LIFE ON THE METABOLIC EDGE: INDIVIDUAL AND MUTUALISTIC METHANOGENIC SYSTEMS

By

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Methanogenesis is the biological production of methane gas by methanogens, single-celled, anaerobic archaea utilizing one of the oldest strategies of life. Methanogens generate the biomass and energy they need to live from simple carbon compounds, producing methane gas. They have evolved in a variety of anaerobic environments and consortia, many forming tight metabolic relationships with other species. Methanogenesis is therefore regulated by interactions both within the cell and between other organisms. This research explores these interactions in two parts.

In the first part, we investigate how methane production is influenced through intracellular interactions involving the coenzyme M-coenzyme B heterodisulfide reductase. Hdr reduces a coenzyme heterodisulfide formed in the terminal step of the pathway, an essential step in methanogenesis. Two classes of Hdr have been identified: a variably expressed cytoplasmic HdrABC and a constitutively expressed membrane-bound HdrED. The generalist methanogen *Methanosarcina acetivorans* contains operons that encode both, making it an ideal model to explore their interactions within the cell. Crosslinking-mass spectrometry shows that HdrED forms a multienzyme complex with two essential methanogenic enzymes, creating a physical link between the carbon fixation pathways and the electron transport system. This complex may function as a biological router for carbon and electrons in

the cell. In contrast, genetic overexpression of the cytoplasmic HdrABC decouples the production of methane from the electron transport chain. This allows increased substrate uptake and methane production without increasing cell growth or biomass.

In the second part, we show a potentially syntrophic relationship between the methanogen *Methanobrevibacter smithii* (*M. smithii*) and the bacterium *Bacteroides thetaiotaomicron* (*B. theta*), then explore how computational software testing techniques can be applied to high-throughput experiments. Syntrophic relationships are mutualistic metabolic relationships in which the members' combined metabolisms perform a process that could not be accomplished individually. We first develop a defined coculture system for high-throughput comparisons of monoculture and coculture phenotypes. We then show that *B. theta* benefits from the presence of *M. smithii*, which removes the products that inhibit fermentation. Finally, we introduce the BioSIMP process, which uses software testing techniques of sampling and machine learning methods to model and predict experimental outcomes.

DEDICATION

This dissertation is dedicated to my parents, Penny and Fred Catlett, for their love, confidence, encouragement and support.

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CHAPTER 1: INTRODUCTION

At least 1 billion species are predicted to live on Earth [1]. Among these one billion species are the single-celled, obligately anaerobic archaea called *Methanogens*. Methanogens live by reducing simple carbon sources to methane gas through the respiratory pathway *methanogenesis*. Unable to tolerate high levels of oxygen, methanogens have adapted to a wide variety of anaerobic environments including soil, swamps, lakes, glaciers, ocean sediment, landfills, animal rumens, and the human gut [2–6]. Their presence plays a critical role in supporting human life both as a part of the global carbon cycle and by influencing human health directly from within the gut.

Methanogens have not evolved in isolated, pure cultures. They have evolved to grow on multiple substrates through several different forms of the methanogenic pathway [7–9], and have incorporated genes and adaptations via lateral gene transfer from other organisms [10,11]. They interact with their environments and the organisms that have co-evolved with them both to their own benefit and to the benefit of other organisms [10,12–16]. It follows that the production of methane through methanogenesis is regulated through both intracellular interactions and interactions with other organisms.

Significance

Methanogens play an important role in the global carbon cycle and climate regulation. Globally, between 400 million and one billion tons of methane are produced by methanogens [7,17,18]. Methane is a major end product of biomass degradation in anaerobic environments, and two percent of the carbon fixed into

biomass by photosynthesis is ultimately converted to methane [19–23]. Methane is also a potent greenhouse gas possessing at least 25 times the 100-year global warming potential of carbon dioxide (CO_2) [24–26]. Although the Earth's atmosphere contains 200 times more carbon dioxide than methane, the proportion of atmospheric methane has been growing steadily over the past 100 years, primarily due to human civilizations' increased dependency on fossil fuels and our disruption of biogenetic sinks such as wetlands, fields, and landfills [27,28].

