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Identyfikacja archeonów potencjalnie zdolnych do bezpośredniego wychwytu elektronów

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Identification of archaea potentially capable of direct electron uptake

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Streszczenie

Fermentacja metanowa jest procesem biologicznym wykorzystywanym w stabilizacji odpadów bogatych w związki organiczne, przy jednoczesnym wytwarzaniem biogazu. Szybkość tego procesu jest często ograniczona przez niskie tempo reakcji metabolicznych u mikroorganizmów syntroficznych. Stabilny i szybki transfer elektronów miedzy bakteriami octanogennymi i archeonami metanogennymi ma kluczowe znaczenie dla wydajnej metanogenezy. Przypuszczalnie bezpośredni międzygatunkowy transfer elektronów jest metabolicznie bardziej korzystny w porównaniu z transferem pośrednim (opartym an wodorze lub kwasie mrówkowym). Jak do tej pory niewiele wiadomo na temat zdolności metanogenów do bezpośredniego międzygatunkowego transferu elektronów. Odkrycie elektrycznie przewodzącej archaelli u *Methanospirillum hungatei* zasugerowało możliwość, że struktura ta może służyć do bezpośredniego wychwytu elektronów.

W ramach niniejszej pracy podjęto próbę identyfikacji archeonów metanogennych potencjalnie zdolnych do bezpośredniego wychwytu elektronów. Identyfikacja takich gatunków może pozwolić na uzyskanie populacji mikroorganizmów o wyższym potencjale metanogennym. Przeprowadzone analizy wykazały obecność homologów białka tworzącego archaelli u archeonów metanogennych należących do klasy Methanomicrobia, w szczególności do rodzaju *Methanosarcina*. Analiza danych literaturowych poddaje jednak w wątpliwość udział archaelli w procesie bezpośredniego wychwytu elektronów. Hipoteza alternatywna mówi, że za bezpośredni wychwyt elektronów są odpowiedzialne struktury powierzchniowe charakterystyczne dla mikroorganizmów z rzędu Methanosarcinales.

Abstract

Anaerobic digestion is an effective biological treatment for stabilizing organic compounds in wastes and in simultaneously producing biogas. It is often limited by the slow reaction rates of different microorganisms' syntrophic biological metabolisms. Stable and fast interspecies electron transfer between acetogenic bacteria and methanogens is crucial for efficient methanogenesis. In particular, direct interspecies electron transfer has been proposed to be metabolically advantageous compared to mediated interspecies electron transfer via hydrogen or formate, but little is known about the diversity of methanogens capable of direct interspecies electron transfer. Discovery of electrically conductive archaella of *Methanospirillum hungatei* suggested a possibility of archaella being a conduit for direct electron uptake. This study tried to explore this possibility and if true, as a conquence would lead to discovery of more methanogenic archae potentially capable of direct electron uptake. In presented study archaella protein analogues were indentified in methanogenic archaea from Methanomicrobia class particularly *Methanosarcina* genus. However analysis of available literature suggests that electrically conductive archaella may not play a direct role in electron uptake. Alternative hypothesis stands that direct electron uptake might be property of cell surface structures unique to methanogens of order Methanosarcinales.

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List of Abbreviations

AD	Anaerobic digestion . . . 1
LCFAs	Long chain fatty acids . . . 2
VFAs	Volatile fatty acids . . . 3
IET	Interspecies electron transfer . . . 5
MIET	Mediated interspecies electron transfer . . . 6
DIET	Direct interspecies electron transfer . . . 6
AQDS	Anthraquinone-2,6-disulfonate . . . 7
e-pili	Electrically conductive pili . . . 9
CM	Conductive material . . . 10
GAC	Granular activated carbon . . . 10
OmcS	Pilin associated c-type cytochrome . . . 10
BRONJ	Bisphosphonate-Related Osteonecrosis of the Jaw . . . 15
BLAST	Basic local alignment search tool . . . 19
NCBI	The National Center for Biotechnology Information . . . 19
MSA	Multiple sequence alignment . . . 19
EMBL-EBI	European Molecular Biology Laboratory-European Bioinformatics Institute . . . 19

1 Introduction

1.1 Anaerobic digestion

Anaerobic digestion (AD) is the breakdown of organic material by microorganisms in the absence of oxygen. Although this takes place naturally in soils, sediments, ruminants, and several other anoxic environments, the term normally describes an artificially accelerated operation in closed vessels called anaerobic digesters, resulting in a relatively stable solid residue. Biogas is generated during AD - mostly methane and carbon dioxide. This gas can be used as a chemical feedstock or as a fuel. AD can treat many biodegradable wastes, including wastes that are unsuitable for composting, such as meat and cooked food[1].

1.1.1 Historical perspective

Anecdotal evidence indicates that biogas was used for heating bath water in Assyria during the 10^{th} century BC and in Persia during the 16^{th} century. Methane generated during anaerobic processes was recorded as mysterious flickering flames in swamps and marshlands known as "will-o-wisp". Jean Baptiste Van Helmont in 1630 first determined that flammable gases could evolve from decaying organic matter. In 1776, Alessandro Volta concluded that there was a direct correlation between the amount of decaying organic matter and the amount of flammable gas emitted. He also determined that certain proportions of this flammable gas were explosive in air. In 1808, Humphry Davy determined that methane was present in the gases produced during the AD of cattle manure. The first digestion plant was built at a leper colony in Bombay, India in 1859. In 1868, Bechamp, a student of Pasteur', attempted to isolate the microorganism responsible for the anaerobic bioconversion of ethanol to methane. In reality, Bechamp's attempts resulted in a co-culture of microorganisms.

The first practical application of AD for energy production took place in

England in 1896 when biogas from sewage sludge digestion was used to fuel street lamps in Exeter. Since that time, the process has received considerable interest to harness its energy producing capabilities[2–4].

1.1.2 Anaerobic digestion process

The AD process takes place through the synergistic action of microorganisms in four stages: hydrolysis, acidogenesis(also known as fermentation), acetogenesis and methanogenesis[1]. Figure 1.1 illustrates these processes.

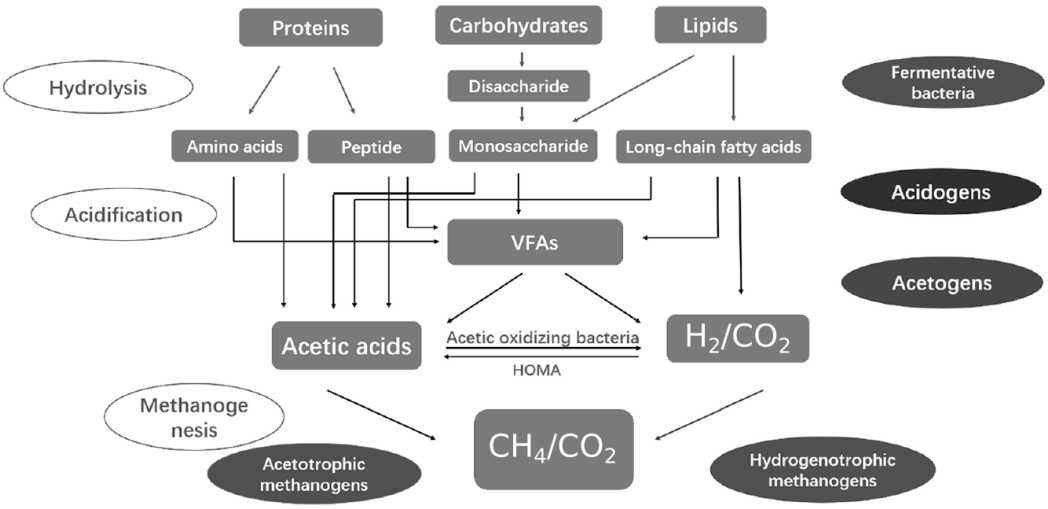


Figure 1.1: Schematic representation of anaerobic digestion[5]

Hydrolysis

Organic biomass contain complex polymers which are inaccessible to microorganisms without being further broken down through hydrolysis. Hydrolysis breaks down large organic macromolecules to smaller components that can be utilized by acidogenic bacteria. Hydrolytic bacteria are able to secrete extracellular enzymes that can convert carbohydrates, lipids, and proteins into sugars, Long chain fatty acids (LCFAs) and amino acids respectively. After enzymatic cleavage, the products of hydrolysis are taken up by acidogenic microorganisms. It is important to note that certain substrates, such as lignin, cellulose, and hemicellulose are difficult to degrade biologically due to their complex structures; enzymes are often added to enhance the hydrolysis of these carbohydrates[6].

Table 1.1: Typical hydrolytic bacteria.[7]

Substrates	Products	Typical species	Gram reaction	Shape	Motility*	Remark
Proteins	Amino acids, sugars	*Clostridium sp.*	+	rod	M	Brewery yeast waste treatment
		Proteus vulgaris	–	rod	M	Directed evolution of a lipase
		Peptococcus sp.	–	rod	M	Fermentation of glutamic acid
		Bacteroides sp.	–	rod	N	Induction of cell populations in peripheral lymph
		Bacillus sp.	+	rod	M	Syntrophic culture with *Clostridium*
		Vibrio sp.	–	rod	M	Determine the effectiveness of freshly grown *vibrio spp.* for acute toxicity
Carbohydrates	Sugars	*Clostridium sp.*	+	rod	M	Palm oil mill effluent treatment
		Acetivibrio cellulolyiticus	–	rod	M	Degradation of cellulose to CH_4
		Staphylococcus sp.	+	sphere	N	Co-metabolism in the presence of glucose
		Bacteroides sp.	–	rod		
Lipids	Higher fatty acids, alcohols, amino acids					
		Clostridium sp.	+	rod	M	Food waste treatment
		Micrococcus sp.	+	sphere	N	Isolation of biosurfactants from *Micrococcus sp.*
	Sugars					
		Staphylococcus sp.	+	sphere	N	
RNA	Purines, pyrimidines					
DNA		*Bacillus sp.*	+	rod	M	

*M, motile; N, non-motile.

Table 1.1 shows substrates, products and characteristics of some typical hydrolytic bacteria.

Acidogenesis

By absorbing the products of hydrolysis through their cell membranes, acidogenic microorganisms are able to produce intermediate Volatile fatty acids (VFAs) and other products. VFAs constitute a class of organic acids such as acetates, and larger organic acids such as propionate and butyrate[6].

