PROGRESS REPORT

3/1/91 TO 6/30/93

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GENETICS OF BACTERIA THAT OXIDIZE ONE- CARBON COMPOUNDS

PRINCIPAL INVESTIGATOR: RICHARD S. HANSON

I. Manuscripts provided with this progress report.

1. Xu, H.H, M. Viebahn and R.S. Hanson (1993a). Identification of methanol-regulated promoter sequences from the facultative methylotrophic bacterium *Methylobacterium organophilum* XX. J. Gen. Microbiol. **139:** 743-752.

2. Xu, H.H, M. Viebahn and R.S. Hanson (1993b). Characterization of regulatory sequences upstream from the *mox* F gene of *Methylobacterium organophilum* XX. Submitted for publication. FEMS Microbiol. Lett.

3. Schnaith, L.M., R.S. Hanson and L. Que Jr. (1993) Dioxygen dependent hydrolysis of pBR322 by a diiron complex at a unique site. Submitted to Proc Natl. Acad. Sci., U.S.

4. Hanson, R.S., B.J. Bratina and R.S. Hanson (1992) Phylogeny and Ecology of methylotrophic bacteria. pp 285-301 In J.C. Murrell and D.P. Kelly (eds.) Microbial Growth on C1 Compounds. Intercept. Andover, U.K.

5. Hanson, R.S. (1992) Introductory chapter. pp 1-21 In J.C.Murrell and H. Dalton (eds.) Methane and Methanol Utilizers. Plenum Press, New York.

II. Regulation of the synthesis of methanol dehydrogenase.

In the past several years researchers have identified at least 20 genes whose products were required for the oxidation of methanol to formaldehyde in three different facultative methylotrophic bacteria. These genes include structural genes for a cytochrome c_L (*mox* G) that is a specific electron acceptor for methanol dehydrogenase (MDH), and the two structural genes that encode the large subunit (*mox* F) and smaller subunit (*mox* I) of MDH . Other genes are required for the synthesis of the prosthetic group of MDH, Pyrroloquinoline quinone (PQQ), and proteins required for assembly of the active MDH in the periplasm. Three genes are believed to be required for incorporation of calcium into the MDH tetramer.

The principal investigator's group has studied the regulation of methanol oxidation in the pink-pigmented-facultative methylotroph *Methylobacterium organophilum* XX. We have mapped several genes and have sequenced the *mox* F gene and sequences upstream of *mox* F. We had tentatively identified several genes required for the transcription of the MDH structural genes in three methylotrophs (Bastein *et al* , 1989. J. Bacteriol. 55: 3124-3130). In the previous proposal, the P.I. proposed to establish an *in-vitro* transcription/translation system to study the function of the regulatory gene products. Further studies demonstrated that the regulation of transcription of these genes was far more complex than imagined at that time and the research plan was modified to determine the number and function of the regulatory genes using genetic approaches.

Howard Xu, a graduate student in the P.I.'s laboratory, constructed a new

promoter-probe vector (pHX200) in order sort out the role of the various genes in regulation of transcription of the structural genes involved in methanol oxidation. The broad-host-range mobilizable vector contains a promoterless *xyl* E gene encoding catechol 2,3-dioxygenase from *Pseudomonas* as *aeruginosa* as a reporter gene. The Shine-Dalgarno sequence of the *xyl* E gene was modified to resemble the *mox* F S-D sequence. Without this modification, expression of the *xyl* E gene from the *mox* F promoter was poor. The vector has a polylinker positioned upstream of the modified S-D sequence. When a 448 bp fragment containing the *mox* F promoter and sequences upstream of the transcriptional start site were inserted into the vector, the expression of *xyl* E was methanol dependent in wild-type *M. organophilum* XX. The construction of this vector and its use in cloning methanol regulated promoter sequences from *M. organophilum* XX are described in more detail in the manuscript by Xu *et al* (1993a).

