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Investigations of the products of \textit{mcrD} and \textit{mcrC}, two genes of the methyl coenzyme M reductase operon from \textit{Methanococcus vannielii}

Stroup, Diane, Ph.D.
The Ohio State University, 1992
INVESTIGATIONS OF THE PRODUCTS OF \textit{mcrD} AND \textit{mcrC}, TWO GENES OF THE METHYL COENZYME M REDUCTASE OPERON FROM \textit{METHANOCOCCUS VANNIELLI}

DISTRIBUTION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the The Ohio State University

By

Diane Stroup, B.S., M.A.

*****

The Ohio State University
1992

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ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. John N. Reeve. Thank you to the other members of my advisory committee, Drs. Charles J. Daniels, Joseph A. Krzycki and F. Robert Tabita. I would like to thank the past and present members of the Reeve laboratory for their intellectual input.

Protein sequencing and oligonucleotide synthesis were performed at the Biochemical Instrument Center by Jane Tolley. The technical assistance of William Swoager, Donald Ordaz, (both of the Ohio State University) and Dr. Henry Aldrich, University of Florida, are gratefully acknowledged.
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<tr>
<td>aa</td>
<td>amino acid(s)</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CoM</td>
<td>coenzyme M, β-mercaptoethane sulfonic acid</td>
</tr>
<tr>
<td>component A</td>
<td>From the purification of methyl reductase, flowthrough of DEAE-cellulose column equilibrated with 20 mM Tris-HCl. Can be resolved into three protein fractions with factor F420 and methyl viologen-reducing hydrogenase activity (A1, A2 and A3) and FAD.</td>
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<tr>
<td>component B</td>
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</tr>
<tr>
<td>component C</td>
<td>From the purification of methyl reductase, 320 mM NaCl eluent of the DEAE-cellulose column. Contains the methyl reductase activity.</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease I</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol (Cleland’s reagent)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ER medium</td>
<td>methanogen minimal medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>F&lt;sub&gt;430&lt;/sub&gt;</td>
<td>factor F&lt;sub&gt;430&lt;/sub&gt;</td>
</tr>
<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
<tr>
<td>gpmcrX</td>
<td>gene product of the mcr open reading frame X</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HTP</td>
<td>7-mercaptoheptanoyl-o-phosphothreonine</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-1-thio-β-D-galactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pairs, 1000 base pairs</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton = 10&lt;sup&gt;3&lt;/sup&gt; Daltons</td>
</tr>
<tr>
<td>l</td>
<td>liter(s)</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium for <em>E. coli</em></td>
</tr>
<tr>
<td>mcr</td>
<td>methyl CoM reductase gene designation</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MR</td>
<td>methyl coenzyme M reductase holoenzyme, α&lt;sub&gt;2&lt;/sub&gt;β&lt;sub&gt;2&lt;/sub&gt;γ&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>nmole</td>
<td>nanomoles, 1 X 10&lt;sup&gt;-9&lt;/sup&gt; moles</td>
</tr>
<tr>
<td>OD&lt;sub&gt;580&lt;/sub&gt;</td>
<td>optical density at absorbance 580 nm</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease A</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SAS</td>
<td>saturated ammonium sulfate</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TRAN&lt;sup&gt;35&lt;/sup&gt;S</td>
<td>hydrolysate of <em>E. coli</em> cells grown on &lt;sup&gt;35&lt;/sup&gt;S&lt;sub&gt;0&lt;/sub&gt;</td>
</tr>
<tr>
<td>Tris</td>
<td>tris-(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>μCi</td>
<td>microcuries</td>
</tr>
</tbody>
</table>
X-gal  5-bromo-4-chloro-3-indolyl-β-D-galactoside
CHAPTER I
INTRODUCTION

Project overview

Methanogens make methane, it is the defining characteristic of the group. To be a methanogen, the organism must have methyl coenzyme M reductase (MR). MR is the enzyme which catalyzes the reaction of methylated β-mercaptoethane sulfonic acid (methyl-CoM) with 7-mercaptoheptanoyl-o-phosphatothreonine (HTP-SH) to yield methane and the heterodisulfide of the cofactors. When the genes for methyl reductase were sequenced, in addition to the three genes which encode the known subunits of the enzyme (mcrA, mcrB and mcrG), two additional open reading frames (ORF’s), mcrD and mcrC, were discovered. Since 1987, five mcr operons have been cloned and sequenced and, in all cases, mcrC and mcrD are present (Allmansberger, et al., 1986; Cram, et al., 1987; Bokranz and Klein, 1987; Bokranz, et al., 1988; Klein, et al., 1988; Weil, et al., 1988). Since the ORF’s were conserved in very diverse organisms, it was, therefore, postulated that the ORF’s were important and perhaps involved in methanogenesis (Sher and Reeve, 1990). The question became, what are the functions of gpmcrD and gpmcrC?

Searches of data bases have yet to turn up sufficient homology to allow for identification of possible roles for mcrC and mcrD. Gene transfer systems are not available for the methanogens and, therefore, genetic approaches are inapplicable to this problem. To investigate possible roles of gpmcrD and gpmcrC, first, a method of detecting them was required. The mcrD and mcrC genes were expressed in E. coli.
the recombinant products purified, antibodies were raised against these gene products and the resulting sera were used as methods of detection.

The \textit{mcr} operon chosen for study is from the mesophile \textit{Methanococcus vannielii} to continue the work initiated by Bruce Sherf. In \textit{Methanococcus vannielii}, \textit{mcr}D has been shown to be expressed as polypeptide (Sherf, 1989) and \textit{gpmcr}D co-immunoprecipitated with MR (Sherf and Reeve, 1990). Using this work as the starting point, various physical and biochemical parameters of \textit{gpmcr}D and \textit{gpmcr}C were investigated.

Even without gene transfer technology, many questions could be addressed experimentally if purified preparations of the proteins and antibodies specific for these gene products were available. Were the gene products of \textit{mcr}C and \textit{mcr}D physically associated with MR, as indicated by the co-immunoprecipitation? What was their steady state levels? Could any regulation be shown for MR, \textit{gpmcr}C or \textit{gpmcr}D? Would the gene products of \textit{mcr}D and \textit{mcr}C or antibodies specific for these gene products effect methanogenesis \textit{in vitro}?

To introduce to these investigations, an overview of the literature available on methanogens and methanogenesis is provided. First, the phylogenetic diversity of the methanogens is stressed, followed by a summary of the relevant characteristics of the most intensely studied methanogenic species that are relevant to this project. The most likely points of \textit{gpmcr}D and \textit{gpmcr}C interaction are described. A discussion of the current understanding of methanogenesis and its coupling to ATP formation is provided, as \textit{gpmcr}D and \textit{gpmcr}C may participate at any point within these complex, interrelated processes.

\textbf{The methanogens are Archaea}

Although the biological nature of the source of some methane has long been appreciated, only recently were methanogens recognized as a cohesive group.
Originally, the methanogens were taxonomically classified with organisms which shared similar morphologies (Balch, et al., 1979). In 1956, Barker proposed grouping the methanogens together on the basis of their distinctive physiology. It was not until 1977, that the true phylogenetic isolation of the methanogens was discovered by C. Woese and associates on the basis of comparing ribonuclease T\(_1\) digestion fragments of the 16S ribosomal RNA (Fox, et al., 1977). In the three domain phylogenetic scheme, the methanogens are classified as Archaea, distinct from the Bacteria and Eucarya (Woese, et al., 1990). The divisions delineated using rRNA cataloging are supported by the distribution of membrane lipids and cell wall compositions (Balch, et al., 1979). The classification based on 16S rRNA cataloging reflects well the different physiologies of these organisms.

RNA cataloging also revealed the genetic diversity of the methanogens. The \(S_{AB}\) values within the methanogens are as low as 0.20 (Methanobrevibacter \(\text{arbophilicus}\) vs. Methanospirillum \(\text{hungatei}\)), whereas Bacillus sp. (Gram positive) vs. Enteric-Vibrio sp. (Gram negative) has an \(S_{AB}\) of 0.27 (Balch, et al., 1979). This diversity is important to consider when comparing the results obtained with different methanogenic species.

The methanogens are divided into three orders: Methanococcales, Methanobacterales and the Methanomicrobiales. Generally, Methanobacterales and the Methanococcales can use formate and carbon dioxide/hydrogen as growth substrates. The members of the Methanomicrobiales can use the widest variety of growth substrates, and because of this diversity, members of this order may be the most important contributors to methane in the environment. Seventy percent of the methane from anaerobic digesters is derived from acetate, a substrate which only the Methanomicrobiales can use (Jetten, 1990).
The Methanococcales

The best studied species of the Methanococcales are Methanococcus vannielli and Methanococcus voltae. The organism used in this study, Methanococcus vannielli, has irregular coccoïd cells, 0.3 to 5μm in diameter which can utilize formate or carbon dioxide and hydrogen. Originally isolated from the marine mud of San Francisco Bay (Stadtman and Barker, 1951), the cells lack a true cell wall, and have instead, a surface protein array (Balch et al., 1979). The 60 kD subunits are arranged in a hexagonal lattice with 10.8 nm spacing (Nusser and König, 1987). Unfortunately, the surface protein's antigenicity and molecular weight interferes with detecting the alpha subunit of MR (60.7 kD)(Cram, et al., 1987) with polyclonal antiserum. Without a cell wall, exposure to oxygen or dilute detergents will lyse Mc. vannielli cells. This has proved to be an advantage for making extracts in this study.

Mc. voltae is similar to Mc. vannielli, but differs in respect to Na⁺ and H⁺(pH) concentration growth optima. Both ions are important for coupling ATP synthesis to methanogenesis. Among other characteristics, Mc. vannielli differs from Mc. voltae in that Mc. vannielli's optimum NaCl concentration is 0.1M, compared to Mc. voltae's 0.4M; and Mc. vannielli's pH range is 7.0-9.0, where Mc. voltae's is more acid, pH 6.5-8.0 (Balch, et al., 1987). The mesophilic Mc. vannielli grows optimally in the range of 35-40°C (Stadtman and Barker, 1951), Mc. voltae grows optimally at 38°C (Whitman, et al., 1982).

Methanomicrobiales

Most of the evidence for the chemiosmotic mechanism for coupling methanogenesis to ATP synthesis comes from data obtained from Methanosarcina barkeri and Gō1. Gō1 has not been classified yet, but is used primarily because everted vesicles can be made from this organism, due to its unusual cell wall structure.
(Jussofie, et al., 1986). Gö1 is similar to the mesophilic *Ms. barkeri* in that it can grow at the expense of methanol, methylamines and acetate. In contrast to Gö1, the wall of the nonmotile *Ms. barkeri* cell is composed of polysaccharide. The pleomorphic cells form packets after division (pseudosarcina) (Balch, et al., 1979).

**The Methanobacteriales**

Among the *Methanobacteriales*, are *Methanobacterium thermooautotrophicum* strains ΔH (Zeikus and Wolfe, 1972) and Marburg (Fuchs, et al., 1978). The cells of both strains are nonmotile rods with cell walls composed of pseudomurein (Balch, et al., 1979). *Mb. thermooautotrophicum* ΔH is the type strain of the species. There are significant differences between these strains, and therefore, results obtained with these two strains should not be considered interchangeable. Touzel, et al., 1992, concluded that *Mb. thermooautotrophicum* strain Marburg is not the same species as ΔH. The authors state that the low (35%) homology between the strains, determined by DNA reassociation, justifies strain Marburg being classified as a different species. They cite Priest, et al., 1987, "that strains within a species should share at least 50 to 60% homology".

**Methanogenesis**

A description of the current understanding of methanogenesis, the enzymatic steps and possible sites of energy coupling, follows. In developing this research project, it was necessary to consider each of the steps within these complex processes as possible sites where the *mcr*D and *mcr*C gene products could be involved.
Methvl coenzyme M reductase

The enzyme which generates methane is methyl coenzyme M reductase. MR's three subunits, alpha, beta and gamma (α, β and γ), are encoded by mcrA, mcrB and mcrG genes, respectively. Five mcr operons have been sequenced. (Methanococcus vannielli, Cram, et al., 1987; Methanococcus voltae, Klein, et al., 1988; Methanobacterium thermoautotrophicum Marburg, Bokranz, et al., 1988; Methanosarcina barkeri, Bokranz and Klein, 1987; and Methanothermus fervidus, Weil, et al., 1988). In all cases, in addition to mcrB, mcrG and mcrA, there are two ORF's, mcrD and mcrC. The gene order was found to be mcrBDCGA. gpmcrB, gpmcrC, gpmcrG and gpmcrA are conserved to approximately the same extent on the amino acid level. Although, gpmcrA is, without exception, the most conserved at the amino acid level (Mc. vannielli vs. Mc. voltae, 91%) (Sherf, 1989). This may reflect gpmcrA containing the F₄₃₀ CH₃CoM, and HTP-SH binding sites (Hartzell and Wolfe, 1986). The gpmcrD's, have diverged to the greatest extent (Mc. vannielli vs. Mc. voltae, 66%). For Mc. vannielli, the mcrD nucleic acid sequence A+T content is distinct as well. mcrD, at 65.6% A+T, is closer to that of the whole genome (69%), than to the other mcr genes (mcrA= 58.1%, mcrB= 58.6%, mcrG= 58.5%, and mcrC= 59.6%) (Cram, et al., 1987).

There are two forms of MR in some species. Rospert, et al., 1990, purified two methyl reductase isozymes from Mb. thermoautotrophicum Marburg. Hennigan, et al., 1991, demonstrated that Mt. fervidus as well as Mb. thermoautotrophicum strain ΔH have two mcr operons, while only one was detected in Mc. vannielli, Ms. barkeri, Methanopyrus kandleri and Methanothrix. Evidence presented in Figure 17, shows Mb. formicicum, a mesophilic organism, also has two, as well as the thermophile, Mb. wolfei, suggesting that two operons correlate with being a Methanobacteria le, not with thermophily nor that all methanogens must have two operons which encode MR. Other evidence demonstrated Methanopyrus kandleri, a Methanobacteria le, has two
as well (J. Palmer, personal communication)

Hartzell and Wolfe, 1986, demonstrated that the information needed to assemble into a methanogenic $\alpha_2\beta_2\gamma_2$ conformation was encoded in the polypeptides from *M. thermoautotrophicum* \(\Delta H\). They showed polypeptides isolated from SDS-polyacrylamide gels could reassemble and, if \(F_{430}\) was added, generate methane from \(CH_3CoM\) and HTP-SH.

The purified MR's from most organisms have native molecular weights of approximately 300 kD. This reflects an $\alpha_2\beta_2\gamma_2$ conformation. Hartzell and Wolfe, 1986, found the $\alpha_1\beta_1\gamma_1$ form inactive but the $\alpha_2\beta_2\gamma_2$ form was methanogenic in an in vitro assay. This evidence strongly supports the view that $\alpha_2\beta_2\gamma_2$ is the minimum complex needed to catalyze the release of methane. However, this does not preclude the possibility that a more complex, higher order form exists in the cell. A $(\alpha_2\beta_2\gamma_2)_n$ complex may not be stable enough to survive purification.

Mayer, et al., 1988, presented evidence for a higher order structure in Gø1, which they named the methylreductosome. Wackett, et al., 1987, calculated the spherical complex was composed of approximately 32 MR (\(\alpha_2\beta_2\gamma_2\)). Additionally, Sherf and Reeve, 1990, demonstrated anti-MR coprecipitated gpmcrD from *Methanococcus vannielii* cell extracts, indicating that the MR's structure may not simply be the $\alpha_2\beta_2\gamma_2$ in the cell. This co-immunoprecipitation is evidence for interaction between MR and gpmcrD. This opened speculation of whether gpmcrD might be involved in assembly of the methylreductosome or in holding it together.

In spite of the compelling but circumstantial evidence provided by the evolutionary persistence of mcrC and mcrD that these ORF's are important, there are no experimental results to support this assertion. To the contrary, MR preparations which contain no detectable gpmcrC or gpmcrD, are methanogenic (Hartzell and Wolfe, 1986). Therefore, the products of these ORF's cannot be absolutely required for MR catalyzed release of methane from methyl-CoM.
Additionally, Ellermann, et al., 1989, reported *E. coli* cell extracts containing β-galactosidase fusions with gpmerD and gpmerC (*M. thermoaerotrophicum* strain Marburg) did not effect methanogenesis *in vitro* when added to the reaction.

