacterium* AM1, and transcriptional termination 5' to the *mox*F genes may have created expression problems. The complemented strains grew on methanol more slowly than wild-type and expressed MeDH activity that was less than wild-type, suggesting that the heterologous MeDH proteins either functioned less well than the *Methylobacterium* AM1 MeDH proteins or were expressed at lower levels. Given the complexity of the Mox system in *Methylobacterium* AM1, it is surprising that complementation was achieved at all. Functional complementation of this *mox*F mutant suggested that the Mox transport, processing and assembly system of *Methylobacterium* AM1 not only functioned with the methanotrophic MoxF proteins, but that similar functions might be present in obligate methanotrophs as well.

b. Mox A. Since the data presented above suggested that Mox functions other than the MeDH structural genes might be present in obligate methanotrophs, we also looked at the *mox*A genes (manuscript in preparation, to be submitted to *J. Bacteriol.*). These three genes (*mox*A1, A2 and A3) appear to be involved in PQQ-apoMeDH processing and assembly (15,16). We are collaborating with C. Unkefer (Los Alamos National Laboratories) in efforts to determine what changes are made to the PQQ in these mutants. Because of their key function, it seemed likely that these genes might be conserved in methylotrophs. Probes from *Methylobacterium* AM1 were generated that were specific to each gene and these were used in hybridization experiments against 9 different strains of methanotrophic bacteria. No specific hybridization could be observed to the *mox*A1 probe at stringencies allowing up to 35% base-pair mismatch, but hybridization to specific bands was observed for the *mox*A2 and A3 probes, although the hybridization to the *mox*A2 probe was weak. The pattern of hybridization to the two probes suggested in some cases that the genes might be contiguous (as they are in *Methylobacterium* AM1) and in others, that they were not (Table 2). The hybridization of the *mox*A3 probe was used to isolate clones from our *M. albus* pVK100 genomic bank, and the putative *mox*A3 gene has been localized to an 0.8 kb fragment (Fig. 1). We have shown that the *mox*A1 and A2 probes also hybridize to the *mox*A3 clone, but the homology in both cases is to a *Sal*I-*Pst*I fragment to the left of the *mox*A3 fragment (as shown in Fig. 1). We have not been able to complement the *mox*A1, A2 or A3 mutant of *Methylobacterium* AM1 with any of several subclones in both orientations that bracket the regions of hybridization. This could be due to problems in expression or problems involving functional complementation. T7 expression studies show that this fragment encodes 5 polypeptides, transcribed left to right as shown in Fig. 1. The region to the left of the *mox*A region encodes a 34 kD polypeptide, the region to the right encodes a 24 kD polypeptide, and the region that hybridizes to the *mox*A probes encodes 3 polypeptides, of 19, 26 and 36 kD. The sizes of the *mox*A1 and A2 gene products in *Methylobacterium* AM1 are not known, but the *mox*A3 gene product is 19 kD (see below). The fragment shown to have homology to *mox*A3 (P_3-E_2) did not produce this polypeptide, but the larger *Bam*HI-*Eco*RI fragment (B_1-E_2) did, suggesting that the

*mox*A3 gene extends to the left of the P_3 site. This is further suggested by the observation that slight homology was observed to the adjoining fragment (Fig. 1).

3) Promoter characterization in methylotrophs

The third goal of the previous proposal was to isolate and characterize promoters from C-1 genes. We have studied one of these from *Methylobacterium* AM1, one from *M. albus* BG8, and we are working on another from *Methylobacterium* AM1.

a. The *mox* FJGI operon of *Methylobacterium* AM1. We have initiated promoter studies in methylotrophs with the *mox*F promoter of *Methylobacterium* AM1. Information from *Tn*5 insertions suggested that the entire *mox*FJGI region might be transcribed as an operon (15,16), and so we have studied this region in more detail. First, we have characterized the gene products of this region (see ref. 22, appended). In brief, we have used the T7 expression system to show that the *mox*FG region encodes 4 polypeptides and we have proposed to designate the genes encoding these *mox*F, J, G and I (Fig. 2). Using antisera we have identified 3 of these polypeptides as the 60 kD MeDH structural polypeptide, the 21 kD cytochrome *c*, and a 12 kD polypeptide that always copurifies with the MeDH and had been assumed by others to be an artifact of purification. These data, as well as more recent work by Nunn et al. (23) suggest that the *mox*I gene product is actually a second subunit of the MeDH. We have shown that in *Methylobacterium* AM1 the *mox*I gene product is unstable in the absence of the *mox*F gene product. The fourth polypeptide has not yet been identified and we have no clues as to its function, except that a polypeptide of similar molecular weight is induced when cells are transferred from succinate to methanol (47).

