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REVIEW

Rumen methanogens: a review

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Abstract The Methanogens are a diverse group of organisms found in anaerobic environments such as anaerobic sludge digester, wet wood of trees, sewage, rumen, black mud, black sea sediments , etc which utilize carbon dioxide and hydrogen and produce methane. They are nutritionally fastidious anaerobes with the redox potential below –300 mV and usually grow at pH range of 6.0–8.0 [1]. Substrates utilized for growth and methane production include hydrogen, formate, methanol, methylamine, acetate, etc. They metabolize only restricted range of substrates and are poorly characterized with respect to other metabolic, biochemical and molecular properties.

Keywords Rumen methanogens · Methanogens · Methane

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Introduction

The domain Archaea consists of Crenarchaeota and Euryarchaeota. The Crenarchaeota are obligate thermophilic organisms and mostly metabolize elemental sulphur. *Sulfolobus acidocaldarius* is a representative species of this domain which thrives in acid hot environments such as hot springs. The Euryarchaeota contains methane forming, extremely halophilic, sulfate reducing, and extremely thermophilic sulfur metabolizing spp. *Methanobacterium formicicum* which produces methane gas from hydrogen gas and carbon dioxide, found in fresh water sediments, marshy soils, and the rumen of cattle and sheep is a representative species of this domain. Methane was first observed as a type of combustible air by the Italian physicist Alexandro Volta in 1776 who collected gas from marsh sediments and showed that it was flammable (the Volta experiment). Later it was subsequently discovered by Beehamp, Popoff, Tappeneiner, Hoppe-Seyler, Sohugen and Omelianski that certain microbial species were responsible for the production of methane. In earlier taxonomic treatments methanogens were grouped among the better characterized bacterial group on the basis of their morphologies. Later they were clustered into a single family Methanobacteriacea. Till now a wide variety of methanogens have been described, their taxonomy based on both phenotypic as well as phylogenetic analysis (comparative 16 S rRNA sequencing), with several orders being recognized.

Classification of methanogens

The biological classification is a hierarchical system that starts with a few categories at the highest level at further

subdivides them at each lower level. The methanogenic bacteria are divided into three orders which are further sub divided into family, genus and species [2].

I Order Methanobacteriales Are very strict anaerobes and are found in anaerobic habitats such as sediments of natural waters, soil, anaerobic sewage digester, gastrointestinal tract of animals (rumen). These consists of short lancet shaped cocci to long filamentous rods, typically gram positive but some cells may be gram variable.

Pseudomurien is the main cell wall content. Substrates utilized as energy source are hydrogen, formate or CO. Any other organic material is not utilized.

Family-Methanobacteriacae consists of generas Methanobacterium and Methanobrevibacter

The Genus Methanobacterium consists of curved, crooked to straight non sporing rods, long and filamentous about 0.5–1.0 μm in width. Non motile or motile due to fimbrae, very strict anaerobes, mesophiles to extreme thermophiles.

Examples of species included in this genus are *M. formicicum, M. bryantii M. thermoautotrophicum*

The Genus Methanobrevibacter consists of short rods or lancet shaped cocci which often occurs in pairs or chain (0.5–1.0 μm in width).Cells are poorly motile or non motile with optimal growth range at 37–39°C.

Examples of species included in this genus are *M. ruminantium, M. smithi, and M. aboriphilus*

II Order Methanococcales consists of gram negative irregular cocci. Cell wall consists of a single layer of protein; H_2 and formate are used as substrate for growth and methanogenesis. Widely found in sediments of natural waters.

Family Methanococcacae consists of genus *Methanococcus*.

Genus Methanococcus consists of regular to irregular cocci which may be single or paired, cells are highly motile.

Examples of species included in this genus are *M. voltae, M. vannieli*

III Order Methanomicrobiales consists of rods or coccus which may be gram positive or gram negative, motile or non-motile. They oxidize hydrogen or formate with reduction of CO_2 to CH_4 via fermentation of methanol, methylamine (trimethylamine and ethyl dimethylamine), and acetate. Does not utilize any carbohydrate, proteinaceous material or organic compound. It is widely distributed in nature. Found in sediments of natural waters, soil, anaerobic sewage digester and GI tract of animals (rumen). *A. Family-Methanomicrobiacae* consists of gram negative cocci or slightly curved or straight rods. Oxidize hydrogen or formate as sole energy source for growth and methane production.

i Genus Methanomicrobium consist of short, straight or slightly curved rods with rounded ends. Cells are motile. Optimum temperature for growth is 38–40°C. Only hydrogen serves as substrate for growth and methane production.

