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Project Summary

Application of Recombinant DNA Technology to Methane Biosynthesis

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A project was conducted to clone the genes encoding the polypeptide subunits of the enzyme methyl-coenzyme M methyl-reductase (methyl CoM-reductase). The experimental approach was to purify the enzyme (initially from *Methanobacterium thermoautotrophicum*, and subsequently from *Methanococcus vannielii*), produce antibodies against the enzyme, and use these antibodies to screen *Escherichia coli* colonies for clones that synthesized antigens with which the anti-methyl CoM-reductase antibodies reacted. The *E. coli* strains contained plasmids or were pre-infected with bacteriophages that had been constructed by *in vitro* DNA recombinant techniques to contain fragments of either *M. thermoautotrophicum* or *M. vannielii* genomic DNA's. The expectation was that the *E. coli* clones that reacted with the anti-methyl CoM-reductase antibodies would contain cloned methanogen DNA sequences encoding part or all of the methyl CoM-reductase polypeptides. However, this technique frequently generated false positive signals. Most of the study period was used in improving the technology to decrease the number of artifactually positive signals and in screening and analyzing positive clones that ultimately were found to contain none of the desired genes. Because enzyme purification and antibody production were very time-consuming and artifactual results were being obtained, alternative approaches were investigated, and experiments were undertaken to identify the sequences in methanogen DNA's that act as regulatory signals for gene expression. This information will be needed in the next stage of this project — the construction of DNA molecules containing methyl CoM-reductase sequences that can be manipulated to direct the synthesis of the enzyme when reintroduced into methanogens.

Although the specific goal of cloning the genes encoding methyl CoM-reductase has yet to be reached, the experiments completed have produced valuable information describing the structures and organization of methanogen genes and the structure of methanogen messenger RNA's.

This Project Summary was developed by EPA's Municipal Environmental Research Laboratory, Cincinnati, OH, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

The anaerobic fermentation of waste biomass to methane concentrates 90% of the solar energy entrapped in this material by photosynthesis into a convenient energy source. As already demonstrated for many other bioprocesses, this bioprocess should be amenable to genetic manipulation using the techniques of genetic engineering. Researchers should be able to delineate the enzymology of methane biogenesis, identify and modify the genes responsible for methane biogenesis, and ultimately introduce such a capability into microbial species currently incapable of methane biogenesis. The substrates used for methane biogenesis are very limited (e.g., acetate, formate, methanol, methylamines, carbon dioxide, and hydrogen), and often the supply of these substrates to methanogens is the rate-limiting step in methane ane production into species capable of

converting a more extensive range of substrates to methane would be a major achievement. Alternatively, genetic information for increased substrate use might be introduced into currently existing methanogens to increase their capabilities. The long-term goals of our research program are to obtain an understanding of the structure and activity of enzymes involved in methane biogenesis and to create improved methanogenic species for use in conversion of biomass to methane. Our immediate objective is to isolate biosynthetic genes from methanogens and to determine their structures and the mechanisms of their regulation. The most abundant enzyme in methanogenic bacteria is methyl-coenzyme M methyl-reductase. This enzyme is responsible for the terminal step in methane biogenesis, in which a methyl group (CH_3-) bound to a cofactor known as coenzyme M is reduced to methane (CH_4). This document summarizes our experiments designed to clone and characterize the genes that encode the subunit polypeptides of methyl-coenzyme M methyl-reductase.

Procedures and Results

Enzyme Purifications

Methyl-coenzyme M methyl-reductase (methyl CoM-reductase) comprises approximately 10% of the protein of methanogens. The holoenzyme form of methyl CoM-reductase is a complex of 3 differently-sized polypeptide subunits ($\gamma_2 \beta_2 \alpha_2$) with a combined molecular weight of approximately 300,000 daltons. It was purified following a published procedure that required the development of anaerobic column chromatography facilities. An anaerobic glove box was modified to accommodate columns and a fraction collector. A refrigeration unit and a spectrophotometer were located adjacent to but outside the anaerobic compartment. Conduits were built that allowed anoxic cooling fluid or column eluates to be circulated through the refrigeration unit or through the spectrophotometer and then be returned to the anaerobic environment.