Construction of this promoter-probe vector was necessary because attempts to clone the *mox* F upstream sequences into other promoter probe vectors containing the *lac* Z or *pho* A genes did not lead to expression of either gene in *M. organophilum* XX. The vector has one shortcoming. There is a promoter upstream of the polylinker which is expressed in *Methylosinsus trichosporium* OB3b,*E. coli, Pseudomonas syringae, Rhizobium meliloti* and some other bacteria but not *M. organophilum* XX and closely related methylotrophs. Dr. Teresa Barta has constructed a modified vector with an omega-fragment upstream of the polylinker. The omega-fragment contains a transcription terminator that makes the plasmid more useful for cloning promoters from a wider range of Gram-negative bacteria.

The construct identified as pHX200V-47 in the manuscript by Xu *et al* (1993a) was used to identify genes that were required for expression of the *mox* F gene. Genes identified as *mox* B,Q,E,M,N and D have been shown to be required for expression of *mox* Fp::*yl* E transcriptional fusions. We have presented a model in the proposal that describes our hypothesis regarding the possible functions of these genes as general or specific activators of methanol regulated genes. Figure 1 of this progress report illustrates this model. It is proposed that *mox* Q and *mox* E gene products are members of a two component regulatory system that result in positive control of *mox* F. The product of *mox* B is also specifically involved in activation of transcription of *mox* F. The products of genes *mox* M, *mox* N and mox D are required for expression of *mox* Fp::*xyl* E transcriptional fusions and mutations in these genes result in the loss of methanol dehydrogenase activity. It is proposed that the product of one or more of these genes is a general activator for methanol inducible genes including *mox* F. Support for this hypothesis is presented in the manuscripts by Xu et al (1993a and 1993b).

Dr. Xu partially purified a DNA binding protein that was shown to bind to a 353 bp fragment upstream of the *mox* F gene. These assays are described in Xu et al 1993b. Since that manuscript was prepared, a smaller fragment (-68 to +10 relative to the transcriptional start site) that was produced by PCR amplification was used in DNA binding assays. DNase I footprinting analysis indicated that nuclotides -63 to -23 relative to the transcriptional start site were protected by the DNA binding protein. The protein did not bind to a DNA sequence prepared by PCR amplification from the -65 to -252 region upstream of *mox* F. The DNA binding protein was detected in crude extracts of cells of wild-type *M. organophilum* XX but not in extracts of mutants with lesions in *mox* Q or *mox* E. Extracts of all other *mox* mutants had DNA binding proteins that bound to the DNA fragment described above. Therefore, we propose that the product of one of these genes is a positively acting regulatory protein that binds to a region labelled symmetry #2 (Figure 1) or more specifically the sequence TGTCT-N_{10}-AGACA that resembles positively acting regulatory protein binding sites (Xu *et al* 1993b). The other gene product may be a sensory protein of a two component regulatory system. We also propose that the product of *mox* B also interacts at this site to specifically activate transcription of *mox* F (Figure 1).

FIGURE 1. A model for the regulation of *mox* F and *mox* W transcription.

The nucleotiide sequence upstream of the *mox* F structural gene can be divided into four distinct structural and functional regions which are named nucleotide segments 1,2,3 and 4. Segment 1 contains the *mox* B promoter and part of a region of dyad symmetry (symmetry #2, TGTCT-N10-AGACA). This sequence is similar to a consensus sequence for procaryotic positive regulator binding sites. Segment 2 also overlaps with the region labelled symmetry #2 and contains two copiesof a septanucleotide (AGAAATG). The third copy of this septanucleotide is located in segment 3. Removal of segment 3 abolished *mox* F expression. Segment 4 contains the -10 and -35 hexamers of the *mox* W promoter. Removal of this segment led to a 59% reduction in the expression of *mox* F::*xyl* E transcriptional fusions. The model proposes that a general positive regulator encoded by *mox* M, *mox* N or *mox* D binds to the conserved septanucleotide located within segment 3 and activates expression of both *mox* F *and mox* W. Other positive regulators, the products of *mox* B and *mox* Q *or mox* E , are proposed to bind to segment 2 (TGTCT-N10-AGACA) and further enhance the expression of *mox* F through interaction with RNA polymerase.