**Mechanism of ATP synthesis**

The evidence available strongly supports the view that ATP production in the *Methanomicrobiales* is coupled to methane production by a chemiosmotic gradient. The production of ATP is uncoupler sensitive, imposed gradients generate ATP and enzymes of the methanogenic pathway have been found to pump H⁺ and Na⁺ (Becher, et al., 1992). For a review, see Blaut, et al., 1990. Vesicles made from Gö1 cells are the only *in vitro* system that has been demonstrated to make ATP as a result of methanogenesis. One mole of ATP was formed by the vesicles when 100 moles of methane were made from H₂ and methyl-coenzyme M. Washed vesicles can generate one mole of ATP by oxidizing two moles of CoM-S-S-HTP, a product of the methyl reductase reaction (Deppenmeier, et al., 1990 and 1991). Imposed proton gradients were also found to be capable of driving ATP synthesis, and as proton extrusion is coupled to methanogenesis in *M. barkeri* cells, ATP production in the *Methanomicrobiales* does appear to result from a chemiosmotic mechanism (Peinemann, et al., 1989).

There is also evidence for ATP-generating ion gradients in the *Methanobacteriales*. *Methanobacterium thermoaerotrophicum*, strain Marburg. Kaesler and Schönheit, 1988, retracted an earlier assertion of uncoupler insensitivity of methanogenesis and ATP levels in these cells (Schönheit and Beimborn, 1985). Their more recent findings (Kaesler and Schönheit, 1988) indicated that the protonophores used did not reduce the membrane potential below the ATP generating threshold which allowed continued methane and ATP production from CO₂ and H₂. However, methanogenesis and ATP level maintenance were still coupled.
When the chemiosmotic gradients were reduced below this threshold, both methane and ATP production were effected. The authors interpreted this result as indicating that the early and late steps of methanogenesis are linked via a membrane gradient. They also showed the production of methane by cell suspensions from HCHO while the ATP levels drop. HCHO reacts with tetrahydromethanopterin non-enzymatically (Kaesler and Schönheit, 1988). The rate of methane production was many times lower than that of whole cells, which may complicate the interpretation of these results, as discussed below.

The Kaesler and Schönheit, 1988, paper would have definitively shown ATP production in methanogens is dependent on the presence of ion gradients, except that they tested *Mb. thermoautotrophicum* and *Ms. barkeri*, but not a representative of the Methanococcales. Several issues cloud the picture. They extrapolated their findings to refute the conclusions of Lancaster (Crider, et al., 1985; Lancaster, 1986) which indicated uncoupler insensitivity of methanogenesis-dependent ATP synthesis in *Mc. voltae*. Kaesler and Schönheit, 1988, claimed that under the conditions used by Lancaster, 1986, the ion gradients would not have been dissipated, as they found in *Mb. thermoautotrophicum* strain Marburg. They do not, however, explain why *Ms. barkeri* should be sensitive under these conditions (as they found), nor why they consider *Mb. thermoautotrophicum* strain Marburg a better model system for Methanococcales than *Ms. barkeri*. *Mb. thermoautotrophicum* strain Marburg's atypical characteristics, detailed below, make it a rather poor paradigm.

Kaesler and Schönheit, 1988, suggested that *Mb. thermoautotrophicum* strain Marburg is less sensitive to uncouplers because of cell wall differences and that *Mb. thermoautotrophicum* strain Marburg can pump protons faster than they can diffuse to equilibrium due to its high rate of methane production. As the Methanococcales lack a pseudomurein cell wall, they are very different than *Mb. thermoautotrophicum*
strain Marburg. The rates of methane production reported for *Mb*. *thermoautotrophicum* strain Marburg are the highest in the literature. Cell suspensions of *Mb. thermoautotrophicum* strain Marburg produced methane at 10 times the rate obtained with *Ms. barkeri* cells (Kaesler and Schönheit, 1988). Purified MR preparations with the highest specific activities are obtained from *Mb. thermoautotrophicum* strain Marburg cells which have been exposed to 100% H₂ before harvesting (Rospert, et al., 1991). In the one experiment where Kaesler and Schönheit, 1988, managed to uncouple methane production and methanogenesis-dependent ATP synthesis, the methane rate was approximately 200 times lower than the rates of the coupled methanogenesis from CO₂ and H₂ (20 vs. 4000 nmoles methane/min/mg).

Experiments with *Methanobacterium thermoautotrophicum* strain ΔH, a *Methanobacteriales*, indicate substrate level phosphorylation is also possible. Keltjens et al., 1988, reported methanogenesis-dependent inorganic pyrophosphate synthesis in cell free extracts. van Alebeek, et al., 1991, found ATP was synthesized from 2,3-diphosphoglycerate independent of the methyl reductase reaction. The physiological significance of these reactions has not been established.

The possible presence of two or more interrelated or alternative gradient-dependent processes complicates the interpretation of results. There is still no explanation of the pH dependent (Perski, et al., 1982) and sodium concentration dependent (Schönheit and Beimborn, 1986) sensitivity of methanogenesis to ionophores. Krzycki and Zeikus, 1984, proposed a model of electron flux from carbon monoxide dehydrogenase to the methanogenic reaction. This model is further supported by the finding that the molar ratio of ATP produced to methane generated is exceptionally high for *Ms. barkeri* oxidizing carbon monoxide (Bott, et al., 1986). This electron movement, and its accompanying proton pumping, would only occur in cells growing on acetate.
In spite of the contradictions, it is reasonable to conclude that membrane gradients play an essential role in methanogenesis, though it is still an open question as to exactly how.

**Known enzymatic steps of methanogenesis**

Many of the intermediate steps of methanogenesis have been investigated in detail (Figure 1). Organisms which can grow on formate, first convert it to carbon dioxide (CO\(_2\)). CO\(_2\) is bound to the C\(_1\) carrier, methanofuran, before it is reduced and transferred to tetrahydromethanopterin. The C\(_1\) moiety undergoes two additional two-electron reductions before it is transferred to coenzyme M. The terminal reaction, which is common to all methanogens regardless of the growth substrate, is the reduction of the methyl group of methyl coenzyme M to methane in a reaction catalyzed by methyl coenzyme M reductase. Each step of the pathway will now be discussed individually.

Methanogens are limited in the range of substrates which they can use as sources of carbon and energy. Carbon dioxide and hydrogen can be used by many methanogens. Most of these can also use formate by virtue of the enzyme formate dehydrogenase. Formate is oxidized to CO\(_2\), yielding two reducing equivalents. Therefore, an organism growing on formate would produce three moles of CO\(_2\) to one mole of methane. One of *Methanococcus vannielii*’s formate dehydrogenases is a molybdenum, iron, and sulfur containing protein with a molecular weight of 105 kD, with the unusual property of not being inhibited by azide. It is, however, sensitive to oxygen, like other formate dehydrogenases from anaerobic organisms. Coenzyme F\(_{420}\) is the electron acceptor (see Figure 2 for structure). The stimulation of growth on formate by selenium lead Jones and Stadtman, 1977, to hypothesize the existence of a selenium requiring formate dehydrogenase. They later showed this to be the case, identifying an isozyme which contains selenium in the form of
Figure 1: Methanogenesis from CO$_2$ and H$_2$. Figure modified from Blaut, et al., 1990.
1, formyl-methanofuran dehydrogenase; 2, formylmethanofuran:tetrahydromethanopterin formyltransferase; 3, 5,10 methenyl-tetrahydromethanopterin cyclohydrolase; 4, 5,10-methylene-tetrahydromethanopterin dehydrogenase; 5, 5,10-methylene-tetrahydromethanopterin reductase; 6, methyl-tetrahydromethanopterin:coenzyme M methyltransferase; 7, methyl coenzyme M reductase; 8, reduced coenzyme F$_{420}$-heterodisulfide oxidoreductase. Abbreviation: HTP-SH; reduced form of 7-mercaptohepanoylthreonine phosphate.
Figure 1
selenocysteine (Jones and Stadtman, 1981; Schauer and Ferry, 1986; Ferry, 1990).

\[ \text{CO}_2 \] is reduced and bound by formyl-methanofuran dehydrogenase to methanofuran (MFR), yielding formyl-MFR. The \( C_1 \) moiety is bound to a primary amine on MFR, which was originally known as the carbon dioxide reduction factor. The structure of MFR is shown in Figure 2. (Leigh, et al., 1984, 1985).

The formyl moiety is transferred from MFR to tetrahydromethanopterin (\( H_4 \text{MPT} \)), forming 5-formyl-\( H_4 \text{MPT} \) by formylmethanofuran:tetrahydromethanopterin formyltransferase (Donnelly and Wolfe, 1986). The structure of \( H_4 \text{MPT} \) is shown in Figure 2. This cofactor, unique to the methanogens, is analogous to the widespread \( C_1 \) carrier, tetrahydrofolate. A cyclohydrolase dehydrates 5-formyl-\( H_4 \text{MPT} \), yielding 5,10-methylene-\( H_4 \text{MPT} \) (Donnelly et al., 1985; DiMarco, et al., 1986), which is then reduced to 5,10-methylene-\( H_4 \text{MPT} \) by a coenzyme \( F_{420} \) dependent dehydrogenase (Hartzell, et al., 1985). Further reduction by 5,10-methylene-\( H_4 \text{MPT} \) reductase, which also requires \( F_{420} \) generates methyl-\( H_4 \text{MPT} \) (Brömmelstroet, et al., 1990). It is interesting to note that the equilibrium of the dehydrogenase in **M. thermoautotrophicum** favors oxidation, under the reaction conditions of Hartzell et al., 1988, even though the organism is using hydrogen and carbon dioxide to grow. \( F_{420} \)-dependent 5,10 methylene-\( H_4 \text{MPT} \) dehydrogenase and reductase have been purified from **M. barkeri** cells grown on methanol. These cells obtain reduced \( F_{420} \) by oxidizing methanol to \( \text{CO}_2 \), apparently by reversing the steps of methanogenesis before the MR reaction (Brömmelstroet, et al., 1991).

Formaldehyde can react non-enzymatically with \( H_4 \text{MPT} \), to enter the methanogenic pathway (Rouviere and Wolfe, 1988). Methanol enters the pathway via specific methyl transferases, enzymes which combine the methyl moiety with the carriers \( H_4 \text{MPT} \) or \( \beta \)-mercaptoethanesulfonic acid (Keltjens and van der Drift, 1986).
Figure 2: Cofactors of the methanogens. Methanofuran and tetrahydromethanopterin figures were from Wolfe, 1985. The diagram of \( F_{420} \) was taken from Jones, et al., 1987. The structure of \( F_{430} \) is a figure in Hausinger, et al., 1984. CoM and HS-HTP (7-mercaptoheptanoylthreonine phosphate) formulas were after Ellermann, et al., 1988.
Methanofuran (MFR)

Tetrahydromethanopterin (H₄MPT)

Factor F₄₂₀

Factor F₄₃₀

⁻S₀₃⁻CH₂⁻CH₂⁻SH
coenzyme M (CoM)

Component B (HS-HTP)
The C₁ moiety is then transferred to β-mercaptoethanesulfonic acid, the final carbon carrier in the pathway (Figure 2), which has been designated coenzyme M (CoM). This methyl transfer was shown to be catalyzed by a protein complex (Sauer et al., 1986) which contains a corrinoid moiety (Taylor and Wolfe, 1974; Van der Meyden, et al., 1983; Poirot, et al., 1987; Kergen et al., 1988). Sauer, et al., 1986, reported that this activity was in a vesicle fraction, suggesting the activity was membrane bound. However, 65% of the methyl transfer activity was found to be soluble and none found in the pellet by Kengen, et al., 1988. Both groups were working with *Mb. thermoaerophilum* strain ΔH.

Methyl-tetrahydromethanopterin:coenzyme M methyltransferase has been shown to be able to pump sodium ions. The translocation of Na⁺ into the lumen of washed inverted vesicles, prepared from G61, accompanied formation of methyl-CoM from methyl-tetrahydromethanopterin in vesicles in which methyl reductase was inhibited by 2-bromoethanesulfonate (Becher, et al., 1992). The role of the Na⁺ chemiosmotic gradient is not clear, although Na⁺ is required for methanogenesis in some species (Perski, et al.,1981,1982). Sodium may be involved in ATP synthesis (there are sodium-ATPases and Na⁺/H⁺ antiporters) or in the stimulation of methanogenesis by the heterodisulfide, a product of the methyl reductase reaction.

**Cofactors of the MR reaction**

Methyl-CoM is the substrate of methyl-CoM methyl reductase. MR is a complex enzyme. The polypeptide subunits which make up MR have CoM (Hausinger et al., 1984), Coenzyme F₄₃₀ (Hartzell and Wolf, 1986), and HTP (Noll and Wolfe, 1986) associated with them.

Coenzyme F₄₃₀ (F₄₃₀), originally called Factor F₄₃₀, was isolated from methanogenic bacteria by Gunsalus and Wolfe, 1978. F₄₃₀ is so called because of its
absorption maximum at 430 nm (and at 274 nm). It is unique to the methanogens. 

$F_{430}$ was found to contain nickel (Ni) by Diekert, et al., 1980a; Diekert, et al.,
1980b; and Whitman and Wolfe, 1980, and therefore, Ni is an essential nutrient for
methanogens (Schönheit, et al., 1979). The nickel porphinoid tetrapyrrole structure
of $F_{430}$, MW= 905, is diagramed in Figure 2. The pathway of assembly of this
cofactor (for review, Friedmann, et al., 1990) was determined by labeling studies with
$^{14}\text{C}$ 3-aminolevulinic acid (Diekert, et al., 1980c) and L-[methyl-$^{14}\text{C}$]methionine
(Jaenchen, et al., 1981a). $F_{430}$ synthesis can be inhibited with levulinic acid
(Jaenchen, et al., 1981b). Ellefson, et al., 1982, recognized $F_{430}$ was the
prosthetic group of MR. Most of the studies of $F_{430}$'s redox behavior have been
done on protein-free $F_{430}$, and, although originally thought to be chemically different
(Keltjens, et al., 1982), free and enzyme-bound $F_{430}$ have been shown to be
identical (Hausinger, et al., 1984; Huster, et al., 1985). The nickel atom can be
reduced to Ni(I) as detected by electron spin resonance spectroscopy, by looking at
the pentamethylester of $F_{430}$ ($F_{430}$M) in aprotic solutions. The redox potential of
$F_{430}$M in dimethyl formamide was found to be -504 mV (Jaun and Pfaltz, 1986).
$F_{430}$ can catalyze the reduction of methyl chloride to methane using
titanium(III)citrate ($E^0$=-480 mV) in aqueous solvent. Jaun and Pfaltz, 1986,
proposed a mechanism in which the Ti(III) reduces the Ni atom to Ni(I) which, in turn,
reduces CH$_3$Cl. It has been observed that B$_{12}$ compounds can also serve as
catalysts for this reduction, but at lower rates. The role of $F_{430}$ in the cell appears,
therefore, to be to pass electrons to the methyl group (Krone, et al., 1989a,b).
However, the donor of electrons is believed to be HTP-SH in the reduction of CH$_3$CoM,
because it is an absolutely required cofactor for the methyl reductase reaction. It is
reasonable to conclude that in methanogens, reduction of the Ni in $F_{430}$ reflects the
activation of the enzyme (i.e., Ni(I) is required for a catalytically active enzyme).
HTP is N-7-mercaptoheptanoyl-O-threonine phosphate. The structure was first identified by Noll, et al., 1986. It has been referred to, in the literature, alternatively as Component B (Gunsalus and Wolf, 1980), cytoplasmic cofactor (Sauer, et al., 1984) and methyl reducing factor (Sauer, et al., 1986), but HTP will be used here. HTP exists in the supernatant of Methanobacterium thermoautotrophicum as a complex UDP-disaccharide. Using fast atom bombardment mass spectrometry and $^1$H-, $^{13}$C-, and $^{31}$P-NMR spectroscopy Sauer, et al., 1990, found the C-6mannosaminuronic acid of a UDP-disaccharide linked to the phosphate group of HTP through a carboxylic-phosphoric anhydride linkage. It is interesting to note that Santos, et al., 1990, found uridine triphosphate accounts for one third the nucleotide triphosphate pool formed when Methanosarcina barkeri cells were fed methanol (ATP being the major component). The accumulation was followed with in vivo $^{31}$P-NMR spectroscopy of cell suspensions. Sauer, 1991, found UDP-5'-diphospho-N-acetylglucosamine to be a noncompetitive inhibitor of the methyl reductase of M. thermoautotrophicum. Since the UDP-disaccharide-HP protected MR from inhibition by UDP-5' diphospho-N-acetylglucosamine, but HTP did not prevent the inhibition, Sauer concluded that the UDP-disaccharide-HP was the methyl reducing factor (MRF). The full chemical name for MRF is uridine 5'-[N-7-mercaptoheptanoyl-O-3-phospho-threonine(2-acetamido-2-deoxy-β-mannopyranuronosyl)acid anhydride]-{(1->4)-O-2 acetamido-2-deoxy-alpha-glucopyranosyl diphosphate. The formula is C$_{36}$H$_{58}$O$_{29}$N$_{5}$P$_{3}$S, with a molecular weight of 1149.21 (Sauer, et al., 1990). The molecule functions as an electron donor to methyl-CoM, forming a heterodisulfide with CoM-SH (Ellermann, et al., 1988).