Purification of the enzyme was followed by polyacrylamide gel electrophoresis (PAGE) of column extracts. The enzymatic assay for the enzyme, namely methane generation from methyl-CoM, was not routinely used. The abundance of the enzyme and known sizes of the subunit polypeptides made identification by PAGE a practical assay. Figure 1 shows the elution profile and a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the material in the column fractions that results from chromatography of methyl CoM-reductase through a DEAE-sephadex column. The three subunit polypeptides (α, β, γ) of methyl CoM-reductase are clearly discernible with molecular weights of approximately 68,000, 45,000, and 38,000 daltons, respectively. Methyl CoM-reductase contains tightly bound nickel atoms, and in one purification, the culture was grown in the presence of ^{63}Ni to provide a specific radioactive label for the enzyme. Purification of the labeled enzyme using the PAGE assay confirmed that these procedures resulted in purification of methyl CoM-reductase as the ^{63}Ni

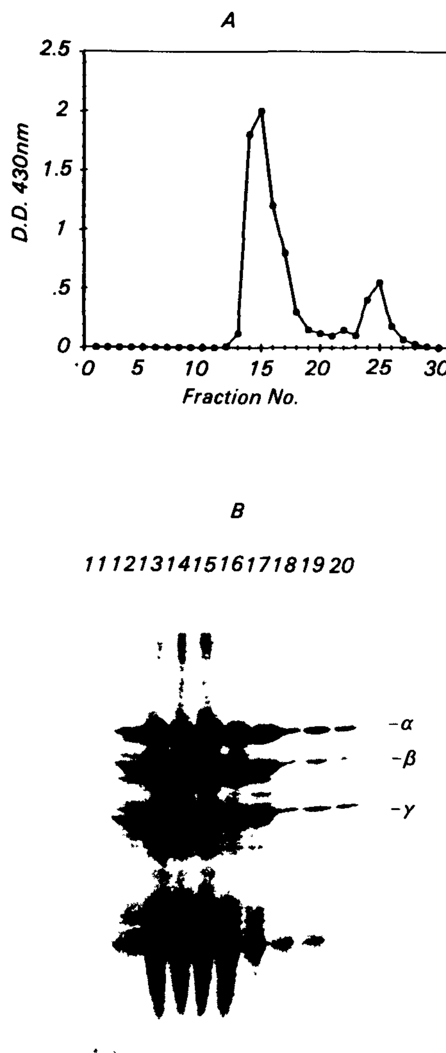

Figure 1. *Elution profile and SDS-PAGE analysis of material in column fractions that result from chromatography of methyl CoM-reductase through a DEAE-sephadex column.*

copurified with the polypeptides identifi as the subunits of methyl CoM-reducta The enzyme was initially purified fro *Methanobacterium thermoautotrophicu* (year 1), which required the use of French pressure cell to rupture the cel Later, enzyme preparations (years 2 a 3) were obtained from *Methanococc vannielii*. This species has only a pr teinaceous cell wall and was much mo readily lysed. In addition, this species h become the species of choice in exp iments designed to develop a genetic e change system for methanogens. Futu work with the cloned methyl Co reductase genes will need this DN transfer technology, and therefore it w decided to concentrate all methanoge related research on this species.

The enzyme preparations obtained fro both *M. thermoautotrophicum* and *vannielii* were shown by PAGE to almost free of contaminating polype tides. But even with the use of silver stai ing to visualize polypeptides in polyacr lamide gels, it was never possible to o tain enzyme preparations entirely free other polypeptides. Several modificatio to the purification procedure we evaluated (e.g., different column materi and changes in salt concentrations us in eluting solutions). Although improv ments were obtained, the preparatio were never absolutely free of conta inating polypeptides. For this reaso preparative PAGE was eventually used the final step in purification. The enzy subunits were separated by SDS-PAG and the regions of the gels that contai ed the individual subunits were cut fro the gel slabs. These gel fragments we then passed through a fine-bore syrin needle to fragment the gel. The resulti gel slurries were incubated in a sm volume of buffer to allow the polypeptid to elute from the gel. These solutions we then used to vaccinate rabbits for antibo production.

