

Studies on Cytochromes and Electron Transport in
Methanosarcina thermophila strain TM-1

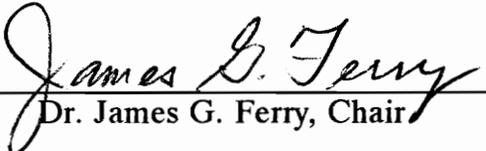
by

Christopher William Peer

Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements
for the degree of

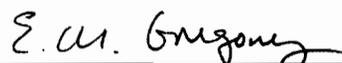
MASTER OF SCIENCE
in
Biochemistry and Anaerobic Microbiology

APPROVED:


Dr. James G. Ferry, Chair



Dr. G. W. Claus



Dr. E. M. Gregory

July, 1993
Blacksburg, Virginia

C.2

LD
5655
4655
1993
P437
C.2

ABSTRACT

Methanosarcina are methanogens capable of growth and methanogenesis from H_2/CO_2 , formate, methanol, methylamines, and acetate. *Methanosarcina* conserve energy by coupling electron transport and methyl transfer to the generation of ion gradients during acetoclastic growth. This work focuses on cytochrome b and heterodisulfide reductase, two proteins involved in energy conservation by electron transport. A procedure was developed for mass cultivation of *Methanosarcina thermophila* strain TM-1 in 12-liter fermentations which produced up to 10 grams wet weight/liter, in order to facilitate biochemical studies. Cytochromes occurring in *Methanosarcina thermophila* were characterized spectrophotometrically using chemical and physiological reactants. This analysis revealed two heme centers, one of which was only reduced by $Na_2S_2O_4$ or carbon monoxide. Partially purified cytochromes were found to be present in a complex and were characterized by electrophoretic and spectrophotometric analysis. The cytochrome containing protein was found to contain two hemes and had an M_r of 28,000 Da. Heterodisulfide reductase was isolated from the soluble fraction by anion exchange chromatography and assayed using methyl viologen as an artificial electron donor. Electron transport from CO to the heterodisulfide of 2-mercaptoethanesulfonic acid (HS-CoM) and 7-mercaptoheptanoylthreonine phosphate (HS-HTP) was reconstituted using carbon monoxide dehydrogenase, ferredoxin, membranes, and heterodisulfide reductase. Both membranes and ferredoxin were required for reduction of the heterodisulfide.

FORWARD

This thesis focuses on the role of cytochromes and heterodisulfide reductase in electron transport during acetotrophic growth of *Methanosarcina thermophila*. Section I contains an introduction and literature review intended to serve as a foundation for understanding the study of methanogenesis and, more specifically, acetate conversion to methane and carbon dioxide. Section II describes the materials and methods used for this work. Section III contains the results of this work. Section IV provides a discussion of the importance of this work to the field. References are included in section V. My curriculum vita is section VI.

ACKNOWLEDGMENTS

This thesis would not have been possible without the guidance and support of my advisory committee, Dr. James G. Ferry, Dr. G. W. Claus, and Dr. E. M. Gregory. I am especially indebted to Dr. James G. Ferry for believing in my potential, for his support, and for giving me the opportunity to begin a career in science. I am also grateful to the Faculty of the Anaerobe Lab for helpful discussions and exchange of ideas. For friendship, help, and support I thank Dr. Bill Claus, Dr. Dennis Bazylnski, Dr. Andrew P. Clements, Matthew T. Latimer, Birgit Alber, D. J. Ferguson, Jenell Decker, Dr. Madeline E. Rasche, Dr. Julie Maupin, Dr. Kavita Singh-Wissman, Dr. Kerry Smith, Dr. Cheryl Ingram-Smith, and Donna Noll. Thanks to my family, Lane, William, Bill and Maura, Dave and Marianne, for support and encouragement.

Lastly, I would like to thank Holly V. Savage for her encouragement, unconditional love, and understanding, without which none of this would have been possible.

TABLE OF CONTENTS

	Page
Title Page	i
Abstract	ii
Forward	iii
Acknowledgments	iv
Table of Contents	v
List of Tables	viii
List of Figures	ix
 Section I:	
Introduction	1
Literature Review	5
Methyl Reduction	10
Activation of Acetate and Cleavage of Acetyl-CoA	12
Methyl Transfer	13
Electron Transport	13
Heterodisulfide Reduction	16
 Section II:	
Materials and Methods	18

Table of Contents (cont.)

Sources of Chemicals	18
Organism	18
Continued Growth with Media Supplement	18
Preparation of Fractions	20
Spectrophotometry	21
Electrophoresis	22
Chromatography	23
Isolation of Cytochrome and Heterodisulfide Reductase	23
Isolation of CO Dehydrogenase and Ferredoxin	24
Synthesis of the Heterodisulfide of HS-CoM and HS-HTP	25
Enzyme Assays	25

Section III:

Results	27
Increased Cell Yields	27
Presence of Cytochromes in TM-1 Membranes	29
Reduction of Cytochromes with Carbon Monoxide	32
Partial Purification of Cytochromes	35
Heterodisulfide Reductase	43
Electron Transport form CO to CoM-S-S-HTP	45

Table of Contents (cont.)

Section IV:

Discussion 47

Section V:

References 53

Section VI:

Curriculum Vita 70

LIST OF TABLES

Page

Section I

Table 1. Unique cofactors of methanogenesis 9

Table 2. Standard free energy change associated with selected
methanogenic fermentations and reactions 11

Section II

Table 3. Media supplement 19

Section III

Table 4. Comparison of fermentations 28

Table 5. Heterodisulfide reductase activity and
distribution from soluble and membrane
fractions of cell extract 44

LIST OF FIGURES

Page

Section I

- Figure 1. Proposed pathway for acetate conversion to methane and carbon dioxide 4
- Figure 2. Pathway for the reduction of CO₂ to methane 8

Section III

- Figure 3. Dithionite-reduced minus H₂O₂-oxidized difference spectrum of membranes 30
- Figure 4. H₂-reduced minus CoM-S-S-HTP oxidized difference spectrum of membranes 31
- Figure 5. CO-binding of Cytochromes 33
- Figure 6. CO-reduced minus CoM-S-S-HTP oxidized Membranes . . 34
- Figure 7. Native PAGE of the isolated cytochrome 37
- Figure 8. Panel A. SDS-PAGE analysis of the isolated cytochrome 38
- Figure 8. Panel B. SDS-PAGE analysis of the isolated cytochrome 39
- Figure 9. SDS-PAGE showing thermal aggregation properties of the 58 and 28 kD bands 40
- Figure 10. Panel A. SDS-PAGE of cell extract, membranes and cytochrome 41

List of Figures (cont.)

- Figure 10. Panel B. SDS-PAGE of cell extract, membranes and cytochrome 42
- Figure 11. Effect of increasing ferredoxin concentration on the production of free thiols from CoM-S-S-HTP using the reconstituted system 46

Section IV

- Figure 12. Proposed revision to the pathway for conversion of acetate to methane by *Methanosarcina* 52

INTRODUCTION

In recent years methanogenesis has received an increased amount of research attention. The importance of methane to global carbon cycling, the novel biochemistry, and several environmental concerns have fueled this increased activity. In 1776 Volta first described "combustible air". It was over 100 years later, in 1886, that Bechamp linked the production of this combustible gas to microbial activity. The methane-producing bacteria then became known through the pioneering work of H.A. Barker (4, 67) and R.E. Hungate (35, 36, 37). The study of methane-producing bacteria began with the development of effective strict anaerobic techniques and primary isolation of methanogens.

Of pressing concern regarding environmental impact is the human production of waste. Methane production has long been known to occur in both sewage digesters and landfills, the latter being harvested at the rate of 70 million cubic yards per year in the United States (68). With the development of the upflow anaerobic sludge blanket (UASB) (15) for use as a digester, many studies on methanogen-containing microbial communities were carried out in the Netherlands. The UASB is one of the most effective ways to reduce the organic content of many agricultural, industrial, and food processing wastes while at the same time producing methane as a useful energy source (15). Methanogens also dehalogenate several toxic chlorinated compounds that are industrial solvents or by-products of

commercial chemical processes (24, 39, 47). Increases in atmospheric methane, a potent greenhouse gas, have been partially attributed to increases in human agricultural activities and deforestation (83).

The first described and most extensively studied pathway for methane production is the reduction of carbon dioxide using hydrogen to produce methane (87). This pathway is probably of primary importance in the rumen where fatty acid concentrations are kept low by intestinal absorption (16, 35, 37). The first described species to use acetate as a substrate for methanogenesis was *Methanosarcina barkeri* (71). This organism, which represents the most metabolically diverse order of methanogens, *Methanomicrobiales*, is capable of growth and methane production on H_2/CO_2 , formate, methanol, methyl amines, and acetate. The study of acetate fermentation to methane was then stimulated by the estimate that over 65% of biologically produced methane was from acetate conversion (58, 73, 85).

The study of acetate consumption as the most environmentally relevant substrate focused on the pathway for conversion of acetate to CH_4 and CO_2 (26). *Methanosarcina thermophila* was chosen by the principal investigator for this work because it lacks the CO_2 reduction pathway and thus the complexity of overlapping alternate pathways. This organism induces production of enzymes needed for conversion of acetate when the growth substrate is changed from methanol to acetate (38). *M. thermophila* is the most favorable for the biochemical study of

acetate conversion because of the relatively high growth rates supported at higher temperatures by this moderate thermophile and the development of the pH auxostat for improved growth yields (74). Findings from *M. barkeri* and *M. thermophila* have been generally accepted as applicable to the other because of the similarities between them.

Major work on the acetate pathway (Fig. 1) has focused on the activation of acetate, cleavage of acetyl-CoA, and the final steps of reductive demethylation of CH₃-CoM. Recently, emphasis has shifted to the study of the role of membranes in energy conservation, as it is clear that ion gradients are the sole means of energy conservation (9). Electron transport driven proton extrusion has been shown in several systems (9, 11, 13), and recent studies have shown that methyl transfer is accompanied by direct sodium ion transport (6).

The work reported here focuses on the role of cytochromes in electron transport and advances the understanding of the proteins that may be involved in energy conservation by generation of a proton gradient.

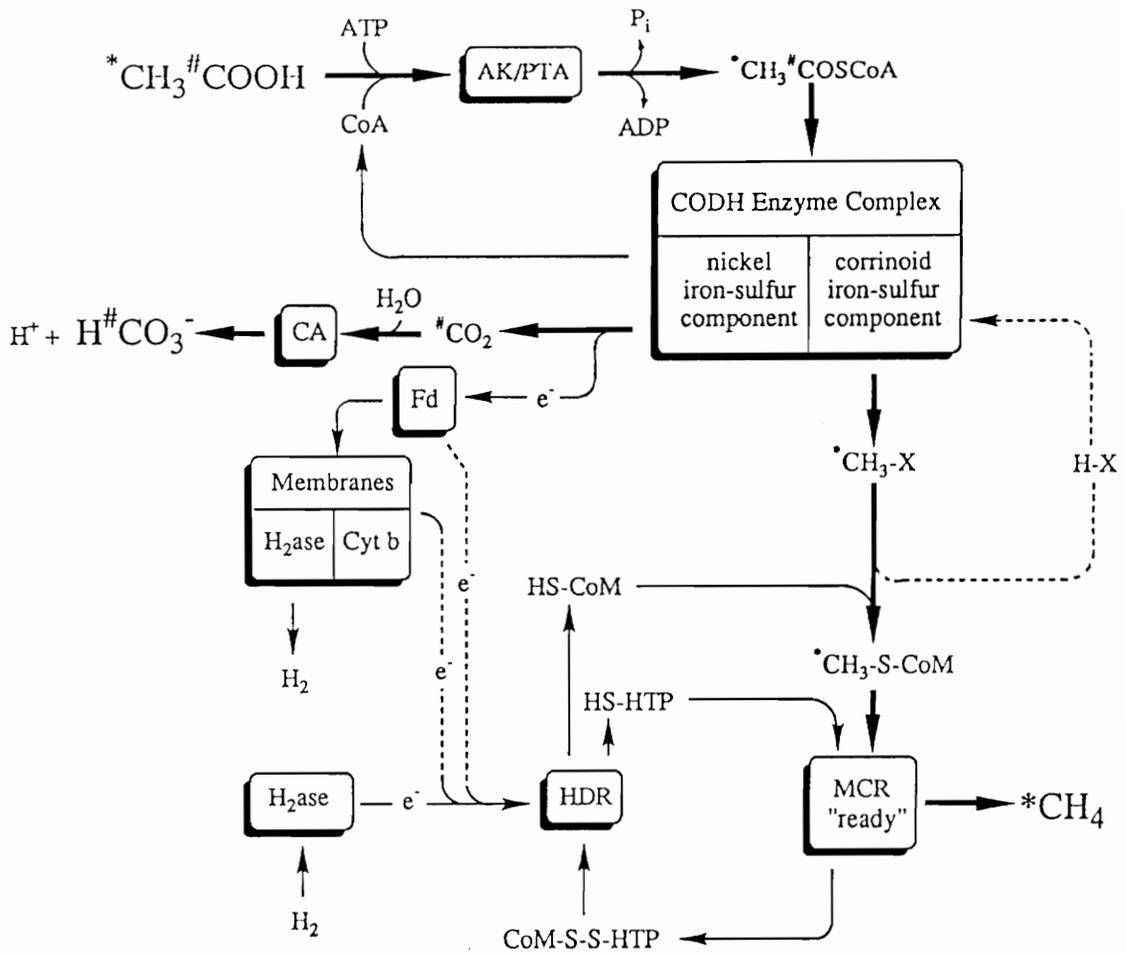


Figure 1. Proposed pathway for the conversion of acetate to CH_4 and CO_2 by *Methanosarcina*. AK, acetate kinase; PTA, phosphotransacetylase; CODH, CO dehydrogenase enzyme complex; Fd, ferredoxin; H₂ase, hydrogenase; Cyt b, cytochrome b; HDR, heterodisulfide reductase; HS-CoM, 2-mercaptoethanesulfonic acid, HS-HTP, 7-mercaptoheptanoylthreonine phosphate. Adapted from Ferry (26)

LITERATURE REVIEW

The methane-producing bacteria, also known as methanogens, comprise a diverse group of organisms belonging to the recently defined Archaea (86). The archaea are thought to be as distantly related to the bacteria as they are to the Eucarya, which raises many questions as to the universal precursor of life and to the distinct evolution of the three domains. The archaea are defined by (i) 16S ribosomal RNA sequence homologies with distinct differences from both the Eubacteria and Eucarya as well as ribosomal structure and subunit composition, (ii) unique membrane phospho- and glyco-lipids which are diether- linked as opposed to ester-linked, and differences in the composition of the cell envelope, and (iii) RNA polymerase subunit composition which resembles the Eucaryotic type with at least eight subunits (41).

