1

PM-1 3 1/2" x 4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGET/S

PIONEERS IN METHYLENE BLUE TESTING SINCE 1974

1000 COUNTY ROAD A, BURNING WOOD, OH 44027 USA
TEL: 812 486 7887 FAX: 812 486 7887 TLA: 2100007400

Acquisitions and
Bibliographic Services Branch

Direction des acquisitions et
des services bibliographiques

395 Wellington Street
Ottawa, Ontario
K1A 0N4

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file / Votre référence

Our file / Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

**THE MOLECULAR BIOLOGY OF METHANOGENS: CELL
LYSIS, PLASMID SURVEY AND THE
CHARACTERIZATION OF A NOVEL PLASMID**

by

STEPHANIE ASALYN MEAKIN

A thesis submitted to
the faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of
Master of Science (Biology)

Department of Biology

Carleton University

Ottawa, Ontario

May, 1992

copyright

1992, Stephanie A. Meakin

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file / Votre référence

Our file / Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-79840-8

Canada

Carleton University
Ottawa, Canada K1S 5B6

Thesis contains black & white
photographs &/or explanatory tables
which when microfilmed may lose their
significance. The hardcopy of the
thesis is available upon request from
Carleton University Library.

The undersigned hereby recommend to
the faculty of Graduate Studies and Research
acceptance of this thesis,

**THE MOLECULAR BIOLOGY OF METHANOGENS: CELL
LYSIS, PLASMID SURVEY AND THE
CHARACTERIZATION OF A NOVEL PLASMID**

submitted by

Stephanie A. Meakin, B.A. (Honours) Biology

in partial fulfilment of the requirements

for the degree of Master of Science.

A handwritten signature in dark ink, appearing to be 'D. Gardner', written over a horizontal line.

Chair, Department of Biology

D. Gardner, Ph.D.

A handwritten signature in dark ink, appearing to be 'R.C. Wyndham', written over a horizontal line.

Thesis Supervisors

Campbell Wyndham, Ph.D.

G. Denis Sprott, Ph.D.

Carleton University

June 8, 1992

ABSTRACT

Methanogens form a diverse group of organisms, of interest because of their physiology, morphology and taxonomy. They play an essential role in the environment in the global recycling of methane and are responsible for anaerobic degradation of natural as well as xenobiotic compounds. This thesis outlines the taxonomy, biochemistry and molecular biology of this group of organisms and presents a generally applicable method of lysis for methanogens, the screening of a number of species for plasmids and the characterization of a novel plasmid pDDK from *Methanosaeata concilli* GP6. pDDK was found to be a circular molecule with a molecular weight of 11.0 kb, 10 copies per cell, a restriction map is also presented. This type of work is important in the development of genetic tools which may unravel many of the questions that have arisen from previous investigations and as well may lead to the use of these organisms in the field of genetic engineering.

ACKNOWLEDGEMENTS

There are many people who made this thesis possible, some gave invaluable financial, scientific and technical assistance while others maintained and assisted my soul. I would like to take this opportunity to thank these people. First, Dr. Kevin Kennedy who allowed me to use time at work to pursue this degree and without such a situation this would not have been possible for me. My committee: Dr. Dennis Sprott my co-supervisor, who always made time, often walking me through a section or problem. My co-supervisor Dr. Campbell Wyndham who took a chance on me and Dr. V.N. Iyer, three respected scientists in their fields and all, helpful, fair professionals. Dr. John Nash who gave expert advise and scientific assistance and taught me much of what I know about molecular biology. Dr. William Murray a good friend and a respected scientist, my role model. The National Research Council where this research was carried out. Kathleen Shaw who maintained many of the cultures used (and me) and Dr. Girish Patel who supplied GP6 and much advise and support. On the emotional front Brad Methven, Brian Agnew, Christian Choquet, Margaret Anne Gidney and the coffee table gang and all my friends whom I'm sure are tired of 'sounds great but I'm working this weekend'. A group of people I have left until last because without them nothing in my life would be possible, my family. This thesis is dedicated to them, my husband David D. Kerr whom the plasmid pDDK is named after, my mom and dad Asalyn and Donald Meakin and my brother Christopher Meakin.

TABLE OF CONTENTS

TITLE PAGE	i
ACCEPTANCE SHEET	ii
ABSTRACT	iii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	v
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF PHOTOGRAPHIC PLATES	viii
TABLE OF ABBREVIATIONS	ix

SECTION I

LITERATURE REVIEW

1.1	ARCHAEOBACTERIA	1
1.2	METHANOGENIC ARCHAEOBACTERIA	6
1.3	TAXONOMY	7
1.4	DIVERSITY OF METHANOGENS	9
1.5	METHANOBACTERIALES	12
1.6	METHANOCOCCALES	14
1.7	METHANOMICROBIALES	14
1.8	ENERGY METABOLISM-METHANOGENESIS	17
1.9	UNIQUE COFACTORS	19

1.10 METHANOGENIC PATHWAYS	21
1.11 CELL WALL STRUCTURE	27
1.12 LIPID COMPOSITION	35
1.13 MOLECULAR BIOLOGY	39
1.14 INTRODUCTION TO PLASMIDS	45
1.15 PLASMID ISOLATION	46
1.16 PLASMID SIZING	49
1.17 RESTRICTION MAPPING	50
1.18 PLASMID COPY NUMBER	50
1.19 METHANOGEN PLASMIDS	52

SECTION II

METHODS AND MATERIALS

2.1 ANAEROBIC CELL CULTURE AND GROWTH CONDITIONS	54
2.2 CELL LYSIS	58
2.3 PLASMID SURVEY	62
2.4 PLASMID ISOLATION	63
2.5 PLASMID pDDK CHARACTERIZATION	64

SECTION III

RESULTS AND DISCUSSION

3.1	CELL LYSIS	66
3.2	SUSPECTED METHANOGEN PLASMIDS AND A NOVEL METHANOGEN PLASMID	76
3.3	ISOLATION AND PURIFICATION OF pDDK	76
3.4	pDDK CHARACTERIZATION	82

SECTION IV

4.1	CONCLUSIONS AND FUTURE PROSPECTS	92
-----	----------------------------------	----

APPENDICES

APPENDIX I	SOUTHERN BLOT PROTOCOL	95
APPENDIX II	HYBRIDIZATION WITH DIGOXIGENIN -LABELED PROBES	96

REFERENCES	101
-------------------	------------

LIST OF TABLES

TABLE	DESCRIPTION	PAGE
TABLE 1	ABBREVIATIONS	xi
TABLE 2	TAXONOMY OF METHANOGENIC BACTERIA	7
TABLE 3	SUMMARY CHARACTERISTICS OF METHANOGENIC BACTERIA	10
TABLE 4	DEGREE OF LYSIS OF FROZEN-THAWED METHANOGEN CELLS	68
TABLE 5	FRAGMENT SIZES USED TO CALCULATE RESTRICTION MAP	83

LIST OF FIGURES

FIGURE	DESCRIPTION	PAGE
FIGURE I	THE PRIMARY KINGDOMS	4
FIGURE II	PATHWAY FOR METHANE FORMATION	23
FIGURE III	THE STRUCTURE OF PSEUDOMUREIN	33
FIGURE IV	MAJOR LIPIDS OF ARCHAEOBACTERIA	37

LIST OF PHOTOGRAPHIC PLATES

PLATE	DESCRIPTION	PAGE
PLATE 1	ANAEROBIC MEDIA APPARATUS	56
PLATE 2	LYSIS OF METHANOGEN CELLS FRESH VS. FROZEN	69
PLATE 3	PRONASE-INDUCED LYSIS (%)	71
PLATE 4	GEL ELECTROPHORESIS OF EXTRACTED DNA FROM METHANOGENS	73
PLATE 5	TOTAL CHROMOSOMAL ELECTROPHORESIS OF GP6	78
PLATE 6	ELECTROPHORESIS OF pDDK	80
PLATE 7	RESTRICTION FRAGMENT ANALYSIS OF pDDK SINGLE AND DOUBLE DIGESTS	84
PLATE 8 i.	AUTORADIOGRAM OF PROBED (<u>Bgl</u> II) SOUTHERN BLOT	86
PLATE 8 ii.	AUTORADIOGRAM OF PROBED (<u>Eco</u> RI) SOUTHERN BLOT	88
PLATE 9	RESTRICTION FRAGMENT MAP	90

TABLE OF ABBREVIATIONS

SYMBOL	DESCRIPTION
tRNA	transfer RNA
rRNA	ribosomal RNA
CsCl	cesium chloride
EtBr	ethidium bromide
CCC	covalently closed circular
ABE BUFFER	ammonium bicarbonate, EDTA
EDTA	ethylenediaminetetraacetic acid disodium salt
DTT	dithiothreitol
SDS	sodium dodecyl sulphate

SECTION I

LITERATURE REVIEW

1.1 ARCHAEOBACTERIA

Our basic concept of life for many years divided cells into two groups: eukaryotes, cells with a well defined nucleus and prokaryotes (eubacteria), which do not have such a nucleus. This held true until in the late 1970's a long known group of organisms were intensively studied and did not seem to belong to either of these basic categories (Woese, 1987; Woese and Wolfe, 1985; Kandler, 1982, Kandler and Zillig, 1986; Woese and Olsen, 1986; Fox, 1980). These organisms are prokaryotic in nature as they do not have a nucleus and morphologically resemble eubacteria. They also have many biochemical features and structural similarities in certain large molecules which structurally resemble eukaryotes. They are as different from prokaryotes as they are from eukaryotes. It was therefore decided to place these organisms in a third lineage, Archaeobacteria (Fig.I). The archaeobacteria (archaeobacteria) were defined as a separate "urkingdom" among the eukaryotes and eubacteria (König, 1988). Recently, Woese et al. (1990) have proposed that these organisms be given domain status as the Archaea, distinct from the Domains of Bacteria and Eucaryota (Fig.I.i).

The Archaeobacterial phylogeny, based primarily on homologies of partial and complete sequences of the 16S ribosomal ribonucleic acid (rRNA), and supported by a host of morphological and biochemical data, appeared as a distinct group. The

prokaryotes were seen to be comprised of two distinct groups: eubacteria, which consist of the traditional bacterial groups including the photosynthetic bacteria, blue-green or cyano-bacteria, actinomycetes and spirochetes; and the archaeobacteria. The archaeobacteria consists of two main phylogenetic branches; the first includes the methanogenic bacteria, the extremely halophilic bacteria, *Thermoplasma spp.*, and the extremely thermophilic archaeobacteria, thermoacidophiles (Jones, 1987); and the second branch contains the sulphur reducing bacteria *Sulfolobus* and the thermophiles, *Thermoproteus*, *Pyrodictium*, *Desulfurococcus*. Methanogen phylogeny is not as compact as the second group as they show much more phenotypic diversity. The archaeobacteria share a number of features with both the eubacteria and the eukaryotes, but they also possess some unique and diverse characteristics (Konig, 1988) with respect to their morphology, nutritional requirements, cell wall composition, and optimal growth temperatures.

Archaeobacteria are terrestrial and aquatic microbes, occurring in anaerobic, hypersaline, hydrothermal and geothermal environments as well as in the digestive tracts of animals and man. They consist of aerobes, anaerobes and facultative aerobes. Archaeobacteria range from mesophilic to thermophilic environments. They have unique biochemical properties including the presence of ether-linked phytanyl lipids, the absence of murein in the cell wall, unique transfer RNA (tRNA) and ribosomal RNA (rRNA) chemical composition and sequence (Jones et al., 1987; Brown et al., 1989).

This thesis will specifically examine the phylogeny, biochemistry and molecular biology of the group of strict anaerobic archaeobacteria called methanogens. It will

address applied problems of cell lysis and DNA purification as well as introduce a novel plasmid. The reason for carrying out this research lies in the unique properties of these organisms; membrane and cell wall components, biochemistry, methane production and their role in the anaerobic decomposition of organic matter. The study of methanogens and the potential genetic engineering of these organisms has importance in the understanding of methane production and cycling, as well the biological remediation of xenobiotic and toxic compounds. Methanogens also promise to be rewarding in other areas of interest such as applications in the pharmaceutical and cosmetic industries (Sprott et al., 1992).

Methane biogenesis is an important process in the global cycling of nutrients and the methanogens inhabit environments where other organisms may be absent due to the extreme anaerobic conditions. In these environments where sulfate and nitrate are absent, carbon dioxide is the terminal electron acceptor in the decomposition of organic matter (Whitman, 1985). Methanogens are also microorganisms of industrial importance. They catalyze the final steps in the anaerobic biodegradation of organic compounds to methane, the biotechnological process used worldwide to detoxify and dispose of waste material and to convert biomass into a convenient, combustible fuel (Reeve, 1988). As well the potential of thermostable enzymes and biocatalyst sources from the extremely thermophilic species make these organisms industrially important. Therefore methanogens are industrially, biochemically and environmentally important, which not only legitimizes this work but also indicates the need for more intensive basic research.

FIGURE 1. THE PRIMARY KINGDOMS

The figure shown here represents an unrooted phylogenetic tree for the three kingdoms. This distance matrix tree is calculated from the aligned sequences of homology between known sequences of 16s rRNA/18s rRNA (Woese and Olsen, 1986). The distance measure bar corresponds to 0.1 mutational events per sequence position.

FIGURE 1 i. UNROOTED PHYLOGENETIC TREE FOR THE THREE PROPOSED DOMAINS

The tree shown here represents the three proposed domain designations of Archaea, Eucarya and Bacteria, branch order and branch length are based upon rRNA sequence comparisons. The numbers on the branch tips correspond to the following groups of organisms, Bacteria: 1) the Thermotogales, 2) the flavobacteria and relatives, 3) the cyanobacteria, 4) the purple bacteria, 5) the Gram+ bacteria, and 6) the green non-sulfur bacteria, Archaea the kingdom Crenarchaeota: 7) the genus *Pyrodictium* and 8) the genus *Thermoproteus*; the kingdom Euryarchaeota: 9) the Thermococcales, 10) the Methanococcales, 11) the Methanobacteriales, 12) the Methanomicrobiales, and 13) the extreme Halophiles, Eucarya: 14) the animals, 15) the ciliates, 16) the green plants, 17) the fungi, 18) the flagellates, and 19) the microsporidia (Woese et al., 1990).

I.

II.

1.2 METHANOGENIC ARCHAEOBACTERIA

Methanogens differ in many respects from other prokaryotes. They are unique with regard to their autotrophic metabolism, utilizing CO_2 and H_2 as their main carbon and energy sources. Some species also use other C_1 or C_2 compounds such as formate, acetate, methanol, and methylamines as electron donors. They contain unique cofactors coenzyme M, methanofuran, methanopterin, component B and F_{420} mediating the methyl transfer and the primary reduction process of the reduction of CO_2 to methane and are obligately anaerobic. Their 16 S rRNA shows very little similarity to that of other prokaryotes. The occurrence of unique tRNA and 5 S rRNA in the translation mechanisms are a few of the variations. Methanogens contain a diverse array of morphological forms such as long rods, lancet shaped rods, cocci, planar cells, and cuboid packets (Konig, 1988). They are widely distributed in anoxic environments such as fresh water sediments, marine sediments, sewage sludge, soil, geothermal heated areas and the intestinal tracts of human beings and animals. Methanogenic bacteria are phylogenetically as distant from other bacteria and cyanobacteria as they are from eukaryotic organisms.

1.3 TAXONOMY

In 1979, the taxonomy of the methanogenic bacteria was reorganized to reflect the phylogenetic relationships determined by 16 S rRNA sequence analysis (Balch et al., 1979). Methanogens are comprised of three major taxa no closer to one another than to the group of extreme halophiles; however, their exact branching order could not be settled by the cataloging approach or by 5 S rRNA sequence analysis. Therefore it was proposed that the methanogens form a deeply branched single phylogenetic unit that exclude the halophiles (Woese and Olsen, 1986) (Fig.I). The group contains three orders, six families, and eleven genera (Table II).

TABLE II TAXONOMY OF METHANOGENIC BACTERIA

(Whitman, 1985; Konig, 1988; OCM, 1990)

	Mol % G+C
Order I. Methanobacteriales	
Family I. Methanobacteriaceae	
Genus I. <i>Methanobacterium</i>	32-50
Genus II. <i>Methanobrevibacter</i>	27-32
Genus III. <i>Methanosphaera</i>	26
Family II. Methanothermaceae	
Genus I. <i>Methanothermus</i>	33
Order II. Methanococcales	

Family I. Methanococcaceae	
Genus I. <i>Methanococcus</i>	31-40
Order III. Methanomicrobiales	
Family I. Methanomicrobiaceae	
Genus I. <i>Methanomicrobium</i>	45-49
Genus II. <i>Methanogenium</i>	51-61
Genus III. <i>Methanospirillum</i>	46-50
Genus IV. <i>Methanoculleus</i>	54-61
Family II. Methanosarcinaceae	
Genus I. <i>Methanosarcina</i>	41-43
Genus II. <i>Methanothrix (Methanosaeta)</i>	52
Genus III. <i>Methanolobus</i>	40
Genus IV. <i>Methanococcoides</i>	42
Genus V. <i>Methanohalophilus</i>	38-41
Genus VI. <i>Methanohalobium</i>	
Family III. Methanoplanaceae	
Genus I. <i>Methanoplanus</i>	39-48
Family IV. Methanocorpusculaceae	
Genus I. <i>Methanocorpusculum</i>	48

These determinations were based on 16 S rRNA homologies and are consistent with a considerable amount of descriptive data including cell wall structure, morphology,

lipid structure, mol % G+C, and nutrition. As new isolates are being discovered which appear to represent new genera, this phylogeny is known to be incomplete.