Production of Antisera

Antisera were obtained by vaccinati of rabbits. The first antigen used was co plete methyl CoM-reductase from *M. th moautotrophicum*. This material contain all three subunit polypeptides, and ther fore the antisera obtained contained an bodies that reacted with all three polype tides. In later experiments, the SDS-PAG separated subunits were used as antige Antibodies raised against the separat subunits have the advantage that positive signal can be related directly the gene encoding the polypeptide us as the antigen. However, they also ha

the potential disadvantage that the separated polypeptides are denatured during SDS-PAGE and therefore may not have all the same antigenic determinants as the native enzyme.

A standard vaccination regime was used in which the initial inoculation was followed 10 weeks later by a booster vaccination. Sera were taken at weekly intervals following the second vaccination, and the presence of anti-methyl CoM-reductase antibodies was assayed. Very variable antibody titers were observed. In some animals, high titers were rapidly obtained and maintained, whereas in other animals, very little useful antiserum was produced. In particular, we obtained high-titer antibody preparations for the two smaller subunits (β, γ) of the *M. vannielii* enzyme, but we were unable to obtain a high-titer antibody preparation from animals vaccinated with the largest (α) subunit polypeptide of this enzyme.

Titration and Evaluation of Antisera

Antisera preparations were analyzed by the standard enzyme-linked immunosorbant assay (ELISA) plate procedure to determine their titers. Sera that contained high titers of anti-methyl CoM-reductase antibodies were then evaluated by Western blotting to determine which polypeptides were recognized by the antibodies contained in the antisera. Lysates of *Escherichia coli, M. thermoautotrophicum,* and/or *M. vannielii* were subjected to electrophoresis alongside preparations of the purified methyl CoM-reductases. Following electrophoresis, the separated polypeptides were blotted onto nitrocellulose paper, which was then immersed in the antiserum being analyzed. Antibodies in the serum bound to the appropriate antigen bound, in turn, to the nitrocellulose. These antibody-antigen complexes were identified by using ^{125}I-labeled sheep-anti-rabbit antisera to bind to the rabbit antibodies in the complexes. The locations of the ^{125}I were then determined by autoradiography. Using this procedure, it was possible to demonstrate that antisera produced by vaccination with the native enzyme contained a mixture of antibodies, including antibodies specific for each of the three polypeptide subunits. As expected, antisera raised against a purified subunit contained antibodies directed against only that polypeptide. The Western blotting experiments also demonstrated that antibodies raised against the *M. thermoautotrophicum* polypeptides recognized and bound to the equivalent (small, medium, or large) polypeptides in preparations of methyl CoM-reductase from *M. vannielii* and vice-versa. These two enzymes must therefore have conserved amino-acid sequences and conserved secondary and tertiary structures. This cross-reactivity is potentially very valuable in that antibody preparations raised against the enzyme from one methanogen can be used to screen for genes cloned from other methanogens. We determined that the cross reactivity extends beyond *M. thermoautotrophicum* and *M. vannielii*, since anti-methyl CoM-reductase antibodies prepared against the enzymes from these two species also bind to methyl CoM-reductase polypeptides in extracts of *Methanobrevibacter smithii, Methanosarcina barkeri, Methanococcus voltae,* and *Methanococcus thermolithotrophicus.*

