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Advances in Applications of Modern Biotechnology Methods in Methanogens

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Advances in Applications of Modern Biotechnology Methods in Methanogens

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Report

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science in Engineering

The University of Texas at Austin

August 2014

Acknowledgements

I would like to thank Dr. Lydia Contreras for her mentoring and guidance throughout this entire project, as well as her continued support. I could not have asked for more in an advisor. I would also like to thank Dr. Kirisits for her help and input with the report, as well as for the opportunity to work with her on research. It is very appreciated. Much of the financial support for my education is provided by the National Science Foundation Graduate Research Fellowship program, making the writing of this report possible. Finally, I would like to thank all of the family members and friends that provided encouragement during this process.

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The University of Texas at Austin, 2014

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Methanogens are autotrophic Archaea that produce methane as a product of their anaerobic metabolism. They are the largest producers of global methane, contributing over 60% of the total methane budget each year. Methane is an extremely potent greenhouse gas, with emissions providing the second-largest contribution to historical global temperature increases after carbon dioxide. Methanogens have become extremely important industrially as because they are used in the production of biofuels, as well as in treating industrial waste for industrial processes. This report will focus on those successful genetic methods and modifications that have been developed for methanogens and how they have started to contribute to understanding methanogen biochemistry.

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1. Introduction

Methane is an extremely potent greenhouse gas, with emissions providing the second-largest contribution to historical global temperature increases after carbon dioxide [1]. It has a global warming potential (GWP) of 34 over a 100-year period, which means that an emission will have 34 times the impact on global temperature of a carbon dioxide emission of the same mass over the following 100 years. Although it has a relatively short lifetime of about 8 years in the atmosphere, methane has a higher efficiency for trapping radiation. Atmospheric methane (CH_4) concentrations have increased by about 150% (1,060 ppb) since 1750 [2]. Methanogens are the largest producers of global atmospheric methane, contributing over 60% of the total methane budget each year [3]. Other sources of atmospheric methane include gases released from the permafrost and glaciers as temperatures rise, wetlands, and plants.

Methanogens are autotrophic Archaea that produce methane as a product of their anaerobic metabolism. They form methane using CO_2 and H_2, formate, methanol, methylamines, or acetate as substrates [4]. Methanogens can be found in a variety of environments, even extreme ones such as arctic lakes. Many are classified as extremophiles because of the conditions they are able to survive in. There are many classes of extremophiles that live all across the world. They are classified by how their particular environmental niche differs from conditions that most organisms live in, such as neutral pH and temperatures between 20 and 45°C [5]. There are 5 orders of

methanogens: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanopyrales, and Methanosarcinae.*

Methanogenesis, the process by which methanogens produce methane, is the last step in the anaerobic decomposition of organic matter. It is the only way that methanogenic bacteria generate ATP. Annually, about 1% of the CO_2 fixed by photosynthetic organisms is remineralized and converted to methane via methanogenesis, resulting in more than 10^9 tons of methane produced per year. Two-thirds of this methane is consumed by methanotrophic bacteria, while the other third is released into the atmosphere [4]. Figure 1, below, illustrates some of the sources and sinks of methane.