1.4 DIVERSITY OF METHANOGENS

Methanogens are obligate anaerobes which share the common property of producing chemolithotrophically or heterotrophically methane as a metabolic product (Jones, 1985; Konig, 1987). Although metabolically restricted, the methanogens exhibit extreme habitat diversity; species have been isolated from virtually every habitat in which anaerobic biodegradation of organic compounds occurs, including freshwater and marine sediments, digestive and intestinal tracts of animals, and anaerobic waste digesters (Jones et al., 1987). Additional isolates have been obtained from extreme environments such as geothermal springs and both shallow and deep-sea hydrothermal vents. Physiologically methanogens contain representatives of extremely thermophilic, moderately thermophilic and mesophilic species (Table III). Although among methanogens there are heterotrophic and autotrophic representatives all presently appear to be organotrophic. They have been found to consist of neutrophiles, alkalophiles and acidophiles (implied by biogenesis of CH_4 in peat samples of pH 2.5, Williams and Crawford, 1985). There have however been no barophiles isolated yet.

The diversity of methanogens is also apparent in their morphology, most morphological types are represented including cocci, groups of cocci (pseudosarcina), rods of various lengths and configurations, and spirillum (Table III).

**TABLE III SUMMARY CHARACTERISTICS OF METHANOGENIC
ARCHAEOBACTERIA** (modified from Jones et al., 1987)

METHANOGEN	MORPHOLOGY	SUBSTRATES	G+C	TEMP	pH	WALL
				OPT.		OPT.
ORDER						
<i>A. Methanobacteriales</i>						
FAMILY						
<i>I. Methanobacteriaceae</i>						
GENUS						
<i>i. Methanobacterium</i>						
<i>M. formicicum</i>	Rod	H ₂ , formate	40.7	37	7.0	pseudomurein
<i>M. bryantii</i>	Rod	H ₂	32.7	38	7.0	pseudomurein
<i>M. thermoautotrophicum</i>	Rod	H ₂	49.7	65-70	7.2-7.6	
						pseudomurein
<i>M. espanolae</i>	Rod	H ₂	34	35	5.6-6.2	
						pseudomurein
<i>ii. Methanobrevibacter</i>						
<i>M. smithii</i>	Rod	H ₂ , formate	31	38	6.9-7.4	

						pseudomurein
<i>M. arbophilicus</i>	Rod	H ₂	29	30-37	7.5-8.0	
						pseudomurein
II. <i>Methanothermaceae</i>						
i. <i>Methanothermus</i>						
<i>M. fervidus</i>	Rod	H ₂	33	83	6.5	pseudomurein, S-layer, protein

ORDER

B. *Methanococcales*

FAMILY

I. *Methanococcaceae*

GENUS

i. *Methanococcus*

<i>M. voltae</i>	Coccus	H ₂ , formate	29.6	32-40	6.7-7.4	protein S-layer
<i>M. jannaschii</i>	Coccus	H ₂	31	85	6.0	protein S-layer

ORDER

C. *Methanomicrobiales*

FAMILY

I. *Methanomicrobiaceae*

GENUS

i. *Methanospirillum*

<i>M. hungatei</i>	Spirillum	H ₂ , formate	45	30-37	6.6-7.4	protein sheath, S-layer
--------------------	-----------	--------------------------	----	-------	---------	-------------------------

II. *Methanosarcinaceae*i. *Methanosarcina*

<i>M. mazei</i>	Coccus	H ₂ , Me, Ac, MeNH ₂	42	40	6.0-7.0	HPS
-----------------	--------	---	----	----	---------	-----

<i>M. barkeri</i>	Coccus, packets ¹	H ₂ , Me, Ac, MeNH ₂	39	35	7.0	HPS, protein
<i>M. acetivorans</i>	Coccus	Me, Ac, MeNH ₂	42	40	6.5-7.0	protein
ii. <i>Methanosaeta</i>						
<i>M. concilli</i>	Rod	Ac	49	35-40	7.1-7.5	protein sheath

note: this table comprises species which were used in this thesis, this table is therefore not a complete listing of all genera.

Abbreviations: Me, methanol; MeNH₂, methylamines; Ac, acetate; HPS, heteropolysaccharide.

¹ growth as cocci or packets depends on growth phase i.e. whether grown on liquid vs. solid media.

1.5 METHANOBACTERIALES

The order Methanobacteriales contains two families, Methanobacteriaceae and Methanothermaceae with thirteen species found in two genera (Table II). All methanogens containing pseudomurein in their cell walls are in this group. They stain Gram+, with diverse nonmotile morphologies, rods, lancet shape and cocci. Cells occur

singly, in chains, or in packets. cell division is by binary fission. In all members tested C_{20} and C_{40} isopranyl glycerol ethers are abundant. They are mesophilic to thermophilic.

Members of the family *Methanobacteriaceae* are short and long, rod shaped organisms. The temperature growth optimum is between 37-70°C. Presently the energy sources known for these organisms is H_2 or formate and the cell walls do not contain an S layer. Three genera are in the family, *Methanobrevibacter*, *Methanobacterium* and *Methanosphaera*. The first occurring as short lancet shaped rods which often form pairs or chains. All members described are mesophilic and grow at neutral pH. DNA base composition is 27-32 mol% G+C. *Methanobacterium* cells are straight, long, sometimes irregular rods. DNA base composition is 50 mol% G+C. The cells are nonmotile and found mesophilic to thermophilic. *Methanosphaera* are coccoid cells found singly or in packets depending on the growth phase. Carbon sources include H_2 , CO_2 , and acetate. The DNA base composition is 26 mol% G+C.

The family Methanothermaceae contains one genus *Methanothermus*. Cells occur as long rods growing optimally above 80°C. The cell wall is composed of a pseudomurein layer and a glycoprotein S-layer. The DNA base composition is 33 mol% G+C. The cells occur only in extremely thermophilic environments. Hydrogen and CO_2 are growth substrates.

1.6 METHANOCOCCALES

The order Methanococcales contains one family, Methanococcaceae and one genus *Methanococcus*. This genus contains eight species all having a morphology of irregular cocci, which stain Gram-. Cell walls are composed primarily of non-glycosylated proteinaceous compounds which allow these organisms to lyse easily in weak detergents. The isopranyl glycerol ether C₂₀ (except for one species) is abundant but the C₄₀ isopranyl ether is usually absent. DNA base composition ranges from 31-40 mol% G+C. All cells are motile with the exception of *Methanococcus deltae*. All the isolates of this group are from coastal and marine environments, they are mesophilic to extremely thermophilic. The requirement for NaCl varies greatly among the species. Among the members, most use H₂ and formate as electron donors and can grow autotrophically (Whitman, 1985). As well most have a growth requirement for selenium.

1.7 METHANOMICROBIALES

The order Methanomicrobiales contains four families, Methanomicrobiaceae, Methanosarcinaceae, Methanoplanaceae and Methanocorpusculaceae and twelve genera.

The first family, Methanomicrobiaceae contains four genera *Methanomicrobium*, *Methanogenium*, *Methanospirillum* and *Methanoculleus* which are restricted to H₂ and formate as electron donors. All morphologies are represented in this order rods,

spirillum, and irregular cocci. Cells stain Gram- and Gram+. The cell walls are proteinaceous, with the lipid content of the only one investigated found to contain both C₂₀ and C₄₀ isopranyl glycerol ethers (Kushwaha et al., 1981). All members use both H₂ and formate as electron donors, H₂/CO₂, acetate, methanol or methylamines can also serve as carbon sources. Isolates have been found in marine and freshwater sediments. The mol% G+C ranges from 45.0-61.2. *Methanomicrobium* are short rods with a subunit cell envelope. Acetate is required for growth and they have complex growth requirements. The DNA base composition is 45-49 mol% G+C. *Methanogenium* occur as highly irregular cocci. Substrates are H₂/CO₂ and formate, most strains requiring acetate as a carbon source. The DNA base composition is 51-61 mol% G+C. *Methanospirillum* are rods which form long filaments. Cells can be surrounded by a SDS-resistant protein sheath, and single cells are septated by spacers. C₂₀ isopranyl diether and C₄₀ isopranyl tetraethers are present. The DNA base composition is 50 mol% G+C.

The family Methanosarcinaceae, contains six genera *Methanosarcina*, *Methanolobus*, *Methanococcoides*, *Methanotherix* (*Methanosaeta*), *Methanohalophilus* and *Methanohalobium*. Cells are coccoid occurring singly or in clumps. Cells stain Gram+ or Gram-. Methanol or methylamines serve as carbon and energy sources. Some species also use H₂/CO₂ or acetate. The genus *Methanosarcina* can grow on acetate, methylamines, methanol and most species on H₂/CO₂; formate is not used. Most species stain Gram+ and contain heteropolysaccharide cell walls. DNA base composition is 40-43 mol%G+C. *Methanolobus* are obligate methylotrophs, cells can only use methanol

and methylamines as energy and carbon sources. The DNA base composition is 40 mol% G+C. *Methanococcoides* share 2-4% DNA homology with *Methanlobus* and share the above description. C₂₀ isopranyl diethers only are found in the cytoplasmic membrane. The DNA base composition is 42 mol% G+C. *Methanothrix* (*Methanosaeta*) cells are thick Gram- rods which form long filaments. Several cells can be surrounded by a SDS-resistant protein sheath. Acetate serves as the sole substrate. Formate is oxidized. C₂₀ isopranyl diethers and hydroxydiether lipids were found in the cell envelope. The DNA base composition is 49-52 mol% G+C.

The family Methanoplanaceae contains one genus *Methanoplanus*, which occur as sharp sided plate-shaped cells which stain Gram-. They possess a glycoprotein S-layer. Acetate is required as a carbon source for growth. H₂ and formate serve as electron donors. The DNA base composition is 48 mol% G+C.

The family Methanocorpusculaceae also contains only one genus *Methanocorpusculum*, which occur as small mesophilic cocci (< 1 µm). They stain Gram-. H₂/CO₂, CO₂, formate and 2-propanol/CO₂ serve as substrates. Acetate, yeast extract and tungstate are required for growth. The DNA base composition is 48 mol% G+C.

1.8 ENERGY METABOLISM: METHANOGENESIS

A brief description of energy metabolism and assimilatory pathways is included here as this information is important for an understanding of the organisms and to proceed with genetic evaluation of these organisms.

Methane-producing microorganisms are ubiquitous and functional in a wide variety of anaerobic environments. The magnitude of methanogen activity in the global cycling of methane is not fully appreciated. It is important to recognize that biogenic methane released to the atmosphere is only a small fraction of that actually produced because the methanogenic bacteria oxidize most of it before it ever reaches the atmosphere. The methane that does reach the atmosphere is equivalent to 0.5% of the annual biological production of dry organic matter, methane is thus one of the most abundant organic compounds on earth, and the microbes that produce it play a major role in the cycling of carbon (Frea, 1982). As well, methanogens play an important role in the anaerobic degradation of organic compounds (Wolfe, 1971). Methane is one of the most abundant organic compounds on earth, and the microbes that produce it play an important role in the cycling of carbon (see Kirsop, 1985 for a review of methanogenesis and Blaut, 1990 for a review of the energetics of methanogens). Microorganisms producing methane as the end product of metabolism benefit by the production of cellular energy during the catabolism of extremely simple substrates at low reduction potentials (Jones et al., 1987).

Methanogens possess a unique mode of energy metabolism: methane generation. Although some anaerobic eubacteria evolve small amounts of methane during their normal metabolism (Postgate, 1969), only methanogenic archaeobacteria are known to couple methane synthesis to energy generation (Jones et al., 1987). Much effort has been devoted to studying the biochemistry and energetics of methanogenesis (Daniels et al, 1984; Keltjens, 1984; Jones et al, 1987, Blaut et al., 1990; Thauer, 1990; Gottschalk and Blaut, 1990) and genes that encode enzymes involved in methanogenesis (Shuber et al, 1986; Allmanberger et al, 1986; Bokranz and Klein, 1987; Cram et al, 1987; Bokranz et al, 1987; Reeve, 1988; Weil et al, 1988; Weil, 1989). Methane formation occurs only under strictly anaerobic conditions and thus methanogenesis is restricted to habitats which are highly anoxic (of low oxidation-reduction potentials). So far, all known methanogens are obligate methane producers, and no secondary energy sources have been identified (Whitman, 1985). The compounds which serve as the substrates methanogens use are produced as end products of various eubacterial and eukaryotic fermentations, and anaerobic oxidations of both complex and simple compounds, these compounds are H_2 , CO_2 , formate, acetate, methanol and methylamines (Jones et al., 1987). Carbon dioxide is a nearly universal substrate for methanogens, the needed electrons usually being derived from H_2 . However, as noted previously a variety of other substrates are converted to methane by certain methanogenic species. When growing on $H_2 + CO_2$, the methanogens are autotrophic with CO_2 serving as both carbon source and electron acceptor. It has been determined from biochemical studies that the typical electron-transport systems of methanogenesis varies in the molecular carriers to traditional carriers

of other anaerobic respiratory processes such as sulfate and nitrate reduction. The electron carriers in methanogenesis require a host of specific coenzymes unique to methanogenic bacteria which serve as cofactors for enzymes which sequentially reduce various C-1 intermediates starting with CO_2 and eventually yielding CH_4 .

The following describes the unique coenzymes involved in methanogenesis, their structures and functions. Secondly, the pathways to methane from CO_2 , formate, methanol and methylamines, and acetate are outlined.

1.9 UNIQUE COFACTORS

The peculiarities of methanogenesis and its coupling to energy conservation led to the evolution of a number of very specialized and unusual coenzymes (Balch et al, 1979; Wolfe, 1985; Jones, 1987; Konig, 1988). To investigate regulation of gene expression it is necessary to identify regulatory units and characterize signals for transcriptional initiation and termination (Konheiser et al., 1984). Structural genes coding for subunits of enzyme complexes lend themselves for studies of gene regulation, since coordinate expression of these genes is most likely unless one or more of the subunits have multiple functions in different metabolic pathways (Konheiser et al., 1984). The following is a description of some of these unique cofactors.

Coenzyme F_{420}

Cells of methanogens can be identified by their strong autofluorescence under oxidizing conditions. The major contributors to this phenomena are coenzyme F_{420} and

the methanopterin derivatives (Jones et al., 1987). The coenzyme F_{420} of methanogens is a deazariboflavin derivative substituted with ribitol, phosphate, and glutamyl residues. It is this coenzyme that gives methanogens fluorescence. F_{420} has been detected in all methanogens but is not found in other organisms with the exception of *Streptomyces*, *Mycobacterium tuberculosis* and *Nocardia aurantia* (Daniels et al., 1985). The function of F_{420} is a low potential electron carrier ($E'_0 = -340$ to 350mV) for two electron transfer reactions and partially replaces ferredoxin. F_{420} is a coenzyme of hydrogenase, the formate dehydrogenase and the CO dehydrogenase systems in methanogens.

F_{430}

F_{430} is a yellow soluble nickel tetrapyrrole which functions in the terminal step of methane reduction, as a part of the methyl reductase system. However, its exact role in the catalytic cycle is not yet clear (Blaut et al., 1990).

Methanofuran and Methanopterin

Methanofuran, is a low-molecular-weight coenzyme that plays an important role in the first step of methanogenesis from CO_2 . It functions as a formyl carrier in the first step of the reduction of CO_2 . The formyl groups are transferred to methanopterin (F_{432}), a derivative related to folic acid. Methanopterin exhibits a bright blue fluorescence, and serves a C-1 carrier during the reduction CO_2 to CH_4 .

Coenzyme M

The oxidation of hydrogen and the reduction of CO_2 to produce methane by methanogens involves a higher unique biochemistry. Coenzyme M (Taylor and Wolfe, 1974) is involved in the methyl transfer reactions in methane bacteria. It is the smallest

known coenzyme and is exceptional in its high sulfur content and acidity. This cofactor is required by the enzyme methyl-coenzyme M reductase present in all methanogens and active in the terminal steps of CO_2 reduction to CH_4 (Balch et al, 1979). Coenzyme M is not found in eubacteria or eukaryotes but is found in high levels in all methanogens.

Cyclodiphosphoglycerate

Cyclodiphosphoglycerate is believed to be involved in the thermoadaptation of enzymes in the group of thermophilic methanogens.

Component B

Like coenzyme M this cofactor is involved in the terminal step of methanogenesis catalyzed by the methyl reductase system, but its precise role is still unknown. Component B resembles the vitamin pantothenic acid and may serve as an electron donor to the methyl reductase system.

1.10 METHANOGENIC PATHWAYS

Despite the common terminal product of the methanogenic metabolism, the pathways leading to methane are different and depend on the substrate utilized (Blaut et al., 1990). The various methanogenic pathways are outlined in the following section.

METHANOGENESIS FROM H₂-CO₂

The pathway of CO₂ reduction with molecular hydrogen has largely been elucidated (Figure II). Methane formation from H₂ + CO₂ is the most common substrate used by almost all methanogens (for reviews see Rouviere and Wolfe, 1988; Jones et al., 1985) and could be considered a type of anaerobic respiration in which CO₂ serves as the electron acceptor. In the first step (Fig. II) CO₂ is reduced to form formylmethanofuran. The formyl moiety is then transferred to tetrahydromethanopterin (H₄MPT), H₂O is cleaved off giving rise to the formation of methenyl-H₄MPT which in turn is reduced to methylene-H₄MPT in an F₄₂₀-dependent reaction. The latter compound is reduced to methyl-H₄MPT with the methyl group subsequently being transferred to the coenzyme M in the transferase reaction. Methyl-coenzyme M is reduced to methane in the terminal methylreductase reaction (Blaut et al., 1990).