In order to further characterize the *mox*FJGI region, we have sequenced the entire 4.7 kb fragment in collaboration with D. Nunn and C. Anthony (Anderson et al., manuscript in preparation, to be submitted to Biochem. J.) and we have mapped the transcriptional start site within this region, using reverse transcriptase (D. Anderson, Ph.D. thesis). Two nucleotides were identified as start sites, at 170 and 171 bases 5' of the first codon of the *mox*F gene (Table 3). The same two nucleotides were identified in mRNA from succinate-grown cells, but the intensity was 20-30 fold less than in mRNA from methanol-grown cells. A putative promoter sequence exists upstream of the transcriptional start site that bears some resemblance to the *E. coli* consensus promoter sequence, but which shows more similarly to sequences found upstream of other *mox*F genes (Table 3). Although this is clearly insufficient data to draw any conclusions about *moxF* promoters, the conservation of sequence and spacing may indicate a role for this sequence in expression. One of the goals of this proposal will be to determine whether these sequences are important for transcription in *Methylobacterium* AM1. No sequences were identified upstream of the transcriptional start site that showed homology to promoters that have been identified in *Pseudomonas* or *Rhodobacter*, or homology to the *rpo*N or *rpo*H recognition sites.

Nucleotide sequence comparison of the *mox*F regions from *Methylobacterium* AM1 and *M. organophilum* XX (27) shows a surprising 96% homology in the coding sequences, which decreases to less than 70% outside the coding sequences, except for the putative promoter region, which is almost 90% homologous. In addition to the similarities in the *mox*F genes, other interesting features have been revealed. Two sets of direct repeat sequences exist upstream of the putative promoter. One of these (starts 201 bp upstream) consists of two identical 9 bp sequences (CCCCTGCGC) separated by 6 bp and the other (starts 278 bp upstream) consists of two identical 10 bp sequences (CCGCCCCCGG) separated by 145 bp. Although in organisms with high %G+C ratios (*Methylobacterium* AM1 is 68% G+C), runs of G's and C's are common, earlier *Tn*5

insertion data suggested that the regions containing these repeats were required for MeDH expression (15, 16). Therefore, it is possible that these sequences have significance, and one of the aims of the current application will be to find out if that is true. Within the coding sequences, other structures could be identified. A 14-bp hairpin structure (ΔG = -36 kcal) exists between the first two genes (*mox*F and *mox*J), and another 9-bp hairpin (ΔG = -10 kcal) is present downstream of the last gene (*mox*I). Again, in genes from high GC bacteria, computer-generated hairpin structures are common, and so these may not be physiologically relevant. However, they could represent points for transcriptional termination, transcriptional attenuation or mRNA decay termination. A similar 13 bp hairpin structure exists downstream of the *mox*F gene in *M. organophilum* XX (27), and recent sequence data by N. Harms from the *Paracoccus denitrificans mox*F region show that a strong hairpin structure is present between *mox*F and a second unidentified downstream ORF (39). This ORF encodes a protein of 29kD and may be analogous to our *mox*J, which encodes a protein of approximately 30kD (22). However, we have found no significant homology between this downstream ORF and our *mox*J gene, either at the nucleotide or amino acid sequence level. We have also found no significant homology between the *Methylobacterium* AM1 *mox*J sequence and any of the proteins in our GenBank database.

Preliminary Northern blot analyses have been carried out, and although the hybridization bands were light, they were reproducible with different RNA preparations. Two transcripts of approximately 2.7 and 4.2 kb, respectively were detected in mRNA from methanol-grown cells, but not in mRNA from succinate-grown cells using three different DNA fragments as probes (Figure 2). A fourth probe covering the 3' end of the region did not detect any transcripts, but this blot was a particularly weak one, and the experiment must be repeated. The smaller transcript appears to be slightly more prominent than the larger. The nucleotide sequence data predict that a transcript of 4.3-4.5 kb would be required for the entire *mox*FJGI region, and that a transcript of 2.7 kb should extend just beyond the *mox*J gene. These data suggest that the *mox*FJGI region is transcribed as an operon. In addition, the position of the probes and the transcriptional start site mapping suggest that both transcripts have similar 5' ends. Further work will be necessary to determine the start and stop points of these two transcripts and their relative half-lives (see Research Plan). It is interesting to point out that in *M. organophilum* XX, the only transcript identified with a 5' *mox*F probe was one of 2.1 kb, the size predicted from sequence data for a transcript covering the *mox*F gene only (27). Therefore, it appears that these two similar systems (see Fig. 3, main text) are transcribed differently. So far, our data are suggestive that mRNA processing or differential stability might be involved in the *mox*FJGI operon, and part of this proposal will address that possibility (see Research Plan).

b. The moxF promoter of *Methylobacterium* AM1. We have used the broad host-range promoter probe vector pGD500 (40) to construct a *lac*Z transcriptional fusion to a 1.55 kb *Xho*I-*Sal*I fragment containing the putative promoter region (Fig. 2), in order to begin defining the functional regulatory sequences. However, we have run into unexpected difficulties with this construction. This fusion directs β-galactosidase synthesis in *Methylobacterium* AM1 in an orientation-specific fashion, suggesting that it contains the *mox*F promoter. However, the activity is the same in methanol- and succinate-grown cells (Table 4), which is surprising, since we have shown that the *mox*F mRNA is 20-30X more abundant in methanol-grown cells than in succinate-grown cells. In cells containing the construction, the chromosomal copy of MeDH is regulated normally, as assessed both by protein level and activity measurements (Table 4). MeDH is constitutive in cells containing the cloned *mox*FJGI operon (*Xho*I-*Bam*HI partial fragment or *Hin*dIII-*Bam*HI fragment, Fig. 2), suggesting that the β-galactosidase level

seen in the pGD500 construction reflects constitutive (non-repressed) expression. In the *mox*FJGI constructions, MeDH is at the same level in cells of wild-type and *mox*F mutant strains. It is not clear why this is so, but the regulation of the cloned genes is clearly abnormal.