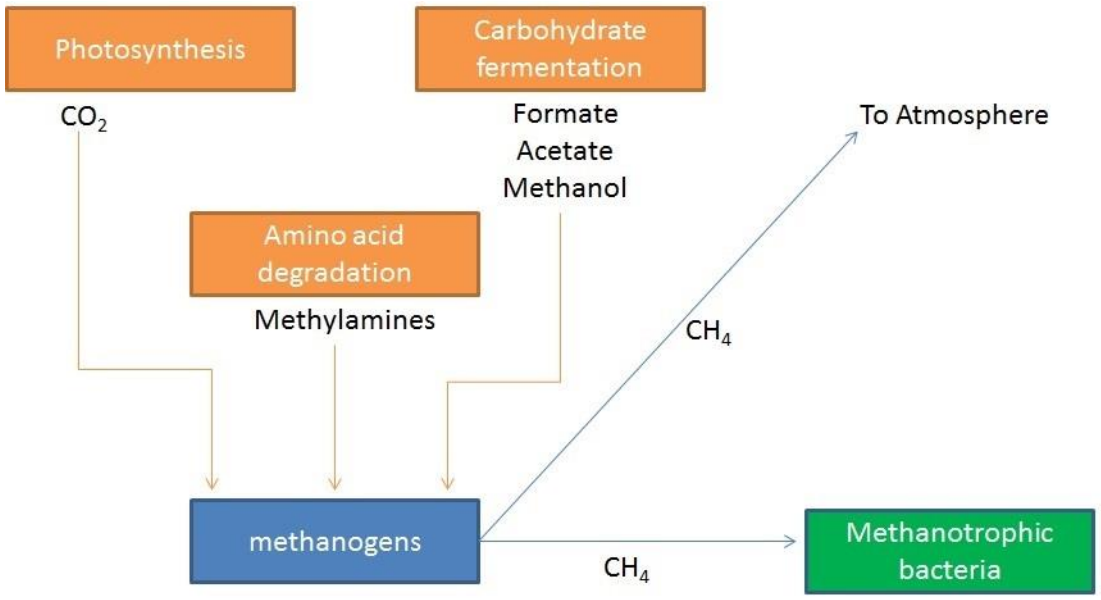

Figure 1 – Methane sources and sinks.

Understanding the process of methanogenesis is important as the concentration of atmospheric methane has been increasing steadily over the last 100 years [1].

Although methanogens are a diverse group of organisms, their methods of producing methane are relatively few. They are restricted to only a few substrates: carbon dioxide, formate, methanol, acetate, and methylamines [4]. The overall reactions for these substrates are shown below in Table 1 [3]. The first methanogenesis reaction from formate was developed in 1933 by Stephenson and Strickland [3].

Table 1 – Methanogenesis substrates.

Energetically Favorable

Substrate	Reaction
Formate	$4HCOO^- + 4H^+ \rightarrow 3CO_2 + CH_4 + 2H_2O$
Carbon dioxide	$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$
Methanol	$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$
Methylamines	$4(CH_3)_3NH^+ + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_4^+$
Acetate	$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$

Although the majority of methane found in nature is produced from acetate, the only class of methanogens that uses acetate as a substrate for methanogenesis is

Methanosarcinales. A member of this class, *Methanosarcina acetivorans,* is the only known organism that can use all of the 5 substrates for energy metabolism. While CO_2 is the most commonly used substrate, formate is the most energetically favorable [3].

Recent discoveries with methanogens have shown their potential for applications in energy production, greenhouse gas reduction, and waste treatment, making methanogens industrially important. However, their applications have been limited until recent years by a lack of knowledge of methanogen biochemistry, as well as the environmental conditions they require for growth, namely an oxygen-free environment because they are obligate anaerobes [4]. This report will focus on those successful genetic methods and modifications that have been developed for methanogens and how they have started to contribute to understanding methanogen biochemistry. The genetic methods and studies leading to their development include plasmid expression systems, gene mutations, and proteomic, transcriptome, and protein studies.

2. Expression Systems and Reporter Systems

Plasmid-based expression systems are commonly used in model organisms to introduce a specific gene into a target cell, and can take control of the cell's mechanism for protein synthesis to produce the protein encoded by the gene. These systems must contain the elements necessary for transcription and translation. Elements necessary might include a strong promoter, the correct translation initiation sequence such as a ribosomal binding site and start codon, a strong termination codon, and a transcription termination sequence. Overall, plasmids are engineered to contain regulatory sequences that act as enhancer and promoter regions and that lead to effective transcription of the target gene [6]. Table 2 lists promoters that have been successfully used as expression systems in methanogens.

Table 2 – Promoters successfully used in methanogens [6].

Constitutive promoters	Description	Inducible promoters
$\mathbf{P_{mcrB}}$	methyl coenzyme M-reductase promoter	Selenium-responsive region of the genome
$\mathbf{P_{hmvA}}$	histone gene promoter from *Methanococcus voltae*	Tetracycline-inducible system
$\mathbf{P_{tbp}}$	polypyrimidine tract-binding protein gene promoter	p1687 (directs the transcription of methyl transferases that demethylate methylamines) [7]
$\mathbf{P_{nif}}$	*nifa* (nitrogen-fixing) gene promoter	

Expression vectors are basic tools for biotechnology and heterologous protein expression. Plasmid-based genetic systems that can be successfully used in methanogens are necessary for continued advances in methanogen physiology and their biotechnological applications [6]. Recent developments in these systems for methanogens have opened many avenues for protein expression and reporter assays and generally increased the capacity for genetic manipulation and more diverse strain construction. Although the common genetic methods and procedures have all been used in the context of model organisms, modifications were necessary to make these protocols applicable to methanogens. For instance, common protocols, such as those for cloning and

transformation, have to be modified to be carried out under strictly anaerobic conditions, since methanogens can only survive anaerobic environments [8].