Examples of species included in this genus are *M. mobile*

ii Genus Methanogenium consists of gram negative, irregular coccoid cells that may or may not require acetate.

Species included in this genus are *M. cariaci, M. marisnigri*

iii Genus Methanospirillum consists of slender rods that continuously form spiral filament, which are motile.

Example of species included in this genus is *M. hungatei*

B. Family Methanosarcinacae consists of large, spherical to pleomorphic gram positive cells (1.5 to 2.5 μm in diameter), often forming packets of various sizes. Planes of division are not always perpendicular. Cells are non-motile, mesophiles to thermophiles. Oxidise hydrogen with reduction of CO_2 to CH_4 by metabolism of methanol, methylamine (di, tri, ethyldimethyl) and acetate. Methane, carbon dioxide and ammonia are formed as end products. Cell wall consists of heteropolysaccharide.

Examples of Genus Methanosarcina is *M. barkeri*

Cell envelopes or cell wall of Methanogens

Methanogens exhibit great diversity in cell envelopes, ranging from simple, nonrigid surface layers consisting of protein or glycoprotein subunits to a rigid "pseudomurein" sacculus, analogous to eubacterial murein [3–6]. Muramic acid or D-amino acids have not been detected till date. According to their major cell wall constituent, cell envelopes of Methanogens may be categorized into three characteristic classes: (i) pseudomurein layer (ii) protein or glycoprotein layer; and (iii) heteropolysaccharides layer.

Order methanobacteriales

Members of the gram-positive *methanobacterium* consists of sharply defined, smooth cell wall 15–20 nm in width and are the only archaebacterial species that possess a *pseudomurein*-type cell wall analogous to eubacterial murein [7, 4]. Pseudomurein differs from eubacterial murein in that (i) L-talosaminuronic acid is substituted for muramic acid (ii) different sequences of amino acids (L configuration of alanine, glutamic acid, and lysine, mainly) are constituents of peptides involved in the glycan polymer cross linking (iii) the chemical bonds between the sugar moieties of alternating N-acetylglucosamine and N-acetyl-talosaminuronicacid are probably 1(1-3) linkages instead of β (1-4) glycosidic linkages which occur in eubacterial murein [8, 6].

Antibiotics such as Vancomycin and Penicillin, which affect eubacterial cell wall biosynthesis by interfering with reactions involving D-alanine, do not affect biosynthesis of methanogen pseudomurein [9]. Eubacterial murein and methanogen pseudomurein appear to be analogous based on function, chemical composition, and primary structure.

In the genus *Methanobrevibacter*, ultra thin section of whole cell of *M. ruminantium* reveal that the cell is covered by a triple layered cell wall of width 30–40 nm. The inner layer is an electron dense layer; the middle layer is electron transparent layer while the outer layer is rough and irregular. The cell wall contents also differed in polypeptide sequence, as the l-ala is replaced by l-threonine and NAG is replaced by N-acetyl galactosamine. It also contains high phosphate level in its cell wall content.

Order Methanococcales

Cell wall consists of a layer of protein or glycoprotein subunits *(S-layer)* with traces of glucosamine external to the cell membrane. In many species it is lysed easily [10] by detergents or solutions of low osmolality. However, some species posses detergent resistant and protease resistant S- layer [11]. The carbohydrate components of the glycoprotein in S-layers vary greatly. No muramic acids or DAP have been yielded from cells. Traces of glucosamine have been found in M. vanielli and M.voltae. One member of the *Methanobacteriales*, the extremely thermophilic *Methanothermus fervidus*, has a pseudomurein cell envelope covered by a layer of protein subunits. Perhaps the S-layer provides greater thermostability for *Methanothermus fervidus* and is an adaptive trait in response to environmental factors.

Electron micrographs of the outer surface of *Methanococcales spp* showed a regular array of protein subunits. Ultra thin sections of whole cell revealed a single layer of cell wall material 18 nm thick (S-layer). On treating cells with 2% SDS at 100°C for 30 min. or disintegration of cells with glass beads followed by incubation with trypsin resulted in complete solublization.

S-layers are not unique to archaebacteria; in fact, protein-containing S-layers are found in diverse species of eubacteria, and little difference in the chemical compositions of S-layers of eubacteria and archaebacteria have been detected. In general, S-layers from both groups are composed of proteins which are rich in acidic amino acids and have a low percentage of sulfur-containing amino acids.

