ABSTRACT

Title of Thesis: EXPRESSION OF RECOMBINANT PROTEINS IN THE METHANE-PRODUCING ARCHAEON *METHANOSARCINA ACETIVORANS*

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Recombinant protein expression is a necessary tool for the investigation of proteins in the post-genomic era. While many systems exist for recombinant protein expression in organisms of the eukaryotic and eubacterial domains, few to none are available in the Archaea. A recombinant protein expression system using the methanogenic archaeon *Methanosarcina acetivorans* was developed which uses the highly regulated *cdh* promoter and allows expression of recombinant protein with optional 6xHis protein fusions to facilitate rapid purification. A protocol for high-density mass cultivation of *M. acetivorans* in a stainless steel bioreactor configured as a pH-auxostat was developed. The *cdh* promoter and alternate promoters were analyzed in attempt to enhance expression of recombinant proteins. The protein expression system was tested on several proteins: the *Methanocaldococcus jannaschii* prolyl tRNA synthetase, the *M. acetivorans* prolyl tRNA synthetase, the *Methanosarcina*

thermophila carbonic anhydrase, and the *Dehalococcoides ethenogenes* tricholorethylene dehalogenase.

EXPRESSION OF RECOMBINANT PROTEINS IN THE METHANE-PRODUCING ARCHAEON *METHANOSARCINA ACETIVORANS*

By

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Introduction

Methanogenesis is an important anaerobic process in the global carbon cycle whereby organic carbon is recycled into the atmosphere as methane gas. In the natural environment, the process requires a consortium of at least three metabolic groups of anaerobic microorganisms. Fermentative bacteria convert complex polymers and monomers to fatty acids and alcohols, which are subsequently converted by acetogenic bacteria, to acetate, formate, methanol, carbon dioxide and hydrogen gas. Finally, the latter products are converted to methane and carbon dioxide by methanogenic Archaea (Sowers 2004). Methanogenesis only occurs in a phylogenetically distinct group of Archaea that grow by the production of methane produced by CO_2 reduction with H_2, by dismutation of acetate, or methylotrophically using substrates such as methanol, methyl-sulfides and methyl-amines. At least two-thirds of global methane is produced via the aceticlastic pathway (Lovley and Klug 1982), where an acetate molecule is dismutated into one methyl group, which is reduced to methane, and a carboxyl group, which is oxidized to CO_2. This globally important process is mediated only by methanogens of the order Methanosarcinales (Ferry 1993). Of these, the most metabolically diverse group is the genus *Methanosarcina*, which possesses the ability to grow by all three methanogenic pathways (Sowers 2000).

Methanosarcina acetivorans was originally isolated from methane-generating marine sediments (Sowers et al. 1984). *M. acetivorans* can grow on acetate, methanol, methyl-amines and methyl-sulfides (Sowers et al. 1984). There is also evidence that *M. acetivorans* can grow non-methanogenically using carbon monoxide

(Rother and Metcalf 2004). Aceticlastic *Methanosarcina* spp. show preferential metabolism of substrates with higher free-energy yields resulting in preferential use of hydrogen and methylotrophic substrates to acetate (Smith and Mah 1978; Zinder and Elias 1985). Also, the low free-energy yield associated with aceticlastic growth results in a much lower growth rate (Smith and Mah 1978).

The modern study of the molecular biology and biochemistry of an organism or group of organisms is greatly enhanced by the use of genomic information and a genetic system. The complete genome of *M. acetivorans* is available (Galagan et al. 2002), as well as the completed genomes of two other *Methanosarcina spp*.: *Methanosarcina mazei* (Deppenmeier et al. 2002) and *Methanosarcina barkeri* (http://genome.jgi-psf.org). Several genetic tools have been developed for use in methanogens of the genus *Methanosarcina* (Sowers and Gunsalus 1988; Apolinario and Sowers 1996; Metcalf et al. 1997; Zhang et al. 2000; Zhang et al. 2002; Pritchett et al. 2004). An efficient genetic system exists for the organism, combining the ability to grown clonal colonies on solidified medium (Apolinario and Sowers 1996), the use of a natural plasmid isolated from *M. acetivorans* to engineer recombinant plasmids containing selectable markers (Sowers and Gunsalus 1988; Metcalf et al. 1997), and a liposome-mediated transformation protocol with a high efficiency of transformation (Metcalf et al. 1997). Other genetic tools that are available for *M. acetivorans* include transposon-mediated random mutagenesis (Zhang et al. 2000) and directed gene disruption techniques, including a recent markerless genetic exchange system (Zhang et al. 2002; Pritchett et al. 2004). Because the genetic system for *M. acetivorans* is among the most advanced available for the

methanogenic Archaea, it serves as a tractable model for the study of the molecular biology of methanogenic Archaea.

Due to advances in sequencing technology in the past decade, an increasing number of microbial genomes have become available. As of January 2004, there were over 150 microbial genomes available, and hundreds more in progress (Nelson et al. 2004). After a genome has been sequenced, the genes are identified and their function is inferred by sequence similarity to known genes. Many of the new genes discovered by genome sequencing are of unknown function. In order fully understand the metabolic capabilities of an organism, the functional analysis of these unknown genes must be performed to determine their role.

Functional analysis of a protein ultimately requires the protein's purification for activity assays. Experiments are conducted to assess protein's function, e.g. its substrates and products, the elements that regulate the enzyme activity, and the rates of activity of the enzyme. Purified proteins are also required for structural analysis in which a protein is crystallized and analyzed by X-ray crystallography. By determining the three-dimensional structure of a protein, the function can be inferred by comparing the structural motifs to a database of structures of proteins with known function . Purified proteins are required for many other biochemical applications, e.g. the metal content analysis of a protein, the identification of prosthetic groups associated with a protein and the identification of the proteins associated with that protein. Purified proteins are also required for many industrial purposes, where they are used for many purposes from food additives to pharmaceuticals.

Prior to genome sequencing, gene discovery generally occurred after enzyme discovery. The investigator studying a specific metabolic capability fractionates cellular proteins, purifying and then characterizing and sequencing the protein from the fractions possessing the desired activity. In this way, the gene for the enzyme of interest can be identified in the genome of the organism. With the wealth of genomic information that is now available, it is common to identify a gene for a protein of interest, amplify it from the organism's genomic DNA, and place it under the control of a strong promoter for expression and subsequent purification. This recombinant protein expression provides abundant material for the study of the gene product directly and requires little initial information about the function of the protein.

Functional and structure analyses of specific proteins also frequently require that the protein be purified in relatively large quantities. These amounts can be obtained by mass culturing of the organism that produces the protein and purifying the protein from the cell material. This method may not be a possibility for proteins that are produced as a small fraction of total cellular protein or for organisms that do not grow rapidly or to high densities. For Archaea, obtaining dense cultures in preparative volumes often involves a great deal of time, expense and difficulty. This is due to the "extreme" habitats to which many Archaea are adapted, e.g. hydrothermal vent systems, hypersaline lakes and polar ice lakes. Organisms from these habitats require specific growth conditions that can be expensive to maintain and have often not been completely optimized in the laboratory, resulting in low yields of cell material. Because of these limitations, large quantities of protein for

subsequent analysis are most commonly produced by expressing the protein in a recombinant system that employs a readily grown, high-yield microorganism.

Therefore, recombinant protein expression is an attractive option because: 1) proteins can be produced in organisms that have fast growth rates and are easy to cultivate (Balbas and Lorence 2004); 2) yields can be increased by producing recombinant proteins under the control of a promoter that allows the protein to be expressed as a greater fraction of the cellular protein (Studier et al. 1990); and 3) recombinant protein expression can allow the investigator to include a translational fusion to aid in protein purification (Palva and Silhavy 1984; Hochuli et al. 1987; Hochuli 1988; Maina et al. 1988).

Most recombinant proteins that have been expressed for structural analysis have been expressed in *Escherichia coli* because genetic manipulations in this organism are relatively easy and inexpensive (Balbas and Lorence 2004). *E. coli* has historically been a model organism for genetic manipulation and investigations of bacterial genetics. Therefore, a multitude of genetic systems for *E. coli* exist, including many systems for the expression of recombinant protein.

There are many other organisms in which recombinant protein expression systems are available and new systems are developed regularly. These include many prokaryotes, e.g. the gram-negative bacterium *Ralstonia eutropha* (Srinivasan et al. 2002) and the gram-positive bacteria *Bacillus subtilis* (Balbas and Lorence 2004), *Lactococcus lactis* (de Ruyter et al. 1996) and *Rhodococcus spp*. (Nakashima and Tamura 2004). Many systems for recombinant protein expression in eukaryotes are also available, including fungal, mammalian, amphibian, insect, plant and yeast cells.

Filamentous fungi of the genus *Aspergillus* are widely used for the expression of recombinant proteins for industrial purposes (Punt et al. 2002). Other widely used systems are the yeast *Pichia pastoris* (Cereghino and Cregg 2000) and insect cells with baculovirus-based expression vectors (Jarvis 1997). No common system exists for recombinant protein expression in the Archaea. Very few examples of published recombinant protein expression in Archaea can be found. Systems have been published describing recombinant expression of homologous proteins in *Haloferax volcanii* and *Halobacterium salinarum* (Jolley et al. 1996; Long and Salin 2000). Although no examples of heterologous recombinant protein expression for subsequent purification in Archaea could be found, genetic markers for plasmid maintenance in archaeal genetic systems often contain heterologous genes; for example the puromycin resistance gene from *Streptomyces albolinger*, *pac*, is used as a selectable marker in the plasmid-based genetic system for *M. acetivorans* and effectively confers resistance to the antibiotic puromycin (Metcalf et al. 1997). Other examples include selectable and phenotypic marker expression and reporter genes expressed in the Archaea (Allers and Mevarech 2005).

The yields obtained using the various protein expression systems vary. Some systems are designed to yield high levels of proteins, while some systems are designed to ensure the correct folding or correct post-translational modifications of the recombinant proteins. In general, the most basic systems result in higher yields of recombinant proteins to compensate for low specific activities and the more complex systems yield proteins that are more likely to have wild-type level activities (Balbas and Lorence 2004). For example, *E. coli* systems can yield up to 1000 mg L^{-1} of

recombinant proteins (Invitrogen.com, accessed December 7, 2005), yeasts and filamentous fungi yield from 0.1 to 300 mg L^{-1} of recombinant proteins (Punt et al. 2002) and mammalian cell expression systems can yield from 0.1 to 50 mg L^{-1} of recombinant proteins (Wurm 2004). Conversely, *E. coli* are not capable of many posttranslational modifications and mammalian cells are capable of the most posttranslational modifications (Balbas and Lorence 2004).

Such a wide range of recombinant protein expression systems exists because recombinant protein expression is often a rate-limiting step in the investigation of many proteins. This is because, while some proteins are expressed in an active form on the first attempt, others are not expressed in an active form and variations in the expression system are required. No protein expression system has yet been developed that works universally for all proteins (Balbas and Lorence 2004).

The most common problem resulting in poor expression of recombinant protein is the formation of inclusion bodies, which are insoluble aggregates of recombinant protein. The exact mechanisms by which these aggregates form is unknown, however, it has been proposed that inclusion body formation is a stress response associated with high-level expression of recombinant protein (Villaverde and MarCarrió 2003). Data on the success rate of recombinant protein expression is not available because negative results are often not reported. One example of the success rate using *E. coli* expression systems comes from the Southeast Collaboratory for Structural Genomics (SECSG), a research group with a mission to achieve high throughput structural characterization of many proteins. Of the 6,397 proteins that they have expressed in *E. coli*, only 23% have been soluble (www.secgc.org,

accessed November 1, 2005). Apart from inclusion body formation, other problems resulting in poor expression include improper folding of the recombinant protein, proteolytic degradation of the target protein, lack of post-translational modifications in the expressed protein, poor expression due to codon usage differences between the native organism and the host organism, and rearrangement of foreign DNA by the host organism.

The multiple systems available utilize a range of tools to enhance protein expression. A simple approach is solubilization of inclusion bodies using strong denaturants and subsequent removal of the denaturants under conditions that allow protein refolding (Nagai et al. 1988). Another approach is the co-expression of various molecular chaperones and foldases with the protein of interest to enhance protein folding and increase solubility (Georgiou and Pascal 1996). The disadvantage of these two methods is that they do not work for all proteins and, when they do work, a number of conditions need to be tested to achieve the correctly folded protein (Middelberg 2002; Sorensen and Mortensen 2005). Another approach is the use of stronger promoters (Studier et al. 1990). This can result in increased expression of recombinant protein, but can still result in inclusion body formation (Sorensen and Mortensen 2005). Gene fusions with genes for proteins that enhance solubility of the protein of interest are also used to enhance solubility of recombinant proteins (Pryor and Leiting 1997; Davis et al. 2000). These enhance the solubility of the protein with a relatively good success rate. The main disadvantage to this technique is that the protein fusion needs to be removed from the protein of interest. Again, this process must be optimized for each protein and the correctly folded protein often cannot be

recovered at high yield after cleavage of the fusion partner (Sorensen and Mortensen 2005).