Table 1.2 shows substrates, products and characteristics of some typical

Table 1.2: Typical acidogenic bacteria.[7]

Substrates	Products	Typical species	Gram reaction	Shape	Motility*	Remark
Amino acids	Valerate, Isovalerate, propionate, butyrate,	*Lactobacillus sp.*	+	rod	N	Production of γ-aminobutyric acid
		Eschericia coli	–	rod	M	Synthesis of nitrobenzocyclophosphamide
	acetate, H_2, Higher fatty acids	*Staphylococcus sp.*	+	sphere	N	
		Bacillus sp.	+	rod	M	
		Pseudomonas sp.	–	rod	M	Biodegradation of mixture of various textile dyes
		Micrococcus sp.	+	sphere	N	
		Eubacterium limosum	+	rod	M	Biotransformation isoflavonoids biochanin
		Clostridium sp.	+	rod	M	Hydrogen production from sucrose
		Zymomonas mobiliz	–	rod	M	Ethanol production
Sugars	CO_2, H_2, formate, acetate, butyrate	*Eubacterium sp.*	+	rod	M	Hydrogen production
	CO_2, H_2, formate, acetate, ethanol, lactate	*Eschericia coli*	–	rod	M	
	Formate, acetate, ethanol, lactate	*Bifidobacterium sp.*	+	rod	N	Production of bacteriocin
	Acetate	*Acetobacterium sp.*	–	ellipse	M	Production of vitamin B_{12}
Fatty acids	Valerate, isovalerate, propionate, butyrate, acetate, H_2	*Closrtidium sp.*	+	rod	M	
Alcohols		*Syntrophomonas wolfei*	–	rod	M	Oxidation of fatty acids

*M, motile; N, non-motile.

acidogenic bacteria.

Acetogenesis

With the production of acetate through acidogenesis, a portion of the original substrate has already been rendered into a substrate suitable for acetoclastic methanogenesis. However, other produced higher VFAs have yet to be made accessible to methanogenic microorganisms. Acetogenesis is the process by which these higher VFAs and other intermediates are converted into acetate, with hydrogen also being produced.

Excessive partial pressure of hydrogen proves to be deleterious to acetogenic microorganisms. However, due to the presence of hydrogenotrophic methanogens, hydrogen is able to be rapidly consumed while maintaining hydrogen partial pressures at a level favorable to acetogenesis. At the same time, lipids undergo a separate pathway of acetogenesis via acidogenesis and β-oxidation, where acidogenesis produces acetate from glycerol and β-oxidation produces acetate from LCFAs[6].

Table 1.3 shows substrates, products and characteristics of some typical acetogenic bacteria.

Table 1.3: Typical acetogenic bacteria.[7]

Substrate	Product	Typical species	Temperature, °C	Gram reaction	Shape	Motility*
Butyrate	Acetate	*Syntrophobacter wolinii*	35–40	–	rod	N
	H_2/CO_2, Formate	*S. fumaroxidans*	35–40	–	rod	N
Propionate	H_2/CO_2, Formate	*Syntrophomonas wolfei*	35–40	–	rod	M
		Pelotomaculum thermopropionicum	50–60†	+	rod	M
		P. schinkii	32–37	+	rod	M
	Butyrate, acetate	*Smithella propionica*	35–40	–	rod	M
H_2, CO_2	Acetate	*Clostridium aceticum*	30–37	–	rod	M

*M, motile; N, non-motile.
†Thermophilic acetogens.

Methanogenesis

Methanogenesis is the final stage of AD. In this stage intermediates produced by previous stages of AD are consumed by methanogenic archaea to produce methane[6].

Table 1.4 shows substrates, products and characteristics of some typical methanogenic archaea.

1.1.3 Syntrophy in methane production

Syntrophy is a form of symbiosis of two metabolically different groups of bacteria, which enables degradation of various substrates[8]

Methanogenic archaea, which are involved in the production of methane exhibit synergistic relationships with Syntrophic bacteria. Syntrophic bacteria can't grow in form of pure cultures, but only when accompanied by microorganisms using hydrogen produced by them. Syntrophic interactions between bacteria and methanogens are the basis to maintain an AD system working efficiently. These microorganisms, with distinct, but complementary metabolic capabilities, exchange electrons for energy purposes, normally through the transfer of small soluble chemical compounds, such as hydrogen or formate, that act as electron shuttles. This is called Interspecies electron transfer (IET).[8]

This interspecies hydrogen/formate transfer process is very important since the overall thermodynamics depends on the capacity of the microbial communities to maintain a low hydrogen partial pressure. Thus, diffusion limitations of these metabolites, between anaerobic bacteria and methanogenic archaea, can

Table 1.4: Typical methanogenic archaea.[7]

			Optimum growth conditions				
Substrates	Products	Typical species	pH	Temperature, °C	Shape	Motility*	Gram reaction
Acetate	CH_4, CO_2	*Methanothrix soehngenii*	7.4–7.8	35–40	Sheathed rod	N	–
		Methanosaeta concilii	7.1–7.5	35–40	Sheathed rod	N	–
		Methanosarcina acetivorans	6.5–7.5	35–40	Coccoid	N	–
H_2, CO_2	CH_4	*Methanobacterium bryantii*	6.9–7.2	37–39	Rod	N	+
		M. thermoautotrophicum	7.2–7.6	65–70†	Rod	N	+
		M. alcaliphilum	8.1–9.1‡	37	Rod	N	–
		Methanobrevibacter arboriphilus	7.8–8.0	30–37	Coccobacillus	N	+
		Methanococcus jannaschii	5.0–7.0	85§	Irregular coccus	M	
		Methanolacinia paynteri	6.6–7.2	40	Irregular rod	N	–
		Methanospirillum hungatei		30–37	Sheathed spiral	M	–
		Methanoplanus endosymbiosus	6.6–7.2	40	Irregular disk	N	–
		M. olentangyi		30–40			
		Methanothermus fervidus	6.5	83§	Rod	M	+
Formate, H_2, CO_2	CH_4, CO_2	*Methanobacterium formicicum*	6.6–7.8	37–45	Rod	N	+
		Methanobrevibacter smithii	7.0	37–39	Rod	N	+
		M. ruminantium	7.0	37–39	Occobacillus	N	+
		Methanococcus voltae	6.5–8.0	35–40	Irregular coccus	M	
		M. deltae		37	Irregular coccus	N	
		M. maripaludis	6.5–8.0	35–40	Irregular coccus	M	
		M. thermolithoautotrophicus	6.5–8.0	65†	Irregular coccus	M	–
		Methanoplanus limicola	7.0	40	Plate	M	–
		Methanogenium cariaci	6.2–6.6	20–25	Irregular coccus	N	–
		M. marisnigri	6.8–7.3	20–25	Irregular coccus	N	–
		M. olentangyi		37	Irregular coccus	N	–
		M. tatii	7.0	37–40	Irregular coccus	N	–
		M. thermophilicum	6.5–7.2	55–60†	Irregular coccus	N	–
		M. bourgense	6.3–6.8	35–42	Irregular coccus	N	–
		Methanocorpusculum aggregans	6.4–7.2	35–37	Irregular coccus	N	–

*M, motile; N, non-motile.
†Thermophilic methanogens.
‡Alkaliphilic methanogens.
§Hyper-thermophilic methanogens.

be important bottlenecks in the anaerobic conversion process.[9]

1.1.4 MECHANISMS OF IET IN METHANOGENS

Interspecies electron transfer (IET) can be classified into two types. Namely,

1. Mediated interspecies electron transfer (MIET)
2. Direct interspecies electron transfer (DIET)

1.1.5 Mediated interspecies electron transfer (MIET)

Mediated interspecies electron transfer (MIET) is the most frequently described mode of IET, whereby an electron carrying compound is transported by diffusion from mediator producing cells to mediator consuming cells along a concentration gradient. The mediator diffusion rate is limited by the concentration gradient at which oxidation and reduction reactions are thermodynamically feasible.[10]. The following subsections briefly explain MIET mechanisms.

MIET via Soluble Molecules

The most studied(in recent past) and widely known mechanism of electron transfer in methanogenic communities is the MIET via hydrogen or formate. Syntrophic bacteria produce hydrogen or formate as a way to dissipate the reducing power, i.e., the electrons formed during the degradation of organic compounds and, in turn, methanogens utilize those molecules as electron donors to reduce carbon dioxide to methane. Therefore, hydrogen/formate act as shuttles between hydrogen/formate forming bacteria and hydrogen/formate utilizing methanogens. At high hydrogen concentrations (>10 Pa), the hydrogenase activity is inhibited and consequently the metabolism of syntrophic bacteria is inhibited as well, while that of the methanogens is stimulated, and vice versa. Formate formation has been detected particularly in co-cultures growing on proteins or fatty acids like propionate and butyrate. Under certain conditions, interspecies formate transfer may prevail because formate has a higher diffusion coefficient, comparing to hydrogen. Syntrophic interactions involving hydrogen or formate as electron shuttles are well described in co-cultures degrading common compounds formed during the AD process such as butyrate, propionate, ethanol, and acetate [9].

MIET via Extracellular Compounds

In numerous anaerobic environments, the interspecies electron transfer can also be mediated by insoluble compounds. Unlike soluble electron shuttles, such as hydrogen or formate, that can diffuse in and out of the cell, insoluble compounds do not penetrate the cell surface. Lovley et al. [11] demonstrated that humus can mediate electron transfer between humics-reducing and humics-oxidizing microorganisms. The electron acceptor properties have been related mainly with the redox active quinone moieties present in humic substances. Several microorganisms were found to reduce humic acids or anthraquinone-2,6-disulfonate Anthraquinone-2,6-disulfonate (AQDS) using hydrogen as electron donor (e.g., the halorespiring bacterium, *Desulfitobacterium* PCE1; the sulfate-reducing bacterium, *Desulfovibrio* G11 and the methanogenic archaea, *Methanospirillum hungatei* JF1) or lactate (Desulfitobacterium dehalogenans and Desulfitobacterium PCE1).[9, 12] Humus can also be reoxidized and act as an electron donor. For example, humic acids can be redox mediators in the anaerobic substrate oxida-

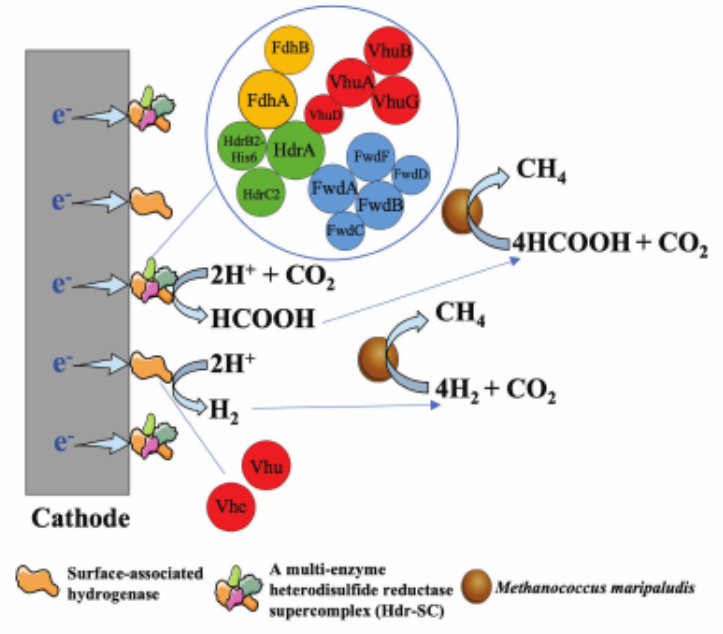


Figure 1.2: Extracellular enzyme dependent MIET[14]

tion coupled to the abiotic reduction of metal oxides such as Fe(III) and Mn(IV), being reoxidized and participating in many cycles.[9, 12]. Some bacteria of the genus *Geobacter* have been reported as quinone reducing microorganisms using Fe(III) as the terminal electron acceptor but other microorganisms share this ability, such as some *Shewanella*, *Desulfitobacterium*, *Desulphuromonas, Geospirillum*, *Wolinella*, and *Geothrix*[13] and the methanogenic archaea *Methanopyrus kandleri*, *Methanobacterium thermoautotrophicum*. The anaerobic oxidation of lactate and hydrogen by *Desulfitobacterium dehalogenans* was obtained with AQDS as mediator, associated with the reduction of goethite[9].