The main model organism for the development of genetic tools among methanogens has been *Methanosarcina acetivorans.* This methanogen is unique in that is capable of using all five substrates for methanogenesis, whereas most methanogens can only use one or two. In a 2005 study, a plasmid-based gene reporter system was developed to construct *lacZ* gene fusions for monitoring intrinsic promoter expression in this methanogen [9]. *M. acetivorans* was transformed with *Escherichia coli/M. acetivorans* shuttle plasmid pWM315 containing a *cdh*-promoter – *lacZ* fusion. The recombinant plasmid was shown to be stable in *M. acetivorans,* demonstrating that plasmids with elements from other organisms can be successfully deployed in a methanogen.

M. acetivorans also is used as a model organism because it is homologous, or has similar biology, to several other methanogenic species, allowing biotechnologies developed for *M. acetivorans* to be successfully transferred to other methanogens with relative ease. An example of this is an inducible system to produce strep-tagged proteins that was developed in *Methanosarcina mazei* [10]. A protein production system for methanogens is needed for the production and purification of methanogenic enzymes because many of those enzymes cannot be expressed in common model organisms, such as *E. coli.*

Another gene reporter system was developed for *M. acetivorans* using β-lactamase from *E. coli* in conjunction with the chromogenic substrate nitrocefin [11]. The gene reporter system was used to monitor gene expression by a synthetic tetracycline riboswitch in *M. acetivorans*. The vector pMR56 was used to place the *bla* gene on the *M. acetivorans* chromosome. The tetracycline riboswitch in this study was the first synthetic riboswitch to be described and used in methanogens. This study also successfully demonstrated a foreign gene being expressed in a methanogen. The results showed that the activity of the *bla* gene was sufficiently stable in *M. acetivorans* to allow visual detection.

A 2010 study investigated another example of an expression system in *M. acetivorans.* The gene for the protein MekB, a broad-specificity esterase from *Pseudomonas veronii*, was fused to a constitutive *M. acetivorans* promoter [12]. The goal of the study was to broaden the already diverse substrate range of *M. acetiovorans* by incorporating a pathway that allowed for the growth and production of methane with non-native substrates, namely methyl acetate and methyl propionate. A DNA fragment containing the promoter-MekB fusion was cloned into the shuttle vector pWM321, which was transformed into *M. acetivorans* using a previously developed liposome-mediated protocol that allows for efficient transformation in methanogens. The mutant strain developed had an 80-fold increase in esterase activity from the wild-type. This study demonstrated successful expression of a bacterial enzyme from an aerobic species in an anaerobic methanogen.

M. acetivorans also has been modified to allow it to incorporate unnatural amino acids (UAAs) into proteins. This ability could be particularly important for applications requiring proteins to be given specific properties that are not necessarily natural. For instance, a system was developed for inserting 3-azidotyrosine (N3-Y) into proteins in *M. acetivorans* [13]. Random mutations were introduced into *M. acetivorans* tyrosyl-tRNA synthetase (TyrRS) around the tyrosine binding pocket, and a TyrRS mutant recognizing (N3-Y) was selected. Rat calmodulin (CaM) containing N3-Y was successfully expressed in *M. acetivorans*. However, expressing CaM containing N3-Y in *E. coli* produced higher yields, indicating that further optimization for use in *M. acetivorans* was needed.

In another system, the promoter p1687 was cloned into the plasmid pWM831 and transformed into *M. mazei*. This promoter directs the transcription of methyl transferases that demethylate methylamines. The gene that codes for β-glucuronidase from *E. coli* was fused to a strep-tag and cloned downstream of the p1687 promoter, allowing activity of the inducible system to be determined by monitoring β-glucuronidase production [7]. This study was another demonstration of successful incorporation of foreign genes into a methanogen, as well as development of a genetic tool capable of being used in other methanogens.

3. Mutagenesis Studies

Mutagenesis of an organism has been a traditional technique to examine the makeup of the biological molecule. Mutations might also produce mutant entities with interesting properties, enhanced or novel functions that could be of commercial use, or ones that allow the molecular basis of a particular cell function to be investigated [6]. Mutagenesis can be accomplished through a combination of techniques, beginning with a plasmid, a bacterial artificial chromosome, or other DNA construct, and proceeding to cell culture [6]. Individual cells are then genetically transformed with the plasmid, with the end goal of producing cells containing the altered gene of interest. Table 3 shows methods successfully used to deliver these plasmids to methanogens.