Therefore, while expressing proteins using a recombinant system is often an attractive option, there are many disadvantages. These disadvantages can be even more pronounced when attempting to express archaeal genes in a eubacterial or eukaryal system. The Archaea differ in their molecular biology as much from the eubacteria as they do from the eukarya (Woese et al. 1990). While Archaea are similar to eubacteria in morphology, archaeal transcriptional machinery, e.g. their large multicomponent RNA polymerase and transcription initiation mechanisms, are more similar to that of the eukarya (Lange and Ahring 2001). Archaeal genes may also vary from eubacterial genes due to differences in sequence and codon usage (Reeve 1993). Also, in-frame amber codons exist in some archaeal genes, encoding for the nonstandard amino acids. For example, pyrrolysine is encoded for by the stop codon UAG and is only found in methanogenic Archaea (Burke et al. 1998; Srinivasan et al. 2002). Proteins containing this nonstandard amino acid would presumably not be expressed in an active form in an organism that did not possess the translational machinery to override this stop codon. In one study, the expression of six archaeal genes was attempted in *E. coli*, four from *Methanocaldococcus jannascii* and two from *Pyrobaculum aerophilum*. All proteins were poorly expressed, however expression was enhanced by the introduction of genes encoding the rare tRNAs (Kim et al. 1998).

In a study by Frankenberg et al. (2001), the *M. jannaschii* 20S proteosome was expressed in *E. coli* and the activity and thermostability of the recombinant

proteosome was compared to the wild-type proteosome. The optimum activity of the recombinant proteosome occurred at 95°C compared to 119°C for the wild-type proteosome. The half-life of the recombinant proteosome was also much shorter compared to the wild-type proteosome. However, when the recombinant proteosome was chemically denatured and then refolded at elevated temperatures, the wild-type activity and half-life was restored. This demonstrates that the folding environment in *E. coli* may not be suitable for some archaeal proteins. In this case, elevated temperature enhanced folding of the hypertheromophilic enzyme (Frankenberg et al. 2001).

In a study by Bayley and Jarrell (1999) flagellin from the mesophile *Methanococcus voltae* was expressed *in E. coli* and *Pseudomonas aeruginosa*. The protein was expressed in both systems, but the leader peptide, which is cleaved in *M. voltae*, was not cleaved in either eubacterium. When the unprocessed peptide was incubated with *M. voltae* membranes, the peptide was cleaved, allowing the researchers to localize the peptidase activity to the *M. voltae* membrane. While the researchers were still able to answer some interesting questions using the unprocessed flagellin, this study demonstrates that the eubacteria used did not contain the correct cellular machinery to correctly process some archaeal genes (Bayley and Jarrell 1999).

Difficulties in expression of archaeal genes in *E. coli* may also arise due to lack of posttranslational modifications of proteins. It is well known that eukaryotic proteins, which require significant levels of posttranslational modifications including proteolytic cleavage, glycosylation and amino acid modifications, are often not well

expressed in eubacteria, which have less complex mechanisms of posttranslational modifications. The mechanisms of posttranslational modifications in the Archaea are not well understood but possess elements that appear eukaryal, eubacterial, and those that are unique to the Archaea (Eichler and Adams 2005). These differences have the potential to cause significant problems when heterologously expressing proteins in organisms from different domains. When *Pyrococcus abyssi* alkaline phosphatase was expressed in *E. coli*, it was present in an active form but with a low yield (Zappa et al. 2003). When genes for rare tRNAs were introduced the yield of the recombinant protein expressed in *E. coli* increased. When the enzyme was expressed in the methylotrophic yeast *Pichia pastoris*, the protein was expressed with a lower yield but with a greater activity (Zappa et al. 2003). This suggests that, while recombinant protein yield can be increased in *E. coli* with enhanced expression of rare tRNAs, the activity of the archaeal protein may have been enhanced due to the translational or posttranslational machinery present in the eukaryal expression system.

In order to enhance expression of archaeal genes, *M. acetivorans* was chosen for development of a recombinant protein expression system. There are several advantages to the use of *M. acetivorans* for recombinant protein expression. Firstly, it is a mesophilic archaeon that can be mass cultured to high densities (Sowers et al. 1984). Secondly, an efficient genetic system and several genetic tools are available for the organism, including an *E. coli-M. acetivorans* shuttle plasmid which is present at 15 copies per cell and is stable over dozens of generations (Sowers and Gunsalus 1988; Apolinario and Sowers 1996; Metcalf et al. 1997; Zhang et al. 2000; Zhang et al. 2002; Pritchett et al. 2004; Apolinario et al. 2005). Thirdly, the species contains

genes for all four known chaperoning systems, a trait that has not been found in any organisms outside of the *Methanosarcina* spp., which should facilitate the correct folding of many heterologous proteins (Macario et al. 2004). Fourth, a well-characterized promoter exists for the *Methanosarcina* spp. that is highly regulated in response to growth substrate. Expression from the *cdh* promoter, which controls transcription from the carbon monoxide dehydrogenase/acetyl coenzyme A operon, is 30-fold higher when the organism is grown on acetate vs. methylotrophic substrates. (Apolinario et al. 2005)

Methanosarcina spp. also utilize a range of metalloenzymes and cofactors that are not synthesized in other organisms. For example, corronoid cofactors are synthesized and incorporated into enzymes involved in the methanogenesis pathway (Stupperich et al. 1990; Weiss and Thauer 1993). Because of this metabolic ability, enzymes requiring corronoid cofactors may be expressed in an active form using the *M. acetivorans* system. Also, *M. acetivorans* is one of the few organisms that has been found to express peptides containing the nonstandard amino acid, pyrrolysine, which is encoded for by UAG, a universal stop codon in eubacterial, eukaryal and archaeal systems. Genes encoding pyrrolysine would be synthesized in a truncated form in systems which did not possess the pyrrolysine tRNA synthetase (Soares et al. 2005).

An expression system using *M. acetivorans* was developed that incorporates the *M. acetivorans* native plasmid, the highly-regulated promoter for *M. thermophila* carbon monoxide dehydrogenase, *cdh,* and a multiple cloning site with coding sequence for optional N- and C- terminal 6xHis fusions. The protein yield obtained

using this system was enhanced by improving the techniques for mass cultivation of acetate-grown *M. acetivorans* in a stainless steel bioreactor. Several recombinant proteins were investigated using this system.

The *M. jannaschii* prolyl tRNA synthetase (MjProRS), which is one of the 21 aminoacyl synthetases which are involved in the charging of tRNA during protein synthesis, was expressed in *M. acetivorans*. This hyperthermophilic protein can be actively expressed in *E. coli* (Lipman et al. 2000), which provided a means of comparing the activities of the products expressed in the archaeal and the bacterial systems.

The *M. acetivorans* mesophilic prolyl tRNA synthetase (MaProRS) was also expressed using this system in order to study the proteins incorporated in the prolyl-tRNA complex during translation. In higher eukaryotes, prolyl tRNA synthetase is one of the nine aminoacyl synthetases that are associated with other peptides. These other peptides appear to be involved in increasing the fidelity of protein translation (Mirande 1991; Norcum and Dignam 1999). In eubacteria, no multi-peptide aminoacyl synthetase complexes are known to exist. MjProRS has been shown to be associated with other peptides, providing evidence for the existence of multi-peptide aminoacyl synthetase complexes in Archaea (Lipman et al. 2003). By purifying the recombinant MaProRS under various conditions in the homologous system, proteins that bind to the enzyme in vivo may be identified.

The carbonic anhydrase from the thermophilic archaeon *Methanosarcina thermophila* (MtCam) was also investigated. Carbonic anhydrases catalyze the hydration of carbon dioxide to bicarbonate and are present in all domains of life.

MtCam is interesting as it belongs to the gamma-class of carbonic anhydrases, which are hypothesized to have evolved at the estimated time of the origin of life (Smith et al. 1999). While this protein has been shown to be a zinc-containing metalloenzyme when expressed in *E. coli*, it has been hypothesized that the native protein may instead contain iron in its active site (Tripp et al. 2004). Expression of this protein in *M. acetivorans*, a mesophilic methanogen that is closely related to *M. thermophila*, will likely provide evidence to resolve this question.

The trichloroethylene (TCE) reductive dehalogenase from *Dehalococcoides ethenogenes* (DeTCE-RD) was also expressed using the *M. acetivorans* expression system. TCE dehalogenases catalyze the conversion of TCE to lesser-chlorinated ethenes (Neumann et al. 1995; Magnuson et al. 1998). Chlorinated ethenes such as TCE are very common groundwater contaminants and are toxic chemicals which pose a public health risk. TCE has been shown to be dechlorinated to the harmless product, ethene, by DeTCE-RD (Magnuson et al. 1998). Yields of dechlorinating bacteria such as *D. ethenogenes* are low and they are very difficult to mass culture, which limits the ability to produce cell material for purifying large quantities of specific enzymes (Maymo-Gatell et al. 1997). Attempts by several laboratories to express an active dehalogenase in a recombinant system have failed (Neumann et al. 1998). *M. acetivorans* may be more likely to produce active dehalogenases than *E. coli* because *D. ethenogenes* and *M. acetivorans* are both obligate anaerobes and are capable of producing corronoid cofactors, which are required for synthesis of the TCE dehalogenase (Magnuson et al. 1998). Also, *M. acetivorans* may possess the molecular chaperones required for biosynthesis of active dehalogenases. If the

dehalogenase expressed in *M. acetivorans* is indeed active, this expression system may be ideal for expression of dehalogenases for other chlorinated compounds that occur as environmental contaminants.

This body of research describes the development of a recombinant expression system in *M. acetivorans*. The characterization of the *cdh* promoter and other promoters with respect to utility in recombinant protein expression was also investigated. A variety of proteins were expressed using the system, and the results obtained will be discussed.

The primary goal of this research is to enhance the genetic tools available for the study of proteins that are difficult to express using existing systems, including archaeal proteins and proteins from obligate anaerobes. To this end, a system for recombinant protein expression in *M. acetivorans* was developed and tested. By analyzing the expression of proteins using this system, it can be determined whether this system is an effective research tool for expression and functional analysis of the target proteins.

Materials and Methods

Cultivation of *M. acetivorans* Using Batch Processes

Methanosarcina acetivorans C2A (DSM 2834) was obtained from sources described previously and maintained as frozen stocks (Sowers and Johnson 1984). Small cultures (10 mL, 100 mL and 1 L) of *M. acetivorans* were grown in marine disaggregating medium (M-medium) prepared anaerobically under an 80% N_2:20%

CO_2 atmosphere (Sowers and Gunsalus 1988). Medium (10 ml) was dispensed into 30 ml anaerobe tubes (Belco) and 100 ml in 160 ml serum bottles, which were sealed under N_2-CO_2 (80/20) with 20 mm butyl stoppers (West Co.) secured with aluminum crimp seals (Wheaton). Medium volumes of 1L were prepared in 2 L round bottom flasks, which were sealed under N_2-CO_2 (80/20) with a rubber stopper secured with 20 gauge steel wire. The medium was sterilized by autoclaving at 121°C for 20 minutes. For phosphate limitation batch experiments medium was prepared without dibasic sodium phosphate and phosphate was added to the desired concentration from an anaerobic, sterile 1 M sodium phosphate solution at the time of inoculation. Solidified medium for colony growth was prepared by adding agar to liquid medium. Solidified medium was prepared in Petri plates and inoculation of solidified medium was performed in an anaerobic glove box as described previously (Apolinario and Sowers 1996). Recombinant strains of *M. acetivorans* (Table 1) were maintained in medium containing 2 μg mL^{-1} puromycin (Invivogen).

Growth of *M. acetivorans* in a pH-auxostat was performed as described by (Sowers et al. 1984) with the exception that a BioFlo IV bioreactor (New England Biolabs) was used. Briefly, M-medium (14 L) was prepared and degassed with N_2 without the addition of bicarbonate buffer. Sodium acetate was added to a concentration of 50 mM and the medium was autoclaved in-place in the stainless steel (SS) bioreactor. Bioreactors were inoculated with 0.5 to 1.0 L *M. acetivorans* grown on 0.1 M sodium acetate. After inoculation, the pH was maintained by the addition of acetic acid. The pH and all other growth parameters were maintained with the BioFlo

Table 1. Plasmids and recombinant *M. acetivorans* strains described in the text.

Protein fused to *cdh* promoter	*M. acetivorans* Strain	Plasmid name	Description
β-galactosidase	KSC8	pEA103	*cdh* promoter fused to *lacZ* for use in characterizing promoter (Apolinario et al. 2005)
none	KSC20	pES1	*cdh* promoter with 6xHis tags flanking multiple cloning site for overexpression
MjProRS	KSC19	pSM1	Gene for MjProRS in pES1 with N-term 6xHis
DeTceA	KSC41	pEA129	*tceA* in pES1 with N- and C-term 6xHis
DeTceAB	KSC42	pEA130	*tceAB* in pES1 with N-term 6xHis on *tceA* and no 6xHis on *tceB*
MtCam	KSC43	pSM12	Gene for MtCam in pES1 without leader sequence, no 6xHis
MaProRS	KSC44	pMaProRS	Gene for MaProRS in pES1 with 6xHis and TAP tags

IV controller. In order to optimize growth conditions, salts (NaCl, $MgSO_4$, KCl, $CaCl_2$ and NH_4Cl), prepared as a 4X solution autoclaved separately in a polypropylene carboy, and a 1,400X filter-sterilized mineral supplement (final supplemental 10X concentration in medium: 25 μM iron, 30 μM cobalt, 10 μM nickel, 8.4 μM molybdenium and 5.8 μM selenium) were added to the bioreactor after sterilization. The recombinant strains of *M. acetivorans* were maintained in the presence of 1 μg mL^{-1} puromycin to maintain transformed plasmids.