Several possible explanations for these observations exist. The lack of proper regulation of the plasmid-borne sequences could be due to titration of a regulatory protein (pGD500 exists in 5-7 copies per cell), but this is unlikely, since it appears that the chromosomal gene is regulated normally. It is not due to readthrough by plasmid promoters, as the background activity of pGD500 is relatively low. Alternatively, it is possible that the *mox*F promoter is constitutive, and that the levels of mRNA are regulated mainly by differential turnover. This also seems unlikely, since the cloned MeDH gene is expressed constitutively, and these constructions are predicted to contain the entire *mox*FJGI transcript. The that sequences upstream of the *Hin*dIII site may be required for repression in the absence of methanol in a cis-acting fashion. This is possible, although the region tested covers almost 2kb of DNA 5' to the transcriptional start site. A few cases in prokaryotes of regulatory sequences as far upstream as 0.6-1.0 kb are known (59) and so this cannot be ruled out. Likewise, downstream regions can sometimes be involved in regulation (59), but in this case the *Hin*dIII-*Bam*HI fragment used includes over 3 kb 3' to the *Sal*I site. It seems most likely this problem is due to an unknown artifact of cloning, having something to do with the higher copy number or the increased supercoiling in the plasmid. We are currently attempting to discriminate between these possible explanations by constructing a chromosomal insertion using the *Xho*I-*Sal*I-*lac*Z fragment cloned into pBR322, a plasmid that is non-replicating but mobilizable in *Methylobacterium* AM1 (Fig. 3). This will result in a complete regulatory region transcriptionally fused to *lac*Z, with the MeDH gene under the control of the *Xho*I-*Sal*I fragment. By measuring both MeDH (protein and activity) and β-galactosidase activity in this construction as compared to controls, we should be able to determine whether the *Xho*I-*Sal*I fragment contains methanol-inducible promoter activity. If we see normally regulated *mox*F protein and β-galactosidase, it would suggest that the problems with the pGD500 construction are due to cloning artifacts (Fig. 3). If we see constitutive β-galactosidase but normal *mox*F protein, that would suggest some difficulty exists with this particular fusion to *lac*Z or that downstream sequences are important. If so, we will generate other *lac*Z fusion constructions, both larger and smaller and test activities in those strains. If we see regulated β-galactosidase and constitutive MeDH, it would suggest that sequences upstream of the *Hin*dIII site are required for normal repression of this system. If this is observed, we will generate other, larger constructions. We have the adjacent *Hin*dIII fragments on both sides cloned and so these constructions will not be difficult. If we cannot obtain a plasmid construction with a properly regulated *lac*Z-promoter fusion, it will make further experiments more difficult, but not impossible (see Research Plan).

No β-galactosidase activity above the background level with vector alone was observed with the *lac*Z-*mox*F promoter fusion plasmid in *E. coli.* This is not surprising, since our expression evidence suggested that this promoter was not recognized in *E. coli.*

c. The *mox*A promoter(s) of *Methylobacterium* AM1. We are currently attempting to define the promoter(s) of the MoxA region in *Methylobacterium* AM1 also. This is an interesting set of genes in relation to the *mox*FJGI system, since the MoxA gene products are not major proteins in the cell, as judged from one-dimensional gel electrophoresis, while it is known that the Mox F, J, G and I gene products are major cell polypeptides (22). Part of the current proposal will focus on the comparison of *mox*F and *mox*A promoters. It is not known whether the three *mox*A genes are cotranscribed,

although *Tn*5 data suggest they are not (15, 16). One always has to be skeptical of these data, however, due to the possibility of internal promoters in the transposon. We are sequencing the entire Mox A region, and it is 90% finished, with the main gaps occurring in the *mox*A2 sequence. The data so far show that the only open reading frames for *mox*A1 and A3 occur left to right, as shown in Fig. 3, (main text), and the putative ORF for *mox*A3 is 19 kD. T7 expression experiments confirm that the *mox*A3 region encodes a 19 kD polypeptide transcribed from left to right as shown in Fig. 3 (main text), but it has not yet been possible to identify the polypeptides from the other two genes. So far, no sequences similar to the putative *mox*F promoter have been identified upstream of any of these genes. However, that may not be surprising, considering the apparent difference in expression levels. Once the sequence is completed and analyzed, it may be possible to identify putative promoter sequences by comparison. Alternatively, these genes appear to be spaced together quite closely, and they may comprise a single transcriptional unit. Northern blot analyses should answer this question (see Research Plan).