Despite the danger of atmospheric methane, when properly harnessed methane is a promising biofuel. It can be generated in anaerobic fermenters or harvested from natural environments and anthropogenic sources such as agricultural waste and landfills [29]. Natural gas is 70 – 90% methane [30,31], and existing infrastructure for the utilization of natural gas can be reapplied to biogas [32,33]. Methods of generating and harvesting biogas from existing production can both reduce the dependence on fossil fuels and prevent the release of methane into the atmosphere [34–37].

Within the gut, methanogens are an important member of the microbiota. Humans depend on methanogens to assist in maintaining digestive health and nutritional balance [38–41], as well as to protect against infection, allergies, cancer, and other digestive disorders [42–49]. Changes in human breath methane can indicate various dietary disorders including obesity, anorexia and inflammatory bowel diseases [50–53]. Understanding how methanogens interact with the gut microbiota could lead to new treatments and insights into human health.

A Literature Review of Methanogenesis: The Beginning

Methanogenesis is one of the oldest metabolic pathways [3,69–73]. Before oxygen became abundant in Earth's atmosphere, life began, spread and flourished. Early microbial life found abundant energy in the form of heat, light, and reactive molecules [74,75]. Hydrogen gas (H2), sulfates, nitrates, and ferrous iron acted as the electron donors and acceptors necessary for cellular respiration [71,76]. Respiration through methanogenesis developed between 4.1 and 3.5 billion years ago, during the Archean era [69,71]. The earliest methanogens most likely reduced carbon dioxide to methane gas while utilizing hydrogen as an electron donor for respiration (hydrogenotrophic methanogenesis, fig 1.1 A), a pathway that has changed very little in many methanogens since [3,69–73,77]. Over time, some methanogens developed the means to produce methane from other sources, such as acetate and methylated compounds (fig 1.1 B, C) [2,19,71–73,76].

Eight hundred million years ago, oxygenic photosynthesis became prominent enough for the accumulation of free O_2 in the atmosphere. Much of the world's anaerobic life subsequently became extinct [75,76,78]. However, methanogens and other obligate anaerobic life continued in places free oxygen could not reach: underground, inside glaciers, in deep oceans, and in the protective centers of microbial communities [2–6,19,71,74,76]. Today methanogens live on the thermodynamic edge of life, adapting pathways virtually unchanged for millions of years to diverse ecosystems such as glacial pools, animal digestive tracts, hot springs, and other extreme environments [6,79–83].