Order Methanomicrobiales

This order consists of Heteropolysaccharide and complex cell envelopes (Third distinguishing cell wall type found in archaebacteria is restricted to *Halococcus* and *Methanosarcina* spp.). A thick, amorphous cell wall structure consisting of acid heteropolysaccharide containing galactosamine, neutral sugars and uronic acids is found in methanogens belonging to the genus *Methanosarcina*, which usually grow in spherical packets. The structural wall in *Methanosarcina* is a polymer of D-glucuronic acid and N-acetylgalactosamine, which is similar to animal chondroitin [4]. The constituents of this polymer in *Methanosarcina* sp. are not sulfated as in the case of *Halococcus* sp. Zeikus *et al* reported that the outer layer appears to be laminated. Zhilina reported a triple layered appearance of cell wall in gas vacuolated strain. They lack the constituents of peptidoglycan [3].

Ultra thin sections of genus *Methanogenium* showed a cell wall of 10nm which consisted completely of protein confirmed by freeze drying the cells and treating with SOD or treating the disintegrated cells with trypsin resulting in complete solublization. No muramic acids or amino sugars were detected [3]. *Methanospirillum hungatei* and *Methanothrix soehngenii* are characterized by complex cell envelopes containing a thin, fibrillar outer sheath surrounding an electron-dense inner wall [12, 13] which covers the cells separated by spacer elements. Isolated sheath material consists of protein (18 amino acids and is resistant to SOD or trypsin treatment) and possibly glycoprotein, as indicated by the presence of amino acids and neutral sugars as sheath hydrolysis products [3, 4]. The inner wall of *Methanothrix* sp. is involved in septum formation during cell division as indicated by electron microscopy; this phenomenon has not been observed in *Methanospirillum spp*. No muramic acids or amino sugars have been detected [3]. Little else is known about this unusual cell wall structure.

In case of methanogens a positive gram reaction is seen if there is the presence of a thick rigid sacculus whereas gram negative reaction reveals its absence [3].

Methanogens found in rumen

Methanogenesis in ruminants has important environmental consequences. Methanogens such as *Methanomicrobium mobile, Methanobacterium formicicum, M. bryantii, Methanobrevibacter ruminantium, M. smithi, Methanosarcina barkeri,* and *M. mazai* have been isolated from rumen by cultural methods. However, molecular methods reveal a considerable genetic diversity of methanogens in the rumen, even within the same ruminant species. Some of the methanogens are non-culturable.

Characteristics of *M. formicicum*

Cell shape Slender, cylindrical with blunt rounded ends. Some cells are unevenly crooked. Some chains and filaments are seen.

Cell size Depending upon the strain and length the cell width may vary from 0.4–0.8 μm.
Motility Non motile.
Gram nature Gram variable.
Major lipids:

a. *neutral (isoprenoid hydrocarbons)* Not determined.
b. *polar (isopranyl glycerol ethers)* $C_{20} + C_{40}$ ethers.

Substrate for growth and methanogenesis H_2 and formate. Acetate, carbohydrate, aminoacid, ethanol, methanol, propionate, butyrate and lactate are not fermented. CO may be fermented in some strains. Some strains do not utilize formate.
Optimum temperature for growth are mesophiles, with optimum growth at 38 and 45°C. No growth at 55°C.
Surface colony characters Colonies are white to gray, flat and filamentous; deep colonies appear as profusely filamented sphaeroid. Incubation period of 3–5 days and temperature of 37°C is required. In about 14 days colonies attain a diameter of 2–5 mm.
Growth in liquid broth Depending upon the strain, either turbidity or granular clumps are seen which do not break even with vigorous agitation.
Host Bovine, ovine.

Characteristics of *M. bryanytii*

Cell shape Slender, cylindrical with blunt rounded ends, often forming chains or filaments with unevenly crooked cells.
Cell size Chains may be up to 10–15 μm in length. Cell width varies from 0.5–1.0 μm.
Motility Non motile and posses fimbrae.
Gram nature Gram positive to Gram variable.
Major lipids:

a. *neutral (isoprenoid hydrocarbons)* $C_{30}H_{50}$, $C_{30}H_{52.}$
b. *polar (isopranyl glycerol ethers)* $C_{20} + C_{40}$ ethers.

Substrate for growth and methanogenesis H_2 is only utilized. Formate is not used. Ammonium ion is essential as source of nitrogen. Acetate, cystiene and B-vitamins are stimulatory for growth.
Optimum temperature for growth 37–39°C.
Optimum pH for growth 6.9–7.2.
Surface colony characters Gray to light gray colonies which are flat with diffuse to filamentous edges. They can reach a diameter of 1–5 mm.
Host Bovine.