d. The moxF promoter of *Methylomonas albus* BG8. We have also begun characterizing the *mox*F promoter of the methanotroph, *M. albus*. A 1.5 kb *Hin*dIII-*Bam*HI fragment at the 5' end of the *mox*F gene has been cloned into pGD500. Various constructions were mated into *M. albus* and tested for β-galactosidase activity. As shown in Table 5, significant activity was observed only with the construction in the correct orientation. We are now constructing a series of deletion fragments within this region, to further define the promoter region (Fig. 4). We have also sequenced this 1.5 kb fragment, as well as an additional 0.5 kb region that includes the 5' end of the *mox*F coding sequences, and we are just beginning studies to map the transcriptional start site. However, a sequence exists 202 bp upstream of the first *mox*F codon within the 1.5 kb fragment having promoter activity, which bears significant resemblance to the putative *mox*F promoter sequence noted above for *Methylobacterium* AM1 and *M. organophilum* XX (Table 3). Part of the current proposal will address the definition of this promoter sequence. In this case as in the *mox*F promoter of *Methylobacterium* AM1, no activity above background was observed for the fusion plasmid in *E. coli*.

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4. Lidstrom, M.E., D.N. Nunn, D.J. Anderson, R.L. Stephens and M.G. Haygood. 1987. Molecular biology of methanol oxidation. In: Microbial Growth on C-1 Compounds, H. van Verseveld and A. Zehnder, eds., Martinus-Nihoff, Amsterdam, pp. 246-254.

In preparation:

5. Anderson, D., T. Morris, D. Nunn, C. Anthony and M. Lidstrom. (in prep) The *mox*FJGI region of *Methylobacterium* AM1. to be submitted to Biochem. J.

6. Kim, Y., P. Arps and M.E. Lidstrom. (in prep) Mox A genes in methanotrophic bacteria. to be submitted to J. Bacteriol.

7. Kuhn, M. and M.E. Lidstrom. (in prep) Analysis of the *mox*F promoter region of *Methylomonas albus* BG8. to be submitted to J. Bacteriol.

8. Henry, S., D. Grbic-Galic, A. DiSpirito and M.E. Lidstrom. (in prep) Trichloroethylene degradation by a Type I methanotroph. to be submitted to Arch. Microbiol.

Table 1. Frequency of drug resistance in different methylotrophs with 5 different Tn5 delivery vehicles.

Plasmid	**Replicon**	**Strains showing Frequency above background**	**Ref.**
pM075	R91-5 (IncP10)	none	19
pGS9	pACYC184	none	34
pSUP5011	pBR325	OBBP (freq. = 10^{-4}) AM1 (freq. = 10^{-3})	35
pSUP102::Tn5-20	pACYC184	none	R. Puhler
pSUP102::Tn5-21	pACYC184	none	R. Puhler

strains tested:

Methylocystis parvus OBBP = OBBP
Methylobacterium AM1 = AM1

Methylomonas A4
Methylomonas albus BG8
Methylomonas MN

Table 2. Hybridization of genomic DNA from methanotrophs to *mox*A2 and *mox*A3 probes from *Methylobacterium* AM1. All fragments are from *Eco*RI digests except those from *Methylomonas* A1, which are from *Pst*I digests.

Strain	Hybridizing Fragments (kb) *mox* A2 probe	*mox* A3 probe
Type I:		
Methylomonas sp. strain A1	10	8.4
Methylomonas albus BG8	5.5	6.0
Type II:		
Methylocystis sp. strain LWY	1.5	3.7
Methylocystis parvus OBBP	1.5	3.7
Methylosinus trichosporium OB3b	5.7	5.2
Methylosinus sporium 5	4.0, 1.9, 1.55	14, 1.2
Type X:		
Methylococcus capsulatus Bath	9.6, 4.7	12, 5.2, 3.6

No detectable hybridization to: *Methylomonas* MN, *Methylobacter capsulatus* Y

Table 3. Comparison of the sequence 5' to the start of *mox*F genes from four different methylotrophs.

	-35		-10	+1	
COLI	TTGACA	XXXXXXXXXXXXXXXXX	TATAAT	XXXXXG	
AM1	CT **AAAGACA**	**TCGCGTCCAATCAAAGCC**	**TAGAAA**	**ATATA***G*---170bp---	first Met
XX	GT **AAAGACA**	**TCTCCTTCAATCAACGCC**	**TAGAAA**	**CGATA** ---171bp---	first Met
BG8	AA **AAAGGAA**	CTTTTCCCGACTCACA	**CGGAAA**	**AGCCA** ---201bp---	first Met
Pd	G **ATCGGA**	CGGGGAAAAACCCCC	**GAGAA**	GTCGG ---167bp---	first Met

bold-face = similarities for AM1 and XX
italics = mapped transcriptional start sites
Mox F = methanol dehydrogenase structural gene

XX = *Methylobacterium organophilum* XX (serine pathway methanol-utilizer) ref. 27
AM1 = *Methylobacterium* sp. AM1 (serine pathway methanol-utilizer)
BG8 = *Methylomonas albus* BG8 (RuMP pathway methane-utilizer)
Pd = *Paracoccus denitrificans* (autotrophic methanol-utilizer) ref. 26

Table 4. Comparative activities in wild-type, *mox*FJGI and *mox*F/*lac*Z fusion containing strains.