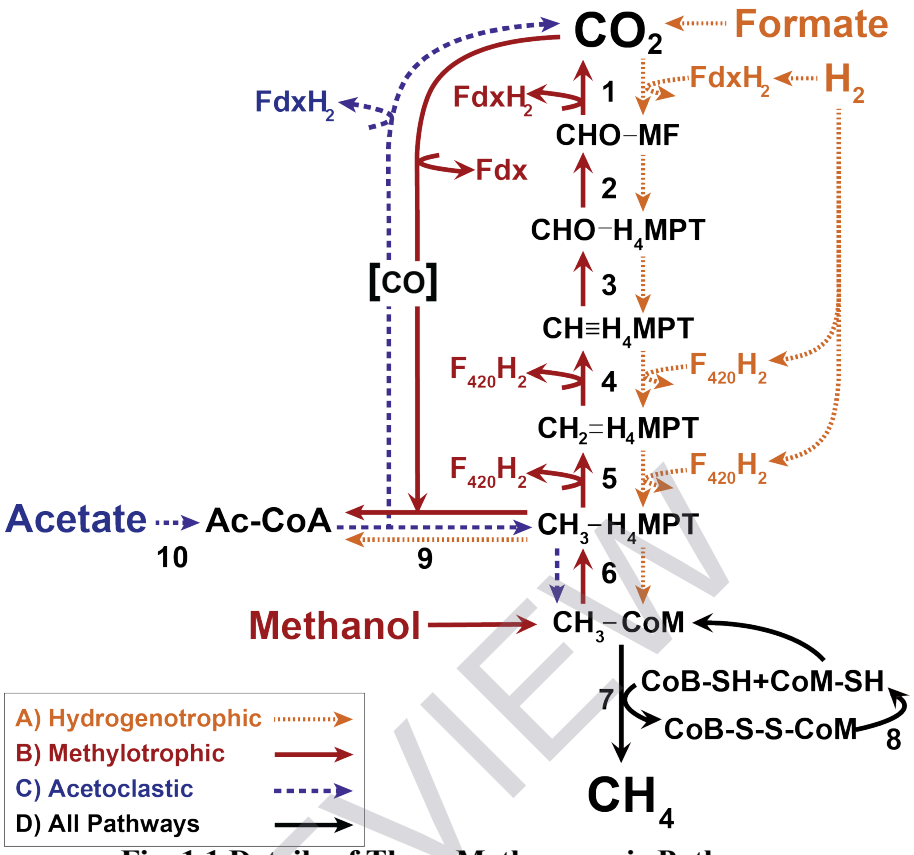

Fig. 1.1 Details of Three Methanogenic Pathways

A) Hydrogenotrophic (orange dotted line), **B) Methylotrophic** (solid red line), **C) Acetoclastic** (blue dashed line). Solid black lines and compounds indicate steps common to two or more pathways.

Enzymes: 1. Fmd, formylmethanofuran dehydrogenase; **2. Ftr**, formylmethanofuran:H_4MPT formyltransferase; **3. Mch**, methenyl H_4MPT cyclohydrolase; **4. Mtd**, F_{420}-dependent methylene-H_4MPT dehydrogenase; **5. Mer**, methylene-H_4MPT reductase; **6**. **Mtr**, methyl-coenzyme M methyltransferase; **7. Mcr**, Methyl-coenzyme M reductase; **8. HDR**, methanophenazine:CoB-S-S-CoM heterodisulfide reductase; **9. ACDS**, acetyl-coenzyme A synthase/CO dehydrogenase; **10. Ack/PTA**, phosphotransacetylase; **11**. **Fdh**, formate dehydrogenase;

Reactants: CO_2, Carbon dioxide; **CO**, Carbon monoxide; **CH_4**, Methane; **CoM-SH**, Coenzyme M thiol, 2-mercapto-ethanesulfonate; **CoB-SH**, Coenzyme B thiol; 7-mercaptoheptanoylthreonine phosphate; **CoB-S-S-CoM,** Coenzyme B-Coenzyme M heterodisulfide; **Ac-CoA**, acetyl-Coenzyme A; **$FdxH_2$**, reduced ferredoxin; **MPH_2**, reduced methanophenazine; **$F_{420}H_2$**, reduced F_{420}; **H_4MPT**, tetrahydromethanopterin; **MF**, methanofuran; **CH_3-H_4MPT**, methyl-tetrahydromethanopterin; **CH_2=H_4MPT**, methylene-tetrahydromethanopterin; **CH≡H_4MPT**, methenyl-tetrahydromethanopterin; **CHO-H_4MPT**, formyltetrahydromethanopterin; **CHO-MF**, formylmethanofuran;

Methanogenic Diversity

Humans have known that decomposition creates flammable gasses for centuries. One of the earliest known studies on this phenomenon was published in the 1620s by Johann Baptista van Helmont. Helmont had observed various chemical reactions release vapors with distinct and independent qualities, including flammability [84,85]. By 1906, Nicolaas Louis Söhngen was detecting methane produced by the decomposition of calcium formate in mixed microbial cultures [86,87]. In 1933, Marjory Stephenson and Lenord Hubert Stickland identified organisms capable of producing methane from formate or carbon dioxide and hydrogen (hydrogenotrophic methanogenesis, fig 1.1A) [86].

Today, seven orders of methanogens have been identified: Methanopyrales, Methanococcales, Methanobacteriales, Methanomicrobiales, Methanocellales, Methanosarcinales and most recently Methanomassiliicoccales [19,88]. The reduction of carbon dioxide with formate or hydrogen (hydrogenotrophic methanogenesis, fig 1.1A) is the most common pathway utilized by methanogenic species [9,89,90]. Methanogens have evolved to grow on other simple substrates, utilizing four additional closely related pathways: methylotrophic on methylated compounds (figs 1.1B), acetoclastic on acetate (fig 1.1C), carboxidotrophic on carbon monoxide and hydrogen gas, and methyl respiration on methylated compounds and hydrogen gas [7–9,73,91–95].

Phylogenetic evidence suggests a single origin of hydrogenotrophic methanogenesis from which the other pathways evolved [71,77,89]. Key enzymes involved in the final reduction to methane gas are conserved across pathways, with even methyl respiration dependent on methyl-CoM reductase (Mcr) and a

heterodisulfide reductase (Hdr) (figs 1 & 2). The acetyl-coenzyme A synthase/CO dehydrogenase (ACDCS) is also present in all pathways, although its function in hydrogenotrophic methanogenesis is not well understood [89,96].