There are three orders of methanogens in the Archaea: Methanobacteriales, Methanomicrobiales, Methanococcales (83). The methanogens share a unique metabolism, but have varied substrates. The Methanomicrobiales, including the genus *Methanosarcina*, show the greatest metabolic diversity and are capable of growth on H₂, methanol, acetate, and mono-, di-, and trimethyl amines. Additionally, several species use primary, secondary and cyclic alcohols as electron donors (84). All bacterial morphologies are represented in the methanogens: rods, cocci, spirilla, sarcina and planar. Methanogens typically inhabit freshwater and

marine anaerobic environments including the digestive tract of ruminants, termites and other animals; sediments, rice paddies, marshes, bogs and tundra peat (83). In these habitats methane is produced by a consortia of three groups of microorganisms. First fermentative organisms break down complex substrates such as proteins, lipids and polysaccharides and ferment the products to fatty acids, H_2 , and CO_2 . Next the homoacetogenic organisms convert some of the fatty acids to formate, acetate, H_2 , and CO_2 . Finally, the methanogenic organisms convert these products to methane (25). The volatile methane rapidly escapes the local environment. This is important in sediments because the H_2 -evolving organisms require a low concentration of hydrogen in the environment so their metabolism remains thermodynamically favorable. The methanogens effectively remove H_2 by consuming free hydrogen in a process known as interspecies hydrogen transfer (16). The methane is released into the gas phase. In digesters the methanogens also play the important role of keeping fatty acid concentrations low by oxidizing H_2 and consuming acetate so that low pH due to acids does not inhibit the digesting organisms. Much of the methane produced in sediments is consumed by obligately aerobic methanotrophs though some escapes into the atmosphere. Several investigators have proposed that anaerobic methane oxidation is carried out by sulfate-reducing bacteria while still in the anaerobic water column, and this affects release of methane from marine environments (59, 70).

The study of acetate conversion to methane lagged far behind work on the

CO₂/H₂ pathway (87). The early studies, aimed at determining the mechanism for methane formation from acetate, used isotope labeled substrates. This work revealed that methane produced from acetate was formed by a different mechanism than methane from CO₂ and that CO₂ was not an intermediate. Furthermore, they found that methane is produced almost exclusively from the methyl carbon of acetate, and that many methanogens used acetate directly for biosynthesis of cell components (17, 75). Stadtman and Barker (75) proposed that carbon from either CO₂ or acetate would form some common precursor that would be evolved to methane, but the identity of this precursor was unknown.

The study of the pathway for CO₂ reduction to methane (Fig. 2) has focused primarily on reduction of the carbon atom (87). This is a series of three 2-electron reductions to form methyl-tetrahydromethanopterin. The methyl group is then transferred to HS-CoM to give CH₃-S-CoM and this is reduced to methane with a final electron pair. The enzymes for each reduction and transfer step have been extensively studied (25).

Several previously unknown cofactors have been described (Table 1) from methanogens that use the CO₂ reduction pathway (87). These cofactors or derivatives are found in all methanogens. Also, the complete sequence of one-carbon reduction and transfer is known. The reducing equivalents for CO₂ reduction are derived from hydrogen. The reduced form of F₄₂₀, (F₄₂₀H₂), is generated by F₄₂₀-reducing hydrogenase, and at least two of the three enzymes

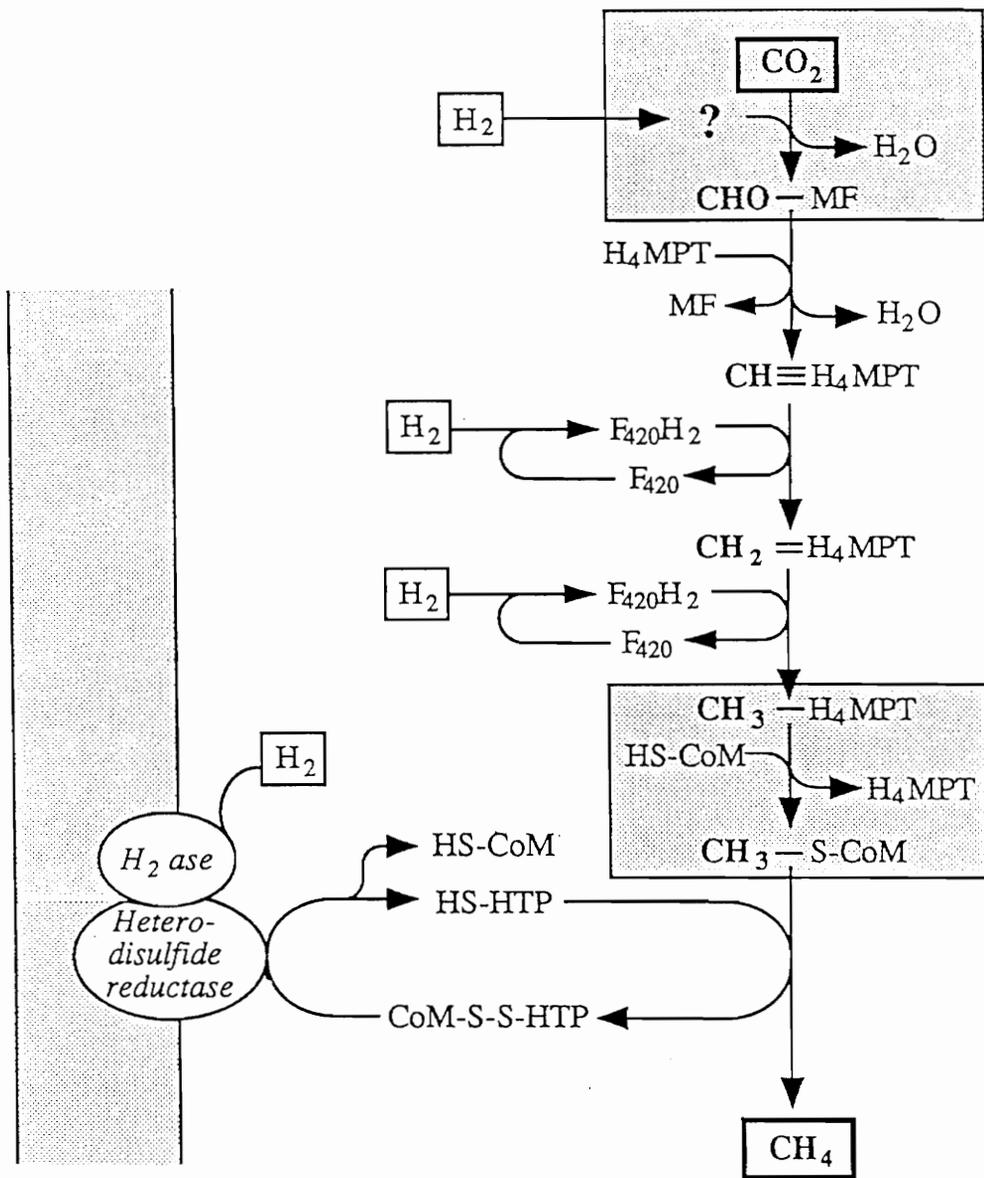


Figure 2. Pathway for the reduction of CO₂ to methane. Grey boxes indicate reactions involved in membrane-dependent energy transduction. MF, methanofuran; H₄MPT, tetrahydromethanopterin; F₄₂₀, Factor 420; HS-CoM, coenzyme M; HS-HTP, 7-mercaptoheptanoylthreonine phosphate; H₂ase, hydrogenase. Adapted from Muller et al. (61)

Table 1. Cofactors unique to methanogenic reactions.*

Trivial name	Structure	Function
CoM	2-mercaptoethanesulfonic acid	methyl carrier
Factor III	5-hydroxybenzimidazolyl cobamide Cobalt Porphyrin ring, (corrinoid)	methyl carrier
H ₄ MPT	tetrahydromethanopterin	C-1 carrier
(H ₄ SPT)	tetrahydrosarcinapterin	
MF	Methanofuran (folate analog)	C-1 carrier
HS-HTP	7-mercaptoheptanoylthreonine phosphate	electron carrier
F ₄₂₀	7,8-didemethyl-8-hydroxy-5-deazaflavin	electron carrier
F ₄₃₀	Nickel porphyrin ring	methyl reduction

* Adapted from Wolfe (87)

needed to reduce CO₂ to the methyl level use F₄₂₀H₂. Energy is conserved in the final steps (Table 2, reaction 5). Methyl-CoM is reduced using HS-HTP to form CH₄ and CoM-S-S-HTP, and the sulfhydryl forms of the coenzymes are regenerated using electrons derived from hydrogen (9, 21, 66).

Methyl Reduction:

The final step of methane evolution received increased attention with the identification of Coenzyme M (HS-CoM, 2-mercaptoethanesulfonic acid) and determination of its role as a methyl carrier (77). The methyl reductase protein was identified, but several other factors were required for activity. These included F₄₂₀-reducing hydrogenase, F₄₂₀ nonreactive (methyl viologen) hydrogenase, flavin and Component B, later identified as 7-mercaptoheptanoylthreonine phosphate (HS-HTP) (32). In all known methanogens the final steps of methyl reduction and methane evolution are the same (45, 69). Once reduced to the methyl level, the methyl group is transferred from H₄MPT to HS-CoM yielding CH₃-S-CoM. This is the common precursor to all biologically produced methane (45, 69). Alternatively methyl groups are transferred directly from methanol or methylamines to HS-CoM. Methyl-CoM is then reduced by methyl-CoM methylreductase (MCR) using an electron provided by HS-HTP to release methane and produce the heterodisulfide CoM-S-S-HTP (3, 63). The sulfhydryl forms of these enzymes are then regenerated by heterodisulfide reductase (HDR) using electrons derived from

Table 2. Standard free energy changes associated with selected methanogenic fermentations and reactions.*

Reaction	Standard Free Energy Change
1. $\text{ADP} + \text{P}_i \rightarrow \text{ATP}$	+32 kJ/mol
2. $4 \text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2 \text{H}_2\text{O}$	-130 kJ/mol
3. $4 \text{CH}_3\text{OH} \rightarrow 3 \text{CH}_4 + \text{CO}_2 + 2 \text{H}_2\text{O}$	-106 kJ/mol
4. $\text{CH}_3\text{COOH} \rightarrow \text{CH}_4 + \text{CO}_2$	-36 kJ/mol
5. $\text{H}_2 + \text{CH}_3\text{-S-CoM} \rightarrow \text{CH}_4 + \text{HS-CoM}$	-85 kJ/mol
6. $\text{H}_2 + \text{CoM-S-S-HTP} \rightarrow \text{HS-CoM} + \text{HS-HTP}$	-42 kJ/mol
7. $\text{CH}_3\text{-S-CoM} + \text{HS-HTP} \rightarrow \text{CH}_4 + \text{CoM-S-S-HTP}$	-43 kJ/mol
8. $\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{H}_2$	-20 kJ/mol

* Adapted from Muller et al. (61)

hydrogen (Table 2, reaction 6) or in acetate utilization, the oxidation of the carbonyl group of acetate (20). Electrons for this reduction can also be derived from methyl group oxidation during growth on methanol or methylamine. Methyl group oxidation is thought to occur by reversal of the CO₂ reduction pathway (61).

H₂-dependent CH₃-S-CoM reduction in resting cells is coupled to an electrochemical proton gradient and supports ATP synthesis (19, 65). ATP can also be synthesized from an artificial pH gradient (65).

Activation of Acetate and Cleavage of Acetyl-CoA:

From thermodynamic considerations it was assumed that acetate must first be activated and two activating enzymes were identified and purified. Acetate kinase first acts by phosphorylating acetate using ATP and consuming one high energy phosphate bond (2). Acetyl phosphate is then converted to acetyl-CoA by phosphotransacetylase, the second of the activating enzymes (57). Cell extracts can use acetyl-PO₄ as a substrate for methanogenesis in place of acetate and ATP (27, 49). In light of the activation energy input, these organisms must have evolved an energy conservation system that approaches or exceeds 50% efficiency (see Table 2, reactions 1,4).

The C-C and C-S bonds of acetyl-CoA are then cleaved by the carbon monoxide dehydrogenase complex (CODH), considered the central enzyme for acetate conversion (79). The CODH complex is composed of 5 subunits and

contains Factor III (corrinoid), Ni, Fe and acid-labile sulfur. The complex can be resolved into two functional units by detergent solubilization and anion exchange chromatography into a Ni-Fe-S subunit and a Co-Fe-S subunit (1). By EPR spectroscopy it has been shown that the Ni-Fe-S subunit binds and oxidizes the CO and transfers the methyl group to the corrinoid of the second component (40).

Methyl Transfer:

The corrinoid-bound methyl group is transferred to H₄SPT (31) and subsequently transferred to HS-CoM. It was proposed that this reaction would be a site for energy conservation and also that a sodium ion gradient would be the high energy intermediate (30, 55). These were postulated based on methyl transfer studies using artificial substrates and the strict requirement of this reaction for sodium (60). These results indicating primary sodium ion translocation were corroborated in *Methanobacterium* (43). Results from *Methanosarcina* strain Go1 show that the H₄SPT:HS-CoM methyltransferase is a membrane-bound primary sodium ion pump (6). This reaction was strictly dependent on sodium and HS-CoM, and inhibitor studies showed that sodium extrusion was primary and electrogenic.

Electron Transport:

Ferredoxin accepts electrons from the CODH complex (80). The electrons

generated from CO oxidation are used to reduce the methyl group of acetate (28, 62). It has long been assumed, since there are no obvious sites for substrate-level phosphorylation in the acetate pathway, that ATP must be generated using a proton motive force derived from this electron transport. This electron transport phosphorylation is in accordance with Mitchell's chemiosmotic mechanism (9).

The specific steps of electron transport system have not been determined, though many studies have identified possible electron carriers and functional attributes. Cytochromes were first postulated to function in electron transport (46) during acetate consumption when it was found that only those methanogens capable of growth on methyl compounds contained cytochromes and that cytochrome levels were elevated when those organisms were cultured on acetate as a substrate (42, 52). As determined by EPR and optical spectroscopy, the membranes of *Methanosarcina barkeri* contain cytochromes, multiple Fe-S centers and possibly a rubredoxin, and it is postulated that these may function in electron transport (46).

Resting cells are capable of oxidizing CO to CO₂ and H₂, and a proton gradient is formed by this process (11, 12, 13). This activity has been reconstructed using CODH, Fd and membranes. This activity is dependent on ferredoxin as the electron carrier from CODH to the membranes (80). Ultimately the electrons derived from the carbonyl moiety must be transferred to heterodisulfide reductase to regenerate the sulfhydryl forms of HS-CoM and HS-HTP.

A proton gradient is formed by the reduction of the heterodisulfide with

electrons from hydrogen (19, 22). A link from hydrogenase to cytochrome has been established by reduction of cytochromes in membranes with hydrogen gas as the reductant (80). One can hypothesize that hydrogen is first evolved by oxidation of the carbonyl group and then oxidized to reduce the heterodisulfide. This sequence of events has not been established for acetate-consuming methanogens. It has been proposed that hydrogen may be involved as an obligate intermediate. This is supported by the recent purification from *M. barkeri* of a membrane-bound H₂:CoM-HTP oxidoreductase complex which contains cytochromes (34). Proof of H₂ as an obligate intermediate is lacking however and several studies provide contrary evidence (44, 50, 56).