Characteristics of *M. ruminantium*

Cell shape Very short lancet shaped to oval rods or coccus, may occur in pairs but usually in chains which may be up to 20 or more cells resembling *Streptococci*.
Cell size Cells are 0.5–1.0 μm in width and 1.0–1.5 μm in length.
Motility Non motile.
Gram nature Gram positive, even in relatively old cultures.
Major lipids:

a. *neutral (isoprenoid hydrocarbons)* $C_{30}H_{50}$, $C_{30}H_{52}$, $C_{30}H_{54}$,$C_{30}H_{56.}$
b. *polar (isopranyl glycerol ethers)* $C_{20} + C_{40}$ ethers.

Substrate for growth and methanogenesis H_2 and formate. Carbohydrate, amino acid, methanol, ethanol, isobutyrate, propionate, valerate, caproate, succinate and pyruvate are not utilized. Acetate, ammonia and sulfide may be essential as important source of cell carbon, nitrogen and sulfur. One or more vitamins are required. It also requires 2-methyl n-butyrate and CoM.
Optimum temperature for growth 37–43°C. Little or no growth at 47°C. Rumen strains do not grow at 33°C.
Optimum pH for growth 6.3–6.8.
Surface colony characters Colonies are off white to yellow in color, translucent, convex and circular with entire margins. They become visible after 3 d of incubation at 37°C and may reach a diameter of 3–4 mm depending upon number of colonies. Colonies in deep agar are lenticular.
Growth in liquid broth Growth is turbid or floc. Vigorous shaking results in breaking of flocs.
Host Bovine.

Characteristics of *M. smithi*

Cell shape Morphologically similar to *M.ruminantium*, with the exception of a single polar flagellum. Short, lancet shaped oval cocci; may occur in pairs or chain.
Cell size Cells are 0.5–1.0 μm in width and 1.0–1.5 μm in length.
Motility Non motile.
Gram nature Strongly Gram positive.
Major lipids:

a. *neutral (isoprenoid hydrocarbons)* $C_{30}H_{50}$, $C_{30}H_{52.}$
b. *polar (isopranyl glycerol ethers)* $C_{20} + C_{40}$ ethers.

Substrate for growth and methanogenesis Either H_2 or formate. Can be cultured in simple chemical defined media, which differentiates it from *M.ruminantium*. Ammonia and acetate are required as major source of cell nitrogen and carbon.
Optimum temperature for growth 37–39°C .
Optimum P^H for growth 6.9–7.4.
Surface colony characters Yellow to white colonies that are translucent, convex, and circular with entire margins.
Host Bovine.

Characteristics of *M. mobile*

Cell shape Straight to slightly curved rods with rounded ends. No chain is seen. May occur as single or in pairs.
Cell size Are 0.7 μm wide and 1.5–2.0 μm long.
Motility Motile with monotrichous flagella.
Gram nature Gram negative.
Major lipids:

a. *neutral (isoprenoid hydrocarbons)* Not determined.
b. *polar (isopranyl glycerol ethers)* Not determined.

Substrate for growth and methanogenesis H_2 and formate. Acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate, succinate, glucose, pyruvate, methanol, ethanol, propanol, isopropanol, and butanolare not utilized as electron donors. It can grow well on media devoid of rumen fluid or extracts of mixed ruminal bacteria.
Optimum temperature for growth 38–40°C.
Optimum P^H for growth 5.9–7.7.
Surface colony characters Colorless to pale yellow colonies which are small, translucent, entire and convex. They require an incubation period of 4 d at 39°C and reach a diameter of 0.7–1.0 mm in 15 d. Deep colonies are lenticular and 0.5–0.7 mm in diameter in 15 d.
Host Bovine.

Characteristics of *M. barkerii*

Cell shape Are spheres mostly occurring in packets of eight or less but sometimes in large mass.
Cell size The diameter of the sphere is 1.5–2.5 μm.
Motility Non motile.
Gram nature Gram positive.
Major lipids:

a. *neutral (isoprenoid hydrocarbons)* $C_{25}H_{46}$, $C_{25}H_{48}$, $C_{25}H_{50}$, $C_{25}H_{52.}$
b. *polar (isopranyl glycerol ethers)* C_{20} ethers.