**Heterodisulfide oxidoreductase**

The heterodisulfide of HTP and CoM is in turn reduced by heterodisulfide oxidoreductase. This step has been associated with ATP synthesis. Hydrogenase and
F_{420}H_{2} dehydrogenase are believed to transfer electrons to membrane bound electron carriers, which translocate protons (Deppenmeier, et al., 1990 and 1991) as these electrons flow to the heterodisulfide oxidoreductase. It is not clear which hydrogenase, methyl viologen-reducing hydrogenase or F_{420} reducing hydrogenase, is involved in H_{2}-dependent reduction. Gö1 vesicles can reduce heterodisulfide without F_{420} (Deppenmeier, et al., 1991). However, the source of reducing power differs between growth on H_{2}, hydrogenases (F_{420} independent), and F_{420} dehydrogenase (from the disproportionation of reduced substrate). The electron acceptor is the heterodisulfide, regenerating the cofactors for the methyl reductase reaction. The heterodisulfide oxidoreductase of Gö1 is membrane associated (Deppenmeier, et al., 1990). However, the activity is soluble in Methanobacterium thermoautotrophicum strain Marburg (Hedderich and Thauer, 1988). The only experiment involving this activity in the Methanococcales was testing Methanococcus thermolithotrophicus membranes for heterodisulfide oxidoreductase activity (Deppenmeier, et al., 1991). None was detected, presumably the heterodisulfide oxidoreductase activity is soluble in these organisms.

If an enzyme’s reaction is associated with pumping of ions across a membrane, it follows that that enzyme interacts directly or indirectly with the membrane. The subcellular locations reported for the heterodisulfide oxidoreductases, as well as hydrogenases and dehydrogenases vary between species. The membranes of all methanogens tested contain electron carrier(s) which cannot be replaced by membranes from non-methanogen organisms in in vitro methanogenesis assays (Deppenmeier, et al., 1989). Enzymes that are soluble may nevertheless, interact transiently with electron carrier/proton pumps in the membrane or may communicate indirectly via other protein(s). Protein/protein interactions are possible roles for gpmcrD and gpmcrC in the cell.
There are, however, many other possible roles which cannot be ruled out. They may participate in enzyme complex assembly or stability, in generating or binding of the complex cofactors, part of the apparatus which reductively activates MR or may regulate gene expression. In the absence of a gene transfer system, a biochemical approach has been taken to investigate these possibilities.

No gene transfer system is available for the methanogens

There is currently no recombinant gene transfer system for the methanogens. For the purpose of this discussion, a gene transfer system is defined as selectable vectors maintained in methanogens coupled with a method to transform DNA into the host strain. Advances, however, are being made. Recombinant plasmids based on pME2001, a plasmid isolated from Methanobacterium thermoautotrophicum strain Marburg, have been constructed for use as shuttle vectors (Meile and Reeve, 1985). Although transformation of a methanogen was first reported for Methanobacterium thermoautotrophicum Marburg, by Worrell et al., 1988, most progress in this area has subsequently been made with Methanococcus species. Transformation protocols using Methanococcus voltae (Micheletti, et al., 1991) and Methanococcus maripaludis (Sandbeck and Leigh, 1991) have been reported, however, the current absence of plasmid vectors limits experiments to those which depend on homologous recombination with the chromosome.
Objective

The objective of this study was to determine if gpmcrD and gpmcrC had identifiable roles in *Methanococcus vannielii*. To accomplish this goal, mcrD and mcrC were expressed in *E. coli*, the products purified and antisera raised against the recombinant products. These reagents were used in the following investigations:

1) Does gpmcrC exist in *Mc. vannielii* as a polypeptide, like gpmcrD?
2) How do the polypeptides localize relative to the other gene products of the mcr operon?
3) Are gpmcrD and gpmcrC abundant proteins? Do their steady state concentrations change with growth phase? Does the abundance of these polypeptides change relative to the abundance of the other gene products of the mcr operon?
4) Are gpmcrC and gpmcrD physically associated with MR?
5) Do anti-gpmcrD or anti-gpmcrC antibodies effect the rate of methanogenesis *in vitro*?

The Appendix contains the published results of a separate study undertaken to investigate properties of the high temperature DNA binding protein, HMf.
CHAPTER II
MATERIALS AND METHODS

Bacterial strains and culture conditions; Methanococcus vannielii (Stadtman and Barker, 1951) cultures were grown in 20 ml anaerobic ER medium (2.7 g NH$_4$Cl, 5 g NaHCO$_3$, 1.25 g Na$_2$CO$_3$, 4.1 g Na acetate·3H$_2$O, 2 mg FeSO$_4$, 0.1 mg resazurin, 0.3 g K$_2$HPO$_4$, 0.3 g KH$_2$PO$_4$, 0.3 g (NH$_4$)$_2$SO$_4$, 0.6 g NaCl, 0.13 g MgSO$_4$·7H$_2$O, 0.08 g CaCl$_2$, 0.24 mg Na$_2$SeO$_3$, 1 mg CoCl$_2$, 0.24 mg Na$_2$MoO$_4$·2H$_2$O, 0.24 mg NiCl$_2$·6H$_2$O, 0.125 g cysteine-HCl, and 0.125 g Na$_2$S·9H$_2$O per liter) (Balch, et al., 1979), in 150 ml serum bottles, with CO$_2$·H$_2$, at a 1:2 ratio, under 40 psi pressure. Escherichia coli strains TB1 [F$^-$ Δ(lac-proAB), ara, thi, strA, lacZΔM15, hsdR, (r$_{K^-}$, m$_{K^+}$)] (Baldwin and Treat, 1984) and DH5α [F$^-$, lacZΔM15, (AlacZYA-argF), (r$_{K^-}$, m$_{K^+}$)] (Focus. 1987. 8:2:9) were used as hosts for the pUC plasmids (Yanisch-Perronet, et al., 1985), pSKS105 (Casadaban, et al., 1983), ptrc99 (Amann, et al., 1988) and the pUR plasmids (Rüther and Müller-Hill, 1983). E. coli HB101 [F$^-$, hsdS20, (r$_{B^-}$, m$_{B^-}$) recA13, leuB6, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, supE44 (λ$^-$)] (Boyer and Poulland-Dussoix, 1969), E. coli BL21(DE3) [F$^-$, hsdS, gal, (r$_{B^-}$, m$_{B^-}$)] (Studier and Moffatt, 1985), were used for the pT7 plasmids (Studier and Moffatt, 1985; Rosenberg, et al., 1987). E. coli was grown in Luria-Bertani (LB) medium [10 g Bacto-tryptone, 5 g Bacto-yeast extract (Difco, Detroit, MI), and 10 g NaCl per liter]
Enzymes: Restriction endonucleases, T4 ligase and T4 kinase were purchased from Bethesda Research Laboratories (Gaithersburg, MD) and used as directed by the supplier. AmpliTaq Taq RNA polymerase was obtained from Perkin-Elmer-Cetus Corporation (Norwalk, CT). Sequenase was obtained from United States Biochemical Corporation (Cleveland, OH). β-galactosidase was from Sigma Chemical Company (St. Louis, MO).

Reagents: X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was purchased from United States Biochemical Corporation (Cleveland, OH). Unlabeled deoxyribonucleotide triphosphates, isopropyl-1-thio-β-D-galactopyranoside (IPTG) and ribonucleotide triphosphates were purchased from Sigma Chemical Company (St. Louis, MO). Monoclonal antibodies against β-galactosidase were purchased from Promega Biochemical Company (Madison, WI). γ-32P-ATP, α-32P-dATP and α-32P-ATP were obtained from I.C.N. (Cosa Mesa, CA), and TRAN35S and 35S-methionine were obtained from Amersham (Arlington Heights, IL). ATP concentrations of cell extracts were determined by the luciferase-luciferin method (Sigma Chemical Company) following the protocol provided by the manufacturer.

Preparation of genomic DNA: Mc.vannielii cells from a 100 ml overnight culture were pelleted by centrifugation for 10 min, at 4°C, in a Sorval GSA rotor at 2,000 rpm. The cells were lysed by resuspending in 1 ml buffer (20 mM Tris-HCl, pH 7.4, 1% dimethyl sulfoxide, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, 0.1 μg proteinase K/ml, and 100 μg RNase/ml) and incubating for 10 min at 37°C. The lysate was phenol extracted gently by adding one tenth volume of 3 M Na acetate, pH 5, and an equal volume of Tris-buffered phenol, pH 7.4. The phases were emulsified by repeatedly inverting the tube on a rotating disk for 10 min. The aqueous phase was removed and the interface reextracted with 300 mM Na acetate, pH 5. The aqueous phases
obtained were combined and then extracted once with a 1:1 mixture of phenol and chloroform and once with chloroform alone. The DNA was precipitated by adding 2.5 volumes of 95% cold ethanol.

**Determination of proteins:** Proteins were determined by the bicinchoninic acid (BCA) (Sigma Chemical Company) method, as described by Smith et al., 1985. When reducing agents were present, proteins were determined by the method of Bradford, 1976.

**Polyacrylamide gel electrophoresis (PAGE) of proteins:** Tris-glycine discontinuous gels were run as described by Laemmli, 1970. Gel electrophoresis in 2 dimensions (2D) was performed by first separating the cell extracts through a polyacrylamide gel without SDS. This gel was then divided vertically and a gel slice layered on top of a second polyacrylamide gel containing SDS after the gel slice had been heated in a microwave oven with 5 vols of 1% SDS, 0.125 M Tris-HCl, pH 6.8.

**Immunoblots of proteins transferred to nitrocellulose:** Polypeptides separated by PAGE were transferred from the gel to nitrocellulose filters (Schleicher and Schuell, Keene, NH) using a BioTrans Model A semi-dry electrophoretic transfer unit (Gelman Sciences, Denmark) according to manufacturer's instructions. The nitrocellulose filters were blocked by incubating with 5% dry milk (w/v) in TBS (20 mM Tris-HCl, pH 7.4, 500 mM NaCl). The filters were then incubated overnight at room temperature with the appropriate antiserum diluted in TBS containing 5% dry milk. After washing 3 times for 20 min with TBS-dry milk, the filters were incubated for 2 hr in a 1:2000 dilution of goat-anti-rabbit antibodies conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, MO). The filters were washed 3 times in TBS for 20 min, and the color developed by adding 30 μl 30% hydrogen peroxide and 50 mg of 4-chloro-1-naphtol (Sigma Chemical Co.) in 100 ml TBS.

**Subcloning and expression of mcrD in E. coli and purification of the recombinant gpmcrD:** The mcrD gene was positioned downstream from the phage T7 gene10
promoter, resulting in plasmid pDSD431 (Figure 3A), which was transformed into E.coli strain BL21(DE3). This host has a copy of the phage T7 DNA dependent RNA polymerase gene, fused to the lac promoter, integrated into the chromosome via a lambda lysogen, so that synthesis of the phage T7 RNA polymerase can be induced by IPTG (Studier and Moffatt, 1985).

Cultures were grown to OD$_{600}$=0.5 at 37°C with vigorous shaking, and induced by the addition of IPTG to 2 mM. After 4 hr, the cells were pelleted, lysed by sonication and the resulting lysates centrifuged at 10,000 rpm in a Sorval SS-34 rotor for 10 min at 4°C (10K supernatant). The recombinant gpmcrD was purified to homogeneity by gel filtration of the 10K supernatant through a 200 ml BioGel P200 (BioRad, Melville, NY) column equilibrated with 20 mM boric acid-borax buffer, pH 8.5, followed by chromatography through a 50 ml DEAE-Sepharose (Pharmacia, Piscataway, NJ) ion exchange column, equilibrated with the buffer used in the P200 column. The protein was eluted from the DEAE-Sepharose column with 100 mM sodium phosphate, pH 8.0, buffer. Recombinant gpmcrD was detected during its purification by following a 18 kD band on stained gels.(Figure 3B). Purified gpmcrD was stored frozen at -70°C in 100 μl aliquots, at a protein concentration of approximately 1 mg /ml.

**Antisera production:** Polyclonal antisera against the recombinant gpmcrD were raised in New Zealand White rabbits. Each 2.5 kilogram (kg) rabbit (8-10 weeks old) received 500 μg of recombinant gpmcrD, emulsified in 1 ml of incomplete Freund’s adjuvant, subcutaneously at 10 sites on the back. One rabbit was boosted with an additional 100 μg of protein, given intravenously, at 2 weeks and sacrificed 10 days later by cardiac puncture. A second rabbit was boosted with 100 μg of gpmcrD given intravenously, at 2, 4 and 10 weeks, and then sacrificed at 12 weeks. Clarified sera were prepared by allowing clot formation at 37°C for 1 h, then at 4°C overnight, followed by centrifugation at 5,000Xg, for 20 min at 4°C. The two separate sera
were aliquoted and stored long term at -20°C and at 4°C as a working reagent after thawing. Aseptic technique was used during preparation of the sera. Antimicrobial agents could not be added as they might have interfered with the in vitro methanogenesis assay.

Recombinant gpmcrD was immobilized by reacting with Reactigel (6X) [1,1 carbonyldiimidazole activated agarose (Pierce Chemicals, Rockford, IL)]. The purified protein (2 mg) was added to 1 ml of Reactigel 6X beads which had been washed in 0.2 M borate buffer, pH 9 and reacted for 48 hr. As gpmcrD was not detected in the supernatant, binding of the recombinant protein to the resin was essentially complete. Any reactive groups that may have remained were blocked by incubating the matrix with 500 mM Tris-HCl, pH 7.4, for 4 hr, at room temperature. Anti-gpmcrD antibodies were affinity purified by mixing the gpmcrD-matrix equilibrated in 10 mM Tris-HCl, pH 7.4, with a 1:10 dilution of serum in the same buffer. The column was washed with 50 column vol. of 10 mM Tris-HCl, pH 7.4, followed by an additional wash with 10 column vol. of 20 mM Tris-HCl, pH 7.4, 500 mM NaCl. Specifically bound antibodies were eluted with 100 mM glycine, pH 2.5, and with freshly prepared 10 mM triethylamine, pH 11.5. The column was washed with 10 mM Tris-HCl, pH 8.5, as an equilibration buffer between the two elution buffers. Antibody containing fractions were combined and the antibodies concentrated by ammonium sulfate precipitation (50% SAS). The pellet was resuspended and dialyzed against PBS (1.1 g Na$_2$HPO$_4$, 0.33 g NaH$_2$PO$_4$·H$_2$O, 8.5 g NaCl per liter). The resulting antibodies were titered by using an ELISA.

**Immobilized antibody columns:** Antibodies were immobilized by crosslinking to protein A-Sepharose beads. IgG was bound to Sepharose beads by mixing 1 ml of heat treated (56°C for 30 min) serum for 1 hr with 0.5 ml Sepharose-protein A (Sigma). The beads were washed twice with 5 ml 100 mM Na borate, pH 8.4. Dimethylpimelimidate (0.026 g) was added to the Sepharose-protein A beads resuspended in 5 ml of 100 mM Na borate buffer, resulting in a final concentration of
20 mM dimethylpimelimidate. The reaction was stopped after 30 min at room temperature by washing with 0.2 M ethanolamine, pH 8.4, for 2 hr at room temperature. The beads were washed in PBS and stored at 4°C in PBS with 0.001% thimerosal. Efficiency of coupling was checked by SDS-PAGE. When the IgG-protein A-Sepharose interaction withstood boiling in 1% SDS sample buffer, the desired covalent coupling had been obtained (Harlow and Lane, 1988).

**TABLE 1: Sequences and locations of oligomers**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence&lt;sup&gt;a,b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCRH136</td>
<td>2096-TAAAGGAGGTAAACATGCCCAGTTAGGA---GAAC-2123</td>
</tr>
<tr>
<td>C15 (R)</td>
<td>2378-GAGCCTAAACTTCCC-2364</td>
</tr>
<tr>
<td>DS16</td>
<td>2603-AGGGCACTATTTGTCGA-2618</td>
</tr>
<tr>
<td>BAS27 (R)</td>
<td>2718-GCCATAAGATCAAGTAACTAAAATGT-2694</td>
</tr>
</tbody>
</table>

<sup>a</sup> 5'→3'

<sup>b</sup> Numbers refer to the nucleotide position designated in the mcr sequences of *M. vanielli* as listed in GenBank (accession number M16893). (R) indicates that the oligomer is homologous to the antisense strand, i.e., complimentary to the coding strand sequence. Bases in the mutagenic primers which differ from the methanogen chromosomal sequences are underlined. MCRH136 and BAS27 were used for mutagenesis of mcrC by PCR. C15 and DS16 were used as sequencing primers. C15 and BAS27 were designed by Sherf (1989).