Table 3 – Methanogen DNA delivery methods.

Organism	Method	Advantages/Disadvantages	Ref.
Methanococcus	Natural competence[1]	Very low efficiency, inconsistent reproducibility	[6]
	PEG-mediated transformation [6]	High efficiency, *Pst* I–like restriction system	[6]
	E. coli conjugation [6]	Easy and rapid protocol, low efficiency	
Methanosarcina	Liposome-mediated transformation	High efficiency, expensive	[6]

Modified methods of mutagenesis have been developed for methanogens because they require a spheroplast transformed by either a polyethylene glycol (PEG) or liposome mediated protocol for efficient gene uptake [15]. Methods using *E. coli* based conjugation have also been developed for methanogens. These *E. coli* based methods are far less

[1] In the natural competence method, naturally competent bacteria actively pull DNA fragments from their environment into their cells. High similarity with the chromosome allows these fragments to change the cell's genotype by homologous recombination, as encompassed by natural transformation [41]

efficient than liposome-mediated ones, but are much more rapid and less expensive, and so might be preferred [15].

One of the first uses of site-directed mutagenesis in a methanogen, *M. acetivorans*, was reported in a 2002 study where homologous recombination-mediated gene replacement was used to construct a variety of mutants with mutations in the proABC locus to investigate genes involved in proline biosynthesis [16]. The goal of this study was to verify that the process of homologous recombination occurs in a reliable and predictable manner in methanogens by looking at genes with a known function. Development of a reliable site-directed mutagenesis method in methanogens allowed for subsequent studies of genes with unknown functions.

Many mutagenesis studies involve deletions or knockouts of a particular gene or genes to investigate their functions. For example, the function of the *nrpR* gene (a transcriptional repressor) was studied by introducing an *nrp* deletion mutation into *Methanococcus maripaludis*, another common model organism for methanogens [17]. *M. maripaludis* was mutated by simple homologous recombination following liposome-mediated transformation. Genome sequencing of *M. maripaludis* revealed the presence of a gene cluster encoding three closely related PII proteins (GlnK1, GlnK2,and GlnB) and two ammonium channel proteins. To test the roles of the *GlnK1* operators in regulation of the *GlnK*1 operon, *lacZ* reporter fusions were constructed and the *nrpR* deletion was introduced into mutants with the reporter fusion. The results of the study showed that

nrpR bound to two of the operators in the operon (*GlnK1, GlnK2*) to repress transcription. This was the first example in Archaea that cited binding of two transcriptional regulator molecules to overlapping binding sites.

Another study looked at the function of *Ehb* (hydrogenase B) in *M. maripaludis* by constructing markerless in-frame deletions in a number of *Ehb* genes. These genes encode the large and small hydrogenase subunits of polyferredoxin and ferredoxin, and an ion translocator. The results supported the hypothesis that Ehb provides low potential reductants for the anabolic oxidoreductases in *M. maripaludis*, providing valuable information about the energy metabolism process in methanogens [18].

Gene deletion has also been use to study other aspects of the methanogenesis. An *M. maripaludis* mutant was constructed by sequential deletion of *fruA* and *frcA (*F_{420} reducing hydrogenases) [19]. In-frame deletions of *hcg* genes were then performed on the mutants. The results of the study provided evidence of the role of the *hcg* gene products in biosynthesis of the H_2-dependent methylene-tetrahydromethanopterin dehydrogenase (Hmd).

Additionally, gene deletion studies have been used to show alternative methanogenesis pathways in *M. maripaludis* [20]. Genes that encoded *Eha*, a hydrogenase enzyme, were in mutants that expressed only one of the three hydrogenotrophic pathways to eliminate all hydrogen metabolism. Then, a single hydrogenase—the F_{420}-reducing hydrogenase— was reintroduced into the hydrogenase-

free strain and observed its effects in isolation from other H_2-metabolizing pathways. The results demonstrated that *Eha* and H_2 are not always essential for growth in hydrogenotrophic methanogens, and that *M. maripaludis* has at least two additional pathways by which the anaplerotic, or intermediate forming, requirements of methanogenesis can be satisfied. These additional pathways provided evidence that the hydrogenotrophic methanogens have unexplored metabolic diversity. While there have been significant advances in this area in methanogens, limitations and challenges still exist. Namely, a high efficiency protocol for transformation has yet to be developed that is economically feasible at a large scale.