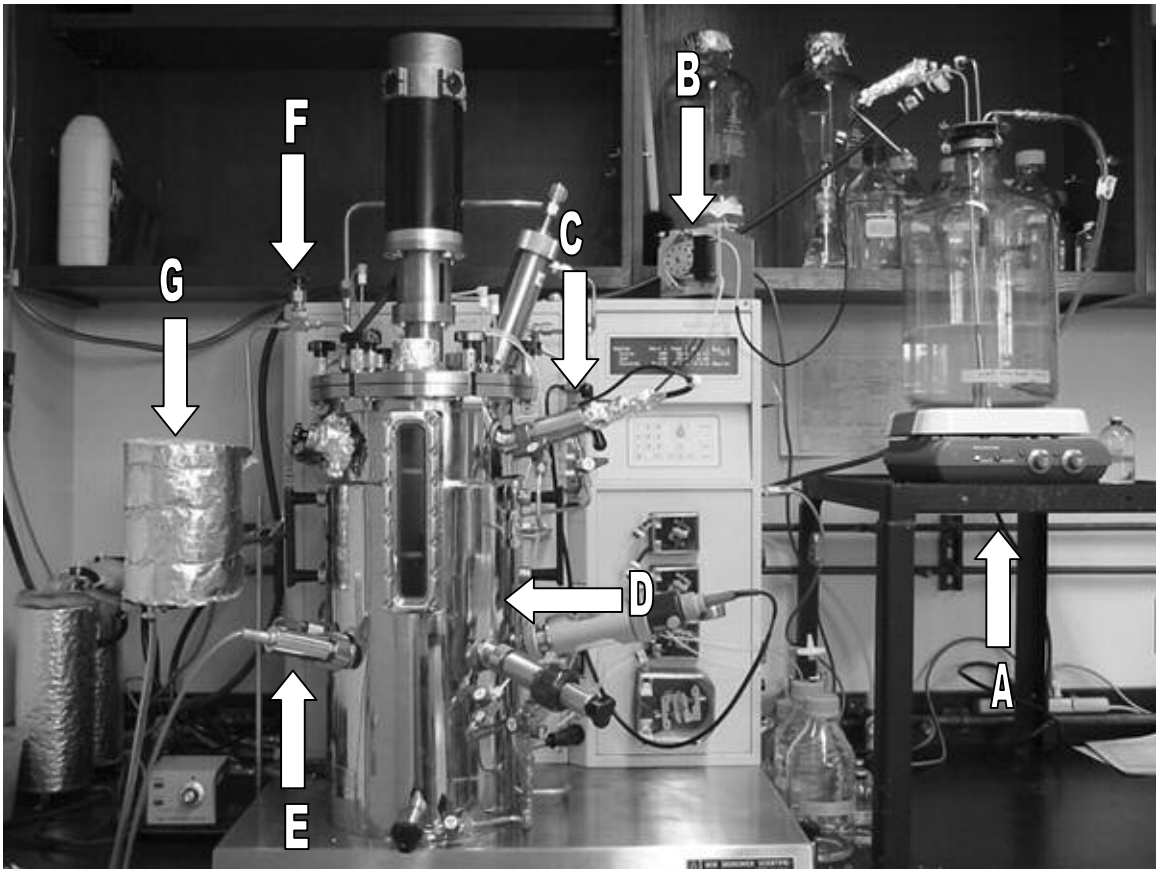
To test for promoter induction, *M. acetivorans* KSC19 (Table 1) was grown in a 20 L bioreactor containing 14 L M-medium, 0.1 M trimethylamine-HCl (TMA) and 0.1 mM sodium acetate. Sterile 4X salts solution, mineral supplement, sodium sulfide and puromycin were added separately to the medium after it was autoclaved. The bioreactor was inoculated with approximately 0.6 L *M. acetivorans* KSC19 grown on 0.1 M TMA. As the culture entered the mid-exponential and stationary

phases of growth, samples (1 L) were removed from the bioreactor at regular intervals and pelleted aerobically by centrifugation for 30 min at 4°C at 10,000 x g. Cell pellets were stored at -80ºC.

Continuous Culture of *M. acetivorans*

Continuous culturing of *M. acetivorans* KSC8 was done in a 20 L BioFlo IV bioreactor configured as a chemostat (Figure 1) by the addition of a SS outflow tube (0.635 cm OD) that was inserted through a top port of the vessel and protruded into the vessel to a level where the medium reached 5.6 L. Outside the vessel the outflow was equipped with a SS cutoff valve immediately above the reactor vessel followed by a 6 meter length of SS tubing (0.635 cm OD) coiled 20 times and surrounded by a 16 cm x 16 cm heating pad. The outflow tube was maintained at 90 ± 5ºC with a Thermolyne type 45500 input controller to prevent backflow contamination of the culture vessel. Bicarbonate-buffered M-medium (6 L) containing 0.1 M sodium acetate and no phosphate was prepared in the vessel. Salts (NaCl, $MgSO_4$, KCl, $CaCl_2$ and NH_4Cl, 4X solution prepared in a glass flask), sodium sulfide and puromycin were added separately. The bioreactor was inoculated with 1 L *M. acetivorans* KSC8 grown on 0.1 M sodium acetate and 4.0 mM phosphate.

Figure 1. BioFlo IV bioreactor configured for continuous culture. "A" indicates the feed carboy. "B" indicates the peristaltic pump controlling the addition of medium. "C" indicates the medium input valve. "D" Indicates the bioreactor, which contains the culture. "E" indicates the cell density probe. "F" indicates the culture outflow valve. "G" indicates the heated coil which sterilizes culture outflow, preventing back contamination of the culture.



Marine medium was prepared separately in a 13.75 L glass carboy containing a magnetic stir bar without the addition of sodium phosphate, cysteine or sodium sulfide. Medium was prepared and sterilized aerobically. A stopper was prepared that fit into the carboy and was fitted with three lengths of 0.635 cm (OD) SS tubing: one length connected externally to a gas line and was fit with a gassing stone extending to the carboy bottom to sparge the medium; one length extended to the

carboy bottom and was connected externally to a peristaltic pump to transfer medium from the carboy to the bioreactor; one length extended into the headspace of the carboy and was externally fit with a 6,894 Pa relief valve to vent the carboy and prevent excess pressurization. This stopper was sterilized separately from the carboy by autoclaving and aseptically inserted into the carboy after the medium had cooled. The carboy was sparged with 80% N_2:20% CO_2 while stirring with a magnetic stir bar. After 1 h of sparging, cysteine-HCl was injected anaerobically into the medium from an anaerobic sterile solution to a final concentration of 0.025% (w/v). After another 1 h, sodium sulfide was injected anaerobically into the medium from an anaerobic sterile solution to a final concentration of 0.025% (w/v). After sparging the medium until it turned clear, the tubing exiting the carboy was clamped and the medium outflow was attached to the BioFlo IV inlet port. Once the culture in the fermentor reached mid-exponential growth the port was opened and medium was pumped into the bioreactor.

Continuous dilution of the culture in the BioFlo IV was achieved by the constant addition of medium from the feed carboy into the BioFloIV, which was controlled by a peristaltic pump (Watson-Marlow). Constant bubbling of the medium in the feed carboy with 80% N_2:20% CO_2 prevented negative pressure in the feed carboy while maintaining anoxic conditions. Constant bubbling of the medium in the BioFloIV with 80% N_2:20% CO_2 maintained positive pressure in the vessel, enabling the culture to be pushed out of the vessel when it reached a level above 5.6 L once the outflow valve was opened; the volume in the BioFloIV was maintained by this mechanism, providing continuous dilution. Contamination of the bioreactor outflow

was prevented by the heated coil connected to the outflow tubing. Contamination of the inflow was prevented using aseptic technique when changing feed carboys and offline measurements were made through a resterilizable sample port. Growth was monitored by a MAX Cell Mass Sensor (Cerex, Inc.) calibrated with offline optical density measurements at 550 nm. The culture was monitored for contamination by light microscopy. A sterile, anaerobic solution of dibasic sodium phosphate was added to both the culture and the medium carboy when the density of the culture began to decrease, indicating potential phosphate limitation. Phosphate concentration was estimated by measuring soluble reactive phosphorous using a colorimetric technique (Murphy and Riley 1962).

Effects of stainless steel on growth of *M. acetivorans*

Duplicate 10 mL cultures of *M. acetivorans* were grown in M-medium containing 0.1 M sodium acetate in 30 mL glass anaerobe tubes. The medium was sealed under N_2-CO_2 with butyl rubber stoppers secured with aluminum crimp seals. Prior to autoclaving and inoculating media, two 5 cm lengths of 0.64 cm (OD), 0.53 cm (ID) type 3-16 stainless steel (SS) tubing (Alltech) were added to 2 replicate sets of tubes. After autoclaving and before inoculating media, all culture tubes were aseptically opened in an anaerobic chamber containing 75% N_2, 20% CO_2 and 5% H_2. Sterile lengths of steel tubing were added to 2 sets of tubes. Sterile mineral supplement solution (final supplemental concentration in medium: 25 μM iron, 30 μM cobalt, 10 μM nickel, 8.4 μM molybdenium and 5.8 μM selenium) was added to one set of tubes autoclaved with lengths of SS tubing, one set of tubes with sterile SS

tubing added after autoclaving, and one set of tubes with mineral supplement alone. The tubes were inoculated anaerobically and incubated at 30 ºC for 2 weeks. At regular intervals, methane was analyzed on a Hewlett Packard model 5890A Gas Chromatograph equipped with a flame ionization detector and 0.32 x 182 cm SS column packed with 80/100 mesh silica gel (Supelco). The oven was run isothermally at 110 ºC with He as the carrier gas. Headspace samples (100 μL) were injected sequentially with 1 min spacing using a gas-tight syringe with valve. Purified methane (Matheson) was used as an external standard. Methanogenesis rates were calculated over a 2 week time period.

Substrate kinetics of *cdh* promoter in batch culture

Substrate kinetics of the *cdh* promoter were investigated using the previously described *M. acetivorans* KSC8, a strain possessing a recombinant reporter plasmid with a *cdh::lacZ* fusion (Table 1; Apolinario-Smith, 2005 #6549]. *M. acetivorans* KSC8 was grown in 100 mL cultures containing either 10 mM TMA and sodium acetate in concentrations ranging from 0 to 0.1 M or in 10 mL cultures containing 10 mM methanol and sodium acetate in concentrations ranging from 0 to 0.15 M sodium acetate (Table 2). Some of these cultures were induced by addition of acetate to an additional concentration of 0.1 M (Table 2). Cultures were incubated at 35ºC, optical density was monitored at 550 nm, and 1 mL samples were extracted anaerobically using a syringe for β-galactosidase activity assays at selected times between 24 and 160 h. Samples were immediately put on ice and pelleted at 14,900 x g for 5 min at

4ºC. β-Galactosidase activity assays were performed as described previously (Apolinario et al. 2005).

Table 2. Results of batch experiments to test the effects of substrates on *cdh* promoter activity over time using *M. acetivorans* KSC8. β-galactosidase (β-gal) activity is expressed as specific activity per μg mL^{-1} protein. "NA" stands for "not applicable".

Methylotrophic Substrate Used	Methylotrophic Substrate (mM)	Initial Sodium Acetate (mM)	Sodium Acetate Added During Mid-Exponential Phase (mM)	Time after Induction (h)	Time between Initial and Final (h)	Initial β-gal Activity	Final β-gal activity	Fold Increase in β-gal Activity (final:intitial)
TMA	10	0	100	1	7	0.50 ± 0.05	1.16 ± 0.16	2.1
TMA	10	10	100	1	7	0.38 ± 0.08	0.80 ± 0.03	2.1
TMA	10	50	100	1	7	0.35 ± 0.08	0.81 ± 0.03	2.3
TMA	10	50	0	NA	5	0.37 ± 0.04	1.27 ± 0.25	3.4
TMA	10	100	0	NA	5	0.39 ± 0.02	2.11 ± 0.00	5.4
Methanol	10	10	100	58	81	0.55 ± 0.03	0.89 ± 0.05	1.6
Methanol	10	50	100	58	81	0.63 ± 0.23	0.95 ± 0.07	1.5
Methanol	10	0	100	58	81	0.65 ± 0.17	0.81 ± 0.01	1.2
Methanol	10	100	0	NA	81	1.12 ± 0.37	1.29 ± 0.10	1.2
Methanol	10	150	0	NA	81	0.93 ± 0.19	1.26 ± 0.09	1.4
Methanol	10	0	0	NA	81	0.75 ± 0.04	0.91 ± 0.08	1.2

Analysis of alternate promoters for protein expression

To test the induciblity of specific genes in *M. acetivorans* after the addition of acetate to a methanol-grown culture, *M. acetivorans* C2A was grown in a 10 mL culture in the presence of 50 mM methanol. When the culture was in mid-exponential phase, sodium acetate was added to the culture to a final concentration of 0.15 M. Prior to the addition of sodium acetate and 1 h and 24 h after the addition of

acetate, 1.5 mL of the culture was removed by syringe and pelleted by centrifugation at 14,900 x g for 5 min at 4ºC. To test the inducibility of specific genes in *M. acetivorans* after the addition of TMA to an acetate-grown culture, *M. acetivorans* C2A was grown in a 100 mL culture containing 50 mM sodium acetate. When the culture was in mid-exponential growth, TMA was added to a concentration of 0.15 M. Prior to the addition of TMA and 1 h and 24 h after the addition of acetate, 10 mL of the culture was removed by syringe and pelleted by centrifugation at 3,200 x g for 10 min at 4ºC. Cultures were grown in the presence of 0.1 M acetate or TMA for use as controls. RNA from these cultures was extracted from 1.5 mL TMA-grown cells and 10 mL acetate-grown cells, which were removed by syringe during mid-exponential growth and pelleted by centrifugation at 14,900 x g for 5 min or 3,200 x g for 10 min, respectively, at 4ºC. Total RNA was extracted immediately from the cell pellets using the RNeasy Mini Kit (Qiagen) per the manufacturer's protocols. After eluting the RNA from the column provided in the RNeasy Mini Kit, the RNA was incubated with 20 U RQ1 DNase (Promega) in the buffer provided by the manufacturer. The DNase was removed by another purification using the RNeasy Mini Kit. RNA was stored in 10 μL aliquots at -20ºC. The RNA was quantified by reading its absorbance at 260 nm and visualized to observe quality by formaldehyde gel electrophoresis. Briefly, a 1.2% agarose (w/v) gel was prepared using diethylpyrocarbonate (DEPC) treated 40 mM MOPS, pH 7.0, containing 10 mM sodium acetate, 1 mM EDTA, and 6.7% formaldehyde (v/v). RNA (1 μg) was incubated in buffer (final concentration: 6.7% formaldehyde (v/v), 50% formamide (v/v), 10 mM sodium acetate, 1 mM EDTA in 40 mM MOPS, pH 7.0) and incubated

at 55 ºC for 15 min. Loading buffer (10 μL, 0.1 mM EDTA, 50% glycerol (v/v), 0.25% bromophenol blue (w/v), 0.25% xylene cyanol (w/v)) was then added, the samples were added to the wells and electrophoresis was carried out at 50 mA for 105 min in 40 mM MOPS, pH 7.0, containing 10 mM sodium acetate and 1 mM EDTA. RNA was visualized by UV after staining with SyBr Gold (Molecular Probes).