Strain (plasmid)	Growth Substrate	β-galactosidase	Methanol dehydrogenase
WT	methanol	-	+++
	succinate	-	+
WT (pGD500)	methanol	+	+++
	succinate	+	+
WT (pGD500::*Xho*I-*Sal*I)	methanol	+++	+++
orientation 1	succinate	+++	+
orientation 2	methanol	+	+++
	succinate	+	+
WT (pRK310::*Xho*I-*Bam*HI)	methanol	-	++
	succinate	-	++
UV26 (pRK310::*Xho*I-*Bam*HI)	methanol	-	++
	succinate	-	++
WT (pRK310::*Hin*dIII-*Bam*HI)	methanol	-	++
	succinate	-	++
UV26 (pRK310::*Hin*dIII-*Bam*HI)	methanol	-	++
	succinate	-	++

orientation 1 = right to left as shown in Fig. 2
orientation 2 = opposite (wrong) orientation

UV26 = MoxF mutant (no protein, no activity)
+++, 10-20X higher than +

Table 5. β-galactosidase activities in *M. albus* containing pGD500 constructs with promoter inserts.

Construct	**β-galactosidase units**
pGD500 (vector)	<1
pGD500::*Hin*dIII-*Bam*HI, orientation 1	400
pGD500::*Hin*dIII-*Bam*HI, orientation 2	20

orientation 1, left to right as shown in Fig. 4
orientation 2, opposite of orientation 1

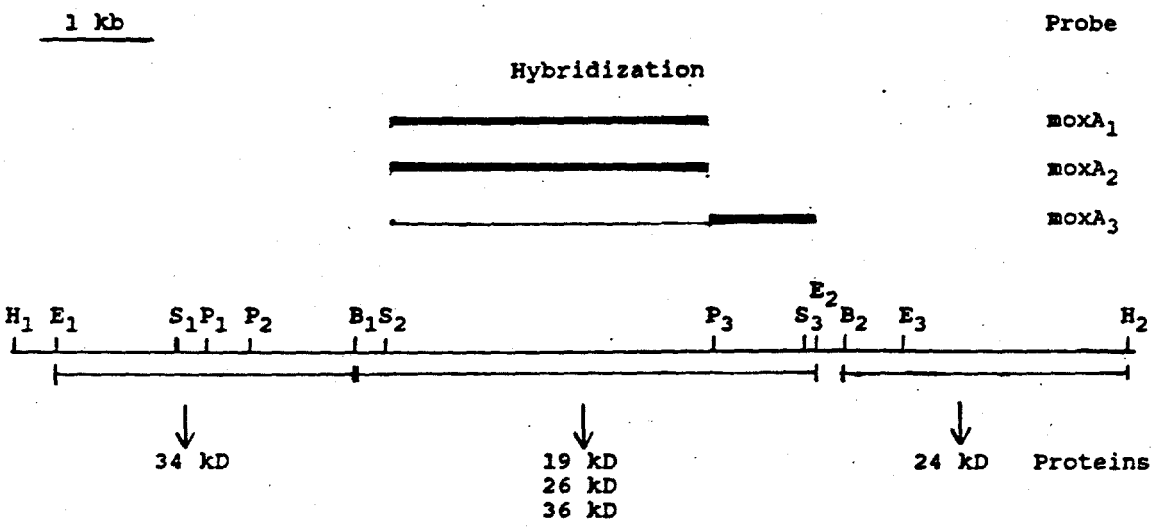


Figure 1. The MoxA region of *Methylomonas albus* BG8. Proteins detected in T7 expression extracts are shown below, and areas of major hybridization to each probe are shown above.