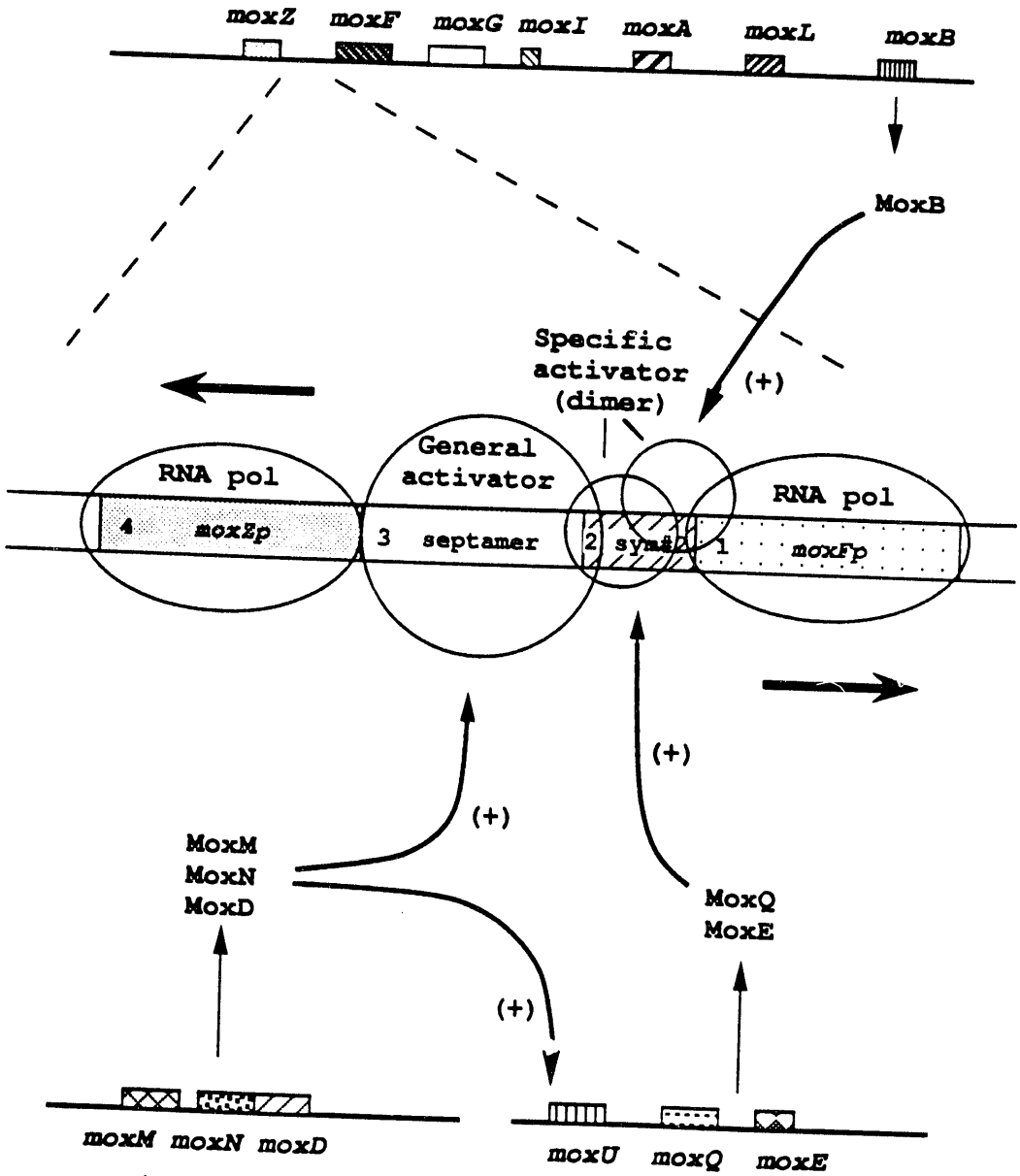
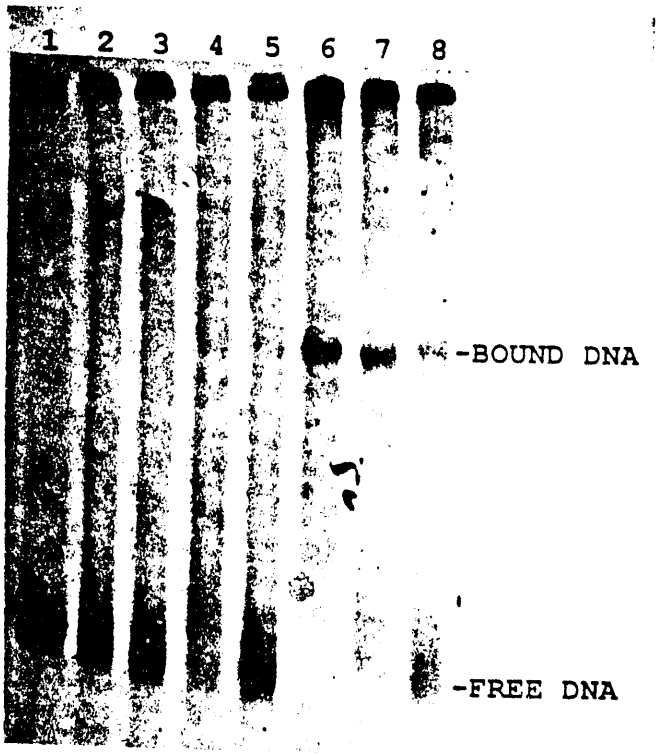