**Cloning and expression of mcrC (including lacZ-mcrC fusion):** Cloning of the mcrC gene is outlined in Figure 4. Sequences were amplified by using the polymerase chain reaction (PCR). Approximately 10 ng of isolated template DNA was diluted to 100μl with 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM MgCl₂, 0.01%(w/v) gelatin, 10 mM DTT, 2 mM dNTP's and 1μM primers. After adding a top layer of mineral oil and heating to 90°C for 2 min, Taq polymerase (1 μl) was added. The reaction was cycled 25 times through 94°C for 1 min, 45°C for 1 min, and 72°C for 1.5 min increased to 2 min over the cycles. The products were gel purified by the Prep-a-Gene method (BioRad).
The gplacZ-mcrC fusion protein was purified as described by Ullmann, 1984. E. coli strain DH5α carrying pDSC2921 were grown in 50 ml LB containing 20 μg ampicillin/ml, at 37 °C with vigorous shaking. The cells were harvested at OD₆₀₀ = 0.8 by centrifuging for 5 min at 5,000 rpm in a Sorval SS-34 rotor. The cells were lysed by sonication after the addition of lysozyme. The lysate was adjusted to 10 mg protein/ml, 20 mM Tris-HCl (pH 7.4), 1.6 M NaCl, 10 mM β-mercaptoethanol, and 10 mM MgCl₂ and loaded onto a 1 ml aminobenzyl thiogalactoside-Sepharose column (Sigma) equilibrated with 20 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1.6 NaCl, 10 mM β-mercaptoethanol. After washing the column with 100 vol of equilibration buffer, the fusion protein was eluted with 100 mM borate, 10 mM β-mercaptoethanol, pH 10.

Antiserum against gplacZ-mcrC was raised as for recombinant gpmcrD. Three rabbits were boosted 4 times.

V8 protease digestion of purified gplacZ-mcrC fusion polypeptide probed with serum directed against the gplacZ-mcrC fusion peptide: Approximately 25 nmoles of β-galactosidase or purified gplacZ-mcrC fusion polypeptide were denatured by boiling for 5 min in 1% SDS and 0.1 and 1 μg of Staphococcal V8 protease added. The samples were then electrophoresed into the 4% stacking gel of a 10-15% (w/v) linear gradient SDS polyacrylamide gel and the power turned off. After 30 min at room temperature the gel was run overnight, the separated polypeptides transferred to nitrocellulose, and probed with a 1:500 dilution of anti-gplacZ-mcrC antiserum.

RNA extraction: RNA was extracted from E. coli and M. vannielii by the diethylpyrocarbonate (DEPC) method of Summers, 1970. Cells from a 1 ml of E. coli culture were pelleted in an Eppendorf Model 5412 microfuge and lysed by incubating with 5 μg lysozyme/ml in 100 μl of 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 50 mM glucose, followed by the addition of 200 μl of 10 mM Tris-HCl, 10 mM NaCl, 1 mM Na citrate, 1.5% (w/v)SDS. 30 μl of DEPC (Sigma) and 250 μl of a saturated NaCl solution were added to each sample and the reaction mixtures incubated on ice for 10 min. The
supernatants obtained after centrifugation for 10 min in a microfuge, were transferred to a clean tube and the nucleic acids precipitated by the addition of 2.5 vol of 95% ethanol and incubating on ice for 10 min. Contaminating DNA was digested by incubation with 30 units of RNase-free DNase I (U.S. Biochemicals) for 5 min at room temperature. The RNA remaining was obtained by phenol/chloroform extraction and ethanol precipitation and resuspended in DEPC treated TE (20 mM Tris, pH 7.4, 0.1 mM EDTA) The nucleic acids were quantitated by measuring the absorbance of solutions at 260 nm.

RNA run-off transcripts directed by T7 promoter in vitro: Template plasmids (1µg) were digested with the appropriate restriction enzyme to linearize the plasmid DNA so that transcription of vector sequences would be minimized. Transcription reactions were carried out by adding to a 25 µl restriction digest: 10 µl of 10X TMG (40 mM Tris-HCl, pH 7.4, 20 mM MgCl₂, 2% glycerol), 10 µl of 1 mg methylated bovine serum albumin/ml, 10 µl of 100 mM dithiothreitol, 1µl of 100 mM GTP, 1µl of 100 mM CTP, 1µl of 100 mM UTP, 1 µl of 10 mM ATP, 1µl of α-3²P]-ATP (100 µCi), 1 µl of T7 DNA dependent RNA polymerase (100 units) and 24 µl of DEPC treated water. After 60 min incubation at 37°C, the reaction was terminated by ethanol addition. Precipitated materials were dissolved in 10 µl of DEPC water and 10µl of 2X RNA sample buffer (80% formamide, 50 mM Tris-borate pH 8.3, 1 mM EDTA, xylene cyanol and bromophenol blue) and loaded onto a 6% polyacrylamide-8M urea gel. Following electrophoresis, radioactive bands were cut from the gel and RNA molecules eluted from the gel fragments into TMG by incubation overnight in the presence of phenol.

Labeling cells for the detection of recombinant proteins synthesized in E. coli: i) Minicells

Minicells were used to evaluate the efficiency of various gpmcrC expression constructs. The minicells were prepared from E. coli DS410 cultures transformed with recombinant plasmids as described by Reeve, 1979.
ii) Rifamipin poisoned \textit{E. coli} cells.

\textit{E. coli} DNA-dependent RNA polymerase was inhibited \textit{in vivo} by the addition of rifampin (Sigma Chemical Co., St. Louis, MO). To induce the synthesis of the T7 RNA polymerase, IPTG was added to a final concentration of 1 mM to cultures of \textit{E. coli} BL21(DE3) growing exponentially at 37°C with shaking in 1 ml of LB containing 20 \(\mu\)g ampicillin/ml, 20 \(\mu\)g chloramphenicol/ml and 4 g glucose/l. Growth was continued for 1 hr. The cells were then pelleted and resuspended in 200 \(\mu\)l of M9 "complete" (12 g \(\text{Na}_2\text{HPO}_4\)/l, 6 g \(\text{KH}_2\text{PO}_4\)/l, 1 g \(\text{NaCl}\)/l, 2 g \(\text{NH}_4\text{Cl}\)/l, 0.5 g \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\)/l, 0.03 g \(\text{CaCl}_2\cdot2\text{H}_2\text{O}\)/l, 1.7 mg thiamine-\(\text{HCl}\)/l, and 4 g glucose/l), 5% LB, and antibiotics, plus 100 \(\mu\)g rifampin/ml (rifampin was prepared by dissolving 100 mg rifampin in 1 ml methanol). These cell suspensions were incubated at 37°C for 45 min, and 100 \(\mu\)Ci of \textit{TRAN}^{35}S label then added, and incubation continued for 30 min. Radioactively labeled cells, so obtained, were pelleted and washed with TN (20 mM Tris-\(\text{HCl}\), pH 7.4, 9 g \(\text{NaCl}\)/l), resuspended in 200 \(\mu\)l of TN containing 1 \(\mu\)g DNasel/ml, 1\(\mu\)g RNaseA/ml and lysed by three cycles of freezing and thawing. The \textit{E. coli} BL21(DE3) host strain used for these experiments carried, in addition to the plasmids shown in Figure 9, pLysS, which directs the synthesis of lysozyme, eliminating the need to add exogenous lysozyme to lyse the cells. The incorporation of \textit{35}S into proteins was measured by determining trichloroacetic acid (TCA) precipitable counts. An aliquot (10 \(\mu\)l) of each lysate was diluted to 1 ml with 8% cold TCA. After incubating on ice for 60 min, the samples were filtered through 13 mm diameter nitrocellulose filters, pore size 0.45 \(\mu\)m. The filters were washed under a gentle vacuum using 5 ml of 8% cold TCA, dried under a heat lamp, submerged in 1 ml of scintillation cocktail (SintaverseE), and the radioactivity counted in a Beckman LS7500 liquid scintillation counter.

\textbf{Purification of MR component C from Methanococcus vannielii:} Components A, B and C of the \textit{in vitro} methanogenic reaction mixture were prepared under anaerobic
conditions as described by Ellefson and Wolfe, 1980. *Mc. vannielii* cells, 5 g (wet weight), were resuspended in 5 ml anaerobic Buffer A (50 mM Tris-HCl, pH 7.0, 10 mM β-mercaptoethanol) and passaged once through a French pressure cell. DNase I (5μg) was added and the extract centrifuged at 100,000Xg for 2 hr at 4°C. The resulting supernatant was subjected to ion exchange chromatography on Whatman DE52 (5 ml of resin) and the flowthrough was designated component A, following the nomenclature of Gunsalus and Wolfe, 1979. The column was then washed with Buffer A containing 100 mM NaCl, and the eluent designated component B. The column was then washed with Buffer A containing 320 mM NaCl and the fractions which contained the MR holoenzyme were combined and designated component C. MR, gpmcrD and gpmcrC were detected in different fractions by western blotting.

**Determining antigen levels:** Antigen levels were quantitated either by scanning western blots using a Soft Laser Densitometer (Zeineth) analyzed by the GelScan computation program or by direct and competitive enzyme linked immunosorbent assays (ELISA). Antisera prepared against MR (α2β2γ2) and the isolated β-subunit of MR were provided by B. Sherf (Sherf, 1989).

To assay MR and gpmcrD by direct antibody capture ELISA, ELISA 96-well plates (Corning, Corning, NY) were coated by incubation overnight at 4°C with the protein sample diluted in PBS. All subsequent steps were performed at room temperature. After washing 5 times with wash buffer (20.2 g NaCl, 0.2 g KH$_2$PO$_4$, 0.92 g Na$_2$HPO$_4$, 0.5 g Tween 20 per liter), the wells were blocked by incubation with 200 μl PBS containing 5% dry milk for 15 min. The blocking solution was decanted and 100 μl of antiserum, diluted 1:200 in PBS containing 5% dry milk, was added to each well. Following incubation for 30 min, the plates were washed 5 times with wash buffer and blocked 5 min by the addition of 200 μl of PBS containing 5% dry milk. The liquid was shaken out of the wells and 100 μl of goat anti-rabbit antiserum conjugated to horseradish peroxidase (Sigma), diluted 1:200 in PBS containing 5% dry milk, were
added. After 30 min, the plates were washed 5 times with wash buffer and twice with 0.1 M citric acid phosphate buffer (7.3 g citric acid, 9.46 g Na$_2$HPO$_4$, pH 5, per liter). 50 µl of a solution containing 8 mg of o-phenylenediamine mixed with 5 µl of 30% hydrogen peroxide in 15 ml of citric acid phosphate buffer were added per well. 75 µl 1 M H$_2$SO$_4$ was added after 15 min to stop color development, and the ELISA plates read using a MR700 Microplate reader (Dynatech Labs, Inc.) measuring adsorption at 450 nm or 492 nm.

For the competitive antibody capture ELISA, 50 ng of purified antigen diluted in PBS to 1 ng protein/µl were used to coat the wells of 96-well ELISA plates. The samples were reacted with the diluted antiserum for 1 hr, and then the preabsorbed antiserum was titered using the coated plates as with the direct ELISA. Samples were quantitated by comparing absorbances to a standard curve. Determinations were done in triplicate.

Rates of methanogenesis by cell suspensions: In a Coy anaerobic chamber, aliquots (1 ml) of Mc. vannielii cultures to be assayed for methane production were transferred to 5 ml vials capped with an open-top closure with a septum. The vials were flushed with H$_2$ and the rate of methane production at 37°C measured as described below for cell free extracts.

Sucrose gradients: Exponentially growing Methanococcus vannielii cells were pelleted and resuspended in a minimal volume of anaerobic PBS, containing 10 µg DNasel/ml, resulting in a final protein concentration of approximately 10 mg/ml. The cells in 250 µl of this suspension were lysed directly on top of 5 ml 5 to 30% (w/v) linear sucrose gradients by the addition of NP-40 detergent to a final concentration of 0.5%. The gradients were centrifuged at 40,000 rpm for 10 hr at 4°C in a SW50.1 rotor (150,000Xg) and then fractionated into 0.5 ml fractions by collecting drops after piercing the bottom of the tube with a 22G winged infusion set. Fractions were assayed for the presence of MR, gpmcrD, gpmcrC by immunoblotting and for total
protein by the BCA assay.

Methanogenesis in vitro: All manipulations were performed under anaerobic conditions. *M. vannielii* cells were pelleted from a 20 l culture growing exponentially (OD$_{580}$=0.4) in ER medium by centrifugation in a Sorval continuous flow rotor. These cells were resuspended in 10 ml of 50 mM MOPS [3-(N-morpholino)propanesulfonic acid], pH 7.0, buffer per gram wet weight and ruptured by passage through a French pressure cell. The resulting lysate was centrifuged for 10 min at 5000Xg at 4°C, and the supernatant obtained frozen at -70°C in 500 µl aliquots in 5 ml vials. The volumes of the assay vials were determined precisely by water weight. Reactions were started by the addition of anaerobic ATP to a final concentration of 12.5 mM, using a Hamilton syringe. Methyl-CoM was added to 10 mM where indicated. The gas phase was made uniformly H$_2$ by flushing the vented vial for 2 min using a gas manifold equipped with a 22G needle. The molar amounts of CH$_4$, CO$_2$, and H$_2$ which accumulated in the headspace during incubation at 37°C were determined at intervals by sampling with a gas-tight Hamilton syringe and analyzing the gases using a Varian 3700 model gas chromatograph, equipped with a thermal conductivity detector and a Porapack N molecular sieving column formed in a stainless steel tube (1/8 inch in diameter and 6 ft in length). Helium was used as the carrier gas. The Hewlett Packard Model 3390A integrator was calibrated using methane, carbon dioxide and hydrogen standards.
CHAPTER III
RESULTS AND DISCUSSION

A) Expression of recombinant mcrD and generation of anti-gpmcrD antisera

Expression of the cloned mcrD gene in E. coli: The recombinant gpmcrD that accumulated in E. coli BL21(DE3) cells containing pDSD431, growing in the presence of IPTG, was purified to homogeneity by gel filtration, followed by DEAE-Sepharose ion exchange chromatography (Figure 3B). The recombinant protein was shown to accumulate to approximately 5% of the cellular protein (Table 2). Purified recombinant protein comigrated during SDS-PAGE with native gpmcrD in extracts of Mc. vannielii cells and cross-reacted with serum directed against the gplacZ-mcrD fusion protein provided by Sherf, 1989. The sequence of the ten amino terminal amino acid residues of the purified protein was determined and shown to be identical with the amino acid sequence predicted for gpmcrD by the sequence of the mcrD gene (Cram, et al., 1987).

Production of gpmcrD-specific antiserum: Purified recombinant gpmcrD was used to immunize New Zealand White rabbits. Antibodies so obtained were shown to bind to a Mc. vannielii protein which comigrated during SDS-PAGE with the purified recombinant protein and with the material that formed a band when assayed by immunoblotting with the anti-gplacZ-mcrD fusion serum. Therefore, mcrD was expressed in E.coli and antibodies specific for the product of the mcrD gene were obtained.
### TABLE 2: Purification of recombinant gpmcrD

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein conc. (mg/ml)</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Purification factor(^b)</th>
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</tr>
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<td>P200</td>
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<td>47</td>
<td>8</td>
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<td>DEAE(^c)</td>
<td>1.2</td>
<td>17.2</td>
<td>21</td>
<td>18</td>
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</table>

\(^a\) The fractionation steps are described and identified in the Materials and Methods and in Figure 3B.

\(^b\) Purification factor calculated by dividing the starting protein mass by the remaining protein mass in the pooled fractions of the gpmcrD peak.

\(^c\) The gpmcrD that elutes from the DEAE-Sepharose migrates as a single band on silver stained gels (Figure 15). Therefore, the fraction was considered to be composed entirely of gpmcrD and used as a standard for measuring gpmcrD concentrations.
Figure 3: Expression of the mcrD gene in E. coli.

A. pDSD431 was constructed by ligating the indicated 600 bp EcoRI/PstI fragment from pMRD107 (Sherf, 1989) into pT7-2 and transforming into E. coli HB101. The resulting 3350 bp plasmid was then transformed into E. coli BL21(DE3), for expression and transcription of the mcrD gene induced by the addition of IPTG. E. coli HB101 (recA⁻) was used for the initial cloning because the transformation efficiency of E. coli BL21(DE3)(recA⁺) was unacceptably low. The numbers indicate the fragment sizes in base pairs (bp). The sequence of the intergenic region between mcrB and mcrD is shown from the translation termination codon of mcrB to the translation initiation codon of mcrD. The sequence presumed to be the ribosome binding site (RBS) for the mcrD gene is underlined. P, PstI; E, EcoRI; S, SmaI; A, Accl.