Substrate-level phosphorylation is precluded because *Methanosarcina* cleave 1 ATP per acetate in activation and gains less than 1 ATP from acetate. It is only possible for ion gradients to be involved in energy conservation since there are no sites for possible substrate level phosphorylation. *Methanosarcina barkeri* contains a proton driven ATPase. Much recent work has focused on the means of energy conservation for ATP synthesis as well as several endergonic reactions that are thought to be coupled to energy consumption (61). This organism must have evolved a very efficient energy conservation system considering the activation energy required and the low free energy change of acetate conversion.

Recently a methyl transferase that acts as a primary sodium pump was purified from *Methanosarcina* strain Go1 (6). The sodium gradient thus generated

is thought to be coupled to endergonic reactions. A H^+/Na^+ antiporter is present and the sodium gradient may drive ATP synthesis indirectly (43). Sodium ions may act directly by substituting for protons in the ATP synthase.

Heterodisulfide Reduction:

Heterodisulfide Reductase (HDR) is the enzyme that carries out the reduction of the heterodisulfide of HS-CoM and HS-HTP and has been shown to be linked in electron transport to hydrogenase and F_{420} -dehydrogenase in *Methanosarcina* Go1 (20, 21, 22, 33). The HDR protein has been shown to be primarily associated with the membranes of *Methanosarcina* strain Go1 from protoplasts (21, 22). In *Methanobacterium thermoautotrophicum* a soluble form of the enzyme was found (33), and a loose association with the cytoplasmic side of the membrane is possible (9). The elucidation of electron transport from the carbonyl group of acetate to CoM-S-S-HTP has become a question of interest, since the origin of electrons from acetate, and the points of consumption by coenzymes is known. As Muller, Blaut, and Gottschalk state in Methanogenesis, there is a current need to identify a ferredoxin-dependent heterodisulfide reductase system since the heterodisulfide reductase systems utilizing $F_{420}H_2$ and H_2 are probably not involved in acetate conversion (61).

According to Stryer (76) cytochromes have long been studied in a variety of organisms including bacteria and the mitochondrion of mammals. Typical studies include the use of spectroscopy to compare the absorption spectrum under different reducing and oxidizing conditions. Stryer also states there are several variations to the heme prosthetic group. In E. coli the most common is heme b (Iron protoporphyrin IX). In b-type cytochromes the prosthetic group is non-covalently bound and has three maxima in the reduced minus oxidized difference spectrum. The alpha band is near 560 nm. C-type cytochromes contain heme b covalently bound via two vinyl side-chains to cysteine residues of the protein. The type of binding to the protein and the immediate environment of the prosthetic group affect both the midpoint potential and the absorption spectrum of the cytochrome. Cytochromes are one electron carriers that are reduced at the iron atom of the heme (76).

Cytochromes in *Methanosarcina* are probably involved in energy conservation through proton translocation during acetate consumption (61). The goal of this study was to investigate the role of cytochromes in electron transport and to determine the electron donor and receiver for the cytochrome system during acetate conversion by *Methanosarcina thermophila*. In addition a method for improving cell yields from 15-liter fermentations was developed to facilitate biochemical studies.

MATERIALS AND METHODS

Sources of Chemicals:

Media components and glycerol were from Fisher Scientific, Pittsburgh, PA. Buffers for cell disruption and chromatography were from Sigma Chemical Co., St. Louis. Reagents for SDS-PAGE, Triton X-100, cytochrome c from horse heart, HS-CoM, BSA, and DTNB were from Sigma. Dimethoxybenzidine was from Eastman Kodak Co., Rochester, NY. The 7-bromoheptanoic acid was from Schweizer-Hall, South Plainfield, NJ. All chemicals were of reagent grade.

Organism:

Methanosarcina thermophila strain TM-1 was grown in 12-liter fermenters on 100 mM acetate at 50°C as previously described (74). Methane production was determined using gas chromatography as described. The fermenters were inoculated with a 1-liter batch culture or by transferring 200-400 ml of culture from another fermenter. The cultures were harvested anaerobically by continuous centrifugation and the cell paste was stored in liquid nitrogen until use.

Continued Growth with Media Supplement:

A media supplement (Table 3) was prepared in a 2-liter round bottom flask

Table 3. Media Supplement: The following components were combined to 1 liter total volume.

NH ₄ Cl	18.9 g	Yeast extract	5.0 g
Trypticase	5.0 g	K ₂ HPO ₄	4.35 g
KH ₂ PO ₄	3.4 g	NiCl·6H ₂ O	0.072 g
CaCl ₂ ·2H ₂ O	0.03 g	Fe(NH ₄) ₂ (SO ₄) ₂ ·6H ₂ O	0.157 g
Vitamins ^a	15 ml	Wolfe's Minerals ^a	100 ml
0.1% resazurin	1.0 ml		
Mineral II ^b	377 ml	PABA solution ^c	1.0 ml.

^a See Wolin et al. (88).

^b Mineral II contains the following components (g/l): KH₂PO₄, 6.0; NH₄Cl, 4.8; NaCl, 12.0; MgSO₄·7H₂O, 2.4; CaCl₂·2H₂O, 1.6. Store at 4°C.

^c PABA (para-aminobenzoic acid) solution contains .0075 g PABA in 100 ml H₂O. Store at 4°C in the dark.

to a total volume of 1 liter. It was then covered and sparged with nitrogen gas for 30 min and autoclaved (30 min, 121°C). The supplement was reduced with cysteine and sodium sulfide (0.25 g each) after autoclaving. When reduced, the media supplement was transferred to the fermentor. Twelve-liter fermenter cultures were supplemented by this procedure after 65 hours of growth. Growth of the culture continued at high rates for another 24 to 30 hrs and was harvested as described above.

Preparation of Fractions:

Cell-free extract was prepared by thawing cells in a Coy anaerobic chamber (atmosphere N₂:H₂, 95:5, v:v). An equal volume of 100 mM bistris-HCl (bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane;2-bis[2-hydroxyethyl]amino-2-[hydroxymethyl]-1,3-propanediol), pH 6.8, 10% glycerol was added with a few crystals of DNase I (grade II, Boehringer Mannheim, Indianapolis). The cell suspension was placed into a french pressure cell, removed from the anaerobic chamber and broken at 20,000 psi into a sealed anaerobic serum bottle. This cell extract was then centrifuged at 6500 × g for 20 minutes at 4°C to remove cell debris and unbroken cells. The supernatant (cell-free extract) was removed and centrifuged at 150,000 x g for 2.5 hrs at 4°C, and this supernatant (soluble fraction) was stored at -20°C until use. The membrane pellet was suspended in 50 mM bistris-HCl (pH 6.8), 10% glycerol (BTG) in a tissue homogenizer, and an equal

volume of 4% (v/v) Triton X-100 in BTG buffer was added. The membranes were solubilized by placing on a rocking platform at 4°C for 4 hrs, centrifuged at 150,000 x g for 1 hour at 4°C and the supernatant (solubilized membranes) was stored at -20°C until use.

To produce membranes by sucrose gradient purification, the cell extract was loaded directly onto a discontinuous sucrose gradient as previously described (80) except using BTG as buffer. Membranes were harvested from the 30-70% sucrose interface.

Spectrophotometry:

Cytochromes were analyzed in rubber stoppered 1.5 ml optical glass cuvettes using a Perkin Elmer Lambda 6 spectrophotometer equipped with an end-on photomultiplier and Perkin Elmer computer software. The scan speed was 20 nm/sec, the spectral bandwidth was 2nm, and the response was set at 3. Cytochromes were reduced with 10 ul of 1.0 M sodium dithionite and oxidized by air or addition of 10 ul of 30% hydrogen peroxide. Cytochromes in membranes purified by sucrose gradient centrifugation were reduced with hydrogen or carbon monoxide (100% gas phase) by flushing the headspace of a rubber stopper-sealed cuvette with the gas for 3 min and incubating at room temperature for 15 minutes. For oxidation with CoM-S-S-HTP the headspace was flushed with N₂, and 65 nmol CoM-S-S-HTP was added in minimal volume. The reduction and oxidation of the

cytochromes were determined from the absorption at 425 and at 558 nm of the difference spectrum, and calibrated against a dithionite-reduced minus peroxide-oxidized difference spectrum (82). The total cytochrome content of the sucrose-gradient membranes was determined from the dithionite vs H₂O₂ difference spectrum using $A_{558}-A_{580}$ or $A_{425} - (A_{400}+A_{450})/2$. The amount of cytochrome involved in the CO reduction, CoM-S-S-HTP oxidation was then determined by the same method of wavelength comparisons. For analysis of the CO binding properties of the cytochrome, the samples were first reduced with dithionite and CO was then bubbled through the sample for 3 min. These spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer as described by Kuhn and Gottschalk (51).

Electrophoresis:

Protein samples were analyzed by PAGE using the Laemlli method (54) with 15% polyacrylamide gels as described by Smith (72). Samples for native (nondenaturing) gels were prepared by omitting SDS from all components. For SDS-PAGE, samples were added to SDS sample buffer, mixed for 30 sec. and incubated at room temperature for 15 min. Protein was stained using Coomassie blue R-250. Dimethoxybenzidine was used to stain for heme as described by Francis (29). To prevent porphyrin ring degradation all samples were prepared without 2-mercaptoethanol unless noted. The gels were stained for heme and

subsequently stained for protein.

Chromatography:

Column chromatography was performed on a standard FPLC (Pharmacia, Upsala, Sweden) plumbed with oxygen-impermeable Saran tubing (0.125 ID, Pyramid Plastics Inc., Hope, AR) to the solvent mixer, and PEEK tubing (.0625" OD x .030" ID, Upchurch Scientific, Oak Harbor, WA) from the mixer to the collection point. Fractions were collected by fitting the tubing with a stainless steel cannula and collecting into an anaerobic crimp-sealed tube. Buffers were prepared in stoppered sidearm flasks. The stoppers were connected to a 10 micron filter on the inside and to norprene tubing (.125 in. ID) with a clamp on the outside by a cannula penetrating the stopper. The norprene tubing was connected to the FPLC after vacuum degassing the buffer at less than 100 millitorrs for 1 hr.

Isolation of Cytochrome and Heterodisulfide Reductase:

For cytochrome isolation detergent solubilized membranes (2 ml, 15 mg protein) were loaded onto a Mono Q 10/10 column (Pharmacia) that was preequilibrated with 50 mM KPO₄ (pH 6.8), 0.2% Triton X-100 and rinsed with two column volumes of the same buffer. Protein was eluted with a 0 to 1 molar NaCl linear gradient, and individual peaks were collected. Fractions were analyzed by staining SDS-polyacrylamide gels for heme-derived peroxidase activity using

dimethoxybenzidine (29) and protein with Coomassie blue. Spectral analysis was also performed on these fractions.

HDR was isolated from the soluble protein on a mono Q 10/10. Two ml of the soluble fraction (60 mg protein) were diluted and mixed with 2 ml of BTG containing 0.2% Triton X-100, 2mM dithiothreitol (DTT). The diluted sample was applied to a mono Q 10/10 column previously equilibrated with 50 mM bistris-Cl (pH 6.8), 10% glycerol, 0.1% triton X-100 (BTGX) containing 2 mM DTT. After washing with 2 column volumes of BTGX plus 2mM DTT, the HDR was eluted by application of BTGX, 2 mM DTT, 200 mM NaCl. The first peak to elute was found to contain heterodisulfide reductase activity.

Other conditions for chromatography were tested. Both cytochrome and HDR bound to hydroxylapatite high resolution (Calbiochem, La Jolla, Ca.) in BTGX, but neither eluted with 1 molar NaCl. HDR could be eluted with 100 mM KPO₄, but a gradient was not performed and no purification was obtained. Cytochrome was bound to the Mono Q column in 20 mM Na-tricine (pH 8.5), 0.2% triton; or 50 mM bistris-Cl (pH 6.8), and could be eluted with NaCl at 0-100 mM concentration, but no purification was obtained.

Isolation of Carbon Monoxide Dehydrogenase and Ferredoxin:

Carbon monoxide dehydrogenase and ferredoxin were purified anaerobically as previously described (48, 78, 81). These were provided by Dr. Madeline

Rasche.

Synthesis of the Heterodisulfide of HS-CoM and HS-HTP:

7-mercaptoheptanoylthreonine phosphate (HS-HTP) and CoM-S-S-HTP were synthesized from 7-bromoheptanoic acid as previously described (10, 63) and CoM-S-S-HTP was assayed for activity by reduction with cell extracts. Heterodisulfide content was determined by reduction to free thiols by heterodisulfide reductase. The enzyme is specific only for the mixed disulfide of HS-HTP and HS-CoM (10, 18, 33). CoM-S-S-HTP solution (13 mM) in 50 mM KPO₄ buffer (pH 8.0) was used for HDR assays and reconstitution experiments.

Enzyme Assays:

Carbon monoxide dehydrogenase and hydrogenase were assayed at room temperature with methyl viologen as an electron acceptor in phosphate buffer as previously described (5, 78).

I developed a rapid spectrophotometric assay for the heterodisulfide reductase (HDR) using methyl viologen as an artificial electron carrier. Methyl viologen absorbs strongly at 603 nm ($\epsilon=11.3 \text{ mM}^{-1}$) when reduced with dithionite. The oxidation of the dye is then coupled to the specific reduction of CoM-S-S-HTP by the heterodisulfide reductase, and the reaction is followed by monitoring a decrease in absorbance at 603 nm. Heterodisulfide reductase was routinely assayed

at room temperature using methyl viologen as an electron donor (34). Reduction of the heterodisulfide was also measured by determination of free thiols using 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB),(23) as described by Clements and Ferry (18). Reconstitution of the CO oxidizing CoM-S-S-HTP reducing system was performed in 8ml amber vials at 50°C using pure CODH, pure Fd, sucrose gradient purified membranes and HDR fraction (Clements 1993). The standard 1.0 ml reaction mixture contained 13.5 µg of CODH, 1.6 µg of Fd, 120 µg of membrane protein, 26 µg of HDR and 1.3 µmol of CoM-S-S-HTP.

Protein was determined by the dye-binding method of Bradford (14) using the BioRad dye reagent (BioRad, Richmond, CA). Bovine serum albumin was used as a standard. Ferredoxin was determined by the BCA assay (Pierce, Rockford, IL) using purified lyophilized Fd as a standard.