METHANOGENESIS FROM FORMATE

Formate does not enter the methanogenic CO₂ reduction pathway directly; it must first be oxidized to CO₂ by formate dehydrogenase, the electrons derived by this reaction

can be used for the reduction steps outlined above for CO₂ reduction (Blaut, et al., 1990). Small amounts of hydrogen are produced during formate conversion however molecular hydrogen does not appear to be an intermediate in methanogenesis from formate (Shauer and Ferry, 1980). Formate dehydrogenase purified from *Methanobacterium formicicum* contains non-heme iron, a molybdopterin cofactor which requires FAD for the reduction of F₄₂₀ (Shauer and Ferry, 1986).

METHANOGENESIS FROM METHANOL AND METHYLAMINES

The conversion of methanol (equation shown above) and methylamines (equation not shown) is characterized by an oxidative and a reductive branch. In the oxidative branch the methyl groups are oxidized to CO₂, and the reducing power gained fuels the reductive branch. In the reductive branch the methyl groups are transferred to coenzyme M, the resulting methyl-CoM is then the substrate of the methyl-CoM methylreductase but differs from the CO₂ reduction pathway, where H₂ serves as the electron donor, the electrons here are derived from the methyl group oxidation (Fig. II b.) (Blaut et al., 1990).

THE CONVERSION OF ACETATE

The pathway of acetate conversion (Fig. II c.) illustrates that prior to cleavage into C₁ moieties, acetate has to be activated. In *Ms. barkeri*, this proceeds by the action of acetate kinase and phosphate acetyltransferase to yield acetyl-CoA. The electrons gained from the oxidation of CO to CO₂ are used to reduce the methyl group to methane (Blaut et al., 1990).

FIGURE II PATHWAYS FOR METHANE FORMATION

A. Pathway of methanogenesis from $H_2 + CO_2$.

B. Pathway of methanogenesis from methanol.

H_4MPT , tetrahydromethanopterin; MF , methanofuran; $HS-CoM$, reduced coenzyme M; $F_{420}H_2$, reduced F_{420} .

C. Pathway of methanogenesis from acetate. $[CO]$, bound CO

1.11 CELL WALL STRUCTURE

The cell envelope structures of the archaeobacteria were one of the first biochemical characteristics studied in detail. From these studies it was apparent that significant differences were present in the archaeobacteria compared to typical bacterial cell envelopes (Kandler and König, 1978; König, 1988). The discussion of cell wall composition is important to this study as the procedure for lysis of a number of different species is dependent on the structure and composition of the membranes and wall. All archaeobacteria lack murein in the cell envelope, although rare in eubacteria some groups also show this feature e.g. *Planctomyces*, *Pirella*, and *Mycoplasma* (König, 1988). Unlike most eubacteria archaeobacteria have no universal cell wall polymer. The unusual chemical composition of the cell envelope of Archaeobacteria show a high degree of resistance against cell wall antibiotics and lytic agents (König, 1988). The archaeobacteria have a collage of cell wall types, they stain Gram- and Gram+, they do not contain muramic acid or D-amino acids. The cell walls of the Gram+ archaeobacteria consist of pseudomurein and methanochondroitin. All the Gram- archaeobacteria have cell envelopes composed of single-layered or more complex crystalline protein or glycoprotein subunits (König, 1988). Single S-layers are found in most organisms of the methanogenic branch. The S layer is commonly a proteinaceous crystalline layer of hexagonally arranged subunits of different molecular weights (König and Stetter, 1986). Glycosylated proteins were not found to be present and the amino acid composition was predominantly non-polar. This layer is the only structural element

outside of the cytoplasmic membrane of most Gram- archaeobacteria (Nuber and Konig, 1986; Sleytr et al. 1986). These S-layers can provide resistance to some of the characteristic environmental conditions these organisms are exposed to such as high salt, low pH, and high temperatures. S-layers can be considered the simplest biological membranes or protein envelopes developed during evolution which can withstand extreme environmental conditions.

Isolation of total cellular DNA for the construction of genomic libraries to clone genes from several species of the methanogenic members of the archaeobacteria domain, has been hindered by the lack of generally applicable lysis method. This is because certain methanogen species contain a cell wall which resists disruption by the traditional chemical and enzymatic methods commonly used to lyse the Gram+ and Gram- species of the eubacteria kingdom.

Methanogens can be classified into several subgroups on the basis of cell wall structure, each exhibiting characteristic behaviors towards various chemicals and enzymes. Cells of the order *Methanobacteriales* have an electron dense cell wall composed at least in part, of pseudomurein (Konig, 1988). Pseudomurein is not susceptible to degradation by lysozyme (Kandler and Konig, 1978) (see Chapter 2.5.1), but can be degraded by an endo -ptidase found in extracts of *Methanobacterium wolfei* (Keiner et al., 1987). Members of the genera *Methanosaeta* ('*Methanothrix*') (Patel and Sprott, 1990), and *Methanospirillum* of the order *Methanomicrobiales* occur as long chains of cells with tubular proteinaceous sheaths. These show unusual recalcitrance against the action of chemical agents and lytic enzymes (Kandler and Konig, 1978;

Beveridge et al., 1985; Patel et al., 1990; Sprott et al., 1986). Lysis can be achieved by incubation of these methanogens at alkaline pH with dithiothreitol, whereas other genera proved resistant (Sprott et al., 1979). Other members of Methanomicrobiales (*Methanosarcina* spp.) have relatively thick cell walls lacking peptidoglycan (Kandler and Hippe, 1977), and are composed of the fibrillar nonsulphated polymer (described in chapter 2.6) methanochondroitin (Kreisl and Kandler, 1986). Many of the methanococci possess a regularly structured, paracrystalline, proteinaceous layer external to the cytoplasmic membrane (Konig, 1988; Nuber and König, 1987). Within the methanogenic bacteria four cell wall types exist. The order Methanobacteriales contains Gram+ cells, which can be long rods, lancet shaped rods, or cocci. The cells of all species exhibit an electron dense cell wall sacculi with a thickness of approximately 15nm. These cell walls are composed of pseudomurein, a new type of peptidoglycan. Historically, the cell wall polymer of typical bacteria was referred to as murein, a muramic acid containing peptidoglycan. This term has been replaced by the more general term peptidoglycan. To distinguish between the two polymer types, the structure in methanogens is referred to as pseudomurein. Morphologically eubacterial murein is very similar to pseudomurein however, biochemically they are very different (Balch, 1979). The genus *Methanobacterium* contains pseudomurein, comprised of two alternating repeats of two amino sugars, N-acetylalosaminuronic acid and N-acetylglucosamine, the former acid being unique to archaeobacteria while the latter makes up one half of the backbone of eubacterial peptidoglycan (König, 1988). The acetylalosaminuronic acid residues are cross linked by amino acids as are the muramic acid groups in peptidoglycan, but the

amino acids are all of the L-form rather than the D-amino acid form found on peptidoglycan (Fig.III). The linkage between the amino sugars in pseudopeptidoglycan is chemically a 1-3 bond rather than the 1-4 bond between acetylglucosamine and muramic acid of peptidoglycan. It is this feature which makes lysozyme ineffectual as it's action is on the B(1-4) linkage. Crystalline proteinaceous S layers associated with the peptidoglycan sacculus are a common feature of the Gram+ eubacterial bacilli and clostridia, among the Methanobacteriales they were only present in the extremely thermophilic members of the genus *Methanothermus*.

The genus *Methanosarcina* have a thick (200 nm), rigid Gram+ wall component (sacculus) consisting exclusively of heteropolysaccharide (Kandler and Hippe, 1977). The major sugars present are N-acetylgalactosamine, glucuronic acid, glucose and a small amount of mannose, a considerable amount of acetate is also present (Konig, 1988). Phosphate and sulfate are absent. Another interesting feature is that the major structural elements, galactosamine and glucuronic acids are similar to the connective tissue component, chondroitin of animal tissues leading to the term methanochondroitin matrix to describe the complex polysaccharide wall of *Methanosarcina*. It is responsible for the aggregating abilities of this group. The difference between the components is that the polysaccharide of *Methanosarcina* is not sulfated.

Methanococcus and *Methanomicrobium* lack carbohydrate in their cell wall and are composed entirely of several distinct proteins. They contain neither a rigid cell wall (sacculus) nor a sheath, but exhibit an outer non-glycosylated protein layer consisting of hexagonal subunits which disintegrate very easily (Jones et al., 1977; Balch, 1979;

Konig, 1988). The S-layers vary with species but are heterologous in their molecular weights and in their antigenicity. They have similar chemical and physical stabilities and amino acid compositions. The acidic amino acid compositions are predominantly composed of hydrophobic amino acids (Konig, 1988). These cell surfaces are more primitive structures than those stable crystalline layers.

Methanospirillum and *Methanothrix* occur in chains forming long filaments. They contain a well defined, double-layered wall composed of protein subunits which form a sheath around the organism. Cell wall preparations show no sacculi with rounded ends, but rather cylindrical sheaths of variable length and brittle structure (Kandler and Konig, 1978). The electron dense outer sheath exhibit a distinct fibrillar surface structure, tubular in nature, composed of hoop-like rings. The parallel fibrils are perpendicular to the longer axis of the sheath and breakage occurs along the fibrils (Kandler and Konig, 1978). The outer wall extends as a surface layer from one cell to another and therefore does not participate in septum formation. Although the sheath is composed of mainly protein and carbohydrate (Kandler and Konig, 1978, 1985; Sprott and McKellar, 1980). The individual cells enclosed in a inner envelope are separated from each other by spacer plugs composed of several layers of periodically arranged material (Shaw et al. 1985; Stewart et al. 1985; Beveridge et al. 1986; Patel et al. 1986). The sheath exhibits unusual resistance to chemical agents and lytic enzymes, treatment with dithiothreitol released spheroplasts but the sheaths remained intact (Sprott et al. 1979). Mercaptoethanol treatment disassembled the sheaths of *Methanospirillum hugatei* into separate hoops and soluble glue peptides (Beveridge et al. 1986b; Sprott et al. 1986).

The hoops can be solubilized by extended incubations with mercaptoethanol and sodium dodecyl sulfate (SDS).

FIGURE III THE STRUCTURE OF PSEUDOMUREIN

Structure of pseudomurein resembles peptidoglycan in the amino acid cross links of the acetylalosaminuronic acid, however the amino acids are in the L form in pseudomurein, as opposed to the D form in peptidoglycan. Shown also is the B (1-3) linkage between the acids, it is this linkage that makes lysozyme ineffectual (this figure is of *Methanopyrus*, variations do exist between species).

1.12 LIPID COMPOSITION

The diversity and unique features of methanogens have been a limiting factor in the development of genetic techniques and the elucidation of genetic features within this group. The lipid structure and components of methanogens is one such feature. Lipid structures may affect the permeability of the cell membrane to the introduction of genetic material. The investigation of methanogen lipids may provide information for the development of techniques of gene transfer or transformation using novel ether lipid liposomes.

A survey of the lipid composition of the methanogens and other bacteria shows that ether-linked polyisoprenoid (branched) chain lipids are the predominant component (Langworthy, 1985). The chemical composition of methanogen and other archaeobacterial membranes differs in their constituents to eubacterial and eukaryotic organisms. The latter organisms' hydrophobic core is composed predominantly of fatty acid ester-linked glycerol lipids. These fatty acid glycerolipids are largely absent from methanogens. The predominant archaeobacterial lipophilic membrane constituents are diphytanyl diether and dibiphytanyl tetraether lipids composed of C₂₀ or C₄₀ carbon units respectively (Langworthy, 1985). The isopranyl glycerol ethers are distinguishing markers for methanogens. Predictions are that the diethers form normal bilayers, and the carbon chain of the tetraether span the membrane forming a monolayer. Seventy to >ninety percent of the lipid content are polar lipids the remaining are neutral lipids. The neutral lipids comprise a wide range (C₁₄ to C₃₀) of isoprenoid hydrocarbons; the

principle compounds being C_{30} , C_{25} and C_{20} acyclic isoprenoid hydrocarbons with a continuous range of hydroisoprenoid derivatives (see Figure IV for a comparison of methanogen lipid structures). For a broader description covering archaeobacteria in general see a recent review on the subject by Kates, 1988.

FIGURE IV MAJOR LIPID STRUCTURES OF METHANOGENS

Major lipids of methanogens: a) glycerol diethers; b) diglycerol tetraethers; c) macrocyclic diether and hydroxydiether (sn-2 and sn-3); d) comparison of the ester linkage of conventional organisms and the ether linkage of archaeobacteria.

(d) Comparison of the ester linkage of conventional organisms and the ether linkage of archaeobacteria

2.9 MOLECULAR BIOLOGY

Although these organisms (methanogens) promise to be extremely important in the study of evolutionary biology and potentially in environmental science, much of the study of their genetics has proceeded slowly. This in part is due to their extreme sensitivity to oxygen and their unique physiology. Historically, genetic studies with methanogens are very recent, beginning in 1982 (Reeve et al., 1982 and Bollschweiler and Klein, 1982). As the technical difficulties which have slowed the genetic work on these organisms are overcome, sufficient information is now available (for a review see Brown et al., 1989) to begin to address significant questions dealing with genome characteristics and the mode of gene organization, regulation and expression in these organisms.

The genome size of methanogens ranges from 1.0×10^9 to 1.8×10^9 daltons. These figures were determined by renaturation kinetics of six methanogens. Comparatively the genome size of methanogens is much smaller than *Escherichia coli* at 2.5×10^9 daltons. The base composition of methanogens is comprised of the standard genetic code of G, C, A, T, with their G+C content ranging from 25.8-61 mol% (Hamilton and Reeve, 1985; Brown et al, 1989) this corresponds to a range equal to all eubacterial species. Codon usage in methanogens is directly related to the availability of A+T and G+C pairs. Methanogens with low G+C contents preferentially use codons with A or U. The codons preferentially used by methanogens are often codons relatively rarely used by *E. coli*, they are AUA, AGA, and AGG (Reeve et al, 1986).

This resembles codon usage similar to the eukaryote *Saccharomyces cerevisiae* (Reeve et al, 1986).

Sequences of cloned methanogen DNA fragments has revealed that genes are flanked by comparatively more non-repeating A and T rich intergenic sequences. As an example, random cloned DNA fragments from *Methanobrevibacter smithii* contained an average of 69% A+T base pairs (Hamilton and Reeve, 1985). These regions may function as gene spacers but it is not yet known if this is a genetic feature of all methanogens (Harris et al, 1986).

The demonstration that cloned methanogen genes can direct the synthesis of functional enzymes in *E. coli* implies that the standard genetic code is used and does not contain introns. Genes that have been cloned from methanogens and that are expressed in *E. coli* care , but not limited to, *hisA*, *argG*, *proC*, *purE* as well as genes that encode sub-units of the enzymes methyl-coenzyme-M methyl-reductase and DNA dependent RNA-polymerase, and genes that encode tRNA's and rRNA's (Reeve et. al. 1986). No firm evidence exists to indicate the presence of introns in methanogen genes which encode polypeptides (Reeve, 1988; Reeve et al, 1986). The discovery of a cloning method that does not require expression in *E. coli* may determine the presence of introns (Reeve et al, 1986).

rRNA and tRNA genes as well as protein encoding genes were cloned from various methanogens either by homology with eubacterial genes or by immunochemical methods using antibodies raised against purified proteins. As well, genes cloned from

methanogens can complement auxotrophic mutants of *E. coli* (Bhatnagar et al. 1986; Kiener et al., 1984; Bertani and Baresi, 1987; Jain and Zeikus, 1987).

As shown for multisubunit proteins, e.g., methyl CoM reductase, the genes of the subunits are transcribed in a polycistronic way (Konheiser et al., 1984; Weil et al., 1988; Konig, 1988).

To facilitate genetic analysis cloning vectors are necessary. Generally the most convenient selective markers integrated into such vectors are antibiotic resistances. Unfortunately methanogens are sensitive to a limited number of drugs (Elhardt and Bock, 1982; Possot et al, 1988; Keiner et al, 1986; Jarsch et al, 1983; Keiner et al, 1984; Hummel and Bock, 1985; Hummel et al, 1985; Haas et al, 1986; Bock and Kandler, 1985; Knox and Harris, 1988). Mutants, however, may serve as genetic markers for the development of a genetic system (Rechsteiner et al., 1986; Bertani and Baresi, 1985; Bowen and Whitman, 1987; Jain and Zeikus, 1987; Santoro and Konisky, 1987; Ladspo and Whitman, 1990; Micheletti et al., 1991). Worrel et al.(1988), transferred 5-fluorouracil resistance between strains of *Methanobacterium thermoautotrophicum*. As well Gerhardt et al.(1990), developed a selectable marker for puromycin resistance and made an important advance toward a shuttle vector with Mip1.