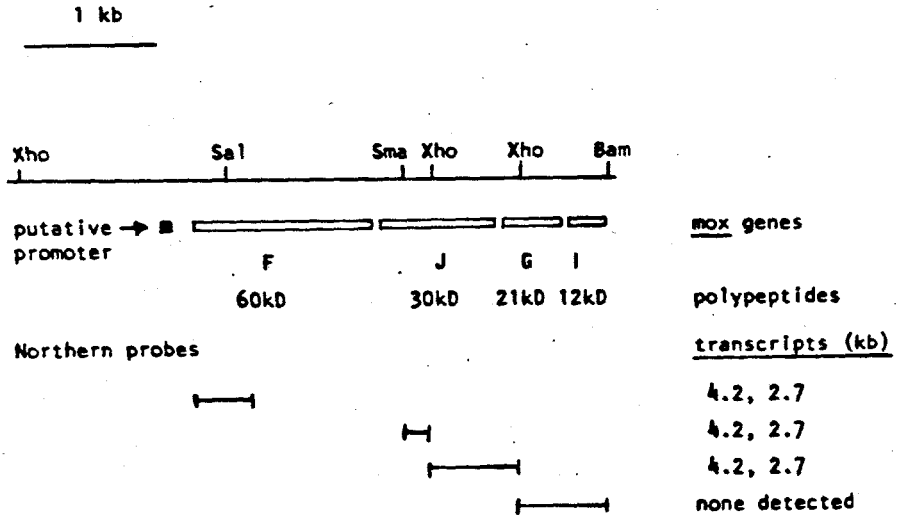
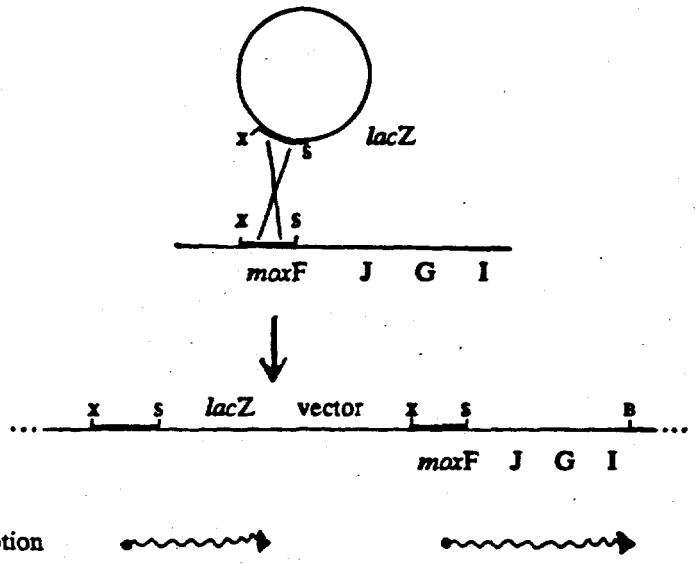


Figure 2. The *mox*FJGI region of *Methylobacterium* AM1.



repeated DNA in black

possible results:

lacZ	MeDH	Conclusion
normal	normal	some cloning artifact exists
const.	normal	some problem with this fusion or downstream sequences important
normal	const.	upstream sequences important
const.	const.	must be a combination of problems

Fig. 3. The proposed chromosomal *mox*F-*lacZ* fusion.

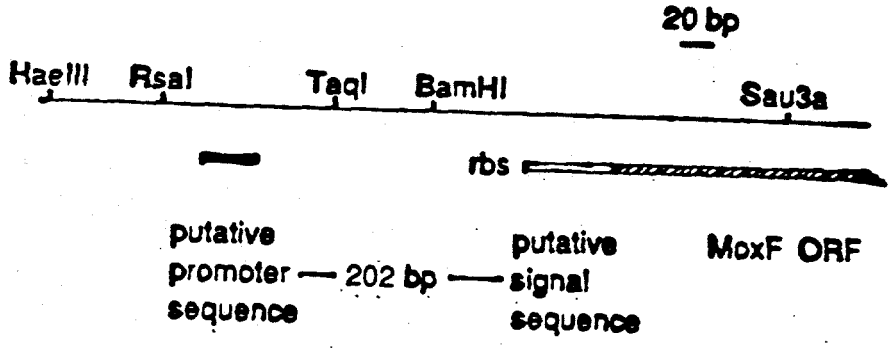


Figure 4. Partial map of the 5' portion of the moxF region from M. albus showing some useful restriction sites. This region has been sequenced, as well as an additional 1 kb upstream.

ANNUAL REPORT SUMMARY

CALIFORNIA INSTITUTE OF TECHNOLOGY - Pasadena, CA 91125

Genetics in Methylotrophic Bacteria
M.E. Lidstrom, Environmental Engineering Science $87,034

The purpose of this project is to use genetic techniques to study the transcriptional regulation of C-1 specific functions in methylotrophic bacteria. The approach involves analysing C-1 specific genes in a facultative methanol utilizer, *Methylobacterium* AM1 and using this organism as a host to study genes encoding similar functions in methane-utilizers. We have focused on methanol oxidation genes, and have cloned the MoxF, A1, A2 and A3 genes from both methanol and methane utilizing bacteria. We have cloned promoter regions from these genes into broad host range promoter cloning vehicles using *lacz* as the reporter gene and are currently characterizing these regions structurally and functionally.

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PROGRESS REPORT
MARCH 1, 1988- MARCH 1 1989

MARY E. LIDSTROM
CALIFORNIA INSTITUTE OF TECHNOLOGY

GENETICS IN METHYLOTROPHIC BACTERIA
DE-FGO3-87ER13753

The purpose of this project is to use genetic techniques to study gene organization and expression in methylotrophic bacteria. In the past year we have continued our work on the methanol oxidation system in the methanol utilizer, *Methylobacterium* AM1 and in the methanotroph, *Methylomonas albus* BG8.