Extracellular enzyme dependent MIET

Hydrogenase and formte dehydrogenase can be released from the living or dead cells of *Methanococcus maripaludis* and then are absorbed on the cathode surface. These surface-associated enzymes can catalyze the formation of hydrogen or formate, which are then rapidly consumed by *M. maripaludis* cells to produce methane (Figure 1.2)[14].

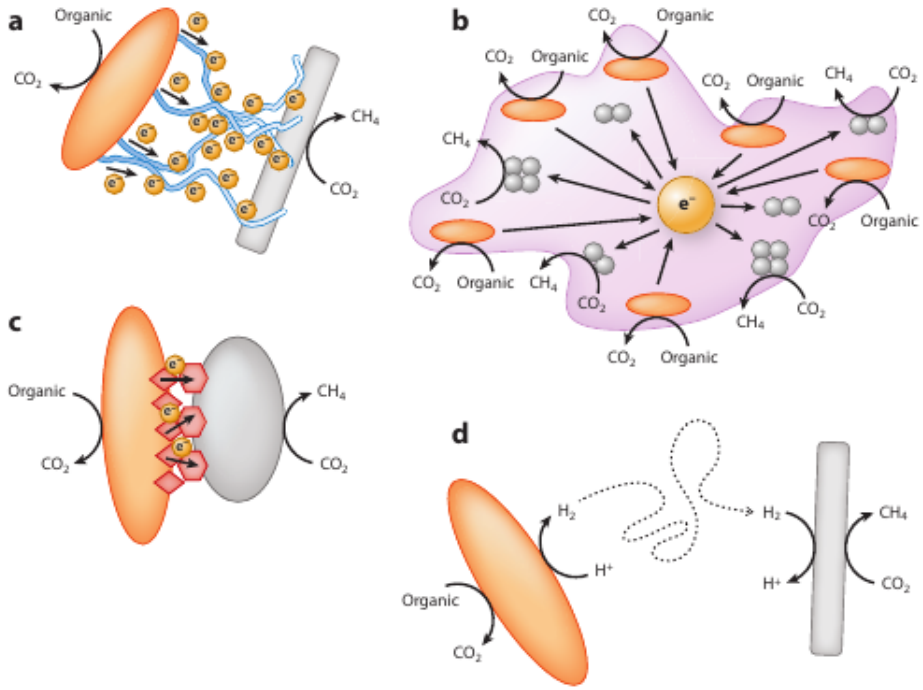


Figure 1.3: Proposed DIET mechanisms[15]

1.1.6 Direct interspecies electron transfer (DIET)

Direct interspecies electron transfer (DIET) has been described in anaerobic environments, involving the formation of an electric current between electron-donating and electron-accepting microbes and without the need to produce and exchange electron carriers (i.e., hydrogen and formate). DIET is analogous to direct extracellular electron transfer, which consists in the electron transfer between cells and a solid-state electron acceptor such as iron and manganese oxides or electrodes[15]. Figure 1.3 shows proposed DIET mechanisms.

DIET promoted by Electrically conductive pili (e-pili)

Direct interspecies electron transfer (DIET) is well studied in bacteria belonging to the genera *Shewanella* and *Geobacter*. DIET was first described in defined co-cultures of *Geobacter metallireducens*, an ethanol oxidizing bacteria, and *Geobacter sulfurreducens*, a fumarate reducing bacteria. These microorganisms establish a syntrophic relationship, where *G. metallireducens* metabolize the ethanol and the *G. sulfurreducens* reduces the fumarate. The ability of this culture for performing DIET was discovered when co-cultures formed with *G.*

sulfurreducens strains lacking the *hyb* gene (thus unable to utilize hydrogen), were able to oxidize ethanol and to reduce fumarate. Under these conditions, interspecies electron exchange between *G. metallireducens* and *G. sulfurreducens* occurred directly via e-pili and without the formation of soluble intermediates. Rotaru et al. [16] showed that *Methanosaeta harundinacea*, a strictly acetoclastic methanogen, can receive electrons directly from *G. metallireducens* to produce methane. The idea that *Methanosaeta* species are acetoclastic specialists, only producing methane from acetate, changed from this point on, since it seems to be able to activate the carbon dioxide reduction pathway for methane production. In this context, the electrons released by *Geobacter* species are transferred, via e-pili, directly to *Methanosaeta*, but the cell machinery involved in electrons uptake by the methanogen is not yet known. These findings gave a new perspective on the interspecies interactions taking place in anaerobic bioreactors producing methane [9].

DIET promoted by conductive materials

The presence of Conductive material (CM) such as Granular activated carbon (GAC), carbon cloth, and biochar appears to promote DIET via a conduction-based mechanism, in which electrons migrate through the CM from electron-donating to electron-accepting cells.[9] Surprisingly, it was observed that the lack of pili and other cell component involved in the exogenous electron transfer can be compensated by the presence of CM, namely GAC, carbon cloth and biochar.[15] This was verified in defined cocultures of pilA-deficient strains of *G. metallireducens* with *Methanosarcina barkeri*, which could not convert ethanol to methane unless in the presence of CM. This methanogenic co-culture in the presence of biochar were able to utilize 86% of the electrons released from ethanol oxidation for methane production, but without biochar no ethanol was consumed and no methane was produced.[17] Similarly, the lack of the Pilin associated c-type cytochrome (OmcS), necessary for extracellular electron transfer in *Geobacter* species, could be compensated by magnetite, another conductive material. Without magnetite *Geobacter* strains lacking genes for OmcS were ineffective in forming viable co-cultures, but in the presence of magnetite, the OmcS deficient mutants performed similarly to the wild type[9, 15].

DIET promoted via outer cell surface

The need for Electrically conductive pili (e-pili) or abiotic conductive materials to serve as interspecies electrical connectors may be alleviated if cells can form tight connections between their outer surfaces. This possibility is evident in co-cultures of *Prosthecochloris aestuarii* and *G. sulfurreducens*. *P. aestuarii*, an anaerobic phototroph, directly accepted electrons from electrodes or DIET to support photosynthesis. *P. aestuarii* and *G. sulfurreducens* formed tight associations with the two species in intimate contact[15].

Hydrogen and Formate IET versus DIET

An interesting approach toward the clarification of the importance of DIET in methanogenesis was presented by Storck et al. [10] who proposed a mechanistic framework that enabled the direct assessment of the relative feasibility of DIET and MIET in a thermodynamically restricted syntrophic system (propionate conversion to acetate and methane), through mathematical modeling. They found that DIET could be more favorable than hydrogen MIET, but substantially less favorable than formate MIET (1 order of magnitude rate difference), assuming a default parameter set based on literature data. The model results also suggested that DIET may be a thermodynamically more feasible alternative to MIET for diverse communities limited by diffusion, which is contrary to experimental observations where nanowire DIET is commonly observed in dense aggregates possibly indicating that co-evolution and co-metabolism are more important than external limitations in the simulated system. These authors also suggested that CM reduce resistivity, and leave only activation losses, making long-range transport even more feasible [9].

1.2 Structure of Archaellum

The archaellum is the swimming organelle in the domain Archaea. Unlike bacterial flagella, archaellum is distinct in terms of molecular composition, mode of action and is has much thinner filament (typically 10–14 nm in diameter)[18]. The following section will briefly explain the structure of archaellum proposed for *Pyrococcus furiosus* by Daum et al. [19] but should be universal to all

motile archaea, as the core of the archaellum machinery is conserved throughout Crenarchaeal, Euryarchaeal and Thaumarchaeal lineages.

Archaella are composed of a limited number of Fla proteins, most of which are encoded in a single Fla operon.[18]

The filament is composed of similar subunits of FlaB3 protein in case of *M. hungatei*. Each FlaB3 subunit comprises two domains: N-terminal a-helix and a C-terminal globular part, similar to that described for archaellins of *P. furiosus* and *Methanocaldococcus jannaschii* which have FlaB0 and FlaB1 respectively in their filament

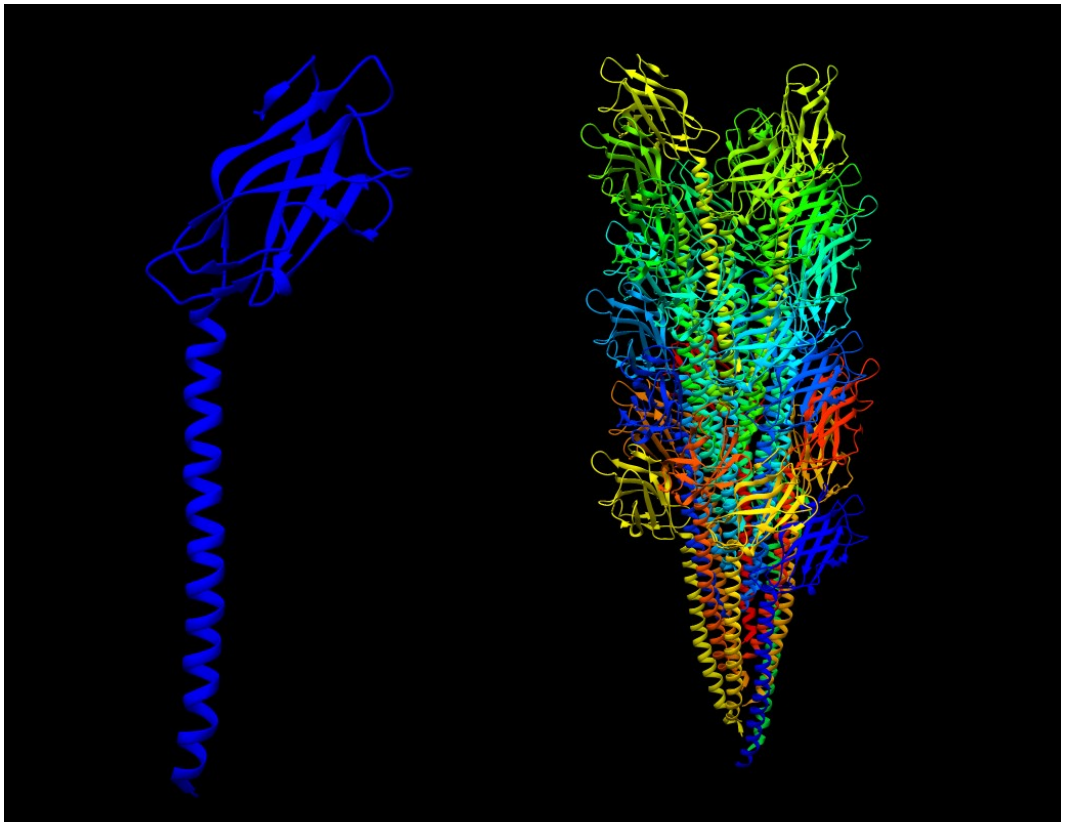


Figure 1.4: Archaellin subunit and filament

Archael filament crosses the S-layer and spans the periplasmic gap. They traverse the periplasm at variable angles of 60–90 degrees between the filament axis and the membrane plane.