Unfortunately, the Western blotting experiments also demonstrated that all antisera contained antibodies that reacted with *E. coli* proteins. This was presumably due to the *E. coli* population present in the environment and the gut of the rabbits used to produce antisera. The presence of anti-*E. coli* antibodies made it impossible to use the antisera preparations directly to screen for methanogen antigens synthesized in *E. coli.* Antisera were therefore mixed with lysates of *E. coli* so that the antibodies that bound to *E. coli* proteins would be removed by adsorption to the proteins in the lysates. This procedure had to be repeated several times before all anti-*E. coli* antibodies were removed from the anti-methyl CoM-reductase antisera preparations. Most of the DNA cloning procedures employed λ-bacteriophage-based vectors. The *E. coli* lysates used to remove *E. coli* antibodies were therefore obtained from λ-infected *E. coli* cells to obviate problems caused by anti-λ antibodies in rabbit antisera. Such anti-λ antibodies were detected in Western blotting experiments. Following removal of the anti-*E. coli* antibodies, the titer of anti-methyl CoM-reductase antibodies was again determined. The antisera also had to be evaluated using the experimental procedures for screening *E. coli* recombinant clones. A preparation of purified methyl CoM-reductase was serially diluted, and an aliquot of each dilution was spotted onto a nitrocellulose filter. The filter was then submerged in the antiserum being tested, and following an incubation period for antigen-antibody interaction, the filter was removed and washed, and the presence of antigen-antibody complexes was determined. Two related procedures for this determination were used. In both cases, the antigen-rabbit-antibody complex was bound by sheep anti-rabbit antibody. The presence of the sheep antibody was then detected by its being either covalently linked to ^{125}I or linked to horseradish peroxidase. The presence of ^{125}I was detected by autoradiography, and the presence of horseradish peroxidase was detected by adding a chromogenic substrate for this enzyme. The ^{125}I-based assay was found to be approximately 10-fold more sensitive (recognizing as little as 10 pg of antigen) than the enzyme-based assay. In the majority of screening experiments, ^{125}I-labeled sheep anti-rabbit antiserum was used. To screen the plaques on lawns of *E. coli*, we used dilutions of rabbit antiserum that could detect less than 0.1 ng of methyl CoM-reductase concentrated in a plaque-sized spot on a cellulose nitrate filter.

Construction of Gene Libraries

The concept of a gene library is that of a population of recombinant DNA molecules in which every gene of the genome of the organism being studied is present in one or more of the DNA molecules. The number of DNA molecules needed for a complete library is determined by the size of the organism's genome and the size of the individual fragments of DNA cloned into hybrid recombinant molecules. We decided to use λ-based vectors that allow the cloning of large 10 to 30 kilobase (kb) DNA fragments and thereby decrease the number of clones needed to constitute a gene library. In the case of methanogens, such a gene library should be less than 1,000 different recombinant molecules. Our initial experiments were to obtain λ1049 and λCharon 30 libraries of *M. thermoautotrophicum* DNA (genome size of $1 \bullet 1 \times 10^9$ daltons). This required producing restriction fragments of *M. thermoautotrophicum* genomic DNA within the size range of 15 to 30 kb. A major problem was that the only mechanisms available to lyse this species incorporated a physical rupturing procedure such as sonication, French pressure cell, or cryoimpacting. With these procedures, it was impossible to obtain sufficiently high-molecular-weight genomic DNA. Without such DNA, we could not produce the large restriction fragments needed for λ cloning. Some recombinant clones were obtained, but it was unlikely that a fully representative gene library was produced. To circumvent this problem, libraries were produced using the plasmid vector pUC8, in which much smaller fragments of DNA (less than 10 kb) generated by a range of restriction

enzymes, could be cloned. Expression of genes cloned in pUC8 can be controlled by the *E. coli lac* promoter also present on pUC8. Later cloning experiments also used plasmid pMF4, in which very small DNA fragments (50 to 200 base pairs) are cloned in a site located between the amino-terminal portion of the λ*cI* gene and the carboxyl region of the *lacZ* gene. If cloning produces an inframe, open-reading frame (probability of 1 in 6), then a polypeptide is synthesized that contains the amino acids encoded by the cloned methanogen DNA sandwiched between the amino terminus of λ*cI* and the carboxyl terminus of *lacZ*. Fusion polypeptides are generally stable and not subject to proteolysis. This stability has been shown to faciltate their detection in lysates of *E. coli* using antibodies to screen gene libraries.

