WARREN LYNWOOD GARDNER
Expression vectors for the methane-producing archaeon *Methanococcus maripaludis*
(Under the Direction of WILLIAM B. WHITMAN)

Methanogens are strict anaerobes that use one and two carbon compounds for methanogenesis. They contain many unusual cofactors and enzymes, and many of their proteins are oxygen-sensitive and are present at low concentrations within the cells. An expression system that overexpresses homologous and heterologous proteins would facilitate research on the unique oxygen-sensitive enzymes in these organisms. However, traditional expression systems may lack the cofactors and maturation enzymes necessary for the expression of the active holoenzymes. Therefore, a major goal of this work was to develop expression vectors.

To develop a shuttle vector, a series of integrative vectors were first constructed. The integrative vectors were based on the pUC-derivative pMEB.2. pMEB.2 provided the puromycin resistance marker for methanococci, an *Escherichia coli* origin of replication, and an ampicillin resistance marker for *E. coli*. A multiple cloning site and the *Methanococcus volta*e histone promoter (P_{hmvA}) were added to form the integrative, expression vector pWLG14. To demonstrate the utility of P_{hmvA}, pWLG14 was used to overexpress the genomic copy of the *Methanococcus maripaludis* acetohydroxyacid synthase.

To form an expression shuttle vector suitable for heterologous genes, pWLG14 and the cryptic plasmid pURB500 from *M. maripaludis* strain were ligated together to form pWLG30. pWLG30 was the first expression shuttle vector for the methanogens. To demonstrate the utility of pWLG30, the *E. coli* β-galactosidase gene was cloned into pWLG30 to yield pWLG30+*lacZ*. Upon transformation into *M. maripaludis*, the

recombinant strain expressed β-galactosidase to the level of 1% of the cellular protein. pWLG30+*lacZ* also provided a convenient vector for the cloning because it provided a blue/white screen in *E. coli*. This vector was then used to express the carbon monoxide dehydrogenase (CODH), pyruvate oxidoreductase, monomethylamine methyltransferase, and a subunit of the F_{420}-reducing hydrogenase in *M. maripaludis*. A transposable element was also used to mutate pWLG30+*lacZ* to identify regions essential for plasmid replication in *M. maripaludis*.

INDEX WORDS: *Methanococcus maripaludis*, Methanogens, Expression, Shuttle vector, Integrative, Acetohydroxyacid synthase, Beta-galactosidase, Pyruvate oxidoreductase, Monomethylamine methyltransferase, Carbon monoxide dehydrogenase, F_{420}-reducing hydrogenase, Translation, Archaea, Cryptic plasmid, Formate, Large scale growth

EXPRESSION VECTORS FOR THE METHANE-PRODUCING ARCHAEON *METHANOCOCCUS MARIPALUDIS*

by

WARREN LYNWOOD GARDNER

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WARREN LYNWOOD GARDNER

Major Professor: William B. Whitman

Committee Members: Michael W. W. Adams

Robert J. Maier

Ellen L. Neidle

Juergen Wiegel

This dissertation has been approved

by the Graduate School of

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Gordhan L. Patel

Dean of the Graduate School

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To my parents, Harford and Linda Gardner, who have always supported my endeavors

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Methanogens are one group of the Archaea. The Archaea is one of three distinct phylogenetic groups based upon sequence similarity of the 16S ribosomal and 18S ribosomal RNAs (40, 41). The Archaea contains three kingdoms: *Crenarchaeota*, *Euryarchaeota*, and *Korarchaeota* (2, 41). Currently, the evidence for the *Korarchaeota* is based solely on 16S ribosomal RNA sequences from environmental samples since a representative organism has not been cultured. Burggraf et al. (1997) provides a detailed description of the members of the *Crenarchaeota* orders that have been cultured. The *Euryarchaeota* contains the halophiles, thermophiles, and methanogens. Methanogens are obligate anaerobes that grow in strongly reducing environments with an oxidation/reduction potential below –330 mV (32). The characteristic of methane formation from carbon dioxide or methyl groups unifies the methanogens (32).

Habits. Methanogens are the most widely distributed cultured archaea in nature. They can be isolated from fresh water and marine sediments, trees, bogs, subsurface oilfields and deep sea hydrothermal vents (29, 32). Bovine rumens, rabbit/hare intestinal tracts, and human feces represent some of the animal reservoirs for methanogens (4, 44). Insect reservoirs for methanogens include termites and cockroachs (20, 26). Methanogens can also colonize artificial environments such as sewage treatment facilities, rice paddies, and solar salt ponds (32). Altogether, methanogens grow over a wide range of environmental conditions.

Salt, pH, and temperature ranges. Collectively, methanogens grow over a wide range of salt (NaCl) concentrations, pHs, and temperatures. Organisms that grow optimally between 2.5 to 5.2 M salt are extreme halophiles (25). Moderate halophiles require 0.5 M to 2.5 M salt for optimum growth (25). *Methanohalobium evestigatum* is

an example of an extremely halophilic methanogen. *M. evestigatum* requires 4.3 M NaCl for optimum growth, and grows over a range of 2.6 M to 5.1 M NaCl (42). *Methanocalculus halotolerans*, which is a moderate halophile with an optimum of 0.85 M NaCl, grows from 0 M to 2.0 M NaCl (29). Methanogens that require low amounts of NaCl are also common in freshwater and other habitats.

Many methanogens grow around a pH of 7. However, alkaliphiles are also described. Alkaliphiles have an optimum pH $\geq$ 8.5 and a maximum pH $\geq$ 10.0 (38). The alkaliphile *Methanosalsus zhilinaeae* grows optimally at a pH of 9.2, and within a pH range of 8.2 to 10.3 (27). Acidophiles, which have a pH optimum of $\leq$ 4.0, are not yet described (10).

Methanogens also have members that grow at extreme temperatures. Numerous thermophiles (optimum temperature (T_{opt}) >50^{o}C, maximum temperature (T_{max}) >60^{o}C) and extreme thermophiles ($T_{opt} \geq$65^{o}C, Tmax >70^{o}C) are described for the methanogens (32, 38). Hyperthermophiles have a T_{opt}>80^{o}C and a T_{max}>85^{o}C for growth (38). *Methanopyrus kandleri* represents the current temperature maximum for a methanogen. *M. kandleri* grows at an optimum of 98^{o}C and a maximum of 110^{o}C (24). Psychrophiles are organisms that have optimum growth temperatures $\leq$ 15^{o}C (7). The first psychrophilic methanogen was described in 1991 for a *Methanosarcina* strain (43). *Methanogenium frigidum* was the first psychrophilic methanogen isolated that grows by CO_2 reduction (18). *M. frigidum* only grows from 17^{o}C until its medium freezes at -2^{o}C (18). The optimum temperature for *M. frigidum* is 15^{o}C.

Methanogenic pathways. Methanogens have three catabolic pathways for the reduction of carbon dioxide or methyl groups to methane (6). These pathways are called

the CO_2-reducing, methylotrophic, and the aceticlastic pathways. Ferry (1999), Deppenmeier et al. (1996), and Thauer (1998) review these pathways in detail.

Methane formation from the CO_2-reducing pathway routinely utilizes hydrogen or formate as the electron donor. Alternatively, some CO_2-reducing methanogens can oxidize primary and secondary alcohols via an alcohol dehydrogenase for the necessary reducing equivalents (5). The CO_2-reducing pathway begins with the formylmethanofuran dehydrogenase and the C-1 carrier methanofuran (35). Molecular hydrogen is oxidized by the F_{420}-non-reducing (NiFe) hydrogenase. The electrons are used by formylmethanofuran dehydrogenase to form N-formylmethanofuran from methanofuran and carbon dioxide. The formyl group is transferred to tetrahydromethanopterin (H_4MPT), which is a C-1 carrier similar to tetrahydrofolate, from N-formylmethanofuran by the formymethanofuran:H_4MPT formyltransferase. The N^5-formyl-H_4MPT is converted to N^5, N^{10}-methenyl-H_4MPT by the N^5, N^{10}-methenyl-H_4MPT cyclohydrolase. The next two reductions, which are catalyzed by F_{420}-dependent methylene-H_4MPT dehydrogenase and F_{420}-dependent methylene-H_4MPT reductase, require reduced coenzyme F_{420} for the reductions of the methenyl group to a methyl group (35). At this point, the methyl group is transferred to coenzyme M (CoM) by methyl-H_4MPT:coenzyme M methyltransferase to yield methyl-CoM and H_4MPT (13). Methyl-coenzyme M reductase catalyzes the last reduction of the methyl group to methane (16). The reactions catalyzed by methyl-CoM reductase involve coenzyme F_{430}, methyl-CoM, and coenzyme B, which is also known as 7-mercaptoheptanoylthreonine phosphate (CoB). The products of the reactions yield methane and the heterodisulfide product, CoM-S-S-CoB. See Thauer (1998) for a proposed mechanism for the reduction of the

methyl group to methane and the formation of CoM-S-S-CoB. The CoM-S-S-CoB is reduced to CoM and CoB by heterodisulfide reductase (16).

Methane generated by the methylotrophic pathway comes from methanol, methylated amines, and methylated thiols (9). The use of monomethylamine as a methanogenic compound is characterized in *Methanosarcina barkeri*. Three proteins are involved in the splitting of monomethylamine after it has entered the cell. The proteins are the monomethylamine methyltransferase (MtmB), monomethylamine corrinoid protein (MtmC), and MT2-type methylcobamide:CoM methyltransferase (MtmA). The MtmB catalyzes the disproportionation of monomethylamine to release ammonia. The methyl group is transferred to the corrinoid in MtmC. MtmC transfers the methyl group to MtmA, which methylates CoM. The remaining portion of the methanogenesis pathway is the same as described above except for the use of H_4SPT (see below). The *M. barkeri* proteins that disproportionate monomethylamine are specific for that substrate (9). *M. barkeri* also contains pathways for each of the following compounds: methanol, dimethylamine, and trimethylamine (16). These three pathways share the MtmA from the monomethylamine pathway for CoM methylation (9). See Ferry (1999) for a review of the methanol, dimethylamine, and trimethylamine pathways. In an environment lacking hydrogen, the methyl group is transferred to H_4SPT for oxidation to carbon dioxide by the same enzymes normally used for the reduction of CO_2 (13). For example, one-fourth of the methyl groups from methanol are oxidized to carbon dioxide (13).

Two-thirds of the methane biogenically produced in nature come from acetate (44). The genera involved in the disproportionation of acetate are *Methanosarcina* and *Methanosaeta* (13). The first step is the conversion of acetate to acetyl-coenzyme A

(acetyl-CoA). These genera utilize different enzymes in these reactions. *Methanosarcina* species use acetate kinase, at the expense of one ATP, to form acetyl-phosphate (13). The acetyl-phosphate is converted by phosphotransacetylase to acetyl-CoA. *Methanosaeta* species use acetate thiokinase, which is also known as acetyl-CoA synthetase, to convert acetate to acetyl-CoA (44). This reaction requires two ATP equivalents (22). The acetyl-CoA is disproportionated by carbon monoxide dehydrogenase/acetyl-CoA synthases (CODH/ACS) in *Methanosarcina* and *Methanosaeta* species. *Methanosarcina* species have two CODH/ACS (17). One CODH/ACS is composed of five subunits, while the other CODH/ACS contains two subunits (17). Both enzymes are able to disproportionate acetyl-CoA (17, 22). The acetyl-CoA is split into carbon dioxide while the methyl group is transferred to tetrahydrosarcinapterin (H_4SPT) to form N^5-methyl-tetrahydrosarcinapterin (21). H_4SPT is structurally similar to H_4MPT and is also a C-1 carrier (21, 39). The methyl group is transferred from methylated H_4SPT to methyl-CoM by the methyl-H_4MPT:CoM methyltransferase (13). Unlike the *Methanosarcina* species, *Methanosaeta soehngenii* only uses the two subunit CODH/ACS to disproportionate acetyl-CoA to carbon dioxide and a methyl group (17). This methyl group is transferred to tetrahydromethanopterin to form N^5-methyl-tetrahydromethanopterin (22). The remaining portion of methanogenesis is the same as described for the CO_2-reducing pathway in both *Methanosarcina* and *Methanosaeta* (Jetten et al., 1992).

Impact on society. Methanogens influence society in many ways. Waste treatment facilities are of major importance. Most sewage treatment facilities contain an anaerobic digestor that uses a consortium of organisms to digest the substrates (44).

Eventually methane and carbon dioxide are produced. Two-thirds of the methane comes from acetate in this environment. This methane can be harvested for energy production from small-scale facilities that use agriculture and domestic wastes (12). Methanogens are also utilized for their ability to dehalogenate organic compounds. In pure cultures, methanogens can dehalogenate 1,2-dichloroethane, chloroform, tetrachloroethylene, tetrachloromethane, and trichlorofluoromethane (14, 15, 23, 28). See Speece (1996) for detailed information on methanogens in treatment facilities and construction of anaerobic treatments of biomass.

Methanogens are also important in the nutrition of domestic animals. In cattle, *Methanobrevibacter* species in the rumen convert carbon dioxide into methane (44). Up to 12% of the feed energy is lost in this manner. To reduce the rate of carbon dioxide reduction to methane, cattle are fed ionophore antibiotics.

Methanogens also have biotechnological potentials. One compound of interest is coenzyme F_{420}, a 5-deazaflavin. This coenzyme is required as an electron donor for synthesis of three antibiotics (12). Methanogens make between 0.1-3 mg of coenzyme F_{420}/g of dry cells. Other sources are *Streptomyces* strains, but coenzyme F_{420} is produced at one-tenth to one-hundredth the level of methanogens (12). Recently, researchers have also identified lipids from methanogens that have medical applications. The lipids have the potential to become part of a drug delivery system (30).

Dissertation goals. Methanogens, which are relatively simple cells with small genomes when compared to eukaryotes, can be compared to bacteria in many biochemical pathways. Developing genetic tools for methanogens increases the ability to efficiently study these pathways at the genetic and biochemical levels.

In *M. maripaludis*, previous work has led to the development of integrative vectors, two antibiotic resistance cassettes, three reporter proteins, a shuttle vector, and a polyethylene glycol-mediated transformation procedure (1, 3, 11, 19, 31, 36, 37). The goals of this work are to develop a series of integrative expression vectors and an expression shuttle vector (Chapter 3). The expression shuttle vector will be used to express heterologous genes (Chapters 5 and 6). Understanding plasmid replication and copy number control can aid the development of future vectors. Thus, the expression shuttle vector was mutagenized to identify essential or important regions within the pURB500 region (Chapter 4). An important feature for an expression system is the ability to grow large amounts of the recombinant organism. Therefore, large scale growth of *M. maripaludis* on sodium formate is also investigated (Chapter 6).

References

1. **Argyle, J. L., D. L. Tumbula, and J. A. Leigh**. 1996. Neomycin resistance as a selectable marker in *Methanococcus maripaludis*. Appl. Environ. Microbiol. **62**:4233-4237.

2. **Barns, S. M., R. E. Fundyga, M. W. Jeffries, and N. R. Pace**. 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc. Natl. Acad. Sci. **91**:1609-1613.

3. **Beneke, S., H. Bestgen, and A. Klein**. 1995. Use of the *Escherichia coli uidA* gene as a reporter in *Methanococcus voltae* for the analysis of the regulatory function of the intergenic region between the operons encoding selenium-free hydrogenases. Mol. Gen. Genet. **248**:225-228.

4. **Biavati, B., B. Sgorbati, and D. Palenzona**. 1992. Plasmid DNA from methanogenic bacteria. Curr. Microbiol. **24**:285-287.

5. **Bleicher, K., G. Zellner, and J. Winter**. 1989. Growth of methanogens on cyclopentanol/CO_2 and specificity of alcohol dehydrogenase. FEMS Microbiol. Lett. **59**:307-312.

6. **Boone, D. R., W. B. Whitman, and P. Rouvière**. 1993. Diversity and taxonomy, p. 35-80. *In* James G. Ferry (ed.), Methanogenesis: ecology, physiology, biochemistry and genetics. Chapman & Hall, New York.

7. **Brock, T. D., M. T. Madigan, J. M. Martinko, and J. Parker**. 1994. Growth and its control, p.321-360. *In* David Kendric Brake (ed.), Biology of microorganisms. Prentice-Hall Inc., New Jersey.

8. **Burggraf, S., H. Huber, and K. O. Stetter**. 1997. Reclassification of the crenarchaeal orders and families in accordance with 16S rRNA sequence data. Int. J. Syst. Bacteriol. **47**:657-660.

9. **Burke, S., S. L. Lo, and J. A. Krzycki**. 1998. Clustered genes encoding the methyltransferases of methanogenesis from monomethylamine. J. Bacteriol. **180**:3432-3440.

10. **Cobley, J. G. and J. C. Cox**. 1983. Energy conservation in acidophilic bacteria. Microbiol. Rev. **47**:579-595.

11. **Cohen-Kupiec, R., C. Blank, and J. A. Leigh**. 1997. Transcriptional regulation in Archaea: *In vivo* demonstration of a repressor binding site in a methanogen. Proc. Natl. Acad. Sci. USA **94**:1316-1320.

12. **Daniels, L**. 1992. Biotechnological potential of methanogens. Biochem. Soc. Symp. **58**:181-193.

13. **Deppenmeier, U., V. Müller, and G. Gottschalk**. 1996. Pathways of energy conservation in methanogenic archaea. Arch. Microbiol. **165**:149-163.

14. **Egli, C., R. Scholtz, A. M. Cook, and T. Leisinger**. 1987. Anaerobic dechlorination of tetrachloromethane and 1,2-dichloroethane to degradable products by pure cultures of *Desulfobacterium* sp. and *Methanobacterium* sp. FEMS Microbiol. Lett. **43**:257-261.

15. **Fathepure, B. Z. and S. A. Boyd**. 1988. Dependence of tetrachloroethylene dechlorination on methanogenic substrate consumption by *Methanosarcina* sp. strain DCM. Appl. Environ. Microbiol. **54**:2976-2980.

16. **Ferry, J. G**. 1999. Enzymology of one-carbon metabolism in methanogenic pathways. FEMS Microbiol. Rev. **23**:13-38.

17. **Fontecilla-Camps, J. C. and S. W. Ragsdale**. 1999. Nickel-Iron-Sulfur active sites: hydrogenase and CO dehydrogenase. Adv. Inorg. Chem. **47**:283-333.

18. **Franzmann, P. D., Y. Liu, D. L. Balkwill, H. C. Aldrich, E. C. de Macario, and D. R. Boone**. 1997. *Methanogenium frigidum* sp. nov., a psychrophilic, H_2-using methanogen from Ace Lake, Antarctica. Int. J. Syst. Bacteriol. **47**:1068-1072.

19. **Gernhardt, P., O. Possot, M. Foglino, L. Sibold, and A. Klein**. 1990. Construction of an integration vector for use in the archaebacterium *Methanococcus voltae* and expression of a eubacterial resistance gene. Mol. Gen. Genet. **221**:273-279.

20. **Gijzen, H. J., C. A. M. Broers, M. Barughare, and C. K. Stumm**. 1991. Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in the cockroach hindgut. Appl. Environ. Microbiol. **57**:1630-1634.

21. **Grahame, D. A. and E. DeMoll**. 1996. Partial reactions catalyzed by protein components of the acetyl-CoA dearbonylase synthase enzyme complex from *Methanosarcina barkeri*. J. Biol. Chem. **271**:8352-8358.

22. **Jetten, M. S. M., A. J. M. Stams, and A. J. B. Zehnder**. 1992. Methanogenesis from acetate: a comparison of the acetate metabolism in *Methanothrix soehngenii* and *Methanosarcina* spp. FEMS Microbiol. Rev. **88**:181-198.

23. **Krone, U. E. and R. K. Thauer**. 1992. Dehalogenation of trichlorofluoromethane (CFC-11) by *Methanosarcina barkeri*. FEMS Microbiol. Lett. **90**:201-204.

24. **Kurr, M., R. Huber, H. König, H. W. Jannasch, H. Fricke, A. Trincone, J. K. Kristjansson, and K. O. Stetter**. 1991. *Methanopyrus kandleri*, gen. and sp. nov. represents a novel group of hyperthermophilic methanogens, growing at 110^{o}C. Arch. Microbiol. **156**:239-247.