Primers were designed to amplify intergenic regions in the mRNA approximately 200 to 350 nucleotides (nt) in length. Primers were designed to amplify the 197 nt beginning 66 nt downstream of the start of transcription in the *M. acetivorans* gene MA2252 (forward primer: 5'-GCGCTTAATGCAGTTTCATTCACC-3'; reverse primer: 5'-GCCAGAGCACCAGCAGGAGG-3'), the 225 nt beginning 45 nt downstream of the start of transcription in MA4634 (forward primer: 5'-CAACTGCACACATATATGGCTCAG-3'; reverse primer: 5'-CCGATGAGCTTTATGACGGAATTG-3'), the 278 nt beginning 282 nt downstream of the start of transcription in MA0528 (forward primer: 5'-GGGGCCGCGACAAGAAGTTC-3'; reverse primer: 5'-GACGGGGTCGATGTGGTGG-3'), and the 349 nt beginning 571 nt downstream of the start of transcription in MA0932 (forward primer: 5'-CCCGTGGGAGAGAATGTCG-3', reverse primer: 5'-CCCTACGGGGGCAGTCCC-3').

Reverse transcriptase PCR (RT-PCR) was done using reagents provided in the Access RT-PCR Kit (Promega). The 50 μL reaction contained 100 ng total RNA, 1.5 mM $MgSO_4$, 0.4 mM dNTPs, 5 U reverse transcriptase, 5 U *Tfl* polymerase and 0.1

mM of both forward and reverse primer in the reaction buffer provided by the manufacturer. Thermocycler conditions were as follows: one reverse transcription step at 45ºC for 45 min, followed by denaturation for 2 min at 94ºC, followed by 40 amplification cycles of 30 s at 94ºC, 1 min at 60ºC, and 1 min at 68ºC, with a final extension time of 7 min at 68ºC. PCR conditions for most probable number (MPN) RT-PCR were identical except that serial dilutions of RNA were added to reactions which contained 10, 1.0 or 0.1 ng total RNA. Amplicons were visualized under UV with agarose gel electrophoresis using 1.2% (w/v) agarose gels stained with ethidium bromide.

Construction of pES1 expression vector

The pES1 expression vector was constructed by first amplifying the 1,114 bp region from 1105 bp upstream to 10 bp downstream of the beginning of the *M. thermophila* TM-1 *cdhABCDE* operon (Genbank accession no. U66032) from pCDH1 (Sowers and Gunsalus 1993). The PCR reaction mixture contained PCR Buffer II (Applied Biosystems), 0.8 mM dNTPs, 1.5 mM $MgCl_2$, 50 ng pCDH1, 100 pmol of each primer, and 1.5 U AmpliTaq DNA polymerase (Applied Biosystems). The PCR reaction conditions were as follows: initial denaturation at 95ºC for 5 min, followed by 30 amplification cycles of 30 s at 95ºC, 1 min at 55ºC, and 1 min at 72ºC, with a final extension time of 5 min at 72ºC. The PCR fragment was directly ligated into the pCR2.1 plasmid using the TA Cloning Kit (Invitrogen) and the resultant plasmid contained the region upstream of the *cdh* promoter in the reverse orientation and was called pCDHPTA-IM. The *cdh* promoter was amplified from this

plasmid with forward primer 5'- GCCCTCTAGATGCAT-3' and reverse primer 5'-TGCCTCTAGAAGCTTTACCT-3', which introduced flanking *XbaI* restriction sites using the same reaction conditions described above. The 1,180 bp PCR product was directly ligated into pCR2.1 using the TA Cloning Kit (Invitrogen), creating a new construct which was named pMH2. The pMH2 plasmid and the pET28b(+) plasmid (Novagen) were digested with *SphI* and *XbaI* (New England Biolabs). The 1,172 and 5,105 bp fragments were isolated using agarose gel electrophoresis from the pMH2 and pET28b(+) plasmid digests, respectively, and purified using the Wizard PCR Purification Kit (Promega). The resultant pET28b(+) vector and *cdh* promoter insert were ligated by overnight incubation at 14ºC with T4 DNA ligase (New England Biolabs). The ligation reaction mixture was transformed into chemically competent *E. coli* BL21 by a $MgCl_2/CaCl_2$ transformation protocol, plated on LB agar plates containing kanamycin and incubated overnight at 37ºC. The resultant colonies were screened by extracting the plasmids using the Wizard Plus MiniPreps DNA Purification System (Promega) and by subsequent restriction analysis. The correct construct was named pMH5 and contained the *cdh* promoter with the coding sequence for 6xHis fusions at the 5' end. Primers were designed to amplify the *cdh* promoter and 6xHis sequence from the pMH5 plasmid. The forward primer 5'-CACACCCGGCGCGCCTAAT-3' and reverse primer 5'-CTCTTCCGGGCGCGCCCATGCC-3' introduced flanking *AscI* sites. The PCR reaction mixture contained PCR Buffer II (Applied Biosystems), 0.8 mM dNTPs, 3 mM $MgCl_2$, 180 ng pMH5, 100 pmol of each primer, 1.5 U AmpliTaq DNA polymerase (Applied Biosystems). PCR reaction conditions were as follows: Initial

denaturation at 95ºC for 5 min, followed by 30 amplification cycles of 30 s at 95ºC, 1 min at 55ºC, and 1 min at 72ºC, with a final extension time of 5 min at 72ºC.

Another plasmid was constructed by digesting the pEA103 plasmid (Apolinario et al. 2005) with *ClaI* (New England Biolabs) to delete a significant portion of the *lacZ* gene. After digestion, the 10,446 bp DNA fragment was excised from an agarose gel, purified as described above and ligated together by incubation with T4 DNA ligase (New England Biolabs). After transforming the plasmid into *E. coli* DH5α-λpir by techniques described above, the colonies were screened by restriction analysis. The construct was designated pEA114. This plasmid was digested with *Eco47III* and *EcoRV* to remove more of the residual *lacZ* gene. The 9,724 bp fragment was excised from an agarose gel, purified as described above and ligated back together and transformed into *E. coli* DH5α-λpir by techniques described above. The transformants were screened by restriction analysis and the confirmed construct was called pEA115.

The amplicon from pMH5 containing the *cdh* promoter and sequence for 6xHis fusions (insert) and the pEA115 shuttle plasmid (vector) were digested with *AscI*. The linearized pEA115 vector was treated with shrimp alkaline phosphatase (USB Corporation) to prevent it from circularizing. The shrimp alkaline phosphatase was denatured by incubating the vector for 16 h at 37ºC. The *AscI* used to digest the insert was denatured by incubation for 20 min at 70°C. The vector and insert were ligated by incubation overnight at 14ºC with T4 DNA ligase (New England Biolabs), transformed into chemically competent *E. coli* DH5α-λpir by a $MgCl_2/CaCl_2$ transformation protocol and plated onto LB agar medium containing ampicillin.

Transformants were screened by subsequent restriction analysis and then by sequencing. The final plasmid construct, designated pES1, contains the *cdh* promoter upstream of a multiple cloning site flanked by 6xHis fusion sequence in a plasmid designed to propagate in and contain selectable markers for both *E. coli* and *M. acetivorans*.

Construction of expression plasmids

Methanocaldococcus jannaschii prolyl tRNA synthetase (MJ1238, MjProRS) was amplified from *M. jannaschii* genomic DNA using the forward primer 5'-TTAATTTGCTAGCAAATAAAAGG-3' and the reverse primer 5'-GTTCATAAATCGGATCCTAAAAATAAG-3', which inserted flanking *NheI/BamHI* restriction sites. These enzyme sites were designed to ensure an N-terminal 6xHis translational fusion when the PCR product was inserted into and expressed using the pES1 vector. The 50 μL PCR reaction contained PCR Buffer II (Applied Biosystems), 3 mM $MgCl_2$, 0.4 mM dNTPs, 1.0 μg *M. jannaschii* genomic DNA, 2 mM of each primer and 1.5 U AmpliTaq DNA Polymerase (Applied Biosystems). After amplification, the PCR product and the pES1 vector were digested with *NheI* and *BamHI* (New England Biolabs). The digested vector was treated with shrimp alkaline phosphatase (USB Corporation). The enzymes were denatured and the PCR product and the vector were incubated with T4 DNA ligase (New England Biolabs) and transformed into *E. coli* DH5α-λpir by a $MgCl_2/CaCl_2$ chemical transformation technique and incubated on LB agar plates containing ampicillin. Colonies were screened by digesting with *NheI* and *BamHI* and then by sequencing

with primers designed to sequence from the multiple cloning site in pES1 from both directions. The MjProRS expression construct was named pSM1.

The *M. acetivorans* prolyl tRNA synthetase (MA3886, MaProRS) expression plasmid was constructed in the laboratory of our collaborator Dr. Ya-Ming Hou at Thomas Jefferson University in Philadelphia, PA. Briefly, MaProRS was amplified from *M. acetivorans* genomic DNA and cloned into the pES1 expression vector with the 6xHis fusion as well as the tandem affinity chromatography (TAP) fusion. The MaProRS expression construct was named pMaProRS.

Cloning of *Methanosarcina thermophila* carbonic anhydrase (MtCam) into the pES1 vector was performed similarly to the cloning of MjProRS. Primers were designed to amplify the MtCam gene from an *E. coli* expression vector containing the MtCam gene (GenBank Accession no. U08885) without its leader sequence that was prepared in the laboratory of Dr. James G. Ferry at Pennsylvania State University (Alber and Ferry 1994). The forward primer 5'-GAAGGAGATCTAGATATGCAGGAAATAACCG-3' and reverse primer 5'-GCTTCAAGCTCATCGATTTATGAAGTTTC-3' inserted flanking *XbaI/ClaI* restriction sites. The PCR amplification was done similarly to the amplification described for MjProRS (PCR Buffer II (Applied Biosystems), 0.8 mM dNTPs, 1.5 mM MgCl2, 70 ng pCCAM, 50 pmol each primer, 0.5 μL AmpliTaq DNA polymerase (Applied Biosystems)). The PCR product was digested with *XbaI* and *ClaI* (insert, 677 bp). The pES1 plasmid (vector) was prepared by digestion with *ClaI* first and then partial digestion with *XbaI* for 5 minutes. The 11,023 bp band was excised from a 0.8% low-melt agarose gel run in TAE buffer for 1.5 h at 50 mA and

purified using the Wizard PCR Prep Kit (Promega). The insert and vector were incubated with T4 DNA ligase (New England Biolabs) and transformed into *E. coli* DH5α-λpir. Colonies were screened by digestion with *XbaI/ClaI* and then by sequencing. The MtCam expression construct was named pSM12.

The TCE reductive dehalogenase from *Dehalococcoides ethenogenes* was cloned by first amplifying the *tceAB* gene (Genbank accession no. AF228507) from *D. ethenogenes* genomic DNA, which was a gift from Dr. Stephen H. Zinder of Cornell University. The gene was amplified with approximately 100 bp of flanking DNA and cloned into a pCR2.1 vector for further cloning. The resultant construct was called pTCE. The genes were amplified from this construct using the forward primer 5'- GAGGTATGCTAGCAGTATGAGTGAA-3', which inserted a *NheI* restriction site 9 bp upstream of the *tceAB* gene and two reverse primers, one (5'-GCACCACCCATAAAGATCTATTC-3') inserted a *BglII* restriction site 16 bp downstream of the *tceA* gene and one (5'-GCTGAGATCTAATCGTGTGTACAGGG-3') inserted a restriction site 75 bp downstream of the *tceAB* gene. The 50 μL PCR reaction mixture contained PCR Buffer II (Applied Biosystems), 0.8 mM dNTPs, 1.5 mM $MgCl_2$, 10 ng pTCE, 50 pmol of each primer and 2.5 U AmpliTaq (Applied Biosystems). The PCR products (inserts) were digested first with *NheI* and then with *BglII* (New England Biolabs). pES1 was digested with *NheI/BamHI* and the 11,382 bp fragment (vector) was excised from an agarose gel and purified using the Wizard PCR Prep Kit (Promega). The vector and insert were ligated together as described above and transformed into

E. coli DH5α-λpir. Transformants were screened by restriction analysis and the *tceA* and *tceAB* expression constructs were named pEA129 and pEA130, respectively.

