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PROGRESS REPORT, FIRST YEAR

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A Study of the Genetics and Regulation of Methane Oxidation

As suggested by the reviewers of this proposal, our initial work has concentrated on the plasmids and genetics in M. ethanolicum. A reprint describing the isolation and characterization of this organism is enclosed. A preprint of a manuscript which will soon be submitted to the Journal of Bacteriology, entitled "Characterization of Plasmids in Methylobacterium ethanolicum" is also enclosed.

I. Characterization of the plasmids in M. ethanolicum

A. Evidence for plasmid-mediated methane oxidation

M. ethanolicum loses the ability to grow on methane upon successive transfer to non-methane substrates. Spontaneous methane (mtn)-negative strains are never revertible and always lack the plasmids which are present in the wild-type strains. Plasmids are not detectible in these strains either by visualization on agarose gels or by hybridization of whole cell DNA with radiolabelled purified plasmid DNA by the technique of Southern (4). These data suggest a gene or genes involved in growth on methane may be plasmid-encoded in M. ethanolicum. R. S. Hanson (personal communication) has isolated putative transconjugants from a cross between mtn^+ M. ethanolicum (plasmids$^+$) and mtn^- M. organophilum (plasmid$^-$). His laboratory is in the process of characterizing the plasmid content of these transconjugants. It seems likely that one or more plasmids in M. ethanolicum is self-transmissible and carries a gene or genes which both

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organisms require for growth on methane. These data have prompted us to characterize the plasmids in M. ethanolicum.

B. Plasmid characteristics

M. ethanolicum contains three plasmids, which can be separated from each other using sucrose gradients. Restriction digests and measurements of plasmid electron micrographs show their molecular weights to be 50, 102 and 118 x 10^6 daltons (md), respectively. We have carried out hybridizations of whole and restricted plasmid DNA with radiolabelled DNA from the 50 md plasmid. The results suggest the 102 md plasmid contains a small region (less than 20%) of homology to the 50 md plasmid, while the 118 md plasmid contains no detectible homologous sequences to this plasmid. We have constructed a restriction map of the 50 md plasmid using the restriction endonucleases EcoR1, Hind III, Bam H1 and Xba 1.

C. Curing rate

An accurate determination of curing rates has been hampered by the background growth of M. ethanolicum on agar plates in the absence of added carbon and energy sources. However, we have recently discovered that a more reliable measure of methane-dependent growth can be obtained if agarose is used to replace agar in plates. Preliminary results suggest that approximately one-third of the colonies which grew up on nutrient agar plates were incapable of growth on methane. Plasmids were not detectible in any of these mtn^- clones. The loss of all three plasmids simultaneously is curious and may suggest a common replication factor.

II. Isolation of Mutants

Our attempts to increase the spontaneous frequency of mutations in M. ethanolicum using ultraviolet light and ethyl methanesulfonate have not been successful. We are currently testing nitrosoguanidine as an alternative.

We have been successful in isolating amino acid auxotrophs using both the mu-Tn5 "suicide" vector, pJB4J1(1), and a ColE1-RP1 hybrid containing Tn7. We are in the process of characterizing these mutants. We are also beginning experiments to isolate mutants unable to grow on methanol and on methane.

III. Genetic Transfer Systems

All attempts to demonstrate transfer of either chromosomal or plasmid markers by genetic transformation have failed, although a wide range of techniques have been tested.

We have obtained transfer of wide host range plasmids from other bacteria into M. ethanolicum using a filter mating technique at the frequencies noted below.

Plasmid	Characteristics	Transfer frequency
RP4	Inc P	10^{-6}
R68.45	Inc P	10^{-6}
pMR5	Inc P rep^{ts}	10^{-6}
pJB4J1	Inc P-mu-Tn5 hybrid	10^{-6}
Sa	Inc W	10^{-3}
pAS8	ColE1-RP1Tra-Tn7 hybrid	10^{-2}

Now that we have auxotrophic markers available, we will begin testing each of these plasmids for their ability to mobilize these markers. The high transfer frequency of Sa and pAS8 make these attractive candidates. Plasmid pAS8 is not

maintained in M. ethanolicum; transfer frequency was determined as the frequency of Tm^r transconjugants (Tn7 marker).

IV. pRK290/pRK2013

Plasmid pRK2013 is a ColE1-RK2 tra hybrid which mobilizes the RK2-derived cloning vector, pRK290. This system has been used successfully in Rhizobium to clone specific genes, mutagenize these cloned DNA fragments in E. coli, and then transfer the mutagenized piece to the original Rhizobium strain. These mutations have been transferred into the Rhizobium DNA by forcing the cloning vector out using an incompatible plasmid and selecting for marker exchange by homologous recombination (2,3). These plasmids together are a powerful genetic tool, providing the researcher with simultaneous cloning, mutagenesis, and gene transfer systems.

We have found that plasmid pRK2013 mobilizes pRK290 into M. ethanolicum from E. coli at a frequency of 10^{-2}. pRK2013 is not maintained in M. ethanolicum. We are beginning experiments to clone pieces of each plasmid from M. ethanolicum into pRK290. These recombinant plasmids will be used firstly, to attempt to obtain complementation of cured (mtn^-) strains, and secondly, to obtain transposon insertions in each plasmid. These experiments have been approved by the local Recombinant DNA Committee (January, 1981).

V. Summary

We are well into the objectives of the original proposal. We have characterized the plasmids present in M. ethanolicum as to size, relatedness and curing rate. We have isolated auxotrophs and are testing the ability of several plasmids to promote mobilization of these markers. We have identified a cloning vector which can be used not only to clone the genes of interest but to isolate

mutants in these genes and place a selectable marker on each of the plasmids. I expect to see considerable advances in the next few months. Specifically, we should isolate a series of methane and methanol mutants, determine which of our plasmids carries the mtn gene(s), and identify methane-specific proteins on two-dimensional O'Farrell gels. In addition, we should be well on our way to the cloning of the methane genes and development of a genetic system. A more detailed appraisal of future experiments is presented in the accompanying renewal proposal.

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