These filaments emerge from a basal density on the cytoplasmic surface of the plasma membrane, which most likely corresponds to the archaellar motor.

Each of these densities is located in gap between the membrane and a sheet-like

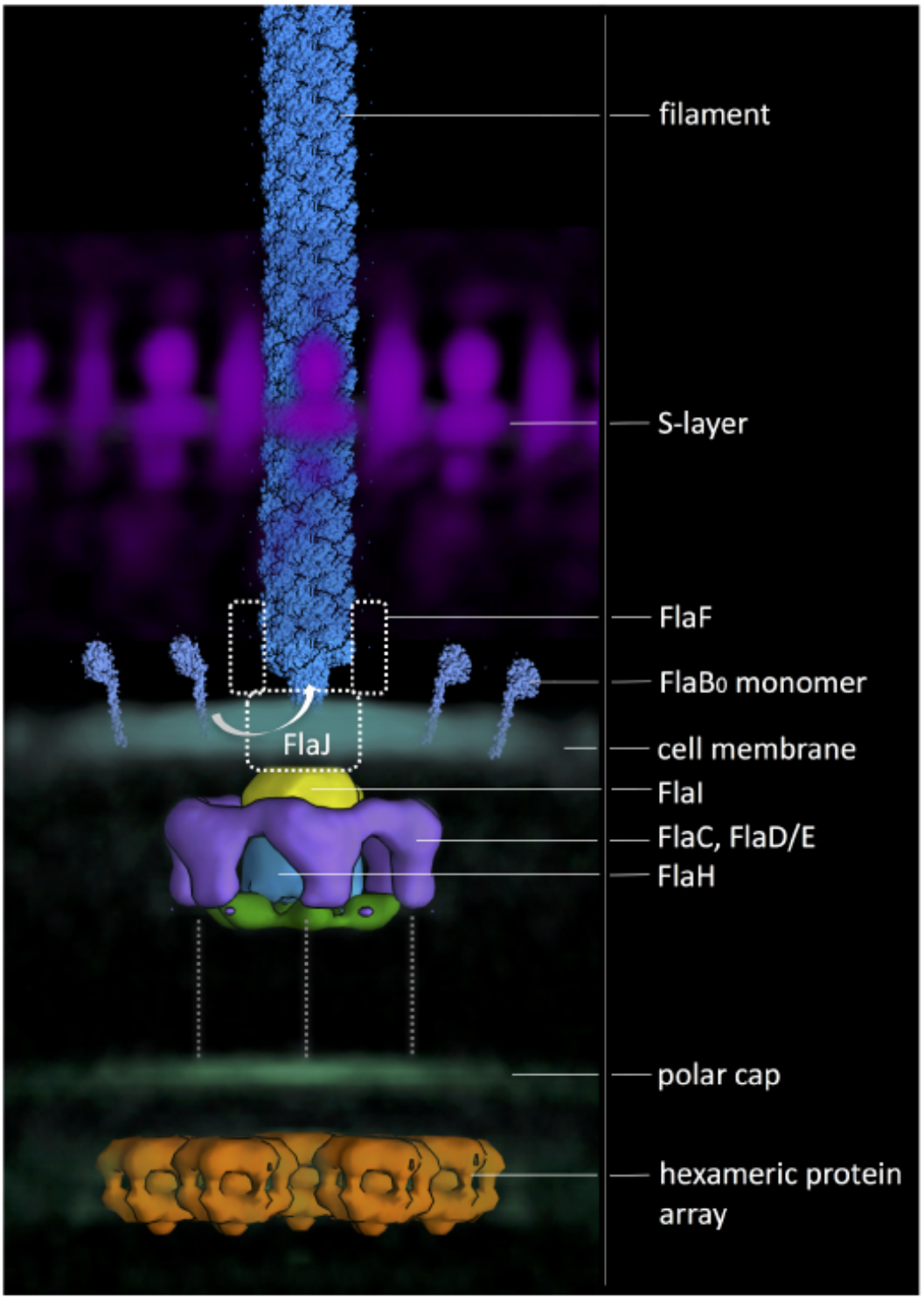


Figure 1.5: Archaellum structure[19]

cytoplasmic structure called polar cap. The polar cap appears to be a hallmark of motile Euryarchaeota, as it has also been observed in related species..

Filament assembly and rotation is powered by the archaellar motor, which is composed of the fully membrane-embedded FlaJ, a bell-shaped cytosolic complex of FlaI and FlaH and a surrounding cytosolic ring, most likely consisting of FlaC and D/E. In the periplasm, the filament is thought to be coordinated by FlaF.

The archaellar motors are juxtaposed to the polar cap, which in *P. furiosus* is only present in combination with an archaellar bundle. This co-localisation indicates that archaella and polar cap are co-regulated and suggests a strong functional connection. It is conceivable that the polar cap functions in concentrating archaella at one cell pole and acts as an anchor to fix the motor complexes in the bilayer to prevent futile rotation. The polar cap is associated with protein complexes that form hexameric protein arrays. While the identity of these complexes is unknown, their localisation next to the archaellar motors suggests that they may be mechanistically linked to motor function.

The first steps of archaellum assembly comprise N- or O-glycosylation and removal of the positively charged N-terminal MAKKG signal peptide from membrane-bound FlaB0 monomers(for *P. furiosus*). Loss of these positive charges primes the individual archaellins for transfer from the lipid bilayer into the growing filament, aided by the membrane protein FlaJ. This process is driven by the amphipathic surface of the individual archaellins and catalysed by ATP hydrolysis through FlaI.

1.3 Possible industrial application of e-pili

Bioenergy

For production of highly efficient microbial fuel cells, electron transfer should occur through biofilms so that microorganisms which are away from the Cathode can recieve electrons from it and increase total current output. E-pili can be useful for such long-range electron transfers and improve overall efficiency of microbial fuel cells[20].

Bioremediation

Shewanella and *Geobacter* have been extensively studied for bioremediation of heavy metals. Discovery of e-pili in these microorganisms has improved their potential in this field. It has been shown that e-pili can play an important role in bioremediation of a heavy metal like uranium [20, 21].

Bioelectronics

Researchers think that e-pili may allow us to develop instruments usable in water and moist environments[22]. Additionally, Leung et al. [23] characterized *Shewanella oneidensis* e-pili and showed that they have enough mechanical strength to be used as a building block for construction of electronic devices[20].

Potential target for pathogenic microorganisms

E-pili have been found in pathogenic biofilms causing Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ)[1] and supposed to play an important role in maintenance and survival of it. This discovery is very important considering the fact that various human pathogenic microorganisms like *Streptococcus pneumoniae* and *Corynebacterium diphtheriae* produce pili which are actively involved in pathogenesis[25]. Exoelectrogens with e-pili play specific role in host immune response. It needs to be studied whether pili are conductive in different pathogenic bacteria and, if so, what role they play in pathogenesis. In the phenomenon called 'bioelectric effect', electrically stimulated pathogenic biofilms showed increased susceptibility to antibiotics and this may happen because of disruption of conductive filaments within them as a result of electrical stimulation[26]. The bioelectric effect also supports the hypothesis that e-pili may play an important role in maintenance of pathogenic biofilms. Thus, e-pili can be a potential target for prevention and treatment of certain diseases[20].

[1] Chronic condition of the oral cavity resulting in mucosal ulceration, exposure of underlying necrotic bone and ensuing secondary complications[24]

1.4 Electrically conductive archaella of *Methanospirillum hungatei*

Methanospirillum hungatei JF1 is a hydrogen and formate utilizing, methane producing archaeon and is the type species of the genus Methanospirillum, which belongs to the family Methanospirillaceae within the order Methanomicrobiales[27]. In 1966, Smith [28] reported the isolation of a new spiral-shaped methanogenic bacterium from sewage sludge. Ferry et al. [29] in 1974 described the genus *Methanospirillum* and the type species was named *M. hungatii* and type strain *M. hungatii* JF-1 (now *M. hungatei* JF-1 [30]) in honour of Robert Edward Hungate[2].These archaea produce colonies that are yellow, circular, and convex with lobate margins; an optical pattern of regular, light and dark striations throughout the colonies is the most unique and distinguishing characteristic[29].

Its morphology is distinct from other methanogens with the ability to form long chains of cells (up to 100 μm in length), which are enclosed within a sheath-like structure, and terminal cells with polar flagella. The genome of *M. hungatei* strain JF1 is the first completely sequenced genome of the family Methanospirillaceae, and it has a circular genome of 3,544,738 bp containing 3,239 protein coding and 68 RNA genes[27].

First atomic model of *M. hungatei* archaella, based on the cryo electron microscopy was built by Poweleit et al. [32] at 3.4 Å resolution. Each archaellum contains $\approx$ 61,500 archaellin subunits organized into a curved helix with a diameter of 10 nm and average length of 10,000 nm. The tadpole-shaped archaellin monomer has two domains, a β-barrel domain and a long, mildly kinked α-helix tail[32]. The discovery of e-pili begged the question if archaellum were conductive. In quest to answer this question, Walker et al. [33] chose *M. hungatei* for their experiment since they were known to reduce extracellular electron acceptors and now thanks to Poweleit et al. [32], the structure of archaellum was also available. Their experiment infact showed that the archaellum of *M. hungatei* was conductive.

This led to questioning of the possibility that, electrically conductive archaellum may be a conduit for direct electron uptake by methanogens.

[2]Pioneer of anaerobic microbial ecology. Developed the techniques for the culturing of anaerobic microbes[31]

2 Objectives

The purpose of this study is to find archaea with potential for Direct interspecies electron transfer (DIET) through electrically conductive archaella by,

1. Selection of proteins enabling direct electron transfer.
2. Identification of microorganisms carrying analogues of selected proteins among organisms living in anaerobic ecosystems.
3. Analysis of potential protein analogues functionality based on sequence alignment.

3 Materials and Methods

3.1 Basic local alignment search tool (BLAST)

The National Center for Biotechnology Information (NCBI) Translated BLAST web interface was used to find analogues of protein sequence of interest[34]. The last date of search was 07/09/2021. The search parameters used are in Table 3.1.

Table 3.1: BLAST parameters

General parameters		**Scoring Parameters**		**Filters**
Expect threshold	0.05	Matrix	BLOSUM62	Low complexity regions filter selected
Word size	2, 3 & 6	Gap Costs	Existence:11 Extension:1	
Max matches in a query range	0	Compositional adjustments	Conditional compositional score matrix adjustment	

3.2 Clustal Omega

Multiple sequence alignment (MSA) was performed using Clustal Omega tool on web interface provided by European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) using default settings[35].

3.3 ESPript 3

ESPript is a program which renders sequence similarities and secondary structure information from aligned sequences for analysis and publication purpose. It was used to visualize MSA results[36].