The first step in gene expression involves recognition of DNA sequences necessary for initiation of transcription. Within the classical prokaryotes, the eubacteria, the recognition and binding site of RNA polymerase has been designated as a promoter (Thomm et al, 1989; Gernhardt et al., 1990). Promoter regions in methanogens have been defined primarily by determining transcription initiation sites and identifying

adjacent conserved sequences. Transcription starts in an A+T rich region, as well the terminal signal is found to be in a A+T rich region. A portion of the conserved sequence ACCGAAAAN **TTTATATA** NTA is similar to the "TATA" box found in eukaryotic promoters. This sequence has been found to be a common element of promoters of stable RNA genes of archaeobacteria (Thomm and Wich, 1988). Although methanogen DNA is expressed efficiently in *E. coli*, it is unlikely that methanogen transcriptional signals are recognized by the eubacterial transcriptional apparatus, since the structure of all methanogen DNA-dependent RNA polymerases are distinct from eubacterial enzymes (Harris et al., 1986). Methanogen DNA-dependent RNA polymerase is composed of 7 to 12 different sized subunit polypeptides, these have complex compositions quite different from those of eubacteria and more closely resemble the eukaryote RNA polymerase (Konig, 1988). They are also similar to the eukaryotes in the structural homologies between the individual subunits (Reeve et al, 1986). Methanogen promoter sequences are yet to be fully defined, but it is conceivable that transcription of methanogen DNA in *E. coli* occurs by the recognition of the A-T rich regions as eubacterial-like promoters (Hamilton and Reeve, 1985). All methanogen RNA polymerases are completely resistant to rifampin and streptolydigin antibiotics which inhibit eubacterial RNA polymerases (Konig, 1988). In fact most antibiotics that specifically affect protein synthesis in eubacteria do not affect methanogen protein synthesis. This is in part due to the macromolecular cell envelope chemistry of methanogens which render these antibiotics ineffectual, therefore they cannot be used as selective agents in genetic experiments involving methanogens (Reeve et al, 1986).

Repeated DNA and insertion elements have been investigated and it was found that in *Methanobacterium thermoautotrophicum* approximately 6% of the genomic DNA consists of repetitive sequences. There is, however, a need for further research in the frequency of rearrangements (Mitchell, 1979). An insertion element ISMI in *Methanobrevibacter smithii* has been reported which structurally resembles insertion sequences found in the eubacteria (Brown et al, 1989).

Eubacterial genes are frequently contained in polycistronic transcriptional units or operons. In contrast eukaryotic genes are not usually transcribed into polycistronic mRNAs. Operons and ribosome binding sites in methanogens appear to be eubacterial-like, multigene transcriptional units and therefore encode typical prokaryotic mRNAs. The translation initiation step is co-ordinated by mRNA-16 S rRNA hybridization, similar to the eubacteria.

Although eubacterial and methanogen ribosomes are similar in size, they differ in shape and there are several steps in methanogen protein synthesis which are different, resembling more closely the eukaryotes. In methanogens and eukaryotes the initiating tRNA carries an unmodified methionine unlike the modified eubacterial methionine residue formylmethionine (Dennis et al, 1986). Methanogen bacterial ribosomes contain three rRNA species 23 S, 16 S, and 5 S as found in eubacteria and chloroplasts. The rRNA encoding genes are physically linked and in the same order as the eubacterial 16 S, 23 S and 5 S rRNA's (Reeve et al, 1986). The number of copies of rRNA encoding genes range from 1 to 4 (Reeve et al, 1986). The archaeobacterial ribosome has a high degree of protein complexity, with the total number of proteins varying between 56

(*Methanobacterium bryantii*) to 64 in a *Sulfolobus*. Comparatively, eubacteria have 52 and there are between 70 - 85 in the eukaryotes (Reeve et al, 1986). The ribosomal proteins of methanogens tend to be very acidic.

Sequencing of methanogen tRNA genes has identified clustered genes, genes as individual transcriptional units, and multigene transcriptional units. Most tRNA genes were found to be clustered in a few operons.

Gene transfer systems reported for Archaeobacteria include mating, (Mevarech and Werczberger, 1985; Rosenshine et.al., 1989) and transformation with genomic DNA (Bertani and Baresi, 1897; Cline, et.al., 1989; Worrell, et.al., 1987; Worrel et al., 1988, Sandbeck and Leigh, 1991), plasmid transformation and bacteriophage infection, in *Halobacterium* (Charlebois, 1987; Hacket and Das Sarma, 1989; Cline and Doolittle, 1987), and transduction(like) gene transfer systems have been found in methanogens (Bertani and Baresi, 1989; Meille et.al., 1990).

A number of extrachromosomal elements as well as restriction enzymes with new restriction sites or consensus sequences have been purified.

It would appear from the current information that gene structure and expression in methanogens follows eubacterial precedents. The long term, successful application of recombinant DNA technologies to the analysis of gene regulation in any biological system requires an efficient transformation protocol. Cloned fragments have elucidated the basic structure and organization of genes, but to determine how these genes are regulated in vivo requires the manipulation of these genes in vitro with subsequent re-introduction and expression in viable cells. Methods of gene transfer in methanogens are

needed to facilitate genetic studies. As these techniques are becoming available the ability to determine the molecular biology of these interesting organisms and their potential benefits is progressing.

1.14 INTRODUCTION TO BACTERIAL PLASMIDS

Bacterial plasmids are double-stranded closed circular DNA molecules that range in size from 1.0 kb to more than 2000 kb (*Rhizobium*). They are found in a variety of bacterial species, where they behave as accessory genetic units that replicate and are inherited independently of the bacterial chromosome (Maniatis, 1989). The reasons for studying plasmids are numerous. They have medical importance as they are in part responsible for the resistance of infectious bacteria to antibiotics (Broda, 1979). Plasmids are also of great value as material for studying the structure and function of genetic material. For this there are two virtues plasmids possess; 1) their relatively small size generally in the range of 0.75 - 100 megadaltons (1 megadalton = 1,500 base pairs = 1.5 kb) sufficient to code for 1-2 average size proteins, which means they are easy to manipulate in vitro compared to intact bacterial chromosomes, and 2) their host cells can usually survive without them. Therefore the properties conferred by a plasmid, the phenotype (i.e. drug resistance, production of colicins, enterotoxins, restriction and modification enzymes, production of antibiotics, and degradation of complex organic compounds) can be easily examined. Since such plasmid-specified functions can often

be selected for, many genetic experiments are possible. Such studies have revealed information of genetic structure, function, replication and bacterial evolution. Plasmids are also powerful experimental tools for molecular biology. Genetic analysis of bacteria is often dependent on the transfer of genes between strains and plasmids can act as transfer agents. Certain plasmids can be transferred between different genera and are used to introduce DNA into novel hosts. The technique is of major importance in genetic engineering and cloning which allows large amounts of a specific gene regulated product to be purified.

1.15 PLASMID ISOLATION

The main problem to overcome in the preparation of plasmid DNA is its separation from chromosomal DNA, since the plasmid usually comprises no more than about 5% of the total DNA (Grinsted and Bennett, 1988). Plasmid DNA is usually isolated as a double-stranded covalently closed circular (CCC) molecule, which twists about itself in a form called a supercoil. A single break or 'nick' in one of the two strands causes the supercoil to convert to an open circular form, if the break occurs in both strands, a linear duplex structure will be formed. Most of the plasmid DNA isolated from bacterial cells is in the supercoiled configuration, which is the most compact form within the cell. Isolation of plasmid DNA can generally be accomplished by making use of the physical properties of the supercoiled DNA molecules and their

small size in comparison with the bacterial chromosome (Hardy, 1986, 1987; Birnbaum and Doly, 1979; Eckhardt, 1978; Holmes and Quigley, 1981; Maniatis, 1982, 1989). As well, plasmid DNA often has a significantly different percentage of guanine-cytosine base pairs in comparison with the chromosome of the host strain. Based on the plasmid DNA form and G+C content the sedimentation rate during ultracentrifugation varies, with the supercoiled form sedimenting more rapidly. Supercoiled molecules also show high sedimentation rates in liquids such as alkaline sucrose. The affinity for certain dyes such as ethidium bromide (EtBr) which intercalates within the DNA molecule, is less with the supercoiled form. Since binding of the dye lowers its density, supercoiled plasmid DNA assumes a different position in a cesium chloride (CsCl) density gradient than chromosomal DNA following equilibrium centrifugation in CsCl-EtBr gradients. To ensure the plasmid molecule remains in the CCC state and not linearized by nicks caused by nucleases, lysis is performed in the presence of ethylene diaminetetra acetic acid (EDTA). EDTA chelates metal ions and so inactivates cation dependent nucleases. Cell extracts can then be saturated with cesium chloride and are then used to separate chromosomal DNA from plasmid DNA during ultracentrifugation (Broda, 1979; Freifelder, 1983; Hardy, 1986, 1987; Maniatis, 1989). The plasmid band can be visualized under a UV light source and removed from the gradient by inserting a syringe through the tube wall. Other methods of plasmid isolation exist and have the benefits of being fast and convenient in the sense that many samples can be tested in one day. These include in-well lysis (Eckhardt, 1978) where the bacteria are lysed by lysozyme followed by a SDS (sodium dodecyl sulphate) treatment within the wells of the agarose

gel electrophoresis apparatus. When the gel is electrophoresed the chromosomal DNA hardly penetrates the gel allowing for the visualization of plasmid DNA. Other rapid methods of plasmid DNA isolation remove the chromosomal DNA first, either by a clearing spin or by selective precipitation (Holmes and Quigley, 1981; Birnbaum and Doly, 1979).

Plasmid DNA molecules of different sizes can also be isolated by electrophoresis on agarose gels, this method provides a way to isolate and size the plasmid DNA, as well as an opportunity to analyze the action of endonuclease and other enzymes on the plasmid DNA (Myers et al, 1976; Broda, 1979; Hintermann, et al, 1981; Schaffer and Sederoff, 1981; Maniatis, 1989; Freifelder, 1983; Hardy, 1986, 1987). Methods exist which also employ the use of ion-exchange, gel filtration chromatography or differential precipitation to separate plasmid and chromosomal DNA's. Most of these methods are not preferentially used; however, differential precipitation with polyethylene glycol can yield plasmid DNA of extremely high purity. Differential precipitation with polyethylene glycol differs from equilibrium centrifugation in CsCl-ethidium bromide gradients in one respect; it does not efficiently separate nicked circular molecules in the closed form of plasmid DNA. A further method of plasmid isolation uses the different effect of high pH (12.0-12.5) on chromosomal and plasmid DNA. High pH denatures chromosomal DNA, but not CCC plasmids (Birnboim and Doly, 1979). If the pH is subsequently lowered and the salt concentration is kept high, chromosomal DNA precipitates, leaving CCC plasmids in solution. Most of the high molecular weight cellular RNA also precipitates under these conditions as well as proteins if the detergent sodium dodecyl

sulphate (SDS) is present (SDS solubilizes proteins). After centrifugation to remove the precipitate, the plasmid DNA is already sufficiently pure (after ethanol precipitation) for some kinds of experiments. It can however, be further purified by cesium chloride-ethidium bromide centrifugation.

1.16 PLASMID SIZING

The sizing of plasmids can be accomplished by a number of methods. For example, electron microscopy by the Kleinschmidt method allows the measurement of the contour length based on a known standard. Alternatively, following electrophoresis in agarose of endonuclease restricted plasmid DNA with known standards, a standard curve of the distances travelled by these standard fragments as a function of the logarithm of their sizes can be constructed. The distance travelled by the sample fragments can then be measured so that their size can be determined from the graph (the various bands can be added together to give the molecular weight). Densitometry can also be employed to differentiate between different fragments having similar sizes. Finally, sucrose gradients based on sedimentation coefficients (S values which relate to the molecular weight), with known standards, may also be used.

1.17 RESTRICTION MAPPING

The physical restriction mapping of plasmids is accomplished using restriction endonucleases and gel electrophoresis. Restriction endonucleases are enzymes that recognize specific sequences of DNA and subsequently cut the phosphodiester backbone of the molecule. The electrophoretic patterns of enzymatic digests of the plasmid DNA are compared and a restriction fragment map is constructed. The most common method for deriving restriction maps is to use two different restriction enzymes. The procedure is to take three samples of a particular DNA species, treat each of two of these with a separate enzyme and one sample with both enzymes (a double digest), and then compare the three sets of fragments. Other methods involve the generation of partial fragments and the radiolabelling with ^{32}P or as will be seen later the use of hybridization with digoxigenin-labelled probes (DIG label).

1.18 PLASMID COPY NUMBER

The copy number of a plasmid is generally defined as the ratio of the number of moles of plasmid DNA to the number of mole equivalents of chromosomal DNA. The number of copies is used to categorize plasmids and to decide upon the replicon to use for gene cloning on the basis of dosage of the cloned gene required. Absolute copy

number as will be seen is not always a useful value. Plasmids that contain 1-2 copies per chromosome are referred to as stringent compared to relaxed plasmids which are present in 15 or more copies per chromosome equivalent. However plasmid copy number will vary with the growth phase. As well, gene expression efficiency is not always directly related, therefore copy number may not always be used at face value. Physical methods are generally used to determine the proportion of a cell's DNA that is of plasmid origin. If the sizes of the plasmid and the chromosome are known the copy number can be calculated from this ratio. This approach requires that the plasmid and the chromosome DNA to be isolated with equal efficiency or at least known efficiencies (Broda, 1979). Plasmid specific RNA and DNA-DNA hybridization can also be used to determine plasmid copy number. Minimum estimates can be achieved by determining the proportion of total DNA that is in the CCC. This method however depends upon the efficient extraction of plasmid DNA as CCC molecules. Alternatively sucrose gradient centrifugation can be used to resolve both CCC and open circular plasmid DNA molecules from chromosomal DNA. Another commonly used technique is the method of Eckhardt (Eckhardt, 1978), to electrophoretically resolve total cell lysates on an agarose gel and then after staining with EtBr, to use UV fluorescence spectrophotometry to determine the relative quantities of plasmid and chromosomal DNA. Estimation of copy number can also be achieved using CsCl-EtBr density gradients and comparison with an internal standard (see Hardy, 1987 for complete descriptions).

1.19 METHANOGEN PLASMIDS

Plasmids and a variety of DNA-containing phages have been characterized from methanogens. *M. thermoautorophicum* strain Marburg contains a 4.5 kbp, high copy number plasmid, pME2001 (Meille et al, 1983). It has been found to form multimers, up to a hexamer. An abundant 611 base transcript from this plasmid contains four ORFs, ranging in length from 234 bases to 90 bases, three of which are preceded by potential ribosomal binding sites (RBS) (Meille et al, 1987). Derivatives of pME2001 have been constructed to replicate in *E. coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *S. cerevisiae* which have the potential to be used as shuttle vectors (Meille and Reeve, 1985). *M. thermoautotrophicum* strain deltaH contains a small amount of a plasmid that hybridizes to pME2001 DNA. A low copy number plasmid 8.7 kb, pURB500 has been isolated and characterized from a new *Methanococcus* isolate (Wood et al, 1985). Plasmids have also been isolated from *M. jannaschii* (pURB800, 64 kb; pURB801, 18 kb) and from a newly isolated related methanococcus, AG86 (pURB900, 20 kb), from a deep sea hydrothermal vent (Zhao et al, 1988). *Methanolobus vulcani* contains a plasmid, pMP1, which integrates into the host chromosome and could be a prophage (Thomm et al, 1983; Wood et al, 1983; Wilharm et al., 1986). *Methanosarcina acetivorans*, an acetotrophic methanogen, contains a plasmid, this plasmid has also been found as a multimer (Sowers and Gunsalus, 1988). Plasmid DNA was also found in three strains of *Methanobacterium thermoformicicum*, a 10.5 kb plasmid, pFZ1 from strain Z-245 was characterized and determined to be cryptic, pFZ2

(10.5 kb) and pFV1 (14 kb) were found in strains FTF and THF respectively (Nolling, 1991). A bacteriophage was isolated from a strain of *Methanosaeta* 'Methanothrix' (Roustan et al., 1986). Three other virus like particles are also known in methanogens PG, 50 kb found in *M. smithii* (Zillig et al, 1988), a 35 kb particle in *Methanococcus* sp. nov. (Zillig et al, 1988) and a 23 kb particle in *Methanococcus voltae* A3 (Wood et al., 1989). This thesis will describe a new methanogen plasmid pDDK found in the organism *Methanosaeta concilli* strain GP6.

SECTION II

MATERIALS AND METHODS

2.1 ANAEROBIC CELL CULTURE AND GROWTH CONDITIONS

Methanogenic bacteria are obligate anaerobes. Anaerobic culture techniques have been developed for the growth of methanogens (Hungate, 1950; Bryant et al, 1968; Bryant and Robinson, 1968; Holdeman, 1972; Aranki, 1972; Wolfe, 1979; Balch et al, 1979; Hippe, 1984; Bernhardt et al, 1987). All procedures from the preparation of media, to the transfer of bacteria as well as all vessels and tubing used are free of oxygen, anaerobic (Plate 1). The gases used are passed through an oxygen scrubber. This is a column maintained at 350° C and filled with copper. Oxygen in the media is removed by percolating with N₂ or being brought to a boil under an atmosphere of 80% H₂/ 20% CO₂. Reducing agents such as cysteine and sulfide are added and the pH is adjusted to the desired level. The liquid media are delivered through tubing to flushed serum bottles or carboys and autoclaved. After cooling, it is again flushed with 80% H₂/20% CO₂, stoppered and pressurized and volatile ingredients such as vitamins, fatty acids and methanol are added through flushed syringes from anaerobic stock solutions. Methods for solid media preparations are also available (Hungate, 1950; Edwards and McBride, 1975; Balch and Wolfe, 1976), as well as fermenter size preparations, but are

not discussed here. The diversity of the methanogens is apparent in the different formulae for media that fulfill growth requirements.

PLATE 1: ANAEROBIC MEDIA SET UP

Laboratory apparatus, diagram of the equipment and set up to work with anaerobic organisms. 1) N₂ cylinder; 2) 20% CO₂/ 80% H₂ cylinder; 3) O₂ scrubber (copper interior kept at 350°C); 4) round bottom flask containing unreduced media; 5) this is brought to a boil over a bunsen burner, this entire process occurs while the round bottom flask is being flushed with nitrogen; 6) once boiling, reducing agents are added usually cysteine and/or sulphide, the pH is adjusted; 7) after transfer to flushed capped serum bottles; 8) the serum bottles containing reduced media are autoclaved, cooled and the vitamins and other volatiles are added; 8) all processes other than autoclaving are performed in a fume hood, pressurized bottles are handled carefully and manipulations are performed behind plexiglass shields for safety.