Transformation of expression vector and plasmids into *M. acetivorans* and induction of the *cdh* promoter

M. acetivorans was transformed with the pES1 vector and expression plasmids as described previously (Metcalf et al. 1997). Transformants were selected and maintained on medium containing puromycin (2 μg mL^{-1}). Transformants were further screened by extracting the plasmid from the recombinant strain grown on 0.1 M TMA using the Wizard Plus Minipreps DNA Purification System (Promega). The plasmids were then transformed into *E. coli* DH5α-λpir by techniques described above. Plasmids were extracted from the transformants and screened by restriction analysis and by sequencing. All transformants selected by growth on puromycin were positive for the presence of the expression plasmid. Medium used in the transformation contained 10 mM TMA and 50 mM sodium acetate. Recombinant strains were transferred 3 times to medium containing 0.1 M sodium acetate to ensure maximum expression of the recombinant protein from the *cdh* promoter.

Protein purification

M. acetivorans KSC19 cells (1L from bioreactor run as pH-auxostat) were pelleted at 10,000 x g for 30 min at 4ºC. The cell pellet was frozen at -80°C and cells were lysed by thawing, resuspension in sonication buffer (0.5 M NaCl, 20 mM Tris, pH 8.0) containing 10 mM imidazole, and sonication on ice. Cell debris was pelleted

at 10,000 x g for 30 min at 4°C, the supernatant was decanted and centrifuged at 100,000 x g for 1 h at 4°C. The supernatant was removed (S100 supernatant) and total protein was measured using the Bradford assay (BioRad). Metal affinity purification was performed at 4°C as follows: S100 supernatant (10 to 50 mg of protein) was incubated with 4 mL Ni-NTA Agarose 50% resin slurry (Qiagen) with shaking. Protein/resin slurries were loaded into columns with silanized glass wool and columns were washed 4 times with 10 mL of sonication buffer containing 20 mM imidazole. Protein was eluted with 10 mL sonication buffer containing 500 mM imidazole and 1 mL fractions were collected. Protein concentration of fractions was measured with the Bradford assay. Proteins were visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels after Coomassie Brilliant Blue staining.

Activity analysis of MjProRS

Activity analyses of MjProRS were performed in the laboratory of Dr. Ya-Ming Hou at Thomas Jefferson University in Philadelphia, PA. *M. acetivorans* cells (4 g) were resuspended in 20 mL buffer A (50 mM Tris pH 7.5, 100 mM NaCl, 2 mM β-mercaptoethanol) and 1 tablet of complete inhibitor cocktail (Roche, Inc.). The cells were sonicated until the cell lysate was fluid and had cleared (approximately 3 times at 50% power for 45 seconds). The suspension was centrifuged at 28,000 x g for 30 minutes and the resulting supernatant was heated at 70°C for 15 minutes, then centrifuged as above for 1 hour. The heated supernatant was incubated with Ni^{2+} affinity resin (His-Link resin, Promega, Inc.) for 30 minutes at room temperature with

shaking. The resin was washed with buffer A 3 times, and the bound MjProRS was eluted at 150 mM imidazole and was judged by SDS-PAGE to be 95% pure. To further assure purity, the MjProRS was purified by FPLC using a mono-Q column (Amersham Biosciences) with a gradient from buffer B (50 mM Tris pH 7.5, 50 mM NaCl, 2 mM β-mercaptoethanol) to 50 % buffer C (buffer B with 1 M NaCl) over 30 minutes. The protein eluted at approximately 20% buffer C. The activity of the purified MjProRS was confirmed by active site titration (Fersht et al. 1975). The standard protocol was followed (Hou et al. 1993) to test the ability of the enzyme to aminoacylate the transcript of Mj $tRNA^{Pro}$ (UGG) with proline, and to obtain the K_M and k_{cat} parameters for tRNAPro.

Activity analysis of DeTCE-RD

The dehalogenase activity of the recombinant strains expressing the *tceA* and *tceAB* genes was tested on whole cells pelleted and resuspended anaerobically in 25 mM bis-tris propane (BTP), pH 7.0 containing 150 mM NaCl, 2 mM cysteine, 2 mM ferrous ammonium sulfate and 1 mM Phenylmethylsulphonylfluoride (PMSF). Reactions were carried out in sealed and degassed glass cuvettes containing 2 mL 25 mM BTP, pH 7.0, containing 150 mM NaCl, 2 mM methyl viologen (MV) and 4 μg protein. The MV was reduced by the addition of titanium (III) citrate (Zehnder and Wuhrmann 1976). TCE (10μL 5% solution (v/v) in ethanol) was added to begin the reaction. Reductive dehalogenation was assayed spectrophotometrically using reduced methyl viologen (MV) as an electron donor (Equation 1; Neumann et al. 1995).

Equation 1:

$R\text{-}Cl + 2\ MV^{+} + H^{+} \rightarrow R\text{-}H + Cl^{-} + 2\ MV^{2+}$

The oxidation of MV was measured at 578 nm (ε_{578} = 9.7 $mM^{-1}cm^{-1}$), 660 nm (ε_{660} = 5.6 $mM^{-1}cm^{-1}$), and 700 nm(ε_{578} = 2.4 $mM^{-1}cm^{-1}$). The rate of reductive dechlorination was calculated as 1 mole chloride removed for each 2 moles of MV oxidized and the rates are reported as μmoles chloride released per minute per mg protein.

Results

Development of a recombinant system for expression of proteins in *M. acetivorans*

A system for expression of recombinant proteins in *M. acetivorans* was successfully developed. This system utilizes a plasmid-based shuttle vector that propagates in and possesses selectable markers for both the archaeon *M. acetivorans* and the bacterium *Escherichia coli* (Figure 2). The plasmid was designed with the promoter for the carbon monoxide dehydrogenase/acetyl coenzyme A synthase (*cdh*) operon upstream of a multiple cloning site (Terlesky et al. 1986; Sowers and Gunsalus 1993). The multiple cloning site is flanked by sequence for 6xHis translational fusions. When fused to the gene of interest, the 6xHis fusion enables the

Figure 2. Expression vector pES1. Figure 2A shows the details of the cloning sites and Figure 2B shows the entire plasmid. Vector contains genes for selection by ampicillin resistance in *E. coli* and puromycin resistance in *M. acetivorans*. The origins of replication are OriR6K and Ori pC2A for *E. coli* and *M. acetivorans*, respectively. Downstream of the carbon monoxide dehydrogenase/acetyl coenzyme A (*cdh*) promoter is a multiple cloning site flanked by sequence for 6xHis tags and the T7 tag for optional protein fusions to aid in protein purification. An N-terminal 6xHis fusion may be cleaved by digestion with thrombin using the thrombin digestion site. After the *cdh* promoter are an archaeal ribosomal binding site (aRBS) and a bacterial ribosomal binding site (bRBS).

Figure 2A.

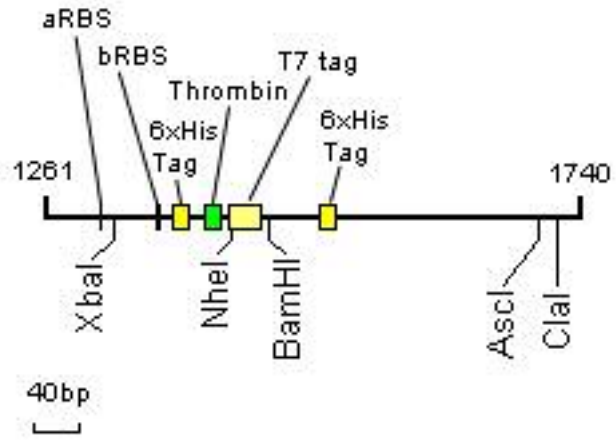
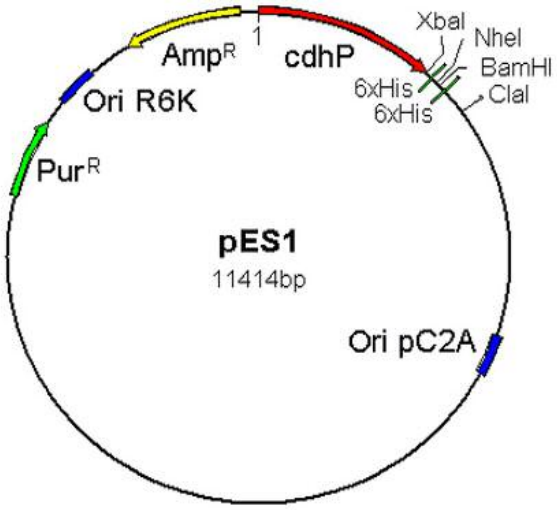


Figure 2B.



protein to be purified through a single-step nickel-affinity column purification. The *cdh* promoter has been shown to be highly regulated in response to growth substrate and it was hypothesized that expression with this promoter could be attenuated or induced by introducing different substrates. Expression from the promoter is 30-fold higher when the organism is grown on acetate vs. the methylotrophic substrates, methanol and trimethylamine (Apolinario et al. 2005). This expression vector can be efficiently transformed into *M. acetivorans* using the protocol developed by (Metcalf et al. 1997). After transforming the expression constructs into the organism and growing the organism on acetate as a growth substrate, expression of the gene of interest is initiated.

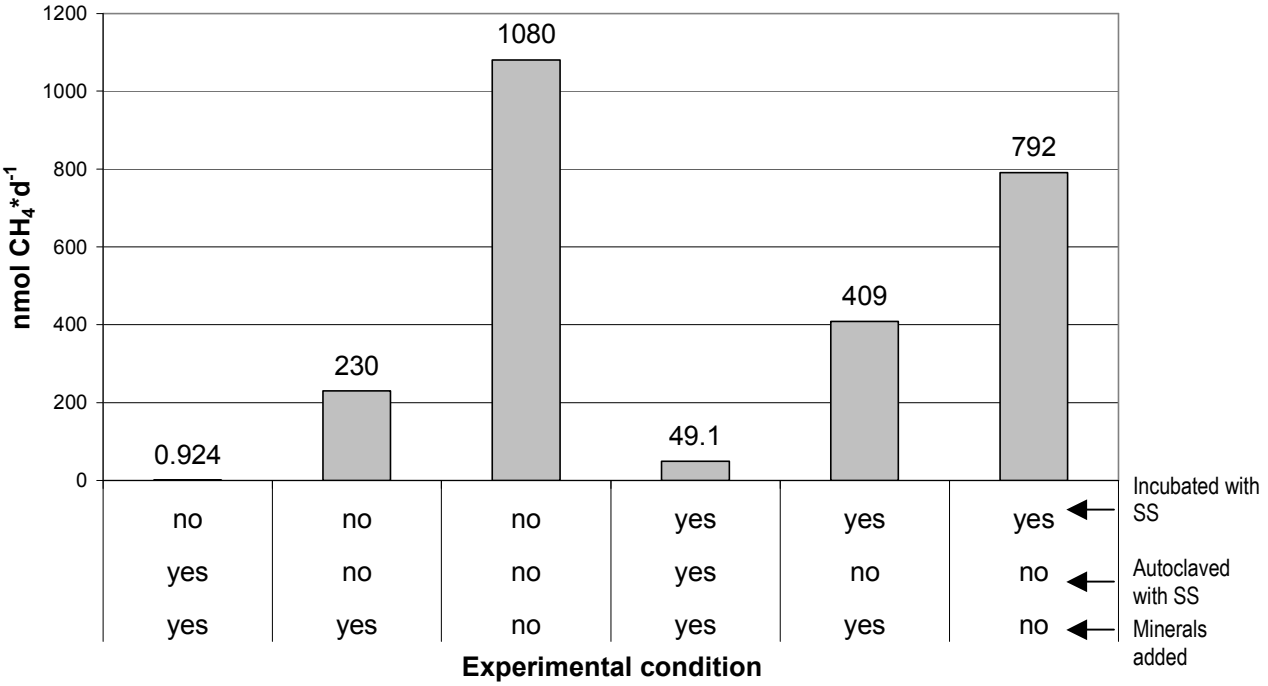
The expression plasmids remained stable in *M. acetivorans* through the process of transformation. After transformation, plasmids were extracted from the recombinant strains and transformed into *E. coli*. After restriction analysis and sequencing of the cloning site, it was determined that all expression plasmids were maintained in the transformed strains. The expression vector has the same plasmid backbone as the pKJ104 and pEA103 plasmids described in Apolinario et al. 2005. The plasmid is present at approximately 15 copies per cell when the organism is grown on either acetate or methanol. The plasmid was also found to be stable for at least 42 generations in the presence of puromycin and up to 22 generations in the absence of puromycin (Apolinario et al. 2005).