B. Purification of recombinant gpMcrD. Photograph of a 12.5 % SDS-polyacrylamide gel stained with Coomassie brilliant blue. Lane 1, low molecular weight size standards (BioRad); lane 2, uninduced cell extract (100 µg); lane 3, cell extract (homogenate) after 4 hr induction by IPTG(100 µg); lane 4, 10,000Xg supernatant (100 µg); lane 5, gpMcrD peak material from the P200 gel filtration column (5 µg); lane 6, gpMcrD peak eluted from DEAE-Sepharose by a linear pH gradient (5 µg). Comparison of homogenate (lane 3) to 10,000Xg supernatant (lane 4) indicated that the recombinant gpMcrD was soluble, i.e., it did not pellet, consistent with gpMcrD not forming inclusion bodies or being membrane associated in E. coli.
A: pDSD431

pMRD107

pUC18

lacP/O

pDSD431

pT7-2

T7 promoter

lacZ

B:

Figure 3
B) Construction of plasmids for mcrC expression in E. coli including lacZ-mcrC gene fusions. Results of expression of mcrC containing plasmids.

Expression of mcrC in E. coli: A mutagenic primer, MCRH136, was used with PCR to introduce a consensus E. coli ribosome binding site upstream of the AUG start codon of the Mc. vannielii mcrC gene (Figure 4). A second primer, BAS27, introduced a HindIII restriction site at the stop codon of mcrC which could then be used to destroy this stop codon during construction a lacZ-mcrC fusion gene. The amplified fragment with mcrC was first cloned into pUC18 and its sequence confirmed. The modified mcrC gene was then subcloned immediately downstream from the T7 promoter in pT7-2 (pDSC432, Figure 7). Another construct, pDSC433, was constructed by ligating the same insert as pDSC432 into pT7-1 which resulted in a plasmid that would transcribe mcrC anti-sense RNA, for use as a riboprobe for the sense strand.

In the absence of a functional assay for gpmcrC or anti-gpmcrC antibodies, the only method available to detect the synthesis of gpmcrC in E. coli strains was to identify a novel band with an apparent Mr of 21 kD following SDS-PAGE. Several different plasmid constructs and E. coli strains where evaluated (Figures 4-6), but with no obvious synthesis of gpmcrC, even though transcription of mRNA from the cloned mcrC gene in E. coli was demonstrated (Figure 8). Synthesis of a 21 kD polypeptide was also not detected in minicells containing plasmids encoding the Mc. vannielii mcr operon (Sherf, 1989).

In an effort to detect the synthesis of even a small amount of gpmcrC, strains of E. coli carrying plasmids in which the mcrC gene had been positioned downstream from the T7 gene10 promoter were induced by IPTG addition to synthesize the T7 DNA dependent RNA polymerase. The E. coli RNA polymerase was then inhibited by the addition of rifampin, and the cultures pulse labeled with TRAN35S. Results are shown in Figure 9B. A plasmid which contained and expressed the mcrD gene using the T7
Figure 4: Subcloning of mcrC: Cloning the PCR amplification product into pUC18.

A: Primers used in PCR to amplify mcrC. The designed E. coli consensus ribosome binding site, translation start and termination codons are underlined. An asterisk (*) marks the transversion (C→G) introduced to create a HpaI site at the termination codon, which was used to construct a fusion gene with lacZ.

B: pDSC81. A gel purified HindIII/BamHI 980 bp fragment of pMCR (Sherf, 1989) was used as the template for PCR. The PCR amplified sequence was subcloned into pUC18 by ligating the undigested, gel purified mcrC containing fragment into pUC18 digested with HincII. Sequences of the junction region are shown, which were confirmed by sequencing. Large type face, PCR primer sequences; small, pUC18 sequences.

C: pDSC82. pET4300 (Cram, et al., 1987) was used as the template for PCR. The amplified fragment was cloned as with pDSC81, however, in the opposite orientation relative to the pUC18 vector.

B, BamHI; H, HindIII, P, PstI; S, SmaI.
Figure 5: Subcloning mcrC into pTrc99A, pT7-2 and pSKS105.

A: Subcloning mcrC into a Ptac expression vector, pTrc99A, which has an IPTG inducible promoter and an E. coli RBS. The 600 bp SmaI/PstI fragment of pDSC81 (Figure 4) was subcloned into pTrc99A which was digested with SmaI/PstI.

No 21 kD protein was detected in pDSC63-containing minicells.

B: Subcloning mcrC into the pT7-2 expression vector, flanked by methanogen chromosomal sequences. The 980 bp HindIII/BamHI fragment of pMRC was ligated into pT7-2 digested with HindIII/BamHI.

This plasmid directed the synthesis of the "15 kD" protein in rifampin treated cells (Figure 8), as did the parent plasmid pMRC in minicells (Sherf, 1989).

C: Subcloning and expression of mcrC into the lacZ fusion vector pSKS105 where the mcrC gene is the 5' sequence, pDCSKS1. E. coli transformed with the ligation reaction of SmaI-digested pSKS105 with pDSC81 (Figure 4) digested with SmaI/HpaI, were screened on X-gal plates yielding 1 light blue, 3 white, 65 blue colonies. The ligation was repeated 4 times with partial digests with similar results. Six independent constructs were investigated. Junctions were confirmed by DNA sequencing, but western blots indicated that the gplacZ synthesized was predominately wild type in size. Synthesis of a gplacZ-mcrC fusion polypeptide was not detected in minicell strain.

B, BamHI; H, HindIII; P, PstI; S, SmaI.
A: pDSC63

ptrc99A Smal/PstI → tac promoter → pDSC81 Smal/PstI

4,900 bp

B: pDSC434

pT7-2 BamHI/HindIII → T7 promoter

165 bp → 9 bp → 9 bp → 195 bp

C: pDSCSKS1

pSKS105 Smal → pDSC81 Hpal partial digest

CCC | AAC ATG | TGGGATCCGT

10,700 bp

Figure 5
Figure 6: Construction of a 5' deletion of mcrC and subsequent subcloning into pSKS105.


B: Subcloning of the truncated mcrC gene, ptruncll. ptruncll is a deletion mutant of pDSC81 (Figure 4), resulting in an N-terminal truncation of gpmcrC. The sequence of the PCR amplified mcrC is shown with its predicted amino acid sequence. The sequence between the 5' HindIII sites was deleted by digesting pDSC81 with HindIII followed by ligation. Digests of the resulting plasmids indicated that most contained the expected deletion. The accuracy of the deletion was verified by sequencing and a representative plasmid named ptruncll.

C: Subcloning the truncated mcrC gene into the lacZ fusion vector, pSKS105. A BamHI/HindIII (sites from the pUC18 polylinker) fragment from ptruncll was subcloned into pSKS105 digested with BamHI/HindIII.

Western blots of cell extracts made from cultures of 5 independent constructs detected the presence of only wild type gplacZ. One construct was confirmed by DNA sequencing.
A:

<table>
<thead>
<tr>
<th>Organism</th>
<th>Consensus Sequence</th>
</tr>
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<tbody>
<tr>
<td><em>M. vanneielli</em></td>
<td>MPVGRKQIVDCRAMGLG3GG</td>
</tr>
<tr>
<td><em>M. voltae</em></td>
<td>---------------------</td>
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<td>-MIGRTIVDCREMGLG3GG</td>
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<td><em>M. thermo.</em></td>
<td>-MIGKCTIVDCREMGLG3GG</td>
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<tr>
<td><em>M. Barkeri</em></td>
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CONSENSUS

```
MPVGRKQIVDCRAMGLG3GG
```

```
MELIDRTQVDCRGAGGLKGG
```

B:

Figure 6
Figure 7: Subcloning and expression of the mcrC gene in pT7-2 and pUR292.

A: Subcloning of the genetically engineered mcrC gene into the T7 expression vector and into the lacZ fusion vector. A BamHI/PstI fragment from pDSC81 was ligated into BamHI/PstI digested pT7-1 and transformed into E.coli. 200 colonies were picked and all hybridized to end-labeled MCRH136 oligo probe (Table 1). Ten plasmids were isolated for restriction mapping and all were shown to have the expected insert. No gpmcrC was detected, however, in silver stained gels following SDS-PAGE of extracts of the expression strain BL21(DE3) containing these plasmids, nor in minicells containing two of these constructs.

pDSC432 was constructed by digesting pT7-2 with BamHI/HindIII and ligating to the 600 bp BamHI/HindIII fragment from pDSC82 (Figure 4). A 25 kD product was detected in E.coli cells when the E.coli RNA polymerase was first inhibited by rifampin, and cells then pulsed with TRAN35S. pDSC433 was constructed by cloning the same BamHI/PstI fragment into pT7-1, resulting in a plasmid with the insert in the opposite direction from pDSC432. This construct was used to generate riboprobes complimentary to the mcrC sense strand which was used to detect mcrC messenger RNA's in northern blots.

pDSC432 was digested with SmaI and PstI and, following the gel purification, the insert was subcloned into pUR291. This vector was first digested with HindIII, the single stranded termini blunted with Mung bean nuclease and then digested with PstI. In the resulting plasmid, pDSC2921, the lacZ gene is 5' to mcrC sequences. This plasmid directs the expression of a 137 kD fusion polypeptide which reacts with antibodies against β-galactosidase. An E.coli strain bearing pDSC2921 was used to generate the gplacZ-mcrC fusion polypeptide which was then used to generate antiserum against gpmcrC epitopes.

B: Sequence of the junction region of pDSC2921. B, BamHI; H, HindIII; P, PstI; S, SmaI
A: pDSC2921

B: sequence of the lacZ-mcrC junction pDSC2921

Figure 7
gene10 promoter (pDSD431) was used as the positive control. Synthesis of the predicted 18 kD gpmC peptide is clearly visible in lane 2. The pT7 construct containing the mcrC gene flanked by its native methanogen sequences (pDSC434), synthesized predominately a polypeptide, that was also seen in minicell expression studies and which comigrated with the polypeptide identified as the "15 kD polypeptide" by Sherf, 1989. The pT7 construct containing the mcrC gene preceded by the engineered consensus E. coli ribosome binding site (pDSC432, lane 4) directed the synthesis of a 25 kD polypeptide which was also synthesized, but in lower amounts, in E. coli cells containing pDSC434 which has the wild type Mc. vannielii ribosome binding site. The 15 kD polypeptide was not detected in E. coli cells containing the plasmid with the engineered RBS (pDSC432).

One of the polypeptides synthesized in minicells containing pET4300 also had an electrophoretic mobility indicative of a molecular mass of 25 kD and was labeled by Cram, et al., 1987, with a question mark. Plasmid pET4300 contains the mcrC gene (see Figure 4). All the available evidence is consistent with the product of the mcrC gene, synthesized in E. coli, migrating during SDS-PAGE with an apparent molecular mass of 25 kD. The molecular mass predicted from the gene sequence was calculated to be 21.2 kD (Sherf, 1989).

Transcription of the mcrC gene in vivo.

Northern slotblots, using riboprobes complimentary to the sense and antisense strands of the mcrC gene were used to probe RNA prepared from Methanococcus vannielii cells. The presence of only of the sense transcript was detected. No antisense transcript was detected in the methanogen. Both sense and antisense transcripts were, however, detected in RNA preparations obtained from E. coli strains carrying plasmids which contained the mcrC gene. A riboprobe (transcribed from pDSC433, Figure 7) for the sense RNA hybridized to the RNA also from the vector without insert but not the antisense probe (transcribed from pDSC432, Figure 7).
These riboprobes have polylinker sequences in common with vector transcripts, which presumably mediated this hybridization. This result was consistent with the interpretation that the pUC-mcrC clones directed the synthesis of the sense and antisense strands of RNA, while the methanogen produced only the sense strand. Inspection of the mcrC sequence revealed an ORF of sufficient length to encode the 15 kD polypeptide located on the opposite DNA strand from the mcrC gene. As this ORF continues into the vector, this is consistent with the slight shifts in electrophoretic mobility of the 15 kD polypeptide which were seen and which were dependent on the orientation of the mcrC gene in different pUC vectors (Sherf, 1989). The 15 kD polypeptide, therefore, appears to be the product of an ORF encoded on the opposite DNA strand relative to mcrC which is presumably synthesized as an artifact of expression in E.coli.

Construction and expression in E. coli of a truncated mcrC gene and the complete mcr operon from Mc. vannielii: Rifampin poisoned E. coli cells synthesized gpmpcrC but not in sufficient amounts to allow its purification (Figure 9). As fusions of the mcrC gene to the 5' end of lacZ, also did not synthesize detectable amounts of a fusion polypeptide (the accuracy of the junction sequences was confirmed by sequencing) it was apparent that something in the mcrC gene or flanking sequence was precluding high levels of mcrC expression in E. coli.

To investigate if 5' sequences were the problem, the mcrC was truncated by deleting sequences between two HincII sites, so that the mcrC ORF began at a downstream methionine, similar to the mcrC gene in Mc. voltae (plasmid pruncI, Figure 6B). This plasmid was used to construct a fusion gene with lacZ. This 5' deletion mutant, pDSKS1 (Figure 6C), also did not direct the expression of detectable amounts of gpmpcrC-lacZ fusion protein. Anti-β-galactosidase was used in western blots to detect the synthesis of this gene product, but without success. The vector-encoded ribosome binding site of lacZ remained available to initiate translation.
When both \textit{mcrD} and \textit{mcrC} were transcribed within the same transcript in \textit{E. coli}, \textit{gpmcrD} was clearly synthesized at much higher levels than \textit{gpmcrC} (figure 10). The low level of \textit{gpmcrC} synthesis must, therefore, be due to poor translation efficiency. Translation of the \textit{mcrD} ORF was, in fact, also higher than that of the \textit{mcrA}, \textit{mcrB} and \textit{mcrG} ORF's despite these genes having ribosome binding sites more complementary to the 3' end of the 16S rRNA of \textit{E. coli} and \textit{M. vanneii} than the RBS of \textit{mcrD} (Sherf, 1989). One possible explanation for this discrepancy could be codon usage.

Looman, et al., 1987, demonstrated a 15 fold difference in β-galactosidase synthesis in \textit{E. coli} when changes were made in the second codon. The CCC codon found at this position in \textit{mcrC} resulted in a low level of translation of β-galactosidase (0.2 units, the best being 1.5 units). The second codon in the truncated \textit{mcrC} gene, GGU, resulted in even less β-galactosidase synthesis (0.1 units). The second codons of \textit{mcrG} (GCA) and \textit{mcrA} (GAA) had scores of 0.6 units, while \textit{mcrB}'s second codon (GTA) scored the same as the highly expressed \textit{mcrD} (ATC), at 0.9 units.

\textbf{C) Results of expression of lacZ-mcrC in \textit{E. coli}: Purification of gplacZ-mcrC from \textit{E. coli}, production of antisera and evaluation of the antibodies so obtained.}

\textbf{Synthesis and isolation of gplacZ-mcrC fusion polypeptide:} To circumvent the problem of low \textit{mcrC} expression in \textit{E. coli}, a \textit{lacZ-mcrC} gene fusion was constructed in which the 5' end of \textit{mcrC} gene was fused to the 3' end of \textit{lacZ}. Translation initiation was therefore determined by \textit{lacZ} sequences. This construction, resulting in plasmid pDSC2921, is outlined in Figure 7. The precision of the gene fusion was confirmed by DNA sequencing. When expression of this \textit{lacZ-mcrC} gene was induced by the addition of IPTG, a polypeptide was synthesized with an electrophoretic mobility consistent with the predicted molecular weight for \textit{gplacZ-mcrC} of 137 kD. Figure 11A is a western blot of extracts from \textit{E. coli} synthesizing \textit{gplacZ-mcrC} (pDSC2921), probed with monoclonal antibodies against β-galactosidase which demonstrates the increase in
Figure 8: Slot blot of RNA isolated from \textit{E. coli} cells containing pT7-1, pDSC434 and pDSC432.

RNA was extracted from \textit{E. coli} BL21(DE3) pLysS strains harboring the plasmids listed below (see Figure 5B for pDSC434 and Figure 7A for pDSC432) as described in Materials and Methods. Cells were grown at 37°C with and without 1 mM IPTG. Formaldehyde denatured RNA (2μg) was loaded into each slot and filtered onto a nitrocellulose sheet equilibrated with 10X SSC (1.5 M NaCl and 0.15 M Na citrate). The filter was baked and incubated with $^{32}$P-end-labeled oligomer, BAS27 (see Table 1) under stringent hybridization conditions (68°C, 5X SSC, 1 mg ficoll/ml, 1 mg polyvinylpyrrolidone/ml, 1 mg BSA/ml, 1 % SDS and 200 μg yeast tRNA/ml). The oligomer BAS27 is a probe for the 3' end of the sense strand of \textit{mcrC}. The material in column A was DNased and in column B was DNased and RNased before binding to the filter. The results demonstrate that the \textit{mcrC} gene cloned in pDSC434 and pDSC432 was being transcribed in \textit{E. coli} cells that were not synthesizing easily detectable amounts of \textit{gpmcrC} and that IPTG induction resulted in increased levels of \textit{mcrC} transcription \textit{in vivo}. The hybridization signals were reduced when the samples were RNase treated, indicating that the signals resulted from the presence of specific RNA.