3.4 SWISS-MODEL

SWISS-MODEL is a fully automated protein structure homology modelling server, accessible via the Expasy web server(Swiss institute of Bioinformatics). This was used to create models of archaella of methanogens found in this study whose protein structures were unavailable[37].

3.5 UCSF Chimera

UCSF Chimera is a program for the interactive visualization and analysis of molecular structures and related data, including density maps, trajectories, and sequence alignments[38]. This program (version 1.15) was used to visualize protein structures of the archaella. It was also used for hydrogenbond analaysis, analysis of protein surface property, analysis of clashes/contacts and superimposition of protein analogues.

4 Results

4.1 Translated BLAST

Translated BLAST search using *M. hungatei* JF-1 archaellum sequence was performed using three different word size parameter. With word size 3 and 6, no target organism was used while with word size 2, search was conducted against a list of methanogenic archae listed in Appendix (page no. 43). All word sizes revealed same result. The 34 methanogenic organisms found are listed in Table 4.1. The organisms found had on average 30% percentage identity match compared to sequence of *M. hungatei* JF-1 with *Methanospirillum sp* J.3.6-F.2.7.3 having highest percentage identity match(54.39%). Most organisms had on average 3 copies of the gene with *Methanosphaerula palustris* E1-9c having the highest number of gene copies (8). Most of the organisms found were of genus *Methanosarcina* (19 out of 34). Few non methanogenic archae were found to have *M. hungatei* archaellum analogues. They are, *Archaeoglobus fulgidus, Archaeoglobus profundus, Geoglobus acetivorans, Thermosphaera aggregans, Desulfurococcus amylolyticus* and *Candidatus Nitrosotenuis aquarius*.

4.2 Multiple sequence alignment

MSA was performed on all methanogenic archae found using clustal omega. when there were multiple strains, type strain was chosen to be portrayed. (Figure 4.1).

It can be observed that the α - helix from N-terminal side is highly conserved. All the sequences have phenylalanine in position 1, 13 and 20 which have been proposed to be the main reason for electrical conductivity of *M. hungatei* archaella. There is quite a bit of variance in sequences that form β barrel on C-terminal side with few small regions being conserved.

MSA was performed on all copies of archaellin genes in *Methanosphaerula*

Table 4.1: List of methanogenic archaea found

	List of methanogenic archaea	% Identity	No. of copies
1	Methanospirillum sp. J.3.6.1-F.2.7.3	54.39%	4
2	Methanospirillum hungatei GP1	49.14%	4
3	Methanoculleus marisnigri JR1	35.83%	2
4	Methanoculleus chikugoensis MG62	35.29%	2
5	Methanolinea sp. EsbE	33.70%	1
6	Methanocella conradii HZ254	33.54%	1
7	Methanoculleus bourgensis MAB1	33.52%	2
8	Methanosphaerula palustris E1-9c	30.98%	8
9	Methanosarcina acetivorans C2A	30.96%	3
10	Methanocella arvoryzae MRE50	30.81%	2
11	Methanosarcina siciliae HI350	30.46%	3
12	Methanococcoides burtonii DSM 6242	30.00%	2
13	Methanosarcina horonobensis HB-1	29.95%	3
14	Methanosarcina mazei LYC	29.57%	3
15	Methanosarcina sp. MTP4	29.28%	2
16	Methanoculleus bourgensis BA1	29.14%	4
17	Methanoregula formicica SMSP	29.05%	4
18	Methanosarcina siciliae T4/M	28.93%	3
19	Methanosarcina siciliae C2J	28.93%	3
20	Methanolacinia petrolearia DSM 11571	28.57%	6
21	Methanoculleus bourgensis MS2T	28.16%	2
22	Methanoregula boonei 6A8	27.60%	2
23	Methanosarcina mazei Tuc01	27.22%	1
24	Methanosarcina mazei SarPi	27.22%	3
25	Methanosarcina mazei Goe1	27.22%	3
26	Methanosarcina mazei WWM610	27.22%	2
27	Methanosarcina mazei S-6	27.22%	3
28	Methanosarcina mazei C16	27.22%	2
29	Methanosarcina mazei JL01	27.22%	2
30	Methanosarcina mazei zm-15	27.22%	2
31	Methanosarcina mazei TMA	27.22%	2
32	Methanosarcina lacustris Z-7289	25.99%	1
33	Methanosarcina sp. WH1	24.43%	1
34	Methanosarcina sp. WWM596	24.43%	1

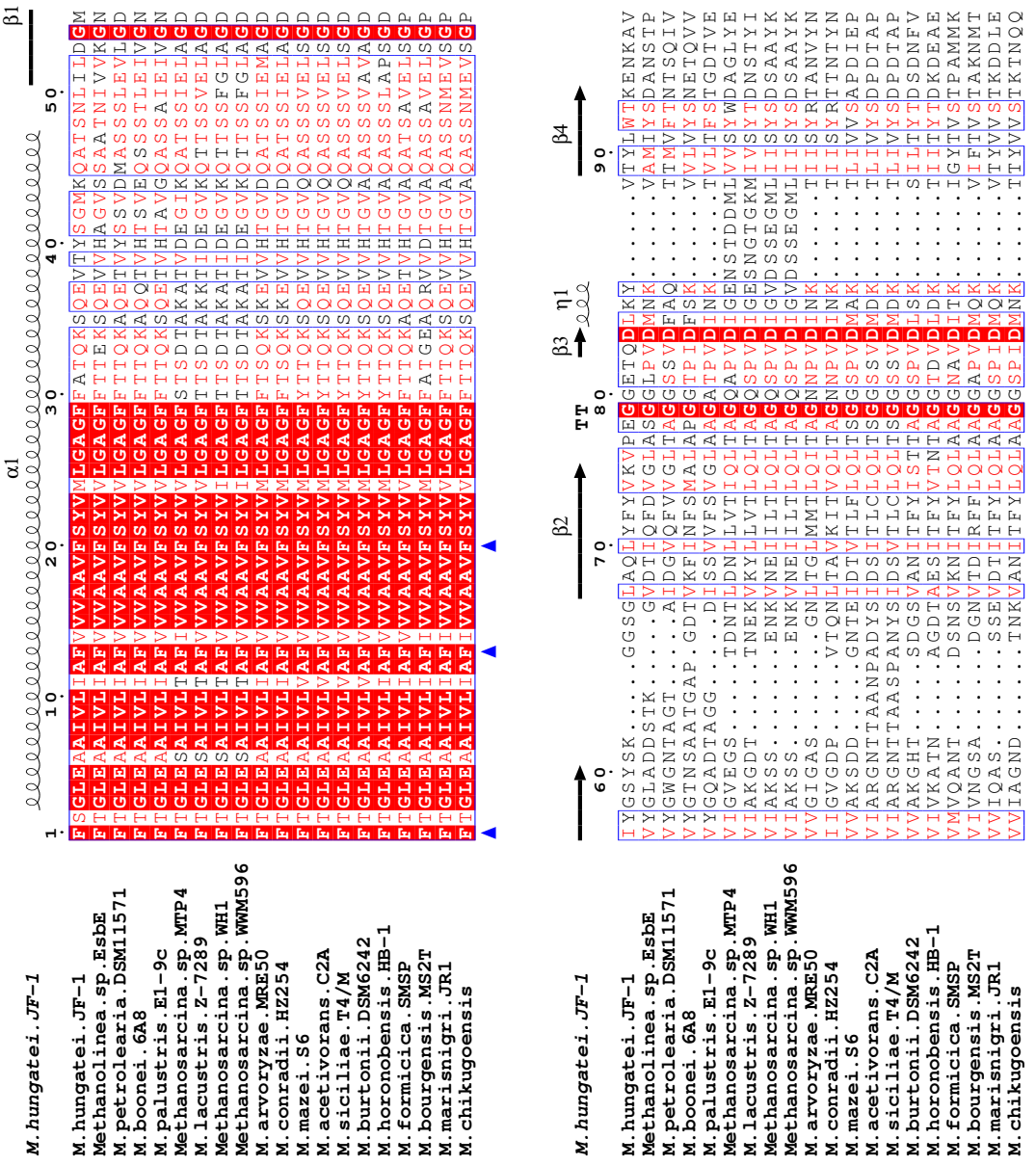


Figure 4.1: (a) Multiple sequence alignment
Blue triangles indicate phenylalanine responsible for electrical conductivity