FIGURE 2. Mobility shift DNA binding assay.

A 353 bp fragment containing the *mox* F promoter region was end-labeled with α-^{32}P-dATP and was used as the probe. Crude protein extract (lane 8) as well as protein partially purified from DNA cellulose (eluted at 0.6 M KCl,and named fraction D)(lanes 2to 7 were added to DNA binding assay mixtures. Lane 1. control (no protein added): Lanes 2 and 3 contained 0.4 ml of fraction D. Lanes 4 and 5 contained 0.75 ml of fraction D and lanes 6 and 7 contained 1.5 ml of fraction D.



Deletion analysis has shown that the region labeled symmetry #1 in Figure 2 of Xu et al 1993b was required for expression of *mox* Fp::*xyl* E transcriptional fusions in *M. organophilum* XX (Xu et al, 1993b). This region is proposed to contain a binding site for a general activator of methanol inducible genes.

The promoter probe vector has been used to clone several other methanol inducible promoters. Cloned DNA fragments carrying enzymes encoding proteins required for formaldehyde assimilation and PQQ biosynthesis were digested with *Sau* 3A and the digestion products were ligated into the *Bam* HI site of pHX200. The ligated plasmids were transformed into *E. coli* JM109 and the tetracyline resistant transformants were mated with wild type *M. organophilum* XX as described by Xu *et al* (1993a). Agar plates containing a mineral salts-methanol-tetracycline medium were sprayed with a solution of catechol and colored colonies were selected and transferred to a mineral salts succinate-tetracycline medium. Clones that expressed *xyl* E only on agar with methanol were selected after spraying with a solution of catechol. Induction by methanol was confirmed using liquid cultures as described by Xu *et al* (1993b). The *E. coli* clones carrying methanol inducible transcriptional fusions were preserved and the inserts of six were sequenced. All contained one or more copies of a conserved septanucleotide sequence (AGAAATG) fund upstream from potential promoters. This is indicated as a potential binding site for a general activator of methanol inducible genes in Figure 1. All of these transcriptional fusions required functional *mox* M, *Mox* N *and mox* D genes. Unlike

mox F:: *xyl* E transcriptional fusions, they were expressed in *mox* B, *mox* Q and *mox* E mutants (unpublished results).