<table>
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<th>Sample</th>
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<td>pT7-1 + IPTG</td>
</tr>
<tr>
<td>5</td>
<td>pDSC434</td>
</tr>
<tr>
<td>6</td>
<td>pDSC434</td>
</tr>
<tr>
<td>7</td>
<td>pDSC434 + IPTG</td>
</tr>
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</tbody>
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Figure 8
Figure 9: Expression of mcrC in rifampin treated E. coli.

A. Structure of the plasmids used in Figure 9B. The vector pT7-2 DNA contained the T7 gene 10 promoter (indicated by the heavy arrow). Restriction sites indicated are B, BamHI; E, EcoRI; H, HindIII; K, KpnI; P, PstI. Remnants of the mcrD and mcrG genes are indicated by the boxes flanking the mcrC gene. The synthetic ribosome binding site constructed as shown in Figure 4, is indicated in pDS432 by SD.

B. Autoradiogram of $^{35}$S labeled E. coli lysates separated by SDS-PAGE. Samples were labeled and prepared for SDS-PAGE as described in Materials and Methods. An equal number of TCA precipitable counts were loaded into each lane of an SDS-polyacrylamide gel. Lanes 1, 2, 3, and 4 contained extracts from E. coli cells containing the plasmids identified for convenience in Figure 9A, as 1, 2, 3 and 4. Arrow 1 indicates the double band formed by $\beta$-lactamase, arrow 2, indicates the putative 25 kD product of mcrC; arrow 3, indicates gpmcrD; and, arrow 4, indicates the "15 kD" polypeptide described by Sherf, 1989.
A:

Lane: 1
pT7-2

2
pDSD431

3
pDSC434

4
pDSC432

B:

1 2 3 4

97.4
66.2
45.0
31.0
21.5
14.4

1 β-lactamase
2 gpmcrC
3 gpmcrD
Figure 10: Cloning and expression of the mcr operon from *M. vannielii* in *E. coli*.

A: Diagram of the mcr operon cloned into pT7-1. Genomic *M. vannielii* DNA was digested with BamHI and a number of other restriction endonucleases predicted (DNA Strider sequence manipulation program) not to cleave within the coding region of the operon. A BamHI site was known to be located approximately 500 bp 3' to the operon (Reeve, personal communication) and by using radioactively labeled pET4300 (Cram, et al., 1987) DNA as the probe, in a Southern hybridization, a SstI site was located approximately 300 bp 5' to the mcr operon in *M. vannielii*. The SstI-BamHI fragment that contained the whole mcr operon had an electrophoretic mobility consistent with a size of 6.3 kb. This fragment was cloned by separating *M. vannielii* chromosomal DNA digested with SstI-BamHI on an agarose gel, isolating the DNA from a window centered at 6.3 kb, and ligating into pT7-1. Transformation into *E. coli* DH5α generated pDS77403. The mcrD gene in pDS77403 was disrupted by digestion at the ClaI site in this gene, filling in the single strand ends with the Klenow fragment of DNA Pol I, and religation which resulted in pDS774036. These plasmids were transformed into the expression strain, *E. coli* BL21(DE3). Sizes are given in kilobase pairs (kb).

B: Coomassie Brilliant Blue stained 10% SDS-polyacrylamide gel following SDS-PAGE of extracts of *E. coli* BL21 (DE3) cells containing: lanes 1 and 2, pT7-1; lanes 3 and 4, pDS77403; lanes 5 and 6, pDS774036; far right lane, component C. The extracts analyzed in lanes 2, 4, and 6 were grown in the presence of 2 mM IPTG for 2 hr at 37°C.

C: The materials shown in Figure 10B were transferred to nitrocellulose and immunoblotted with antiserum raised against the MR holoenzyme (Cram, et al., 1987). The levels of synthesis of *gpmcrA*, *gpmcrB* and *gpmcrG*, were not
changed by the frameshift mutation in \textit{mcrD} (pDS774036, lane 6). If this mutation is also nonpolar in the methanogen it may be useful for chromosomal disruption experiments in \textit{M. vanniielli}.

Probing these \textit{E. coli} extracts with the anti-gplacZ-mcrC antibodies resulted in many spurious bands (see Figure 13). These cross-reacting \textit{E. coli} bands obscured the MR bands.
Figure 10
Figure 11: Expression of the lacZ-mcrC gene fusion in E. coli and purification of the resulting fusion peptide, gplacZ-mcrC. Polypeptides were separated by SDS-PAGE through 10% polyacrylamide gels.

A: Western blot probed with a 1:5,000 dilution of anti-β-galactosidase monoclonal antibodies. Equal culture volumes were loaded in each lane. Lane 1, host strain, E. coli DH5α (ΔlacZYA), without plasmid; lane 2, E. coli DH5α containing the pUR292 vector; and, lane 3, E. coli DH5α containing pDSC2921. Note the molecular weight shift from 116 kD for β-galactosidase to 137 kD, the predicted molecular weight of the fusion polypeptide, gplacZ-mcrC (116 kD+ the mol. wt. of gpmcrC 21 kD= 137). The lower bands in lane 3 are believed to result from degradation products.

B: Purification of the gplacZ-mcrC fusion polypeptide. Coomassie brilliant blue stained gel of the sequential steps used in the purification of gplacZ-mcrC fusion polypeptide from E. coli DH5α (pDSC2921) using an ABTG affinity column, as described in Materials and Methods. Lane 1, molecular weight markers, lane 2, extract of E. coli DH5α containing pUR292 (vector alone)(100 µg); 3, extract of E. coli DH5α containing pDSC2921 (mcrC insert)(100 µg); 4 and 5, flowthrough of ABTG affinity columns (100 µg); 6 and 7, elution peaks of two preparations of fusion protein (20 µg). Lanes 4 and 6 corresponds to one binding and elution of the fusion peptide to the ABTG column, and lanes 5 and 7 to a second run. The materials in tracks 6 and 7 were combined to immunize rabbits to obtain anti-gplacZ-mcrC antibodies. The lower bands in lanes 6 and 7 are believed to result from degradation products.
Figure 11
molecular mass, from 116 kD for β-galactosidase alone, to 137 kD for the β-galactosidase plus gpmerC fusion polypeptide.

This fusion polypeptide was purified by taking advantage of its β-galactosidase moiety. An extract of E. coli cells containing plasmid pDSC2921 was passed through an amino-benzyl-thio-galactoside (ABTG) affinity column which bound specifically β-galactosidase by being an uncleavable substrate analog. The column was then washed and the gplacZ-mcrC fusion polypeptide eluted with 100 mM Na borate, pH 10. The two separate preparations of the gplacZ-mcrC protein were obtained that were combined and used to immunize New Zealand White rabbits and are shown in Figure 11B.

The antiserum obtained from these rabbits were shown to react with gpmerC epitopes by two procedures. Anti-β-galactosidase antibodies were removed by adsorption to excess soluble, purified β-galactosidase and this sera was then used for immunodetection following electrophoresis of purified β-galactosidase and the gplacZ-mcrC fusion protein. The native β-galactosidase band was not detected with the preadsorbed antiserum whereas the gplacZ-mcrC band remained. The same result was obtained from all three rabbits immunized with the gplacZ-mcrC fusion polypeptide. There were, therefore, antibodies present in the antiserum that bound epitopes in the fusion polypeptide than were not present in β-galactosidase (Figure 12). These were presumably antibodies which bound specifically gpmerC epitopes.

The second procedure employed a partial digestion of β-galactosidase and the gplacZ-mcrC fusion protein with Staphylococcal V8 protease and an immunoblot of the resulting peptides separated PAGE and transferred to nitrocellulose. Antibodies in the anti-gplacZ-mcrC antiserum bound to additional bands in the lanes of V8 protease digested gplacZ-mcrC that were not found in V8 protease-digested β-galactosidase lanes. The antiserum, therefore, contained antibodies that bound epitopes in addition to those found in β-galactosidase, i.e., gpmerC specific epitopes.
Figure 12: Demonstration of anti-gpmcrC antibodies in antiserum raised against gplacZ-mcrC.

The figure shows western blots of purified β-galactosidase (5 μg, lane 1) and purified gplacZ-mcrC fusion protein (5.8 μg, lane 2) following SDS-PAGE and transfer from a 10% polyacrylamide gel onto nitrocellulose.

A: Antiserum from rabbit number 1 immunized with gplacZ-mcrC was reacted with proteins on the nitrocellulose filter at 1:200 dilution.

B: Antiserum from rabbit number 1, preincubated with 50 μg purified β-galactosidase (Sigma) per 25 μl serum, before reacting with filter at 1:200 dilution. The signal from the β-galactosidase band is reduced by preadsorption of the antiserum with β-galactosidase, whereas the 137 kD band (gplacZ-mcrC) was unaffected.
Figure 12
Figure 13: Binding of the anti-<i>gplacZ-mcrC</i> antibodies to a polypeptide from <i>M. vanielleii</i> cells and binding to a number of bands in <i>E. coli</i> extracts. The figure is a western blot of cell extracts separated by SDS-PAGE, transferred to nitrocellulose and probed with antiserum raised against the <i>gplacZ-mcrC</i> fusion polypeptide. At 1:1000 dilution of the antiserum, one antibody binding band predominates in extracts of <i>M. vanielleii</i> cells (lanes 1, 2 and 3), whereas antibodies bind to a number of <i>E. coli</i> proteins. Lane 1, the supernatant following anaerobic ultracentrifugation at 100,000Xg (for 2 hr at 4°C) of a lysate of <i>M. vanielleii</i> cells obtained by passage through a French pressure cell (200 µg protein); lane 2, pellet resulting from 100,000Xg centrifugation; lane 3, unfractionated extract of <i>M. vanielleii</i> cells (starting material); lane 4, induced extract of <i>E. coli</i> carrying pT7-1 (approximately 100 µg protein); lane 5, same, but with pDSC432 (see Figure 7); 6, same but with pDSC434. The <i>E. coli</i> cultures were grown for 2 hr in the presence of 2 mM IPTG. The arrow indicates the 25 kD putative <i>gpmcrC</i>.
Figure 13

gpmC →
With antisera against gpmcrD and gpmcrC available to detect these polypeptides and purified gpmcrD and MR available for use as mass standards, studies were undertaken to localize and quantitate these gene products in *Mc. vanielli*.

**Demonstration and subcellular localization of gpmcrC in *Mc. vanielli* cells:** The anti-\( \text{gplacz-mcrC} \) antiserum contained antibodies that bound to a polypeptide from *Mc. vanielli* cells that migrated with an electrophoretic mobility indicative of a molecular weight of 25 kD during SDS-PAGE (Figure 13). This polypeptide also comigrated with the gene product identified as recombinant gpmcrC, synthesized in *E. coli*, providing strong evidence that mcrC was expressed, resulting in the synthesis of a polypeptide, gpmcrC in *Mc. vanielli*.

**Identification of gpmcrD and gpmcrB in extracts of *Mc. vanielli* by two dimensional gel electrophoresis:** Two dimensional electrophoresis separated gpmcrD from MR (Figure 14). Identifying the gpmcrD spot was facilitated by having recombinant protein as a positive control, but as there was no recombinant gpmcrC available for use as a control, a spot could not be positively identified with gpmcrC. It was, in fact, impossible to demonstrate that gpmcrC entered the first dimension (non-denaturing) even using acid native gels.

**Following gpmcrD and gpmcrC during purification of MR from *Mc. vanielli* cells by the published protocol:** Components A, B and C are DEAE column fractions. Component A is the material in crude cell extract that does not bind to DEAE resin at pH 7, component B is the 100 mM NaCl wash of the resin, and component C is the material which elutes at 320 mM NaCl. Component C contains, amongst other materials, MR. Results of following gpmcrD during fractionation are shown in Figure 15. gpmcrD was found in component A, none could be detected in component C.
was found in component C. Whereas MR and gpmcrD were found predominantly in the supernatant fraction of anaerobic extracts subjected to centrifugation at 100,000×g, approximately half of the gpmcrC was detected in the pellet fraction (Figure 13). A small amount of MR was detected in this pellet, and this pellet material was found to methanogenic when boiled cell extract was added. The supernatant was also methanogenic, and the rate of methanogenesis was stimulated by the addition of the pellet, as previously observed (Deppenmeier, et al., 1990).

Component C from *M. vanneili* could not make methane, nor could it be activated by Ti(III)citrate, DTT, B12 or cell extracts (which contain gpmcrD). This prevented further cell fractionation and activity reconstitution experiments.

**Quantitation of MR and gpmcrD antigen levels:** Previously (Ellefson and Wolfe, 1981), MR had been estimated to be 5-10% of the total cellular protein, based on the intensity of staining of proteins in polyacrylamide gels following SDS-PAGE and yields of enzyme after purification. Although gpmcrD levels had never been measured, gpmcrD was assumed to be synthesized in lower amounts than MR because there was no polypeptide with a molecular mass of 18 kD in extracts of *M. vanneili* cells that formed a prominent band following SDS-PAGE. The relative concentrations of MR to gpmcrD, and if this relationship changed over growth in a batch culture, were therefore determined.

The amount of MR, as a percentage of total cell protein, was determined by ELISA and by densitometry of western slotblots. The levels of gpmcrD were determined by competitive ELISA. The results obtained are given in Table 3. MR was found to be 6% of the total protein at early time points and accumulated to 11% by late exponential growth phase in batch cultures. gpmcrD concentration was found to be 100 times lower, at 0.04%-0.06%. Western blots of extracts taken at different phases of growth, separated by SDS-PAGE, confirmed the accumulation of these proteins relative to total protein.
From the results of these experiments, the calculated molar ratio of MR to gpmcrD was $30 \alpha_1\beta_1\gamma_1$ MR to 1 gpmcrD (given that gpmcrD has a molecular mass of 18 kD and is 0.04% of the total cellular protein and $\alpha_1\beta_1\gamma_1$ MR has a molecular mass of 150 kD and is 10% of the total cellular protein). The pattern of synthesis of gpmcrD parallels that of MR, although the amounts synthesized are lower. The pattern of synthesis of gpmcrC also paralleled the synthesis of MR and gpmcrD, but the amount of gpmcrC could not be quantitated as purified gpmcrC was not available for use as a standard.

**TABLE 3: Quantitation of MR and gpmcrD during growth in batch culture**

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<th>MR$^a$</th>
<th>gpmcr$^b$</th>
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<tbody>
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<td>early exponential 0.33</td>
<td>6</td>
<td>0.04</td>
</tr>
<tr>
<td>mid exponential  0.65</td>
<td>11</td>
<td>0.06</td>
</tr>
<tr>
<td>late exponential 1.10</td>
<td>10</td>
<td>0.04</td>
</tr>
</tbody>
</table>

$^a$ Percentages calculated from the results of western slot blots (+/- 1%). Direct ELISA results, which include results from cells taken from cultures at $\text{OD}_{580}$ below 0.2, confirmed that the percentage of MR relative to total protein is approximately double at the late exponential phase of growth compared to cells in the early exponential growth phase.

$^b$ Percentages calculated from the results of competitive ELISA (+/- 0.01%).

**Methanogenesis by cell suspensions:** Although MR increased as a percentage of total protein in cells over growth in batch cultures (Table 3), the rate of methane production was highest at early growth stages. The results obtained by measuring the rate of methane production by cells taken from cultures at different growth phases are shown in Figure 16. A polynomial curve fit (line B, Figure 16), predicts the observed points better than an exponential curve fit (line A). These results indicate
that after peaking early, as the cells shifted up, the rate of methanogenesis per OD unit decreased and then leveled off during the exponential (or the so-called balanced) growth phase (up to an OD$_{580}$ of approximately 0.7) and then decreased further in a stepwise fashion as the cells in the culture shifted down into stationary phase.

These results should be considered preliminary as overall the specific methanogenic activity of the cell suspensions was low. The highest specific activity measured for cell suspensions (OD$_{580}$=0.1) was approximately 200 nmoles/min/mg protein. This was approximately 20 times the specific activities of the extracts which were assayed at that time, which were 5-10 nmoles/min/mg. Similar rates of methanogenesis were reported for 	extit{Ms. barkeri} (300-600 nmoles/min/mg) by Kaesler and Schönheit, 1988, however, these rates are only ~1/60th of the 4000 to 6000 nmoles/min/mg obtained with cell suspensions of 	extit{Methanobacterium thermoautotrophicum} strain Marburg (Kaesler and Schönheit, 1988).