The deepest rooting orders of Methanopyrales and Methanococcales are primarily hydrogenotrophic [9,89,90]. Only the order Methanomassiliicoccales has completely lost the ability to grow on CO_2 and H_2 [88,89]. Members of Methanosarcinales, Methanobacteriales and Methanomassiliicoccales can grow on methylated substrates through methylotrophic methanogenesis or methyl respiration [9,89,90] but only Methanosarcinales can grow on acetate, despite acetate being the source of nearly two-thirds of biologically produced methane gas [9,97]. Methanosarcinales is the only order whose members have incorporated cytochromes and the menaquinone analogue methanophenazine (MP) into methanogenesis, facilitation increased energetic efficiency through an improved electron transport chain and increased electrochemical potential across the membrane [9,19] (fig 1.3).

Hydrogenotrophic Methanogenesis

With or without cytochromes, hydrogenotrophic methanogenesis couples the oxidation of formate or hydrogen gas (H_2) to carbon dioxide reduction through a modified Wood–Ljungdah pathway [73,98] dependent on several carbon carrier coenzymes: methanofuran (MF), tetrahydromethanopterin (H_4MPT) and coenzyme M (2-mercaptoethanesulfonate, CoM) (fig 1.1A). Hydrogenases transfer electrons from hydrogen or formate to the electron carriers ferredoxin (fdx) and F_{420}, which in turn reduce CO_2 to methane in seven steps (fig 1.1A). First, CO_2 is reduced and activated by methanofuran, forming formyl-MF (CHO-MF). The formyl group is

then passed to tetrahydromethanopterin (H_4MPT) for the stepwise reduction of the formyl-group to methyl-H_4MPT. The membrane-bound enzyme methyl-coenzyme M methyltransferase (Mtr) catalyzes the transfer of the methyl group to the coenzyme M (CoM, 2-mercaptoethanesulfonate), using the energy of the transfer to pump sodium ions across the cell membrane, driving ATP production. The methyl-S-coenzyme M reductase (Mcr) catalyzes the final step using electrons donated by Coenzyme B (CoB, 7-mercaptoheptanoylthreonine phosphate), reducing the bound methyl group from CH_3-CoM to methane (CH_4) and creating a disulfide of CoM and CoB: CoM-S-S-CoB. To restart the cycle, this disulfide is reduced by a heterodisulfide reductase (Hdr) (fig 1.1A). All told, one mole of carbon dioxide is reduced to methane through this pathway, allowing the synthesis of 1-3 moles of ATP [7,9,19]:

(1.1) $CO_2 + 4H_2 = CH_4 + 2H_2O$ $\Delta G'_0 = -131$ kJ/mol CH_4

Most methanogens lack cytochromes and rely on cytoplasmic hydrogenases that couple the oxidation of hydrogen to the reduction of electron carriers ferredoxin and F_{420}. The sodium gradient generated by Mtr is primarily responsible for ATP generation, although in some species sodium translocating hydrogenases Eha and Ehb may be present as well [9,19,95]. Energy is conserved primarily through the cytoplasmic, flavin-containing heterodisulfide reductase HdrABC (fig 1.2A). HdrABC couples the endergonic reduction of ferredoxin ($E^{0'}$ = -500 mV) to the exergonic reduction of the CoM-S-S-CoB disulfide ($E^{0'}$ = -140 mV) through flavin-driven electron bifurcation, which allows the coupling of acceptor/donor potentials between 0 and -500 mV [19,56,99]. Flavoproteins (FP) can be reduced multiple times with different redox potentials: The reduction of FP to FPH, the reduction of

FP to FPH_2 and the reduction of FPH to FPH_2. FPH_2 can likewise be oxidized by two different electron acceptors. This coupling allows the reduction of the CoM-S-S-CoB disulfide to drive the reduction of CO_2 without depending on an ion gradient [19,100,101].