2.2 CELL LYSIS: GENERAL

The diversity in cell wall structure (Section 1.11) explains why methanogens vary greatly in the ease with which their cell walls can be disrupted. For example, certain methanococci lyse readily when suspended in solutions of low ionic strength, while mechanical methods such as multiple passages through a French pressure cell may be ineffective for others such as *Methanobrevibacter smithii* (pers. obs.).

For genetic studies, total genomic DNA must be extracted in a form where it is relatively unsheared or damaged by cellular nucleases. A number of enzymatic and chemical procedures have been developed for the release of unsheared high molecular weight DNA from eubacterial cells (Marmur, 1961; Sambrook et al., 1989). However, these procedures are often ineffective against the nonmurein containing methanogens (Keiner et al., 1987). The usefulness of the pseudomurein endopeptidase of *Methanobacterium wolfei* is limited to sensitive strains such as *Methanobacterium thermoautotrophicum* (Keiner et al., 1987), in which there are no protecting wall layers external to the pseudomurein. In addition, the enzyme is not commercially available, and since it is O₂ sensitive must be freed of contaminating endonucleases by anaerobic purification methods (Keiner et al., 1987). Spontaneous formation of osmotically-sensitive cell forms during growth have been reported for *Methanobacterium bryantii* (Jarrell et al., 1982) and *Methanosarcina barkerii* (Davis and Harris, 1985), but nuclease activity is likely to degrade the DNA during slow autolysis of the cells. Also, it has been found (pers. obs.) that procedures for DNA isolation from methanogens which

involve the grinding of frozen cell pastes (Smucker and Pfister, 1975; Weil et al., 1988; Jarrell et al., 1991) or French pressure cell treatment of cells (Worrel et al., 1988), give low yields of DNA which is often sheared. Therefore a method was developed which was generally applicable to a wide range of methanogens, that produced DNA that could be restricted by restriction endonucleases and used laboratory reagents that were commonly available.

BACTERIAL STRAINS

The methanogens used in this study were obtained from the National Research Council of Canada culture collection and are listed in Table III pg.10 of this paper. *Methanobacterium thermoautotrophicum* strain H, *Methanobacterium formicicum* strains MF and G2R, *Methanobacterium bryantii* strains M.o.H and M.o.H.G, *Methanobacterium espanolii* GP9, *Methanospirillum hungatei* GP1, *Methanosaeta concilli* GP6, *Methanobrevibacter smithii*, *Methanobrevibacter arborophilicus*, *Methanothermus fervidus*, *Methanosarcina mazei*, *Methanosarcina acetivorans*, *Methanosarcina barkeri*, *Methanococcus voltae* and *Methanococcus jannaschii*. *Methanobacterium espanolii* GP9 and *Methanosaeta concilli* GP6 were grown according to the procedure of Patel et al. (1990) respectively. Growth media and growth conditions for the other methanogens were as described by Sprott et al. (1990).

CONDITIONS OF CELL LYSIS

All strains were grown anaerobically to the late exponential growth phase. Ten (10) ml samples in growth medium were collected by centrifugation. The samples were pelleted in a Sorval SS-34 rotor at 7,000 rpm for 10 min., one half of the samples (four) were frozen as cell paste at -20° C and thawed at 23° C, the other half were used fresh.

All samples were gently resuspended in 10 ml ABE buffer (ammonium bicarbonate [Anachemia] 50 mM pH 8.0; EDTA, ethylenediaminetetraacetic acid, disodium salt [Boeringer Mannheim] 50 mM), and an O.D. 660 was taken maintaining the initial fixed volume of 10 ml (Beckman spectrophotometer). Each of the four samples of fresh and frozen cells were treated differently to determine which chemicals were the contributing lysis agents and which steps in the following procedure series was necessary. Each sample was subjected to the following treatments: i) single treatments with one of pronase, sodium dodecyl sulphate (SDS) or dithiothreitol (DTT); ii) pronase treatment, followed by treatment with DTT and then by SDS; iii) pronase treatment, followed by SDS treatment; iv) pronase treatment , followed by DTT treatment; and, v) ABE only. Pronase (Boehringer Mannheim, predigested to inactivate nucleases (Sambrook et al., 1989) in 50 mM Tris-HCL, pH 8, for 1 h at 37°C) was used at a final concentration of 40 ug. ml⁻¹, for 1 h at 37°C; DTT (Boeringer Mannheim) at a final concentration of 200 ug. ml⁻¹, at 23°C for 15 min; and, SDS (Sigma) at a final concentration of 200 ug. ml⁻¹, at 55°C for 15 min. After each treatment the optical density at 660 nm was recorded, and the percentage of cell lysis (% lysis) determined.

The percent lysis is defined by dividing the optical density at each relevant step by the initial optical density, multiplying by 100 and expressing the result as a percentage.

DNA ISOLATION

In order to recover the total genomic DNA, the cell debris was precipitated by the addition of potassium acetate to the lysed cells at a final concentration of 0.3 M, followed by incubation at -20°C for 30 min. The sample was centrifuged at 12 100 X g for 10 min, and the supernatant was recovered. The DNA was precipitated by the addition of 1/5 vol. of 10M ammonium acetate (IBI chemicals) and 2.5 vols. of absolute ethanol. DNA was recovered by centrifugation at 12 100 X g for 10 min and resuspended typically in 500 ul of 10 mM Tris-HCL (Sigma), and 10 mM EDTA, pH 8. DNA purity and concentration were determined, using UV absorbance. Concentration was determined by a calculation using the O.D. at $UV_{\lambda 280}$;

$O.D. 280 \times \text{dilution factor} \times 50$ (for double stranded DNA) = mg/ml or ug/ul
Purity is used to determine the possible contamination by protein and is determined by a ratio of the O.D. using UV at 260 to 280. The ideal is a ratio of 1.9-2.0 for DNA.

DNA MANIPULATION

To determine the restriction of the DNA, the samples were digested in a 20ul vol. with the restriction endonuclease *BglII* and *PstI* (Gibco/BRL) as directed by the manufacturer. After restriction digestion, immediately prior to gel electrophoresis, contaminating RNA was removed by the addition of 1 ul of RNase A (Boehringer

Mannheim; 10mg.ml⁻¹ in 300mM sodium acetate, pH 4.5). The sample was incubated at 23°C for 2 min, whereupon loading dye was added. Restricted and unrestricted DNA samples were subjected to electrophoresis and visualized as described by Sambrook et al. (1989).

2.3 PLASMID SURVEY (SCREENING)

Plasmid DNA, as was described in previous sections is extrachromosomal DNA that can be used as vectors for gene transfer. The concept of the archaeobacteria forming one of the three domains of living organisms (Woese et al., 1990) has generated considerable interest in identifying and characterizing archaeobacterial extrachromosomal elements in order to study gene transfer in and develop transformation systems for these unique organisms (Nolling et al., 1991). Furthermore, detailed knowledge of these extrachromosomal elements contributes to understanding their evolution and their possible interaction with the chromosome of their hosts. Therefore the investigation of plasmid containing methanogenic species is of interest.

Total genomic DNA of all species (Table III) tested for lysis were screened for the presence of extrachromosomal DNA. After extraction of total genomic DNA (Meakin et al., 1991), 10 ul samples were restricted as per manufacturers (Boeringer and Mannheim) instructions with Pst I for 2 h at 37°C. 10ul samples of each species were electrophoresed in TAE (Tris-acetate-EDTA; 40 mM Tris, 20 mM Acetate, 2 mM EDTA, pH 7.8) containing 1.0% agarose (Sigma) for 12 h at 20v and 30mA. The agar

gel was stained in a solution of 0.5 ug/ml ethidium bromide for 30 min and destained in distilled water for 30 min. After staining, the gels were visualized and photographed under a UV light source. The photographs were analyzed for the presence of specific bands to indicate extrachromosomal DNA.

2.4 PLASMID ISOLATION

Following the screening of the methanogen species tested (Table III) the DNA suspected of being extrachromosomal DNA, occurring in *Methanosueta concilii* strain GP6 was isolated. The method of DNA isolation is not the usual method of CsCl-EtBr ultracentrifugation as is commonly employed for isolating plasmid DNA. It was, however, found to be the only efficient method (see discussion). Total genomic DNA from *M. concilii* GP6 was electrophoretically resolved as described in section 2.3. The band suspected of being plasmid DNA was excised from the gel by cutting it out using a razor blade, under a UV lightsource and pooled. These bands containing the suspected plasmid DNA were then electro-eluted using a Bio-Rad Model 422 electro-eluter as per manufacturers instructions. The resulting DNA was again pooled in Tris EDTA buffer(usually gel slices from 5-20 wells of agarose gels), a 10 ul sample of this DNA was electrophoretically resolved as per above description to verify the purity of the band excised and the remainder stored at 4° for further investigation.

2.5 PLASMID pDDK CHARACTERIZATION

In order to characterize the plasmid DNA, size, copy number, and restriction mapping were investigated. The size of the plasmid was determined by electrophoresis of Pst I and Eco RI digested (as per manufactures instructions) plasmid DNA against known standards of lambda Hind III (Boeringer Mannheim) and 1kb standard (Boeringer Mannheim). The sizes of the restricted fragments of the DNA of interest were determined by relating gel mobility to the fragments of the standards. Sizes were determined by using a standard curve of the fragments in the standards (see section 1.16).

For copy number determination of the plasmid, now designated pDDK, several dilutions of total DNA isolated from strain GP6 were analyzed by agarose gel electrophoresis. By comparing the intensity of the plasmid band and the chromosomal DNA bands, the copy number can be determined with the assumption that the size of the chromosomal DNA of *M. concilii* GP6 is approximately 1.1×10^9 Da (Nolling et al. 1991). $1 \text{ MDa} = 1,500 \text{ bp}$

Restriction mapping is a useful tool in determining potential cloning sites, therefore, the mapping of restriction enzyme positions within the plasmid as initial characterization is necessary. Restriction fragment mapping was achieved by again a novel approach. First, single digestions of the plasmid DNA were electrophoretically resolved in an agarose gel with standards as described above, restriction enzymes used (as per manufactures instructions) were Bgl II, Hpa I, Eco RI and Pst II. Double

restriction digests of the plasmid DNA (BglII/HpaI; BglII/EcoRI; BglII/PstI; HpaI/PstI; HpaI/EcoRI; PstI/EcoRI) were also electrophoretically resolved in an agarose gel under the same conditions and with the same standards. All gels were stained as described above and photographed with a ruler under UV light. After initial investigation of both sample types, single digestions and double digestions were run simultaneously on two agarose gels. (NOTE: the gels used for the Southern blots had a very low concentration of pDDK as the DIG is extremely sensitive, overloading of the gel leads to poorly defined bands, therefore, the gels used for the Southern blots are not included here as no bands were visible). From initial single digests two bands were excised, a 6.9 kb band from the single Bgl II digestion and a 7.2 kb band from the Eco RI single digestion, electrophoretically resolved in low melting point agar (Sigma) and stored in sealed Eppendorf tubes at 4°. Southern blots of the agarose gels containing the double digested DNA were obtained (see Maniatis, 1989 and appendix I). The excised single bands from the low melting point agar gels were used as the probes for the Southern blots (Southern, 1975). The method of labelling used was chemiluminescence with digoxigenin (DIG) (Amersham, as per manufactures instructions, labelling protocol appendix II). Following labelling the probes were hybridized to the filters obtained from the southern blotting and autoradiographed using the DIG antibody-chemiluminescence treatment (Appendix II). From the autoradiogram the position of the probe DNA's in the double restricted fragments could be determined therefore enabling the construction of the restriction fragment map.

SECTION III

RESULTS AND DISCUSSION

3.1 CELL LYSIS

The results of the various treatments with frozen-thawed cells are shown in Table IV. The complete process of pronase, DTT and SDS was necessary to obtain maximum lysis for several of the strains (Meakin et al., 1991)(Table 4). In the case of *Methanothermus fervidus*, where % lysis was less than for most of the other strains tested, repeated freeze/thawing did not substantially increase lysis. Furthermore, in certain methanogens (e.g., *Methanobacterium* strain G2R and *Methanobacterium bryantii* M.o.H.G), the pretreatment of frozen-thawed cells with SDS was less effective at 35°C compared to 55°C, which led to the use of 55°C routinely. Treatment with SDS alone lysed cells well in the cases of *Methanobacterium bryantii* strain M.o.H, *Methanospirillum hungatei* strains GP1 and JF1, *Methanosarcina barkeri* and *Methanosaeta concilii*. In several cases, freeze/thawing of cells in ABE was sufficient for lysis. Not surprisingly, two of these strains were *Methanococcus* species, which lyse quite readily in the absence of salt and osmotically stabilizing solutions (e.g. sucrose)(Jones et al., 1983). *Methanosarcina mazei* lysed readily, whereas *Methanosaeta concilii* and *Methanosarcina acetivorans* exhibited partial lysis. *Methanosarcina acetivorans* is of marine origin, and known to lyse in the absence of stabilizing salts (Sowers et al., 1983). Freeze-thawing sensitized the cells of several methanogens to the

combination of pronase, DTT, and SDS treatments (Plate 2, Table 4). This effect was less pronounced for the four remaining methanogens listed in Table III but not shown in Plate 2.

Pronase greatly influenced the lysis of *Methanobrevibacter smithii*, as well as the lysis of several other methanogens (Plate 3, Table 4). Pronase action was limited to cells which had been frozen and thawed, suggesting that freeze-thaw damage had exposed sites to enzyme attack. This data is supportive of a previous report showing that pronase contains a proteolytic enzyme capable of inducing lysis of *Methanobacterium formicicum* (Bush, 1985), and specifically strain Gø1 of *Methanosarcina barkerii* (Jussøfie, 1986).

Although the maximum lysis of each strain varied from 51-99%, enough DNA was extracted from 0.25g of each sample to perform several restriction analyses. In most cases, the genomic DNA was amenable to enzyme restriction analyses with PstI (Plate 4 a.), however, with *Methanothermobacter fervidus* [mol G+C 33% (Stetter et al., 1981)] and *Methanobacterium smithii* [molG+C 32% (Balch et al., 1979)], BglII (Plate 4 b.) was more effective than PstI. Several of these DNA's tested in this experiment have been restricted with other restriction enzymes or ligated with vector plasmids in the construction of genomic libraries (pers. com. Dr.J.Nash).

Recently Jarrel et. al. 1991, published a method of lysis for a range of methanogen species. This included liquid nitrogen freezing and grinding, although this method has been reported to produce DNA which could be restricted by endonucleases, personal observations have found this to be a time consuming method with variable results, often shearing the DNA.

ABLE 4 Degree of lysis of frozen-thawed methanogen cells.

Species:	pronase DTT SDS	pronase DTT	pronase SDS	lysis (%)			SDS	ABE only
				pronase	DTT	SDS		
<u>Methanobacterium bryantii</u> M.O.H	51	51	51	49	34	60*	10	
<u>Methanobacterium bryantii</u> M.O.H.G	61	57	63*	55	42	47	34	
<u>Methanobacterium capanole</u> GP9	95*	67	56	64	48	32	9	
<u>Methanobacterium formicicum</u> MF	74*	49	62	41	62	30	2	
<u>Methanobacterium strain</u> G2R	63*	39	55	38	15	29	21	
<u>Methanobacterium thermoautotrophicum</u> GC1	60*	23	29	26	20	25	10	
<u>Methanobrevibacter arboriphilus</u>	79*	59	75	54	55	47	25	
<u>Methanobrevibacter smithii</u>	81*	69	76	66	36	26	7	
<u>Methanococcus jannaschii</u>	99*	n.d. ^b	n.d.	n.d.	n.d.	n.d.	99*	
<u>Methanococcus voltae</u>	98*	n.d.	n.d.	n.d.	n.d.	n.d.	85	
<u>Methanosaeta concillii</u> GP6	87*	86	68	59	61	78	49	
<u>Methanosarcina acetivorans</u>	98*	n.d.	n.d.	n.d.	n.d.	n.d.	49	
<u>Methanosarcina barkeri</u>	81*	59	58	54	37	74	38	
<u>Methanosarcina mazei</u>	99*	n.d.	n.d.	n.d.	n.d.	n.d.	77	
<u>Methanospirillum hungatei</u> GP1	95*	19	92	15	15	92	15	
<u>Methanospirillum hungatei</u> JF1	95*	20	92	4	19	93	12	
<u>Ethanothermus fervidus</u>	54*	45	43	41	23	29	22	

Degree of lysis is expressed as %lysis as described in the text. Frozen-thawed cells, suspended in

E buffer, were subjected to the various procedures used to lyse the cells as described in the text.

n.d., not done.

minimum requirements for most successful lysis.

PLATE 2: LYSIS OF METHANOGEN CELLS FRESH VS. FROZEN

Lysis of methanogen cells comparing fresh (closed bars) and frozen-thawed (hatched bars) cells. The cells were lysed by treatment with pronase, DTT and SDS as described in the text, to both cells that had been frozen and cells that were used fresh.

PLATE 3: PRONASE-INDUCED LYSIS (% difference)

The effect of pronase on the lysis of fresh (closed bars) or frozen-thawed (hatched bars) cells of several methanogens. Fresh and frozen-thawed cells of each methanogen were divided into two aliquots. One aliquot was treated with pronase, while the other served as a control lacking pronase. All cell suspensions were treated with DTT and SDS, as described in the text, and lysis measured. Results are expressed as the difference in % lysis caused by the presence of pronase.