25. **Kushner, D. J**. 1978. Life in high salt and solute concentrations: halophilic bacteria, p. 317-368. *In* D. J. Kushner (ed.), Microbial life in extreme environments. Academic Press, Inc., London.

26. **Leadbetter, J. R. and J. A. Breznak**. 1996. Physiological ecology of *Methanobrevibacter cuticularis* sp. Nov. and *Methanobrevibacter curvatus* sp. nov., isolated from the hindgut of the termite *Reticulitermes flavipes.* Appl. Environ. Microbiol. **62**:3620-3631.

27. **Mathrani, I. M., D. R. Boone, R. A. Mah, G. E. Fox, and P. P. Lau**. 1988. *Methanohalophilus zhilinae* sp. nov., an alkaliphilic, halophilic, methylotrophic methanogen. Int. J. Syst. Bacteriol. **38**:139-142.

28. **Mikesell, M. D. and S. A. Boyd**. 1990. Dechlorination of chloroform by *Methanosarcina* strains. Appl. Environ. Microbiol. **56**:1198-1201.

29. **Ollivier, B., M.-L. Fardeau, J.-L. Cayol, M. Magot, B. K. C. Patel, G. Prensier, and J.-L. Garcia**. 1998. *Methanocalculus halotolerans* gen. nov., sp. nov., isolated from an oil-producing well. Int. J. Syst. Bacteriol. **48**:821-828.

30. **Patel, G. B. and G. D. Sprott**. 1999. Archaeobacterial ether lipid liposomes (archaeosomes) as novel vaccine and drug delivery systems. Crit. Rev. Biotechnol. **19**:317-357.

31. **Sniezko, I., C. Dobson-Stone, and A. Klein**. 1998. The *treA* gene of *Bacillus subtilis* is a suitable reporter for the archaeon *Methanococcus voltae*. FEMS Microbiol. Lett. **164**:237-242.

32. **Sowers, K. R**. 1995. Methanogenic Archaea: an overview. p. 3-13. *In* K. R. Sowers and H. J. Schreier (ed.). Archaea: a laboratory manual, Methanogens. Cold Spring Harbor Laboratory Press, Planeview, New York.

33. **Speece, R. E**. 1996. Principles of anaerobic treatment, p. 25-68. *In* Anaerobic biotechnology for industrial wastewaters. Archae Press, Nashville, Tn.

34. **Thauer, R. K**. 1998. Biochemistry of methanogenesis: a trbute to Marjory Stephenson. Microbiology **144**:2377-2406.

35. **Thauer, R. K., R. Hedderich, and R. Fischer**. 1993. Methanogenesis from CO_2 and H_2, p. 209-252. *In* James G. Ferry (ed.), Methanogenesis: ecology, physiology, biochemistry and genetics. Chapman& Hall, New York.

36. **Tumbula, D. L., T. L. Bowen, and W. B. Whitman**. 1997. Characterization of pURB500 from the archaeon *Methanococcus maripaludis* and construction of a shuttle vector. J. Bacteriol. **179**:2976-2986.

37. **Tumbula, D. L., R. A. Makula, and W. B. Whitman**. 1994. Transformation of *Methanococcus maripaludis* and identification of a *Pst*I-like restriction system. FEMS Microbiol. Lett. **121**:309-314.

38. **Wiegel, J**. 1998. Anaerobic alkalithermophiles, a novel group of extremophiles. Extremophiles **2**:257-267.

39. **White, R. H. and D. Zhou**. 1993. Biosynthesis of the coenzymes in methanogens. p. 409-444. *In* James G. Ferry (ed.), Methanogenesis: ecology, physiology, biochemistry and genetics. Chapman & Hall, New York.

40. **Woese, C. R. and G. E. Fox**. 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc. Natl. Acad. Sci. USA **74**:5088-5090.

41. **Woese, C. R., O. Kandler, and M. L. Wheelis**. 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc. Natl. Acad. Sci. USA **87**:4576-4579.

42. **Zhilina, T. N. and G. A. Zavarzin**. 1987. *Methanohalobium evestigatus*, n. gen., n. sp., the extremely halophilic methanogenic *Archaebacterium*. Dokl. Akad. Nauk USSR **293**:464-468.

43. **Zhilina, T. N. and G. A. Zavarzin**. 1991. Low temperature methane production by a pure culture of *Methanosarcina* sp. Dokl. Akad. Nauk USSR **317**:1242-1245.

44. **Zinder, S. H**. 1993. Physiological ecology of methanogens, p. 128-206. *In* James G. Ferry (ed.), Methanogenesis: ecology, physiology, biochemistry and genetics. Chapman & Hall, New York.

CHAPTER II

ARCHAEAL PLASMIDS[1]

[1] Gardner, W. L. 2000. Encyclopedia of Life Sciences, in press.

Introduction

Based on 16S and 18S ribosomal RNA sequences, Woese and Fox (1977) proposed the three domains of life known as Archaea, Bacteria, and Eucarya. The Archaea are comprised of two kingdoms. One kingdom is the *Crenarchaeota*, which consists of *Sulfolobales* and *Thermoproteales*. The second kingdom, the *Euryarchaeota*, is composed of methanogens, extreme halophiles and the orders *Thermoplasmales* and *Thermococcales* (Woese *et al.*, 1990; Stetter, 1996; Schleper *et al.*, 1995b). Plasmids have been found in members of both kingdoms. Isolation of these plasmids is important for fundamental understanding of archaeal replication mechanisms, identification of origins of replication, and construction of shuttle vectors for basic research and biotechnology.

Archaeal plasmids

Euryarchaeota

Methanogens

Methanogens are strict anaerobes that use one carbon compounds, hydrogen, and acetate for the production of methane and energy. These anaerobes have been isolated from a variety of locations, including freshwater and marine sediments, the intestinal tracts of cattle, humans, and termites, and bioreactors and waste treatment facilities. Methanogenic isolates have been found to grow over a wide range of temperatures, including psychrophilic and hyperthermophilic. This variety of sources has been important to the basic understanding of the diversity in methanogens.

The isolation of plasmids from methanogens began with the description of pMP1 by Thomm *et al.* (1983). pMP1 is a 7.0 kilobase (kb) cryptic plasmid purified from a coccoid methanogen strain PL-12/M, which was isolated from a fumarole located off of Vulcano Island, Italy. PL-12/M was the only isolate out of 15 isolates to contain a plasmid. The identification of pMP1, which was never sequenced, confirmed the existence of plasmids in methanogens. Later that year, pME2001 was described from the thermophile *Methanobacterium thermoautotrophicum* strain Marburg ("*Methanothermobacter*

marburgensis strain Marburg") (Wasserfallen *et al.*, 1999). Isolation of pME2001 dispelled a proposal that plasmids could only be purified from newly isolated methanogens and not from strains that had been maintained in the laboratory. Different multimeric forms of this plasmid, ranging from monomers to hexamers, were found by electron microscopy (Meile *et al.*, 1983). In an effort to construct a cloning vector for a *Methanobacterium* species, pME2001 was sequenced (see Table 1) (Bokranz *et al.*, 1990). *Methanobacterium thermoautotrophicum* ΔH ("*Methanothermobacter thermautotrophicus*" strain ΔH), a close relative to strain Marburg, does not contain plasmids (Smith *et al*., 1997, Wasserfallen *et al*., 1999). If the host range of pME2001 is broad enough, vectors derived from it could be used to study basic physiology within both of these well studied methanogens. To date, vectors based on pME2001 have not been constructed.

Modular structures were found among plasmids isolated from *Methanobacterium* species. *Methanobacterium thermoautotrophicum* strain ZH3 and *Methanobacterium thermoautotrophicum* strain Marburg are closely related, with 99.9% similarity between their 16S rRNA sequences. Each strain also contains a plasmid (Meile *et al*., 1983; Stettler *et al.*, 1994). These plasmids, pME2200 from strain ZH3 and pME2001 from strain Marburg, were homologous but not identical. pME2200 and pME2001 shared a common 3.8 kb region with 99.9% sequence similarity. pME2200 contained three additional regions when compared to pME2001. These 183 bp, 364 bp, and 1058 bp regions did not contain open reading frames (ORF), and were considered nonessential for plasmid replication. Both pME2001 and pME2200 were cryptic plasmids that may be co-ancestoral rather than derived from an individual plasmid (Stettler *et al.*, 1994).

Another example of modular structure comes from three plasmids identified in *Methanobacterium thermoformicicum* strains. Two of the plasmids, pFZ1 and pFZ2, from strains Z-245 and FTF, respectively, appeared identical by hybridizations and restriction endonuclease digestions. The third plasmid, from *M. thermoformicicum* strain THF, pFV1 was larger and possessed similarity to pFZ1 and pFZ2 (Nölling *et al.*, 1991).

Upon sequencing, pFV1 and pFZ1 were found to contain an 8.2 kb region with greater than 91% sequence identity (Nölling *et al.*, 1992). In addition, pFV1 possessed a restriction and modification system composed of the DNA methyltransferase *Mth*TIM and the restriction endonuclease *Mth*TIR. This type II deoxyriboendonuclease cleaved at GGCC sequences after the second guanosine residue. *Mth*TIM methylated the third residue to form GG^{me}CC (Nölling and de Vos, 1992a). Similar to pFV1, pFZ1 encoded the DNA methyltransferase *Mth*ZIM modification system for the nucleotide sequence CTAG (Nölling *et al.*, 1991; Nölling and de Vos, 1992b). Both the Z-245 and FTF strains of *M. thermoformicicum* utilized this restriction/modification system. Southern hybridizations further identified a region of pFZ2 homologous to the methyltransferase gene *mthZIM* of pFZ1. An ORF on pFZ1 in the reverse orientation of *mthZIM* may also encode the restriction enzyme for this modification system, *Mth*ZIR (Nölling and de Vos, 1992b). This ORF has yet to be characterized biochemically to confirm this hypothesis. In addition to these shared sequences, both pFZ1 and pFV1 also contained regions that hybridized to the genomic DNA of *Methanobacterium thermoautotrophicum* strain ΔH and five *M. thermoformicicum* strains (Nölling *et al.*, 1993; Smith *et al*., 1997). The mechanism of replication was not identified for these plasmids. However, both single-stranded plasmid DNA and initiator proteins predicted for rolling circle mechanisms were undetectable in cells containing pFV1 and pFZ1, suggesting that the plasmids replicated by a theta mechanism (Nölling *et al.*, 1992).

The first shuttle vector developed for a methanogen utilized the cryptic plasmid pURB500 (Table 1) from *Methanococcus maripaludis* strain C5 (Tumbula *et al.*, 1997). Strain C5 was isolated from Airport Marsh on Sapelo Island, Georgia, in an area that contained the short form of *Spartina alterniflora* as the major flora (Whitman *et al.*, 1986). The study that isolated strain C5 was conducted to identify plasmid-containing methanococci for the development of a genetic system. These methanogens have a relatively short doubling time, a simple cell morphology, and are easily grown. pURB500 was the

only plasmid described in the 21 methanococcal strains screened. This plasmid was found to be maintained at about three copies per genome in strain C5 (Wood *et al.,* 1985). Plasmid dimers were observed by electron microscopy. Tumbula and Whitman (1997) proposed that pURB500 may replicate by using a theta mechanism to generate the dimers. Although pURB500 was sequenced (see Table 1) none of the 18 putative ORFs possessed a high degree of similarity to known proteins (Tumbula *et al.*, 1997). Two areas of complex secondary structure that might be origins of replication were identified. However, subcloning of the pURB500 plasmid has yet to yield a minimum replicon containing only these regions. The host range of pURB500 appears to be restricted to strains of *M. maripaludis* (Tumbula *et al.*, 1997).

Of the methane produced in the environment, 70% of the methane comes from aceticlastic methanogenesis. Aceticlastic methanogenesis occurs when acetate is disproportionated into methane and carbon dioxide. Interest in this transformation lead to a search of nine acetotrophic and three obligate methylotrophic methanogens for plasmids (Sowers and Gunsalus, 1988). A total of three plasmids were identified in strains maintained as laboratory cultures for over 5 years. The three strains, *Methanosarcina acetivorans* strain C2A, isolate C2B, and isolate C2C, were obtained from sediments collected at the Scripps Submarine Canyon off the coast of La Jolla, California. The sizes and restriction endonuclease maps of the plasmids were similar, so only the plasmid (pC2A) from *M. acetivorans* strain C2A was further characterized. pC2A was a low copy number plasmid with about 6 copies/cell (Sowers and Gunsalus, 1988). Sequencing identified 4 ORFs larger than 120 amino acids. One ORF had homology to a family of recombinases; a second ORF had homology to a replication initiation protein. The other ORFs did not possess known homologs. pC2A was used to construct a shuttle vector for *M. acetivorans* and *Escherichia coli*. The shuttle vector, pWM307 (see below), had a broad host range and was able to replicate in seven *Methanosarcina* species (Metcalf *et al.*, 1997).

Plasmids have also been identified in hyperthermophilic methanococci. Two plasmids have been isolated from *Methanococcus jannaschii* strain JAL-1 ("*Methanocaldococcus jannaschii*"), and one plasmid has been isolated from *Methanococcus fervens* strain AG86 ("*Methanocaldococcus fervens*") (Zhao *et al*., 1988; Jeanthon *et al.*, 1999;Whitman *et al.*, 1999). Strain AG86, which is closely related to *M. jannaschii* JAL-1, contains one plasmid called pURB900 (Zhao *et al*., 1988). Based upon restriction digestions and hybridizations, the 20 kb pURB900 was not related to the plasmids from *M. jannaschii* JAL-1. Both *M. jannaschii* plasmids were sequenced during a total genome sequencing project (Bult *et al.*, 1996). pURB800 (also known as MJECL) possessed 44 ORFs, five of which had homology to known proteins. There were regions of nucleotide sequence similarity among the putative genes that suggested recent recombination events between pURB800 and the genome. The origin of replication was not identified in this plasmid. The smaller *M. jannaschii* plasmid pURB801 contained 5 ORFs, none of which had homology to known proteins. The origin of replication was also not identified in pURB801. Neither pURB800 or pURB801 possessed homology to pURB500 from *M. maripaludis* strain C5. Development of pURB800 or pURB801 into shuttle vectors has not been reported.

Methanogenic isolates from mesophilic and thermophilic molasses stillage fermentors as well as rabbit feces were screened for plasmids (Biavati *et al.*, 1992). Of the 79 strains examined, five strains contained plasmids. A 26.5 kb plasmid was found in *Methanosarcina barkeri* strain DSM 800, which had previously been described as lacking plasmids and was used as a negative control for this experiment (Sowers and Gunsalus, 1988). This example illustrates the difficulty of identifying plasmids. Biavati *et al.* (1992) suggested that the methodology for plasmid isolation contributed to the discovery of the plasmid in DSM 800 and that low copy number plasmids could also be underrepresented by their methodology.

Including the study of Biavati *et al.* (1992), 137 methanogenic strains have been screened for plasmids. Plasmids reported in sixteen of these strains or 12%. Of the sequenced plasmids described, none contained ORFs with homology to known antibiotic resistance markers. This lack of antibiotic resistance markers was not surprising because archaea are insensitive to many of the antibiotics for bacteria and eucarya.

Halophiles

In the *Euryarchaeota* the organisms collectively referred to as the halophiles include extreme halophiles, or organisms that grow optimally at 2.5 to 5.2 M salt, as well as moderate halophiles, or organisms that grow with 0.5 M-2.5 M salt (Kushner, 1978). The extremely halophilic methanogens are not usually included in this largely aerobic group. In 1963, the initial identification of plasmid DNA was confirmed in two extremely halophilic archaea (Joshi *et al*., 1963). Since that discovery, plasmids have been found in many halophilic archaea, where multiple plasmids are common and can comprise up to 31% of the deoxyribonucleic acid in some species (Moore and McCarthy, 1969).

The moderate halophile, *Haloferax volcanii* strain DS2 (formerly known as *Halobacterium volcanii*), contained four different plasmids that contribute up to 25% of the cellular DNA (Holmes *et al.*, 1995). These plasmids were the 86 kb pHV1, the 6.4 kb pHV2, the 442 kb pHV3, and the 690 kb pHV4 (Charlebois *et al.*, 1987; Charlebois, R. L., 1991). Only pHV2 was studied in great detail. About 6 copies/chromosome were present in the cell. Sequence analysis identified four ORFs with sizes greater than 189 amino acids and numerous smaller ORFs. None of the ORFs possessed homology to known proteins (Charlebois *et al.*, 1987). pHV51 was a natural variant of pHV2 that possessed an insertion element. This cryptic plasmid was developed into the shuttle vector pWL102 (see below). This vector replicated in three different halophilic genera that spanned the 16S rRNA phylogenetic tree for halophilic archaea and included *Haloferax, Halobacterium,* and *Haloarcula* (Lam and Doolittle, 1989; Blaseio and Pfeifer, 1990; Cline and Doolittle, 1992).

Haloferax strain Aa 2.2 possessed two plasmids. The smaller 10.5 kb cryptic plasmid was named pHK2 and was maintained at 7 to 8 copies/cell. With the addition of a selectable marker, pHK2 was transformed into *H. volcanii*. Both pHK2+marker and pHV2 were recovered from the transformed cells (Holmes and Dyall-Smith, 1990). While never completely sequenced, a 3359 bp fragment of pHK2 was found to contain the minimum region required for replication. This region contained three ORFs and four inverted repeats. Although ORF1 had homology to rolling-circle initiator replication proteins, single-stranded DNA replication intermediates could not be detected (Holmes *et al.*, 1995).

Plasmids initially were found in *Halobacterium* based upon the observation that many species contained satellite DNA with a different mol% G+C than genomic DNA (Moore and McCarthy, 1969). In *Halobacterium salinarium* strain 5, a 66.7 kp plasmid was found to be associated with gas vacuole production. This strain also contained two additional plasmids of 39.4 kb and 130.3 kb that were never linked to a phenotype (Simon, 1978). Gas vacuole production was also linked to the 150 kb plasmid pHH1 from *Halobacterium halobium* strain NRC817 (Weidinger *et al.*, 1979; DasSarma and Arora, 1997). This plasmid was maintained at 4 copies per chromosome (Weidinger *et al.*, 1979). Other *Halobacterium halobium* strains also contained plasmids with homology to pHH1. *H. halobium* strain DSM670, *H. halobium* strain DSM671 and *Halobacterium cutirubrum* maintained plasmids which varied in size from 75.8 kb to 150 kb with homology to pHH1 (Pfeifer *et al.*, 1981). These strains have not been characterized further.

H. halobium contained a dynamic population of plasmids formed by a high rate of deletions (Pfeifer *et al.*, 1988; Pfeifer and Blaseio, 1989). pHH9, a 5.7 kb derivative of pHH1, was formed through successive spontaneous deletions of pHH1 (Pfeifer and Blaseio, 1989). This plasmid had a copy number identical to pHH1 and presumably contained all the information necessary for stable replication (Blaseio and Pfeifer, 1990). Sequencing and subcloning parts of pHH9 identified a minimum replicon of 2.9 kb. This DNA fragment contained an 1,893 bp ORF and a 350 bp A+T rich region. The ORF did

not possess homologs in the databases. The A+T rich region was similar to origins of replication for some bacterial plasmids (Pfeifer and Ghahraman, 1993). A shuttle vector was developed from pHH9 (see below). This vector, pUBP2, had a broad host range and replicated in *Halobacterium halobium*, *Haloferax volcanii*, *Haloarcula hispanica*, and *Haloarcula vallismortis* (Cline and Doolittle, 1992).