III. Studies of the regulation of the synthesis of soluble methane monooxygenase in *Methylosinus trichosporium* OB3b.

Dalton and his coworkers first described the production of sMMO during growth of some methanotrophs in media with low levels of copper. We have shown that the rapid oxidation of trichloroethylene, other halogenated hydrocarbons and some halomethanes occurred only in cells grown on low levels of copper. The oxidation of TCE correlated well with the production of protein components as measured on Western blots. We had cloned structural genes encoding some components of sMMO and have used these probes to detect these genes in methanotrophs. We and J.C. Murrell at Warwick University, U.K. observed that the genes which encoded protein components of sMMO and the ability to produce sMMO was restricted to a limited number of methanotrophs. Our probes have also been employed by collaborators (Prof. Gary Saylor's group at the University of Tennessee) to detect methanotrophs that possess these genes in environmental samples. We have also used the probes to characterize methanotrophs in bioreactors and environmental samples (Hanson *et al*, 1992).

J.C. Murrell's group has mapped and sequenced the structural genes encoding sMMO components from *Methylosinus trichosporium* OB3b and *Methylocapsulatus capsulatus* (BATH).

The information presented above is reviewed in more detail in the proposal.

During the current grant period, Dr. Barta has cloned a PCR amplified 452 bp DNA fragment located upstream of the *mmo* X gene of *M. trichosporium* OB3b into a modified form of pHX200 (Figure 2). The amplified region is shown by the arrows at the top of the figure. The transcriptional start site for mmo X is identified (T) as is the original translational start site (ATC) that has been modified from the original ATG so that this region contained a restriction site. The modified vector has an omega cassete inserted upstream of a polycloning site to prevent transcription of *xyl* E from a promoter located somewhere upstream of the cassete. This promoter causes *xyl* E expression in some bacteria including *M. trichosporium* OB3b. The fusion in the vector shown at the bottom of the figure is not expressed in *E. coli* or *P. syringae* but is expressed in *M. trichosporium* OB3b. This transcriptional fusion should be very useful for defining sites upstream of *mmo* X essential for transcription and for the isolation of mutants that lack regulatory proteins essential for activation or repression of the transcription of genes encoding sMMO protein components.

IV. Reinvestigation of the ability of *Methylobacterium organophilum* to grow as a facultative methanotroph.

Ms. Marieke Viebahn, who is employed at the Technische Fachhochschule Berlin, Germany, spent a one year leave in the P.I's laboratory. She succeeded in growing *M. organophilum* XX on methane as a sole carbon and energy source. This bacterium also grows with methanol and several other organic compounnds as a sole carbon and energy sources (Patt *et al.* 1976 in Bibliography of the proposal). The bacterium oxidized methane and synthesized intracytoplamic membranes when grown with methane but not when grown with methanol, succinate or other organic substrates. The ability of this bacterium to oxidize methane was rapidly and permanently lost when grown on alternative substrates. T We have found no evidence for loss of a plasmid in strains that lose the ability to grow with methane. For these reasons, some members of the scientific community have been reluctant to accept the ability of this bacterium to use methane on the grounds that the methanotrophic culture may not have been pure. We had originally gone to great lengths to exclude the presence of other bacteria but decided to more rigorously exclude the possibility.

FIGURE 3. Construction of a promoter probe vector for studying expression of sMMO genes