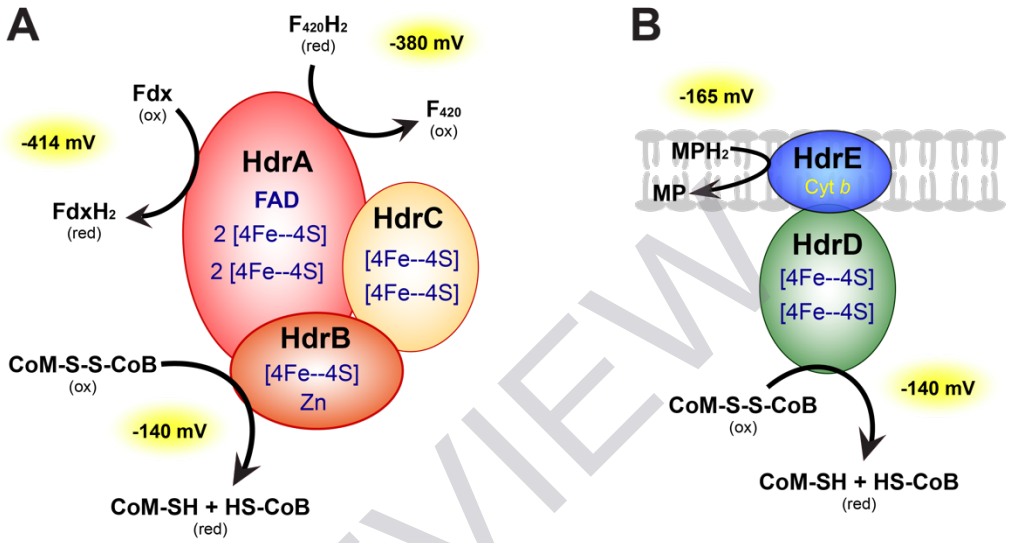

Fig. 1.2 Heterodisulfide Reductase

A) HdrABC: cytoplasmic heterodisulfide reductase (Hdr) found in methanogens without cytochromes. Reduces ferredoxin and F_{420} potentially through electron bifurcation coupled to the reduction of the CoM-CoB heterodisulfide.
B) HdrED: membrane bound heterodisulfide reductase found in methanogens with cytochromes and methanophenazine. Couples the oxidation of methanophenazine to the reduction of the CoM-CoB heterodisulfide.

Cytochrome-containing methanogens utilize a membrane-bound electron transport chain and create electrochemical potential through the translocation of sodium ions and protons across the membrane (fig 1.3A) [9,19,95]. This electrochemical potential is used to drive both ATP generation and reduction of ferredoxin by the ferredoxin-dependent hydrogenase Ech. Ech uses the proton motive force drive the endergonic reduction of internal ferredoxin ($E^{0'}$ = -500 mV) with electrons from H_2 ($E^{0'}$ = -414) outside the cell. The cytochrome-containing

heterodisulfide reductase HdrED helps build the electrochemical proton potential across the membrane by coupling the oxidation of the CoM-CoB heterodisulfide ($E^{0'}$ = -140 mV) with the reduction of methanophenazine (($E^{0'}$ = -1651 mV) (figs 1.2B, 1.3A). The oxidized methanophenazine is reduced by cytochrome-containing Vho, oxidizing H_2 on the outside of the membrane and increasing the proton potential [9,19,95].