Another minimal region for replication was identified in a plasmid from another strain of *H. halobium*. Strain NRC-1 contained two plasmids of 350 kb, pNRC200, and 200 kb, pNRC100. Strain NRC-1 also contained a population of smaller plasmids derived from pNRC100. A minimum replication region of only 3,874 bp was identified for pNRC100. This region contained an 3,027 bp ORF with homology to replication proteins and an A+T rich region. The ORF also had homology to an ORF from the *H. volcanii* plasmid pHV2. However these ORFs were not homologous to the ORFs from pHH1. This 3.9 kb region of pNRC100 allowed plasmid replication in strain NRC-1 and *H. volcanii* strain WFD11. A shuttle vector was made by ligating the 3.9 kb fragment to an *E. coli* plasmid containing a selectable marker for *Halobacterium* and *Haloferax.* The vector was not stable in the absence of the antibiotic selection in *H. volcanii*. So even though the plasmid replicated, portions required for plasmid stability were lacking (Ng and DasSarma, 1993).

A large number of other plasmids have been described in *Halobacterium* strains (see Table 1). *Halobacterium* strain GN101 possessed the 1.7 kb pHGN1 and four large plasmids of 39, 43, 49, and 65 kb (Ebert *et al.*, 1984). Strain GRA contained a 38 kb plasmid and a 65 kb plasmid. Strain SB3 had a 52 kb plasmid and a 34 kb plasmid with homology to an insertion sequence on pHH1. Strain SB3 also possessed the 1.7 kb plasmids pHSB1 and pHSB2. Strain GRB maintained the 1.7 kb pGRB1 and two large plasmids at 35 kb and 65 kb (Ebert *et al.*, 1984). A single-stranded intermediate of pGRB1 was found in the host's cells, but the mechanism of replication was not determined (Sioud *et al.*, 1988). pHGN1 and pHSB1, the 1.7 kb plasmids from strain SB3, were also thought

to replicate through single-stranded intermediates (Hall and Hackett, 1989). These small plasmids, which were sequenced, all possessed a homologous ORF that was proposed to be a replication protein even though no direct evidence was reported (Hackett *et al.*, 1990). Another common feature among the plasmids was the sequence GATTT(C/G). pGRB1, pHSB1, and pHGN1 contained multiple copies of this element (Hackett *et al.*, 1990). The location of the hexanucleotide element corresponded to similar regions among the plasmids. This sequence was thought to be common in bacterial origins of replication and speculated to be the origin for these plasmids (Hackett *et al.*, 1990).

During sequencing of pHSB1, pHGN1, and pGRB1 some positions appeared to contain more than one nucleotide (Akhmanova *et al.*, 1993). Thus, what were thought to be single plasmids were populations of closely related plasmids. Two forms of heterogeneity were described for these plasmids. Macroheterogeneity, or a large region with multiple nucleotide changes, was found in a 250 bp region of pHSB1 and pHSB2, which were 80% identical at the nucleotide level. Microheterogeneity, an isolated nucleotide change between plasmids, was detected in the sequence analysis of pHSB1 that lead to the discovery of a pHSB1 population. Microheterogeneity was not described for pHSB2. No hybrids between pHSB1 and pHSB2 were detected. This observation was interesting since many *Halobacterium* species had high recombination rates. pHGN1 from strain GN101 also contained microheterogeneity (Akhmanova *et al.*, 1993). pHSB1, pHSB2, and pHGN1 were also thought to be maintained at a high copy number. pGRB1 was maintained in *Halobacterium* strain GRB at 180 copies/cell. pGRB1, after being developed as a shuttle vector for use with *E. coli*, also replicated in *H. halobium* strain R1 following transformation (Krebs *et al.*, 1991). Additionally, *H. halobium* strain R1 maintained pHSB2 after transformation (Akhmanova *et al.*, 1993).

Thermoplasmales* and *Thermococcales

Within the *Euryarchaeota* are the orders *Thermococcales* and *Thermoplasmales*. *Thermococcales* is composed of the genera *Thermococcus* and *Pyrococcus* (Woese *et al.*,

1990). With the exception of a few thermophiles, these organisms are hyperthermophiles with optimum growth occurring at 80^{o}C or higher. *Thermoplasmales* currently consists of the genera *Thermoplasma* and *Picrophilus*. Both genera are thermophiles with growth optimums near 60^{o}C. These organisms are also hyperacidophilic and capable of growth below a pH of 1 (Schleper *et al.*, 1995b).

In 1995, two articles reported the isolation of plasmids from the *Thermoplasmales*. pTA1 from *Thermoplasma acidophilum* strain HO-1012 was a 15.2 kb cryptic plasmid maintained at 7-13 copies/cell (Yasuda *et al.*, 1995). *T. acidophilum* strains HO-63 and HO-121 also contained plasmids that hybridized to pTA1. An additional three strains contained plasmids that were not further characterized. Twelve *T. acidophilum* strains were screened for metal and novobiocin resistance. No significant differences were found between the plasmid-free and the plasmid-containing strains (Yasuda *et al.*, 1995). Plasmids were also found in 8 strains of *Picrophilus oshimae* (Schleper *et al.*, 1995b). Six strains contained an 8.3 kb plasmid. Another strain contained an 8.8 kb plasmid, while the last strain carried both plasmids. Restriction analysis was not reported for these plasmids, but Southern hybridizations suggested that the 8.3 kb and 8.8 kb plasmids shared homologous regions (Schleper *et al.*, 1995b). The closely related species *Picrophilus torridus* did not contain plasmids (Schleper *et al.*, 1996).

Many plasmids have been isolated from the order *Thermococcales*. Screening of the *Thermococcales* was conducted to increase the diversity and number of plasmids for basic research and biotechnological applications. During the screening of 52 strains from a Pacific hydrothermal vent, plasmids were found in eleven strains (Benbouzid-Rollet *et al.*, 1997). Of these 11 strains, six were chosen for further characterization since they had a higher concentration of plasmid DNA. By restriction fragment length polymorphisms (RFLPs), strains I559, I560, and I690 were shown to be related to *Thermococcus stetteri*. Each strain contained a 3.5 kb and a 24 kb plasmid. Through the use of Southern hybridizations, the small plasmids were shown to hybridize to each other but not to the

larger plasmids. Likewise, the large plasmids also hybridized to each other. A 5.3 kb plasmid (pGN31) was found in *Thermococcus* sp. strain GE31. pGN31 did not hybridize to the other plasmids within this study (Benbouzid-Rollet *et al*., 1997). In addition, plasmids were found in two *Pyrococcus abyssi* strains. *P. abyssi* strain GE23 contained an unique 16.8 kb plasmid called pGN23. This strain had 79% DNA hybridization to the type strain *Pyrococcus abyssi* strain GE5 (Benbouzid-Rollet *et al.*, 1997).

A plasmid, pGT5, was also found in *Pyrococcus abyssi* strain GE5, an organism from a Pacific hydrothermal vent (Erauso *et al.*, 1993). pGT5 was maintained at 25-30 copies/chromosome (Erauso *et al.*, 1996). It was 3,444 bp, and was the only *Thermococcales* plasmid sequenced. From the nucleotide sequence, two open reading frames were identified. When translated, ORF1 possessed motifs similar to proteins involved in rolling-circle replication mechanisms. ORF2 did not have homology to proteins within the databases. However, based on location and putative identification of ORF1 to the pC194 family of plasmids that replicate by a rolling-circle mechanism, it was proposed that ORF2 may be involved in recombination (Erauso *et al.*, 1996). Plasmids that utilize a rolling-circle mechanism contain two origins of replication. The plus origin is the site recognized by a replication protein that binds and then nicks the plasmid (Jannière *et al.*, 1993). This nick allows the synthesis of single-stranded (ss) DNA from the plasmid. After the ssDNA synthesis is complete, the replication protein terminates the reaction and forms a single-stranded product and a double-stranded plasmid. The minus origin is then used for the conversion of the single-stranded intermediate into a double-stranded plasmid (Jannière *et al.*, 1993). A region of 11 nucleotides identical to the double-stranded origin of replication used by other rolling-circle type plasmids was identified in pGT5 (Erauso *et al*., 1996). A region of low identity corresponding to the second origin of replication was also suggested. To confirm a rolling-circle mechanism of replication, a single-stranded DNA intermediate was identified in cell extracts for the positive strand of the plasmid (Erauso *et al.*, 1996). In addition, *P. abyssi* strain GE27, possessed a 3.5 kb plasmid (pGN27) that

was thought to be closely related if not identical to pGT5 (Benbouzid-Rollet *et al*., 1997). pGT5 was the basis for a shuttle vector for *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, and *Escherichia coli* (see below; Aravalli and Garrett, 1997).

Crenarchaeota

The order *Sulfolobales* within the *Crenarchaeota* is composed of the genera *Sulfolobus*, *Acidianus*, *Metallosphaera*, *Sulfurisphaera*, and *Stygiolobus* (Stetter, 1996; Fuchs *et al.*, 1996; Kurosawa *et al.*, 1998). Species of the genus *Sulfolobus* oxidize hydrogen, elemental sulfur, and sulfide under autotrophic growth conditions in media of low ionic strength. The genus *Acidianus* contains salt tolerant autotrophs that also oxidize sulfides, elemental sulfur, and hydrogen. Under anaerobic conditions, members of this genus reduce elemental sulfur to sulfide while oxidizing hydrogen (Segerer *et al.*, 1986).

Plasmids were discovered in the chemolithoautotroph *Acidianus ambivalens* strain LEI10 in 1985. Strain LEI10, formerly known as *Desulfurolobus ambivalens*, was isolated at pH 2.3 and 94^{o}C in a solfataric waterhole in Iceland and contained three plasmids (Zillig *et al.*, 1986; Fuchs *et al.*, 1996). The most abundant plasmid pDL10, previously known as pSL10, was 7.7 kb. Although pDL10 was initially thought to be involved in the reduction of elemental sulfur to hydrogen sulfide, cured strains were capable of sulfur reduction, and this hypothesis was disproved (Zillig *et al.*, 1985). pDL10 had around 50 copies/chromosome under anaerobic conditions (Zillig *et al.*, 1994). Under aerobic conditions, cells contained 20-fold less plasmid (Zillig *et al.*, 1986). Plasmids similar to pDL10 were also found in 20 out of 22 isolates of *A. ambivalens* (Zillig *et al.*, 1996). Although the sequence of pDL10 appears in the databases (see Table 1), the description of the nucleotide sequence has not been published.

Three plasmids were identified during a screening of Icelandic solfataric field isolates belonging to the order *Sulfolobales*. These plasmids came from *Sulfolobus islandicus* strains HEN7H2 and REN1H1. pHE7 from *S. islandicus* strain HEN7H2 was maintained at 15 copies/chromosome and had a size of 7 kb. This cryptic plasmid has never

been sequenced. *S. islandicus* strain REN1H1 contained the plasmids pRN1 and pRN2 at 20 and 30 copies/chromosome, respectively (Zillig *et al.*, 1994). pRN1 had a host range of *S. islandicus* and *Sulfolobus solfataricus* but not *Sulfolobus acidocaldarius* (Zillig *et al.*, 1994). *S. solfataricus* was proposed as a genetic model for crenarchaeotes since electroporation and plating techniques were developed, and pRN1 was the first plasmid completely sequenced from the *Crenarchaeota* (Keeling *et al*., 1996). pRN1 contained 6 ORFs within its 5350 bp. One of the ORFs had homology to the repressor protein (CopG). CopG controls the expression of a protein that initiates plasmid replication in bacteria. The remaining ORFs did not possess homology to known proteins. The replication origin for this plasmid was not identified (Keeling *et al*., 1996). pRN2, which was recently sequenced, had the same host range as pRN1 (Zillig *et al.*, 1994). Three ORFs in pRN2 were in the same orientation in pRN1. The first ORF was the CopG homolog. The two remaining ORFs were conserved between the two plasmids but did not have any other homologs. Noncoding regions of the plasmids were also conserved in five distinct blocks. The fifth block contained multiple copies of the interon CTAACTCT close to a polycytosine track. While this unusual structure was conserved between pRN1 and pRN2, no functioning role was identified (Keeling *et al.*, 1998).

The *Sulfolobales* order contained the first example of an archaeal plasmid that underwent horizontal transfer via a contact-mediated mechanism. The plasmid was the 41 kb pNOB8 from *Sulfolobus* isolate NOB8H2. The cryptic pNOB8 had around 20 copies per genome when the cells were grown in a liquid culture. Upon plating on a solid medium, cells contained only 2-5 copies of pNOB8/genome. Schleper *et al.* (1995a) proposed that this low copy number may be representative of environmental growth conditions. Mating allowed pNOB8 to be transferred to different *Sulfolobus* species. In 3 strains of *S. solfataricus* and 3 strains of *S. islandicus*, the copy number was 20 to 40 copies of pNOB8/genome in liquid culture. The reduction in copy number was repeated upon plating these strains. In *S. islandicus* strain HEN7H2, which possessed both pNOB8 and the non-

conjugative pHE7, only pNOB8 was transferred (Prangishvili *et al.*, 1998). Reisolation of plasmid DNA from the host and recipients showed that the cells contained wild type pNOB8 and smaller pNOB8-derived plasmids. The major variant of the smaller plasmids was named pNOB8-33. pNOB8-33 was found only in recipient strains and not in isolate NOB8H2 and was missing about 8 kb of the 41 kb plasmid (Schleper *et al.*, 1995a; She *et al.*, 1998). pNOB8-33 was still able to replicate and be transferred among *Sulfolobus* species.

pNOB8 was recently sequenced due to the interest in archaeal conjugation and the development of genetic tools for *Sulfolobus.* pNOB8 was a 41,229 bp plasmid containing multiple repeats. The largest perfect repeat was 85 bp separated by 7942 bp. This repeat was identified to be the point of recombination that yielded pNOB8-33. About 50 ORFs were identified in pNOB8. Of these ORFs, less than 20% possessed homology to known proteins. However, homologs were identified to transposases (see section on Insertion sequences) and proteins involved in partitioning plasmids to daughter cells. Some of these latter ORFs were lost upon the formation of pNOB8-33, which may have contributed to its instability (She *et al.*, 1998).

pNOB8 has also been electroporated into *S. solfataricus* (Schleper *et al.*, 1995a). An expression system for *S. solfataricus* strain PH1 was developed by cloning the strong promoter for ribosomal protein S12 from *S. acidocaldarius* and the beta-galactosidase gene from *S. solfataricus* strain PI into pNOB8 (Elferink *et al.*, 1996). This expression vector was called pNOB8:*lacS.* However, this expression system was not stable. After transformation of pNOB8:*lacS* into *S. solfataricus* PH1, both large and small colonies that contained beta-galactosidase (*lacS*) were discovered. Using Southern hybridizations, these larger colonies were found to have loss the pNOB8, but contained *lacS* integrated into the genome. The smaller colonies initially maintained the expression vector, but restreaking frequently generated plasmid-minus colonies (Elferink *et al.*, 1996).

Since the description of pNOB8, eleven additional conjugative plasmids were identified during the screening of nearly 300 strains of *Sulfolobus islandicus* (Prangishvili *et al.*, 1998). These plasmids were proposed to belong to a single family which includes pNOB8. While not all of the plasmids were able to initiate conjugation, all could be mobilized during conjugation. For instance, although the 6.0 kb plasmid pING3 was nonconjugative, it could be mobilized by pING6. Many of the plasmids were closely related and some plasmids, such as the pINGs and pSOG2/4 plus clones, formed subfamilies. Nine of the eleven new plasmids were between 25.0 kb and 26.6 kb. The largest new plasmid was 36.5 kb. Although some of the new plasmids were incompatible, others were stably maintained in the same strain, albeit at different copy numbers (Prangishvili *et al.*, 1998). Towards the understanding of compatability, members of the pING family are being sequenced (Stedman *et al.*, manuscript in preparation).

Currently, all of the plasmids from the *Crenarchaeota* were from *Sulfolobales*. The plasmid size ranges from 5.3 kb to 41 kb. Most of the plasmids were maintained at a high copy number in the original host. Some plasmids had an initially high copy number after transferal into a new *Sulfolobus* isolate. However, the copy number decreased during subsequent growth of the isolate. None of the sequenced plasmids contained identifiable antibiotic resistance genes, and all of the plasmids were cryptic. For the most recent review, see Zillig *et al.*, 1998.

Insertion sequences and Transposons

Insertion sequences

Insertion sequences (IS) are self-contained genetic elements that are capable of insertion into new regions of DNA (Joset and Guespin-Michel, 1993). IS can also contain gene(s). Bacterial IS frequently encode transposases essential for transposition of the element. IS can also influence the expression of neighboring gene(s) outside of the element (Galas and Chandler, 1989). Many IS have been discovered in the Archaea.

Using libraries from *Halobacterium halobium* strains NRC-1 and R-1, over 50 IS families were identified on genomic DNA and plasmids (Sapienza and Doolittle, 1982). IS were proposed to contribute to the rapid rearrangements that occurred in the plasmids from this genus (Pfeifer *et al.*, 1988; Pfeifer and Blaseio, 1989). Three plasmids from *Halobacterium* species were found to contain insertion sequences (Dyall-Smith and Doolittle, 1994), and the 150 kb pHH1 from *H. halobium* strain NRC817 had seven different types of insertion sequences. Many of these elements were present in multiple copies on the plasmid (Derkacheva *et al.*, 1993). The 200 kb pNRC100 from *H. halobium* strain NRC-1 contained three of the same IS found in pHH1 plus one additional IS (Ng and DasSarma, 1993). Upon sequencing, no IS were identified in pGRB1 from *Halobacterium* strain GRB. Upon passage through *H. halobium* strain R-1, pGRB1 acquired an additional region of DNA that corresponded to a new IS, called ISH11. The 1,068 bp ISH11 was typical of the IS found in *H. halobium* species. It contained an inverted repeat of 15 bp at the terminal ends of the IS and an ORF that corresponded to 334 amino acids (Krebs *et al.*, 1990). Although a function was not proposed for the putative protein, other IS contained an ORF that appeared necessary for transposition (Pfeifer and Blaseio, 1990). ISH27 had observed transpositions into pHH4 and pHH6. When Northern hybridizations were performed on *H. halobium* ORF1167 from ISH27, messenger RNA for this ORF was detected in *H. halobium* (Pfeifer and Blaseio, 1990). Evidence for ORF1167's involvement in transposition was discovered during the analysis of ISH51 transposition events (see below). For reviews of individual elements see Derkacheva *et al.* (1993) and Pfeifer (1987).

Haloferax volcanii also contained IS. Of the 20-30 copies of the ISH51 found, five were located on the 86 kb plasmid pHV1 (Hofman *et al.*, 1986; Schalkwyk *et al.*, 1992). The ISH51 family was found to have at least three members (Hofman *et al.*, 1986; Pfeifer and Blaseio, 1990). ISH51-3, found by a recent transposition into pHV2, was the third ISH51 element sequenced (Pfeifer and Blaseio, 1990). ISH51-3 had 91% identity in 1200

nucleotides to ISH27-1 from the *H. halobium* plasmid pHH4. ISH51-3 contained a homolog of ORF1167, which was thought to be essential for transposition. Two members of the ISH51 family, ISH51-1 and ISH51-2, contained mutations that disrupted the ORF1167 homolog (Pfeifer and Blaseio, 1990). ISH51-1 and ISH51-2 were thought to be non-transposable elements (Hofman *et al.*, 1986). ISH51-3, which contained a 21 nucleotide deletion at the 3'-terminal end of ORF1167 homolog when compared to ISH27, was observed to transpose into pHV2 (Lam and Doolittle, 1989, Pfeifer and Blaseio, 1990).

Plasmids from methanogens were also found to contain insertion sequences. The first insertion sequences were found in pFV1 and pFZ1 from *Methanobacterium thermoformicicum* strains THF and Z-245, respectively. FR-I was an IS element that resided in pFV1 and not pFZ1 (Nölling *et al.*, 1992). This element was also found in the genomes of *Methanobacterium thermoautotrophicum* strain ΔH and four *Methanobacterium thermoformicicum* strains, but not strain Z-245. FR-I was 1,501 bp with direct repeats at both ends of the element. The direct repeats were the palindromic sequence of AAATTT. Although pFZ1 lacked FR-I, this sequence was in a similar position, and may represent the integration site for FR-I. Upon comparison to the genomic-derived elements from *M. thermoformicicum* strains THF and ΔH, two conserved regions, 1,118 bp box 1 and 383 bp box 2, were identified. Those regions encoded ORFs which didn't have homologs in the databases. The second IS sequence was located in both plasmids. FR-II was 2,510 bp in pFZ1 and 3,034 bp in pFV1. The difference in size was caused by the addition of a 524 bp direct repeat in pFV1. The ORFs found in FR-II didn't possess any homologs in the databases (Nölling *et al.*, 1993).