All the 	extit{Mv. vannielii} cell suspensions were treated identically and therefore, although low in relative terms, the changes in rates of methanogenesis observed almost certainly do reflect changes in physiology and are not artifacts of the assay conditions. Combined with the results of the quantitation of antigen levels which showed an accumulation of MR, it appears that the specific activity of the MR in vivo decreases over growth, indicating another factor is limiting, possibly reducing equivalents. A similar pattern of high initial rates of methanogenesis that dropped off was also found during growth of a batch culture of 	extit{Methanothermus fervidus}.

Another aspect of this result is that some methanogens have two MR isozymes. In the only organism assayed, 	extit{Mb. thermoautotrophicum} strain Marburg, both are expressed early in growth, but one is no longer made by mid-exponential growth (Bonacker, et al., 1992). So, instead of an additional factor being limiting, possibly this pattern reflects an early, very active MR isozyme being replaced later during growth, by a less active form. Kinetic studies with the 	extit{Mb. thermoautotrophicum} isozymes
Figure 14: Two dimensional electrophoretic separation of extracts of *Mc. vannielii* cells.

A. The figure shows a silver stained gel of 250 μg of 100,000Xg supernatant fraction. Electrophoresis in the first dimension was through a 6-10% linear gradient native polyacrylamide gel and in the second dimension was through 10-15% polyacrylamide gel containing SDS.

B. Western blot of the polypeptides separated as shown in Figure 14A, reacted with antibodies in a mixture of antisera raised against *gpmcrB*, *gpmcrD* and *gplacZ-mcrC*. *gpmcrB* and *gpmcrD* are indicated.
Figure 14
Figure 15: Purification of MR from *Methanococcus vannieli*.

A: The polypeptides present in fractions obtained during purification of MR using the protocol described by Gunsalas and Wolfe, 1979, were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, cell extract; lane 2, component A; lane 3, component B; lane 4, component C; lane 5, purified recombinant gpmcrD.

B: Fractions shown in Figure 15A analyzed by immunoblotting using antibodies directed against gpmcrB and gpmcrD. gpmcrD was found in component A, which is the flowthrough of a DEAE-cellulose column to which MR bound. MR eluted from the DEAE-cellulose in 320 mM NaCl, resulting in the fraction designated component C (lane 4). However, no gpmcrD was found in this fraction.

β, β-subunit of MR; D, gpmcrD.
Figure 16: Rate of methane production by cell suspensions taken at different stages of growth in batch cultures.

The data presented were from four different cultures. 20 ml of ER medium in 150 ml serum bottles was inoculated with 1 ml of an overnight *M. vanneiili* culture and incubated at 37°C without shaking. In a Coy anaerobic chamber, samples were withdrawn with a 1ml syringe and transferred to a 5 ml screwcap vial with an open-top cap with a septum. The gas phase was made H₂ and the vials incubated at 37°C.

Rates of methanogenesis were determined by quantitating the methane present in the headspace with a gas chromatograph over time. Line A, exponential curve fit. Line B, polynomial (to the 6th power) curve fit. This indicates that the rate of methane production per OD₅₈₀ did not have a constant rate of change. The best description of the rate of methanogenesis over growth is that it leveled off during balanced growth, with a maximum at very early time points and a minimum when the cells had entered stationary phase.
Figure 16
Figure 17: Screening for two mcr operons by Southern hybridization with a degenerate oligomer probe.

A: Sequence of oligomer probes (23-mers). The mixture of probes was designed to be complimentary to a conserved region of the mcrB gene. The location of this region is indicated by the amino acid numbers and its sequence given above the oligomer sequence (Weil, et al., 1989). The sequence shown from _Mt. fervidus_ (Weil, et al., 1988), with the degeneracies introduced into the probe indicated (V.J. Steigerwald, unpublished).

B: Autoradiogram of chromosomal DNA of methanogens bound to a nitrocellulose filter and probed with the [32P]-labeled oligomers shown in Figure 17A. Genomic DNA from the indicated methanogens were treated with the restriction endonuclease _EcoRI_. _M. formicicum, M. barkeri, Methanothrix_, and _Methanopyrus kandleri_ DNA’s were the gifts of T. Darcy, J. Krzycki, Y. Kagamata and J. Palmer, respectively. 3 X 10^7 cpm (specific activity of 1.3 X 10^7 cpm/µg DNA) of end-labeled probe were added to 10 ml of hybridization solution (0.75 M NaCl, 0.075 M Na citrate, 0.1 % SDS, 5% dry milk and 1µg salmon sperm DNA/ml) and the filter was incubated for 21 hr at 55°C. Dots indicate bands which remain at high stringency (Figure 17C)

C. High stringency blot of _EcoRI_ digested methanogen genomic DNA. The conditions were the same as in Figure 17B, except that the hybridization temperature was 65°C. The DNA sources are indicated above each lane.
A:

\[ \begin{array}{c}
\text{mcrB} & \text{mcrD} & \text{mcrC} & \text{mcrG} & \text{mcrA} \\
\end{array} \]

\[401-D--A--G--T--Q--M--F--S-410\]

Mt. fervidus: 5' GATGCTGGAAACACCAATGTTTTC 3'

Probe: 3' CTACGACCATGGGTCTACAAGAG 5'

B:

Figure 17
Figure 17 (continued)

C:
Figure 18: Recombinant gpmcrD does not bind DNA in vitro. Purified proteins were mixed with 1 μg of Rsal/EcoRI digested pET1000 (Cram, et al., 1988) diluted in 20 μl TEP (20 mM Tris-HCl, 1mM Na$_2$HPO$_4$, 1 mM EDTA (pH 8). The mixtures were separated by electrophoresis through an 8% polyacrylamide gel and then silver stained. Lane 1, 1μg gpmcrD, no DNA; lanes 2 and 7, 1μg pET1000 digested with Rsal and EcoRI; lane 3, 0.1 μg gpmcrD and pET1000; lane 4, 1μg gpmcrD and pET1000; lane 5, 1μg HMf and pET1000; lane 6, 1μg bovine serum albumin (BSA) and pET1000.

The DNA bands in HMf lane (lane 5) were retained at the top of the gel, whereas in the gpmcrD containing lanes (lanes 3 and 4), the mobilities of the DNA molecules were unchanged. The promoter region of the mcr operon is on the 231 bp EcoRI/Rsal fragment indicated by the arrow as the bottom band. Its electrophoretic mobility was not changed by the presence of gpmcrD.
indicate that there are differences their in activities (Bonacker, et al., 1992).
However, there is no evidence that *M. vannielii* has two MR isozymes. Methanogenesis
by *Mt. fervidus*, which apparently has two MR isozymes (Figure 17), follows the same
rate pattern as *M. vannielii*, which apparently only has one MR (Figure 17). The
relevance of one versus two MR isozymes to this pattern of methanogenesis is,
therefore, uncertain.

**gpmcrD does not bind DNA:** The low level of gpmcrD relative to MR suggested a
regulatory role, however, the sequence of gpmcrD does not have predicted DNA
interaction domains and the recombinant gpmcrD, purified from *E. coli*, showed no
affinity for DNA in vitro (Figure 18). The plasmid pET1000 (Cram, et al., 1988), which
contains the promoter for the *M. vannielii* mcr operon, was digested with EcoRI, and
incubated with gpmcrD, and the reaction mix then subjected to PAGE. Mobility shifts
of the DNA fragments in the gpmcrD lanes were not observed, although the positive
control, binding by HMF, did alter the mobility of the DNA bands. BSA was used as a
negative control. If gpmcrD is a regulatory protein, it does not act by non-specific
binding to DNA or by binding the mcr promoter.

**E) Interaction of gpmcrD with MR holoenzyme**

Initial evidence that gpmcrD interacts with MR at the protein level was provided
by Sherf and Reeve, 1990, who showed that gpmcrD and MR could be co-
immunoprecipitated. To investigate this interaction, the following experiments were
undertaken.

**Immobilized antibody columns:** MR and gpmcrD bound an affinity column made with
antibodies raised against gpmcrD, however, subsequently, it proved impossible to elute
gpmcrD from this column, even with 8 M urea dissolved in PBS. The conditions used to
affinity purify anti-gpmcrD antibodies from the immobilized gpmcrD denatured the proteins which bound the antibody column. That gpmcrD and MR were bound to the column was demonstrated by boiling the resin in 1% SDS, separating the proteins by SDS-PAGE and western blotting. However, these harsh conditions required for elution made this method unsuitable for purifying gpmcrD-MR complexes. Binding of MR to immobilized gpmcrD could not be demonstrated.

Immobilized antibody columns containing anti-gplacZ-mcrC antibodies did not bind detectable amounts of gpmcrC from extracts of Mc. vanniellii cells. The antiserum also did not mediate immunoprecipitation of gpmcrC, which suggests that the antibody titer was too low for use as a reagent in these types of experiments. Attempts to purify and concentrate the anti-gpmcrC antibodies were unsuccessful.

**Sucrose gradients:** Separation of proteins in lysates of Mc. vanniellii cells by centrifugation through a 5-30% (w/v) sucrose gradient resulted in two peak localizations of gpmcrD (Figure 19). Namely, on top of the gradient and at a position indicating sedimentation through the gradient within a complex with a molecular mass of approximately 300 kD. The each lane of the western probed with anti-gpmcrD in Figure 19 was loaded with approximately equal protein to demonstrate the enrichment of gpmcrD in fraction 4. When equal volumes of fractions were loaded, the gpmcrD at the top of the gradient became apparent. However, more total protein is in fractions 6-9, so the gpmcrD was not enriched in these fractions as it was in fraction 4. The αβ2γ2 complex of MR co-sedimented with the 300 kD complex containing gpmcrD, indicating that MR and gpmcrD could be part of the same complex.

The presence or absence of oxygen did not alter these sedimentation patterns, nor did the detergents Tween-20 or NP-40. When the sample was boiled in 1% SDS before centrifugation, all peptides were found near the top of the gradient, as expected for SDS disassembled polypeptide complexes. Freeze-thaw, French pressing or allowing the lysate to stand, resulted in some α, β and γ subunits of MR disassociating.
from $\alpha_2\beta_2\gamma_2$ complexes and sedimenting as monomers. More gpmcrD migrated into the gradient when the culture was harvested at mid-exponential rather than at stationary growth phase. Some gpmcrD was found at the top of the gradient, regardless of the conditions used for cell lysis. The amount of gpmcrD in each fraction from a gradient was quantitated by competitive ELISA, revealing that the molar ratio of gpmcrD to MR in the fraction containing the complex was 1:100-150. This is lower than the overall ratio of total gpmcrD to total MR (1:15) in unfractionated cell extracts (Table 3).

The sedimentation pattern of gpmcrD may have resulted from aggregation of gpmcrD. Aggregation is a characteristic of chaperonins, like T55 of Sulfolobus shibatae, which migrates at 20S in glycerol gradients. Rubisco-binding protein, groEL and hsp60 form 14mers in solution (Trent, et al., 1991). A 14mer of gpmcrD would be predicted to have a molecular mass of 252 kD, similar to where gpmcrD migrated into the gradient. To determine if MR and gpmcrD were physically associated, sucrose gradients were run in the presence of anti-gpmcrD antiserum and normal serum was used in control gradients. Following fractionation of the gradient, material in each fraction was immunoprecipitated by the addition of protein-A immobilized on Sepharose beads. The beads were washed and boiled in sample buffer which contained 1% SDS. The resulting solution was subjected to SDS-PAGE, transferred to a nitrocellulose filter, and assayed by western blotting with serum directed against the $\beta$ subunit of MR. The antiserum directed against gpmcrD precipitated MR from the sucrose gradient fractions which contained both MR and gpmcrD, whereas the control serum did not (Figure 20). This provided convincing evidence that gpmcrD was physically associated with the $\alpha_2\beta_2\gamma_2$ form of MR within these gradients within a 300 kD complex.

Effects of gpmcrD and anti-gpmcrD antibodies on rates of methanogenesis in vitro: Having demonstrated that gpmcrD was physically associated with MR in cell extracts, it seemed possible that antibodies directed against gpmcrD might inhibit
Figure 19: Sucrose gradient sedimentation of *Mc. vannielii* cell extracts. Top, a lysate of exponentially growing *Mc. vannielii* cells was loaded on top of a 5-30% sucrose gradient and centrifuged for 10 hr at 150,000Xg. Fractions 1-9 were collected from the bottom of the tube, and the polypeptides in a 5 µl aliquot from each fraction separated by SDS-PAGE (12.5%) and silver stained. Lane E contained 2 µl of the starting material; lane P, the resuspended pellet from the bottom of the gradient tube; rD contained 1µg of purified recombinant gpMrO.

Bottom, western blots of the material in the gradient fractions shown above, probed using anti-gpMrB (upper) or anti-gpMrD (lower) antisera. In the upper immunoblot, 20 µl aliquots from each sucrose fraction were separated by SDS-PAGE through a 10% polyacrylamide gel. In the lower blot, TCA-precipitated material from 500 µl of fractions 1-5, and 9, and 250 µl from 6-8, were loaded in each lane of a 15% SDS-PAGE gel, so that approximately equal protein was loaded in each lane. α, gpMrA; β, gpMrB; γ, gpMrG; D, gpMrD.
Figure 19
Figure 20: Immunoprecipitation of MR using anti-gpmcrD antiserum. Sucrose gradients (See Figure 19) were run in the presence of anti-gpmcrD antiserum (lanes 7-11), or normal rabbit serum (lanes 2-6) and the material in each fraction that bound to the antibodies was subsequently immunoprecipitated by addition of protein-A immobilized on Sepharose beads. The washed beads were boiled in electrophoresis sample buffer and the material released was subjected to SDS-PAGE, transferred to nitrocellulose, and assayed with antiserum raised against the β subunit of MR (gpmcrB). Lane 8 and 9 show the gpmcrB that was co-precipitated from the sucrose gradient by anti-gpmcrD. gpmcrB was not precipitated by normal rabbit serum, as shown in lanes 3 and 4. Lane 1, purified component C; lanes 2 and 7 contained material from fractions 2 of the respective sucrose gradients; lanes 3 and 8, contained material from fractions 3; lanes 4 and 9, contained material from fractions 4; lanes 5 and 10, contained material from fractions 5; and lanes 6 and 11, contained material from fractions 6. The band above β-subunit of MR is the heavy chain of IgG (55kD), αgpmcrD, anti-gpmcrD antiserum.
Figure 21: Inhibition of methanogenesis in vitro by anti-gpmcrD antibodies.

A. Representative inhibition experiment. Affinity purified anti-gpmcrD antibodies (5 μg) were added to 550 μg of cell extract (diamond). Affinity purified antibodies directed against HMf, a DNA binding protein, were used in the control (circle). The rate of methanogenesis from methyl-CoM was determined by incubating the extracts in vials at 37°C and sampling the headspace at intervals after the addition of ATP and methyl-CoM.

B. Methane production by extracts (1600 μg) from which gpmcrD had been removed by immunoprecipitation with anti-gpmcrD antiserum (diamond), normal serum was used in the control (circle). Excess antiserum was incubated overnight at 4°C with extracts of *M. vannielii* cells. Antigen-antibody complexes were removed from solution by addition of anaerobic protein-A Sepharose beads, mixing gently for 10 min and allowing the beads to settle. The methanogenic activity of the resulting supernatant was determined and both the pellet and supernatant were assayed for gpmcrD by immunoblotting.
Figure 21
methanogenesis in vitro. Anti-gpmcrD antibodies were therefore added to extracts of *Mc. vannielii* cells, obtained by passage of cell suspensions through a French pressure cell, and the rates of methane production were measured. Table 4 gives the general characteristics of methanogenesis by the extracts obtained from *Mc. vannielii* cells and Figure 20 shows representative results of inhibition experiments. Preincubating extracts with affinity purified anti-gpmcrD antibodies then adding ATP and CH$_3$CoM, resulted in 5-23% inhibition of methanogenesis. In five different experiments the anti-gpmcrD antibodies inhibited methanogenesis on average 15% in comparison with control reactions containing unrelated rabbit antibodies.

Addition of the purified recombinant gpmcrD did not effect the rate of methanogenesis in vitro, however, preabsorbing the anti-gpmcrD antiserum with gpmcrD removed the inhibitory activity of the antiserum. The rates of methanogenesis by different extracts, in the absence of antiserum, varied by approximately 10%. Some inhibition of methanogenesis by anti-gpmcrD antibodies was always observed provided that the cells were harvested during exponential growth. During the course of this study, inhibition was observed in 21 separate experiments with antiserum and affinity purified IgG raised against gpmcrD, when compared with preimmune serum and affinity purified anti-HMF antibodies (an unrelated, DNA binding protein, see appendix). It can, therefore, be concluded that an inhibitory effect of anti-gpmcrD antibodies on methanogenesis in vitro was clearly documented.