RESULTS

Increased Cell Yields

Cell growth during fermentations was monitored by determining the rate of methane production using gas chromatography. In conventional fermentations by the method of Sowers (74), using a pH auxostat, the cells entered late exponential growth phase after approximately 70 hours as determined from the rates of methane production. Maximal methane production in these fermentations reached 6000-8000 nmol CH₄/sec/liter. Cell yields for the 12-liter fermentation averaged 34 grams wet weight after three days of growth (Table 4). Cell growth in 12 liter fermentations continued in log phase for up to 30 additional hours when the media supplement (Table 3) was added. The continued exponential phase growth was confirmed by gas chromatography measurements. Fermentations that were continued by supplementation yielded up to 128 grams in the four day fermentation and methane production rates reached 14,700 nmol CH₄/sec/l. Table 4 compares methane production and cell yields for the conventional (74) and supplemented fermentations (see material and methods).

Table 4. Comparison of fermentations.^a

	Conventional ^b	Supplemented ^c
Time until harvest ^d	70 hours	90-95 hrs.
Maximal CH ₄ production	8300 nmol/sec/l	14,700 nmol/sec/l
Avg CH ₄ production	4900 ± 1600 nmol/sec/l	10,300 ± 3000 nmol/sec/l
Maximum cell yield	58 g	128 g
Avg cell yield	34.3 ± 14.5 g	93.1 ± 18.4 g

^a Values are from 12 conventional fermentations and 7 supplemented fermentations.

^b By the method of Sowers (74).

^c By the supplementation procedure described in text, using media supplement (Table 3).

^d Cultures were harvested during late exponential growth phase

Presence of cytochromes in TM-1 Membranes:

Membranes were purified by discontinuous sucrose gradient centrifugation of cell extracts. The dithionite-reduced minus H_2O_2 -oxidized difference spectrum (Fig. 3) is typical of b-type cytochromes and has absorbance maxima at 558 nm (alpha band), 532 nm (beta band) and 425 nm (gamma or Soret band). The alpha and gamma bands had shoulders at 563 and 436 nm respectively (Fig. 3 inset), which suggested the presence of two heme centers. The spectrum is clearly indicative of b-type cytochromes and did not indicate the presence of c-type cytochromes. However, an acetone-HCl extract was not prepared and the presence of small amounts of c-type cytochrome cannot be excluded.

H_2 -reduced minus air-oxidized difference spectra (Fig. 4) were similar to those in Figure 3 which suggested H_2 replaced dithionite as a reductant and confirmed earlier reports (80) that membranes contain a hydrogenase linked to b-type cytochromes. H_2 -reduced cytochromes were completely oxidized by the heterodisulfide CoM-S-S-HTP (Fig. 4) and were not further oxidized by H_2O_2 (data not shown). These results are consistent with b-type cytochromes involved in electron transport from H_2 to CoM-S-S-HTP in *M. thermophila*. Similar results were recently reported for *M. barkeri* (34).

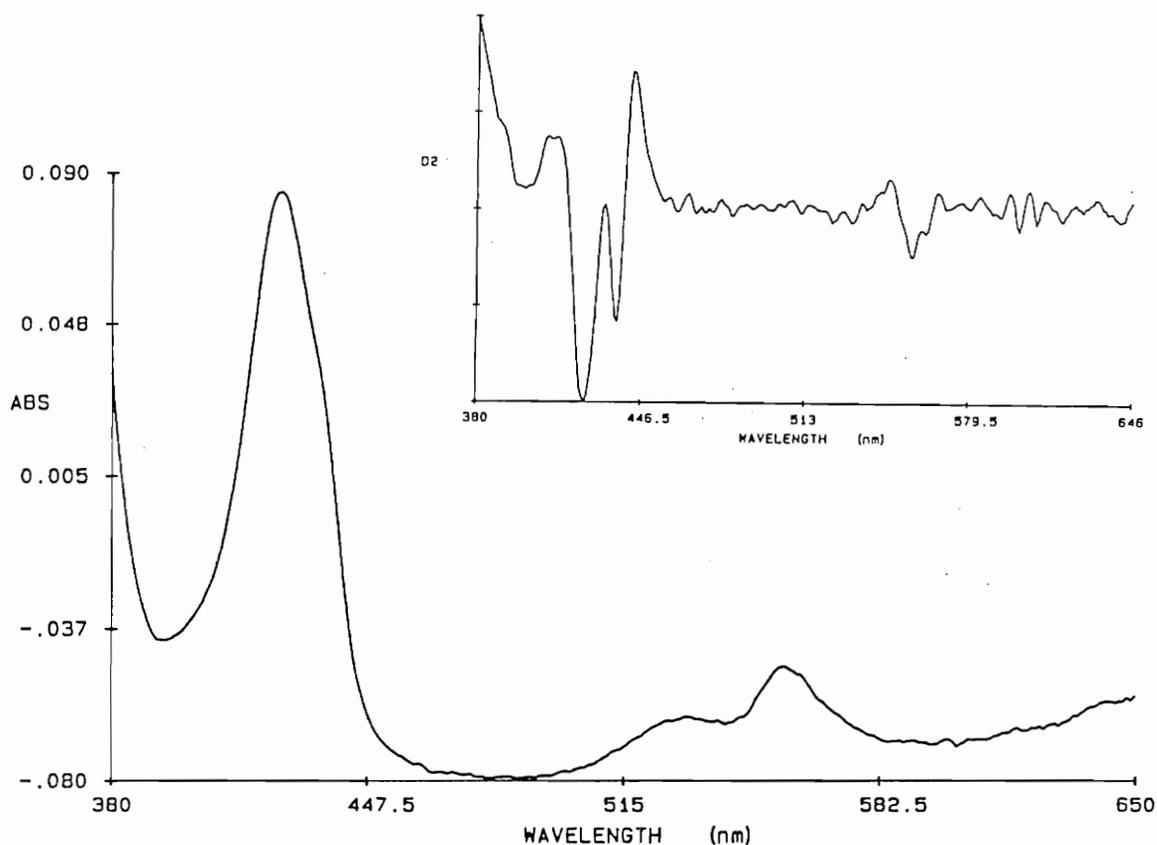


Figure 3. Dithionite-reduced minus H_2O_2 -oxidized difference spectrum of *M. thermophila* membranes. The difference spectrum shows cytochromes in membranes that were prepared by discontinuous sucrose gradient centrifugation. Second derivative (inset) was generated from the difference spectrum using Perkin Elmer computerized scanning software. The sample contained 2.1 mg protein/ml.

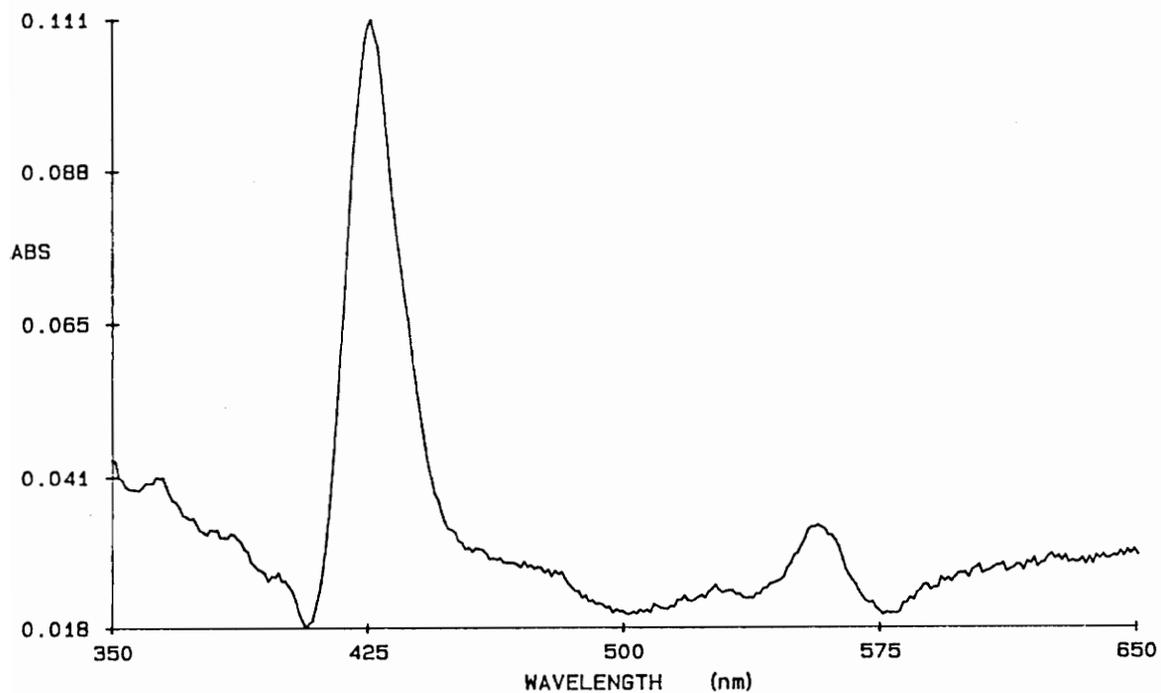


Figure 4. H₂-reduced minus CoM-S-S-HTP oxidized difference spectrum. Membranes were treated with 100% H₂ gas phase after isolation and subsequently oxidized with CoM-S-S-HTP under a N₂ gas phase. The sample contained 1.7 mg protein/ml

Reduction of Cytochromes with Carbon Monoxide:

The effects of carbon monoxide on the spectral properties of the cytochromes were investigated. Detergent solubilized membranes were reduced with dithionite, and CO was bubbled through the sample for 3 min (Fig 5A). The dithionite-reduced/CO-treated minus dithionite-reduced difference spectrum (Fig 5B) had maxima at 568, 546, 414 and 395 nm and minima at 552, 432, and 406. This result shows that CO reacts with dithionite-reduced cytochromes in detergent solubilized membranes when CO is present in the headspace and bubbled through the solution.

Figure 6 shows the difference spectrum of CO-reduced minus CoM-S-S-HTP oxidized membranes. In this experiment, membranes were incubated with 100% CO in the headspace, but CO was not bubbled through the solution. This spectrum was nearly identical to the dithionite-reduced minus H₂O₂-oxidized spectrum (Fig. 3), a result which indicates complete reduction of the cytochromes with CO and complete oxidation by CoM-S-S-HTP. There was no evidence of CO binding to the cytochromes when only the headspace contained CO. Cytochromes were not oxidized by the homodisulfide of HS-CoM (data not shown), which indicated that the reaction was dependent on heterodisulfide reductase activity associated with membranes. These results are consistent with b-type cytochromes involved in the electron transport chain coupling oxidation of CO by CODH and reduction of

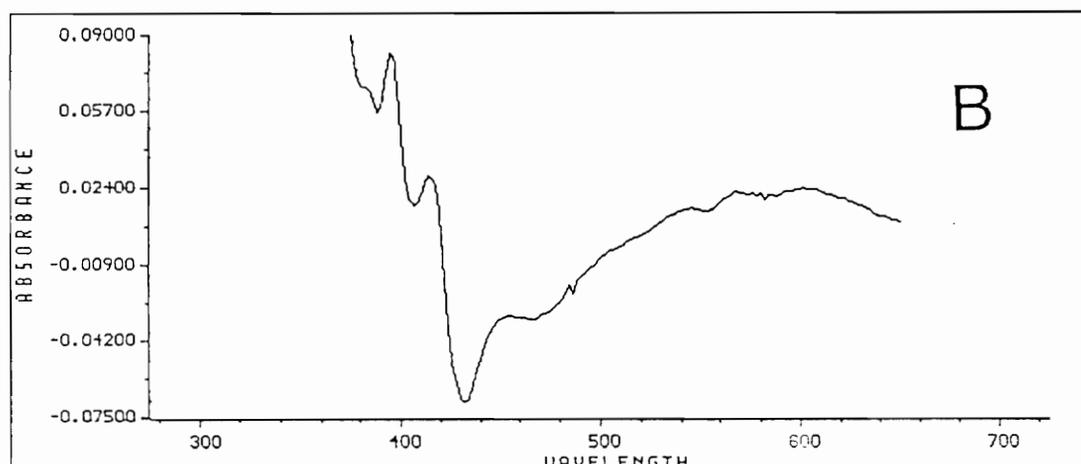
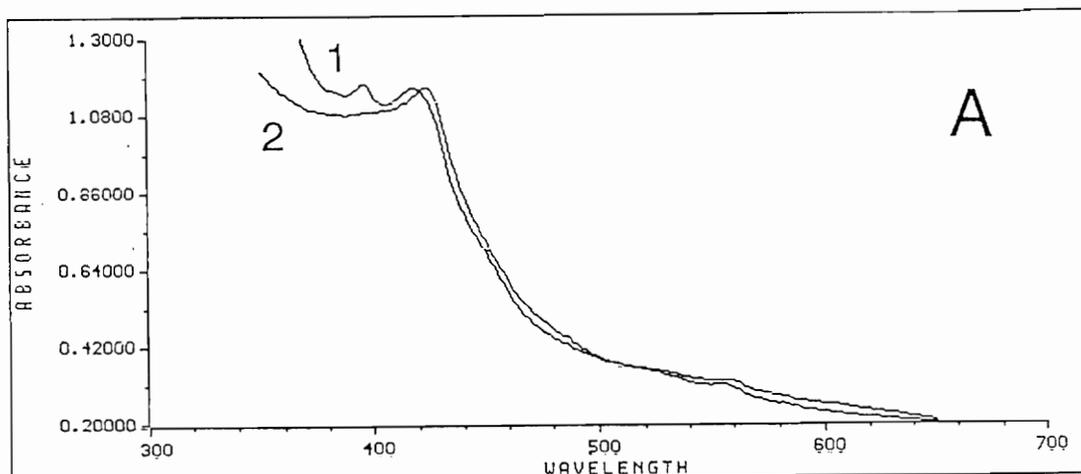


Figure 5. A. Spectra of dithionite-reduced/CO treated, and dithionite-reduced membranes. Triton X-100 solubilized membranes (5 mg protein/ml) were reduced with 10 μmol dithionite (2) and were subsequently treated with CO bubbling for 3 minutes (1). The spectrum of CO treated membranes (2) shows a prominent peak at 395 nm typical of reduced corrinoids with the cobalt atom in the Co^{+1} redox state.

B. Dithionite-reduced plus CO minus dithionite-reduced difference spectrum of detergent solubilized membranes from *M. thermophila* TM-1.