In order to increase the yield of recombinant protein, a system for cultivation of acetate-grown *M. acetivorans* to high densities in a stainless steel (SS) bioreactor

was developed. The organism had previously been grown to high densities in a glass bioreactor configured as a pH-auxostat (Sowers et al. 1984). In this system, the organism is grown in unbuffered medium kept at a constant pH by the addition of acetic acid. There is a stoichiometric release of hydroxyl anions as acetate is converted to methane. By lowering the pH with acetic acid, the pH and substrate concentration remain constant throughout growth, which enables the culture to be grown to high densities (Sowers et al. 1984).

Cultivation to high densities was not achieved in a SS bioreactor, because the growth of culture was either inhibited by a leachate not present in glass bioreactors or by sequestering of trace metals by adsorption to the SS vessel. The cell yield previously reported for *M. acetivorans* grown in a glass bioreactor configured as a pH-auxostat was 3.5 g wet weight per L (Sowers et al. 1984), while the cell yield was at least 5-fold less in the SS bioreactor (Table 3). To determine if the SS was the factor, experiments were done to test the inhibition of *M. acetivorans* cultures grown in medium autoclaved with a 0.635 (OD) x 0.533 (ID) cm length of SS tubing vs. cultures autoclaved prior to adding SS tubing. It was shown that the growth rate of the organism was 10-fold higher in medium that did not contain the SS during autoclaving (Figure 3). Using this observation, medium was prepared in the bioreactor without salts (NaCl, $MgSO_4$, KCl, $CaCl_2$ and NH_4Cl). A 4X concentrated salts solution was prepared and autoclaved separately from the rest of the medium components in a polypropylene carboy and added to the bioreactor after it was sterilized and cooled. Using this method the density of the culture increased by approximately 4-fold (Table 3).

Figure 3. Effect of stainless steel (SS) heated by autoclaving and then incubated with marine medium, SS incubated with marine medium autoclaved without SS, and 10-fold increased mineral supplement on the growth (as measured by methanogenesis rate) of *M. acetivorans*. Increased mineral supplement results in a final concentration of 25 μM iron, 30 μM cobalt, 10 μM nickel, 8.4 μM molybdenium and 5.8 μM selenium in the medium.



To determine whether trace nutrients become limiting due to adsorption to the SS vessel the effect of supplementing minerals on the growth of *M. acetivorans* in the presence of stainless steel was also tested. The minerals that were supplemented were iron, molybdenum, cobalt, selenium and nickel. These were chosen because each has been shown to enhance the growth of methanogens (Schonheit et al. 1979; Diekert et al. 1981; Sowers and Ferry 1985; May and Patel 1988; Mukhopadhyay et al. 1999). There was not a large difference in the growth rate of the organism when 10-fold greater minerals were added to small batch cultures (Figure 3), however it was

hypothesized that minerals may be limiting at a later stage in the growth of the organism. Fermentor runs were done with 5- and 10-fold higher mineral concentration than normally used in the fermentation medium and the density of the culture increased by approximately 2-fold (Table 3).

Table 3. Effect of sterilizing salt solution separately in a polypropylene carboy and increasing mineral concentration of medium when growing *M. acetivorans* in a stainless steel bioreactor configured as a pH-auxostat. Mineral concentration in normal medium is 2.5 μM iron, 3.0 μM cobalt, 1.0 μM nickel, 0.8 μM molybdenium and 0.6 μM selenium.

Bioreactor type	Salt solution heated in bioreactor	Mineral supplement added (fold normal medium)	Maximum cell yield (OD_{550})	Final yield ($g\ L^{-1}$)	Reference
Glass	Yes	1	Not reported	3.5	(Sowers et al. 1984)
Stainless Steel	Yes	1	0.3	0.74	This study
Stainless Steel	No	1	1.3	1.5	This study
Stainless Steel	No	5	2.3	2.0	This study
Stainless Steel	No	10	2.4	2.2	This study

By combining post-addition of salts with trace metal supplement the cell density of the culture increased by 10-fold. This system, combined with the expression system for recombinant proteins in *M. acetivorans* provided the means to produce recombinant protein in preparative yields.

Optimization of the expression system

While the overexpression system developed for *M. acetivorans* enables expression of recombinant protein when the organism is grown with acetate as a growth substrate, the yield of recombinant protein is lower than yields with comparable systems, e.g. bacterial systems. The yield of MjProRS expressed in the *M. acetivorans* system was approximately 1 mg L^{-1}. This is low for a prokaryotic expression system but similar to yields typically obtained with eukaryotic expression systems. In order to enhance expression from the *cdh* promoter, experiments were done to test the whether expression could be induced by the addition of acetate to a culture growing on a methylotrophic substrate. This could enhance expression of the recombinant protein due to the higher growth rate of the organism on methylotrophic substrates, as well as allowing for the induction of expression of proteins that may be toxic to the cell.

To test the inducibility of the *cdh* promoter, experiments were done with the *M. acetivorans* strain expressing MjProRS (*M. acetivorans* KSC19). *M. acetivorans* KSC19 was grown as a batch culture in a bioreactor with 0.1 M trimethylamine-HCl (TMA) as a growth substrate and a small amount (0.1 mM) of acetate to stimulate *cdh* promoter activity. When the culture reached mid-exponential phase, acetate was added to a final concentration of 100 mM. The cells were lysed and protein was purified from samples taken from the bioreactor prior to the addition of acetate and from 1 to 30 hours after the addition of acetate. The amount of MjProRS detected

after nickel-affinity purification was below detection in all samples (data not shown), indicating that no significant expression from the *cdh* promoter occurred.

In order to test the inducibility of the *cdh* promoter under a wider variety of conditions, a *M. acetivorans* strain expressing the *cdh* promoter was fused to the *lacZ* gene, which codes for β-galactosidase (Apolinario et al. 2005). Cultures were grown on 10 mM TMA plus 0 to 100 mM acetate (Table 2). Additional acetate was added to a concentration of 100 mM to some of the cultures in mid-exponential phase in an attempt to induce expression from the *cdh* promoter. In the cultures where acetate was added during mid-exponential phase in order to induce expression from the *cdh* promoter, β-galactosidase activity increased approximately 2-fold 1 h after induction. After this increase, the β-galactosidase activity did not increase after an additional 134 h incubation. In the cultures where no additional acetate was added during mid-exponential phase, β-galactosidase activity increased by 3.4- to 5.4-fold. No further increase in β-galactosidase activity resulted from additional incubation of up to 136 h. This shows that the expression from the *cdh* promoter did increase during the growth of *M. acetivorans* on mixed substrates. However, the initial β-galactosidase activity was low, so the fold increase did not reflect a high level of expression from the *cdh* promoter.

A similar experiment was also conducted with 10 mL cultures grown on 10 mM methanol and 10 to 150 mM sodium acetate (Table 2). Additional acetate was added to a concentration of 100 mM to some of the cultures in mid-exponential phase. The fold increase in the cultures where acetate was added in during mid-exponential growth and where no additional acetate was added was from 1.2- to 1.6-

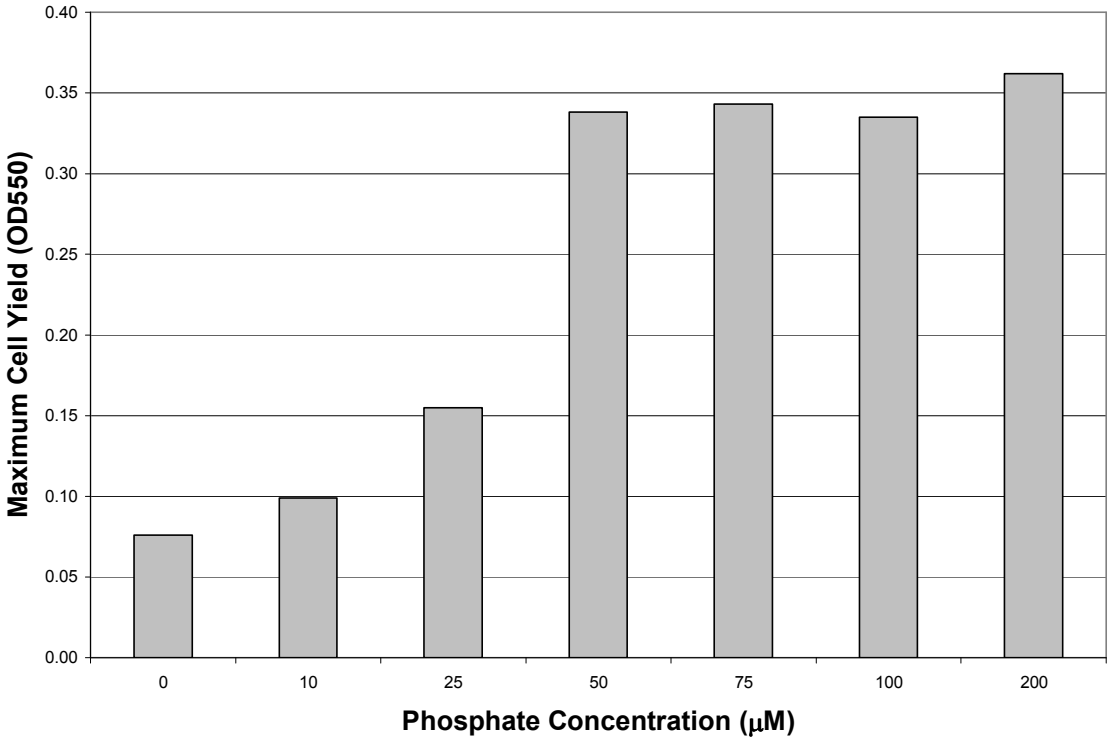
fold. No additional increase in β-galactosidase activity resulted from additional incubation of the cultures for 256 h. The increase in β-galactosidase activity when the cultures where grown with methanol-acetate mixed substrate was less than the increase in β-galactosidase activity when the cultures where gown with TMA-acetate mixed substrate. Again, the initial β-galactosidase activity was quite low and the fold increase in β-galactosidase activity did not reflect high expression from the *cdh* promoter.

To further test the effects of changing substrates over time on the expression from the *cdh* promoter when grown with varying amounts of mixed substrates, *M. acetivorans* was grown in a chemostat. *M. acetivorans* KSC8, the *cdh::lacZ* fusion strain, was used so that the expression levels from the *cdh* promoter could be monitored by β-galactosidase activity (Apolinario et al. 2005). The purpose of this study was to test *cdh* expression when the organism was grown with different levels of acetate. Additionally, *cdh* expression upon gradual addition of a methylotrophic substrate was to be tested.

In order to maintain steady-state conditions for the culture, nutrient limitation is required to control growth rate. It is common to use carbon, nitrogen or phosphate limitation in chemostat studies. However, both carbon and nitrogen could not be used in this study, since carbon source was a variable and *Methanosarcina* spp. fix gaseous nitrogen (Belay et al. 1984; Murray and Zinder 1984; Postgate 1984), which is continuously bubbled into culture in the anaerobic bioreactor. Because of this, phosphate limitation was used. In batch studies with *M. acetivorans* cultures containing low concentrations of phosphate, 25 μM phosphate was shown to be

limiting (Figure 4). When the phosphate was diluted to this concentration in chemostat culture, however, there were no signs of nutrient limitation. After further diluting the culture, washout began to occur, indicating nutrient limitation. When phosphate was added back to the culture, growth was not stimulated, even when phosphate was at concentrations greater than what was found to be limiting in batch cultures. Due to the difficulty of growing *M. acetivorans* in a phosphate-limited chemostat culture, the kinetic analysis of the *cdh* promoter could not be completed. The cultures remained dormant for several months and did not recover.

Figure 4. Effect of phosphate concentration on maximum cell yield in a phosphate-limited batch cultures of *M. acetivorans* grown on acetate.



Due to the relatively low level of recombinant protein expressed and the inability to induce rapid protein expression from the *cdh* promoter, other genes which may have promoters that can be induced by the addition of a different growth substrate were tested. The promoters chosen for testing were 1) promoters that may be induced upon addition of acetate to methanol-grown cultures and 2) promoters that may be induced upon addition of TMA to acetate-grown cultures. The former group was identified by microarray analysis of acetate- vs. methanol-grown *M. acetivorans* to be highly up-regulated in response to growth on acetate (personal communication with Robert P. Gunsalus). The genes selected were MA2252 and MA4634, which code for hypothetical proteins. The genes in the latter group were genes for TMA-methyltransferases, MA0528 and MA0932, which were expected to be significantly up-regulated due to the preferential growth of *M. acetivorans* on TMA vs. acetate.

In order to test whether these promoters could be induced by addition of a second growth substrate, *M. acetivorans* was grown on one substrate to mid-exponential phase, and then a high level of another growth substrate was added (Table 4). RNA was extracted from the cultures prior to the addition of the second growth substrate, and then one and twenty-four hours after the addition of the second substrate. RNA was also extracted from a control culture was grown on only the second substrate. Reverse-transcriptase PCR (RT-PCR) was conducted to amplify the intergenic region of the genes listed above (Table 4).

Table 4. Genes tested for ability to be induced when the organism, grown on a single substrate, is exposed to another during growth. Experiments were conducted by growing *M. acetivorans* on one substrate and adding the second substrate during mid-exponential phase. RNA was extracted prior to addition of the second substrate, one hour after and twenty-four hours after the addition of the second substrate. RT-PCR was performed to detect the presence of the transcript of the genes tested on all RNA extracts. RT-PCR products that were detectable on ethidium bromide stained agarose gels are indicated by (+). RT-PCR products that were detectable when the RNA was diluted to 1.0 ng per reaction are indicated by (++). RT-PCR products that were not detectable are indicated by (-).