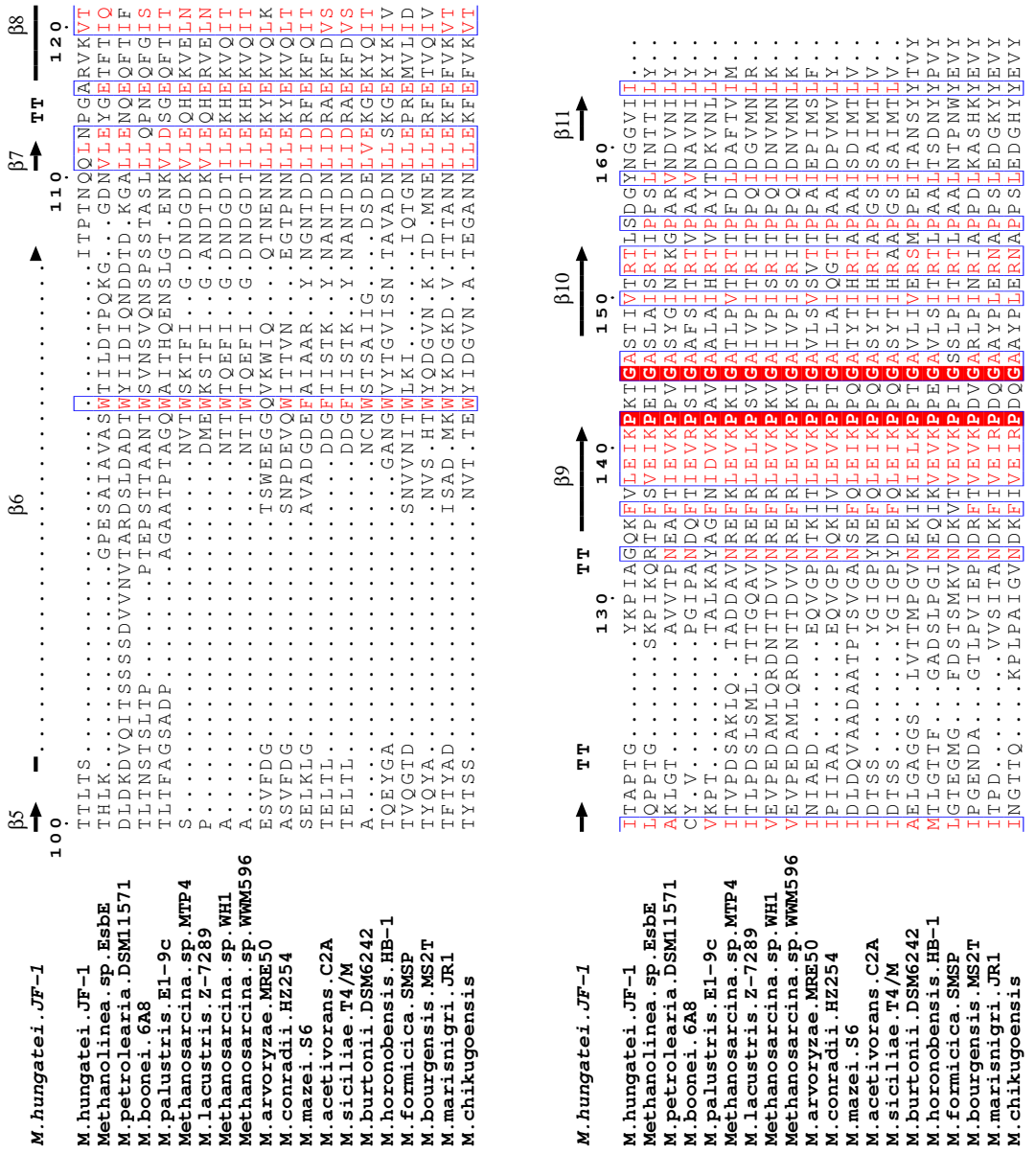


Figure 4.1: (b) Multiple sequence alignment

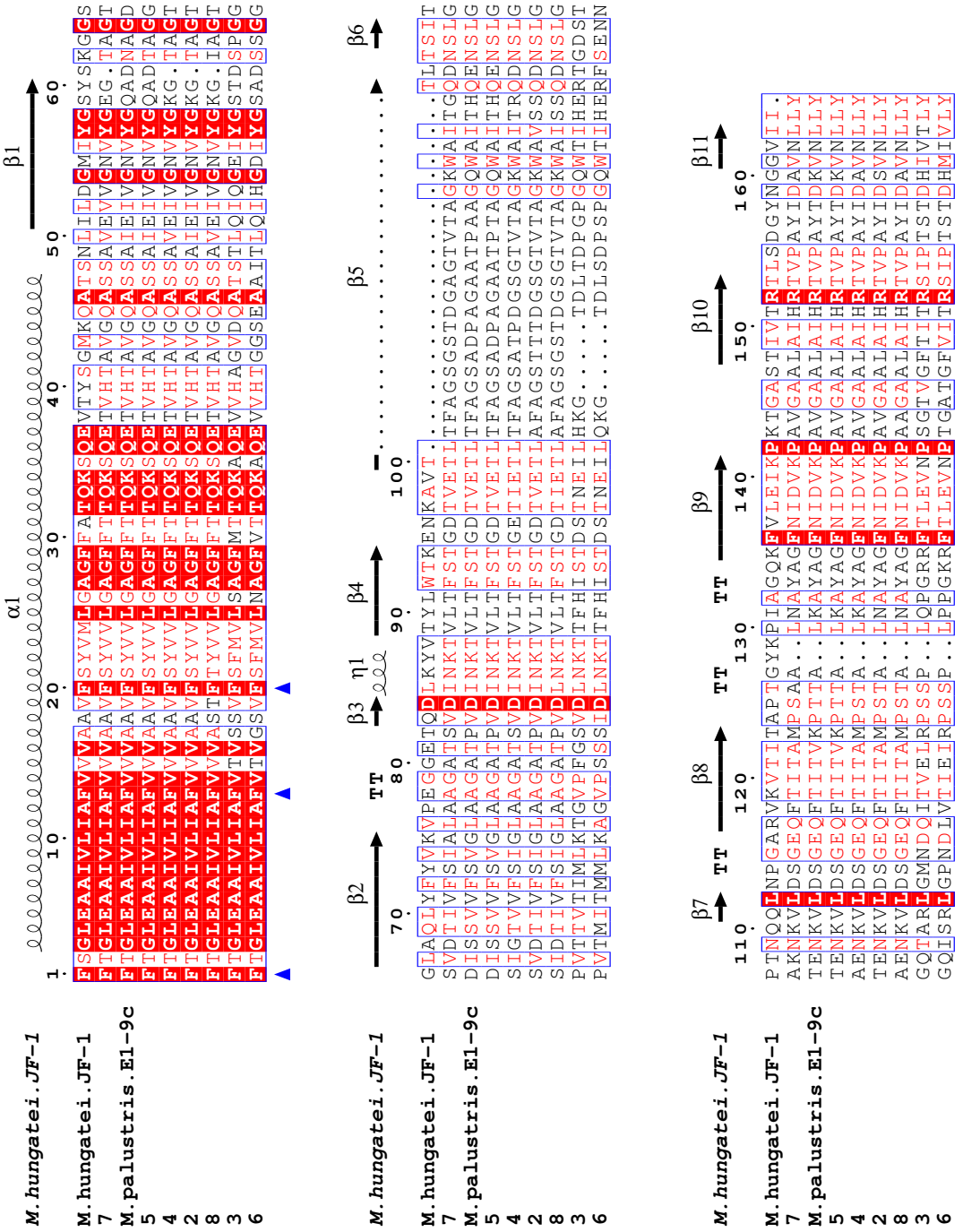


Figure 4.2: Multiple sequence alignment of *Methanosphaerula palustris* E1-9c
Sequences numbered in order of appearance in the genome.

palustris E1-9c in order to check if the copies are identical. *M. palustris* was chosen since it had the most amount of copies. Interestingly, it yielded results similar to previous MSA with α - helix being conserved and variability in β barrels (Figure 4.2). Two copies were more variable compared to others (3 and 6). More MSA were performed with those two copies in order to find possibility of horizontal gene transfer but yielded no result. However, the search was limited so one can't rule out the possibility.

4.3 Phylogenetic tree analysis

Based on MSA results, phylogentic tree was constructed (Figure 4.3) and compared with phylogentic tree constructed by Adam et al. [39] using bayesian phylogeny grounded on a 41 gene supermatrix. The 41 genes consist of 36 genes from the Phylosift marker genes list provided by Darling et al. [40], plus RNA polymerase subunits A and B, and three universal ribosomal proteins (L7-L12, L30, S4) from Liu et al. [41]. The tree is rooted according to Raymann et al. [42] (Figure 4.4).

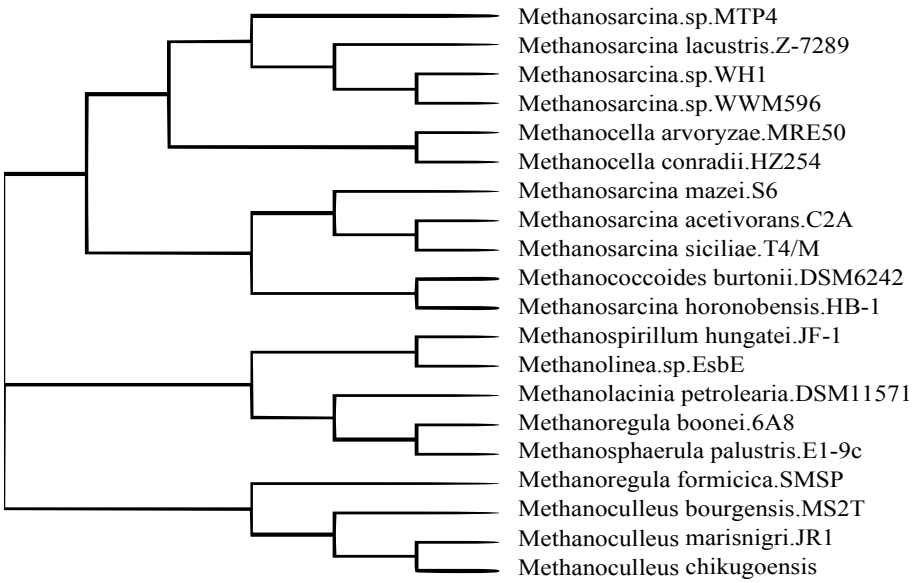


Figure 4.3: Phylogenetic tree based on MSA

Phylogenetic tree constructed based on archaellin sequence and reference phylogenetic tree are similar.

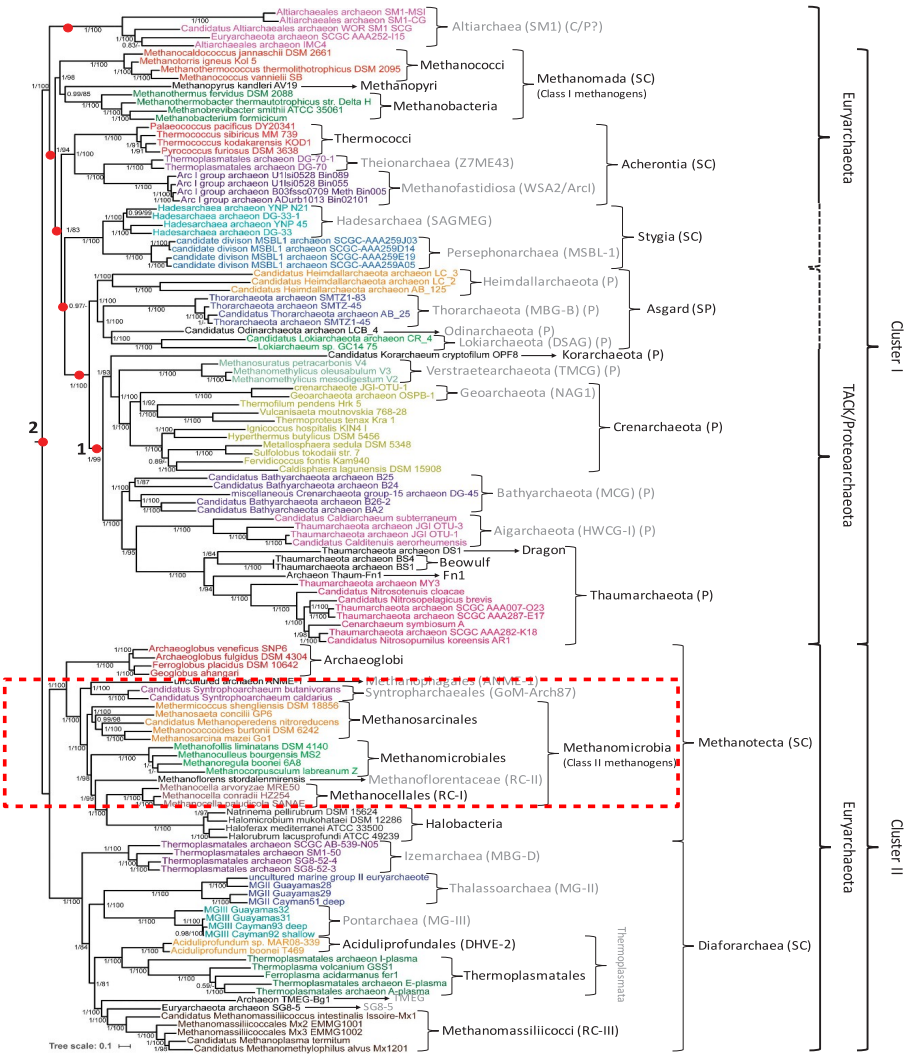
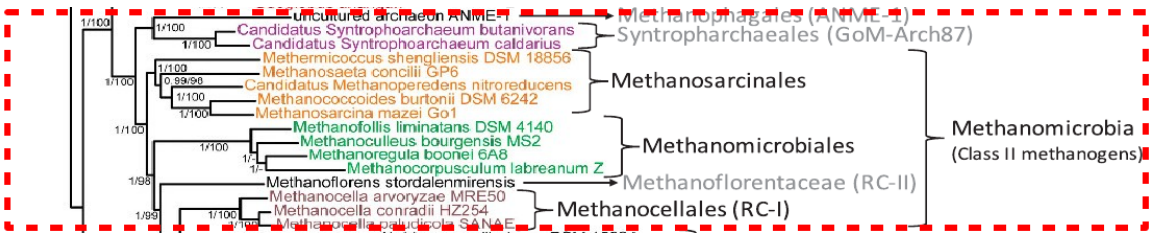


Figure 4.4: Reference phylogenetic tree [39]



4.4 Hydrogen bond analysis

Hydrogen bond analysis was performed using chimera in hopes that it will shed some light on stability of archaella. Results revealed that the hydrogen bonds were mostly intra-subunit. Bonds between two subunits have been visualized in Figure 4.5. One can notice a single bond near the tip of α - helix.