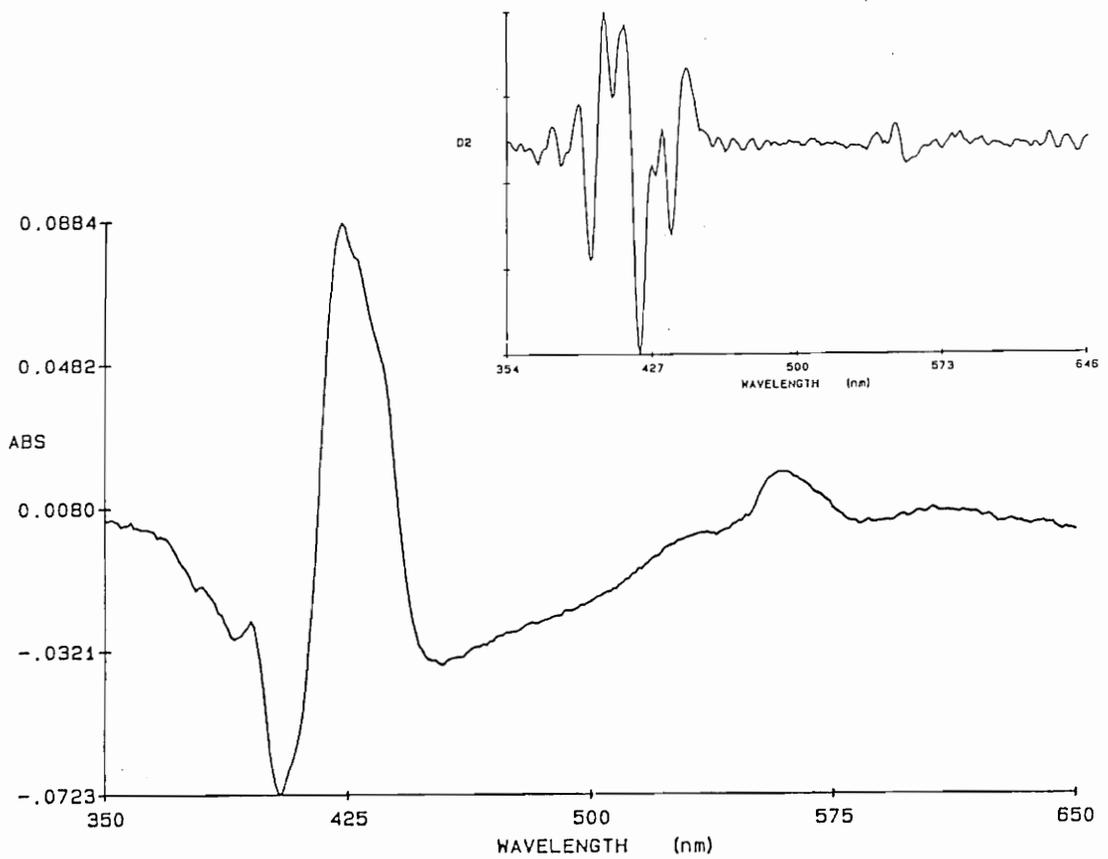


Figure 6. CO-reduced minus CoM-S-S-HTP oxidized membranes. Membranes were isolated and oxidized (see text), then reduced by incubation with a CO atmosphere and reoxidized with CoM-S-S-HTP under a N₂ gas phase. Inset shows second derivative. The sample contained 1.05 mg protein/ml.

CoM-S-S-HTP by the heterodisulfide reductase.

The spectrum of CO-reduced membranes (not shown) contained an additional absorbance maximum at 395 nm that was not present in H₂-reduced samples. This absorbance is also apparent in the dithionite-reduced/CO-treated spectrum of solubilized membranes (see Fig. 5A). Absorbance at 395 nm is typical of corrinoids in which the cobalt atom is reduced to the Co⁺¹ redox state (40). These results suggest that CO, but not H₂, donates electrons to a membrane-associated corrinoid-containing protein.

Partial Purification of Cytochromes

In an initial attempt to purify the cytochromes, Triton X-100 solubilized membranes were fractionated on a Mono Q anion exchange column. Fractions were analyzed for cytochromes spectrophotometrically and by the more sensitive method of staining polyacrylamide gels for heme-derived peroxidase activity. After loading the sample, the column was washed with 2 column volumes of 50 mM KPO₄ (pH 6.9) containing 0.2% Triton X-100. The cytochromes did not bind and were eluted isocratically. The difference spectrum of this fraction (not shown) had the same absorbance maxima as the membranes (see Fig. 3), indicating the same cytochrome content. The column was then developed with a linear gradient of NaCl (0 to 1 M) and small amounts of cytochrome were present in the fraction collected from 0-100 mM NaCl. When analyzed by nondenaturing PAGE only one

major protein band was detected in the isocratically eluted cytochrome fraction, and this band stained for heme (Fig. 7). Since there was only one band on the native gels, it is possible that cyt b is in a complex with other proteins. The SDS-polyacrylamide gels of the cytochrome fraction revealed six major bands, M_r 58, 47, 40, 28, and 20 kD (Fig. 8A). of which stain only the 28 kD stained for heme (Fig. 8B). The 0-100 mM NaCl fractions containing small amounts of cytochrome showed a different protein banding pattern on SDS-PAGE but again only contained heme in the 28 kD band. Since the samples were not boiled it is not possible to conclude that this 28 kD band represented a single polypeptide. When boiled, proteins in the 28 and 58 kD bands formed a high molecular weight aggregate that did not enter the separating gel (Fig. 9). This thermal aggregation is characteristic of membrane-associated proteins.

Cell free extracts, membranes, solubilized membranes and the Mono Q fraction that contained cytochrome were electrophoresed as described for Fig. 8 and the gels were stained for heme-derived peroxidase activity. The three samples had only one band, at 28 kD, that stained for heme (Fig. 10). When isolation of cytochrome was carried out as described above but with 50 mM bistris-Cl (pH 6.8) or 20 mM Na-tricine (pH 8.5) buffer substituting for KPO_4 , cytochrome bound to the column matrix and was eluted in 0-100 mM NaCl. The cytochrome isolated by these procedures was electrophoresed in SDS-PAGE as described for Fig. 8, and again only the 28 kD band stained for heme (data not shown). These results

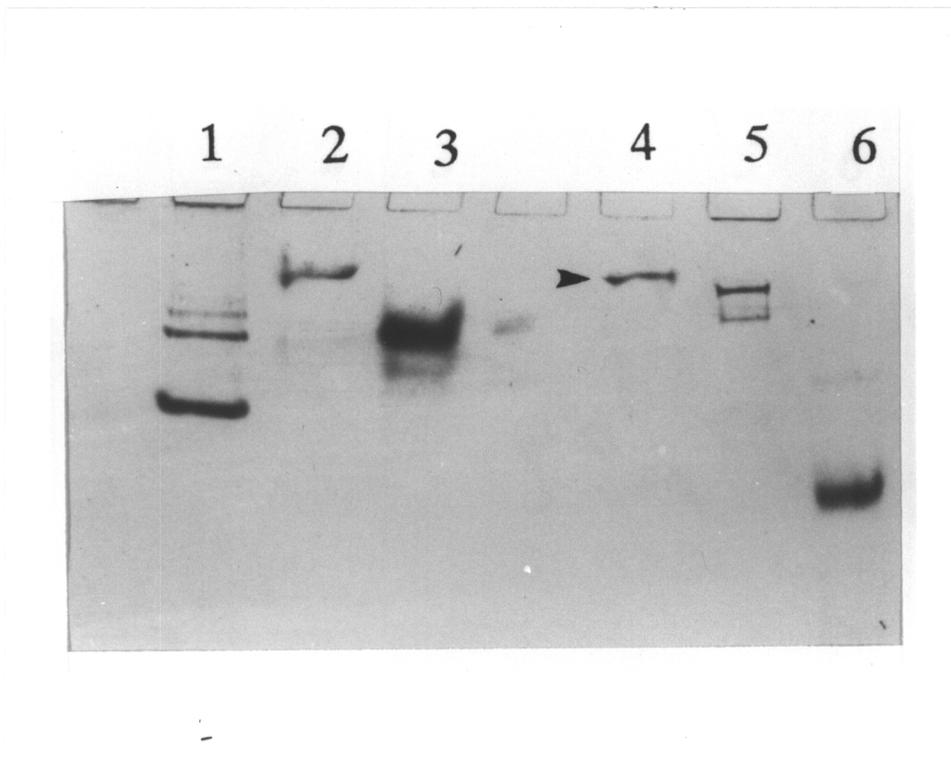


Figure 7. Native PAGE of the cytochrome containing Mono Q column fraction. Samples were not boiled and were prepared without SDS. Following electrophoresis the gel was stained for heme-derived peroxidase activity (not shown) with dimethoxybenzidine (see ref. 29). The band in lane 4 indicated by the arrow and the corresponding band in lane 2 stained for this activity. Subsequently the gel was stained with Coomassie blue R-250 (shown). Lane 1, BSA; lane 2,4, cytochrome fraction; lane 3, carbonic anhydrase; lane 5, urease; lane 6, chicken egg albumin.

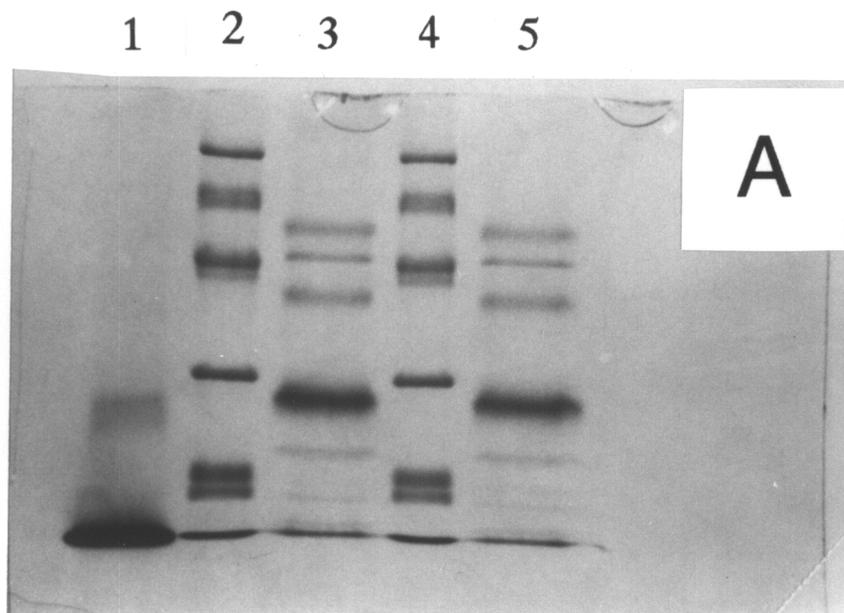


Figure 8. Panel A. SDS-PAGE analysis of the isolated cytochrome fraction. Protein was denatured in SDS at room temperature and separated on a 12% polyacrylamide gel. The gel was stained for heme (Panel B) and subsequently stained for protein (Panel A) as in Fig. 7. Lane 1, horse heart cytochrome c; lanes 2 and 4, BioRad low molecular weight standards; lanes 3 and 5, *M. thermophila* cytochrome b fraction from Mono Q column. The intensity of cyt-c, lane 1, and the 28 kDa band, lanes 3 and 5, is enhanced by prior heme staining.

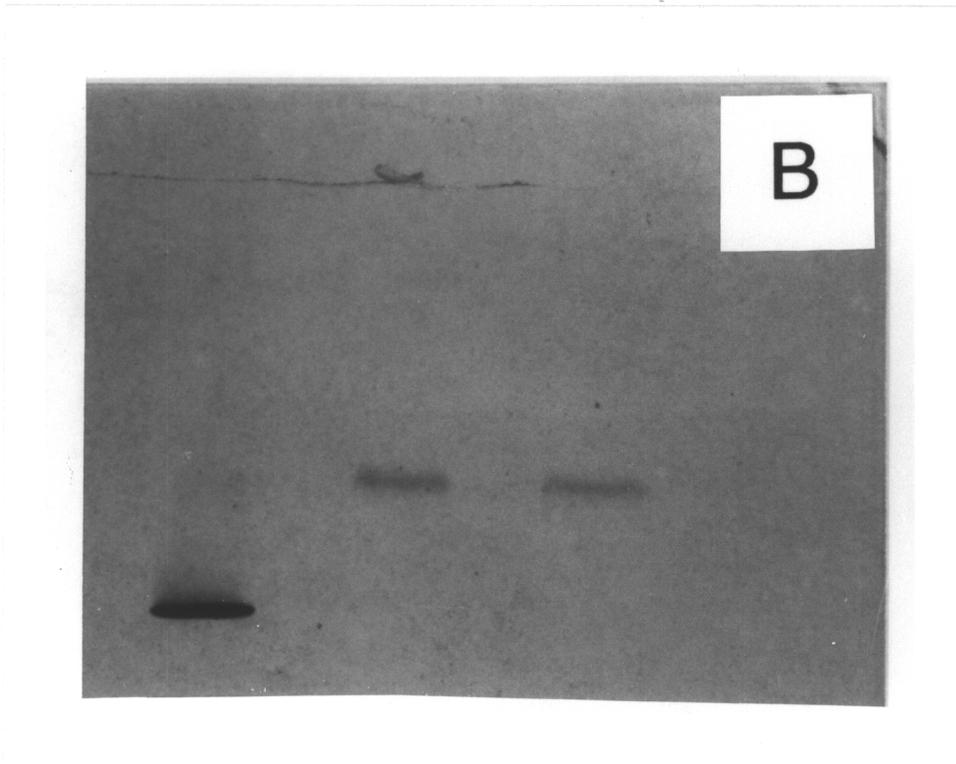


Figure 8. Panel B. **SDS-PAGE analysis of the isolated cytochrome fraction.** Protein was denatured in SDS at room temperature and separated on a 12% polyacrylamide gel. The gel was stained for heme (Panel B) and subsequently stained for protein (Panel A) as in Fig. 7. Lane 1, horse heart cytochrome c; lanes 2 and 4, BioRad low molecular weight standards; lanes 3 and 5, *M. thermophila* cytochrome b fraction from Mono Q column. The intensity of cyt-c, lane 1, and the 28 kDa band, lanes 3 and 5, is enhanced by prior heme staining.

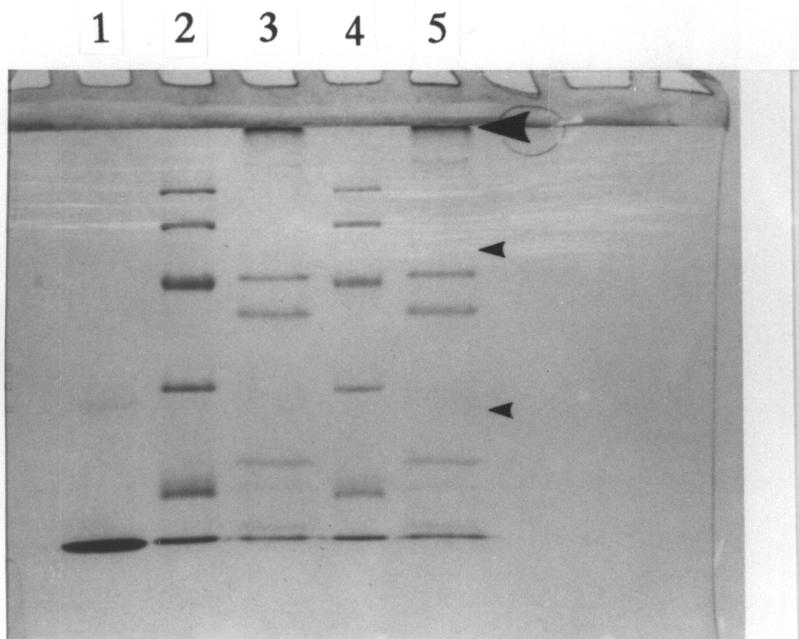


Figure 9. SDS-PAGE showing thermal aggregation of the 58 and 28 kD bands. All samples were heat treated at 95°C for five minutes in SDS-containing sample buffer with 2-mercaptoethanol. Electrophoresis conditions were the same as in Fig. 8. No heme was detected by the DMB staining as predicted (see ref. 29). A high molecular weight aggregate was formed by the 28 and 57 kD proteins and can be seen at the top of the gel. Arrows indicate absence of the 58 and 28 kD bands and the aggregate.