Substrate for growth and methanogenesis H_2 is used. Methanol, methylamine, acetate and CO are fermented with the formation of CH_4 and CO_2. Growth and methanogenesis are more rapid in methanol than in acetate media. Carbohydrate, amino acid, formate, ethanol, propionate, butyrate are not fermented.
Optimum temperature for growth Are mesophiles to thermophiles.
Optimum P^H for growth 7.0.
Surface colony characters Deep colonies are whitish and 0.5–1.0 mm in diameter in methanol agar with inorganic salts.
Growth in liquid broth Growth may occur as zoogloeal masses on as flocculent sediments with active gas formation.
Host Carpine, bovine

Both CoM requiring and non requiring strains of *Methanobrevibacter* have been isolated from bovine rumen [14, 15]. Lovely et al. [14] isolated two strains of *Methanobrevibacter* from high dilutions of bovine rumen fluid. The Co M Synthesizing strain had simple nutritional requirements and higher growth rate as compared to non synthesizing strain; also it did not reacted with the antiserum against type strain of *M. rumination*. Similarly four CoM requiring and two CoM non requiring strains of *M. ruminantium* have been isolated by Miller et al. [15]. However none of the strain reacted with antiserum against the type strain of *M. ruminantium*. These observations confirm that both CoM requiring and non requiring strains of *M. ruminantium* are present in high concentration in bovine rumen content.

Culture of methanogens

Methanogens require very low redox potential and are perhaps the strictest anaerobic bacteria known. They can be cultured only by procedures that ensure culture in the absence of oxygen. Many workers have developed procedures for cultivating Methanogens. The *Hungate* culture technique, with modifications, has proven to be an excellent method for isolating fastidious anaerobes. The methods described by Bryant [16] for culturing larger quantities of cells have proven acceptable for most Methanogenic species. Bryant et al. [16, 17] cultured methanogens using glass test tubes (anaerobic culture tubes) that are tightly sealed with neoprene, butyl, or synthetic, but not gum, rubber stoppers. A gas mixture of 80% H_2 and 20% CO_2 is used which is made free of oxygen by passing it through heated copper filings. Some investigators also prefer a 50:50 mixture of H_2 and CO_2 [16] because this gas mixture is more dense and not as easily displaced by air when culture containers are opened. The tubes are gassed while incubation or substrate addition. Macy et al. [18] described the syringe method for substrate or media addition or inoculation in "Hungate type" tubes that are screw-capped and sealed with flanged rubber stoppers.

Serum bottles or various glass containers fitted with serum bottle necks have been described for cultivation of methanogens by Miller and Wolin [19]. The bottles are sealed with metal seal after closing with butyl rubber stoppers. This is advantageous over non sealed stoppers because non sealed stoppers are often blown out of culture tubes as the result of active fermentation of methanol. Here all inoculations and transfers are done with a hypodermic syringe and needle. This technique is also better due to less fragility and easy handling of serum bottles.

Use of Freter type anaerobic glove box equipped with an inner ultralow oxygen chamber has been described by

Edwards and McBride [20] for isolation and growth of methanogens. The inner chamber maintains the redox potential necessary for growth of methanogens and is used for incubation of plates in pressure cooker containers which are specially modified for high atmospheric pressure of H_2 and CO_2 as the inner chamber, is periodically flushed with H_2 and CO_2 (80:20). Cultures are plated in the outer anaerobic glove box and immediately placed in the inner chamber. This method is considerably more expensive than Hungate procedures; however, it offers unique advantages. For example, it requires less skill and manual dexterity, and it allows for routine genetic procedures such as replica plating.

Balch and Wolfe [21] have described a new method for cultivating H_2 oxidizing methanogens by applying high gas pressure (2 to 4 atmospheres of H_2 and CO_2). This avoids the need of gassing the culture repeatedly. Specialized gassing manifold, glass culture tubes (for liquid cultures) and anaerobic incubators (for agar plate cultures) have also been used by these workers for isolating the organism.

Herman Knoll and Wolfe [22] described the isolation of methanogenic bacteria using agar bottle plate. The bottle solved the problem of water exudates from agar medium and provided convenience of streaking, adding or sampling a defined gas atmosphere.

Identification and Quantification of Methanogenic Bacteria

F_{420} present in methanogens can be exploited to identify the bacteria. Methanogenic colonies fluoresce when exposed to long wavelength UV radiations due to the presence of cofactor F_{420} [20]. During the active growth of methanogens the F_{420} exists in particular oxidized state [20]. This oxidized form is excited by the long wavelength UV radiations resulting in fluorescence. Results of fluorescent microscopy can be enhanced by a proper selection of excitation and barrier filters. Also, methanogenic bacteria can be identified by their bright fluorescence under UV microscopy [23].