1. Sequence of the *mox*FJGI operon of *Methylobacterium* AM1

We had previously reported expression studies that demonstrated the presence of four Mox genes in the MoxFG region of *Methylobacterium* AM1 with gene products of 60, 30, 21 and 10 kD. These were identified as the methanol dehydrogenase large subunit, an unknown protein, the cytochrome c_L structural gene and the small methanol dehydrogenase-associated peptide that may be a second subunit (see enclosed reprint, Anderson & Lidstrom). In collaboration with D. Nunn and C. Anthony (Univ. of Southampton), we have sequenced the entire 4.5 kb region encompassing the *mox*FJGI operon of *Methylobacterium* AM1, confirming the presence of these four open reading frames. Several interesting features have emerged from this analysis: 1) the *mox*F gene is over 96% identical to the *mox*F gene from *Methylobacterium organophilum* XX at the nucleotide sequence level, 2) each of the genes is separated by 50-100 bp, 3) each contains a putative signal sequence with strong homologies to each other and 4) a strong hairpin structure (putative terminator) exists 50 bp downstream of the operon. A region of approximately 1 kb upstream of the operon has been sequenced also, and a putative promoter region has been identified (see next section). A manuscript describing this work is in preparation and will be submitted to Biochem. J.

2. Promoter for the *mox*FJGI operon of *Methylobacterium* AM1

Reverse transcriptase has been used to map the transcriptional start site for the *mox*FJGI operon of *Methylobacterium* AM1. A single start site is observed, located 170 bp upstream of the translational start in mRNA from both succinate and methanol grown cells. The amount of the mRNA in methanol-grown cells is approximately 10-fold greater than in succinate-grown cells. Therefore, this operon is transcriptionally regulated in *Methylobacterium* AM1. Initial data from Northern blots suggest that the entire region is transcribed as a unit, but smaller size classes are also observed, suggesting that internal processing may occur. Directly upstream of the transcriptional start site is a putative promoter sequence that is identical to a putative promoter sequence previously identified by Machlin & Hanson for the *M. organophilum* XX *mox*F gene. We have cloned a 1.55 kb fragment that includes this putative promoter into the promoter probe vehicle pGD500, in a transcriptional fusion to *lac*Z. About 40-fold higher β-galactosidase activity is observed with constructions carrying this fragment in the correct orientation than in the opposite orientation, and methanol grown cells show 5-10 fold higher activity than succinate grown cells. Both methanol dehydrogenase activity and the MoxF, J, G and I proteins

are similarly induced. A manuscript describing this work is currently being prepared for submission to J. Bacteriol.

3. Mox A1, A2 and A3 genes of *Methylobacterium* AM1

We have sequenced the entire MoxA region of *Methylobacterium* AM1, and have identified putative open reading frames for each of the genes. We have so far only confirmed these for the *mox*A3 gene, by expression in *E. coli*, but these studies are continuing. All three genes are transcribed in the same direction. The next step in this project will be to clone and characterize the promoter regions for these three genes.

4. MoxF promoter in *Methylomonas albus* BG8

We have sequenced the region upstream of the *mox*F gene of the methanotroph, *M. albus* BG8 and have identified a putative promoter sequence that shows substantial homology to the putative promoter region for the *Methylobacterium* AM1 *mox*F gene. Preliminary attempts to map the transcriptional start site have not been successful, apparently due to poor quality mRNA preparations, but we are continuing these efforts. We have cloned a 1.2 kb fragment including this putative promoter sequence into pGD500, and have demonstrated orientation-specific β-galactosidase activity. Experiments are in progress to further define this putative promoter.

5. Transposon mutagenesis

We have screened a variety of transposon delivery vehicles for Tn5 insertion in several methanotrophs and methanol-utilizers. Only one of these plasmids, pSUP5011, shows promise for further studies. This plasmid was constructed by R. Simon. It contains a ColE1 replicon and has the *mob* genes from RK2 inserted into a non-coding region of Tn5. In *Methylocystis parvus* OBBP and *Methylobacterium* AM1, Km^r colonies were observed at frequencies several orders of magnitude above controls. We have optimized this process by altering mating parameters and we are currently determining mutation frequencies.

6. Degradation of trichloroethylene

Since the long term goals of this project include utilization of methanotrophs for commercial purposes, we have collaborated with Deane Little (Oak Ridge National Laboratories) to study a newly isolated methanotroph capable of cooxidation of methane and trichloroethylene. This work is described in an enclosed reprint (Little et al.).

RENEWAL PROPOSAL

The goals of the original proposal have changed only slightly. We will continue to characterize the Mox genes and clone and sequence putative promoter regions.

PUBLICATIONS

Little, C.D., A.V. Palumbo, S.E. Herbes, M.E. Lidstrom, R.L. Tyndall and P.J. Gilmer. 1988. Trichloroethylene biodegradation by a methane-oxidizing bacterium. Appl. Environ. Microbiol. 54:951-956.

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.