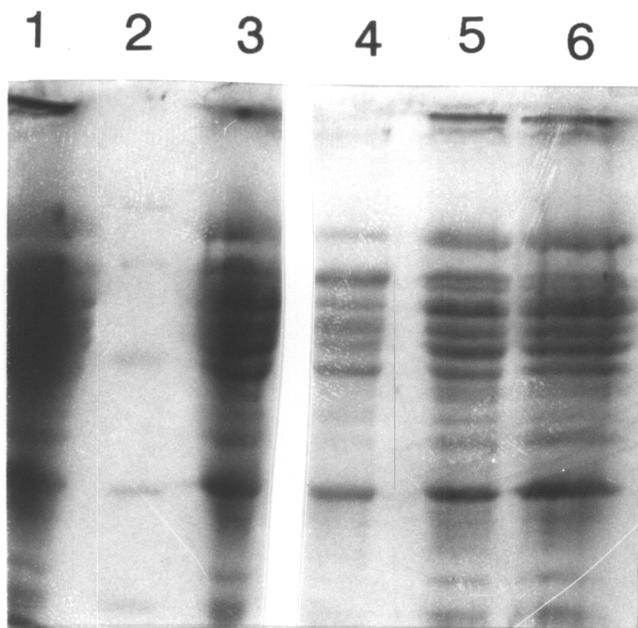


Figure 10. Panel A. SDS-PAGE analysis of cell-free extract, membranes, and the cytochrome fraction. Samples were prepared as described for Fig. 8 and stained for heme (panel B) and protein (panel A). Lanes 1, 3, isolated cytochrome fraction; lane 2, BioRad low molecular weight markers; lane 4, Cell-free extract; lane 5, membranes; lane 6, solubilized membranes. When stained for protein, the 28 kD band showed increased intensity from prior heme staining.

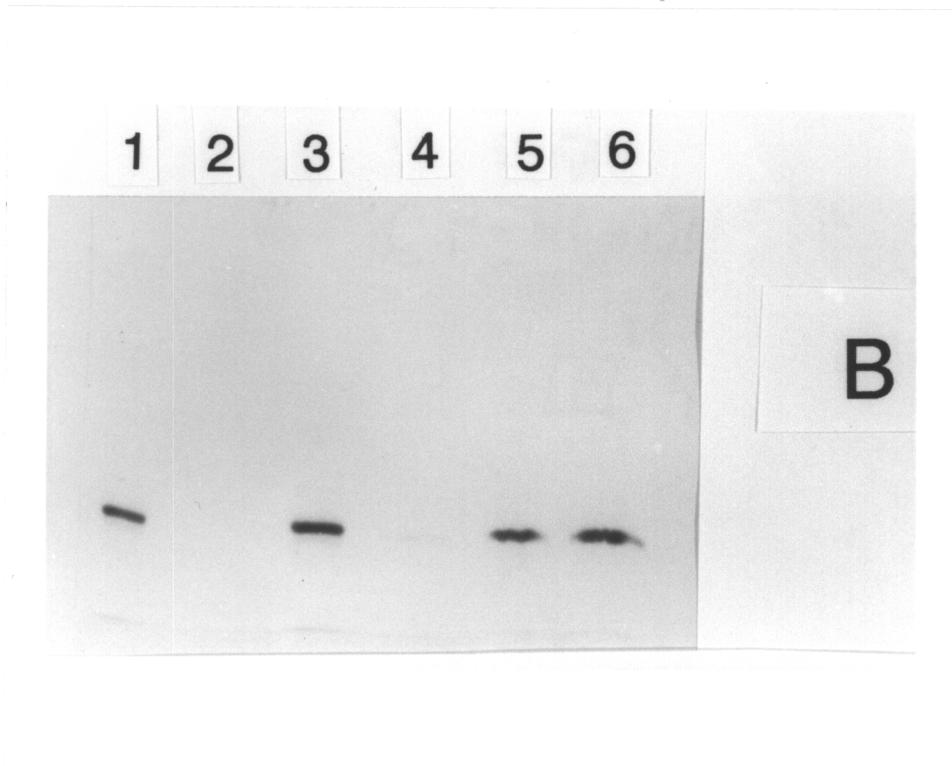


Figure 10. Panel B. SDS-PAGE analysis of cell-free extract, membranes, and the cytochrome fraction. Samples were prepared as described for Fig. 8 and stained for heme (panel B) and protein (panel A). Lanes 1, 3, isolated cytochrome fraction; lane 2, BioRad low molecular weight markers; lane 4, Cell-free extract; lane 5, membranes; lane 6, solubilized membranes. When stained for protein, the 28 kD band showed increased intensity from prior heme staining.

suggest that the two heme centers seen in difference spectra are present in a 28 kDa protein.

Heterodisulfide Reductase:

Cell extracts were fractionated by discontinuous sucrose gradient centrifugation. Heterodisulfide reductase activity was assayed in the membrane and cytoplasmic fractions using methyl viologen as an electron donor. The soluble fraction contained 83% of the activity (Table 5).

Soluble protein from ultracentrifugation of *M. thermophila* extracts was separated on a Mono Q 10/10 anion exchange column. The sample was diluted two-fold in BTG buffer containing 0.2% Triton X-100 and loaded onto a column pre-equilibrated with BTGX. Heterodisulfide reductase was eluted by application of 200 mM NaCl in BTGX. By this procedure 73% of the activity present in the soluble fraction was recovered. The specific activity was 1.83 U/mg, representing a two-fold increase over the soluble fraction (data not shown). Glycerol was required to stabilize the activity. No heterodisulfide reductase activity could be recovered when the fractionation was attempted in bistris-HCl without glycerol (data not shown).

Table 5. Heterodisulfide reductase activity and distribution from soluble and membrane fractions of cell extract.

	Specific ^a Activity	Total ^b Activity	Percent
Soluble Fraction	1.77 ± 0.66 (n=8)	235 U	83%
Membranes	6.83 ± 1.05 (n=9)	48 U	17%
Total		283 U	100%

^a Units of activity per mg protein

^b 1 unit = 1 umol CoM-S-S-HTP reduced per min

Electron transport from CO to CoM-S-S-HTP:

Electron transport from CO to CoM-S-S-HTP was reconstituted by combining CODH, ferredoxin, membranes and the HDR fraction. It is known that CODH oxidizes CO and reduces ferredoxin. The route of electrons for reduction of the heterodisulfide is thought to involve membranes (61). The HDR catalyzes the last step of the reaction sequence, reduction of the heterodisulfide. Dithionitrobenzoic acid (DTNB) was used to quantitate free thiols produced by reduction of the disulfide bond of CoM-S-S-HTP. After the fractions were combined, 30% CO was added to the headspace and the reaction started by transferring the reaction vial to a 50°C reciprocating water bath.

The reaction was dependent on the concentration of ferredoxin (Fig. 11). The reaction was also strictly dependent on membranes for activity. Heterodisulfide reductase fraction was required and increasing concentrations increased the rate of the reaction (data not shown). These results suggest that ferredoxin donates electrons to the membranes for reduction of CoM-S-S-HTP. Also this demonstrates that redox components of the *M. thermophila* membranes can transfer electrons to a soluble heterodisulfide reductase. This electron transport system was specific for reduction of CoM-S-S-HTP and the homodisulfide of HS-CoM was not reduced.

Based on these experiments, I propose that cytochromes function to transport electrons originating from CODH to the heterodisulfide reductase.

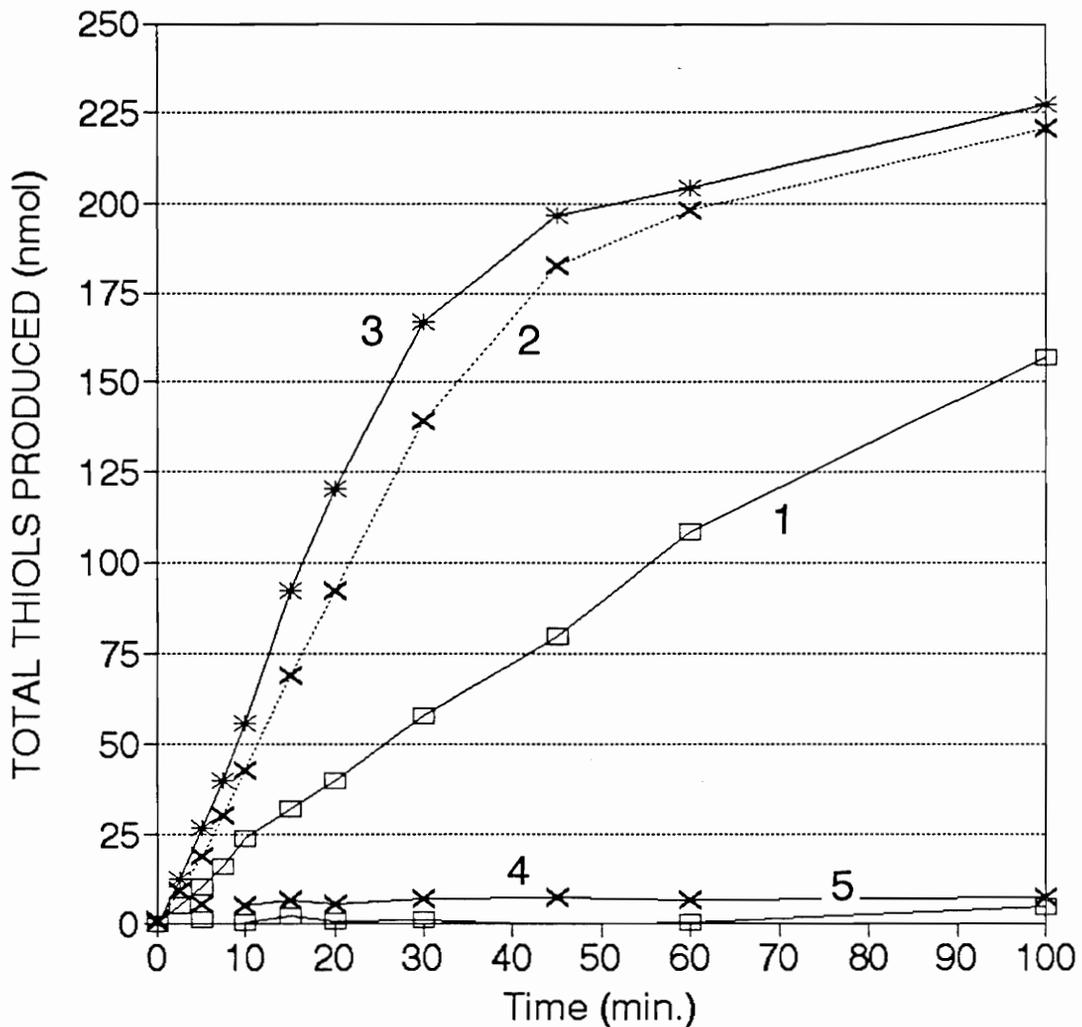


Figure 11. Effect of increasing ferredoxin concentration on production of free thiols from CoM-S-S-HTP using the reconstituted system. Reactions in 8 ml amber vials with 30% CO headspace were carried out at 50°C in a reciprocating water bath at 180 rpm. Production of free thiols was quantitated using DTNB. (1) The standard reaction contained 13.5 μg CODH, 1.6 μg Fd, 120 μg membrane protein, 26 μg HDR; (2) standard reaction with 50 μg Fd; (3) standard reaction with 100 μg Fd; (4) standard reaction minus membranes; (5) standard reaction minus Fd.

DISCUSSION

Cytochromes from methane-producing bacteria were assumed to be involved in methyl group oxidation, since they are present only in methylotrophic methanogens. Kemner first proposed cytochromes function in electron transport from CO to methyl-CoM (46). Recent evidence further supports a role in electron transport during growth on methyl compounds (61).

Evidence that cytochromes participate in heterodisulfide reduction by hydrogen has been shown directly by purification of the cytochrome-containing H_2 :CoM-S-S-HTP oxidoreductase complex from membranes of acetate-grown *M. barkeri* (34). The physiological significance of this complex has not yet been resolved. It is unclear whether hydrogen is involved as an obligatory intermediate through a hydrogen cycling mechanism during growth on acetate. Whether H_2 :CoM-S-S-HTP oxidoreductase is involved during acetate conversion is not known, since *M. barkeri* is capable of growth on H_2/CO_2 the complex may be constitutively expressed and used only when supplied with H_2 from the environment. Recall that the H_2/CO_2 pathway provides the most available free energy of all methanogenic substrates (Table 2), so constitutive expression of H_2 :CoM-S-S-HTP oxidoreductase could be beneficial to scavenge any available H_2 from the environment. Additionally, when supplied with H_2 and a methyl compound *Methanosarcina* are capable of using the H_2 to reduce the methyl compounds to methane (64, 89). This conserves more energy than oxidizing one

of the four methyl groups to generate reducing equivalents.

As shown in this work, HDR activity is found mostly in the soluble fraction, and glycerol was required to stabilize activity. I report here higher total recovery of activity than reported for *M. barkeri* (34). It is possible that the activity reported underestimates total activity in *M. barkeri*. Moreover, determination of cellular location of HDR from *M. barkeri* may be inaccurate by failing to quantitate HDR in the soluble fraction, since glycerol may act to stabilize HDR dissociated from the membrane during cell lysis. It is possible that acetate-grown cells of *M. thermophila* do not require a tight association of HDR to the membrane. Reconstitution experiments indicated that membrane bound electron carriers donated electrons to a soluble heterodisulfide reductase. This is consistent with a loose or peripheral association between HDR and the membranes.

The subunit composition of the functional cytochrome system in acetate metabolism in *M. barkeri* and *M. thermophila* is unresolved. The functional $H_2:CoM-S-S-HTP$ oxidoreductase purified from *M. barkeri* is composed of nine different polypeptides. The heme containing band was 23 kD. The cytochromes isolated from *M. thermophila* TM-1 are a complex with as few as six polypeptides of which the 28 kD band contained heme. The cytochrome from TM-1 was not tested for $H_2:CoM-S-S-HTP$ oxidoreduction activity. Subunits of the complex from both *M. barkeri* and *M. thermophila* aggregate to a high molecular mass upon boiling

rendering it impossible to conclude the heme containing band represented a single polypeptide. However, under several different sets of conditions the heme staining band was still apparent at 28 kD. The spectra revealed at least two heme groups and this suggests that the heme groups are bound by the polypeptide(s) in the 28 kD band. This result is similar to the b-type cytochrome purified from the archaeobacteria *Sulfolobus acidocaldarius* which had an Mr of 30,000 and contained two heme groups determined spectrophotometrically (7).