The problem of obtaining large DNA molecules from *M. thermoautotrophicum* was bypassed when cloning experiments were undertaken with DNA from *M. vannielii*. Cells of this species can be lysed by simply adding 1% SDS. This procedure avoids mechanical cell disruption and thus the concomitant breakage of DNA. Gene libraries were constructed from *M. vannielii* genomic DNA using the bacteriophage vectors λCharon 35 (λCh35) and λgt11 and the plasmid vectors pUC8 and pMF4. The λ-based molecules were packaged *in vitro* into λ particles and used to infect *E. coli*. The λgt11 recombinants could be shown to contain inserts by using X-*gal* indicator plates. Insertion of DNA into the cloning site of λgt11 inactivates the *lacZ* gene of this vector and gives white plaques on X-*gal* plates. *E. coli* infected with the vector alone produces blue plaques. The number of recombinants obtained using the bacteriophage vectors λCh35 or λgt11 to clone *M. vannielii* DNA varied between $5x10^3$ and $1x10^5$ clones; plasmid libraries of either *M. vannielii* or *M. thermoautotrophicum* DNA contained approximately $1x10^5$ to $1x10^6$ different clones. Based on the number of clones in each library and the average size of the insert DNA, all libraries of *M. thermoautotrophicum* or *M. vannielii* genomic DNA were expected to contain copies of all gene sequences.

Screening of Gene Libraries

Plaques produced by infection of *E. coli* with λ recombinant phages or colonies of *E. coli* containing plasmid-based recombinant molecules were screened for the presence of antigens that could bind the anti-methyl CoM-reductase antibodies. The first experiments resulted in very large numbers of positive clones; however, this result was quickly recognized as stemming from the presence of anti-*E. coli* antibodies in the antisera preparations (see above). When the anti-*E. coli* antibodies were removed, only a few recombinant clones in each λ library gave positive signals. These were chosen for further study.

Analysis of Positive Clones

Phages from plaques containing antigens that bound anti-methyl CoM-reductase antibodies were plaque-purified and then produced as high-titer phage stocks. DNA was purified from these phages, and restriction enzyme analyses were perfomed to determine the size and restriction maps of the cloned DNA's. Although several completely different recombinant phages were obtained from both the *M. thermoautotrophicum* and *M. vannielii* libraries, we also obtained several independently constructed phages that contained common and overlapping restriction fragments. The latter result was expected because partial restriction digests were used in the construction of the λ recombinant phages. We also obtained phages containing common restriction fragments of *M. vannielii* DNA when different preparations of antisera were used in the screening protocol. These results were encouraging, as they demonstrated that antisera raised against methyl CoM-reductase facilitated the consistent isolation of the same fragments of methanogen-derived DNA's. Figure 2 pictures an electrophoretic separation of DNA fragments in restriction enzyme digests of λgt11-based recombinant phage DNA's. The large DNA fragments at the top of the gel are the λ vector DNA's; the smaller DNA fragments that migrate further into the gel during electrophoresis are the cloned fragments of methanogen DNA's. Southern blotting procedures were used to demonstrate that the cloned DNA's had, in fact, originated in the genomes of the methanogens used as the sources of DNA's. However, isolation of additional, unrelated phages was also a concern in that it was clear that many of these could not contain the desired genes, even though they gave strong positive signals in the antigen:antibody screenings. Many attempts were made to identify the polypeptides that were synthesized in *E. coli* following infection with the different λ recombinant phages that interacted with the anti-methyl CoM-reductase antibodies. Western blotting of infected cells, immune precipitation of infected cell lysates, and PAGE analysis of proteins synthesized in infected minicells all failed to unambiguously identify the polypeptide(s) that gave the positive signals in the plaqu screenings. Infection of minicell demonstrated that many of the recombir ant phages did direct the synthesis c novel polypeptides in *E. coli*; with thi procedure, however, only the molecula weights (not the functional activities) c these pahge-encoded polypeptides coul be determined.