**PLATE 4: GEL ELECTROPHORESIS OF EXTRACTED DNA FROM
METHANOGENS**

Gel electrophoresis of extracted DNA from methanogens. DNA samples were electrophoretically resolved through 0.8% agarose gels in Tris-borate buffer at 70V for 3.5 h (panel A), or 1.0% agarose in Tris-acetate buffer at 30V for 16 h (Panel B), and stained with ethidium bromide. Numbers to the left represent DNA sizes (in bp) of the DNA standard. Starting with lane 2, the first sample in each pair is uncut DNA, while the second sample is digested DNA. Panel A (PstI-restricted DNA): lane 1, DNA standard (1kb ladder, Gibco/BRL); lanes 2-3, *M. espanolae* GP9; lanes 4-5, *M. smithii*; lanes 6-7, *M. concilii* GP6 (NOTE: the presence of pDDK in this picture after restriction with the endonuclease PstI could be the result of partial cutting) ;lanes 8-9, *M. fervidus*; lanes 10-11, *M. hungatei* JF1; lanes 12-13, *M. voltae*; lanes 14-15, *M. acetivorans*. Panel B (BglII-restricted DNA): lane 1, 1kb ladder; lanes 2-3, *M. fervidus*; lanes 4-5, *M. smithii*; lane 6, phage lambda DNA digested with HindIII.

Martin and Savage (1988) noted that the DNA, in crude lysates of the obligate anaerobe *Roseburia cercicola*, degraded upon exposure to air. Although this possibility was not critically evaluated in the case of methanogen DNA, the inclusion of DTT to aid in cell lysis may have had an additional role in the protection of the DNA. As a precaution, DTT may be included during the initial pronase digestion.

Presented here is a simple method to readily isolate restrictable genomic DNA from a wide range of species of methanogenic bacteria. The method involves harvesting mid-exponential phase cells, freeze-thawing them, and lysing them in ammonium bicarbonate-EDTA buffer, pH 8, by sequential treatments with pronase (37°C for 1h), DTT (23°C for 15 min) and SDS (55°C for 15 min). After removal of all the cell debris and SDS precipitation with potassium acetate, followed by centrifugation, DNA is recovered by precipitation with ethanol. Although not all the steps of this procedure are necessary (e.g. freeze-thawing or pronase treatment) for complete lysis of all the strains tested, their inclusion does not handicap the successful isolation of genomic DNA. Furthermore, the addition of pronase probably acts to degrade any contaminating DNases which would decrease the recoverable yield of DNA. Purity and concentrations were determined for all species, *M. concilii*, for example average a 260:280 ratio of 1.9, and a concentration of 4.360 ug/ul.

3.2 SUSPECTED METHANOGEN PLASMIDS AND A NOVEL METHANOGEN PLASMID

The plasmid screening showed a extrachromosomal band in *Methanosaeta concilii* GP6, named pDDK (see Plate 4 a. lane 7). Other species which indicated the possible presence of plasmids were *Methanobacterium espanolae* GP9, *Methanospirillum hungatei* JF1 and *Methanosarcina mazei*. The data for the suspected extrachromosomal elements in the four species listed, is not shown as this data was taken from the analysis of a number of preparations and subsequent agarose gel resolutions. The evidence was the presence of bands other than the chromosomal DNA band, further analysis would be necessary to confirm these findings. At this time it was decided to concentrate on one plasmid and pDDK from *Methanosaeta concilii* was chosen. This plasmid was chosen as it was an organism isolated in Dr. G. Patel's laboratory at N.R.C., it is also the only pure culture of an acetoclastic, mesophilic methanogen that can use only acetate as a carbon source.

3.3 ISOLATION AND PURIFICATION OF pDDK

Once pDDK was identified from the screening as a possible extrachromosomal element, it was purified from the chromosomal DNA. The first method attempted was CsCl-EtBr ultracentrifugation; after several unsuccessful attempts to separate the plasmid

from the chromosomal DNA other methods were explored. The reason for the unsuccessful separation by CsCl-EtBr ultracentrifugation remains unknown. Perhaps the plasmid form was nicked and became linear, such that it no longer behaved as a circular molecule. This altered mobility during centrifugation could result in its co-migration with the chromosomal DNA. It was at this time known from the screening procedure that the plasmid pDDK separated in gel electrophoresis and migrated very slowly forming the band above the chromosomal DNA. This indicated that the plasmid may be in a multiple state such as a trimer, tetramer or more or that the plasmid was very large > 20.0 kb. If this was the case it would be difficult to separate by ultracentrifugation. Migration of DNA using electrophoresis is size dependent up to approximately 20.0 kb., above this size DNA molecules tend to run with common mobility regardless of size. Pulse-field electrophoresis which is commonly used for large molecule separation (Serwer, 1990) was explored using a Hoeffer PC 750 pulse controller. This method separated the plasmid pDDK from the chromosomal DNA in the same manner as traditional gel electrophoresis. It was therefore used only to separate the plasmid band for excision as was traditional gel electrophoresis. It became apparent that a method to purify the plasmid was to electrophoretically resolve total DNA preparations from *Methanosueta concilii* GP6 (see Plate 5), excise the plasmid band and elute it from the agarose. This was performed as described in the methods resulting in a reasonably pure solution of pDDK (see Plate 6). The purity and concentration of pDDK were calculated as described in the methods and found to have a 260:280 ratio of 1.6-1.9 and a concentration of 0.53 ug/ul - 1.45 ug/ul (depending on preparation).

PLATE 5: TOTAL CHROMOSOMAL ELECTROPHORESIS OF GP6

1.0% agarose gels in 0.5% TAE buffer: 30v, 25mA overnight. Lane 1. 1 kb standard; Lanes 2-14. 30 ul total GP6 DNA; Lanes 15. Lambda HindIII. This gel is shown to describe the method of first electrophoretically resolving total *Methanosaeata concilii* GP6 DNA, then excising the plasmid band and eluting the plasmid DNA from these gel slices.

PLATE 6: ELUTED pDDK SAMPLE

1.0% agarose gel in 0.5% TAE; 30 mA, 25 V overnight, Lane 1. 1 kb standard; Lane 2. eluted pDDK (10 ul). The standard in this picture is inappropriate for the size of the plasmid band, it was included only as a standard to ensure the gel conditions were correct; this picture is included to show only that pDDK was purified from the chromosomal DNA and the preparation after elution contained pDDK.

1 2

2036

3.4 CHARACTERIZATION OF pDDK

The characterization of pDDK involved determining the size, restriction map, form and copy number of the plasmid. The size was determined to be 11.0 kb. This determination was from the addition of restriction fragments (see Plate 7), compared to standard fragments. The form was determined to be circular. Evidence supporting this claim was from the hybridization studies undertaken to complete the restriction map. The pattern of the restriction digest fragments (undigested, single digests and double digests) after probing on the autoradiograms revealed that the molecule was circular. The restriction map was constructed from data collected as described in the methods section (see autoradiograms, Plate 8 i., ii. and Table 5) and is shown in (Plate 9). The copy number was determined to be 1/5 of the chromosomal DNA; at 11.0 kb = approximately 10 copies per cell.

As mentioned in section 1.18, plasmids have been found in a number of methanogens. They are variable in size, ranging from 65 kb to 4.5 kb, pDDK at 11 kb is within this range. The copy numbers of methanogen plasmids also vary and as of yet all are cryptic, as no plasmid functional genes have been elucidated to date.

TABLE V FRAGMENT SIZES USED TO CALCULATE RESTRICTION MAP

i. SINGLE DIGESTS			
<u>BglII</u>	<u>HpaI</u>	<u>PstI</u>	<u>EcoRI</u>
6.900	6.100	2.950	7.600
1.800	4.500	2.680	3.300
1.635	2.500	1.880	1.300

ii. DOUBLE DIGESTS					
<u>BglII/HpaI</u>	<u>BglII/EcoRI</u>	<u>BglII/PstI</u>	<u>HpaI/PstI</u>	<u>HpaII/EcoRI</u>	<u>PstI/EcoRI</u>
6.600	3.050	1.800	2.900	16.500	2.650
3.750	2.560	5.450	2.600	4.600	0.080
3.400	1.850		1.850	3.250	
	1.720				

iii. SOUTHERN BLOTS HYBRIDIZED WITH DIG LABELLED PROBES					
A. <u>BglII</u> PROBE (6.90 kb)					
<u>BglII/HpaI</u>	<u>BglII/EcoRI</u>	<u>BglII/PstI</u>			
4.072	3.900	2.205			
3.059	2.980	1.900			
B. <u>EcoRI</u> PROBE (7.20 kb)					
<u>BglII/EcoRI</u>	<u>HpaI/EcoRI</u>	<u>PstI/EcoRI</u>			
4.072	4.361	3.059			
2.100	3.990	2.027			
2.027					

21 of/de 2

PM-1 3 1/2"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

PHOTOMASKS IN METHYLENE BLUE TESTING SINCE 1974

Microlith

1800 COUNTY ROAD 8, BURNING WOOD, OH 44027 USA
TEL: 914 426 7857 FAX: 914 426 7857 TLE: 5100000000

PLATE 7: RESTRICTION FRAGMENT ANALYSIS OF pDDK

Restriction digestion was performed as described in the methods section. 1.0% agarose gel, in 0.5% TAE buffer; 30 mA, 25 V overnight. Lanes 1: 1 kb standard, Lane 2-11: 10ul of pDDK used, Lane 2: BglII, Lane 3: HpaI, Lane 4: PstII, Lane 5: EcoRI, Lane 6: BglII/HpaI, Lane 7: BglII/EcoRI, Lane 8: BglII/PstI, Lane 9: HpaI/ PstI, Lane 10: HpaI/ EcoRI, Lane 11: PstI/ EcoRI, Lane 12: Lambda HindIII standard.

PLATE 8 i. AUTORADIOGRAMS OF DIG-PROBED SOUTHERN BLOTS
USING BglIII PROBE

Undigested pDDK, and double restriction digested pDDK electrophoretically resolved as per method section, southern blotted and probed with Dig labelled excised band a) BglIII probe, Lane 1. pDDK uncut; Lane 2. pDDK BglIII; Lane 3. pDDK BGLII/HpaI; Lane 4. pDDK BglII/EcoRI; Lane 5. pDDK BglII/PstI.

**PLATE 8 ii. AUTORADIOGRAMS OF DIG-PROBED SOUTHERN BLOTS
USING EcoRI PROBE**

Undigested pDDK, and double restriction digested pDDK electrophoretically resolved as per methods section, Southern Blotted and probed with Dig labelled excised band. b) EcoRI probe, Lane 1. pDDK uncut; Lane 2. pDDK EcoRI; Lane 3. pDDK EcoRI/BglII; Lane 4. EcoRI/HpaI; Lane 5. EcoRI/PstI.

PLATE 9: RESTRICTION FRAGMENT MAP OF pDDK

Constructed restriction fragment map of pDDK.

..... Bgl II probe
--- Eco RI probe

SECTION IV

CONCLUSIONS AND FUTURE PROSPECTS

Methanogens and the process of methanogenesis have revealed novel biological and physiological properties. Many of these novelties are specializations for a unique form of energy metabolism, a phenomenon common to the archaeobacteria (Jones et al., 1987). The diversity of biochemical and phylogenetic characteristics exhibited in the methanogens, once unravelled will reveal information of great interest to evolutionary and molecular biologists as well as the bacterial physiologists. The general areas of phylogeny, coenzyme, metal and membrane biochemistry have already proven this to be the case. The future prospects in molecular biology and genetic engineering are quickly emerging. It is the merging of all the scientific disciplines and their discoveries which may lead to utilization of these organisms for environmental, industrial and medical advancements.

The purpose of this thesis was to investigate the molecular biology of these organisms with the goal to increase the information available on methods of lysis and the presence of plasmids. This information will add to the increasing knowledge becoming available.

The method of lysis in this paper allows for a large number of species with variable cell ultrastructures to be readily lysed. This is important in screening processes for plasmids and extraction of chromosomal DNA for further genetic studies. The

characterization of the plasmid pDDK is only the beginning of possible future genetic studies on the host organism *Methanosaeta concilii*. More importantly the protocols presented here may be used to study other undiscovered methanogen plasmids. This as well as the advances in solid media culturing may lead to the development of a genetic exchange system by the identification of selectable traits. The subsequent problem of successful transfer and the identification of transfer of DNA from the donor strain to the recipient strain may be aided by the discovery of a plasmid capable of acting as a vector.

There remains much work to be done on all aspects of these unique organisms. Future work from this thesis would involve the sequencing of the plasmid pDDK, once sequenced the possible function of genes carried by the plasmid may be elucidated by comparing the sequence to a bank of known genes from methanogens or eubacteria. As well as hybridization studies of it to the host chromosomal DNA may reveal information of the plasmid function and evolution. Further hybridization studies of pDDK to other known plasmids may also be of interest in determining the origins of the plasmid as well may reveal information in possible function. Other work would involve the curing of plasmid pDDK. This could be accomplished by successive transfers of the culture under conditions stress; using temperature, pH, or metabolic requirements variations. As this plasmid is now available cloning experiments to determine if pDDK could be used as a vector may be attempted. Another interesting series of experiments would be to determine if the acetoclastic ability to utilize acetate as a carbon source is conferred on the plasmid. To examine this question other acetoclastic organisms such as *M. barkeri*, *M. mazei*, *M. acetivorans* could be examined. It is known that *M. acetivorans* contains

a plasmid pC2A, as well *M. mazei* is suspected of carrying a plasmid, these along with *M. barkeri* should be examined indepth to determine if infact the plasmid is conferring the ability to use acetate.

APPENDIX 1

SOUTHERN BLOT PROTOCOL

- 1) After electrophoresis of DNA samples, the gel is stained and destained as per methods and photographed.
- 2) Denature the gel in 0.5 M NaOH and 1.5 M NaCl for 30.0 min. shaking at room temperature.
- 3) Neutralize gel in 1.0 M $\text{NH}_4\text{Acetate}$ and 0.02 M NaOH for 30.0 min. shaking at room temperature.
- 4) Cut 2 pieces of Hybond Nylon filter (Manufacturer) and 6 pieces Whatman filter paper (Manufact) the same size as the gel.
- 5) Place the three pieces of the Whatman filter paper soaked in the neutralizing solution on a flat hard surface, being careful to role out any air bubbles with a pipette. Place the gel DNA side up on the filter paper. On top of the gel place the Hybond filter (use gloves to avoid fingerases) and the remaining soaked Whatman filter paper. Place a dry sponge on top of the Whatman and a hard flat surface above this (a plastic or glass plate works well), place weights on the plastic plate and allow transfer to proceed overnight (2 hrs. is sufficient if necessary).
- 6) Separate the gel and the filter and rinse the filter in 2X SSC to remove bits of agarose. Expose the Hybond filter to UV light for 5 min. to fix.
- 7) The filter is now ready for hybridization. (see appendix II, for protocol)

APPENDIX II

(pers. comm. J. Nash)

Hybridization with DIGOXIGENIN-labelled probes (Part 1).1. Labelling of the probe DNA

a. Solutions:

- DIG 10x dNTP labelling mixture.

1 mM dATP	2.5 µl of 10 mM dATP
1 mM dCTP	2.5 µl of 10 mM dCTP
1 mM dGTP	2.5 µl of 10 mM dGTP
0.65 mM dTTP	1.75 µl of 10 mM dTTP
0.35 mM DIG-dUTP	8.75 µl of 1 mM DIG-dUTP (Boehringer Mannheim)
dH ₂ O	7 µl

- React 2 10x buffer from BRL

- Random primers (from Amersham or New England Biolabs, or Boehringer Mannheim or Gibco-BRL - they're all much the same).

- Probe DNA (50 to 1000 ng is optimum, but 10 ng should do).

b. Reaction:

- Denature the probe DNA in a 10 to 32.5 µl volume (made up with dH₂O) by boiling for 5 min at 100 °C, followed by 5 min on ice.

- Set up the following reaction tube:

React 2 10x	5 µl
denatured DNA	10 to 32.5 µl
DIG 10x dNTP labelling mixture	5 µl
random primers	5 µl
dH ₂ O	to 50 µl final volume (incl. Klenow)
Klenow polymerase	2.5 µl

Incubate at 37°C for at least 3 hr, but 16 hr is optimum. If incubation is for an extended period, add 100 µl of light mineral oil to the tube. Extended incubations generate/synthesize more probe fragments.

Remove the mineral oil by spotting the whole sample onto clean Parafilm. The oil will disperse leaving the aqueous sample as a drop on the Parafilm. Transfer the sample to a clean Eppendorf tube.

Add:	4 μ l	0.5 M EDTA, pH 8
	12.6 μ l	4M LiCl
	375 μ l	cold absolute ethanol.

Precipitate at -70°C for 30 min (usually EtOH precipitations are done at room temp. This is different). Spin at 4°C for 15 min. Wash with 70% ethanol and absolute ethanol. Resuspend in 50 to 100 μ l TE buffer.

Probe concentration can be estimated by spotting on ethidium bromide plates.

Use the probe at 20 to 100 ng / ml hybridization solution. Denature the probe by boiling at 100°C and chilling on ice before use.

The probe can be reused over and over again. If it is in a large volume of hybridization solution, increase the boil and chill times to 15 min. Store the probe frozen between uses.

2. Filter hybridization and autoradiography.

Avoid scratching the filters or drying them out. This leads to BAD background problems! Volumes of pre-hyb., hyb., and wash solutions are given per 100 cm^2 filter (10 cm x 10 cm). Adjust your volumes proportionally.

- Remove the debris from the filter(s) (if it's a colony/phage lift) by incubation with 5XSSC, 0.1% SDS at 65°C , with shaking. Remove extra debris using a gloved finger.
- An hour before the pre-hybridization, make the hybridization solution. Make approx. 24 ml per 100 cm^2 filter - a circular Petri plate filter is approx 50 cm^2 .