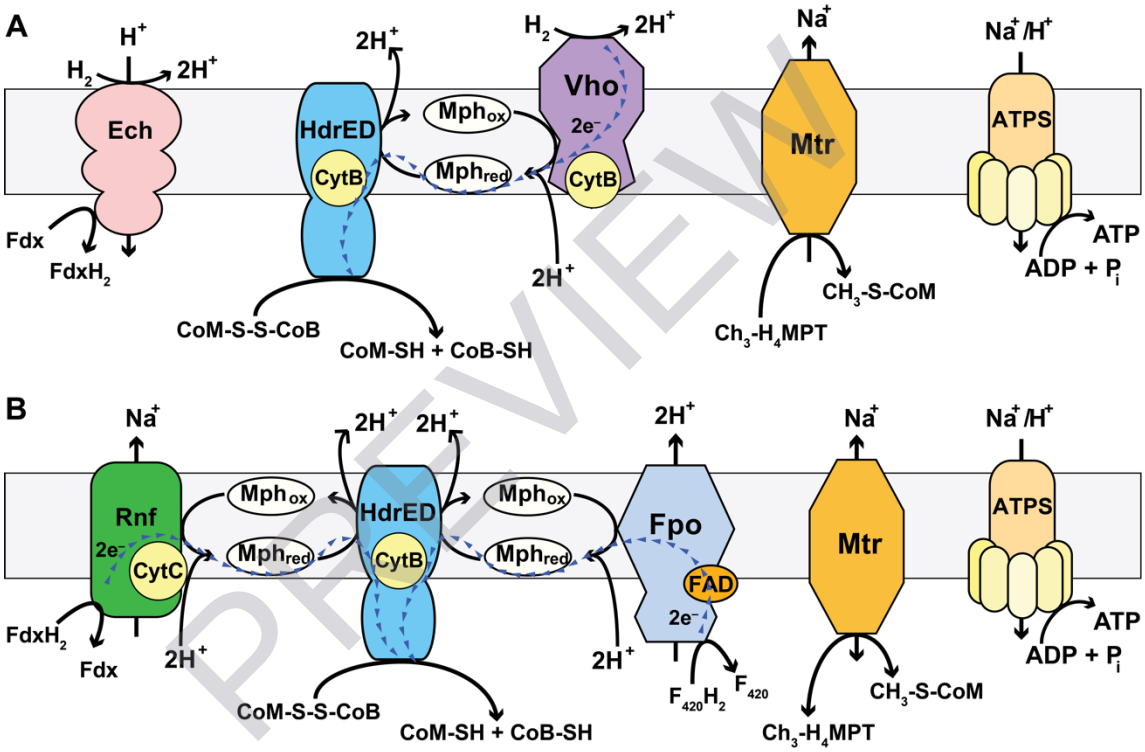

Fig. 1.3 Membrane-bound enzymes in cytochrome-containing methanogens
Methanogens with cytochromes utilize several membrane-bound enzymes that contribute to the formation of electrochemical potential for ATP production.
A) Hydrogenotrophic methanogens: **Ech**, ferredoxin-dependent hydrogenase; **Vho** F_{420}-non-reducing hydrogenase; **HdrED**, heterodisulfide reductase; **Mtr** methyl-coenzyme M methyltransferase; **ATPS**, ATP synthase
B) Generalist methanogen *Methanosarcina acetivorans* utilizes different enzymes depending on the substrate. **HdrED** is constitutively expressed.
Methylotrophic methanogenesis: **Fpo**, F_{420} dependent dehydrogenase; **Mtr** imports a sodium ion.
Acetoclastic methanogenesis, Rnf, sodium-pumping ferredoxin:methano-phenazine oxidoreductase; **Mtr** exports a sodium ion..

Methanogens with cytochromes grow faster and show higher growth yields than methanogens without cytochromes under the same conditions [19]. However, methanogens with cytochromes cease oxidation of H_2 at concentrations 10 times higher than those without, and do not compete well for H_2 under most environmental conditions [6]. However, many methanogens with cytochromes have the ability to grow on multiple substrates. Some species such as *Methanosarcina acetivorans* (*M. ace*) have lost the ability to grow on H_2 and CO_2 completely and rely entirely on other substrates [55,102].

Carboxidotrophic Methanogenesis

Carboxidotrophic methanogenesis is closely related to hydrogenotrophic methanogenesis. Carbon monoxide is a nearly universal intermediate in methanogenesis [95,103]. Methanogens with and without cytochromes contain the enzyme complex acetyl-coenzyme A synthase/CO dehydrogenase (ACDS). The dehydrogenase active site reduces CO_2 and binds CO, making it available to be combined with Hs-CoA and a methyl group transferred from H_4MPT to form acetyl-CoA. ACDS is critical for carbon fixation in hydrogenotrophic and methylotrophic methanogenesis, and in the demethylation of acetyl-CoA in acetoclastic methanogenesis (fig 1.1). Hydrogenotrophic methanogens have been suggested to first oxidize carbon monoxide to carbon dioxide, then reduce the carbon monoxide along the Wood–Ljungdah pathway as in hydrogenotrophic methanogenesis using H_2 as a reductant [73,98]. For additional information on carboxidotrophic methanogenesis, see Schöne and Rother 2018 [95].