In spite of the uncertainty as to which if any, of the recombinant phages cor tained the methyl CoM-reductase gene we decided to concentrate our efforts o a DNA fragment from *M. vannielii* foun cloned in several different recombinar phages. This DNA fragment was shown b Southern blotting to be highly conserve in the genome of the related methanoge *M. voltae*. This was expected for a DN sequence encoding what appears to be highly conserved enzyme. A researc group in Marburg, West Germany, ha already reported cloning the genes er

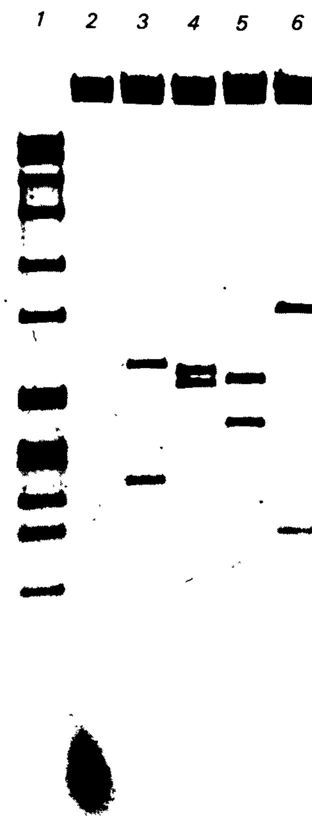

Figure 2. Restriction analysis of λgt1 recombinant phage DNA.

coding methyl CoM-reductase from *M. voltae*. We therefore argued that if the sequence of the DNA we had cloned from *M. vannielii* was present in the methyl CoM-reductase genes cloned by the Marburg group, we would be able to assume that we had also cloned a methyl CoM-reductase gene, but from *M. vannielii*. The DNA sequence we determined (see full report) contains an open reading frame of 122 codons. Unfortunately, it does not appear in the sequence of the *M. voltae* methyl CoM-reductase genes. Our antibody screening indicated it should be part of the gene encoding the small subunit of methyl CoM-reductase. Comparison of the *M. vannielii* and *M. voltae* sequences indicated no similarity between our sequence and theirs for the smallest polypeptide of the *M. voltae* enzyme. We must therefore conclude that although this DNA, when cloned in *E. coli*, causes the synthesis of an antigen that specifically reacts with antibodies raised against the purified, smallest subunit polypeptide of methyl CoM-reductase of *M. vannielii*, the DNA probably does not encode this polypeptide. Discussions of this anomolous result with several investigators experienced in using the antibody screening procedure have elicited many descriptions of similar artifactually positive results. The conclusion appears to be that although this technique does allow the identification and isolation of desired genes, it frequently requires extensive screening of positive clones before the correct positive is found. A second, independent screening procedure is therefore very useful to sort through the clones identified as positives by the antibody screen. The most frequently used second screening employs a synthetic DNA probe.

Determination of an Amino Acid Sequence for Design of a DNA Probe

In 1983, we first recognized the seriousness of the problem of false positives generated by the antibody screening procedure. We therefore decided to obtain an amino acid sequence of the amino terminus of one of the methyl CoM-reductase polypeptides. This sequence could then be used to design a DNA probe that would be used by DNA:DNA hybridization to screen our gene libraries for the homologous DNA sequence. Two immediate practical problems were: (1) the need to obtain sufficiently large amounts of the purified enzyme for amino acid sequencing, and (2) gaining access to an amino acid sequencing facility. Neither of these problems could be solved at The Ohio State University (O.S.U.), as facilities for growth of large cultures of methanogens and equipment for amino acid sequence determinations were not then available. Large cultures were therefore grown in collaboration with the University of Iowa, and purified polypeptides were sent to the amino acid sequence-determining facility at the University of Michigan. We visited both of these institutes to help with and learn procedures. Unfortunately no useful sequence information was obtained. The University of Michigan facility was unable to obtain a satisfactory amino acid sequence. We have therefore developed the facilities to repeat this approach at O.S.U. and have begun to prepare sufficient enzyme for use in amino acid sequence determination.