ANNUAL REPORT SUMMARY

CALIFORNIA INSTITUTE OF TECHNOLOGY - Pasadena, CA 91125

Genetics in Methylotrophic Bacteria

M.E. Lidstrom, Environmental Engineering Science $84,996

The purpose of this project is to use genetic techniques to study the transcriptional regulation of C-1 specific functions in methylotrophic bacteria. The approach involves analysing C-1 specific genes in a facultative methanol utilizer, *Methylobacterium* AM1 and using this organism as a host to study genes encoding similar functions in methane-utilizers. We have shown that the methanol oxidation system in AM1 requires 12 gene functions, at least 7 of which appear to be non-regulatory. We have begun studying these 7 functions in methanotrophs, and our data suggest that these are conserved in methanol and methane utilizing bacteria, at both genetic and functional levels. We are currently cloning promoter regions from these genes into broad host range promoter cloning vehicles using *lacz* as the reporter gene. These will be characterized and used to study transcriptional regulation in response to environmental parameters.

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PROGRESS REPORT
JULY 1, 1987 - MARCH 1 1988

MARY E. LIDSTROM
CALIFORNIA INSTITUTE OF TECHNOLOGY

GENETICS IN METHYLOTROPHIC BACTERIA
DE-FGO3-87ER13753

The purpose of this project is to use genetic techniques to study gene organization and expression in methylotrophic bacteria. In the past year we have continued our work on the methanol oxidation system in the methanol utilizer, *Methylobacterium* AM1, and we have begun studying this system in methanotrophs.

1. Development of an expression system for methylotrophic genes.

One of our difficulties in studying methylotrophic genes has been the lack of a heterologous gene expression system for determining gene products on DNA fragments. Dawn Anderson in my laboratory has used a dual T7 promoter/T7 polymerase expression system, that has been successful for expressing genes of *Pseudomonas* species in *E. coli*. She has optimized a procedure that now results in detectable expression of methylotrophic genes in *E. coli*.

2. Identification of Mox genes in *Methylobacterium* AM1.

Using the T7 expression system described above, Dawn Anderson has shown that the *mox*FG region encodes four polypeptides. Three of these have been identified using antisera and Western blots as the large subunit of the methanol dehydrogenase, the cytochrome c_L and a small protein that copurifies with the methanol dehydrogenase. This small protein was always assumed to be a purification artifact, but our data suggest it is involved in methanol oxidation. We have also shown that it is unstable in the absence of the large subunit. The function of the fourth polypeptide has not been identified, and we are currently attempting to construct mutants in this gene to assess their phenotype. This work suggests that 12 genes are required for methanol oxidation in *Methylobacterium* AM1. This project is described in the enclosed preprint, in press in *J. Bacteriol.*

3. Characterization of the *mox*A1, A2 and A3 genes of *Methylobacterium* AM1.

Besides the genes noted above, three other Mox genes identified in *Methylobacterium* AM1 are likely to be conserved in methylotrophs. These are the *mox*A1, A2 and A3 genes, which appear to encode factors necessary for proper cofactor/apoprotein assembly. David Nunn, previously supported by DOE funds, is now in Chris Anthony's laboratory in Southampton. He has shown that the A2 and A3 gene functions involve modifying the PQQ, and A1 appears to involve assembly. Young Kim and Tina Morris in my laboratory have studied these genes in more detail, mapping them more precisely and initiating sequencing. The sequencing is about half finished at this point and we expect the final sequence to delineate the size of the gene products as well as potential regulatory sequences. Tina Morris has expressed these three genes in the T7 expression system, and her preliminary evidence suggests that three small polypeptides are encoded in this region. She is currently determining the location of each of these.

4. Survey of *mox*A genes in methanotrophs.

Because it seems likely that the *mox*A gene functions will be conserved in methylotrophs, we have surveyed homology to these genes in methanotrophs. Young Kim has isolated suitable

probes for each of the three genes and used these in Southern blots against genomic digests of several Type I and Type II methanotrophs. The *mox*A1 gene shows very little hybridization, even at low stringency, the *mox*A2 gene shows some hybridization, and the *mox*A3 gene shows substantial hybridization. The patterns of hybridization suggest that unlike in *Methylobacterium* AM1, these genes are not located on the same DNA fragment in any of these methanotrophs.

5. Cloning of the *mox*A3 gene from *Methylomonas albus* BG8.

The hybridization observed with the *mox*A3 probe has been used to clone a putative *mox*A3 gene from a genomic clone bank of *Methylomonas albus* BG8 DNA using colony blots. The homology has been mapped to an 0.8 kb region. Peggy Arps is currently attempting to complement the *Methylobacterium* AM1 *mox*A3 mutant with subclones of this DNA, and to determine the gene product(s) using the T7 expression system.

RENEWAL PROPOSAL

The goals of the original proposal have changed only slightly. Because of the success of the T7 expression system, we have discovered more Mox genes in *Methylobacterium* AM1. Therefore, some effort will continue to be made defining this system in more detail. The goals concerning the methanotrophic genes will remain unchanged. That is, we will characterize the genes, and clone and sequence putative promoter regions.

PUBLICATIONS

Anderson, D.J. and M.E. Lidstrom. The *mox*FG region encodes four polypeptides in the methanol-oxidizing bacterium *Methylobacterium* sp. strain AM1. J. Bacteriol., in press.