Gas Chromatographic Analysis (GC) is also used for identification and quantification of methanogens. Isolated cultures of methanogens actively produce methane by oxidation of H_2 and reduction of CO_2.Tubes containing broth are inoculated, incubated and tubes are then observed for methane as head gas by gas chromatography. This method is also used for counting methanogens by MPN (Most Probable Number). For MPN analysis tubes of three consecutive dilutions are inoculated in triplicate. After incubation the tubes containing more than 100 ppm of methane are counted as positive. A more rapid, sensitive, and convenient GC procedure for analysis of ^4C-labeled and unlabeled metabolic gases has also been described [24]. In this method gases are detected by thermal conductivity detector and the effluent is directly channeled into a gas proportional counter for radioactivity measurement. Thermal conductivity detection is often more useful than flame ionization detection because H_2, CH_4, and CO_2 can be accurately quantified on the same column, where as Flame Ionization Detector is only limited to CH - containing compounds.

Real-time polymerase chain reaction (PCR) using a broad-range (universal) probe and primers set is also used for the quantification of methanogens [25].

Single Strand Conformation Polymorphism (SSCP) based genetic probes of small subunit rRNA genes have been described [26]. DNA is extracted from rumen fluid collected from cow and amplified with Ex Taq DNA polymerase (TAKARA). Primers used for PCR are M301F and M915R; Ar1000F and Ar1500R. The PCR product is purified and digested by exonuclease to yield ssDNA. This ssDNA is reamplified by PCR and is then sequenced. Applying SSCP 22 clones were sequenced.

FISH (Fluorescent in situ Hybridization technique) This is one of the modern methods to study the complex microbial communities. Here rRNA targeted fluorescent oligonucleotide probes are used. Whole cell FISH is detected by confocal scanning laser microscopy (CSLM) [27]. A new method of quantification for Methanogens by fluorescence in situ hybridization (FISH) based on the measurement of specific binding (hybridization) of 16S rRNA-targeted oligonucleotide probe Arc915, has been described by Stabnikova et al. [28]. Specific binding of probe per 1 ml of microbial sludge suspension from anaerobic digester linearly correlated with concentration of autofluorescent cells of Methanogens. However this method is not applicable for diluted suspensions of Methanogens.

The detection and quantification of Methanogens by the above mentioned methods have certain limitations like there are methanogenic bacteria that have no F_{420} or only low levels (The genus *Methanosaeta*) [29] or, conversely, there are non methanogenic bacteria that exhibit similar UV fluorescence. Therefore further proof is required for proper identification. Also this method is not applicable for aggregate forming cells (The genus *Methanosarcina*) [30]. The plate counting method and the MPN method is time consuming [31, 32] as Archaea are slow grower. Also it requires special laboratory equipment and can evaluate only viable cells. The Real time (quantitative) PCR method is not suitable for dense suspension of methanogens. The disadvantage of FISH described by Amman *et al* is extraction of RNA and use of radioactive labels.

Methanogenesis

Methanogenesis is the production of methane by methanogenic bacteria by utilizing simple substrate at low reduction

potential, to produce cellular energy. Although some Eubacteria have also been reported to produce methane [33], only methanogens have been reported to couple methane generation to energy production. Less than 1 ATP is derived by cells from each molecule of methane produced. Some unique enzymes are present in methanogens which carry out the process of methanogenesis. Ralph Wolfe in early 1970s first started studying the methanogenic reduction of CO_2 [34]. In the next 20 years six new coenzymes were discovered. By the early 1990s the pathway of methanogenesis was elucidated. The process of methanogenesis (Fig. 1) requires seven coenzymes and eight enzymes.

The various coenzymes involved in methanogenesis are *Coenzyme 420 – the* (N-(N-L-lactyl-y-glutamyl) L-glutam-