The *M. barkeri* cytochrome complex contained two heme centers determined by low temperature spectroscopy (34). Further, the cytochromes were only 50% reduced with H₂ and this was explained as inactivation of the unreduced cytochrome. In contrast, the two major cytochrome peaks from *M. thermophila* were reduced fully with CO. As shown in Fig. 3 (dithionite-reduced) and Fig. 6 (CO-reduced), there is a prominent shoulder at 436 nm in the Soret band which is not apparent in the H₂-reduced membranes (Fig. 4). The physiological significance of this is unknown; however, it does imply that there is a heme center that can be reduced by CO but not H₂. This may account for the incomplete reduction of cytochromes reported in the purified complex from *M. barkeri*. It is possible that there is a "core" cytochrome complex that may be involved in proton translocation. Under growth on different substrates other proteins could be produced that interact with the complex and supply low potential electrons from various sources, depending on the growth substrate. If this were the case it could explain the

inconsistencies between the work on cytochromes from *M. thermophila* and *M. barkeri*.

Spectral analysis of CO-treated membranes suggested the presence of corrinoids associated with the membranes. Since membranes contain a low CODH activity, this could account for the corrinoid. The Ni-Fe-S component of CODH oxidizes CO and reduces the corrinoid of the Co-Fe-S component. However, a predominantly soluble location has been determined for the CODH complex (78). The presence of corrinoid could implicate a methyltransferase homologous to the corrinoid-containing methyltransferase isolated from *M. barkeri* (6) that acts as a primary Na⁺ pump. It is interesting that the cobalt atom of the corrinoid could be reduced with CO but not H₂. The corrinoid-containing methyl transferase from *M. barkeri* requires H₂ and ATP for reactivation by reduction of the cobalt atom to the Co⁺¹ redox state.

Early work showed that cytochromes were present only in methylotrophic methanogens, and cytochromes were present at higher levels when these organisms are grown on acetate (53). Electrons for reduction of CoM-S-S-HTP are derived from hydrogen or F₄₂₀H₂ when other methyl compounds or H₂ is supplied as the growth substrate. The unique aspect of electron transport during acetate metabolism is the source of electrons (61). This is consistent with a role for

cytochromes in electron transport from the carbonoyl moiety of acetate to CoM-S-S-HTP. The identification of a ferredoxin-dependent heterodisulfide reductase is critical for furthering the understanding of electron transport in acetate catabolism. The purification of cytochrome- containing H_2 :CoM-S-S-HTP oxidoreductase does not address this aspect of electron transport.

It is reported here that there is both a requirement for and concentration dependence of the ferredoxin in coupling CO oxidation to CoM-S-S-HTP reduction. Since H_2 may be involved as an intermediate in this reaction, our results are consistent with cytochromes participating in H_2 :CoM-S-S-HTP oxidoreduction. The work presented in this thesis does not prove cytochrome b involvement in electron transport during acetoclastic methanogenesis but is consistent with other work in support of this hypothesis. This and other work in the past several years has led to a revision in the proposed pathway for conversion of acetate to methane and carbon dioxide by *Methanosarcina* (Fig. 12)

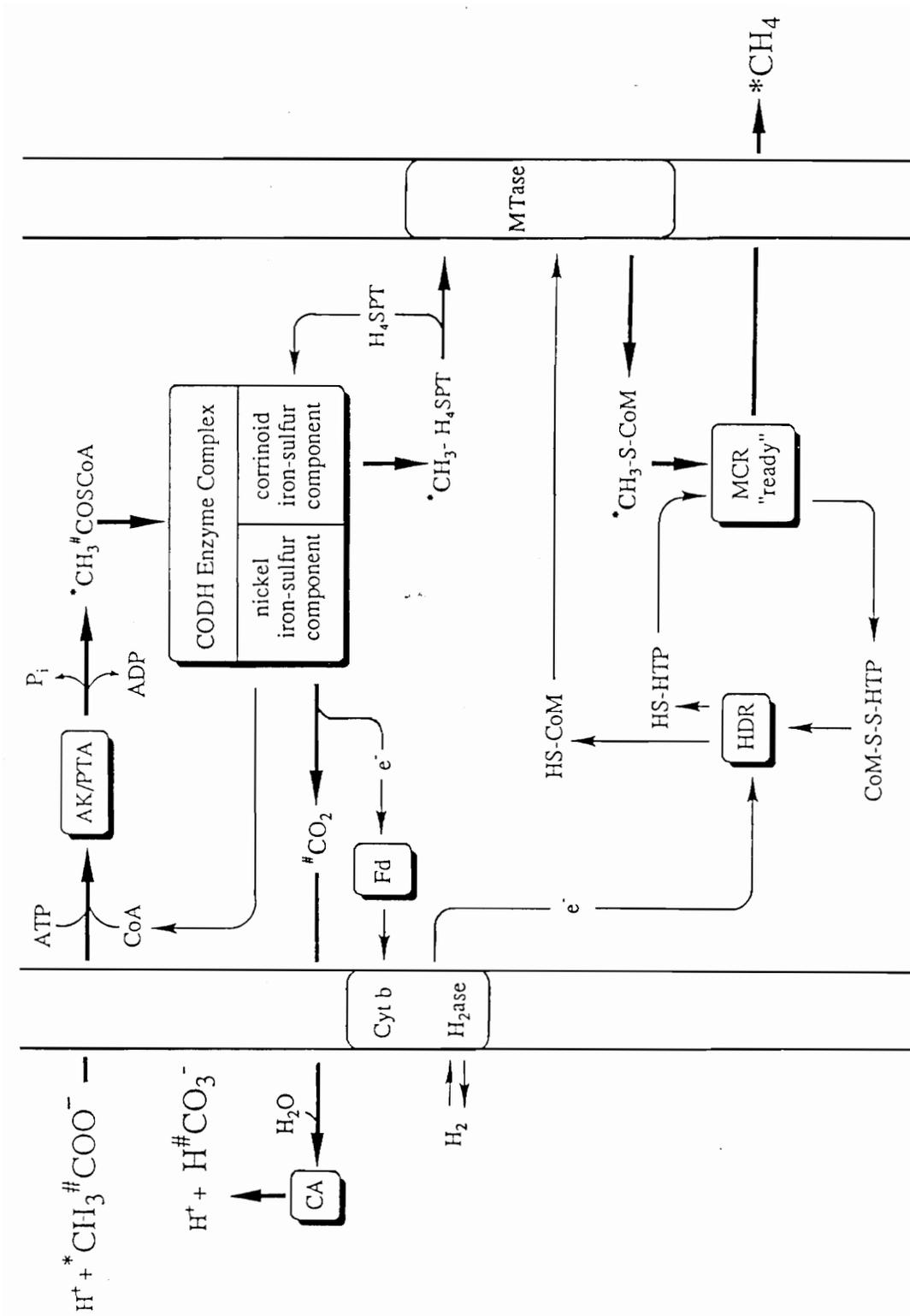


Figure 12. Proposed revision to the pathway for conversion of acetate to methane by *Methanosarcina*.

REFERENCES

1. **Abbanat, D. R., and J. G. Ferry.** 1991. Resolution of component proteins in an enzyme complex from *Methanosarcina thermophila* catalyzing the synthesis or cleavage of acetyl-CoA. Proc. Natl. Acad. Sci. U.S.A. 88:3272.
2. **Aceti, D. J., and J. G. Ferry.** 1988. Purification and characterization of acetate kinase from acetate-grown *Methanosarcina thermophila*. J. Biol. Chem. 263:15444.
3. **Ankel-Fuchs, D., R. Bocher, R. K. Thauer, K. M. Noll, and R. S. Wolfe.** 1987. 7-Mercaptoheptanoylthreonine phosphate functions as component B in ATP-independent methane formation from methyl-CoM with reduced cobalamin as electron donor. FEB. 213:1, 123-127.
4. **Barker, H. A.** 1936. On the biochemistry of the methane fermentation. Arch. Microbiol. 7:404-419
5. **Baron, S. F., and J. G. Ferry.** Purification and properties of the membrane-

associated coenzyme F₄₂₀-reducing hydrogenase from *Methanobacterium formicicum*. J. Bacteriol. 171:7, 3846-3853.

6. **Becker, B., V. Muller, and G. Gottschalk.** 1992. The methyl-tetrahydromethanopterin:coenzyme M methyltransferase of *Methanosarcina* strain Go1 is a primary sodium pump. FEMS Micro. Letters. 91:239-244.
7. **Becker, M., and G. Schafer.** 1991. Purification and spectral characterization of a b-type cytochrome from the plasma membrane of the archaebacterium *Sulfolobus acidocaldarius*. FEBS. 291:2, 331-335.
8. **Blaut, M., and G. Gottschalk.** 1984. Protonmotive force-driven synthesis of ATP during methane formation from molecular hydrogen and formaldehyde or carbon dioxide in *Methanosarcina barkeri*. FEMS Microbiol. Lett. 24:103-107.
9. **Blaut, M., V. Muller, and G. Gottschalk.** 1992. Energetics of methanogenesis studied in vesicular systems. Jour. Bioenergetics and Biomembranes. 24:6, 529.
10. **Bobik, T. A., and R. S. Wolfe.** 1988. Physiological importance of the

heterodisulfide of CoM and 7-mercaptoheptanoylthreonine phosphate in the reduction of carbon dioxide to methane in *Methanobacterium*. Proc. Natl. Acad. Sci. (USA) 85:60-63

11. **Bott, M., B. Eikmanns, and R. K. Thauer.** 1986. Coupling of carbon monoxide oxidation to CO₂ and H₂ with phosphorylation of ADP in acetate-grown *Methanosarcina barkeri*. Eur. J. Biochem. 159:393-398.
12. **Bott, M., and R. K. Thauer.** 1987. Proton-motive-force-driven formation of CO from CO₂ and H₂ in methanogenic bacteria. Eur. J. Biochem. 168:407-412.
13. **Bott, M., and R. K. Thauer.** 1989. Proton translocation coupled to the oxidation of carbon monoxide to CO₂ and H₂ in *Methanosarcina barkeri*. Eur. J. Biochem. 179:469-472.
14. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.
15. **Brummeler, E. T., L. W. Hulshoff Pol, J. Dolging, G. Lettinga, and A. J. B.**

- Zehnder. 1985. Methanogenesis in an upflow anaerobic sludge blanket reactor at pH 6 on an acetate-propionate mixture. *Appl. Environ. Microbiol.* 49:6, 1472-1477.
16. Bryant, M. P., E. A. Wolin, M. J. Wolin, and R. S. Wolfe. 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. *Arch. Microbiol.* 59:20-31.
17. Buswell, A. M., and F. W. Sollo. 1948. The mechanism of methane formation. *J. Am. Chem. Soc.* 70:1778
18. Clements, A. P., R. H. White, and J. G. Ferry. 1993. Structural characterization and physiological function of component B from *Methanosarcina thermophila*. *Arch. Microbiol.* 59:, in press.
19. Deppenmeier, U., M. Blaut, and G. Gottschalk. 1989. Dependence on membrane components of methanogenesis from methyl-CoM with formaldehyde or molecular hydrogen as electron donors. *Eur. J. Biochem.* 186:317-323.
20. Deppenmeier, U., M. Blaut, A. Mahlmann, and G. Gottschalk. 1990.

Membrane-bound $F_{420}H_2$ -dependent heterodisulfide reductase in methanogenic bacterium strain Go1 and *Methanobus tindarius*. FEBS. 261:1:, 199-203.

21. **Deppenmeier, U., M. Blaut, A. Mahlmann, and G. Gottschalk.** 1990. Reduced coenzyme F_{420} :heterodisulfide oxidoreductase, a proton-translocating redox system in methanogenic bacteria. Proc. Natl. Acad. Sci. (USA). 87:9449-9453.
22. **Deppenmeier, U., M. Blaut, and G. Gottschalk.** 1991. H_2 :heterodisulfide oxidoreductase, a second energy-conserving system in the methanogenic strain Go1. Arch. Microbiol. 155:272-277
23. **Ellman, G. L.** 1958. A colorimetric method for determining low concentrations of mercaptans. Arch. Biochem. Biophys. 74:443-450.
24. **Fathepure, B. Z., and S. A. Boyd.** 1988. Reductive dechlorination of perchloroethylene and the role of methanogens. FEMS Microbiol. Lett. 49:149-156.
25. **Ferry, J. G.** 1992. Biochemistry of methanogenesis. Crit. Rev. Biochem. Mol.

Biol. 27:6, 473-503.

26. **Ferry, J. G.** 1993. Fermentation of acetate. *In* J. G. Ferry (ed.), Methanogenesis. 1993. Chapman Hall, New York, N.Y.
27. **Fischer, R., and R. K. Thauer.** 1988. Methane formation from acetyl phosphate in cell extracts of *Methanosarcina barkeri*. FEB. 228:2, 249-253.
28. **Fischer, R., and R. K. Thauer.** 1990. Ferredoxin-dependent methane formation from acetate in cell extracts of *Methanosarcina barkeri* (strain MS). FEBS Lett. 269:368-372.
29. **Francis, R. T., and R. R. Becker.** 1984. Specific indication of hemoproteins in polyacrylamide gels using a double staining process. Analytical Biochem. 136:509-514.
30. **Gottschalk, G., and M. Blaut.** 1990. Generation of proton and sodium motive forces in methanogenic bacteria. Biochem. Biophys Acta. 1018:263-266.
31. **Grahame, D.** 1991. Catalysis of acetyl-CoA cleavage and

- tetrahydrosarcinapterin methylation by a carbon monoxide dehydrogenase-corrinoid enzyme complex. *J. Biol. Chem.* 266:33, 22227-22233.
32. **Gunsalus, R. P. and R. S. Wolfe.** 1980. Methyl coenzyme M reductase from *Methanobacterium thermoautotrophicum*: resolution and properties of the components. *J. Biol. Chem.* 255:1891-95.
33. **Hedderich, R., and R. K. Thauer.** 1988. *Methanobacterium thermoautotrophicum* contains a soluble enzyme system that specifically catalyzes the reduction of the heterodisulfide of coenzyme M and 7-mercaptoheptanoylthreonine phosphate with H₂. *FEBS Lett.* 234:1, 223-227.
34. **Heiden, S., R. Hedderich, E. Setzke, and R. K. Thauer.** 1993. Purification of a cytochrome b containing H₂:heterodisulfide oxidoreductase complex from membranes of *Methanosarcina barkeri*. *Eur. J. Biochem.* 213:529-535
35. **Hungate, R. E.** 1967. Hydrogen as an intermediate in the rumen fermentation. *Arch. Microbiol.* 59:158-164.
36. **Hungate, R. E.** 1969. A role tube method for cultivation of strict anaerobes. *In* J. R. Norris and D. W. Ribbons (eds.), *Methods in Microbiology*, vol. 3b.

Academic Press, Orlando, Fl.