Isolation of mRNA for Use as a Probe or Synthesis of cDNA

Methyl CoM-reductase constitutes approximately 10% of the total protein in methanogens, and therefore we assumed that the mRNA encoding the polypeptides should be abundant. If such mRNA molecules could be purified, they could be used to locate complimentary DNA sequences cloned in a gene library by functioning as probes in DNA:RNA hybridizations. Alternatively, if these mRNA molecules had 3'poly-A sequences, they might serve as templates to synthesize cDNA using reverse transcriptase. This cDNA could then be cloned and used as a probe in DNA:DNA hybridization screenings of the genomic DNA libraries. A project was therefore initiated to isolate and characterize mRNA molecules from *M. vannielii*. This effort required development of purification protocols and procedures to determine the size, stability, and polyadenylation status of mRNA's from this species. The results of this work demonstrate that methanogen mRNA's closely resemble eubacterial mRNA's. These mRNA's do not offer the opportunity of synthesizing cDNA's, as there is only limited polyadenylation. Gel electrophoresis of purified mRNA's did not show bands of enriched mRNA's as was expected of mRNA's encoding very abundant polypeptides. We have therefore begun to develop an *in vitro* translation system so that extracted mRNA's can be translated to facilitate identification of the polypeptides they encode. Electrophoresis and/or sucrose gradients will be used to subdivide mRNA preparations into fractions containing differently sized mRNA's. The products of translation of each fraction will be assayed for the presence of methyl CoM-reductase antigens using the anti-methyl CoM-reductase antibodies. If we can identify an mRNA fraction that is enriched for the mRNA encoding methyl CoM-reductase, the mRNA's in this fraction will be made radioactive. This labeled mRNA preparation will then be used as a probe using DNA:RNA hybridization to screen the recombinant clones that gave positive signals in the antibody:antigen screening protocols already completed.

Structural Analysis of Cloned Genes

In parallel with the attempts to clone the genes encoding methyl CoM-reductase, we have characterized methanogen genes that, when cloned in *E. coli* or *Bacillus subtilis*, complement auxotrophic mutations in these eubacterial species. The goals of these experiments are: (1) to obtain precise details of the structure of regulatory elements such as promoters and ribosome-binding sequences, and (2) to determine the overall organization of genes within the genomes of methanogens. This information will be essential for designing logical genetic engineering approaches to manipulating the genes encoding methyl CoM-reductase once these genes are available.

Studies on these methanogen genes that complement auxotrophic mutations have shown that these genes resemble eubacterial genes in being organized into operons and having strong ribosome binding sequences. Codon usages differ radically from both *E. coli* and *B. subtilis*. We have identified conserved methanogen sequences that may be promoters. Comparisons of DNA sequences of related genes cloned from different methanogenic species have allowed us to estimate evolutionary divergence and suggest taxonomic relationships. Details of the procedures and results obtained in these studies are provided in publications from our laboratory listed in the reference section of the full report.

Conclusions

The specific goal of cloning the genes that encode the subunit polypeptides of methyl-coenzyme M methyl-reductase has not been reached. We have obtained a number of positive clones in terms of their ability to synthesize antigens that interact with antibodies raised against the subunits of methyl CoM-reductase. These clones must be screened further to determine whether any contain the desired recombinant DNA molecules. The experiments

undertaken to isolate and characterize methanogen mRNA's have provided the first detailed description of archaebacterial mRNA structures and have generated the RNA substrates for development of an *in vitro* translation system. Analyses of cloned methanogen genes, which complement auxotrophic mutations in *E. coli* and *B. subtillis*, have resulted in publications containing the first DNA sequences of methanogen-derived, protein encoding genes. In addition, we have isolated and characterized the first methanogen insertion element (ISM1), and we have provided the first description of regulatory elements used in expression of methanogen genes. Although it is disappointing that the methyl CoM-reductase genes of *M. vannielii* are not yet in hand, we feel that the technical problems encountered can be overcome. Nonetheless, the results of our research to date have already provided a firm foundation for future applications of recombinant DNA technology to methane biogenesis.

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The complete report, entitled "Application of Recombinant DNA Technology to Methane Biosynthesis," (Order No. PB 87-102 265/AS; Cost: $9.95, subject to change) will be available only from:

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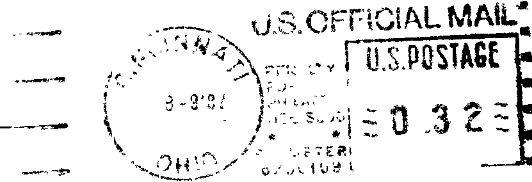

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