The genomic sequencing of *M. jannaschii* revealed a putative IS in pURB800 (Bult *et al.*, 1996). This IS belonged to a 11 member *M. jannaschii* family known as ISAMJ1. The pURB800 IS was named ISAMJ1-C, and was the smallest member of the family at 265 bp. ISAMJ1-C did not appear to encode a protein and had a 16 bp inverted repeat at the terminal regions of the element (Bult *et al.*, 1996).

To date, insertion elements have been discovered on only one plasmid from the *Crenarchaeota.* pNOB8 from *Sulfolobus* contained two putative insertion sequences. ORFs 406 and 413 appeared to encode transposases. ORF 406 had 89% identity at the amino acid level to the Ro2 elements from *Sulfolobus solfataricus* strain P2 but did not contain terminal inverted repeats (She *et al.*, 1998). The lack of repeats was also similar to the Ro2 elements from *S. solfataricus* strain P2 (Sensen *et al.*, 1996). Repetitive sequences are frequently involved in the transposition of insertion sequences. The second potential IS was ORF 413, which had 28% identity at the amino acid level to the IS256 family. The IS256 family is a group of transposases from bacteria. The putative IS256 homolog had an imperfect inverted repeat of 32 bp flanking the ORF (She *et al.*, 1998). Neither ORF 406 or ORF 413 were proven experimentally to be mobile insertion sequences.

Transposons

Transposons are genetic elements that are usually comprised of insertion sequences, genes required for transposition, and accessory gene(s) (Joset and Guespin-Michel, 1993). These mobile elements can be used to identify genes by insertional inactivation, add exogenous genes, or map the location of genes within a genome. Unfortunately, no natural archaeal transposons are known. Artificial transposons were developed for *Haloarcula hispanica* (Dyall-Smith and Doolittle, 1994). These transposons used the insertion sequences ISH2, ISH26, and ISH28 from *Halobacterium halobium.* These ISH elements were chosen because they had been sequenced, were very mobile, and didn't require a specific integration sequence. The *Haloferax volcanii* gene for mevinolin resistance was cloned into each IS, and the artificial transposon was ligated into an *Escherichia coli* plasmid. Upon transformation into *H. hispanica* and selection on medium containing mevinolin, no transformants were recovered with the element constructed from ISH2. However, the artificial elements constructed from ISH26 and ISH28 had average transformation frequencies of 60 and 840 CFUs per microgram of DNA. Subsequent experiments confirmed that *H. hispanica* did not contain ISH26- or ISH28-like elements

prior to transformation and that the transformants contained the artificial elements. Upon analysis of individual transpositions, some isolates appeared to have duplicated the transposon region, and in one case the *E. coli* plasmid was cointegrated. This cointegration was thought to occur after the primary integration event or possibly by transposition of a plasmid dimer (Dyall-Smith and Doolittle, 1994).

Archaeal Shuttle Vector Development and Use in Biotechnology

Archaeal shuttle vector development is important because assembly of an active enzyme may require an archaeon's unusual cofactors and enzymatic pathways. The vectors must contain replicons for *Escherichia coli* and at least one archaeon, genes that encode antibiotic resistance, and species-specific promoters for controlled expression of genes. There is significant interest in these vectors for both basic research and biotechnology.

Shuttle vectors are a recent occurrence for thermophiles and methanogens. A challenge for the thermophiles has been the identification of selectable markers and thermostable reporters. pAG1, the first thermophilic shuttle vector (Table 2), utilized the alcohol dehydrogenase (ADH) from the thermophile *Sulfolobus solfataricus* as a selectable marker. The vector was also found to replicate in *Pyrococcus furiosus* and *Sulfolobus acidocaldarius* (Aravalli and Garrett, 1997). A thermostable beta-galactosidase from *Sulfolobus solfataricus* was utilized as a reporter (Elferink *et al.*, 1996). For methanogens, the obstacle was the absence of transformation systems. Uptake of genomic DNA by *Methanococcus voltae*, for example, was inefficient with only 2-100 transformants/microgram of genomic DNA (Bertani and Baresi, 1987). Recently, *Methanococcus maripaludis* and *Methanosarcina acetiovorans*, using a polyethylene glycol or liposome-mediated methodology for the respective organism, were transformed with plasmid DNA (Tumbula *et al.*, 1997; Metcalf *et al.*, 1997). These transformations had efficiencies of 10^7 to 10^8 transformants/microgram of

plasmid using pDLT44 for *M. maripaludis* or pWM307 for *M. acetiovorans*. However, neither shuttle vector has been tested for *in vivo* heterologous gene expression. The plasmid pWLG30, which was similar to pDLT44, expressed the *E. coli* beta-galactosidase gene in *M. maripaludis* (Gardner and Whitman, 1999).

Ventosa and Nieto (1995) reviewed the potential applications of halophiles and their products. An example was the membrane protein bacteriorhodopsin. Bacteriorhodopsin formed a hexagonal array on the membrane surface and composed up to 75% of the membrane dry weight of *Halobacterium halobium*. This protein was a light-driven proton pump, and was used for ATP synthesis in *H. halobium* (Nicholls and Ferguson, 1992). Commercial products included the production of sensors and data storage devices. pMPK35, a shuttle vector used in *E. coli* and *H. halobium*, was able to express bacteriorhodopsin at 25-40% of wild type levels in *H. halobium* strains lacking the gene (Krebs *et al.*, 1991). This vector will allow intensive site directed mutagenesis studies with bacteriorhodopsin.

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References

Akhmanova AS, Kagramanova VK, and Mankin AS (1993) Heterogeneity of small plasmids from halophilic Archaea. *Journal of Bacteriology* **175**: 1081-1086.

Aravalli RN and Garrett RA (1997) Shuttle vectors for hyperthermophilic archaea. *Extremophiles* **1:** 183-191.

Benbouzid-Rollet N, López-García P, Watrin L, Erauso G, Prieur D, and Forterre P (1997) Isolation of new plasmids from hyperthermophilic Archaea of the order Thermococcales. *Research in Microbiology* **148:** 767-775.

Bertani G and Baresi L (1987) Genetic transformation in the methanogen *Methanococcus voltae* PS. *Journal of Bacteriology* **169:** 2730-2738.

Biavati B, Sgorbati B, and Palenzona D (1992) Plasmid DNA from methanogenic bacteria. *Current Microbiology* **24:** 285-287.

Blaseio U and Pfeifer F (1990) Transformation of *Halobacterium halobium*: Development of vectors and investigation of gas vesicle synthesis. *Proceedings of the National Academy of Science of the USA* **87:** 6772-6776.

Bokranz M, Klein A, and Meile L (1990) Complete nucleotide sequence of plasmid pME2001 of *Methanobacterium thermoautotrophicum* (Marburg). *Nucleic Acids Research* **18:** 363.

Bult CJ, White O, Olsen GJ, Zhou L, Fleischmann R D, Sutton GG, Blake JA, FitzGerald LM, Clayton RA, Gocayne JD, Kerlavage AR, Dougerty BA, Tomb JF, Adams MD, Reigh CI, Overbeek R, Kirkness EF, Weinstock KG, Merrick JM, Glodek A, Scott JL, Geoghagen NSM, Weidman JF, Fuhrmann JL, Nguyen D, Utterback TR, Kelley JM, Peterson JD, Sadow PW, Hanna MC, Cotton MD, Roberts KM, Hurst MA, Kaine BP, Borodovsky M, Klenk HP, Fraser CM, Smith HO, Woese CR, and Venter JC (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. *Science* **273:** 1017-1140.

Charlebois RL (1991) Detailed physical map and set of overlapping clones covering the genome of the archaebacterium *Haloferax volcanii* DS2. *Journal of Molecular Biology* **222:** 509-524.

Charlebois RL, Lam WL, Cline SW, and Doolittle WF (1987) Characterization of pHV2 from *Halobacterium volcanii* and its use in demonstrating transformation of an archaebacterium. *Proceedings of the National Academy of Science of the USA* **84:** 8530-8534.

Cline SW and Doolittle WF (1992) Transformation of members of the genus *Haloarcula* with shuttle vectors based on *Halobacterium halobium* and *Haloferax volcanii* plasmid replicons. *Journal of Bacteriology* **174:** 1076-1080.

DasSarma S and Arora P (1997) Genetic analysis of the gas vesicle gene cluster in haloarchaea. *FEMS Microbiology Letters* **153:** 1-10.

Derkacheva NI, Kagramanova VK, and Man'kin AS (1993) Genetic variability in halophilic archaebacteria (a review). *Molecular Biology* **27:**287-295.

Dyall-Smith ML and Doolittle WF (1994) Construction of composite transposons for halophilic Archaea. *Canadian Journal of Microbiology* **40:** 922-929.

Ebert K, Goebel W, and Pfeifer F (1984) Homologies between heterogeneous extrachromosomal DNA populations of *Halobacterium halobium* and four new halobacterial isolates. *Molecular and General Genetics* **194:** 91-97.

Elferink MGL, Schleper C, and Zillig W (1996) Transformation of the extremely thermoacidophilic archaeon *Sulfolobus solfataricus* via a self-spreading vector. *FEMS Microbiology Letters* **137:** 31-35.

Erauso G, Marsin S, Benbouzid-Rollet N, Baucher TBM-F, Zivanoic Y, Prieur D, and Forterre P (1996) Sequence of plasmid pGT5 from the archaeon *Pyrococcus abyssi*: Evidence for rolling-circle replication in a hyperthermophile. *Journal of Bacteriology* **178:** 3232-3237.

Erauso G, Reysenbach AL, Godfroy A, Meunier J-R, Crump B, Partensky F, Baross JA, Marteinsson V, Barbier G, Pace NR, and Prieur D (1993) *Pyrococcus abyssi* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *Archives of Microbiology* **160:** 338-349.

Fuchs T, Huber H, Burggraf S, and Stetter KO (1996) 16S rDNA-based phylogeny of the archaeal order Sulfolobales and reclassification of *Desulfurolobus ambivalens* as *Acidianus ambivalens* comb. nov. *Systematic and Applied Microbiology* **19:** 56-60.

Galas DJ, and Chandler M (1989) Bacterial insertion sequences. *In* Berg DE and Howe MM (eds) Mobile DNA. pp. 109-162. American Society for Microbiology, Washington, D.C.

Gardner WL and Whitman WB (1999) Expression vectors for *Methanococcus maripaludis*: overexpression of acetohydroxyacid synthase and β-galactosidase. *Genetics* **152**: 1439-1447.

Hackett NR, Krebs MP, DasSarma S, Goebel W, RajBhandary UL and Khorana HG (1990) Nucleotide sequence of a high copy number plasmid from *Halobacterium* strain GRB. *Nucleic Acid Research* **18**: 3408.

Hall MJ and Hackett NR (1989) DNA sequence of a small plasmid from *Halobacterium* strain GN101. *Nucleic Acids Research* **17:** 10501.

Hofman JD, Schalkwyk LC and Doolittle WF (1986) ISH51: a large, degenerate family of insertion sequence-like elements in the genome of the archaebacterium, *Halobacterium volcanii*. *Nucleic Acid Research* **14:** 6983-7000.

Holmes ML and Dyall-Smith ML (1990) A plasmid vector with a selectable marker for halophilic archaebacteria. *Journal of Bacteriology* **172**: 756-761.

Holmes ML, Pfeifer F, and Dyall-Smith ML (1995) Analysis of the halobacterial plasmid pHK2 minimal replicon. *Gene* **153:** 117-121.

Jannière L, Gruss A, and Ehrlich SD (1993) Plasmids. *In* Sonehshein AL, Hoch JA, and Losich R (eds), *Bacillus subtilis* and other gram positive bacteria: biochemistry, physiology, and molecular genetics. pp. 625-644. ASM, Washington DC.

Jeanthon C, L'Haridon S, Reysenbach AL, Corre E, Vernet M, Messner P, Sleytr UB, and Prieur D (1999) *Methanococcus vulcanius* sp. nov., a novel hyperthermophilic methanogen isolated from East Pacific Rise and identification of *Methanococcus* spp. DSM 4213 as *Methanococcus fervens* sp. nov. *International Journal of Systematic Bacteriology* **49**: 583-589.

Joset F and Guespin-Michel J (1993) Glossary. *In* Carr NG (ed), Prokaryotic Genetics: genome organization, transfer, and plasticity. pp. 433-441. Blackwell Scientific Publications, Boston.

Joshi JG, Guild WR, and Handler P (1963) The presence of two species of DNA in some halobacteria. *Journal of Molecular Biology* **6:** 34-38.

Kagramanova VK, Derckacheva NI, and Mankin AS (1988) The complete nucleotide sequence of the arcaebacterial plasmid pHSB from *Halobacterium*, strain SB3. *Nucleic Acids Research* **16**: 4158.

Keeling P, Klenk H-P, Singh RK, Feeley O, Schleper C, Zillig W, Doolittle WF, and Sensen CW (1996) Complete nucleotide sequence of the *Sulfolobus islandicus* multicopy plasmid pRN1. *Plasmid* **35:** 141-144.

Keeling PJ, Klenk H-P, Singh RK, Schenk ME, Sensen CW, Zillig W, and Doolittle WF (1998) *Sulfolobus islandicus* plasmids pRN1 and pRN2 share distant but common evolutionary ancestry. *Extremophiles* **2:** 391-393.

Kurosawa N, Itoh YH, Iwai T, Sugai A, Uda I, Kimura N, Horiuchi T, and Itoh T (1998) *Sulfurisphaera ohwakuensis* gen. nov., sp. nov., a novel extremely thermophilic acidophile of the order *Sulfolobales*. *International Journal of Systematic Bacteriology* **48**: 451-456.

Krebs MP, Hauss T, Heyn MP, Rajbhandary UL, and Khorana HG (1991) Expression of the bacterioopsin gene in *Halobacterium halobium* using a multicopy plasmid. *Proceedings of the National Academy of Science of the USA* **88:** 859-863.

Krebs MP, RajBhandary UL, and Khorana HG (1990) Nucleotide sequence of ISH11, a new *Halobacterium halobium* insertion element isolated from the plasmid pGRB1. *Nucleic Acids Research* **18:** 6699.

Kushner DJ (1978) Life in high salt and solute concentrations: halophilic bacteria. *In* Kushner DJ (ed), Microbial life in extreme environments. pp. 317-368. Academic Press, Inc., London.

Lam WL and Doolittle WF (1989) Shuttle vectors for the archaebacterium *Halobacterium volcanii*. *Proceedings of the National Academy of Science of the USA* **86:** 5478-5482.

Meile L, Kiener A, and Leisinger T (1983) A plasmid in the archaebacterium *Methanobacterium thermoautotrophicum*. *Molecular and General Genetics* **191:** 480-484.

Metcalf WW, Zhang JK, Apolinario E, Sowers KR, and Wolfe RS (1997) A genetic system for Archaea of the genus *Methanosarcina*: Liposome-mediated transformation and construction of shuttle vectors. *Proceedings of the National Academy of Science of the USA* **94:** 2626-2631.

Moore RL, and McCarthy BJ (1969) Characterization of the deoxyribonucleic acid of various strains of halophilic bacteria. *Journal of Bacteriology* **99:** 248-254.

Ng W-L and DasSarma S (1993) Minimal replication origin of the 200-kilobase *Halobacterium* plasmid pNRC100. *Journal of Bacteriology* **175:** 4584-4596.

Nicholls DG and Ferguson SJ (1992) Bacteriorhodopsin and the purple membrane of halobacteria. *In* Bioenergetics 2, pp.182-187. Academic Press INC. San Diego, CA.

Nölling J, v. Eeden FJM, Eggen RIL, and d. Vos WM (1992) Modular organization of related archaeal plasmids encoding different restriction-modification systems in *Methanobacterium thermoformicicum*. *Nucleic Acids Research* **20:** 6501-6507.

Nölling J, v. Eeden FJM, Eggen RIL, and d. Vos WM (1993) Distribution and characterization of plasmid-related sequences in the chromosomal DNA of different thermophilic *Methanobacterium* strains. *Molecular and General Genetics* **240:** 81-91.

Nölling J, Frijlink M and d. Vos WM (1991) Isolation and characterization of plasmids from different strains of *Methanobacterium thermoformicicum*. *Journal of General Microbiology* **137:** 1981-1986.

Nölling J and d. Vos WM (1992a) Characterization of the Archaeal, plasmid-encoded type II restriction-modification system *Mth*TI from *Methanobacterium thermoformicicum* THF: homology to the bacterial *Ngo*PII System from *Neisseria gonorrhoeae*. *Journal of Bacteriology* **174:** 5719-5726.

Nölling J, and d. Vos WM (1992b) Identification of the CTAG-recognizing restriction-modification systems *Mth*ZI and *Mth*FI from *Methanobacterium thermoformicicum* and characterization of the plasmid-encoded *mthZIM* gene. *Nucleic Acids Research* **20:** 5047-5052.

Pfeifer F (1987) Genetics of halobacteria. *In* Rodriguez-Valera F (ed), Halophilic bacteria. pp.105-133. CRC Press, Inc., Florida, USA. 2.

Pfeifer F and Blaseio U (1989) Insertion elements and deletion formation in a halophilic archaebacterium. *Journal of Bacteriology* **171:** 5135-5140.

Pfeifer F and Blaseio U (1990) Transposition burst of the ISH27 insertion element family in *Halobacterium halobium. Nucleic Acid Research* **18:** 6921-6925.

Pfeifer F, Blaseio U, and Ghahraman P (1988). Dynamic plasmid populations in *Halobacterium halobium. Journal of Bacteriology* **170:** 3718-3724.

Pfeifer F and Ghahraman P (1993) Plasmid pHH1 of *Halobacterium salinarium*: characterization of the replicon region, the gas vesicle gene cluster and insertion elements. *Molecular and General Genetics* **238:** 193-200.

Pfeifer F, Weidinger G, and Goebel W (1981) Characterization of plasmids in halobacteria. *Journal of Bacteriology* **145:** 369-374.

Prangishvili D, Albers S-V, Holz I, Arnold HP, Stedman K, Klein T, Singh H, Hiort J, Schweier A, Kristjansson JK, and Zillig W (1998) Conjugation in Archaea: frequent occurrence of conjugative plasmids in *Sulfolobus. Plasmid* **40:** 190-202.

Sapienza C and Doolittle WF (1982) Unusual physical organization of the *Halobacterium* genome. *Nature* **295:** 384-389.

Schalkwyk LC, Charlebois RL and Doolittle WF (1992) Insertion sequences on plasmid pHV1 of *Haloferax volcanii*. *Canadian Journal of Microbiology* **39:** 201-206.

Schleper C, Holz I, Janekovic D, Murphy J, and Zillig W (1995a) A multicopy plasmid of the extremely thermophilic archaeon *Sulfolobus* effects its transfer to recipients by mating. *Journal of Bacteriology* **177:** 4417-4426.

Schleper C, Puehler G, Holz I, Gambacorta A, Janekovic D, Santarius U, Klenk H-P, and Zillig W (1995b) *Picrophilus* gen. nov., fam. nov.: a novel aerobic, heterotrophic thermoacidophilic genus and family comprising Archaea capable of growth around pH 0. *Journal of Bacteriology* **177:** 7050-7059.

Schleper C, Pühler G, Klenk H-P, and Zillig W (1996) *Picrophilus oshimae* and *Picrophilus torridus* fam. nov., gen. nov., sp. nov., two species of hyperacidophilic, thermophilic, heterotrophic, aerobic Archaea. *International Journal of Systematic Bacteriology* **46:** 814-816.

Sensen CW, Klenk H-P, Singh RK, Allard G, Chan CC-Y, Liu QY, Penny SL, Young F, Schenk ME, Gaasterland T, Doolittle WF, Ragan MA, and Charlebois RL (1996) Organizational characteristics and information content of an archaeal genome: 156 kb of sequence from *Sulfolobus solfataricus* P2. *Molecular Microbiology* **22:** 175-191.