Hybridization solution (per 100 ml):

5x SSC	25 ml 20x SSC
0.1% N-lauroyl sarcosine, Na salt (NLS)	2 ml 5% NLS
0.2% SDS	2 ml 10% SDS
1% Boehringer Mannheim blocking agent	2 g of powder
or 10 ml of blocking agent 10x (see recipe below)	
dH ₂ O	to 100 ml.

If powdered blocking agent is used, stir at 50° to 70° for 1 hr. If blocking agent 10x is used instead, incubate at 50° to 70° for 30 min.

- Prehybridize in hybridization solution, with shaking, at 68°C for > 1 hr. Use 20 ml hyb. sol. / 100 cm² filter. Pour off pre-hyb. sol. immediately before adding hyb. sol. PLUS probe.
- Hybridize in hyb. sol. PLUS 10 to 100 mg/ml denatured probe at 68°C for 6 hr to overnight (preferred), with gentle shaking. Use 2.5 ml hyb. sol. PLUS probe per 100 cm² filter.
- After hybridization, transfer the filters to a glass dish containing 2xSSC, 0.1% SDS. Store the probe for reuse.

The washes are:

Two washes in 2x SSC, 0.1% SDS at room temp. for 10 min.
One wash in 1x SSC, 0.1% SDS at 68°C for 10 min.

Use 50 ml wash sol. per 100 cm² filter. Wash with shaking.

The filters are now ready for the antibody treatment.

3. Antibody treatment.

- Wash filters briefly (2 to 5 min) in buffer 1M at room temperature with shaking. Use 50 ml per 100 cm² filter.

Buffer 1M: 100 mM maleic acid
150 mM NaCl, pH 7.5. Usually made as a 5x concentrate.

- Incubate filters in buffer 2M at room temp, with gentle shaking for > 30 min. Use 100 ml per 100 cm² filter.

Buffer 2M: 10% blocking agent 10x in buffer 1M,
i.e. for 100 ml of buffer 2M, use 10 ml blocking agent 10x + 20 ml buffer 1M
5x + 70 ml dH₂O.

Blocking agent 10x:

Add 50 g blocking agent powder (Cat. 1096 176, Boehringer Mannheim) to a 500 ml measuring cylinder. Add 100 ml buffer 2M, and dH₂O to 500 ml. Stir at 60°C until the powder has all dissolved. Autoclave the solution, and dispense aseptically into 40 ml lots. Store at 4°C.

- Incubate the filters in buffer 2M PLUS antibody. Antibody (Anti-digoxigenin-Ap, FAB fragments Cat. 1093274, Boehringer Mannheim) should be diluted at 75 mU/ml -i.e. diluted 1: 10 000. Incubate filters with gentle shaking for > 30 min at room temp. Use 20 ml per 100 cm² filter.
- Wash the filters twice for 15 min at room temp, with gentle shaking, in buffer 1M. Use 100 ml per 100 cm² filter.
- Equilibrate filters for > 2 min in buffer 3. Use 20 ml per 100 cm² filter.

Buffer 3: 100 mM Tris, pH 9.5
100 mM NaCl
50 mM MgCl₂

4. Chemiluminescence treatment.

Use either Lumiphos 530 or AMPPD, there's no real difference, results-wise.

- Lumiphos 530 protocol:

Put 2 to 3 ml Lumiphos in a large watchglass. Wet filter (probe side down) in the solution. Seal in heatseal bags (don't use ziplock, it inactivates the Lumiphos). Incubate at 37° for 15 min. Expose to film for 15 to 60 min. The light signal is active for approx 24 hr, so re-exposure is possible.

Store the used Lumiphos in a scintillation vial in the dark. it is reusable.

- AMPPD protocol:

AMPPD comes at 10 mg/ml (3.5 mM). Dilute it 1:100 in buffer 3. Incubate filters in AMPPD for a few min. Seal the filters in a heatseal bag. Incubate at 37° for 5 to 15 min. Expose to film for 15 to 60 min. AMPPD is also reusable. Store it at 4° in the dark. The signal is also active for approx 24 hr.

REFERENCES

Allmansberger, R., C. Bollschweiler, U. Konheiser, B. Muller, E. Muth, G. Pasti and A. Klein. 1986. Arrangement and expression of methyl CoM reductase genes in *Methanococcus voltae*. Syst. Appl. Microbiol. 7, 13-17.

Arkani, A. and R. Freter. 1972. Use of anaerobic glove boxes for the cultivation of strictly anaerobic bacteria. Am. J. Clin. Nutri. 25, 1329-1334.

Balch, W.E. and R.S. Wolfe. 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. 32, 781-791.

Balch, W.E., G.E. Fox, L.J. Magrum, C.R. Woese and R.S. Wolfe. 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. 43, 260-296.

Bernhardt, G., R. Jaenicke and H.D. Ludemann. 1987. High pressure equipment for growing methanogenic microorganisms on gaseous substrates at high temperature. Appl. Environ. Microbiol. 53, 1876-1879.

Bertani, L. and G. Baresi. 1984. Study of methanogens by genetic techniques. SERI/STR-231-2291, Solar Energy Research Institute.

Bertani, G. and L. Baresi. 1987. Genetic transformation in the methanogen *Methanococcus voltae* PS. J. Bacteriol. **168**: 2730-2738.

Beveridge, T.J., M. Stewart, R.J. Doyle, and G.D. Sprott. 1980. Unusual stability of the *Methanospirillum hungatei* sheath. J. Bacteriol. **162**, 728-737.

Beveridge, T.J., G.B. Patel, B.J. Harris and G.D. Sprott. 1986 a. The ultrastructure of *Methanothrix concilii*, a mesophilic acetoclastic methanogen. Can J. Microbiol. **32**, 703-710.

Beveridge, T.J., B.J. Harris, G.B. Patel and G.D. Sprott. 1986 b. Cell division and filament splitting in *Methanothrix concilii*. Can. J. Microbiol. **32**, 779-786.

Beveridge, T.J., B. Harris and G.D. Sprott. 1987. Septation and filament splitting in *Methanospirillum hungatei*. Can. J. Microbiol. **33**, 725-732.

Bhatnagar, L., J.G. Zeikus and J.P. Aubert. 1986. Purification and characterization of glutamine synthetase from the archaebacterium *Methanobacterium ivanovi*. J. Bacteriol. **165**, 638-643.

Bhatnagar, L., M.K. Jain, J.G. Zeikus and J.P. Aubert. 1986. Isolation of auxotrophic mutants in support of ammonia assimilation via glutamine synthetase in *Metanobacterium ivanovi*. Arch. Microbiol. **144**, 350-354.

Blaut, M., V. Muller and G. Gottschalk. 1990. Energetics of Methanogens. In: The Bacteria vol. XII. Bacterial Energetics Ed. T.A. Krulwich. Academic Press, Inc. New York, N.Y. Pg. 505-537.

Birnbaum, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA, Nucleic Acids Res. **7**, 1513-1523.

Bock, A. and O. Kandler. 1985. Antibiotic sensitivity of archaeobacteria. In: The Bacteria vol.8 Archaeobacteria. eds. C.R. Woese and R.S. Wolfe. Academic Press, Inc., New York. pg. 525-544.

Bollschweiler, C. and A. Klein. 1982. Polypeptide synthesis in *Escherichia coli* directed by cloned *Methanobrevibacter arboriphilus* DNA. Zentralbl. Bakteriол. Parasitenkd. Infektionskr. hyg. Abt. 1 Orig. Reihe C. **3**, 101-109.

Bokranz, M. and A. Klein. 1987. Nucleotide sequence of methyl coenzyme M reductase gene cluster from *Methanosarcina barkeri*. Nucleic Acids Res. **15**, 4350-4351.

Bokranz, M. G. Baumner, R. Allmansberger, D. Ankel-Fuchs and A. Klein. 1988. Cloning and characterization of the methyl coenzyme M reductase genes from *Methanobacterium thermoautotrophicum*. J. Bacteriol. **170**(2), 568-577.

Bowen, T.L. and W.B. Whitman. 1987. Incorporation of exogenous purines and pyrimidines by *Methanococcus voltae* and isolation of analog-resistant mutants. Appl. Environ. Microbiol. **53**(8), 1822-1826.

Broda, P. 1979. Plasmids. W.H. Freeman. Oxford. pp.1-22.

Brown, J.W. and C. Daniels and J. Reeve. 1989. Gene structure, organization, and expression in archaebacteria. CRC in Microbiology, **10**(4), 287-337.

Bryant, M.P. and I.M. Robinson. 1961. An improved nonselective culture medium for rumen bacteria and its use in determining diurnal variation in of numbers of bacteria in the rumen. J. Dairy Sci. **44**, 1446-1456.

Bryant, M.P., B.C. McBride and R.S. Wolfe. 1968. Hydrogen-oxidizing methane bacteria. I. cultivation and methanogenesis. J. Bacteriol. **95**, 1118-1123.

Bush, J.W. 1985. Enzymatic lysis of the pseudomurein containing methanogen *Methanobacterium formicicum*. J. Bacteriol. **163**: 27-36.

Charlebois, R., W.L. Lam, S. Cline and W.F. Doolittle. 1987. Characterization of pHV2 from *Halobacterium volcanii* and its use in demonstrating transformation of an archaeobacterium. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8530-8534.

Cline, S.W. and W.F. Doolittle. 1987. Efficient transfection of the archaeobacterium *Halobacterium halobium*. *J. Bacteriol.* **169**, 1341-1344.

Cline, S.W., W.L. Lam, R. Charlebois, L.C. Schalkwyk and W.F. Doolittle. 1989. Transformation methods for halophilic archaeobacteria. *Can. J. Microbiol.* **35**, 148-152.

Cram, D.S., B.A. Sherf, R.T. Libby, P.J. Mattaliano, K.L. Ramachandran and J.N. Reeve. 1987. Structure and expression of the genes, merBDGCA, which encode the subunits of component C of methyl coenzyme M reductase in *Methanococcus vannielii*. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3992-3996.

Daniels, L. 1984. Biological methanogenesis: physiological and practical aspects. *Trends in Biotechnol.* **2**, 91-98.

Daniels, L., N. Balchiet and K. Harmon. (1985). Widespread distribution of a 5-deazaflavin in actinomyces and related bacteria. *Syst. Appl. Microbiol.* **65**, 12-17.

Danson, M.J. 1989. Central metabolism of the archaeobacteria; an overview. *Can. J. Microbiol.* **35**, 58-64.

Davis, R.P. and J.E. Harris. 1985. Spontaneous protoplast formation of *Methanosarcina barkeri*. *J. Gen. Microbiol.* **131**, 1481-1486.

Dennis, P.P. 1986. Molecular biology of archaeobacteria. *J. Bacteriol.* **168**, 471-478.

Eckhardt, T. 1978. A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. *Plasmid*, **1**, 584-588.

Elhardt, D. and A. Bock. 1982. An in vitro polypeptide synthesizing system from methanogenic bacteria: sensitivity to antibiotics. *Mol. Gen. Genet.* **188**, 128-134.

Fox, G.E., E. Stackebrandt, R.B. Hespell, J. Gibson, J. Maniloff, T.A. Dyer, R.S. Wolfe, W.E. Balch, R. Tanner, L. Magrum, L.B. Zallen, R. Blakemore, R. Gupta, L. Bonen, B.J. Lewis, D.A. Stahl, K.r. Luehrsen, K.N. Chen, C.R. Woese. 1980. The phylogeny of prokaryotes. *Science* **209**, 457-463.

Frea, J.I. 1982. Methanogenesis: Its role in the carbon cycle. In: Microbial Chemoautotrophy. eds. W.R. Strohl and O.H. Tuovinen. Ohio State U. Press, Columbus. pg. 228-253.

Freifelder, D. 1983. Molecular Biology A Comprehensive Introduction to Prokaryotes and Eukaryotes. Jones and Bartlett Pub. Inc. Boston. pps. 701-807.

Gernhardt, P., O. Possot, M. Foglino, L. Sibold and A. Klein. 1990. Construction of an integration vector for use in the archaeobacterium *Methanococcus voltae* and expression of an eubacterial gene. *Mol. Gen. Genet.* **221**, 273-279.

Gottschalk, G. and M. Blaut. 1990. Generation of proton and sodium motive forces in methanogenic bacteria. *Biochim. Biophys. Acta*, **1018**, 263-266.

Grinsted J. and M. Bennett. 1988. Preparation and electrophoresis of plasmid DNA. In: Methods in Microbiology, vol. 21 Plasmid Technology. Eds. J. Grinsted and P.M. Bennett. Academic Press, London. pg. 143-154.

Haas, E.S., L.A. Hook and J.N. Reeve. 1986. Antibiotic resistance caused by permeability changes of the archaeobacterium *Methanococcus vanniellii*. *FEMS Microbiol. Lett.* **33**, 185-188.

Hackett, N.R. and S. DasSarma. 1989. Characterization of the small endogeneous plasmid of *Halobacterium* strain SB3 and its use in transformation of *H. halobium*. *Can. J. Microbiol.* **35**, 86-91.

Hamilton P.L. and J.N. Reeve. 1985. Sequence divergence of an archaebacterial gene cloned from a mesophilic and a thermophilic methanogen. *J. Mol. Evol.* **22**, 351-360.

Hamilton, P.L. and J.N. Reeve. 1985. Cloning and expression of archaebacterial DNA from methanogens in *Escherichia coli*. In: Microbial Chemoautotrophy. pg. 291-308.

Hamilton, P.L. and J.N. Reeve. 1985. Structure of genes and an insertion element in the methane producing archaebacterium *Methanobrevibacter smithii*. *Mol. Gen. Genet.* **200**, 47-59.

Hamilton, P.L. and J.N. Reeve. 1986. Molecular biology of archaebacteria. In: Microbiology pg. 337-348. (ed.) L. Leive. American Society for Microbiology, Washington, D.C.

Hardy, K.J (ed). 1987. Plasmids a practical approach. IRL Press. Washington, D.C.

Hardy, K.J. 1986. Aspects of Microbiology 4, Bacterial Plasmids 2nd ed. American Society for Microbiology. Washington, D.C.

Harris, J.E., D.M. Evans, M.R. Knox and D.B. Archer. 1986. Genetic approaches with methanogens important in mesophilic digestion. In: Progress in Biotechnology 2, Biology of anaerobic bacteria. pg. 123-137. (eds.) H.C. Dubourguier. Elsevier, New York.

Hintermann, G., H.M. Fischer, R. Crameri and R. Hutter. 1981. Simple procedure for distinguishing CCC, OC, and L forms of plasmid DNA by agarose gel electrophoresis. Plasmid. 5: 371-373.

Hippe, H. 1984. Maintenance of methanogenic bacteria. In: Maintenance of microorganisms. A Manual of Laboratory Methods. pg. 69-81. Academic Press, New York.

Holdeman, L.V. and W.E.C. Moore (ed). 1972. Anaerobe laboratory manual. Virginia Polytechnic Institute and State University, Blacksburg.

Holmes, D.S. and H. Quigley. 1981. Anal. Biochem. 114, 193-197.

Hummel, H., M. Jarsch and A. Bock. 1985. Unique antibiotic sensitivity of protein synthesis in atchaebacteria and the possible structural basis. In: Molecular biology of archaeobacteria. pg. 370-374.

Hummel, H., U. Bar., G. Heller and A. Bock. 1985. Antibiotic sensitivity pattern of in vitro polypeptide synthesis systems from *Methanosarcina barkeri* and *Methanospirillum hungatei*. System. Appl. Microbiol. **6**, 125-131.

Hungate, R.E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14**, 1-49.

Jain, M.K., and G. Zeikus. 1987. Methods for the isolation of auxotrophic mutants of *Methanobacterium ivanovii* and initial characterization of acetate auxotrophs. Appl. Environ. Microbiol. **53**(6), 1387-1390.

Jarrell, K.F., J.R. Colvin and G.D. Sprott. 1982. Spontaneous protoplast formation of *Methanosarcina barkerii*. J. Bacteriol. **149**, 346-353.

Jarrell, K.F., D. Faguy, A. Herbert M. Kalmokoff. 1991. A General method of isolating high molecular weight DNA from methanogenic Archaea. (not yet pub.)

Jarsch, M., J. Altenbucher and A. Bock. 1983. Physical organization of the genes for ribosomal RNA in *Methanococcus vannielii*. Mol. Gen. Genet. **189**, 41-47.

Jones, W.J., J.A. Leigh, F. Mayer, C.W. Woese, and R.S. Wolfe. 1983. *Methanococcus jannaschii* sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. Arch. Microbiol. **136**: 136-261.

Jones, W.J., J.R. Nagle and W.B. Whitmann. 1987. Methanogens and the diversity of archaebacteria. Microbiol. Rev. **51**: 135-177.

Jusselie, A., F. Mayer and G. Gottschalk. 1986. Methane formation from methanol and molecular hydrogen by protoplasts of new methanogenic isolates and inhibition by dicyclohexylcarbodiimide. Arch. Microbiol. **146**, 245-249.

Kandler, O., and H. Hippe. 1977. Lack of peptidoglycan in the cell walls of *Methanosarcina barkeri*. Arch. Microbiol. **113**:57-60.

Kandler, O. and H. König. 1978. Chemical composition of the peptidoglycan-free cell walls of methanogenic bacteria. Arch. Microbiol. **118**:118-152.

Kandler, O. 1982. ed. Archaebacteria. Gustav Verlag, Stuttgart.

Kandler, O. and H. König. 1985. Cell envelopes of archaebacteria. In: The Bacteria, vol. VIII: Archaebacteria. (eds.) C.R. Woese and R.S. Wolfe. pp. 413-458 Academic Press Inc. New York, N.Y.