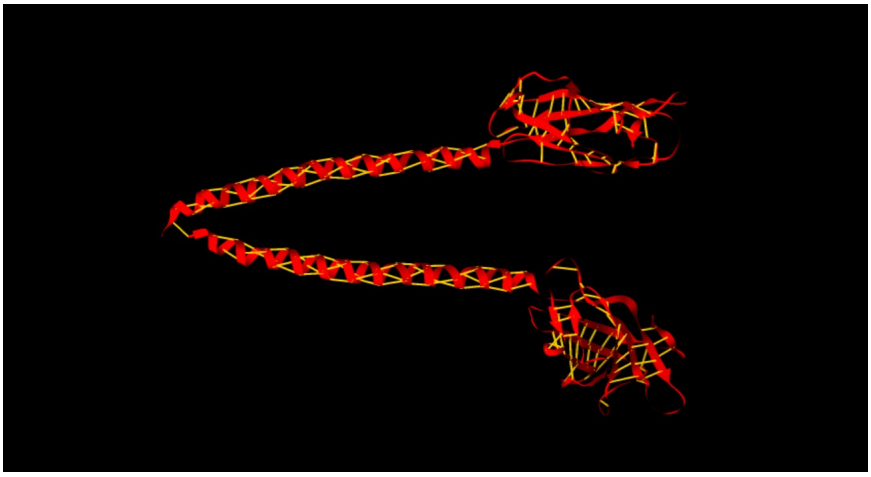


Figure 4.5: Hydrogen bond visualization
Hydrogen bonds coloured in yellow

4.5 Protein surface property analysis

In further attempt to understand reasons behind archaella stability, protein surface hydrophilic-hydrophobic property was visualized using chimera (Figure 4.6). Results revealed a hydrophobic α - helix which forms the core of the archaella and hydrophilic β barrel which forms the external surface.

4.6 Phenylalanine overlaps

Clashes/contacts were analysed using chimera to study proximity of phenylalanine in archaella structure of *M. hungatei*. The results showed clashes/contacts between phenylalanine from different subunits of the archaellum filament. The phenylalanine in these subunits were individually visualized. They form a christmas tree like structure, tightly packed in the core of the archaellum shown in Figure 4.7.

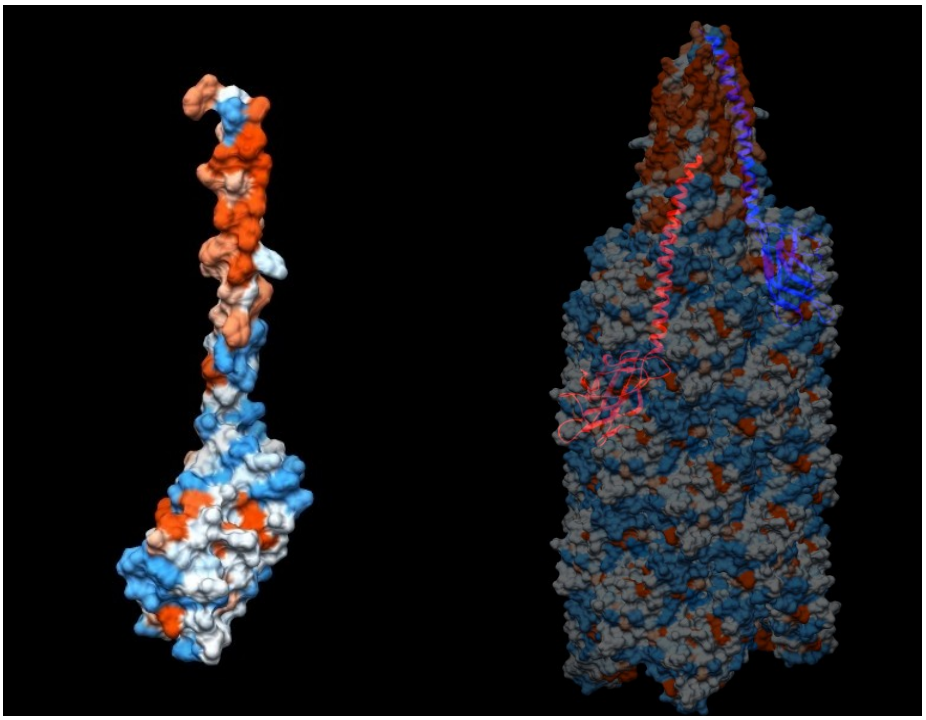


Figure 4.6: Hydrophobicity of protein surface
Single subunit on left

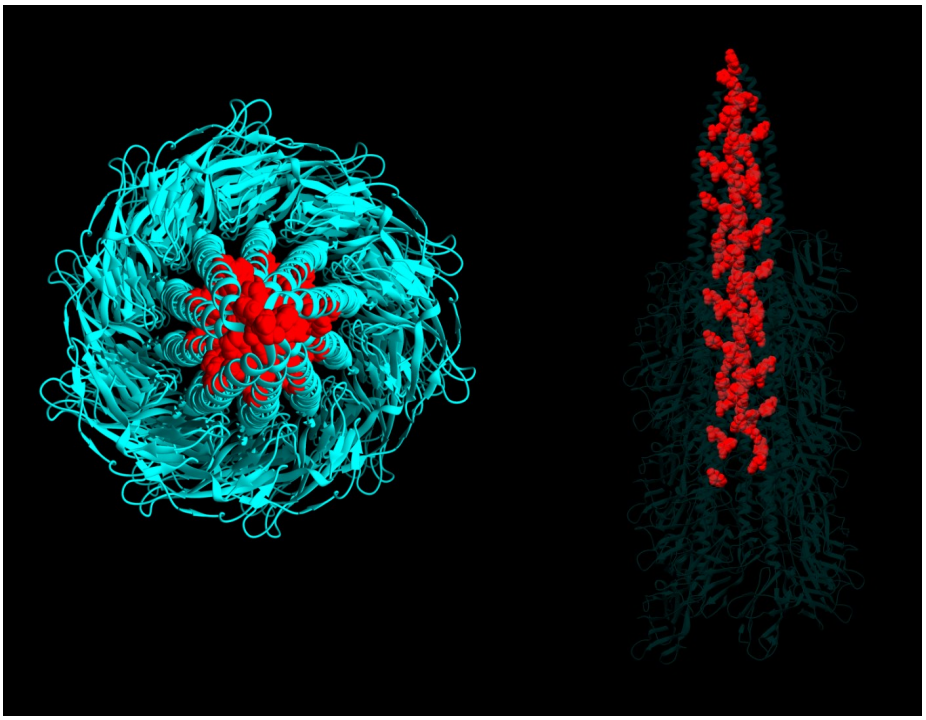


Figure 4.7: Phenylalanine visualization

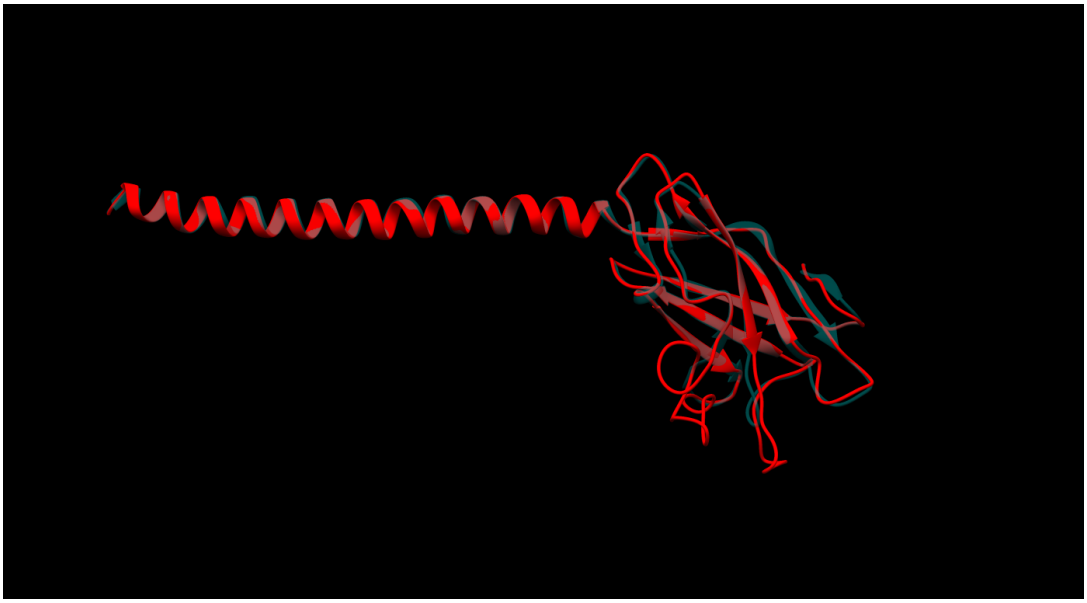
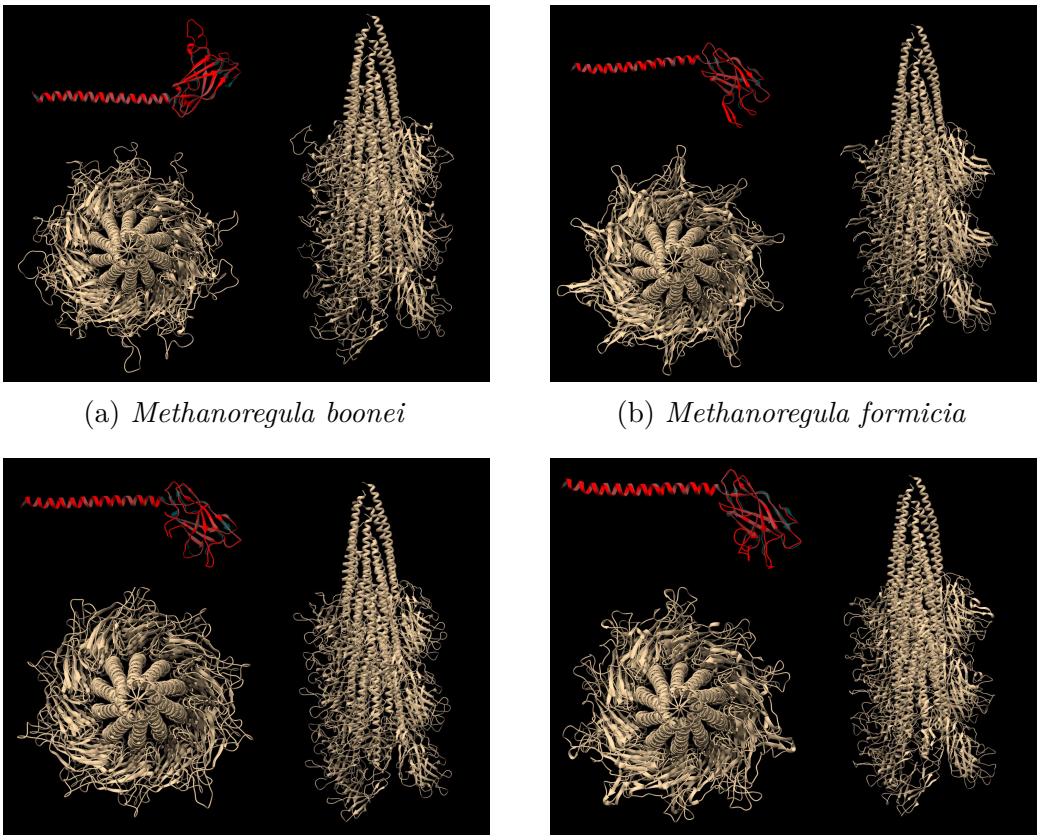