The concentration of gpmcrD in the extracts of *Mc. vannielii* used for the methanogenesis assays was estimated to be approximately 0.04% $\pm$ 0.02% of the total protein by Western blotting and by competitive ELISA. There was, therefore, approximately 0.2 µg of gpmcrD per 550 µg extract. As sufficient antibody was added to each reaction mixture to bind 25 µg of gpmcrD, the less than total inhibition of methanogenesis observed was not the result of insufficient antibody, nor was inhibition increased with increased antibody concentration.
Methanogenesis in vitro was inhibited to approximately the same extent when
\textit{gpmcrD} was removed by immunoprecipitation or by antibody binding (Figure 21). The
average inhibition in quadruplicate samples was 18%, ranging from 17% to 19%. The
material remaining in the supernatant, and used for methanogenesis in vitro after
immunoprecipitation, was tested for residual \textit{gpmcrD}. A trace of \textit{gpmcrD} was
detected by western blotting, but at concentrations too low for quantitation.

The difference in the extent of inhibition resulting from addition of antibody to
extracts and following the removal of \textit{gpmcrD} from extracts by immunoprecipitation
was not significant. The extracts used in the inhibition experiments were the same as
those used in the immunoprecipitation experiments, however, considerable activity was
lost during the manipulations involved in the immunoprecipitation procedure. For
example, in an ‘antibody added’ experiment, the rate of methane production in the
affinity purified anti–HMf antibody control vial was measured at 88.8 nmoles/min/mg,
and in the affinity purified anti–\textit{gpmcrD} antibody vial was 68.6 nmoles/min/mg,
indicating an inhibition of 23%. However, using the same extract, the average rate of
methanogenesis in the normal serum control vial following the antibody precipitation
protocol was 3.6 nmoles/min/mg, and in the anti–\textit{gpmcrD} experimental vial was 2.9
nmoles/min/mg, the inhibition being 19%.

The rate of methanogenesis from added methyl-CoM (MR’s substrate) was 4-fold
higher than from \textit{CO$_2$} and \textit{H$_2$}, however, the extent of inhibition of this activity by anti-
\textit{gpmcrD} antibodies was approximately the same. Although the results obtained with
the anti–\textit{gpmcrD} antibodies do suggest that \textit{gpmcrD} must play a role in
methanogenesis, the precise site at which \textit{gpmcrD} is involved could not be further
determined. The large variation in experimental rates measured in different
experiments relative to the effect of anti–\textit{gpmcrD} antibodies, the difficulty in obtaining
sufficient HTP-SH, and the inability to activate component C precluded further
progress.
Table 4: Summary of the characteristics of methanogenesis in vitro by extracts of *Methanococcus vanneili*.

1. BES (2-bromoethanesulfonic acid) sensitive.
2. 0.5% (w/v) NP-40 (cmc= 0.03) had no effect on the rate.
3. Rates of 30-90 nmoles methane/min/mg protein in extracts from CH₃CoM and H₂
   
   Compare to rates from CH₃CoM and HS-HTP (given in nmoles/min/mg) of:
   i) 33 for Gö1 (Peinemann, et al., 1989); 18 for Gö1 (Deppenmeier, et al., 1989)
   ii) 100 for *Methanosarcina barkeri* (Kaesler, et al., 1989)
   iii) 46 (Ellerman, et al., 1988); 190 (Rospert et al., 1991) for *Methanobacterium thermoautotrophicum*
   iv) 2000 for H₂-reduced *Methanobacterium thermoautotrophicum* (Rospert, 1991)

4. Methane generated from carbon dioxide and hydrogen in crude extracts, by membrane pellet supplemented with boiled cell extract and by 150,000Xg supernatant. Molar ratio of methane produced to carbon dioxide consumed was 1:1

5. Role of ATP:
   i) not required for methanogenesis in crude extracts or remix, but stimulated 10 fold.
   ii) not required for methanogenesis in 100,000Xg supernatant, provided exogenous CH₃-CoM. In presence of ATP, methanogenesis from CO₂+H₂.
   Suggests ATP activated a step which preceded the MR reaction,
   iii) stimulation: ATP=ADP>NaCl>no additions (determined using supernatant) ATP and ADP concentration was 12.5 mM; [NaCl]=18.75 mM so that an equal amount of Na⁺ was added. Suggests the presence of a soluble ADP+ADP=ATP+AMP activity. NaCl stimulation indicated isotonic strength was not optimized in the extracts.

6. Interface between 100,000Xg supernatant and pellet could make methane
   i) pellet needed to be supplied with deproteinized extract.
   ii) estimates of MR levels suggested that the specific activity was higher in the interface and membranes, as compared to the supernatant.
   iii) membranes stimulated methanogenesis by supernatant (provided an electron carrier, cannot exclude the possibility that this fraction provided the means of generating reducing power from ATP, and hence the stimulatory activity.
CHAPTER IV
DISCUSSION

The association of gpmcrD with the MR holoenzyme and its role in methanogenesis

MR was found to comprise 6-10% of total cellular protein in \textit{Mc. vannielli} cells and apparently, accumulated over growth. The level of gpmcrD was found to be 0.04-0.06% of cellular protein and gpmcrD accumulated like MR (Table 3). The molar ratio was calculated to be $15 \alpha_2\beta_2\gamma_2$ MR to 1 gpmcrD. As the methylreductosome was calculated by Wackett et al., 1987, to contain $32 \alpha_2\beta_2\gamma_2$ MR, it is possible that gpmcrD is a minor component of this large complex, possibly functioning to hold this complex together. If the methylreductosome complex actually does exist \textit{in vivo}, but is too fragile to isolate intact, gpmcrD might disassociate from this complex during MR purification. This would be consistent with the observation that gpmcrD is not found in preparations of purified MR.

The molecular mass of the MR, as measured on the sucrose gradients, was approximately 300 kD, corresponding to an $\alpha_2\beta_2\gamma_2$ conformation, and gpmcrD was associated with this form, in a ratio of 1:100 to 150 (Figure 19). Unlike the subunits of MR, some gpmcrD was always found on the top of the gradient, regardless of the conditions of breakage. This suggests that the interactions of the $\alpha$, $\beta$ and $\gamma$ subunits of MR are stronger than the interaction of gpmcrD with MR. Alternatively, the same result would be obtained if not all gpmcrD were complexed with MR in \textit{Mc. vannielli}. If this is correct, the number of MR holoenzymes that could have an associated gpmcrD molecule in the cell, may be less than the theoretical maximum (ie, the molar ratio of MR to gpmcrD). This does not necessarily argue against gpmcrD being part of
methylreductosome. Not all MR must be complexed into higher order structures in the cell.

One possible role for gpmpcrD would be to assist in the assembly of active MR, without necessarily being part of the final complex, therefore, playing neither a catalytic nor structural role. However, gpmpcrD cannot be absolutely required for assembly of the α2βγ2 MR holoenzyme because Hartzell and Wolfe, 1986, showed that gel purified gpmpcrA, gpmpcrB and gpmpcrG were able to re-assemble into active enzyme in vitro. These isolated polypeptides assembled into an α2βγ2 complex which was methanogenic if F43Q was added to the assembly mixture and the enzyme was reductively activated. In vitro, gpmpcrD may, however, accelerate this process.

Mathematically, not all MR holoenzymes can be complexed with gpmpcrD, which may explain the low percentage of inhibition (20%) of methanogenesis in vitro by the antibodies directed against gpmpcrD (Figure 21). Only 7.5% of MR holoenzymes could bind a gpmpcrD molecule directly at a molar ratio of 1:15 (Table 3). This does not appear to be sufficient to account for the 15-20% inhibition observed, even if the anti-gpmpcrD antibody completely inhibited the methanogenesis of every MR-gpmpcrD complex. The possibility exists that MR complexed with gpmpcrD is more active than MR alone. Alternatively, the inhibitory effect of the anti-gpmpcrD antibodies may not be directly on the MR catalyzed reaction, but the antibodies may inhibit MR's interaction with other enzymes.

There are many points at which gpmpcrD could interact with the complex methanogenic machinery. Organisms which cannot utilize CO2, like Methanosphaera stadtmannae (Wijngaard, et al., 1991), have not yet been screened for the presence of gpmpcrD and gpmpcrC. The enzymatic steps intervening between CO2 and CH4 cannot be formally excluded as points for interaction of gpmpcrD on this basis. However, as anti-gpmpcrD antibodies inhibit methanogenesis in vitro from CH3-CoM and CO2 to the same extent, the steps of carbon flow between CO2 and CH3-CoM
apparently can be eliminated as possible points of involvement of gpmcrD.

Some remaining possible roles for gpmcrD include linking the soluble MR with membrane-bound ion pumping methyl transferases (Becher, et al., 1992) or heterodisulfide oxidoreductase. Alternatively, gpmcrD could, instead of protein/protein interactions, assist the binding of the cofactors. As discussed in the Introduction, HTP exists as a nucleotide disaccharide \textit{in vivo}, although experiments reported to date which study methanogenesis \textit{in vitro} have used HTP without the UDP and sugar moieties. It is possible that gpmcrD plays a role in the specificity of cofactor binding, or in the regulation or release of the products of the MR reaction. No information is available on the mechanism of the MR reaction, so it is difficult to design experiments to address these possibilities at this time.

In addition to the possibilities described above, one possible point of gpmcrD interaction which might have been amenable to investigation is the reductive activation of the Ni atom of F$_{430}$. The time required for this activation is believed to cause the lag observed before methanogenesis begins in extracts prepared from \textit{Mb. thermoautotrophicum}. Unfortunately, the hypothesis that anti-gpmcrD interfered with this activation could not be tested immediately because such a lag was never observed with the \textit{Mc. vannieli} extracts; inactive, purified MR from \textit{Mc. vannieli} could not be reductively re-activated; and, the anti-gpmcrD antibodies did not bind to gpmcrD in extracts of any of the methanogens that did demonstrate this activation.

One way to test if anti-gpmcrD antibodies interfere with activation, may be to try to block the inhibition by anti-gpmcrD antibodies by adding polylysine, based on the analogy that polylysine blocks the interaction of cytochrome c with cytochrome c reductase (Stryer, 1981). First, it would be necessary to determine if the activation phenomenon which is observed with extracts of \textit{Mb. thermoautotrophicum} can be blocked with polylysine. Then, in \textit{Mc. vannieli}, if anti-gpmcrD antibodies are
interfering with this activation, by first blocking the activation with polylysine, there should be no difference between the rates of methanogenesis of extracts treated with anti-gpmcrD antibodies and in the controls.

Though the results obtained provide evidence that gpmcrD has a role in methanogenesis, they do not define that role. Mutants are now needed to answer the question: "What does gpmcrD do in the cell?"

Recent advances in transformation of *Methanococcus* species (Micheletti, et al., 1991; Sandbeck and Leigh, 1991) have allowed gene disruption experiments to be undertaken for the first time in a methanogen. A linearized plasmid encoding the entire mcr operon with mcrD disrupted (Figure 10), could be transformed into a *Methanococcus* cell, and then, through homologous recombination, the mcrD gene should be inactivated. If viable, such mutants could be selected by their resistance to pseudomonic acid if the pseudomonic acid resistance gene (Jenal, et al., 1991) was linked to the mutant mcrD gene (Figure 10). A wild type mcr operon, similarly linked to the pseudomonic acid resistance gene, could be used as the control. In *E. coli*, translation of the genes downstream from mcrD was not affected by the frameshift mutation (Figure 10). If the mutation is nonpolar when the genes are expressed in *M. vannielii*, the mutation should only be lethal if gpmcrD is essential in the growth conditions used. If mutant mcrD is lethal, these experiments could be performed in a methanogen which has two operons for MR.

**Possible role of gpmcrC**

The antiserum raised against the gplacZ-mcrC fusion polypeptide demonstrated that the mcrC gene was expressed as a polypeptide in *M. vannielii* (Figure 13). Although, there is currently no evidence linking gpmcrC to any activity of the cell, gpmcrC does have two pairs of lysine residues near its carboxyl terminus which are predicted to be on the hydrophilic face (surface) of an amphipathic alpha helix (Figures 22 and 23). This motif has been implicated in protein-protein interactions of
Screening for polypeptides which interact with gpmcrC in vivo might provide some insight into the role of gpmcrC. It may be possible to use the in vivo transcription activation with fusion polypeptides method described by Fields and Song, 1989, to find polypeptides (possibly MR or gpmcrD) which interact with gpmcrC. In this yeast based system, the protein of interest is fused to the GAL4 DNA binding region. The GAL4 activating region is fused to either a specific second protein to be tested for affinity to the protein of interest or to a randomly generated clone bank for screening. The genes for the fusion polypeptides are moved into a yeast strain which has the GAL1 gene fused to lacZ, downstream from the upstream activating region for the genes encoding enzymes required for galactose utilization. If the second protein associates with the protein of interest, both domains are brought into proximity and mediate the transcription of the lacZ gene. The cells are then assayed for β-galactosidase activity.

Considerations for future experiments

Homology searches did not reveal sufficient similarity of gpmcrC or gpmcrD to known proteins for their functions to be identified (FASTA, Pearson and Lipman, 1988). Whether or not gpmcrC (or gpmcrD) is involved in reductive activation of MR will require mutants in the activation pathway or an activatable in vitro system. Holliger, et al., 1992, suggest a model of $F_{430}$-catalyzed dechlorination where the reductive activation machinery for MR provides the reducing equivalents. This suggests a way to isolate mutants defective in the reductive activation enzymes. Since some of the reducing power for activation of MR must be diverted to the reductive dechlorination reaction, cells which are less tolerant of chlorinated compounds may be defective in providing reductive activation. Total blockage of the pathway would, presumably, be lethal. Lack of growth on otherwise sub-inhibitory levels of chlorinated compounds would be the phenotype. When shuttle vectors become available, strains defective in activation could be used to determine if gpmcrC
(or gpmcrD) can compliment any of the lesions in activation. Complementing plasmids would restore resistance to the chlorinated compounds, allowing for a simple selection scheme.

Conclusions

There is not yet sufficient evidence to determine the roles, if any, of gpmcrD and gpmcrC. This work described detection methods for the gene products of mcrD and mcrC. The relative levels of expression of gpmcrD were measured and compared to MR. The molar ratio of gpmcrD to MR was calculated to be 1:15. This work also shows the level of MR changes over growth, as does the rate of methane production of cell suspensions. Due to the inherent difficulty and complexity of methanogenesis in vitro, the development of a transformation system for Mc. vannielii would greatly simplify determining the functions of gpmcrD and gpmcrC.
Figure 22: Predicted protein folding of gpmcrC. Polypeptide was folded using the Protlyze program (Scientific and Educational Software, 1987). This program is based on Chou and Fasman, 1978a, 1978b and 1979; and Garnier, et al., 1978, considerations. Results obtained with both considerations are shown for comparison. The figure shows the predicted alpha helical regions of gpmcrC examined in Figure 23. Icons used for the different conformations are given at the bottom of the figure.
Predicted folding of gpm\textsubscript{crC} with Chou-Fasman considerations

Predicted folding of gpm\textsubscript{crC} with Garnier-Osguthorpe-Robson considerations

Legend
\( \text{\textbullet} \) = Helix \hspace{1cm} \( \text{\textbackslash\textbackslash} \) = Sheet \hspace{1cm} \( \text{\textsquare} \) = Turn \hspace{1cm} \( \text{\textcircle} \) = Coil

Figure 22
Figure 23: Predicted structure of conserved alpha helical regions of gpmcrC.
A: Sequence of the C-terminal amino acids of gpmcrC arrayed on the alpha helical wheel. Location of amino acids in the linear sequence shown are relative to the initiator methionine given by the amino acid number (Sherf, 1989). Alpha helical wheel after Murre, et al., 1989, the numbers on the wheel indicate the linear order of the amino acids. 181-SPQ-183 are not predicted to be part of the alpha helix and are therefore placed on unnumbered radii. Boxed residues are conserved in all gpmcrC molecules. Amino acid residues marked with an asterisk (*) are conservative substitutions. As the carboxyl terminal amino acid is ionized, glycine198 is given a negative charge. The charge on Histidine196 is ambiguous because it is determined by the residue's local environment.
B: Detail of the alignment of the C-terminal amino acids of the five sequenced gpmcrC's (Sherf, 1989). Letters in the consensus line indicate residues conserved in all cases, an asterisk indicates a conservative substitution, and a dash indicates a residue which is not conserved.
C: Predicted structure of an alpha helical region of gpmcrC which also has amphipathic character. Its highly conserved nature suggests it has functional significance, perhaps providing intra- or inter-molecular interactions.
A:

181-SPQKIDEIIESIKKHLG-198

(pK=6.5)

141-PVDQMDFAKIGKTLDVMP-159

PVD-EDFA--G*KT--VMP

Figure 23
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