Methylotrophic Methanogenesis

Some members of Methanosarcinales have developed the ability to grow exclusively from methylated compounds (methylotrophic methanogenesis, fig 1.1B) without requiring H_2 or formate as a reductant (methyl respiration) [94]. These cytochrome-containing methanogens first activate methyl groups with the substrate-specific corrinoid methyltransferases MT1. A second methyltransferase MT2 transfers the methyl group to HS-CoM [103,104]. To gain reducing power, methyl-S-CoM is oxidized through a reverse Wood–Ljungdah pathway to carbon dioxide. The oxidation of one mole of methyl-S-CoM reduces two moles of F_{420} and one mole of ferredoxin. For every three moles of methyl-S-CoM that is reduced to methane gas, one mole is reduced to carbon dioxide [9,95]:

(1.2) $4CH_3OH = 3CH_4 + CO_2 + 2H_2O$ $\Delta G'_0 = -106$ kJ/mol CH_4

Unlike in hydrogenotrophic methanogenesis, the sodium ion motive force drives the transfer of the methyl group from methyl-S-CoM to H_4MPT by Mtr. ATP generation is instead driven by the proton motive force generated through the heterodisulfide reductase HdrED and the F_{420} dependent dehydrogenase Fpo (fig 1.3B). In addition to HdrED, *Methanosarcina acetivorans* contains a cytoplasmic heterodisulfide reductase, HdrABC that closely resembles the reductases used by methanogens without cytochromes (fig 1.2A). *M. ace* also contains the sodium ion pump Rnf, however it is downregulated when grown on methylated substrates and can be deleted without effect [105,106].

Methyl respiration

Methanogens in Methanomassiliicoccales have can developed the ability to grow on methylated substrates through methyl respiration. These methanogens are distinct from the other orders due to the lack of the Wood–Ljungdah pathway and Mtr enzyme [88–90]. Methyl respiration is a cycle of the reductive steps of methylotrophic methanogenesis. Hydrogen gas is used to reduce the methylated compounds and to facilitate the transfer of the methyl group to SH-CoM. Methanomassiliicoccales lack cytochromes, so the CoM-CoB heterodisulfide is likely reduced by a cytoplasmic HdrABC/Mvh complex that utilizes electron bifurcation. Electrochemical potential for ATP generation is potentially produced by an unusual Fpo/hdrD complex [73,88].

Acetoclastic Methanogenesis

70% of environmental methane is produced from acetate through the acetoclastic pathway, however only members of the genra *Methanosarcina* and *Methanosaeta* can grow exclusively on acetate [90,107–110]. Acetoclastic methanogens activate acetate to acetyl-phosphate by the acetate kinase Ack, then the phosphostransacetylase Pta transfers the acetyl group to CoA, forming acetyl-CoA. ACDCS (CO dehydrogenase/acetyl-CoA synthase) transfers the methyl group to H_4MPT, binding carbon monoxide (CO) in the process. The majority of the Wood–Ljungdah pathway is bypassed, but methyl-H_4MPT is reduced to methane, with Mtr generating a sodium gradient as the methyl group is transferred to CoM as in hydrogenotrophic methanogenesis (fig 1.1C) [111,112]. Electrons from the oxidation of CO to CO_2 drive the final reduction of the CoM-S-S-CoB disulfide to the thiol

cofactors. Acetoclastic methanogenesis has one of the lowest change in free energy of all the methanogeneic pathways, as shown by equation 1.3 [105,113]:

(1.3) $CH_3–COOH = CH_4 + CO_2$ $\Delta G'_0 = -36$ kJ/mol CH_4

Because the change in free energy is so small, energy conservation must be very efficient. As in other pathways, ATP generation is driven by the translocation of sodium ions and proton ions. The electron transport chain in *Methanosarcina* depends on the electrochemical potential generated by Mtr and HdrED. *Methanosarcina acetivorans* also upregulates the the sodium-pumping ferredoxin:methano-phenazine oxidoreductase Rnf (fig. 1.3B) [106]. *Methanosaeta* lack HdrED, and appear to rely entirely on Mtr to create an ion gradient for ATP generation [113].

Methanogenesis Within Microbial Consortia

Methanogens did not evolve in isolation, but as members of microbial communities [76]. In these communities, cells of different species and domains grow and interact with their environments and each other [97]. In some communities, the cells can become interdependent and specialized, in much the same way cells within plants and animals have evolved to fulfill specific, interdependent functions [59]. Interactions between cells in the community can be as simple as waste products from one species feeding another, and as complex as the signaling mechanisms used in quorum sensing. In some cases, species develop a mutually beneficial symbiotic relationship, known as syntrophy [22,59,114].

Syntrophy is a form of metabolic mutualism in which interactions benefit both species. A syntrophic relationship is characterized by a close metabolic