Gene tested	Initial growth substrate (50 mM)	Growth substrate added (150 mM)	Level of expression with initial substrate	1 h after addition of second substrate	24 h after addition of second substrate	Level of expression with only second substrate
Conserved hypothetical protein (MA2252)	Methanol	Acetate	+	+	+	+
Conserved hypothetical protein (MA4634)	Methanol	Acetate	+	+	+	+
TMA methyl-transferase (MA0528)	Acetate	TMA	+	+	+	++
TMA methyl-transferase (MA 0932)	Acetate	TMA	-	-	-	+

For MA2252 and MA4634, the genes were expressed in all experimental conditions: before the addition of acetate, 1 h and 24 h after the addition of acetate, and on cells grown on acetate alone. The amount of expression, as determined with MPN RT-PCR were not higher on the acetate-grown cells than on the methanol-grown cells, indicating that these genes were not expressed more highly on methanol vs. acetate, which contrasted with the microarray analysis that identified these genes as up-regulated.

Different results were obtained for MA0528 and MA0932. MA0528 was expressed in all experimental conditions; however the amplicon resulting from RT-PCR on the TMA-grown culture was more dominant than that resulting from RT-PCR of the acetate-grown culture before and after the addition of TMA. MA0932 was not found to be expressed in the acetate grown cells either before or after the addition of TMA. It was found to be expressed in the TMA-grown cells. This suggests that the expression of this gene may not be rapidly up-regulated in response to TMA. Based on these results, none of the genes tested appeared to have promoters that were rapidly induced, and were therefore deemed to be unsuitable for inducible expression in a recombinant protein expression system.

Expression of recombinant proteins

Several recombinant proteins have been expressed in *M. acetivorans* using the expression system described above. The first protein expressed was the *M. jannaschii* prolyl tRNA synthetase (MjProRS). This protein comes from a hyperthermophilic archaeon and had been actively expressed in *E. coli* (Lipman et al. 2000). The expression vector for MjProRS was readily constructed by amplifying the gene from *M. jannaschii* genomic DNA with primers designed to clone the gene into pES1 to incorporate a 6xHis translational fusion at the 5' end of the gene. MjProRS was expressed in *M. acetivorans* and purified by nickel affinity chromatography (Figure 5). The activity of the *M. acetivorans* recombinant protein was comparable to the activity of the *E. coli* recombinant protein, showing that the *M. acetivorans* expression system was equivalent to the *E. coli* system (Table 5). The amount of

recombinant MjProRS purified from *M. acetivorans* was approximately 2% of the total cellular protein and approximately 1 mg per L culture, based on direct protein measurements.

Figure 5. SDS-PAGE showing purification of MjProRS from *M. acetivorans* by nickel affinity chromatography.

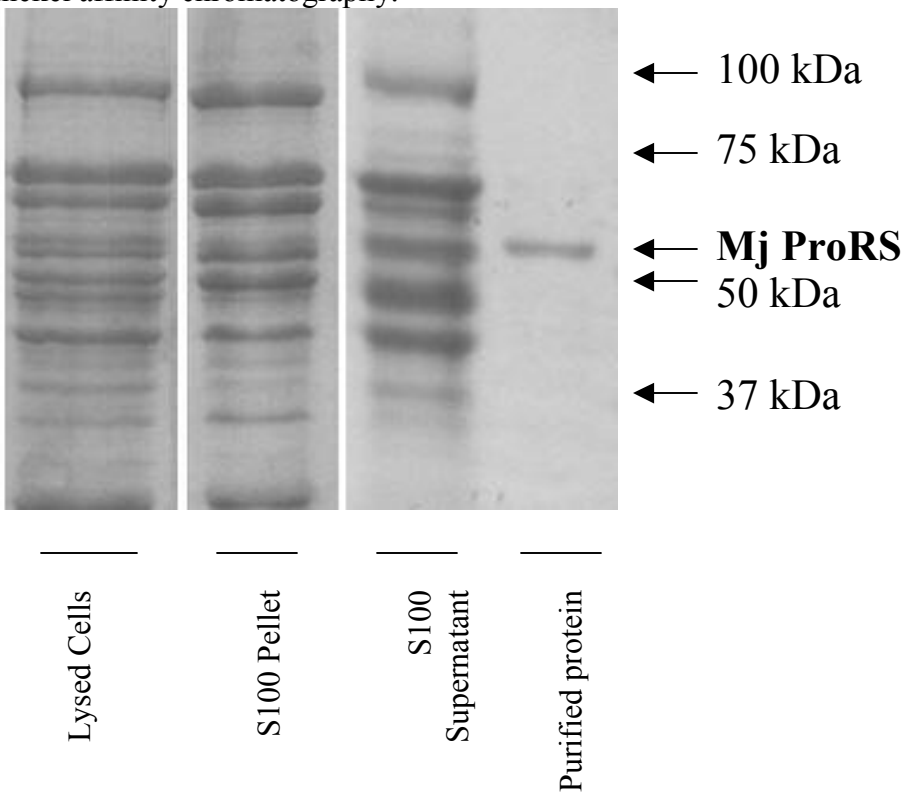


Table 5. Kinetic parameters of the recombinant MjProRS expressed in *M. acetivorans* compared to the recombinant MjProRS expressed in *E. coli*. The activity assays were conducted in the laboratory of our collaborator, Ya-Ming Hou at Thomas Jefferson University in Philadelphia, PA.

Recombinant Protein	K_M (μM)	K_{cat} (sec^{-1})	K_{cat}/K_M (μM^{-1} sec^{-1})
ProRS from *M. acetivorans*	0.52 ± 0.15	0.32 ± 0.12	0.60 ± 0.11
ProRS from *E. coli*	0.30 ± 0.09	0.27 ± 0.00	0.99 ± 0.30

The prolyl tRNA synthetase from *M. acetivorans* (MaProRS) was also expressed using the *M. acetivorans* protein expression system. This enzyme was cloned into the pES1 expression vector with a tandem affinity purification (TAP) translational fusion (Rigaut 1999). By expressing a protein with the TAP fusion, native proteins that complex with the recombinant protein may be purified and characterized. Protein complexes are purified by affinity of the TAP fusion protein to an IgG-matrix. The protein complex is then removed from the matrix by TEV protease cleavage. In an additional purification step, the eluted protein is incubated with calmodulin-coated beads in the presence of calcium and eluted the addition of EGTA. The protein complexes can then be analyzed by SDS-PAGE and identified by several available methods (Rigaut 1999). By expressing MaProRS with the TAP fusion, the recombinant protein expression system can be used to the study the proteins involved in the prolyl tRNA synthetase complex that forms in vivo in *M. acetivorans*. This research is ongoing and the protein expression experiments are currently being conducted at the laboratory of a collaborator, Dr. Ya-Ming Hou, at Thomas Jefferson University in Philadelphia, PA.

The *M. thermophila* carbonic anhydrase (MtCam) is being expressed with no protein fusion using the *M. acetivorans* expression system. This enzyme has been actively expressed in *E. coli* and has been shown to be a zinc-containing metalloenzyme (Kisker et al. 1996). However, Tripp, et al. (2004) have shown that enzyme activity is increased when enzyme is reconstituted anaerobically with iron (Tripp et al. 2004). The enzyme was expressed in *M. acetivorans* to determine which metal is associated with the enzyme when the protein is expressed in a methanogen.

The metal content analysis of this enzyme is ongoing in the laboratory of a collaborator, Dr. James Ferry, at Pennsylvania State University in University Park, PA.

The *D. ethenogenes* TCE reductive dehalogenase (DeTCE-RD) was also expressed using the *M. acetivorans* expression system. There have been no reported cases of a recombinant dehalogenase that has been actively expressed. The gene for the enzyme, *tceA*, is upstream of a membrane anchor protein, *tceB* (Magnuson et al. 2000). Expression plasmids for the *tceA* gene and the *tceA* and *tceB* genes were made by cloning into the pES1 expression vector with an N-terminal 6xHis fusion on the *tceA* gene in both plasmids. For each of these strains, enzyme activity analyses were conducted to test for dehalogenase activity on cell suspensions of the recombinant strains. Both strains expressing the DeTCE-RD were shown to dechlorinate TCE at a rate significantly higher than strains not expressing DeTCE-RD, and the strain expressing *tceAB* dechlorinates at a rate slightly higher than the strain expressing *tceA* alone (Table 6). This research is ongoing and it is not yet known whether purification of the enzyme using the 6xHis fusion will yield active dehalogenase.

Table 6. Dehalogenase activity of whole cell suspensions of *M. acetivorans* strains expressing no recombinant gene, and the *D. ethenogenes* genes, *tceA* and *tceAB*.

***M. acetivorans* strain**	**Recombinant gene expressed**	**Specific activity (μmole min^{-1} mg $protein^{-1}$)**
KSC20	none	0.19 ± 0.03
KSC41	*tceA*	0.56 ± 0.07
KSC42	*tceAB*	0.70 ± 0.06

Discussion and Conclusions

The protein expression system in *M. acetivorans* is the first system that I am aware of for expression of heterologous recombinant protein for subsequent purification in an archaeon. Other systems have been developed to express homologous proteins in the haloarchaea *Haloferax volcanii* and *Halobacterium salinarum* (Jolley et al. 1996; Long and Salin 2000), but these systems have high intracellular solute concentrations, which is inhibitory to most proteins from non-halophilic organisms. The system uses an *E. coli-M. acetivorans* shuttle plasmid which has been shown to be stable after transformation into the methanogen, present at a stable copy number and maintained after dozens of transfers in the presence of puromycin (Apolinario et al. 2005). The utility of the system is improved by the ability to express proteins with a 6xHis fusion to aid in protein purification. This system provides a new tool for the investigation of proteins that are difficult to express in an active form in other systems. Because *M. acetivorans* possesses genes for all four of the known chaperoning systems, the proportion of properly folded proteins expressed using this system may be higher than with other systems (Macario et al. 2004). *M. acetivorans* possesses the ability to introduce archaeal posttranslational modifications in proteins which may not be processed properly if the protein is expressed in eubacterial or eukaryal systems (Eichler and Adams 2005). *M. acetivorans* also possesses the ability to synthesize complex metalloenzymes and cofactors used by anaerobic bacteria, such as corroniod cofactors (Stupperich et al. 1990; Weiss and Thauer 1993). *M. acetivorans* also expresses pyrrolysine-containing proteins, therefore it can accurately express proteins containing this nonstandard

amino acid (Soares et al. 2005). These combined benefits of the system may allow for the recombinant expression of a wide array of enzymes from different organisms in *M. acetivorans*.

While the expression level of the recombinant enzyme is low (approximately 1 mg L^{-1} for MjProRS) compared to *E. coli* expression systems, which can express recombinant enzyme as up to 1000 mg L^{-1}, the recombinant enzyme expressed in this system is still present at levels which enable its purification and characterization. Higher yields of recombinant protein do not directly correlate with correct folding and posttranslational modifications and often systems which result in lower yields, e.g. eukaryotic systems, enable the expression of recombinant enzymes with higher activities.

The ability to mass culture acetate-utilizing methanogens in stainless steel (SS) bioreactors using the pH-auxostat has been significantly improved. Previously, a technique for cultivation of *M. acetivorans* to high densities in a bioreactor configured as a pH-auxostat was developed (Sowers et al. 1984). This technique was developed in a glass bioreactor and the high cell densities could not be reproduced in a SS bioreactor. The inhibition of growth by marine medium autoclaved in SS vessels is shown (Figure 2). One difference between the use of glass and SS bioreactors is that grade SA240-316L SS alloy is composed of iron with 17% Cr, 12% Ni, 2.5% Mo, 2% Mn, and traces of Si, C, P and S. It has been shown that under high temperature and high salt conditions, which exist when the SS bioreactor is sterilized containing marine medium, metals may leach from SS (Muraleedharan 2002). It was hypothesized that leaching from SS bioreactor was somehow inhibiting

M. acetivorans growth. By autoclaving the marine salts separately in a polycarbonate container and adding after sterilization of the bioreactor, the yield of acetate-grown *M. acetivorans* was improved substantially (Table 3). As the culture grows to high densities and substrate does not limit growth in the pH-auxostat, other nutrients essential for growth may become limiting. It was also shown that adding supplemental minerals increased the cell yield in the pH-auxostat (Table 3). This suggests that mineral availability may become limiting at a later stage in the pH-auxostat. Further research could be done to identify the specific limiting nutrients and growth yield could be enhanced further by supplementing specific minerals. This improved technique for mass cultivation may expand to other acetate-utilizing and formate-utilizing methanogens, which can also be mass-cultured using the pH-auxostat technique (Sowers and Schreier 1995).

While attempts to induce the promoter used for this system were unsuccessful, some interesting observations regarding the nature of the *cdh* promoter and the growth of *M. acetivorans* can be made. Expression from the *cdh* promoter does indeed increase when acetate is added to a culture grown on TMA or methanol. This shows that expression from the *cdh* promoter is not completely inhibited by the presence, or recent presence, of other growth substrates. The cultures were grown with 10-fold lower amounts of the methylotrophic substrates than is usually used for optimal growth. Therefore, methylotrophic substrates may have been depleted, allowing for growth on acetate and reducing repression of the *cdh* promoter. Interestingly, the increase in the expression of the *cdh* promoter was higher in the cultures where no additional acetate was added to the culture in mid-exponential

phase. This may be due to low levels of oxygen added along with the acetate or due to some sort of osmotic effect on the growth of the culture resulting from excess salts.