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1   ATCGATCCGGCGCGCGGTACCCGTCTCGCATTGCGCAACGCCGTCGCCACCGCATCGTCATTGATGACCGTCGAATGCGCGGTCACT
88  CATATTCCGCCGAGCGATCCGACATACGGCTTCGATCCGCATCTCGCCGCTGCGACGCGTGAAGATCCGCGCAGCTGAAGCAGCGAA
175 TGGCGAAAGAATGAAAGCAGCTGCGGCGACTGTTCGCCGCAGCGTTTTTTTATGCGCGGATGCGCCCGTGATTTCATCGAATGCAGT
262 CATCATCACGATACGTATGGTTCTTTGACATGTGTATCGCGCATCGCCATAGCACGATCATTTTTACGCGTCGCACGTAGTTGCGAA
349 TGCGACGAATCCCAACAAATACCGATGAAATCTATTATCCGCAGCGAGTGGCACAGGCCTTGCCAAATAAGAAGCGTCGACGCTTCC
436 GCGCAGATGCGCAAGGACGAGAGACGTTTCGAAAACCCGAGGTTTGGAAAGCGGCGACGCGGTTCGGATGATCGACGCGCGCAATGA
523 GCGCGAGCACGACGGTCCGAAACAAAAGAAAACTCTGTCAGGAGGAACAAGGATCCCG
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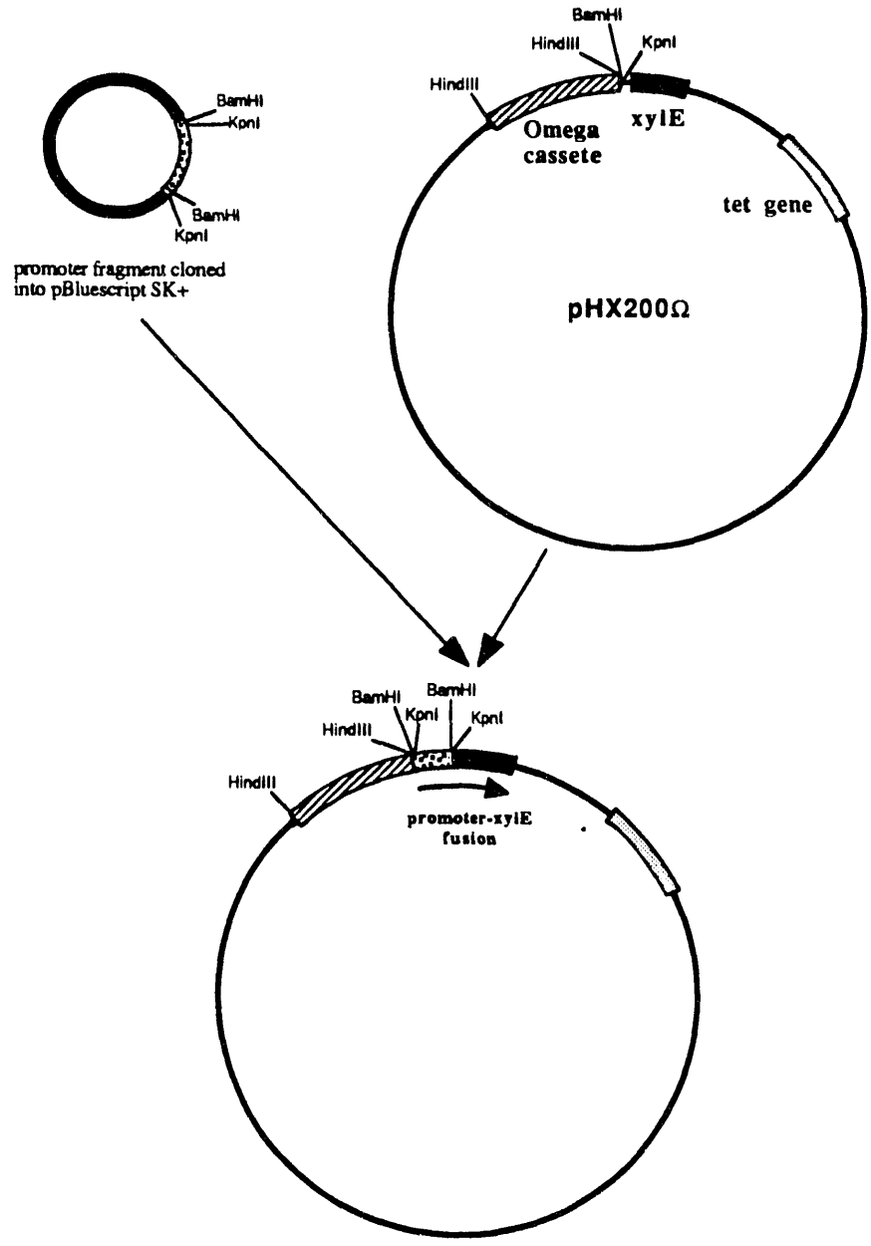
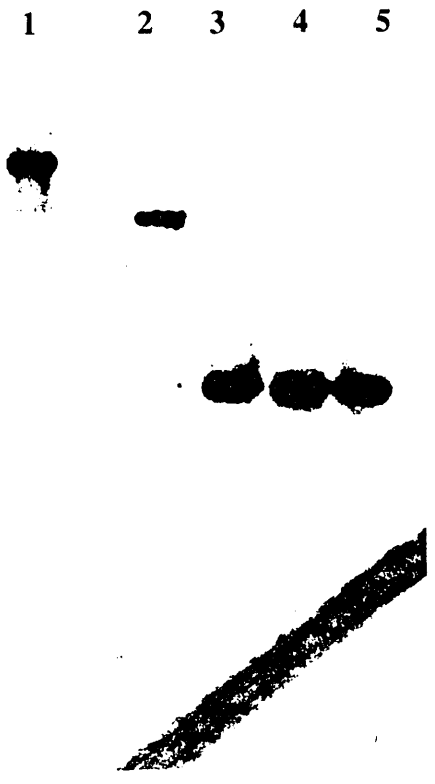