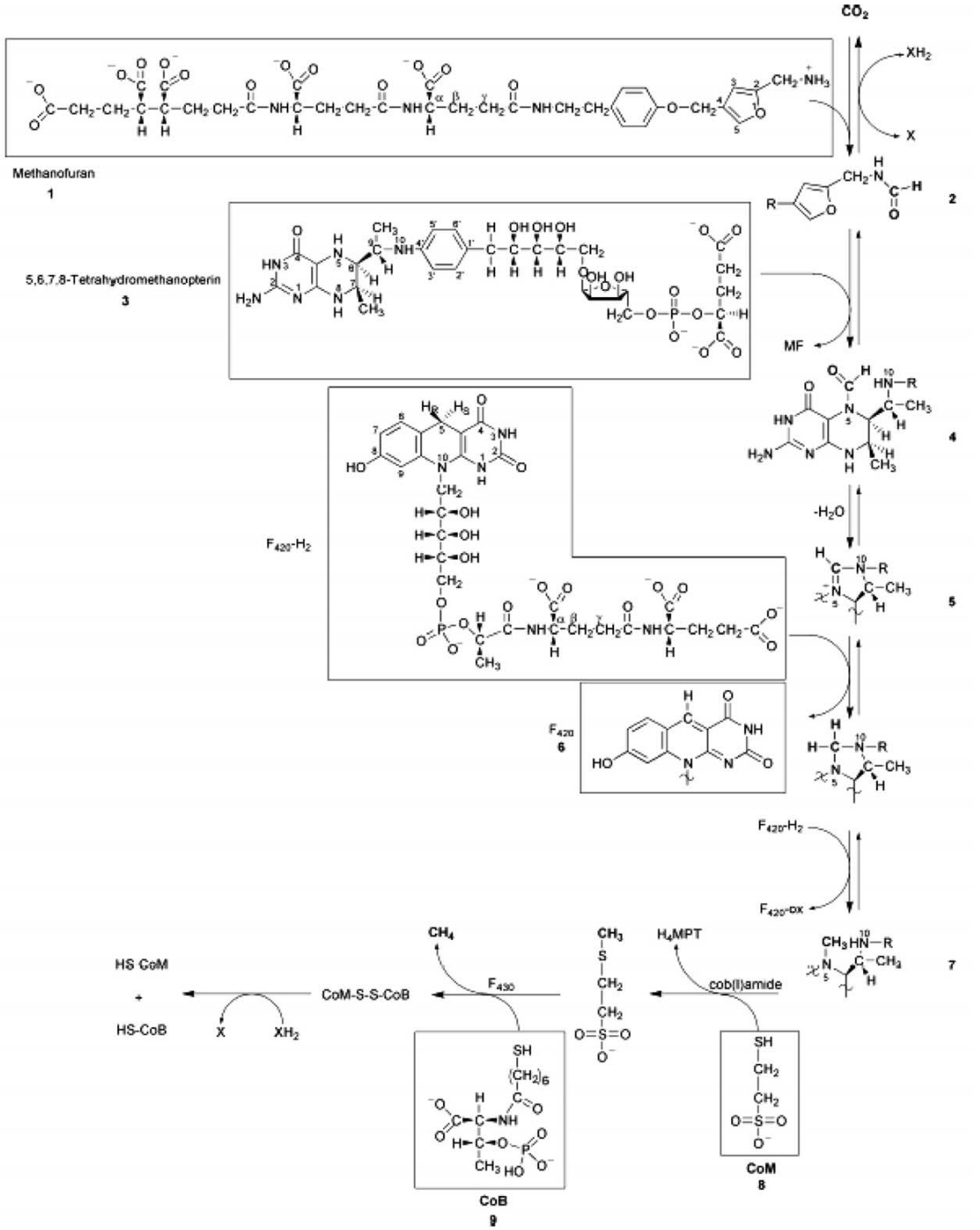

Fig. 1 Methanogenesis by the reduction of CO_2. (Elucidation of methanogenic coenzyme biosyntheses: from spectroscopy to genomics David E. Graham and Robert H. White)$_2$.

ic acid phosphodiester of 7, 8-didemethyl) 8-hydroxy-5-deazariboflavin 5 phosphate was first isolated from M*ethanobacterium strain M.o.H*. It has an absorption maxima at 420nm and hence its name. It gives blue green fluorescence in oxidized state. The fluorescence is lost upon reduction. It is present in methanogens at levels ranging from 1.2 mg/kg of dry cell wt. in *M.ruminantium* to 65 mg/kg of dry cell wt. in *M. thermoautotrophicum*.

Earlier this coenzyme was believed to be unique to hydrogen metabolizing euryarchaea. Later it was also identified in *Halobacteria* and some Gram positive bacteria. All the F_{420} found in these organisms differ in their glutamate side chains though they are functionally interchangeable and have comparable spectroscopic properties. Some cyanobacteria, mosses, green algae, midge produce DNA photolyase containing 8-hydroxy 5-deazariboflavin cofactor. F_{420} with additional glutamyl residue linked to distal glutamate is found in *M. barkerii* [36].