4. Protein and Protein Purification Studies

Expression and subsequent purification of proteins directly from methanogens have several advantages for studying their native properties. The intracellular environment, including pH, redox balance, cofactors, chaperones, and posttranslational modification machinery required for proper maturation and activity of specific proteins, is often lacking during recombinant expression in non methanogenic organisms. Codon bias and improper localization are often problematic, and proteins from the anaerobic methanogen species are often ill equipped to function in aerobic environments [6]. Also, creating expression platforms and plasmid expression systems for methanogens requires understanding of their transcriptional and translational processes. This section will focus on more recent methanogen protein studies (within the last 5 years).

The recent increase in genome sequences for methanogens has provided an increased number of methanogenic proteins with unknown functions or mechanisms [14]. The accuracy of sequence-based functional annotations is often variable, particularly if the sequence homology to a known function is low [22]. In a 2011 study, nuclear magnetic resonance was investigated as a method to provide more accurate annotation of new sequences. The method was investigated by testing functional assignments of putative enzymes that may be of variable accuracy.

A 2012 study investigated the role of the fused corrinoid/methyl transfer protein CmtA during carbon monoxide (CO)-dependent growth of *M. acetivorans* [23]. The

results of the study support a role for CmtA in the CO-dependent pathway of methanogenesis when cultured with growth-limiting partial pressures of CO. The results also support the need for further biochemical investigations to determine the function of other fused corrinoid/methyltransferase (FCMT) homologs in *M. acetivorans* and other methanogenic species reported.

Understanding how proteins interact with each other is essential to figuring out the complex mechanisms behind energy metabolism in methanogens. In an initial characterization of specific protein interactions centered on heterodisulfide reductase (*Hdr)* in *M. maripaludis,* it was shown that electron bifurcation is essential for the energy conservation mechanism that is utilized by hydrogenotrophic methanogens [20]. The bifurcation likely takes place at the flavin associated with the α subunit of heterodisulfide reductase (HdrA). In *M. maripaludis* the electrons bifurcation come from either formate or H_2 using formate dehydrogenase (Fdh) or Hdr-associated hydrogenase (Vhu). However, the way enzymes bind to HdrA to deliver electrons was previously unknown. This study provided information on that binding and showed evidence that the δ subunit of hydrogenase (VhuD) is central to the interaction of both enzymes with HdrA.

Transfer RNA-guanine transglycosylases from *M. acetivorans* were studied for their involvement in archaeosine (G^+) synthesis [24]. Transfer RNAs contain a large number of modified nucleosides, which are thought function in the efficiency and fidelity of protein biosynthesis. Archaeosine tRNA-guanine transglycosylase (ArcTGT) catalyzes

the base-exchange reaction from guanine to 7-cyano-7-deazaguanine ($preQ_0$) in G^+ synthesis. ArcTGT is classified into full-size or split types. While the full-size type forms a homodimeric structure, the split type has been assumed to form a heterotetrameric structure with of two kinds of peptides. This study represents the first definitive evidence of this heterotetrameric structure. This tRNA transcript is expected to be useful as a substrate for studies seeking the enzymes responsible for G^+ biosynthesis.

5. Large Scale Genomics Studies

The accelerated rate and ease at which DNA sequence data can now be generated has led to a recent increase in the number of genome sequences available for methanogens. Coupled with large-scale functional genomics approaches, such as proteomics and transcriptomics, genome sequences can provide valuable information about methanogen biology and function [25]. To date, these large scale studies have elucidated the functions behind complex methanogenesis mechanisms.

Unlike the genome, the transcriptome can vary with external environmental conditions. In this way, analysis of the transcriptome at different growth and environmental conditions can provide valuable information about cellular functions by looking at dynamic and conditional gene activation. This analysis can be done by many methods including high-throughput DNA microarray methods, as well as next-generation sequencing methods, such as Tn- and RNA-seq [26]. Likewise, proteomic analyses can provide a direct measure of which, how much, and when proteins are being produced in a cell. As a result, fundamental biology and adaptive responses of microorganisms can be studied by determining cellular pathways and processes functioning in a cell or community using proteomic data [25].