Segerer A, Neuner A, Kristjansson JK, and Stetter KO (1986) *Acidianus infernus* gen. nov., sp. nov., and *Acidianus brierleyi* comb. nov.: facultatively aerobic, extremely acidophilic thermophilic sulfur-metabolizing archaebacteria. *International Journal of Systematic Bacteriology* **36:** 559-564.

She Q, Phan H, Garrett RA, Albers S-V, Stedman KM, and Zillig W (1998) Genetic profile of pNOB8 from Sulfolobus: the first conjugative plasmid from an archaeon. *Extremophiles* **2:** 417-425.

Simon RD (1978) *Halobacterium* strain 5 contains a plasmid which is correlated with the presence of gas vacuoles. *Nature* **273**: 314-317.

Sioud M, Baldacci G, Forterre P, and de Recondo A-M (1988) Novobiocin induces accumulation of a single strand of plasmid pGRB-1 in the archaebacterium *Halobacterium* GRB. *Nucleic Acids Research* **16**: 7833-7842.

Smith DR, Doucette-Stamm LA, Deloughery G,Lee H, Dubois J, Aldredge T, Bashirzadeh R, Blakely D, Cook R, Gilbert K, Harrison D, Hoang L, Keagle P, Lumm W, Pothier B, Qiu D, Spadafora R, Vicaire R, Wang Y, Wierzbowski J, Gibson R, Jiwani N, Caruso A, Bush D, Safer H, Patwell D, Prabhakar S, McDougall S, Shimer G, Goyal A, Pietrokovski S, Church GM, Daniels CJ, Mao J-I, Rice P, Nölling J, and Reeve JN (1997) Complete genome sequence of *Methanobacterium thermoautotrophicum* ΔH: functional analysis and comparative genomics. *Journal of Bacteriology* **179:** 7135-7155.

Sowers KR and Gunsalus RP (1988) Plasmid DNA from the acetotrophic methanogen *Methanosarcina acetivorans*. *Journal of Bacteriology* **170:** 4979-4982.

Stetter KO (1996) Hyperthermophilic procaryotes. *FEMS Microbiology Reviews* **18:** 149-158.

Stettler R, Pfister P, and Leisinger T (1994) Characterization of a plasmid carried by *Methanobacterium thermoautotrophicum* ZH3, a methanogen closely related to *Methanobacterium thermoautotrophicum* Marburg. *Systematic and Applied Microbiology* **17:** 484-491.

Thomm M, Altenbuchner J, and Stetter KO (1983) Evidence for a plasmid in a methanogenic bacterium. *Journal of Bacteriology* **153:** 1060-1062.

Tumbula DL, Bowen TL, and Whitman WB (1997) Characterization of pURB500 from the Archaeon *Methanococcus maripaludis* and Construction of a Shuttle Vector. *Journal of Bacteriology* **179:** 2976-2986.

Ventosa A and Neito JJ (1995) Biotechnological applications and potentialities of halophilic microorganisms. *World Journal of Microbiology & Biotechnology* **11:** 85-94.

Wasserfallen A, Nölling J, Pfister P, Reeve J, and de Macario EC (1999) Phylogenetic analysis of 18 thermophilic *Methanobacterium* isolates supports the proposals to create a new genus, *Methanothermobacter* gen. nov., and to reclassify several isolates in three species, *Methanothermobacter thermautotrophicus* comb. nov., *Methanothermobacter wolfeii* comb. nov., and *Methanothermobacter marburgensis* sp. nov. *International Journal of Systematic Bacteriology* In Press.

Weidinger G, Klotz G, and Goebel W (1979) A large plasmid from *Halobacterium halobium* carrying genetic information for gas vacuole formation. *Plasmid* **2:** 377-386.

Whitman WB, Boone DR, and Koga Y (1999) Order *Methanococcales* Balch and Wolfe 1981, 216^{vp}. In Press in Bergey's Manual of Systematic Bacteriology.

Whitman, W. B., J. Shieh, S. Sohn, D. S. Caras, and U. Premachandran. 1986. Isolation and characterization of 22 mesophilic methanococci. *Systematic and Applied Microbiology* **7:** 235-240.

Woese CR and Fox GE (1977) Phylogenetic structure of the prokaryotic domain: The primary kingdoms. *Proceedings of the National Academy of Science of the USA* **74:** 5088-5090.

Woese CR, Kandler O, and Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Science of the USA* **87:** 4576-4579.

Wood AG, Whitman WB, and Konisky J (1985) A newly-isolated marine methanogen harbors a small cryptic plasmid. *Archives of Microbiology* **142:** 259-261.

Yasuda M, Yamagishi A, and Oshima T (1995) The plasmids found in isolates of the acidothermophilic archaebacterium *Thermoplasma acidophilum*. *FEMS Microbiology Letters* **128:** 157-162.

Zhao H, Wood AG, Widdel F, and Bryant MP (1988) An extremely thermophilic *Methanococcus* from a deep sea hydrothermal vent and its plasmid. *Archives of Microbiology* **150:** 178-183.

Zillig W, Arnold HP, Holz I, Prangishvili D, Schweier A, Stedman K, She Q, Phan H, Garrett R, and Kristjansson JK (1998) Genetic elements in the extremely thermophilic archaeon *Sulfolobus. Extremophiles* **2:** 131-140.

Zillig W, Kletzin A, Schleper C, Holz I, Janekovic D, Hain J, Lanzendörfer M, and Kristjansson JK (1994) Screening for *Sulfolobales*, their plasmids and their viruses in Icelandic solfataras. *Systematic and Applied Microbiology* **16:** 609-628.

Zillig W, Prangishvilli D, Schleper C, Elferink M., Holz I., Albers S., Janekovic D., and Götz D (1996) Viruses, plasmids and other genetic elements of thermophilic and hyperthermophilic *Archaea. FEMS Microbiology Reviews* **18:** 225-236.

Zillig W, Yeats S, Holz I, Böck A, Gropp F, Rettenberger M, and Lutz S (1985) Plasmid-related anaerobic autotrophy of the novel archaebacterium *Sulfolobus ambivalens*. *Nature* **313:** 789-791.

Zillig W, Yeats S, Holz I, Böck A, Rettenberger M, Gropp F, and Simon G (1986) *Desulfurolobus ambivalens*, gen. nov., sp. nov., an autotrophic archaebacterium facultatively oxidiing or reducing sulfur. *Systematic and Applied Microbiology* **8:** 197-203.

Table 1 Sequenced archaeal plasmids

Host(s)[a]	Plasmid	Size (bp)	Accession number
Methanosarcina acetivorans strain C2A*	pC2A	5,467	U78295
Methanobacterium thermoautotrophicum strain Marburg*	pME2001	4,439	X17205
Methanobacterium thermoformicicum strain THF*	pFV1	13,513	X68366
Methanobacterium thermoformicicum strain Z-245*	pFZ1	11,014	X68367
Methanococcus jannaschii strain JAL1*	pURB800/ MJECL	58,407	L77118
Methanococcus jannaschii strain JAL1*	pURB801/ MJECS	16,550	L77119
Methanococcus maripaludis strain C5*	pURB500	8,285	U47023
Halobacterium strain GN101*	pHGN1	1,765	X16460
Halobacterium strain GRB* *Halobacterium halobium* strain R1	pGRB1	1,781	X52610
Halobacterium strain SB3*	pHSB1	1,735	X07128
Halobacterium strain SB3* *Halobacterium halobium* strain R1	pHSB2	1,781	X66324
Haloferax volcanii strain DS2*	pHV2	6,354	J03014
Pyrococcus abyssi strain GE5*	pGT5	3,444	U49503
Acidianus ambivalens strain LEI10*	pDL10/ pSL10	7,598	AJ225333
Sulfolobus islandicus strain REN1H1* *Sulfolobus solfataricus* strain P1	pRN1	5,350	U36383
Sulfolobus islandicus strain REN1H1*	pRN2	6,959	U93082
Sulfolobus isolate NOB8-H2*	pNOB8	41,229	AJ010405

[a]Asterisk indicates source.

Table 2 Engineered vectors for the Archaea

Plasmid	Size (kb)	Host(s)
pAG1	4.730	*Pyrococcus furiosus* *Sulfolobus acidocaldarius* *Escherichia coli* strain JM109
pDLT44	12.691	*Methanococcus maripaludis* strain JJ *Escherichia coli* strain XL1-Blue MRF'
pMPK35	4.5	Halobacterium *halobium* strain R1 *Halobacterium* strain GRB *Escherichia coli*
pUBP2	12.3	*Halobacterium halobium* *Haloferax volcanii* strain WFD11 *Haloarcula hispanica* *Haloarcula vallismortis* *Escherichia coli* strain DH5α
pWL102	10.5	*Haloferax volcanii* strain DS2 *Haloarcula hispanica* *Haloarcula vallismortis* *Halobacterium halobium* strain PO3 *Escherichia coli* strain DH5α
pWM307	8.715	*Methanosarcina acetivorans* strain C2A *Methanosarcina barkeri* strains Fusaro, MS, W *Methanosarcina mazei* strain S-6 *Methanosarcina thermophilia* strain TM-1 *Methanosarcina siciline* strain C2J *Methanosarcina* spp. WH1 and WH2 *Escherichia coli* strain DH5α/λpir

CHAPTER III

EXPRESSION VECTORS FOR *METHANOCOCCUS MARIPALUDIS*: OVEREXPRESSION OF ACETOHYDROXYACID SYNTHASE AND ß-GALACTOSIDASE[2]

[2] Gardner, W. L. and W. B. Whitman. 1999. Genetics. 152:1439-1447.

ABSTRACT

A series of integrative and shuttle expression vectors were developed for use in *Methanococcus maripaludis*. The integrative expression vectors contained the *Methanococcus voltae* histone promoter and multiple cloning sites designed for efficient cloning of DNA. Upon transformation, they can be used to overexpress specific homologous genes in *M. maripaludis*. When tested with *ilvBN*, which encodes the large and small subunits of acetohydroxyacid synthase, transformants possessed 13-fold higher specific activity than the wild type. An expression shuttle vector, based on the cryptic plasmid pURB500 and the components of the integrative vector, was also developed for the expression of heterologous genes in *M. maripaludis*. The ß-galactosidase gene from *Escherichia coli* was expressed to about 1% of the total cellular protein using this vector. During this work, the genes for the acetohydroxyacid synthase (*ilvBN*) and phosphoenolpyruvate synthase (*ppsA*) were sequenced from a *M. maripaludis* genomic library.

INTRODUCTION

Methanogens are important catalysts in the global carbon cycle. These strictly anaerobic archaea are the source of most of the earth's atmospheric methane, a potent greenhouse gas, and are responsible for processing 1-2% of the carbon fixed every year during photosynthesis (REEBURGH *et al.* 1993). Given the enormity of this biogeochemical process, it is not surprising that methanogens are found in many different types of environments, ranging from psychrophilic to hyperthermophilic conditions (for a review see BOONE *et al.* (1993)). In this regard, they are the most cosmopolitan archaea-- abundant in extreme environments such as deep-sea hydrothermal vents as well as temperate environments such as the gastrointestinal tracts of man and animals.

Methanococcus maripaludis strain JJ is typical of other hydrogenotrophic methanococci. It was isolated from the sediment of a salt water marsh and grows by the conversion of carbon dioxide and hydrogen gas or formate into methane (JONES *et al.*

1983a). Amino acids and acetate are assimilated, but they are not significantly metabolized, indicating that methanogenesis is the sole or major energy source (WHITMAN *et al.* 1987). An important characteristic of *M. maripaludis* is its relatively rapid doubling time of 2.3 hours at the optimal temperatures of 35-39° (JONES *et al.* 1983a). Thus, it is one of the fastest growing mesophilic methanogens.

M. maripaludis has also proven amenable to genetic analysis (TUMBULA and WHITMAN 1999). Integrative and shuttle vectors have been developed for *Methanococcus* species (COHEN-KUPIEC *et al.* 1997; GERNHARDT *et al.* 1990; TUMBULA *et al.* 1997). These vectors are efficiently transformed into *M. maripaludis* by a polyethylene glycol-based method (TUMBULA *et al.* 1994). Like other methanogens, *M. maripaludis* contains many oxygen-sensitive enzymes with complex prosthetic groups. These enzymes are frequently not expressed in an active form in *E. coli*. Thus, a genetic system in *M. maripaludis* might be suitable for the expression of genes encoding these types of enzymes. We report here the development of expression shuttle and integrative vectors for *M. maripaludis* that use the *M. voltae* histone promoter, P_{hmvA}.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions: *Methanococcus maripaludis* JJ was obtained from W. J. Jones. The plasmids used in this study are listed in Table 1. *M. maripaludis* was grown at 37° on 275 kPa of H_2:CO_2 (80:20) in McN (mineral medium), McC (complex medium minus the vitamin solution) or McNA (McN plus 10 mM sodium acetate) (WHITMAN *et al.* 1986). The sodium bicarbonate in the medium was reduced from 5 g l^{-1} to 2 g l^{-1} during growth at 100 kPa in 1 L Wheaton bottles. Transformation of *M. maripaludis* with plasmid DNA was described previously (TUMBULA *et al.* 1994). For selection of puromycin resistant methanococci, a stock solution of 500 μg ml^{-1} puromycin dihydrochloride (Sigma) in distilled water was filter-sterilized and added to the medium at a final concentration of 2.5 μg ml^{-1}. Strictly anaerobic techniques were used for medium preparation and cultivation.

The *E. coli* strains were grown at 37° on low salt Luria-Bertani (LB) medium with the NaCl at 50% of the regular concentration (MANIATIS *et al.* 1982). Antibiotic concentrations were 60 μg ml^{-1} for ampicillin and 50 μg ml^{-1} for kanamycin in both liquid and solid LB medium. ß-galactosidase was used as a blue/white screen on LB agar plates with isopropylthio-ß-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) according to SAMBROOK *et al.* (1989). Plasmids were transformed into *E. coli* strains by electroporation with a Bio-Rad Gene Pulser (Bio-Rad Laboratories) using the settings of 200 Ω, 2.5 kV, and 25 μF with cuvettes (0.2 cm gap width).

Growth of *E. coli* and *M. maripaludis* was measured at 600 nm with a spectrophotometer (Spectronic 20).

Plasmid purification from *M. maripaludis* and *E. coli*: Plasmid isolation from *M. maripaludis* was begun by centrifuging a 5 ml culture at 16000 *g* at 4° followed by resuspension of the cells in 100 μl of methanococcal medium. After resuspension, the Wizard miniprep kit (Promega) minus the resuspension step was used for preparation of the plasmid DNA for transformation into *E. coli*. pURB500 was isolated from *M. maripaludis* C5 as described previously (TUMBULA *et al.* 1994). Plasmids from *E. coli*, grown with selective conditions, were isolated with the Wizard miniprep kit.

Construction of *M. maripaludis* genomic library: *M. maripaludis* genomic DNA was isolated using a modified procedure from SAITO and MIURA (1963). *M. maripaludis* was grown in 150 ml of McN to an A_{600}=0.8. The cells were harvested by centrifugation at 7700 *g* and 4° for 15 minutes in a Beckman JA-21 rotor and washed once in 28 ml of sterile 0.4 M NaCl. The cell pellet was then resuspended in 3 ml of 0.15 M NaCl + 0.1 M EDTA (pH=8.0). The cell suspension was frozen by plunging the centrifuge tube into an ethanol/dry ice bath. The frozen suspension was slowly thawed at 18°, at which time the cells lysed. The cell extract was then transferred to a Corex tube, and 5 ml of 50% phenol saturated with 0.15 M NaCl/0.1 M EDTA (pH=8.0) and 50%

chloroform:isoamyl alcohol (24:1 v/v) were added. The solution was gently mixed by inversion prior to centrifugation at 8200 *g* for 10 minutes. The red aqueous phase was extracted with another 5 ml of phenol/chloroform/isoamyl alcohol. Two volumes of cold 100% ethanol were then added to the aqueous phase, and the suspension was gently inverted before centrifugation at 4° for 15 minutes at 8200 *g*. The supernatant was decanted, and the pellet was dried at room temperature. The pellet was resuspended in 100 microliters of 15 mM NaCl/1.5 mM trisodium citrate (SSC) and stored at 4°. Additional SSC was added every 24 hours until the DNA was resuspended.

To make a genomic library for *M. maripaludis*, the genomic DNA (12 µg in 100 microliters) was partially digested with 0.01 units of *Sau*3A I at 37° for 5 minutes. The reaction was terminated by the addition of phenol/chloroform/isoamyl alcohol (pH=8.0, Ameresco). The resulting linear DNA fragments ranged from 1 to 6 kilobase pairs (kbp) by gel electrophoresis. The DNA was precipitated upon the addition of one-half volume of 7.5 M ammonium acetate and two volumes of cold 100% ethanol followed by centrifugation. After decanting the supernatant, the pellet was resuspended in 10 µl of distilled water. Five µg of digested DNA (5 microliters) were ligated with 2 µl of Zap Express (Stratagene) that was predigested with *Bam*H I in a 10 µl reaction. After the ligation, the mixture was packaged and amplified once in *E. coli* XLOLR (Stratagene) before storage at -80° according to the company's directions.

Isolation and sequencing of the *M. maripaludis ilvBN*: The *M. maripaludis* genomic library was screened with a polymerase chain reaction (PCR) product of the acetohydroxyacid synthase (AHAS) large subunit gene (*ilvB*) from *Methanococcus aeolicus* using pTLB30 as the template. The PCR contained the primers 5'-GAACGGAGCAGAGGC-3' and 5'-ACCAACCATACCAAGGGC-3', 5 units *Taq* DNA polymerase, 1 mM dATP, 1 mM dGTP, 1 mM dCTP, 0.65 mM dTTP, and 0.35 mM digoxigenin-deoxyuridine-5'-triphosphate (DIG-dUTP) from Boehringer Mannheim. The annealing temperature was 48°, and the extension time was 2 minutes at 72°. This DIG-

dUTP labeled PCR product was used to screen plaque lifts from the library according to Boehringer Mannheim protocols. The hybridization temperature was 63°. The isolated phagemid was then converted to the plasmid form (pWLG1). *Sac* I was used to subclone a 3.8 kbp fragment of pWLG1. This fragment was ligated into a *Sac* I digested pUC18 yielding pWLG3. The remaining portion of pWLG1 was religated together to form pWLG2. These three plasmids were sequenced by primer walking utilizing API sequencers at the Molecular Genetics Facility, University of Georgia. All oligonucleotides were made by Integrated DNA Technologies (Coralville, Iowa). Sequence analysis was performed with the GCG software package (Genetics Computer Group, Madison, WI). FRAMES was used to identify open reading frames (ORFs) using ATG, GTG, or TTG as the start codons. GAP was used to calculate the percent identities between the methanococcal proteins. PILEUP was used to identify the truncated regions for *ppsA* and *ilvN.*

Plasmid construction: The integrative vectors developed in this study were based on pMEB.2 (Figure 1). To remove the alpha-complementation fragment of *lacZ,* pMEB.2 was digested with *Nde* I and *Sac* I followed by mung bean nuclease treatment and blunt end ligation (Figure 1). This deletion allowed a unique *Nde* I site to be introduced into the multiple cloning site (MCS). The MCS was added following digestion of the plasmid with *Eco*R V and *Eco*R I and dephosphorylation with CIAP (calf intestine alkaline phosphatase). The phosphorylated oligonucleotides, 5'-AATTCAAGCATCATATGAAGCATACGCGTCTTAAGAGATCTCATGAT-3' and 5'-ATCATGAGATCTCTTAAGACGCGTATGCTTCATATGATGCTTG-3', were annealed and ligated into the vector forming pWLG11. The *Eco*R I fragment of Mipuid, which contained the *Methanococcus voltae* histone promoter (P_{hmvA}) and ribosome binding site and the *E. coli* ß-glucuronidase (*uidA*), was then cloned into the *Eco*R I site of pWLG11. Removal of *uidA* by *Nde* I digestion formed the *M. maripaludis* integrative expression vector pWLG13. To form pWLG13+5'-*ilvB,* the first 641 base pairs of the *M. maripaludis ilvB* was cloned into pWLG13. First, the 5' end of *ilvB* was amplified by PCR

using the primers 5'-AAAAAAACATATGAAAGGAGCAGAGGCTATGATG-3' and 5'-AAAAAAAGATCTCCGCCTGCAATAATAACAGGTCTT-3'. These primers contained *Nde* I and *Bgl* II sites at the 5' and 3' ends respectively. The PCR contained 2.5 units of *Pfu* DNA polymerase (Stratagene), and was performed an annealing temperature of 55^{o} for 1 minute and an extension time of 2 minutes at 72^{o}. The PCR product was drop dialyzed against distilled water with VSWP membranes (Millipore) before digestion with *Nde* I and *Bgl* II for 4 hours. The enzymes in the reactions were removed using the Wizard clean-up kit (Promega).