37. **Hungate, R. E., W. Smith, T. Bauchop, and J. C. Rabinowitz.** 1970. Formate as an intermediate in the rumen fermentation. *J. Bacteriol.* 102:389-397.
38. **Jablonski, P. E., A. A. DiMarco, T. A. Bobik, M. A. Cabell, and J. G. Ferry.** 1990. Protein content and enzyme activities in methanol- and acetate-grown *Methanosarcina thermophila*. *J Bacteriol.* 172:3, 1271-1275.
39. **Jablonski, P. E., and J. G. Ferry.** 1992. Reductive dechlorination of trichloroethylene by the CO-reduced CO dehydrogenase complex from *Methanosarcina thermophila*. *FEMS Microbiol. Lett.* 96:55-60.
40. **Jablonski, P. E., W. P. Lu, S. W. Ragsdale, and J. G. Ferry.** 1992. Characterization of the metal centers of the corrinoid/iron-sulfur component of the CO dehydrogenase enzyme complex from *Methanosarcina thermophila* by EPR spectroscopy and spectroelectrochemistry. *J. Biol. Chem.* 268:325-329.
41. **Jones, W. J., D. P. Nagle, and W. B. Whitman.** 1987. Methanogens and the diversity of archaeobacteria. *Microbiol. Rev.* 51:1, 135-177.

42. **Jussofie, A., and G. Gottschalk.** 1986. Further studies on the distribution of cytochromes in methanogenic bacteria. *FEMS Microbiol. Lett.* 37:15-18.
43. **Kaesler, B., and P. Schonheit.** 1989. The role of sodium ions in methanogenesis. *Eur. J. Biochem.* 184:223-232.
44. **Kalmage, B., and M. Blaut.** 1992. Characterization of cytochromes from *Methanosarcina* Strain Go1 and their involvement in electron transport during growth on methanol. *J. Bacteriol.* 174:12, 3921-3927.
45. **Keltjens, J. T., and G. D. Vogels.** 1993. Conversion of methanol and methylamines to methane and carbon dioxide. *In* J. G. Ferry (ed.), *Methanogenesis*. 1993. Chapman Hall.
46. **Kemner, J. M., J. A. Krzycki, R. C. Prince, and J. G. Zeikus.** 1987. Spectroscopic and enzymatic evidence for membrane-bound electron carriers and hydrogenase and their relation to cytochrome b function in *Methanosarcina barkeri*. *FEMS Microbiol. Lett.* 48:267-272.
47. **Krone, U. E., R. K. Thauer, and H. P. C. Hogenkamp.** 1989. Reductive dechlorination of chlorinated C1-hydrocarbons mediated by corrinoids.

Biochemistry. 28:4908-4914.

48. **Krzycki, J. A., and J.G. Zeikus.** 1984. Characterization and purification of carbon monoxide dehydrogenase from *Methanosarcina barkeri*. *J. Bacteriol.* 158:231-237.
49. **Krzycki, J. A., L. J. Lehman, and J. G. Zeikus.** 1985. Acetate catabolism by *Methanosarci barkeri*: Evidence for involvement of carbon monoxide dehydrogenase, methyl coenzyme M, and methylreductase. *J. Bacteriol.* 163:2, 1000-1006.
50. **Krzycki, J. A., J. B. Morgan, R. Conrad, and J. G. Zeikus.** 1987. Hydrogen metabolism during methanogenesis from acetate by *Methanosarcina barkeri*. *FEMS Microbiol. Lett.* 40:193-198.
51. **Kuhn, W., K. Fiebig, B. Walther, and G. Gottschalk.** 1979. Presence of a cytochrome b_{559} in *Methanosarcina barkeri*. *FEBS Letters.* 105:2 271-274.
52. **Kuhn, W., K. Fiebig, H. Hippe, R. A. Mah, B. A. Huser, and G. Gottschalk.** 1983. Distribution of cytochromes in methanogenic bacteria. *FEMS Micro Letters.* 20:407-410.

53. Kuhn, W., and G. Gottschalk. 1983. Characterization of the cytochromes occurring in *Methanosarcina* species. Eur. J. Biochem. 135:89-94
54. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680-685.
55. Lancaster, J. R. 1989. Sodium, protons, and energy coupling in methanogenic bacteria. J. Bioenergetics Biomembranes. 21:6, 717.
56. Lovely, D. R., and J. G. Ferry. 1985. Production and consumption of H₂ during growth of *Methanosarcina* spp. on acetate. Appl. Envir. Micro. 49:1, 247-249
57. Lundie, L. L., and J. G. Ferry. 1989. Activation of acetate by *Methanosarcina thermophila*, purification and characterization of phosphotransacetylase. J. Biol. Chem. 264:31, 18392-18396.
58. Mackie, R. I., and M. P. Bryant. 1981. Metabolic activity of fatty acid-oxidizing bacteria and the contribution of acetate, propionate, butyrate, and CO₂ to methanogenesis in cattle waste at 40 and 60°C. Appl. Environ. Microbiol. 41:1363-1373.

59. **Martens, C. S., and R. A. Berner.** 1977. Interstitial water chemistry of anoxic Long Island Sound sediments. *Limnol. Oceanogr.* 22:10-25.
60. **Muller, V., C. Winner, and G. Gottschalk.** 1988. Electron-transport-driven sodium extrusion during methanogenesis from formaldehyde and molecular hydrogen by *Methanosarcina barkeri*. *Eur. J. Biochem.* 178:519-525.
61. **Muller, V., M. Blaut, and G. Gottschalk.** 1993. Bioenergetics of methanogenesis. In J. G. Ferry (ed.), *Methanogenesis*. 1993. Chapman-Hall, New York, N.Y.
62. **Nelson, M. K., and J. G. Ferry.** 1984. Carbon monoxide-dependent methyl coenzyme M methylreductase in *Methanosarcina* spp. *J. Bacteriol.* 160:2, 526-532.
63. **Noll, K. M., M. I. Donnelly, and R. S. Wolfe.** 1986. Synthesis of 7-mercaptoheptanoylthreonine phosphate and its activity in the methyl-CoM methylreductase system. *J. Biol. Chem.* 262:2, 513-515.
64. **O'Brien, J. M., R. H. Wolkin, T. T. Moench, J. B. Morgan, and J. G. Zeikus.** 1984. Association of hydrogen metabolism with unitrophic or mixotrophic

growth of *Methanosarcina barkeri* on carbon monoxide. J. Bacteriol. 158:1, 373-375.

65. **Peinemann, S., M. Blaut, and G. Gottschalk.** 1989. ATP synthesis coupled to methane formation from methyl-CoM and H₂ by vesicles of the methanogenic bacterial strain Go1. Eur. J. Biochem. 186:175-180.
66. **Peinemann, S., R. Hedderich, M. Blaut, R. K. Thauer, and G. Gottschalk.** 1990. ATP synthesis coupled to electron transport from H₂ to the heterodisulfide of 2-mercaptoethanesulfonate and 7-mercaptoheptanoylthreonine phosphate in vesicle preparations of the methanogenic bacterium strain Go1. FEBS Lett. 263:1, 57-60.
67. **Pine, M. J., and H. A. Barker.** 1955. Studies on the methane fermentation. XII. The pathway of hydrogen in the acetate fermentation. J. Bacteriol. 21:644-648.
68. **Rathje, W. L.** 1991. Once and future landfills. Nat. Geograph. 179:5, 116.
69. **Rouviere, P. E., C. H. Kuhner, and R. S. Wolfe.** 1990. Biochemistry of the methyl coenzyme M methylreductase system, p. 259-267. In J. P. Belaich

(ed.), *Microbiology and biochemistry of strict anaerobes involved in interspecies hydrogen transfer*. Plenum Press, New York.

70. **Reeburgh, W. S., and D. T. Heggie.** 1977. Microbial methane consumption reactions and their effect on methane distributions in freshwater and marine environments. *Limnol. Oceanogr.* 22:1-9.
71. **Schnellen, C. G. T. P.** 1946. *Onderzoekingen over der methaangisiting*. Ph.D. Thesis, Technical University of Delft, Rotterdam, The Netherlands.
72. **Smith, J. A.** 1987. Electrophoretic separation of proteins, p. 10.2.1-10.2.9. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, K. Struhl (eds.), *Current protocols in molecular biology*. 1987. Greene Publishing Assoc. New York.
73. **Smith, P. H., and R. A. Mah.** 1966 Kinetics of acetate metabolism during sludge digestion. *Appl. Microbiol.* 14:368-371.
74. **Sowers, K. R., M. J. Nelson, and J. G. Ferry.** 1984. Growth of acetotrophic, methane-producing bacteria in a pH auxostat. *Curr. Microbiol.* 11:227-230.

75. **Stadtman, T. C., and H. A. Barker.** 1948. Studies on the methane fermentation. VII. Tracer experiments on the mechanism of methane formation. *Arch. Biochem.* 21:256.
76. **Stryer, L.** 1988. *Biochemistry*, ch. 17,22. W. H. Freeman and Co. New York.
77. **Taylor, C. D. and R. S. Wolfe.** 1974. Structure and methylation of coenzyme M (HSCH₂CH₂SO₃). *J. Biol. Chem.* 249:4879-85.
78. **Terlesky, K. C., M. K. Nelson, and J. G. Ferry.** 1986. Isolation of an enzyme complex with carbon monoxide dehydrogenase activity containing corrinoid and nickel from acetate-grown *Methanosarcina thermophila*. *J. Bact.* 168:3, 1053-1058.
79. **Terlesky, K. C., M. J. Barber, D. J. Aceti, and J. G. Ferry.** 1987. EPR properties of the Ni-Fe-c center in an enzyme complex with carbon monoxide dehydrogenase activity from acetate-grown *Methanosarcina thermophila*, evidence that acetyl-CoA is a physiological substrate. *J. Biol. Chem.* 262:15392.
80. **Terlesky, K. C., and J. G. Ferry.** 1988. Ferredoxin requirement for electron

transport from carbon monoxide dehydrogenase complex to a membrane-bound hydrogenase in acetate-grown *Methanosarcina thermophila*. J. Biol. Chem. 263:9, 4075-4079.

81. **Terlesky, K. C., and J. G. Ferry.** 1988. Purification and characterization of a ferredoxin from acetate-grown *Methanosarcina thermophila*. J. Biol. Chem. 263:9, 4080-4082.

82. **Wakefield, L. M., A. E.G . Cass, and G. K. Radda.** 1984. Isolation of a membrane protein by chromatofocusing: Cytochrome b₅₆₁ of the adrenal chromaffin granule. J. Biochem. Biophys. Meth. 9:331-341

83. **Whitman, W. B.** 1985. Methanogenic bacteria, p. 3-84. In C. R. Woese, R. S. Wolfe (eds.), The Bacteria, A Treatise on Structure and Function, Vol VIII. 1985. Academic Press, Inc. Orlando, Fl.

84. **Widdel, F., P. Rouviere, and R. S. Wolfe.** 1988. Classification of secondary alcohol-utilizing methanogens including a new thermophilic isolate. Arch. Microbiol. 150:477.

85. **Winfrey, M. R., and J. G. Zeikus.** 1979. Anaerobic metabolism of immediate methane precursors in Lake Mendota. *Appl. Environ. Microbiol.* 37:244-253
86. **Woese, C. R., O. Kandler, and M. L. Wheelis.** 1990. Toward a natural system of organisms. Proposal for the domains archaea, bacteria, and eucarya. *Proc. Natl. Acad. Sci. U.S.A.* 87:4576.
87. **Wolfe, R. S.** 1991. My kind of biology. *Annu. Rev. Microbiol.* 45:1-35.
88. **Wolin, E. A., M. J. Wolin, and R. S. Wolfe.** 1963. Formation of methane by bacterial extracts. *J. Biol. Chem.* 38:996-1008.
89. **Zinder, S. H., and A. F. Elias.** 1985. Growth substrate effects on acetate and methanol catabolism in *Methanosarcina* sp. strain TM-1. *J. Bacteriol.* 163:1, 317-323.

CURRICULUM VITA

Christopher William Peer

Department of Biochemistry and Anaerobic Microbiology
Virginia Tech
Blacksburg, VA 24061

EDUCATION:

- 1991-1993: Virginia Tech. Master of Science, Department of Biochemistry and Anaerobic Microbiology.
Thesis: Studies on Cytochromes and Electron Transport in *Methanosarcina thermophila* TM-1.
- 1987-1991: Virginia Tech. B.S. Biology, Minor in Chemistry
- 1986-1987: Castleton State College, Castleton, VT. Mathematics major.
- 1986: Morris Knolls H.S. Denville, NJ. Diploma

WORK EXPERIENCE:

Research Assistant (June 1991 to present) Master's Degree candidate in the Dept. of Anaerobic Microbiology, Virginia Tech.

Graduate Teaching Assistant (Spring '92)

Taught a senior-level Pathogenic Bacteriology Lab under the direction of Dr. Noel Krieg. Responsibilities included: preparing lectures, modifying and preparing lab experiments, writing tests and quizzes, and assigning grades for 25 students.

Lane Business Systems (Summers and School breaks 1982 to 1991)

Computer Programming, Systems Operations for the Lane 2100 and Lane 115 series Micro Bond Traders, Lane Business Systems.

Computer Assembly and Repair, Lane Business Systems

Camelback Ski Area (1985 to 1986) Junior Ranger Patrol

Work cited in:

Structure and Physiological Function of Component B from *Methanosarcina thermophila*. AP Clements, RH White, JG Ferry. Archives of Microbiology, 159:3 (Mar 1993) 296-300

Fermentation of Acetate. J.G. Ferry in Methanogenesis. Ed. J.G. Ferry. Chapman-Hall, 1993.

In preparation:

Identification of a Ferredoxin Dependent Heterodisulfide Reductase and Reconstitution of a CO-dependent Electron Transport System from Acetate Grown *Methanosarcina thermophila*.

CW Peer, ME Rasche, JG Ferry

PROFESSIONAL AFFILIATIONS:

American Association for the Advancement of Science

The American Society for Microbiology

Virginia Branch ASM

American Institute of Biological Sciences

ACADEMIC HONORS/AWARDS:

Phi Kappa Phi National Honor Society

Phi Sigma National Biological Honor Society

Gamma Beta Phi National Honor and Service Society

Recipient of three one-semester tuition waivers, Department of Anaerobic Microbiology based on Research and Academic achievement

Nominee- GM Volunteer Spirit Award

PERSONAL:

Date of Birth: 8/27/68 (Denville, NJ)

EXTRACURRICULAR ACTIVITIES:

Certified Emergency Medical Technician
Volunteer- Montgomery Regional Hospital
Volunteer- New River Valley Free Medical Clinic
Project Coordinator- Humane Society of Montgomery County
SGA Senator for the College of Agriculture and Life Sciences
Chapter Advisor, Member- Alpha Tau Omega Fraternity
Virginia Tech Rugby Football Club
Virginia Tech Isshinryu Karate Club
Castleton State College Ski Team



Christopher W. Peer