These large scale genomics approaches have recently been used to study cold adapted behavior in several methanogenic organisms [27], [28] . Studying how methanogens respond to cold temperatures is especially important for their applications

in low temperature anaerobic digestion [27], [29]. One study revealed 20 proteins, with several functions including cellular information processing and chaperones, which showed differential expression in response to cold temperatures in *Methanosarcina barkeri* [27]. In the study, proteomic data was taken at 37°C and then again at 15°C and compared between the two temperatures. The 20 proteins identified showed differential expression of 1.5-fold or greater. The functions of these proteins included methanogenesis, protein folding, and oxidative stress relief, indicating a widespread response to cold shock in *M. barkeri.* The upregulation of a methyltransferase protein, which catalyzes trimethylamine reduction to methyl-coenzyme M, was particularly interesting, as it indicated ability to convert methylamine to methane at 15°C was key to *M. barkeri'*s survival at low temperatures.

Another study looked at the psychrophilic methanogen *Methanolobus psychrophilus* and its cold adapted gene expression [28]. Methanogenesis and biosynthesis are all downregulated by lower temperatures in *M. psychrophilus*, much like in a previously studied psychrophilic, or cold-adapted, methanogen, *Methanococcoides burtonii. M. psychrophilus* also exhibited other characteristics common to psychrophiles, including abundant two-component system (TCS) genes and upregulation of transcription machinery genes at low temperatures. Two-component signal transduction systems serve as a basic stimulus-response coupling mechanism to allow organisms to sense and respond to changes in many different environmental conditions [30]. Typically, TCSs consist of a membrane-bound sensor histidine kinase (HK) and a cognate response

regulator (RR) and catalyze a phosphotransfer between the two [30]. Increased transcription is thought to be because more enzyme proteins are needed to compensate for low activities at low temperature. This study provided insight into the complex process behind the survival of organisms living in extreme cold environments.

Methanogenic response to other environmental stresses has also been studied using a proteomics approach. For example, the response of protein levels in *M. maripaludis* in response to nutrient limitation was studied in 2010. Three different nutrient limitation conditions were studied: H_2, ammonia, and N_2 limitation. Six to ten percent of the proteome changed significantly with each nutrient limitation [31]. Many proteins related to methanogenesis increased under hydrogen limitation, possibly allowing for the activation of alternate methanogenesis pathways for other substrates in addition to facilitating more processing of the decreased hydrogen. This in conjunction with another limitation study indicates an ability of hydrogenotrophic methanogens to grow in the complete absence of H_2, meaning that *M. maripaludis* is more metabolically diverse than previously thought. The response to phosphate limitation supports the hypothesis that *M. maripaludis* has three alternative phosphate transporters, all of which showed increased expression under phosphate limitation. The results suggested a wider response to nutrient limitation than previously thought and also provided information that was used for the development of a regulatory network model [31].

These studies can also elucidate the functions of different methanogenic proteins, as well as facilitate the discovery of novel proteins [32] A top-down proteomics approach revealed the presence of several previously unseen protein forms in *M. acetivorans* [32]. Ninety-nine proteins were newly identified using top-down tandem mass spectrometry, in addition to 200 proteins predicted to be present in *M. acetivorans* that were confirmed. These new proteins include 15 with incorrectly predicted start sites from a previous protein identification in which 101 proteins were identified, five previously unannotated proteins, a few co- or post-translational modifications (PTMs), and the use of a very rare AUA codon for a start methionine (Met) instead of the standard start AUG. This study provided useful information to be applied to automated genome annotation in Archaea.

Functional genomics approaches have also been used to determine the number of essential genes in methanogenic organisms. Essential gene studies are important for giving insight into specific questions regarding methanogens as well as more general questions about the genetics, biochemistry, and physiology of archaea [33]. Essential genes are ones that are absolutely required for an organism's survival. A whole genome analysis of gene function in *M. maripaludis* revealed 121 hypothetical ORFs that were classified as possibly essential and likely play fundamental roles in information processing or metabolism in methanococcal organisms that are not established outside this group of prokaryotes [33]. Many of the identified genes were involved in transcription and translation and thought to be essential for growth.