Following digestion of pWLG13 with *Nde* I and *Bgl* II, the PCR product was ligated in to form pWLG13+5'-*ilvB.* The sequence of the PCR product was confirmed by sequencing with the primers that flanked the insertion site, pMEB.2seq1, 5'-AGGCACCCCAGGCTTTACAC-3' (5' end), and pMEB.2seq2, 5'-GCGTTTTTTATTACCTACTA-3' (3' end).

To replace the *Nde* I site near the histone promoter of pWLG13 with a *Nsi* I site, the promoter was PCR amplified using the primers 5'-ATCTGCGAATTCAGCTGATCGATCAAAATATAACATAAATAACATAGGTTTAA-3' (P_{hmvA}*Nsi*I1) and 5'-GAAGATCTTCAGCGCTAAACATGCATTTCACCTATTAGTTATCTATAAAATTATA-3' (P_{hmvA}*Nsi*I2). The first 12 nucleotides of P_{hmvA}*Nsi*I1 contained an *Eco*R I site and 5' extension for *Eco*R I digestion. Restriction sites for *Pvu* II and *Cla* I were also added immediately upstream of the *M. voltae* histone promoter. P_{hmvA}*Nsi*I2 possessed a *Bgl* II site plus a two nucleotide extension that allowed endonuclease digestion. The PCR annealing temperature was 43^{o}, and the extension time was 1 minute at 72^{o}. The PCR product was digested with *Eco*R I and *Bgl* II for directed ligation into pWLG11, which had been digested with the same enzymes and treated with CIAP. The product, pWLG14, contained the *Nsi* I site as part of the start codon downstream of the histone promoter.

The *E. coli lacZ* gene was engineered by PCR for ligation into pWLG14. The PCR contained pTer7 as the template and the primers 5'-CCAATGCATGACCATGATTACGGATTCACTGG-3' and 5'-GAAGATCTTTCCTTACGCGAAATACGGGCAG-3' to introduce flanking *Nsi* I and *Bgl* II sites. It was performed with 2.5 units of *Taq* DNA polymerase and an annealing temperature of 52.3° and an extension time of 3.75 minutes at 72°. The *lacZ* product was cloned into pGEM-T, yielding pGEM-T+*lacZ.* This vector was digested with *Nsi* I and *Bgl* II to release the *lacZ* fragment. After treatment of pWLG14 with *Nsi* I and *Bgl* II followed by CIAP, the *lacZ* fragment was ligated in to yield pWLG18. Upon electroporation into *E. coli* XL1-Blue MRF' (Stratagene), pWLG18 was identified by ß-galactosidase production on plates that contained X-gal and IPTG.

pWLG30, the expression shuttle vector, was constructed by ligation of a partial *Eco*R I digestion of pURB500 with a complete *Eco*R I digestion of pWLG14 (Figure 2). The ligation mixture was then transformed into *M. maripaludis*. The transformants were then inoculated into McC + puromycin broth to select for plasmids that contained both a methanococcal origin of replication from pURB500 and the *pac* cassette from pWLG14. Plasmids were then prepared from the broth culture of the transformants and electroporated into *E. coli* XL1-Blue MRF'. The location and orientation of pWLG14 within pURB500 was determined by digestion with *Sac* II and *Eco*O109 I and confirmed by sequencing. To form pWLG30+*lacZ*, pWLG30 was digested with *Nsi* I, *Bgl* II, and *Xba* I. *Bgl* II lowered the background of the plasmids that were digested by only *Nsi* I or *Xba* I. pWLG18 was digested with *Nsi* I and *Xba* I, and the *Nsi* I-*Xba* I fragment containing *lacZ* was gel purified. After ligation and transformation into *E. coli* XL1-Blue MRF', pWLG30+*lacZ* was found by screening for blue colonies on LB+ampicillin+X-Gal plates. The sequencing primers designed for confirming cloned DNA in pWLG30 or pWLG30+*lacZ* were 5'-ACTCTCCAGAATACATAAAA-3' (pWLG30seq1) and pMEB.2seq2.

Assays of acetohydroxyacid synthase (AHAS) activity: The wild type strain JJ1 or a strain transformed with pWLG13+5'-*ilvB* was grown in 100 ml of McNA in modified Wheaton bottles at 100 kPa of H_2:CO_2 to an A_{600}=0.60-0.75. The cells were harvested by centrifugation under anaerobic conditions (SHIEH *et al.* 1988). The pellets were resuspended in 100 µl of dilution solution per 100 ml of medium. The dilution solution, which was sparged with N_2 for 1 hour, contained 1 mM cysteine hydrochloride, 1 mM DL-dithiothreitol, 1 mg bovine pancreatic DNase I (Boehringer Mannheim), 7.5 ml NaCl solution (293 g/L), 50 ml distilled water, and 50 ml general salts solution (WHITMAN *et al.* 1986). The cells were placed in an aluminum seal vial on ice and flushed with hydrogen gas for 2 minutes before freezing at -20° for 2 hours. Upon thawing, the cells had lysed, and the cell extract was dialyzed in 12-14,000 MWCO tubing overnight at 4°. The dialysis buffer was the basic purification buffer that had been sparged with N_2 and autoclaved (XING and WHITMAN 1994). The AHAS assays were performed at 37° as previously described (PARK *et al.* 1995). For protein quantification by the bicinchoninic acid protein assay (Pierce), the dialyzed extract was first diluted 1:50 in 0.1 M sodium hydroxide, boiled for 10 minutes, and cooled on ice.

Assays of ß-galactosidase activity: The ß-galactosidase assay was performed with 2-nitrophenyl-ß-D-galactopyranoside (ONPG) and a temperature of 37° (MILLER 1992). *E. coli* XL1-Blue MRF' containing pWLG30+*lacZ* was grown overnight in low salt LB broth medium + ampicillin without IPTG. These cells were inoculated into fresh LB broth + ampicillin with and without 1 mM IPTG and grown to A_{600}=0.64-0.70 before being assayed. *M. maripaludis* was grown on McC medium + puromycin unless stated otherwise. *M. maripaludis* assays used the same conditions as *E. coli*.

SDS-PAGE: Performed according to the method of LAEMMLI (1970) for a 10% polyacrylamide gel.

Nucleotide sequence accession numbers: The GenBank accession numbers for the *M. maripaludis ilvBN* and *ppsA* were AF118061 and AF118060, respectively. The

accession numbers for pWLG11, pWLG13, pWLG14, and pWLG30 were AF134196, AF134197, AF134198, and AF134199, respectively.

RESULTS AND DISCUSSION

Cloning of the *ilvBN* genes of *M. maripaludis*: The two subunits of acetohydroxyacid synthase (AHAS), which catalyzes an early step in branched-chain amino acid biosynthesis, are encoded by the *ilvBN* genes in *Methanococcus aeolicus* (BOWEN *et al.* 1997). Screening of the *M. maripaludis* genomic library identified one plaque that hybridized with the *M. aeolicus ilvB* probe. Upon conversion of the phagemid to the plasmid form, the plasmid pWLG1 was found to contain about 7 kbp of *M. maripaludis* genomic DNA. A total of 4838 base pairs (bp) was sequenced, and three ORFs with homology to known genes were identified (Figure 3A). Two ORFs encoded the large and small AHAS subunits (E.C. 4.1.3.18), *ilvB* and *ilvN*, respectively. The deduced amino acid sequence of the large subunit (IlvB), which contained 587 amino acids, had 68.1%, 72.9%, and 31.4% sequence identity to the *M. aeolicus*, *Methanococcus jannaschii* (MJ0227), and *M. jannaschii* (MJ0663) IlvB homologs. The low amino acid sequence similarity to MJ0663 was consistent with the assignment of this ORF to another function (BOWEN *et al.* 1997). Fourteen nucleotides separated *ilvB* from *ilvN*. Upon comparison to the *M. aeolicus* gene, *ilvN* appeared to be truncated at the 3' end to form a deletion equivalent to eight amino acids. This conclusion was supported by the presence of a *Sau*3A I site and the beginning of the pBK-CMV vector before a stop codon in the *ilvN* sequence. The deduced amino acid sequence of the small subunit (IlvN) from *M. maripaludis* was 71% and 73% identical to the *M. aeolicus* and *M. jannaschii* IlvN homologs. The *ilvBN* genes from *M. aeolicus*, which were separated by 172 bp, and *M. maripaludis* were linked (Bowen *et al.* 1997). In contrast, the *M. jannaschii* AHAS genes were unlinked (BULT *et al.* 1996).

Enzyme activity corresponding to a phosphoenolpyruvate synthase (E.C. 2.7.9.2; pyruvate, water dikinase) has been described in cellular extracts of *M. maripaludis* (SHIEH and WHITMAN 1987), and a gene encoding phosphoenolpyruvate synthase (*ppsA*) appeared

immediately upstream of *ilvB* . When compared to the *M. jannaschii*, *Pyrococcus furiosus*, and *Staphylothermus marinus* homologs, the *M. maripaludis* PpsA was between 29 to 38 amino acids shorter at the N-terminus. In addition, an initiation codon was not observed at the 5'-end of the cloned ORF, but a *Sau*3A I site was present, suggesting that the cloned *ppsA* had been truncated and that the cloned DNA in pWLG1 was chimeric (Figure 3A). Alternatively, it is also possible that the *ppsA* cloned was a pseudogene naturally present in the genome. In contrast, *ilvB* did not appear to be truncated because the 5'-end of the gene encoded an amino acid sequence nearly identical to the N-terminal sequence of the *M. aeolicus* AHAS (XING and WHITMAN 1994). The *M. maripaludis* PpsA was 72% identical at the amino acid level to the *M. jannaschii* PpsA (MJ0542). The *M. jannaschii ppsA* contained an internal insertion that translated into a putative intein of 412 amino acids (BULT *et al.* 1996). At the intein/extein junction, a mechanistically important cysteinyl residue was found in the first position of the second extein (CHONG *et al.* 1996, BULT *et al.* 1996, Figure 3B). The deduced amino acid sequence of the *M. maripaludis* PpsA was nearly identical to the *M. jannaschii* homolog near the intein/extein junction, and the mechanistically important cystienyl residue was conserved. However, the putative intein was absent.

Overexpression of *ilvBN* in *M. maripaludis*: AHAS is normally expressed at the low levels typical of biosynthetic genes within the methanococci (XING and WHITMAN 1987). Because this enzyme is both unstable and O_2-labile, it would be much easier to characterize biochemically if it could be overexpressed. To overexpress this gene, pWLG13+5'-*ilvB* was constructed. In this plasmid, the start codon of *ilvB* overlapped with the *Nde* I site 4 bp downstream of the ribosome binding site and promoter (P_{hmvA}) for the *M. voltae* histone (Figure 1). This plasmid also contained 641 bp of the 5'-end of *ilvB* to allow for efficient homologous recombination into the genomic copy of *ilvB.* Following transformation into *M. maripaludis*, integration of the pWLG13+5'-*ilvB* into the genome would place a truncated *ilvB* gene under control of the native promoter and place the intact *ilvBN* operon under the control of P_{hmvA}. To test this hypothesis, the specific activity of AHAS in extracts

of a pWLG13+5'-*ilvB* transformant was determined (Table 2). Compared to extracts of wild type cells, AHAS specific activity was 13-fold higher in the transformant. The anticipated specific activity was calculated from the expression of the *Methanothermus fervidus* histone. In this organism, the number of histone molecules was 1-2 x 10^4 molecules per genome, and the genome possessed two genes (STROUP and REEVE 1992). The level of expression per gene would then be expected to be about 0.5-1 x 10^4 molecules. Based upon the specific activity and molecular weight of the *M. aeolicus* AHAS (XING and WHITMAN 1994), the same level of expression of the *M. maripaludis ilvBN* operon would yield a specific activity of 0.08-0.17 units (mg of protein)$^{-1}$, or very close to the observed value. This result also demonstrated that the *M. voltae* promoter was active in *M. maripaludis*.

To facilitate cloning into the integration vectors, pWLG18 was constructed with *lacZ* downstream of the P_{hmvA} to provide an opportunity for blue/white screening of clones in *E. coli* (Figure 1). Because *lacZ* contains a *Nde* I site, pWLG18 was constructed with an *Nsi* I site immediately downstream of the P_{hmvA} and overlapping the start codon. The availability of a vector with a *Nsi* I site also provides an alternative method for cloning genes that possess *Nde* I sites near their 5'-end and would not be suitable for cloning in pWLG13.

Construction of the expression shuttle vector: To express heterologous genes in *M. maripaludis*, an expression shuttle vector was constructed from pWLG14 and pURB500 (Figure 2). pURB500 is a cryptic plasmid from *M. maripaludis* C5 and contains a methanococcal origin of replication, an *Nde* I site, and three *Eco*R I sites (TUMBULA *et al.* 1997). The *Nde* I site precluded use of this same site near the P_{hmvA}. Therefore, pWLG14 was chosen because it contained a *Nsi* I site downstream of P_{hmvA}. The expression vector was then constructed by ligation of a partial *Eco*R I digestion of pURB500 into pWLG14 and transformation of *M. maripaludis* to select for the methanococcal origin of replication and puromycin resistance. Plasmids isolated from two independent transformants contained the same *Sac* II and *Eco*O109 I endorestriction maps,

indicating that pWLG14 was inserted in the same *Eco*R I site of pURB500 as utilized in pDLT44 (Figure 2, TUMBULA *et al.* 1997). The expression shuttle vector pWLG30 was then further modified by adding *lacZ* to allow for blue/white screening in *E. coli* (Figure 2).

E. coli XL1-Blue MRF' containing pWLG30+*lacZ* produced blue colonies in the presence of X-gal. The production of ß-galactosidase was independent of the addition of IPTG, indicating that expression was not from the *lac* promoter. Enzymatic assays of *E. coli* in LB broth plus ampicillin detected ß-galactosidase activity slightly above background levels. Weak expression with P_{hmvA} in *E. coli* was also observed by BENEKE *et al.* (1995) using Mipuid. Like pDLT44, pWLG30 transformed *M. maripaludis* with a high efficiency even though the plasmid yields were low from *E. coli*. pWLG30, isolated from *M. maripaludis*, was checked for rearrangements after transformation back into *E. coli*. No rearrangements were observed.

Expression of *lacZ* in *M. maripaludis* and *E. coli*: The expression shuttle vector pWLG30 was tested for *in vivo* expression of the ß-galactosidase gene (*lacZ*) from *E. coli*. *M. maripaludis* does not contain ß-galactosidase, and activity was not detectable in wild-type cells (COHEN-KUPIEC *et al.* 1997). However, ß-galactosidase was readily detectable in cell free extracts of a transformant bearing pWLG30+*lacZ* (Table 3). A protein band corresponding to the expected size of ß-galactosidase was found by SDS-PAGE in the transformants of pWLG30+*lacZ* but not in cells transformed with pWLG30 alone (Figure 4).

The specific activity of ß-galactosidase varied with growth phase of the culture. During growth on H_2:CO_2, exponential growth was usually observed only at low cell densities, i.e. A_{600} <0.4 (JONES *et al.* 1983b). At higher cell densities, the rate of H_2 transfer from the gas to liquid phases probably limited growth, and arithmetic growth was observed (Figure 5A). In cultures of the transformant, the specific activity of ß-galactosidase increased 3-fold from exponential to stationary phase. During stationary phase, where absorbance (600 nm)=1, the ß-galactosidase specific activity was 7.5 micromol min^{-1} (mg of

protein)$^{-1}$. Purified ß-galactosidase has a specific activity of 600 micromol min^{-1} (mg of protein)$^{-1}$ (Boehringer Mannheim). Thus the ß-galactosidase appeared to represent about 1% of the cellular protein of the transformant. Whether or not the increase in specific activity was due to changes in the level of expression from P_{hmvA} or in the copy number of the plasmid was not determined.

The stability of pWLG30 in methanococci is important for production of large amounts of recombinant protein. In one experiment, pWLG30+*lacZ* was transferred every three weeks in McC+puromycin medium for 3 months. Upon electroporation into *E. coli*, all of the >500 colonies examined on LB-amp+X-gal-containing medium were blue, indicating that the *lacZ* had been maintained. Restriction endonuclease mapping of the plasmid from one of the clones failed to detect any differences with the original plasmid. To further address this issue, the levels of ß-galactosidase were tested after multiple transfers in media without puromycin (Figure 5B). In both mineral (McN) and rich (McC) media, the levels of ß-galactosidase activity rapidly dropped after four serial transfers in media without puromycin. Although the initial specific activity in mineral medium was lower than in complex medium, the relative rate at which activity was lost was about 20-25% per transfer in both media. If the loss in ß-galactosidase activity was an indication of loss of the plasmid, the availability of the amino acids and other components of the rich medium appeared to have little affect on this process. In contrast, in the presence of puromycin, the level of ß-galactosidase activity was maintained for at least four transfers, and the apparent decline was not statistically significant (Figure 5B).

Summary: To our knowledge, this report is the first description of an expression shuttle vector for the methanogenic archaea. Because these strict anaerobes contain many oxygen sensitive enzymes and unusual coenzymes, they are good candidates for an expression system for enzymes that are not expressed in an active form in *E. coli*. Among the methanogens, the methanococci may be especially useful in this regard. Their rapid growth facilitates genetic manipulations. They can be cultured on a large scale on formate,

which substantially reduces the hazards and expense of cultivation with H_2:CO_2. Thus, even though the levels of expression are only about 1% of the total cellular protein, it is possible to obtain large amounts of protein.

In addition to expressing homologous enzymes for biochemical studies, the integrative expression vectors may be useful for manipulating the physiology of *M. maripaludis*. For instance, a spontaneous mutation in *Methanosarcina barkeri* overexpressed the genes encoding pyruvate oxidoreductase and allowed the mutant to grow on pyruvate as an electron donor (BOCK and SCHÖNHEIT 1995). With the integrative expression vector, it would be possible to recreate such a mutant in *M. maripaludis* to determine if it also allows heterotrophic growth on pyruvate.

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LITERATURE CITED

BENEKE, S., H. BESTGEN, and A. KLEIN, 1995 Use of the *Escherichia coli uidA* gene as a reporter in *Methanococcus voltae* for the analysis of the regulatory function of the intergenic region between the operons encoding selenium-free hydrogenases. Mol. Gen. Genet. **248**: 225-228.

BOCK, A.-K., and P. SCHÖNHEIT, 1995 Growth of *Methanosarcina barkeri* (Fusaro) under nonmethanogenic conditions by the fermentation of pyruvate to acetate: ATP synthesis via the mechanism of substrate level phosphorylation. J. Bacteriol. **177**: 2002-2007.

BOONE, D. R., W. B. WHITMAN, and P. ROUVIÈRE, 1993 Diversity and taxonomy of methanogens, pp. 35-80 in Methanogenesis: ecology, physiology, biochemistry, and genetics, edited by J. G. FERRY. Chapman & Hall, New York.

BOWEN, T. L., J. UNION, D. L. TUMBULA, and W. B. WHITMAN, 1997 Cloning and phylogenetic analysis of the genes encoding acetohydroxyacid synthase from the archaeon *Methanococcus aeolicus*. Gene. **188**: 77-84.