The increase in expression from the *cdh* promoter was greater when acetate was added to TMA-grown cells than when added to methanol-grown cells. This could be due to increased repression of the *cdh* promoter by methanol metabolism. The growth rate of *M. acetivorans* is higher on methanol than on TMA, yet the response to acetate in fold increase in β-galactosidase activity was more rapid when the cells were initially grown with TMA. These observations suggest that there are differences in the mechanisms by which the *cdh* promoter is repressed on TMA and methanol metabolism.

While increased expression from the *cdh* promoter was found when the cells were grown on mixed substrates, the increased expression is not sufficient to result in substantial increase in expression from *cdh* promoter because, compared to *cdh* expression on acetate, it is very low. Expression from the *cdh* promoter is 30-fold higher in acetate-grown cells than TMA- or methanol-grown cells (Apolinario et al. 2005). Initial expression levels in the experiments described above were comparable with expression levels of the *cdh* promoter grown on only methanol or TMA (Apolinario et al. 2005). Therefore, even the largest fold increase observed would account for six-fold lower expression, which would not be sufficient for use in an inducible protein expression system.

Although expression using the *cdh* promoter was not rapidly induced, the expression from the promoter is inducible depending on the growth substrate of the organism. Using the *cdh::lacZ* reporter strain of *M. acetivorans*, it has been shown

that the highest levels of expression from the *cdh* promoter are not achieved until 3 to 4 weeks after the culture is transferred to medium containing acetate as a sole carbon source (data not shown). This demonstrates that expression from the *cdh* promoter is inhibited for an extended period after growth on acetate is initiated. Using the expression system described, expression from the *cdh* promoter does result in recombinant protein yields that are suitable for purification and characterization of the protein of interest. Therefore, rapid induction is not required for the recovery of active recombinant proteins in preparative yields.

In the analysis of other genes that may have promoters that could be integrated into an overexpression system, no useful promoters were identified. These results, however, yielded some interesting discussion points. For the genes that were identified as up-regulated on acetate vs. methanol by microarray analysis, the RT-PCR results failed to confirm any significant up-regulation. This demonstrates the need for confirmation of microarray results by RT-PCR or another system, for example promoter fusions to reporter genes or proteomic analysis. RT-PCR is semi-quantitative, i.e. the amount of amplicons produced is relative to the abundance of the mRNA target, especially when fewer PCR amplification cycles are used, or when low amounts of RNA are added to the reaction mixture. In order to make the RT-PCR more quantitative, serial dilutions of RNA were used to investigate the level at which the RNA target was diluted below the concentration required for amplification. The result of this was that the target mRNA was not more abundant in the acetate-grown cells than in the TMA-grown cells. One caveat of this approach was that total RNA was used in the RT-PCR reactions as opposed to mRNA. However, it is difficult to

purify mRNA from total RNA and to determine the proportion of the target mRNA to total RNA or mRNA in the sample, so the abundance of the mRNA target measured using this technique is only an estimate.

The RT-PCR results obtained in the experiments which measured the abundance of methyltransferase transcripts in cultures grown on acetate with the addition of TMA are also interesting. For the MA0528 transcript, the gene was present in the acetate grown cells both before and after the addition of TMA. This implies that this methyltransferase may be constitutively expressed. The MA0932 transcript, however, was not detected in the acetate-grown cells either before or after the addition of TMA, but it was detected in the cells that had been grown on TMA with no addition of acetate. This implies that this methyltransferase may not be expressed in the presence of acetate, or may be induced by addition of TMA after a period longer than 24 h.

No conditions were found to rapidly induce expression from any of the promoters investigated. Other conditions could be tested in order to test whether the promoters could be rapidly induced. Also, other promoters could be used for recombinant protein expression. Rapid induction is not required if the promoter is highly regulated, as demonstrated with the *cdh* promoter. Another promoter which is highly regulated in the methanogens is the *nifH* promoter. The *nifH* gene encodes nitrogenase reductase, which is a requisite for nitrogen fixation. In Cohen-Kupiec et al. (1997), the *Methanococcus maripaludis nifH* promoter was fused to the *lacZ* gene and transformed into *M. maripaludis*. The expression from the *nifH* promoter was found to be approximately 45-fold higher when the organism was grown on nitrogen

gas vs. ammonia as a nitrogen source (Cohen-Kupiec et al. 1997). The use of the *nifH* promoter many enhance expression of recombinant proteins in *M. acetivorans* and allow for expression when the organism is grown on substrates other than acetate. Other promoters that could potentially be used to direct recombinant gene expression were demonstrated in Zhang et al. (2000). The mariner transposon was put under the control of three different constitutively expressed promoters: the *mcrB*, *serC* and *orf1* promoters. The expression levels of these promoters had previously been reported to be high, medium and low, respectively. In the study, the expression of the transposon gene in *M. acetivorans* was required to achieve recombinant colonies. The transposon which was regulated under the *mcrB* promoter resulted 4-fold more colonies than those which were regulated under the other promoters. By using promoters which are constitutively expressed at known levels, the authors were able to control the yield of recombinant colonies (Zhang et al. 2000). These promoters may therefore be used to control the yield of recombinant protein in *M. acetivorans* regardless of the growth conditions of the organism.

The chemostat experiments provide some interesting evidence on the nature of phosphate limitation in *M. acetivorans*. It has been shown in other organisms that response to phosphate limitation and phosphate addition in cultures may not occur rapidly due to the storage of phosphate intercellularly as polymers. Phosphate limitation in cultures when other nutrients are in excess has also been shown to cause the enlargement of cells because their biomass can increase, but they cannot divide due to the insufficient phosphorous for DNA replication. Some increase in the size of the *M. acetivorans* cells was indeed observed in the phosphate-limited chemostat

cultures. The bioavailability of phosphorous may have also been affected by the increase in minerals in the medium and by the interactions between the phosphorous and the SS bioreactor. Differences between batch phosphate limitation experiments and phosphate limitation experiments in continuous culture may have also been confounded by intrinsic differences in the definitions of nutrient limitation. In the batch experiments, phosphate limitation was defined as the amount of phosphorous that caused a decrease in cell yield, whereas phosphate limitation in continuous culture was defined as the concentration of phosphate in the medium that resulted in a decrease in growth rate.

Because of the inability to achieve regulation of the growth rate of *M. acetivorans* in continuous cultures limited by phosphate concentration, the use of a different limiting nutrient should be investigated. Nitrogen limitation is another nutrient that is often used to control growth rate in chemostat cultures. This nutrient was not used in the study described above because *M. acetivorans* fixes gaseous nitrogen, which is continuously bubbled into the culture to maintain positive pressure and anaerobic conditions in the bioreactor. Also, carbon source was a variable in the chemostat experiment. Methylamines were to be used as the methylotrophic carbon source; however these compounds are also a source of nitrogen. In order to use nitrogen limitation to control growth rate, *M. acetivorans* would need to be grown in a bioreactor in the absence of nitrogen gas or methylamines. Argon or helium could substitute for nitrogen gas as the gas phase in the bioreactor. Also, methanol could be used in the place of methylamines for the methylotrophic substrate. Special care would need to be used to ensure that the concentrations of methanol in the culture

remained constant, since this carbon source is particularly volatile. The use of nitrogen as an alternate limiting nutrient may enable chemostat cultivation *of M. acetivorans* to be achieved and kinetic analysis of the *cdh* promoter may be preformed.

The utility of the *M. acetivorans* protein expression system has been shown by the diversity of proteins that have been expressed. The heterologous proteins that have been expressed include one from a thermophilic methanogen, *M. jannaschii*, two from mesophilic methanogens, one from *M. thermophila* and one from *M. acetivorans* itself, and one from a gram-positive anaerobic bacterium, *D. ethenogenes*.

The recombinant protein yield for MjProRS obtained using this system (approximately 2%) is lower than in most *E. coli* expression systems, where expressed proteins often represent 20% or more of the cellular protein (Balbas and Lorence 2004). The yield has not been determined for the other proteins expressed using the *M. acetivorans* system. This yield, however, is comparable to some eukaryotic expression systems, which serve as an alternative for expression of difficult-to-express archaeal proteins that require post-translational modifications (Balbas and Lorence 2004). Therefore, the expression of recombinant proteins in *M. acetivorans* is a useful tool for proteins which are not expressed in an active form using more simple systems.

These proteins have been expressed with and without 6xHis fusions. The 6xHis fusion enables one-step purification of the recombinant protein. This allows for proteins to be expressed and purified easily with little or no information regarding

their function. This contributes and excellent tool to use to aid in the analysis of genomic information in *Methanosarcina* spp. and other similar organisms. In addition to 6xHis fusions for use in the purification of recombinant proteins, the MaProRS protein shows the ability to use the TAP protein fusion to purify protein complexes to study in vivo protein complex formation in *M. acetivorans*. If successful, TAP proteins fusions could be an excellent tool for the study of protein complexes in *M. acetivorans* and other Archaea. The *M. acetivorans* system is ideal for the TAP fusion system because the system requires relatively low levels of expression of the recombinant protein so that the yield of protein complexes that can be purified is increased (Rigaut 1999). The use of the TAP system for MaProRS expression is ideal because the ProRS enzyme has been shown to be associated with other peptides in the Archaea (Lipman et al. 2003). This demonstrates yet another unique molecular mechanism in the Archaea. While there are no known peptides to be associated with eubacterial aminoacyl tRNA synthetases, many are associated with ProRS in the higher eukaryotes. The peptide that has been shown to be associated with the achaeal ProRS is different than that found in the higher eukaryotes (Lipman et al. 2003). Further investigation into the activity of this peptide and its interaction with the aminoacyl tRNA synthetase will contribute to the investigation of achaeal aminoacyl tRNA synthetase complexes.

The MtCam expression demonstrates the ability to use this system to aid in characterization of archaeal enzymes with metal-containing active sites, which may not be accurately expressed in eubacterial systems. *Methanosarcina* spp. are obligately anaerobic and are adapted to much lower redox potentials than *E. coli*.

This may result in differences in metalloenzyme biosynthesis even when *E. coli* is grown anaerobically. By expressing this *M. thermophila* enzyme in *M. acetivorans*, which is closely related and more efficiently transformed genetically than *M. thermophila*, it can be determined with more certainty whether zinc or iron is the metal in the active site of the native enzyme. It was shown that the activity of the enzyme greatly increased when the metal in the active site was replaced with iron (Tripp et al. 2004). The activity may, however, have been reduced by the process of altering the enzyme active site. By purifying the enzyme with its metal-containing active site intact, the activity of the native enzyme can more accurately be estimated.

The DeTCE-RD expression demonstrates the expression of a group of eubacterial enzymes that has not been expressed in an active form using the available eubacterial systems (Neumann et al. 1998). While *M. acetivorans* is evolutionarily divergent from *D. ethenogenes*, it still has much of the same physiology. Both *D. ethenogenes* and *M. acetivorans* are able to synthesize corronoid cofactors, an ability that is not present in *E. coli*. *D. ethenogenes* and *M. acetivorans* also are both obligate anaerobes which exist in sediments. The biology of *D. ethenogenes* is a very interesting subject of study due to its ability to dechlorinate PCE completely to the non-toxic compound, ethene (Maymo-Gatell et al. 1997). *Methanosarcina* spp. also have been shown to decholorinate chlorinated ethenes co-metabolically due to their possession of complex metal cofactors, however it occurs at a slower rate (Fathepure et al. 1987). The strains expressing the DeTCE-RD were shown to dechorinate TCE at a rate significantly higher than the strain not expressing the dehalogenase (Table 6). The dechlorination activity demonstrated by the strain expressing both the *tceA* and

tceB genes is slightly higher than the activity of the strain exprssing only the *tceA* gene (Table 6). This may be indicative of the ability of *M. acetivorans* to recognize the twin arginine translocation signal (Hutcheon and Bolhuis 2003), effectively localizing the enzyme to the membrane where it may be anchored by the *tceB* gene product. The dehalogenase may not have been anchored and may have been localized elsewhere in the *tceA* expression strain and dehalogenase activity may have been reduced due to lower solubility of the enzyme.

A system for recombinant protein expression in the methanogenic archaeon *M. acetivorans* has been developed. This tool can be used to produce active recombinant proteins from the Archaea and other anaerobes. It is not, however, without its disadvantages. Recombinant protein expression is much lower than expression systems which use strong eubacterial promoters. Also, construction of *M. acetivorans* expression plasmids must be performed in *E. coli* prior to transformation into *M. acetivorans*. *M. acetivorans* is also an obligate anaerobe and genetic transformation must be done in an anaerobic glove box. Optimal expression of recombinant protein under the control of the *cdh* promoter requires that the expression strains be adapted to growth on sodium acetate, a process which requires several weeks. Additionally, high yield of recombinant protein requires the use of a fermentation facility with bioreactors that can be configured as a pH-auxostat. Many of these disadvantages are easily overcome in a well-equipped anaerobic microbiology laboratory. The main disadvantage to the difficulty of using the *M. acetivorans* expression system may well be the amount of time required to complete the process from construction of the expression vector to purification of recombinant

protein. However, this amount of time could easily be spent optimizing expression of a protein using eubacterial and eukaryal systems if the protein was not expressed actively on the first attempt.

Therefore, the *M. acetivorans* expression system may be used to yield archaeal and other proteins in an active and native form which are difficult to express using other systems. This system contributes to the genetic toolbox already available for *M. acetivorans* and enhances its ability to serve as a model organism for the study of the methanogenic Archaea.

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