Kandler, O. and W. Zillig. 1985. eds. Archaeobacteria. Gustav Fischer Verlag, Stuttgart.

Kates, M. 1988. Structure, physical properties, and function of archaeobacterial lipids. In: Biological Membranes: Aberrations In Membrane Structure and Function. (eds.) Karnovsky, M.L., A.M. Leaf and L.C. Bolis. pg. 357-384. Alan R. Liss. Inc., New York.

Keltjens, J.T. 1984. Coenzymes of methanogenesis from hydrogen and carbon dioxide. *Antonie van Leeuwenhoek J. Microbiol.* **50**, 383-396.

Kiener, A., C. Holliger and T. Leisinger. 1984. Analogue-resistant and auxotrophic mutants of *Methanobacterium thermoautotrophicum*. *Arch. Microbiol.* **139**, 87-90.

Kiener, A., T. Rechsteiner and T. Leisinger. 1986. Mutation to pseudomonic acid resistance of *Methanobacterium thermoautotrophicum* leads to an altered isoleucyl-tRNA synthetase. *FEMS Microbiol. Lett.* **33**, 15-18.

Kiener, A., H. König, J. Winter, and T. Leisinger. 1987. Purification and use of *Methanobacterium wolfei* pseudomurein endopeptidase for lysis of *Methanobacterium thermoautotrophicum*. *J. Bacteriol.* **169**: 1010-1016.

Kleinschmidt, A.K. 1968. Monolayer techniques in electron microscopy of nucleic acid molecules. *Methods in Enzymol.* **12**, 361-377.

Knox, M.R. and J.E. Harris. 1988. Isolation and characterization of mutants of mesophilic methanogenic bacteria resistant to analogues of DNA bases and nucleosides. *Arch. Microbiol.* **149**, 557-560.

Kirsop, B.H. 1990. Methanogenesis. *CRC Critical Reviews in Biotechnology.* **1**(2), 109-159.

Kleinschmidt, A.K. (1986). Monolayer techniques in electron microscopy of nucleic acids. *Methods Enzymol.* **12**: 361-377.

Konheiser, U., G. Pasti, C. Bollschweiler and A. Klein. 1984. Physical mapping of genes coding for two subunits of methyl CoM reductase component C of *Methanococcus voltae*. *Mol. Gen. Genet.* **198**, 146-152.

Konig, H. and K.O. Stetter. 1986. Studies on archaeobacterial S layers. *System. Appl. Microbiol.* **7**, 300-309.

Konig, H. 1988. Archaeobacterial cell envelopes. *Can. J. Microbiol.* **34**: 395-406.

Konig, H. 1988. Archaeobacteria. In: Biotechnology, vol. 66. Special microbial processes. eds. H. J. Rehn and G. Reed. Cte Publishers, New York, New York. pg.697-728.

Kreisl, P., and O. Kandler. 1986. Chemical structure of the cell wall polymer of *Methanosarcina*. Syst. Appl. Microbiol. 7:293-299.

Kushwaha, S.C. M. Kates, G.D. Sprott, and I.C.P. Smith. 1981. Novel polar lipids from the methanogen *Methanospirillum hungatei* GP1. Biochim. Biophys. Acta. 664, 156-173.

Ladapo, J. and W.B. Whitman. 1990. Method for the isolation of auxotrophs in the methanogenic archaeobacteria: role of acetyl-CoA pathway of auxotrophic CO₂ fixation in *Methanococcus maripaludis*. Proc. Natl. Acad. Sci. U.S.A. 87, 5598-5602.

Langworthy, T.A. 1985. Lipids of Archaeobacteria. In: The Bacteria, Vol. VIII, Archaeobacteria. (eds.) C.R. Woese and R.S. Wolfe. 459-498. Academic Press Inc. New York, N.Y.

Maniatis, T., E.F. Fritsch and J. Sambrook. 1982, 1989. Molecular Cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mavarech, M. and R. Werczberger. 1985. Genetics transfer in *Halobacterium volcanii*. J. Bacteriol. **162**, 461-462.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J.Mol. Biol. **3**:208-218.

Martin, J.H. and D.C. Savage. 1988. Degradation of DNA in cells and extracts of the obligately anaerobic bacterium *Roseburia cericola* upon exposure to air. Appl. Env. Microbiol. **54**, 1619-1621.

Meakin, S.A., J.H.E. Nash, W.D. Murray, K.J. Kennedy and G.D. Sprott. 1991. A generally applicable technique for the extraction of restrictable DNA from methanogenic bacteria. J. Microbiol. Methods. **14**, 119-126.

Meille, L. A. Kiener and T. Leisinger. 1983. A plasmid in the Archaeobacterium *Methanobacterium thermoautotrophicum*. Mol. Gen. Genet. **191**: 480-484.

Meille, L. and J.N. Reeve. 1985. Potential shuttle vectors based on the methanogen plasmid pME2001. Biotechnology, pg. 69-72.

Meille, L. P. Abendschein and T. Leisinger. 1990. Transduction in the archaeobacterium *Methanobacterium thermoautotrophicum* Marburg. **172**(6), 3507-3508.

Micheletti, P.A., K.A. Sment and J. Konisky. 1991. Isolation of a coenzyme-M auxotrophic mutant and transformation by electroporation *Methanococcus voltae*. J. Bacteriol.

Mitchell, R.M., L.A. Loeblich, L.C. Klotz and A.R. Loblich. 1979. DNA organization of *Methanobacterium thermoautotrophicum*. Science. **204**, 1082-1084.

Nolling, J., M. Frijlink and W.M. De Vos. 1991. Isolation and characterization of plasmids from different strains of *Methanobacterium thermoformicicum*. J. Gen. Microbiol., **137**, 1981-1986.

Myers, J.A., D. Sanchez, L.P. Elwell and S. Falkow. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**(3), 1529-1537.

Nuber, E. and H. Konig. 1986. S layer studies on three species of *Methanococcus* living at different temperatures. Can. J. Microbiol. **33**, 256-261.

Nuber, E. and H. Konig. 1987. Cell envelope studies on three species of *Methanococcus* living at different temperatures. Can. J. Microbiol. **33**: 256-261.

Patel, G.B. G.D. Sprott, R.W. Humphrey, and T.J. Beveridge. 1986. Comparative analyses of the sheath structures of *Methanothrix concilli* GP6 and *Methanospirillum hungatei* strains GP1 and JF1. *Can. J. Microbiol.* **32**: 623-631.

Patel G.B., and G.D. Sprott. 1990. *Methanosaeta concilii* gen.nov., sp. nov. ("*Methanothrix concilli*") and *Methanosaeta thermoacetophila* nom. rev., comb. nov. *Int. J. Syst. Bacteriol.* **40**: 79-82.

Patel, G.B., G.D. Sprott, and J.E. Fein. 1990. Isolation and characterization of *Methanobacterium espanolae* sp. nov., a mesophilic, moderately acidophilic methanogen. *Int. J. Syst. Bacteriol.* **40**: 12-18.

Possot, O., P. Gernhardt, A. Klein and L. Sibold. 1988. Analysis of drug resistance in the archaebacterium *Methanococcus voltae* with respect to potential use in genetic engineering. *Appl. Environ. Microbiol.* **54**(3), 734-740.

Postgate, J.R. 1969. Methane as a minor product of pyruvate metabolism by sulphate-reducing and other bacteria. *J. Gen. Microbiol.* **57**:293-302.

Rechsteiner, T., A. Kiener and T. Leisinger. 1986. Mutants of *Methanobacterium thermoautotrophicum*. *Syst. Appl. Microbiol.* **7**, 1-4.

Reeve, J.N., N.J. Trun and P.T. Hamilton. 1982. Beginning genetics with methanogens. In: Genetic Engineering of Microorganisms for Chemicals. (eds.) A. Hollaender, R.D. DeMoss, S. Kaplan, J. Konisky, D. Savage and R.S. Wolfe. pg. 233-244. Plenum Publishing Corp., New York.

Reeve, J.N., P.T. Hamilton, G.S. Beckler, C.J. Morris and C.H. Clarke. 1986. Structure of methanogen genes. *System. Appl. Microbiol.* **7**, 5-12.

Reeve, J.N. 1988. Molecular biology of methanogens and methanogenesis. In: Genetics and molecular biology of industrial microorganisms.

Rosenshine, I., R. Tchelet and M. Mevarech. 1989. The mechanism of DNA transfer in the mating system of an archaeobacterium. *Science*. **245**, 1387-1389.

Roustan, J.L., J.P. Touzel, G. Prensier H.C. Dubourguier and G. Albagnac. 1986. Evidence for a lytic bacteriophage for *Methanotherix* sp. In: Biology of Anaerobic bacteria. eds. H.C. Dubourguier et. al., Elsevier Science Publishers, The Netherlands.

Rouviere, P. and R.S. Wolfe. 1988. Novel biochemistry of methanogenesis. *J. Biol. Chem.* **263**, 7913-7916.

Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Second edition. Cold Spring Harbor Laboratory Press. Cold Spring harbor, New York.

Sandbeck, K.A. and J.A. Leigh. 1991. Recovery of an integration shuttle vector from tandem repeats in *Methanococcus maripaludis*. Appl. and Environ. Microbiol. **57**(9), 2762-2763.

Santoro, N. and J. Konisky. 1987. Characterization of bromoethanesulfonate resistant mutants of *Methanococcus voltae*: evidence of a coenzyme M transport system. J. Bacteriol. **169**, 660-665.

Schaffer, H.E. and R.R. Sederoff. 1981. Improved estimation of DNA fragment lengths from agarose gels. Anal. Biochem. **115**, 113-122.

Schauer, N.L. and J.G. Ferry. 1980. Metabolism of formate in *Methanobacterium formicicum*. J. Bacteriol. **142**, 800-807.

Schauer, N.L. and J.G. Ferry. 1986. Composition of the coenzyme F₄₂₀-dependent formate dehydrogenase from *Methanobacterium formicicum*. J. Bacteriol. **165**, 405-411.

Serwer, P. 1990. Sieving by agarose gels and its use during pulsed-field electrophoresis. *Biotech and Genetic Engineering Reviews*. Vol.8, pg. 319-343.

Shaw, P.J., G.J. Hills, J.A. Henwood, J.E. Harris and D.A. Archer. 1985. Three dimensional architecture of the cell sheath and septa of *Methanospirillum hungatei* J. *Bacteriol.* **161**, 750-757.

Shuber, A.P., E.C. Orr, M.A. Recnay, P.F. Schendel, H.D. May, L. Schauer and J.G. Ferry. 1986. Cloning, expression and nucleotide sequence of the formate mutants of *Methanobacterium formicicum*. *J. Biol. Chem.* **261**, 12942-12947.

Sleytr, U.B. and M. Sara. 1986. Ultrafiltration membranes with uniform pores from crystalline bacterial cell envelope layers. *Appl. Microbiol. Biotech.* **25**: 83-90.

Sleytr, U.B., P. Messner, M. Sara and D. Pum. 1986. Crystalline envelope layers in archaeobacteria. *System. Appl. Microbiol.* **7**, 310-313.

Smucker, R.A. and R.M. Pfister. 1975. Liquid nitrogen cryo- impacting: a new concept for cell disruption. *Appl. Microbiol.* **30**, 445-449.

Sowers, K.R. S.F. Baron and J.G. Ferry. 1984. *Methanosarcina acetivorans* sp. nov., an acetotrophic methane producing bacterium isolated from marine sediments. Appl. Environ. Microbiol. 47, 971-978.

Sowers, K.R. and R.P. Gunsalus. 1988. Adaptation for growth at various saline concentrations by the archaebacterium *Methanosarcina thermophila*. J. Bacteriol. 170, 998-1002.

Sprott, G.D., J.R. Colvin and R.C. McKellar. 1979. Spheroplasts of *Methanospirillum hungatei* formed upon treatment with dithiothreitol. Can. J. Microbiol. 32, 847-854.

Sprott, G.D. and R.C. McKellar. 1980. Composition and properties of the cell wall of *Methanospirillum hungatei*. Can. J. Microbiol. 26, 115-118.

Sprott, G.D., T.J. Beveridge, G.B. Patel, and G. Ferrante. 1986. Sheath disassembly in *Methanospirillum hungatei* strain GP1. Can. J. Microbiol. 32: 847-854.

Sprott, G.D., I. Ekiel, and C. Dicaire. 1990. Novel, acid labile, hydroxydiether lipid cores in methanogenic bacteria. J. Biol. Chem. 265: 13735-13740.

Sprott, G.D., C.G. Choquet, G.B. Patel and I. Ekeil. 1992. Formation of stable liposomes from lipid extracts of archaeobacteria. U.K. Informal Patent Application.

Stetter, K.O., M. Thomm, J. Winter, G. Wildgrubber, H. Huber, W. Zillig, D. van Ecovic, H. Konig, P. Palm and S. Wunderl. 1981. *Methanothermus fervidus*, sp. nov., a novel extremely thermophilic methanogen isolated from an Icelandic hot spring. Zentralbl. Bakteriол. Parasitenkd. Infektionskr. Hyg. Abt. I. Orig., C2, 166-178.

Stewart, M., T.J. Beveridge and G.D. Sprott. 1985. Crystalline order to high resolution in the sheath of *Methanospirillum hungatei*: a cross-beta structure. J. Mol. Biol. 183, 509-515.

Taylor, C.D. and R.S. Wolfe. 1974. Structure and methylation of coenzyme M (HS-CH₂-SO₃). J. Biol. Chem. 249, 4879-4885.

Thauer, R.K. 1990. Energy metabolism of methanogenic bacteria. Biochim. Biophys. Acta. 1018, 256-259.

Thomm, M., J. Altenbuchner and K.O. Stetter. 1983. Evidence for a plasmid in a methanogenic bacterium. J. Bacteriol. 153(2): 1060-1062.

Thomm, M. and G. Wich. 1988. An archaeobacterial promoter element for stable RNA genes with homology to the TATA box of higher eukaryotes. *Nucleic Acids Res.* **16**: 151-163.

Thomm, M., G. Wich, J.W. Brown, G. Frey, B.A. Sherf and G.S. Beckler. 1989. An archaeobacterial promoter sequence assigned by RNA polymerase binding experiments. *Can. J. Microbiol.* **35**, 30-35.

Weil, C.F., D.S. Cram, B.A. Sherf, and J.N. Reeve. 1988. Structure and comparative analysis of the genes encoding component C of methyl coenzyme M reductase in the extremely thermophilic archaeobacterium. *Methanothermobacter thermautotrophicus*. *J. Bacteriol.* **170**: 4718-4726.

Weil, C.F., B.A. Sherf and J.N. Reeve. 1989. A comparison of the methyl reductase genes and gene products. *Can. J. Microbiol.* **35**, 101-108.

Whitman, W.B. 1985. Methanogenic Bacteria. In: Woese, C.R., Wolfe R.S. (eds.), *The Bacteria*, Vol.8: Archaeobacteria. pg. 3-65, Academic Press, New York, N.Y.

Wilhelm, T., M. Thomm and K.O. Stetter. 1986. Genetic analysis of plasmid pMP1 from *Methanobrevibacter vulgatus*. *System. and Appl. Microbiol.* **7**, 401.

Williams, R.T. and R.L. Crawford. 1985. Methanogenic bacteria, including an acid tolerant strain, from peatlands. *Appl. Env. Microbiol.* **50**, 1542-1544.

Woese, C.R. 1987. Bacterial evolution. *Microbiol. Rev.* **51**: 221-271.

Woese, C.R., O. Kandler and M.L. Wheels. 1990. Towards a natural system of organisms: Proposal for the Archaea, Bacteria and Eucarya. *Proc. Natl. Acad. Sci.* **87**: 4576-4579.

Woese, C.R. and G.J. Olsen. 1986. Archaeobacterial Phylogeny: Perspectives on the Urkingdoms. *System. Appl. Microbiol.* **7**, 161-177.

Woese, C.R. and R.S. Wolfe. 1985. (eds.): The Bacteria, Vol. VIII: Archaeobacteria. Academic Press Inc. New York, N.Y.

Wolfe, R.S. 1971. Microbial formation of methane. *Adv. Microb. Physiol.* **6**, 107-146.

Wolfe, R.S. 1979. Methanogens: a surprising microbial group. *Antonie van Leeuwenhoek.* **45**, 353-364.

Wolfe, R.S. 1985. Unusuai coenzymes from methanogens. *Trends Biochem. Sci.* **10**, 396-399.

Wood, A.G., A.H. Redburg, D.R. Cue, W.B. Whitman and J. Konisky. 1983. Complementation of *argG* and *hisA* mutations of *Escherichia coli* by DNA cloned from the archaeobacterium *Methanococcus voltae*. *J. Bacteriol.* **156**, 19-20.

Wood, A.G., W.B. Whitman and J. Konisky. 1985. A newly isolated methanogen harbors a small cryptic plasmid. *Arch. Microbiol.* **142**, 256-261.

Worrel, V.E., D.P. Nagle, D. McCarthy, and A. Eisenbraun. 1988. Genetic transformation system in the archaeobacterium *Methanobacterium thermoautotrophicum* Marburg. *J. Bacteriol.* **170**: 653-656.

Zhao, H., A.G. Wood, F. Widdel and M. P. Bryant. 1988. An extremely thermophilic *Methanococcus* from a deep sea hydrothermal vent and its plasmid. *Arch. Microbiol.* **150**: 178-183.

Zillig, W.P., W.D. Reiter, F. Gropp, G. Puhler and H.P. Klenk. 1988. Comparative evaluation of gene expression in archaeobacteria. *Eur. J. Biochem.* **173**, 474-482.

END

2|5 0|5 93

FIN