DNA from cells grown on different substrates. The DNA was treated with different restriction enzymes. The sizes of fragments from cells grown on different media were indistinguishable (Figure 4). Southern blots were prepared and were hybridized with a 2.5 kb fragment containing the gene for the large subunit of MDH (*mox* F). The restriction fragments that hybridized to the *mox* F gene were identical in DNA extracted from cells of *M. organophilum* XX grown with methane and methanol and DNA extracted from cells grown on Nutrient Agar (Figure 3). If another bacterium in a mixed culture oxidized methane to methanol which served as the substrate for growth of *M. organophilum* XX, this bacterium should be the dominant bacterium in the culture grown with methane and different restriction fragments should be detected in the different cultures. The *mox* F gene hybridized with DNA from all the methylotrophs we have tested. It appears to be highly conserved.

This bacterium remains interesting as an exception to the general observations that methane oxidizing bacteria are obligate methylotrophs and do not grow with multicarbon substrates. The difficulties encountered with growing this strain, however have precluded its use as the bacterium of choice for genetic studies of the regulation of methane monooxygenase synthesis. We will continue to define the conditions required for good expression of MMO and will if possible determine which type of MMO is present in this bacterium. In the meantime, *Methylomonas* 761 appears to be a better choice for genetic studies of the particulate MMO as related in the proposal.

FIGURE 4 : A Southern blot containing DNA restriction fragments derived from *Pvu* II treated DNAs of methylotrophic bacteria and hybridized with ^{32}P-labeled *mox* B gene.

The DNAs in each lane were obtained from bacteria grown on the following carbon sources: Lane 1, *Methylobacterium extorquens* AM1 grown with methanol: Lane 2, *M. trichosporium* OB3b grown on methane; Lane 3, *M. organophilum* XX grown in nutrient broth: Lane 4: *M. organophilum* XX grown with methanol; Lane 5, *M. organophilum* XX grown with methane.



V. Cleavage of plasmid pBR322 by a synthetic diiron catalyst that oxidizes methane.

As described in the proposal and the accompanying manuscript by Schnaith *et al* (1993), we have observed that a synthetic diiron catalyst in combination with H_2O_2 and a reductant will oxidize methane and also hydrolyzes plasmid pBR322 at a site near the ampicillin resistance gene. The catalyst behaves like a restriction enzyme, production overlapping ends with 3'-hadroxy and 5'-phosphate groups. DNA ligase rejoins the ends to form covalently closed circular plasmid molecules. Hydrolysis requires supercoiled plasmid.

The reasons for pursuing this work as part of this project are presented in the proposal.

VI. Ph.D. students completing their degrees while supported by this grant.

H. Howard Xu completed his Ph.D. degree in May, 1993 and has been awarded a U.S. Deptartment of Energy Distinguished Postdoctoral Fellowship for work in the laboratory of Prof. Robert Tabita in the Department of Microbiology at the Ohio State University, Columbus, Ohio.

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