BULT, C. J., O. WHITE, G. J. OLSEN, L. ZHOU, R. D. FLEISCHMANN, G. G. SUTTON, J. A. BLAKE, L. M. FITZGERALD, R. A. CLAYTON, J. D. GOCAYNE, A. R. KERLAVAGE, B. A. DOUGERTY, J. F. TOMB, M. D. ADAMS, C. I. REIGH, R. OVERBEEK, E. F. KIRKNESS, K. G. WEINSTOCK, J. M. MERRICK, A. GLODEK, J. L. SCOTT, N. S. M. GEOGHAGEN, J. F. WEIDMAN, J. L. FUHRMANN, D. NGUYEN, T. R. UTTERBACK, J. M. KELLEY, J. D. PETERSON, P. W. SADOW, M. C. HANNA, M. D. COTTON, K. M. ROBERTS, M. A. HURST, B. P. KAINE, M. BORODOVSKY, H. P. KLENK, C. M. FRASER, H. O. SMITH, C. R. WOESE, and J. C. VENTER, 1996 Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science **273**: 1017-1140.

CHONG, S., Y. SHAO, H. PAULUS, J. BENNER, F. B. PERLER, and M.-Q. XU, 1996 Protein splicing involving the *Saccharomyces cerevisiae* VMA intein. J. Biol. Chem. **271**: 22159-22168.

COHEN-KUPIEC, R., C. BLANK, and J. A. LEIGH, 1997 Transcriptional regulation in Archaea: *In vivo* demonstration of a repressor binding site in a methanogen. Proc. Natl. Acad. Sci. USA **94**: 1316-1320.

GERNHARDT, P., O. POSSOT, M. FOGLINO, L. SIBOLD, and A. KLEIN, 1990 Construction of an integration vector for use in the archaebacterium *Methanococcus voltae* and expression of a eubacterial resistance gene. Mol. Gen. Genet. **221**: 273-279.

JONES, W. J., M. J. B. PAYNTER, and R. GUPTA, 1983a Characterization of *Methanococcus maripaludis* sp. nov., a new methanogen isolated from salt marsh sediment. Arch. Microbiol. **135**: 91-97.

JONES, J. W., W. B. WHITMAN, R. D. FIELDS, and R. S. WOLFE, 1983b Growth and plating efficiency of methanococci on agar media. Appl. Environ. Microbiol. **46**: 220-226.

LAEMMLI, U. K., 1970 Cleavage of structural protein during the assembly of the head of the bacteriophage T4. Nature **227**: 680-685.

MANIATIS, T., E. F. FRITSCH, and J. SAMBROOK, 1982 Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

MILLER, J. H., 1992 A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, New York.

PARK, S. H., R. XING, and W. B. WHITMAN, 1995 Nonenzymatic acetolactate oxidation to diacetyl by flavin, nicotinamide and quinone coenzymes. Biochim. Biophys. Acta. **1245**: 366-370.

REEBURGH, W. S., S. C. WHALEN, and M. J. ALPERIN, 1993 The role of methylotrophy in the global methane budget, pp. 1-14 in Microbial growth on C_1 compounds, edited by J. C. MURRELL and D. P. KELLY. Intercept Ltd, Andover.

SAITO, H. and K.-I. MIURA, 1963 Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta. **72**: 619-629.

SAMBROOK, J., E. F. FRITSCH, and T. MANIATIS, 1989 Molecular cloning: a laboratory manual (2nd edition). Cold Spring Harbor Laboratory Press, New York.

SHIEH, J., M. MESBAH, and W. B. WHITMAN, 1988 Pseudoauxotrophy of *Methanococcus voltae* for acetate, leucine, and isoleucine. J. Bacteriol. **170**: 4091-4096.

SHIEH, J. S., and W. B. WHITMAN, 1987 Pathway of acetate assimilation in autotrophic and heterotrophic methanococci. J. Bacteriol. **169**: 5327-5329.

STROUP, D., and J. N. REEVE, 1992 Histone HMf from the hyperthermophilic archaeon *Methanothermus fervidus* binds to DNA in vitro using physiological conditions. FEMS Micro. Biol. Lett. **91**: 271-276.

TUMBULA, D. L., T. L. BOWEN, and W. B. WHITMAN, 1997 Characterization of pURB500 from the archaeon *Methanococcus maripaludis* and construction of a shuttle vector. J. Bacteriol. **179**: 2976-2986.

TUMBULA, D. L., R. A. MAKULA, and W. B. WHITMAN, 1994 Transformation of *Methanococcus maripaludis* and identification of a *Pst*I-like restriction system. FEMS Microbiol. Lett. **121**: 309-314.

TUMBULA, D. L. and W. B. WHITMAN, 1999 Genetics of *Methanococcus*: possibilities for functional genomics in archaea. Submitted to Mol. Microbiol.

WHITMAN, W. B., J. SHIEH, S. SOHN, D. S. CARAS, and U. PREMACHANDRAN, 1986 Isolation and characterization of 22 mesophilic methanococci. System. Appl. Microbiol. **7**: 235-240.

WHITMAN, W. B., S. SOHN, S. KUK, and R. XING, 1987 Role of amino acids and vitamins in nutrition of mesophilic *Methanococcus* spp. Appl. Environ. Microbiol. **53**: 2373-2378.

XING, R. and W. B. WHITMAN, 1987 Sulfometuron methyl-sensitive and -resistant acetolactate synthases of the archaebacteria *Methanococcus* spp. J. Bacteriol. **169**: 4486-4492.

XING, R. and W. B. WHITMAN, 1994 Purification and characterization of the oxygen-sensitive acetohydroxy acid synthase from the archaebacterium *Methanococcus aeolicus*. J. Bacteriol. **176**: 1207-1213.

Table 1. Plasmids used in this study[a]

Plasmids	Properties	Reference
Mipuid	contains *M. voltae* histone promoter with *uidA*, ampicillin resistance (amp^r)	BENEKE *et al.* 1995
pBK-CMV	amp^r	Stratagene
pGEM-T	amp^r	Promega
pGEM-T+*lacZ*	*lacZ* with flanking *Nsi* I and *Bgl* II sites, amp^r	This work
pMEB.2	pUC derivative that contains puromycin resistance cassette (*pac*) for methanococci (pur^r)	GERNHARDT *et al.* 1990
pTer7	contains *E. coli* wild-type *lacZ*, amp^r	From ELLIOT ALTMAN
pTLB30	contains *M. aeolicus* acetohydroxyacid synthase genes (*ilvBN*), amp^r	BOWEN *et al.* 1997
pUC18	amp^r	
pURB500	cryptic plasmid of *M. maripaludis* C5	TUMBULA *et al.* 1997
pWLG1	contains *M. maripaludis ilvBN* and *ppsA*, kanamycin resistance (kan^r)	This work
pWLG2	3.8 kbp deletion of pWLG1, kan^r	This work
pWLG3	pUC18 plus 3.8 kbp insert from pWLG1, amp^r	This work
pWLG11	pMEB.2 derivative with multiple cloning site, amp^r, pur^r	This work
pWLG12A	pWLG11 plus *M. voltae* histone promoter (P_{hmvA}) with *uidA*, amp^r, pur^r	This work
pWLG13	integrative expression vector with *Nde* I site downstream of P_{hmvA}, amp^r, pur^r	This work
pWLG13+5'-*ilvB*	contains *M. maripaludis ilvB* fragment	This work

pWLG14	integrative expression vector with *Nsi* I site downstream of P_{hmvA}, amp^r, pur^r	This work
pWLG18	pWLG14 with *lacZ* for blue/white screen	This work
pWLG30	expression shuttle vector for *M. maripaludis*, amp^r, pur^r	This work
pWLG30+*lacZ*	contains *lacZ* for *E. coli* blue/white screen	This work
pZeRO-2	requires *E. coli* Top10, kan^r	Invitrogen

[a]Ampicillin (amp) and kanamycin (kan) are for selection in *E. coli*. Puromycin (pur) is for selection in *M. maripaludis*.

Table 2. Overexpression of the acetohydroxyacid synthase in *M. maripaludis* transformed with pWLG13+5'*ilvB*[a].

Vector	Specific Activity[b]
None	0.011
pWLG13+5'*ilvB*	0.150

[a]Cells were grown to an absorbance of 0.7-0.9 in McNA.

[b]micromol acetolactate formed min^{-1} (mg of protein)$^{-1}$. Average of triplicate assays.

Table 3. Expression of ß-galactosidase in *M. maripaludis* by the shuttle vector[a].

Vector	Specific Activity[b]
pWLG30	<0.02[c]
pWLG30+*lacZ*	4.5

[a]Cells were grown to an absorbance of 0.45-0.48 in McC medium + puromycin.

[b]micromol min^{-1} (mg of protein)$^{-1}$. Average of duplicates.

[c]No activity was detected.

Figure 1:--Construction of the integrative vectors pWLG13, pWLG14, and pWLG18. Only the unique or otherwise useful restriction sites are shown. pMEB.2 was digested with *Nde* I and *Sac* I to remove *lacZ*' before a multiple cloning site (MCS) was added to form pWLG11. pWLG14 was constructed by the addition of P_{hmvA} between the *Eco*R I and *Bgl* II sites of pWLG11. *lacZ,* modified by PCR to contain *Nsi* I and *Bgl* II restriction sites, was ligated into pWLG14 to form pWLG18. pWLG12A was developed from pWLG11 by the addition of P_{hmvA} and *uidA* from Mipuid. The *uidA* was released from pWLG12A by an *Nde* I digestion to form pWLG13.

The MCS was designed so that multiple restriction enzymes can be utilized in a single buffer. Using either *Nde* I or *Nsi* I plus a second restriction enzyme allows directed cloning into the vectors. Addition of a third endonuclease, with a site between the first endonuclease sites, can lower the background associated with the religation of a single digestion. P_{hmvA} can also be replaced using *Eco*R I (or *Cla* I in pWLG14) and *Nsi* I or *Nde* I.

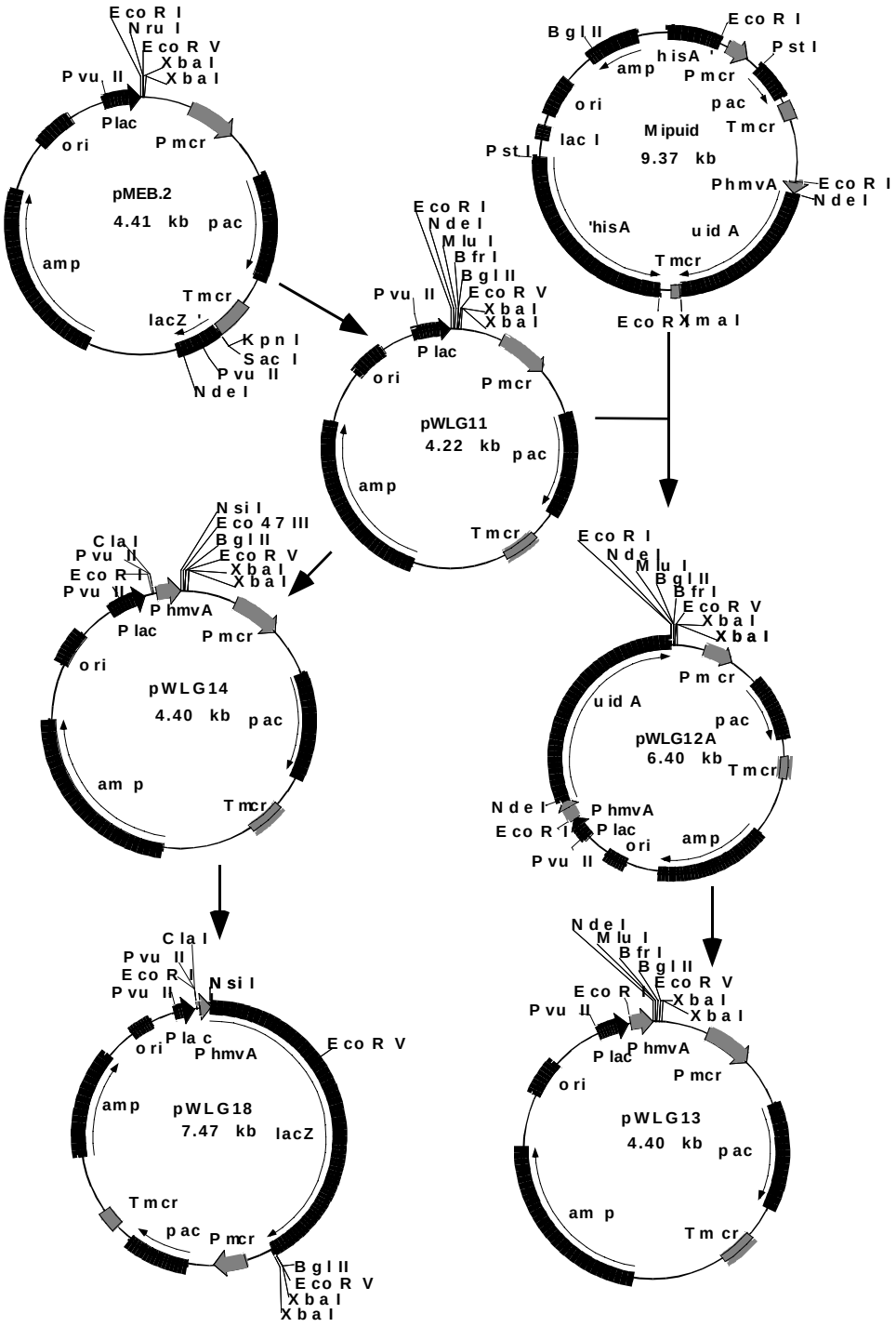

EcoR I
Nru I
EcoR V
Xba I
Xba I
Pvu II
Plac
ori
Pmcr
pMEB.2
4.41 kb pac
amp
Tmcr
lacZ'
Kpn I
Sac I
Pvu II
Nde I
BglII
EcoR I
pstI
amp
hisA'
Pmcr
ori
pac
lac I
Mipuid
Tmcr
9.37 kb
pstI
PhmvA
EcoR I
Nde I
'hisA
uid A
Tmcr
EcoR I
Sma I
EcoR I
Nde I
Mlu I
Bfr I
BglII
Pvu II
EcoR V
Xba I
Xba I
Plac
ori
Pmcr
pWLG11
4.22 kb pac
amp
Tmcr
Nsi I
Eco47 III
BglII
EcoR V
Xba I
Xba I
Cla I
Pvu II
EcoR I
Pvu II
PhmvA
Plac
Pmcr
ori
pWLG14
4.40 kb pac
amp
Tmcr
EcoR I
Nde I
Mlu I
BglII
Bfr I
EcoR V
Xba I
Xba I
Pmcr
uid A
pac
pWLG12A
6.40 kb
Tmcr
Nde I
PhmvA
EcoR I
Plac
ori
amp
Pvu II
Cla I
Pvu II
EcoR I
Pvu II
Nsi I
ori
Plac
PhmvA
EcoR V
amp
pWLG18
7.47 kb lacZ
Tmcr
pac
Pmcr
BglII
EcoR V
Xba I
Xba I
Nde I
Mlu I
Bfr I
BglII
EcoR V
EcoR I
Xba I
Pvu II
Xba I
Plac
PhmvA
Pmcr
ori
pWLG13
4.40 kb pac
amp
Tmcr

Figure 2:--Construction of methanococcal expression shuttle vectors pWLG30 and pWLG30+*lacZ.* pWLG30 was constructed by ligation of a partial *Eco*R I digestion of the cryptic methanococcal plasmid pURB500 into the *Eco*R I site of pWLG14. *lacZ* from pWLG18 (Figure 1) was then added between the unique *Nsi* I and *Bgl* II sites of pWLG30. The low level of *lacZ* expression of pWLG30+*lacZ* forms a blue/white screen for clones in *E. coli*. The unique *Cla* I site in pWLG14 and pWLG30 allows the P_{hmvA} to be replaced with other promoters in future studies.

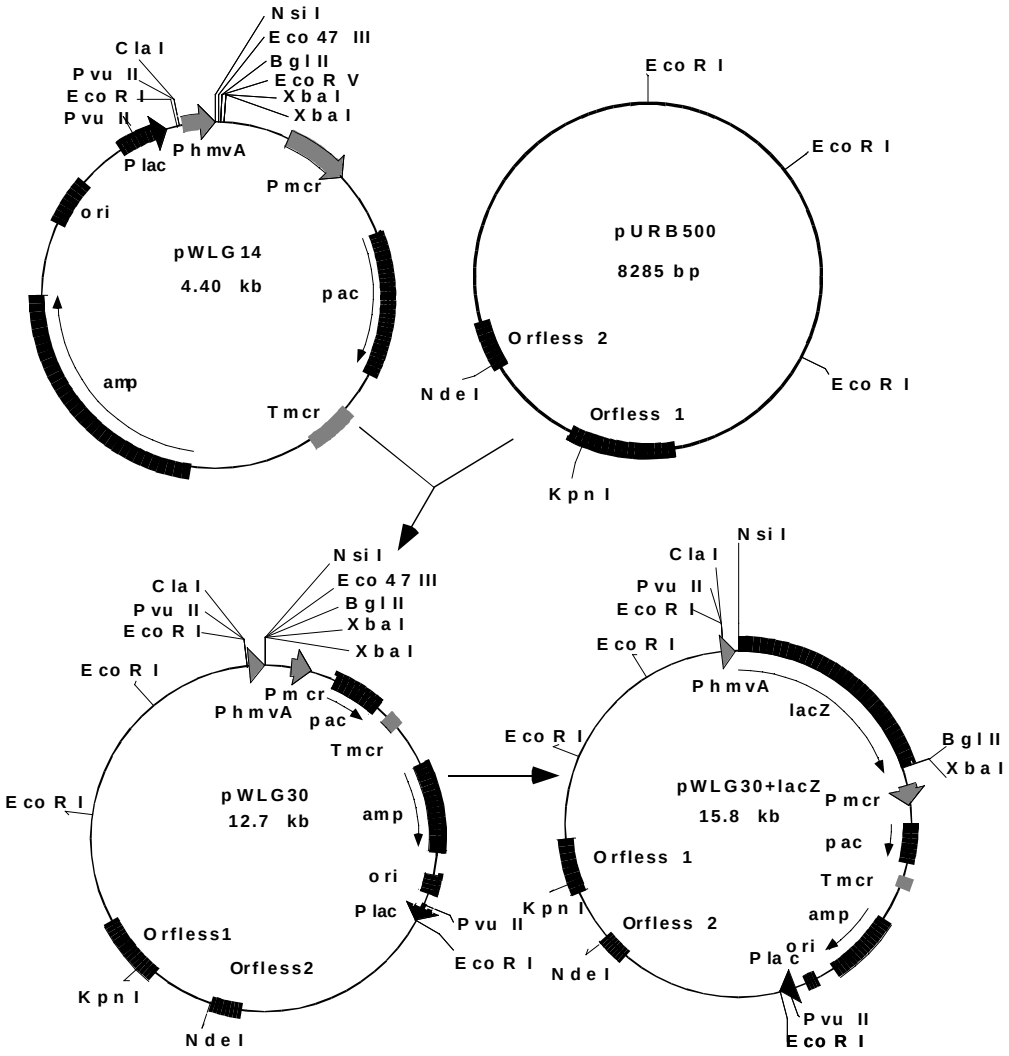

Nsi I
Eco 47 III
Cla I
Bgl II
Pvu II
EcoR V
EcoR I
Xba I
Pvu II
Xba I
PhmvA
Plac
Pmcr
ori
pWLG14
4.40 kb
pac
amp
Tmcr
EcoR I
pURB500
8285 bp
EcoR I
Orfless 2
NdeI
Orfless 1
EcoR I
KpnI
Nsi I
Cla I
Eco 47 III
Pvu II
Bgl II
EcoR I
Xba I
Xba I
EcoR I
Pmcr
PhmvA
pac
Tmcr
EcoR I
pWLG30
12.7 kb
amp
ori
Plac
Pvu II
EcoR I
Orfless1
Orfless2
KpnI
NdeI
Nsi I
Cla I
Pvu II
EcoR I
EcoR I
PhmvA
lacZ
EcoR I
BglII
XbaI
pWLG30+lacZ
15.8 kb
Pmcr
pac
Orfless 1
Tmcr
KpnI
Orfless 2
amp
ori
Plac
NdeI
Pvu II
EcoR I

Figure 3.--A. Sequenced region of pWLG1 containing the *M. maripaludis* genes encoding AHAS (*ilvBN*) and PEP synthase (*ppsA*). The junction between *ppsA* and *ilvB* indicating the position of the *Sau*3A I site likely to be involved in formation of a chimera is shown. The arrow corresponds to the position of the putative intein splice junction in the *M. jannaschii ppsA*. B. Deduced amino acid sequence of PpsA from *M. maripaludis* and *M. jannaschii* near the intein splice junction. Arrow represents putative intein splice junction.