Figure 4.8: Superposition of individual subunit of *M. hungatei* and *Methanosarcina mazei*

4.7 Homology based modelling

Since there were no structures available for the methanogens found in this study, homology based structural modelling was done using swiss-model. The models were analysed using chimera to check possibility of these organisms to form an archaella. Individual subunits were superimposed onto structure of *M. hungatei* to see the similarity (Figure 4.8) and all the previous mentioned analysis were done. Based on this it could be deduced that the probability of these organisms to form a stable archaella is high. Figure 4.9 shows models built using swiss-model with individual subunit superimposed with *M. hungatei*. The models portrayed were chosen based on their position in phylogenetic tree (Figure 4.3; Page no. 26) for diversity.



(a) *Methanoregula boonei* (b) *Methanoregula formicia*

(c) *Methanosarcina lacustris* (d) *Methanosarcina mazei*

Figure 4.9: Homology based modelling

5 Discussion and Conclusion

5.1 Electromethanogenesis

The list of methanogenic archaea obtained in this study was compared to comprehensive list of organisms involved in electromethanogenesis provided by Blasco-Gòmez et al. [43]. The result was neither inclusive of all organisms found in this study nor exclusive. If it were to be exclusive or inclusive of all the organisms found in this study, it would've strongly suggested the role of conductive archaellum as a conduit for direct electron uptake by these methanogens. Further literature search was performed to check for methanogens that were not on the list of organisms associated with electromethanogenesis but yielded no results. However it was noted that all organisms of order Methanosarcinales found in this study were on the list of organisms associated with electromethanogenesis.

5.2 DIET through E-pili

The study by Yee and Rotaru [44] lists all the experimentally proven syntropic DIET pairs till date and expands on it by additional six pairs. On comparison, it was found that not all methanogens that formed DIET pairs were part of results of this study suggesting presence of electrically conductive archaellum not being a necessity for DIET through E-pili. Moreover study by Yee and Rotaru [44] contradicts the idea of archaellum being conduit for direct electron uptake in DIET pairs by confirming and expanding on previous study by Rotaru et al. [16] that strict hydrogenotrophs like *M. hungatei* are incapable of forming DIET pairs with electrogens. All the methanogens that formed DIET pairs were of order Methanosarcinales and the study concluded that DIET is conserved only to this order. The study further hypothesized that the ability of Methanosarcinales for direct electron uptake might be property of cell surface structures.

5.3 DIET through conductive material

DIET through conductive materials have been observed in few methanogens[15] which on examination have revealed that all the methanogens are of order Methanosarcinales. Study conducted by Salvador et al. [45] showed that carbon nanotubes acelerated methane production in pure cultures of methanogens including *M.hungatei* but indicated no evidence of DIET.

Study conducted by Holmes et al. [46] tested DIET pair *Methanosarcina acetivorans* and *Geobacter metallireducens* with and without presence archaellum in *M. acetivorans*. The pair without archaellum did not form efficient DIET. Introduction of GAC showed to mitigate this problem which is known to be substitute for e-pili[15]. However the study proposed that further experiments need to be performed and traditional roles of archaella, such as conferring motility and facilitating attachment might be the reason for said observation rather than the possibility of archaellum of *M. acetivorans* being conductive.

The above observations made indicate a strong possibility that electrical conductive archaella might not have any direct role to play in DIET in favor of hypothesis proposed by Yee and Rotaru [44] that DIET is property of cell surface structures unique to methanogens of order Methanosarcinales. The function of electrically conductive archaella of *M. hungatei* may merely be to facilitate cell attachment by dissipating charge barriers between cells and minerals/electrodes as suggested by Walker et al. [33]

5.4 Other findings

This study confirms that in euryarchaeotes archaella are characterized by the presence of multiple archaellin proteins as stated by Meshcheryakov et al. [18] and that such multiplicity is assumed to be important for assembly of functional archaella, since deletion of individual archaellin genes often leads to non archaellated cells. They reported $\geq$5 copies being the norm but in this study it was found that *M. acetivorans* which has only three copies of archaellin gene was observed to form archaella[46]. Some methanogens in results of this study have only one copy. This needs to be investigated whether they will form archaella.

The phylogenetic tree constructed based multiple squence alignment of archaellin showed that the organisms found are evolutionarily closely related which was confirmed by comparing to work by Adam et al. [39].

Regarding the structure of the archaellum, this study confirms that archaellins universally possess highly structurally conserved and mostly hydrophobic N-terminal sequences which form inward facing α helix while C-terminal outward facing portion of the β barrel is hydrophilic.[18, 19, 32]. This property and the highly conserved metal binding site discovered by Meshcheryakov et al. [18] seems to be the primary reason for archaella stability.

5.5 Conclusion

This study indicates a strong possibility that electrically conductive archaella might not play any direct role in DIET and supports the hypothesis that DIET is property of cell surface structures and is unique to methanogens of order Methanosarcinales.

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7 Appendix

Methanimicrococcus blatticola
Methanobacterium aarhusense
Methanobacterium alcaliphilum
Methanobacterium arcticum
Methanobacterium beijingense
Methanobacterium bryantii
Methanobacterium congolense
Methanobacterium espanolae
Methanobacterium ferruginis
Methanobacterium flexile
Methanobacterium formicicum
Methanobacterium ivanovii
Methanobacterium kanagiense
Methanobacterium lacus
Methanobacterium movens
Methanobacterium oryzae
Methanobacterium palustre
Methanobacterium petrolearium
Methanobacterium subterraneum
Methanobacterium thermaggregans
Methanobacterium uliginosum
Methanobacterium veterum
Methanobrevibacter acididurans
Methanobrevibacter arboriphilus
Methanobrevibacter curvatus
Methanobrevibacter cuticularis
Methanobrevibacter filiformis
Methanobrevibacter gottschalkii
Methanobrevibacter millerae
Methanobrevibacter olleyae
Methanobrevibacter oralis
Methanobrevibacter ruminantium
Methanobrevibacter smithii
Methanobrevibacter thaueri
Methanobrevibacter woesei
Methanobrevibacter wolinii
Methanocalculus chunghsingensis
Methanocalculus halotolerans
Methanocalculus pumilus
Methanocalculus taiwanensis
Methanocaldococcus fervens
Methanocaldococcus indicus
Methanocaldococcus infernus
Methanocaldococcus jannaschii
Methanocaldococcus villosus
Methanocaldococcus vulcanius
Methanocella arvoryzae
Methanocella conradii
Methanocella paludicola
Methanococcoides alaskense
Methanococcoides burtonii
Methanococcoides methylutens
Methanococcus aeolicus
Methanococcus maripaludis
Methanococcus vannielii
Methanococcus voltae
Methanocorpusculum aggregans
Methanocorpusculum bavaricum
Methanocorpusculum labreanum
Methanocorpusculum parvum
Methanocorpusculum sinense
Methanoculleus bourgensis
Methanoculleus chikugoensis
Methanoculleus marisnigri
Methanoculleus palmolei
Methanoculleus receptaculi
Methanoculleus submarinus
Methanoculleus thermophilus
Methanofollis aquaemaris
Methanofollis ethanolicus

Methanofollis formosanus
Methanofollis liminatans
Methanofollis tationis
Methanogenium boonei
Methanogenium cariaci
Methanogenium frigidum
Methanogenium marinum
Methanogenium organophilum
Methanohalobium evestigatum
Methanohalophilus euhalobius
Methanohalophilus halophilus
Methanohalophilus mahii
Methanohalophilus portucalensis
Methanolacinia paynteri
Methanolinea mesophila
Methanolinea tarda
Methanolobus bombayensis
Methanolobus oregonensis
Methanolobus profundi
Methanolobus psychrophilus
Methanolobus taylorii
Methanolobus tindarius
Methanolobus vulcani
Methanolobus zinderi
Methanomethylovorans hollandica
Methanomethylovorans thermophila
Methanomicrobium mobile
Methanoplanus endosymbiosus
Methanoplanus limicola
Methanoplanus petrolearius
Methanopyrus kandleri
Methanoregula boonei
Methanoregula formicicum
Methanosaeta concilii
Methanosaeta harundinacea
Methanosaeta pelagica
Methanosaeta thermophila
Methanosalsum zhilinae
Methanosarcina acetivorans
Methanosarcina baltica
Methanosarcina barkeri
Methanosarcina horonobensis
Methanosarcina lacustris
Methanosarcina mazei
Methanosarcina semesiae
Methanosarcina siciliae
Methanosarcina thermophila
Methanosarcina vacuolata
Methanosphaera cuniculi
Methanosphaera stadtmanae
Methanosphaerula palustris
Methanospirillum hungatei
Methanospirillum lacunae
Methanothermobacter crinale
Methanothermobacter defluvii
Methanothermobacter marburgensis
Methanothermobacter tenebrarum
Methanothermobacter thermautotrophicus
Methanothermobacter thermoflexus
Methanothermobacter thermophilus
Methanothermobacter wolfeii
Methanothermococcus okinawensis
Methanothermococcus thermolithotrophicus
Methanothermus fervidus
Methanothermus sociabilis
Methanotorris formicicus
Methanotorris igneus
Methermicoccus shengliensis
Methanospirillum psychrodurum
Methanobrevibacter boviskoreani
Methanobacterium movilense
Methanomethylovorans uponensis
Methanocalculus natronophilus
Methanohalophilus levihalophilus
Methanosarcina soligelidi
Methanococcoides vulcani
Methanospirillum stamsii
Methanomassiliicoccus luminyensis
Methanoculleus horonobensis
Methanoculleus hydrogenitrophicus