F_{420} is a low potential electron carrier [38]. The reduction potential of F_{420}/F_{420}-H_2 is -340to -350mV, between the redox potential of NAD (P)/NAD (P) H and $2H+/H_2$. It is similar to nicotinamide cofactors as it functions in two electron transfer reactions. It also shows some similarities with riboflavin as both riboflavin and F_{420} are synthesized from a shared precursor, also the 8 hydroxy 5 deazariboflavin moiety of F_{420} is structurally similar to riboflavin.

The activities of formate dehydrogenase [29, 41], CO dehydrogenase [37], hydrogenase [38–40] $NADP^+$ reductase [39, 41, 42], pyruvate synthetase and α-ketoglutarate synthetase [43,44] are coupled to oxidation /reduction of F_{420}.

F_{390}-In oxygen stressed methanobacterium cells a new chromophore derived from F_{420} was discovered [45]. Structurally they are F_{420} adducts. They have adenosine-5 phosphate /guanosine-5 phosphate linked to 8 hydroxy groups. They act as alarmone [45] in response to oxygen stress. They also activate the enzyme $NADP^+$ reductase and play an important role in metabolism.

Coenzyme M – The CoM (2-mercaptoethanesulfonic acid) is the smallest known organic cofactor. It was first characterized in 1971 in *Methanobacterium strain M.o.H*. as one of the several enzymes involved in methanogenesis [46]. Taylor and Wolfe described the structure of oxidized $(S\text{-}Co\ M)_2$ disulfide. Till 1999 it was considered unique to methanogens until it was discovered in *Xanthobacter* as a cofactor in alkane oxidation pathway. It acts as terminal methyl carrier in methanogenesis.

Coenzyme B – It is a colorless cofactor. It was earlier called component B as it was identified as one of the three chromatographically separated fractions required to reconstitute MCR (Methyl Coenzyme reductase). Its structure was determined as 7-mercaptohepta-moylthreonine phosphate. It acts with Coenzyme M in the final step of methanogenesis. It contains a thiol group and an L-threonine phosphate group which is specifically recognized by MCR. The thiol group displaces methane from M methyl Co M and L- threonine phosphate group binds to basic amino acid in MCR.

Methanofuran (MFR or carbon diooxide reduction factor) – It was first obtained from the cell extracts of *Methanobacterium thermoautotrophicum*. Earlier it was named as CO_2 reduction factor [47]. Later its structure was determined and was renamed as Methanofuran [48]. It is the only cofactor known to contain furan moiety. It is found in all methanogens at level ranging from 0.5–2.5 mg/kg of cell dry wt–[49]. Five different Methanofuran cofactors are produced by Archaea. The central core structure consist of 4-[N-(7-L-glutamyl-7-L-glutamyl)-P-(B-amino-ethyl) phenoxy methyl]-2-(amino methyl) furan, to which additional structures are attached by an amide bond to the ά-amino of terminal glutamyl residue. The MFR isolated from *Methanobacterium thermoautotrophicum* consisted of core structure attached to 1, 3, 4, 6-hexanetdra carboxylic acid (HTCA). MFR found in *Methanosarcani barkeri* consist of 2 γ-linked glutamic acid [19] while to hydroxyl HTCA is found in MFR found in *M. smithi*.

MFR react with CO_2 in the first of methanogenesis and forms an N- carboxymethanofuran. This cabamate is reduced to formyl methanofuarn by enzyme formyl methanofuran dehydrogenase.

Methanopterin- It was originally identified in *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri*. The cofactor in *Methanosarcina barkeri* was known as *sarcinopterin*. The structures of both the cofactor are similar except their dicarboxylic acid side chains sarcinopterin contain a glutamyl residue esterified to the hydroxyl glutarate moiety.

Structurally Methanopterin is related to folic acid [8, 50] as it consists of the same pteroic acid core found in folates. Pteroic acid core is attached to L-glutamates in folates, whereas methanopterin consists of pteroic acid core whose carboxylic acid is replaced by ribitol containing side chain also, the reduced form of both the cofactors, H_4MPT and H_4F are biologically active substances. However, both are well differentiated by enzymes that are specific for them.

In the process of methanogenesis MPT acts as intermediate C1 carrier in the reduction of formyl group to methyl group. H_4MPT is also required by *M. thermoautotrophicum* to synthesize acetate, whereas the methylene tetrahydromethanopterin is required for serine synthesis [51].

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