Another large scale genome analysis in *M. maripaludis* allowed for the development of a systems level predictive model for global gene regulation of methanogenesis [34] . This is the first systems scale model developed for a hydrogenotrophic methanogen. The model demonstrates regulatory affiliations within methanogenesis as well as between methanogenesis and other cellular functions, and has predicted the presence of previously unknown genes. The model was constructed by first generating a comprehensive list of coding and noncoding RNAs through comparative analysis of the complete transcriptome and proteome. This list was combined with over 50 transcriptome profiles from steady-state conditions and during dynamic cellular response to a spectrum of environmental perturbations to build the model. The model was able to accurately predict transcriptional responses of all the genes in the network. The ultimate goal of the study was to use the model as a framework to formulate novel hypotheses regarding gene functions and regulation. From the perspective of metabolic engineering for industrial applications, this model provides a map for potential systems level consequences resulting from targeted manipulation of specific steps in methanogenesis.

6. Rising Interests in Methanogens

Methanogens have become extremely important industrially as they are used in the production of biofuels, as well as in treating waste for municipal and industrial processes [5].Increased knowledge of methanogen physiology and biochemistry from the studies previously discussed will allow for successful development of these and future biotechnological applications of these organisms.

For greenhouse gas reduction, methanogens are being used in carbon dioxide capture and storage systems (CCS) to reduce CO_2 emissions to the atmosphere. In these systems CO_2 is captured from point sources, such as plants burning fossil fuels, and sequestered in geological reservoirs, such as depleted oil or natural gas reservoirs [35]. A recent study investigated how the methanogen *Methanothermobacter thermoautotrophicus* behaved in a CO_2 reservoir. The results indicated that *M. thermoautotrophicus* was capable of recovering energy from the captured CO_2 by using it to produce methane within a depleted oil reservoir [36].

Another application of methanogens in reducing carbon dioxide emissions involves the process of electromethanogenesis. Electromethanogenesis was studied in the hydrogenotrophic organism *Methanobacterium autotrophicus* [37]. In this process, a methanogenically-catalyzed electrode was used to convert CO_2 to methane. Instead of using molecular hydrogen, *M. autotrophicus* used a proton and electrical current from the electrode as reducing power to reduce CO_2 to methane according to the reaction

$$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O.$$

The goal of that research is to establish a system to convert stored CO_2 to methane, which can be used as energy, allowing CO_2 reservoirs to be converted into large-scale energy storage. The study showed that when used alone with an electrode, *M. autotrophicus* had a 20% CO_2-to-methane conversion. However, when it was co-cultured with other microorganisms, the conversion could be increased up to 96%. Successful development of this technology could allow for storage of energy from renewable power sources, such as solar panels.

In addition to energy applications, methanogens are being applied in biotechnology for treatment and reduction of waste. Upflow anaerobic sludge blankets (UASB) containing both hydrogenotrophic and aceticlastic methanogens have successfully been used to treat solid wastes in municipal wastewater [38]. In addition to cleaning the water, the methanogens in the UASBs produce methane that can be converted into biogas. A recent study demonstrated that the dissolved methane they produce can be collected by a degassing membrane in the reactor [38]. UASBs have also been used in the treatment of wastewater containing tetramethyl ammonium hydroxide (TMAH), a toxic corrosive chemical used widely in the semiconductor and opto-electronic industries [39]. *Methanomethylovorans hollandica* and *Methanosarcina mazei* co-cultured in an UASB were shown to degrade TMAH to methane and ammonium. In another waste reduction study, *M. thermoautotrophicus* was shown to accelerate the

degradation of cellulosic waste when used with cellulolytic bacteria, allowing more energy to be recovered from solid organic waste [40].

7. Conclusions

The interest in methanogens due to the advances in applications discussed in the previous section has caused a great increase in studies on methanogen genetics. Many common genetic techniques have been modified to accommodate environmental conditions required by methanogens. The most common modification is that all procedures must be carried out in an oxygen-free environment. Usually, oxygen contamination is avoided by performing experiments in an anaerobic chamber, where oxygen is removed using hydrogen gas and a catalyst, such as palladium [36]. The use of highly flammable hydrogen gas creates safety concerns that must also be addressed when working with methanogens.

As many studies have found that methanogens are more metabolically diverse, meaning able to perform methanogenesis using a wider range of substrates, future studies can focus on using alternative carbon sources for methanogenesis. These studies will be useful in developing the waste reduction applications of methanogens, such as treating industrial wastewater. Also, further studies on how methanogens can be used in the capture of carbon dioxide will be useful due to rising levels of CO_2 in the atmosphere. The methane produced by methanogens during all of these processes can be converted into biofuel and used as an alternative energy source.

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