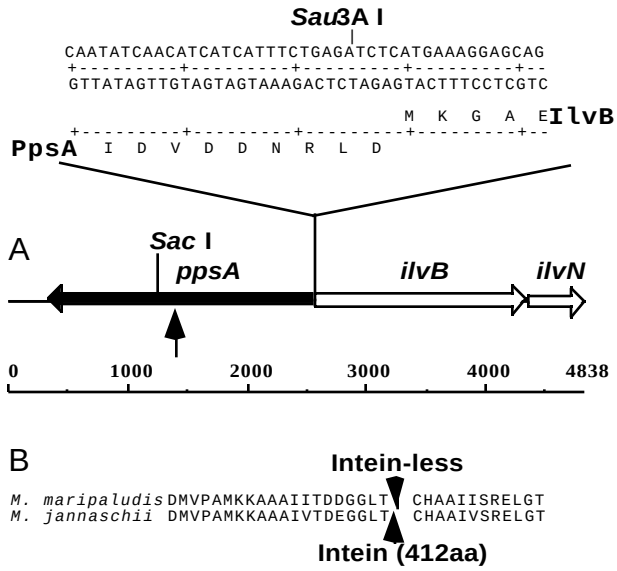

Sau3A I
CAATATCAACATCATCATTTCTGAGATCTCATGAAAGGAGCAG
GTTATAGTTGTAGTAGTAAAGACTCTAGAGTACTTTCCTCGTC
M K G A E IlvB
PpsA I D V D D N R L D
A
Sac I
ppsA
ilvB
ilvN
0
1000
2000
3000
4000
4838
B
Intein-less
M. maripaludis DMVPAMKKAAAIITDDGGLT CHAAIISRELGT
M. jannaschii DMVPAMKKAAAIVTDEGGLT CHAAIVSRELGT
Intein (412aa)

Figure 4.-- Overexpression of ß-galactosidase in *M. maripaludis* by the expression shuttle vector pWLG30. SDS-PAGE of total protein from *M. maripaludis* stained with Coomassie blue. Wild-type cells (lane B), cells transformed with pWLG30 (lane C), cells transformed with pWLG30+*lacZ* (lane D); arrow is a protein with the expected M_r of ß-galactosidase (116.3 kDa). Bio-Rad low molecular weight standards (lane A): rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa), and bovine carbonic anhydrase (31.0 kDa). The transformants were grown in McC medium+puromycin to an absorbance of 0.5, and the wild type was grown to an absorbance of 0.8 in McC medium.

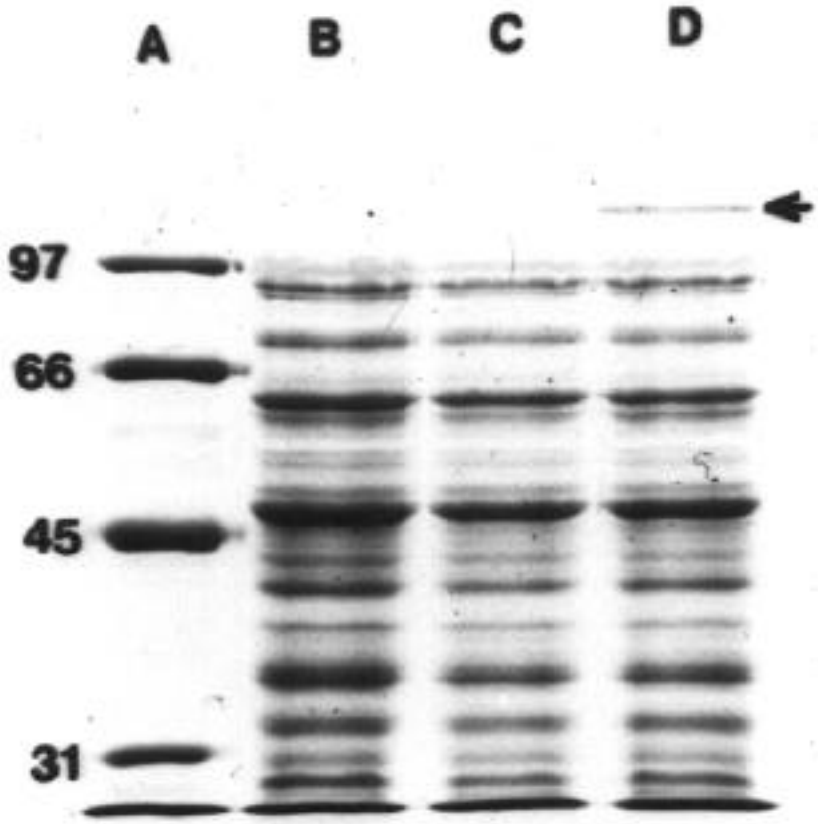
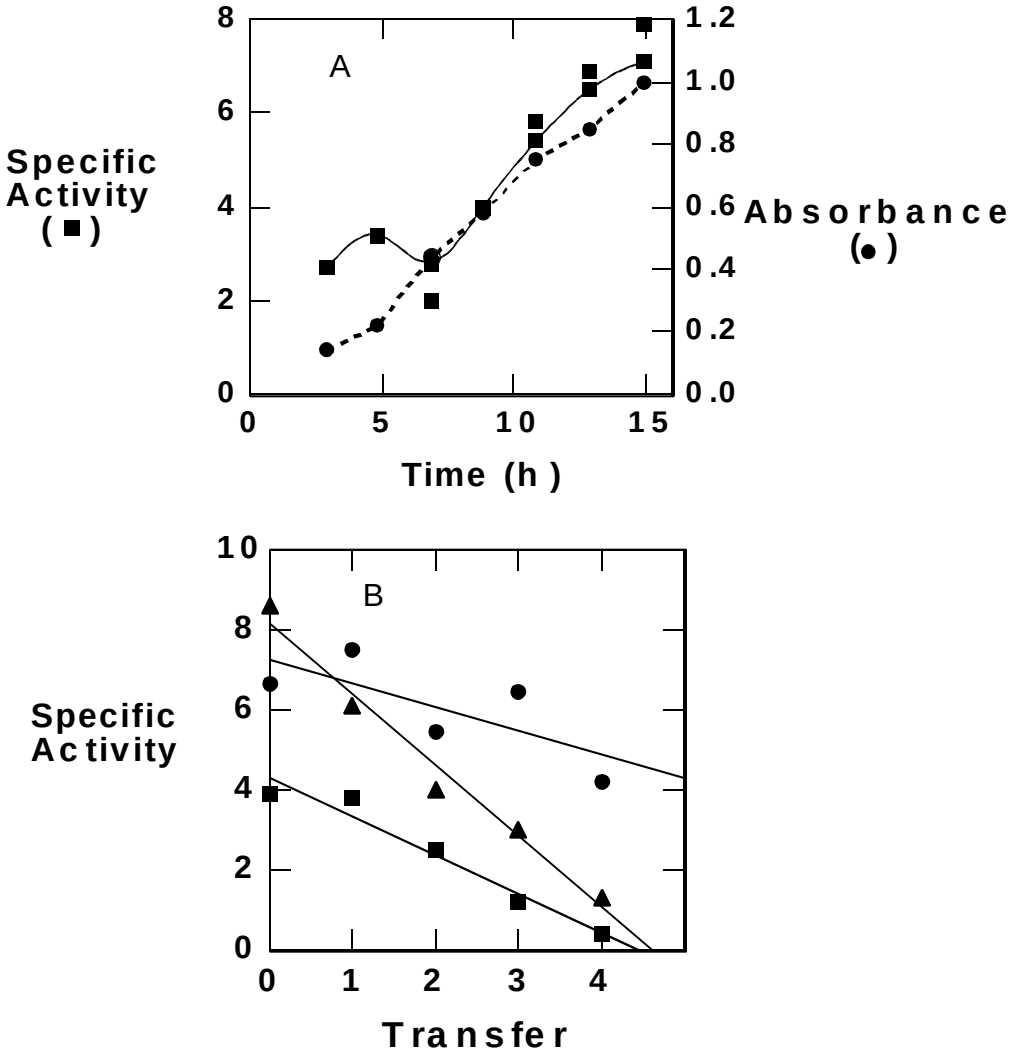

A
B
C
D
97
66
45
31

Figure 5.-- Expression of ß-galactosidase in a transformant of pWLG30+*lacZ*. Specific activity was in micromol of ONPG transformed min^{-1} (mg of protein)$^{-1}$. A. Effect of growth phase on the specific activity. Cells, 4% v/v, were inoculated into McC+puromycin medium. B. Stability of pWLG30+*lacZ* in *M. maripaludis* upon serial transfers of the culture. *M. maripaludis* was grown to an absorbance (600 nm) of 0.62-0.85 in McN (■), 0.40-0.58 in McC (▲), and 0.44-0.66 in McC+puromycin (●). The inoculum was 4%. Every 24 h, the culture was transferred to fresh medium, and the specific activity was determined after 14 h of growth. The average number of generations in the three media after transfer 1, 2, 3, and 4 were 5, 10, 14.5, and 23 respectively. Data points were the average of duplicate assays. For McN, McC, and McC+puromycin media, correlation coefficients of the specific activity with the number of transfers were 0.99, 0.98, and 0.74, respectively. Critical values for significance at P=0.05 and 0.01 were 0.88 and 0.96, respectively.


A
Specific Activity (■)
Absorbance (●)
Time (h)
0
2
4
6
8
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
5
10
15
B
Specific Activity
Transfer
0
2
4
6
8
10
0
1
2
3
4

CHAPTER IV

IDENTIFICATION OF ESSENTIAL REGIONS OF THE CRYPTIC PLASMID pURB500 IN *METHANOCOCCUS MARIPALUDIS*[3]

[3] Gardner, W. L. and W. B. Whitman. To be submitted.

Keywords: essential, cryptic plasmid, replication, expression, archaea, anaerobic, shuttle vector, translation, and methanogen

Abstract

A Tn7-based transposon was used to mutate pWLG30+*lacZ* to identify regions of the cryptic pURB500 replicon essential for replication and copy number control in *Methanococcus maripaludis*. After mutation, the plasmids were transformed into *M. maripaludis* and β-galactosidase assays performed to measure the plasmid levels within the cells. Two plasmids containing the transposon in ORF1 of pURB500 failed to transform *M. maripaludis*, which suggested that ORF1 was required for replication. Mutations in six additional open reading frames (ORFs) showed a small, but significant difference in β-galactosidase levels in *M. maripaludis*.

Introduction

Plasmids are extra-chromosomal elements found in the all three domains of life: *Archaea*, *Bacteria*, and *Eukarya* (10). Recent research on plasmids have examined the minimum regions required for replication, mechanisms of replication, and their role in evolution. Plasmids also provide the basis of many genetic vectors. Physically, plasmids are either circular or linear. See Hinnebusch and Tilly (1993) for a review of linear plasmids.

Autonomous replication and copy number control are features of plasmids. Replication origins are defined in three ways. These definitions are based on the level of knowledge about the replication process. The most general definition is a *cis*-acting region that is the minimum size sufficient to direct self-replication (10). The second definition involves the region where the plasmid DNA melts to begin autonomous

replication. The most specific definition is the base or bases where the leading strand of DNA replication initiates. Currently, circular plasmids are known to replicate by three different mechanisms: rolling-circle, theta, and strand-displacement.

The rolling-circle mechanism requires two types of replication origins, a plasmid encoded Rep protein, and replication machinery from the host (13). Plasmid replication begins with the Rep protein binding to the double strand origin (dso) and nicking the DNA. The dso may contain interons. Interons are two or three sets of directly repeated nucleotides, that are the sites of Rep protein binding. After nicking and relaxing the plasmid, host proteins then begin to replicate the plasmid at the 3'-OH generated at the nicking site while displacing the parental plasmid strand containing the bound Rep protein. The single-stranded parental DNA is immediately bound by ssDNA binding proteins during synthesis of a complementary strand. Upon reaching the newly synthesized dso, the Rep protein cleaves the single stranded parental DNA to form a circular single stranded plasmid and a relaxed double stranded plasmid. The single stranded plasmid is presumably converted to the double stranded form by the host proteins since no plasmid encoded proteins are known to be involved in this step. The synthesis of the lagging strand happens after a single stranded intermediate is formed, and the synthesis of the leading and lagging strands are uncoupled (10). Initiation of replication may begin again or a DNA gyrase may supercoil the plasmid (1). Plasmids that utilize this replication mechanism do not have a partitioning system since they are maintained at a high copy number in their natural host (13).

The theta mechanism is most extensively researched with plasmids from the gram-negative bacteria (10). In many cases, this mechanism requires a Rep protein for

replication initiation at the origin. However, ColE1 replication is independent of a plasmid encoded Rep protein. See Davison (1984) for a review. The origin region may also contain additional sites that allow host proteins, such as the DnaA initiator protein, to bind to the plasmid (10). Many origin regions contain iterons, but not all iterons are origins. Some iterons, which can be located outside of the origin region, function to control replication but are not required for replication initiation. After the origin is bound by a Rep protein and, in many cases, the host-encoded DnaA, the DNA is melted, and a complex of host-encoded proteins is formed. A helicase unwinds the double stranded plasmid DNA, and leading strand synthesis begins. Lagging strand synthesis also occurs at this time. The RNA primers, which are required for lagging strand synthesis are produced by either plasmid-encoded or host-encoded proteins. The direction of the synthesis is usually unidirectional, but bidirectional cases are known (10). Replication termination requires another protein complex, which is composed of proteins from the host, in some plasmids.

Strand-displacement mechanism is the replication method used by the IncQ plasmid family, which is also known as Inc P4 (10; 14). These plasmids are isolated from *Escherichia coli* and some species of *Neisseria*, *Proteus*, *Pseudomonas*, *Providencia*, and *Salmonella* (14). The plasmids have a broad host range, a size range of 8.3 kilobase pairs (kb) to 14 kb, and a medium copy number of 4 to 12 plasmids per chromosome (14). Much of the knowledge about strand-displacement is based on the plasmid RSF1010. General genetic characteristics for the IncQ family include a requirement for a complex origin and three plasmid-encoded replication proteins, RepABC (10). The origin is composed of three 20 nucleotide iterons with a 174

nucleotide segment that contains a 28 nucleotide GC-rich region and a 31 nucleotide AT-rich region. Replication begins with the RepC, which has initiator activity, binding to the origin. The DNA helicase RepA binds to the DNA next to RepC and melts the DNA until two single-stranded origins are exposed. The primase RepB adds RNA primers so that replication can begin using the host's replication machinery. Replication continues from both single-stranded regions so plasmid RSF1010 is replicated bidirectionally (10). See Sakai and Komano (1996) and Haring and Scherzinger (1989) for more extensive reviews.

Although a few plasmids have been sequenced from the *Crenarchaeota*, there is little experimental evidence for the mechanisms of plasmid replication. pRN1 and pRN2 are two cryptic, sequenced plasmids from *Sulfolobus islandicus* strain REN1H1 (23; 24). They are maintained at approximately 20 and 35 copies per chromosome for pRN1 and pRN2, respectively (40). Both pRN1 and pRN2 have putative genes with homology to CopG and a helicase-like protein (23; 24). CopG is a regulatory protein found in bacterial plasmids that utilize the rolling circle mechanism (9). The actual mechanism for replication is not known. Another completely sequenced plasmid is pNOB8, which is 41.2 kb, from *Sulfolobus* isolate NOB8-H2 (34). This conjugative plasmid is maintained at 20 copies per chromosome, and has putative genes with high similarity to bacterial partitioning systems (33; 34). However like pRN1 and pRN2, the mechanism that is used for replication is not known.

The *Euryarchaeota* contain many completely sequenced plasmids. Within the halophiles, there is a group of sequenced, high copy number 1.7 kb plasmids named pHGN1, pGRB1, and pHSB1 (16; 17; 22). pGRB1, which is maintained at around 180

copies per chromosome, is related to pHSB1 and pHGN1 (16; 25). Each plasmid contained an ORF that might be involved in replication, but the ORF was not characterized further. pGRB1 forms a single-stranded, circular intermediate during replication (35). The replication mechanism is thought to be either strand-displacement or rolling-circle (35). pHK2, a 10.5 kb plasmid from *Haloferax* sp Aa2.2, is maintained at 7 to 8 copies per cell (20). The minimal region of 3359 bp contains one ORF. This ORF has 30% sequence identity to the ORFs in pHGN1, pGRB1, and pHSB1 (21).

In addition to the small plasmids, halophiles have plasmids that are 150 kb to 200 kb in size. The minimum origin of replication is known for two of these plasmids. *Halobacterium salinarium* contains the 150 kb pHH1 (31). pHH1 requires only 2.9 kb of the 150 kb plasmid for replication in *Haloferax volcanii*. Analysis of this sequence revealed one ORF which is required for replication and does not have any homologs (31). The minimum origin of replication of the 200 kb pNRC100 is 3.9 kb (29). Sequencing the 3.9 kb fragment identified one ORF named *repH*. *repH* is expressed in *H. halobium* and *H. volcanii* (29). RepH has a low identity (24%) to an ORF in pHV2, which is a cryptic plasmid (6; 29).

pGT5 is the only sequenced plasmid from the *Thermococcales*. The 3.4 kb plasmid from *Pyrococcus abyssi* is maintained at 25-30 copies/chromosome (12). This plasmid has two ORFs within its sequence. ORF1 has motifs reminiscent of bacterial Rep proteins. The second ORF, which does not have homology to known proteins, is hypothesized to be involved in recombination events. A single-stranded form of the plasmid, which corresponds to the plus strand, is found within cell extracts of *P. abyssi*

(12). The confirmation of a single-stranded intermediate suggests that pGT5 uses a rolling-circle replication mechanism.

A large number of plasmids from the methanogens have been sequenced including: pC2A (27), pFV1 (30), pFZ1 (30), pME2001 (4), pURB500 (37), pURB800 (5), and pURB801 (5). The 5.5 kb pC2A from *Methanosarcina acetivorans* has four ORFs that can encode proteins with 120 amino acids residues or greater (27). The deduced amino acids from one ORF, which is known as *ssrA*, has extensive homology with a family of site-specific recombinases. The deduced amino acid sequence from a second ORF has low sequence similarity to a RepA protein. The remaining ORFs did not encode proteins with significant similarity to sequences in the databases. Three long inverted sequences are in the pC2A sequence. With this evidence, pC2A is thought to use a rolling-circle mechanism for replication (27).

pFV1 (13.5 kb) and pFZ1 (11.0 kb) are from *Methanobacterium thermoformicicum* strains THF and Z-245, respectively (30). These two plasmids are highly related at the nucleotide level, excluding the extra 2,499 bp in pFV1. Since these plasmids are highly related, a similar replication mechanism may be expected (30). These plasmids possess two inverted repeats that are different in only 11 positions over 520 nucleotides. One ORF is also conserved between the two plasmids at 99% identity. Nölling et al. (1992) propose that pFV1 and pFZ1 replicate by a rolling-circle mechanism but are unable to detect single-stranded intermediates.

pME2001 is a 4.4 kb cryptic plasmid found in *Methanobacterium thermoautotrophicum* (Marburg) (4). While this plasmid is isolated with multimeric forms from *M. thermoautotrophicum*, a replication mechanism has not been suggested

from the physical or genetic data (26). pURB500 is discussed below. The *Methanococcus jannaschii* pURB800 and pURB801 are 58.4 kb and 16.6 kb, respectively (5). Of the 44 ORFs in pURB800, none are associated with plasmid replication mechanisms. However, pURB800 contains two ORFs for putative histones. The 16 ORFs in pURB801 lacked any significant similarity to known proteins.

pURB500 is a cryptic plasmid from *M. maripaludis* strain C5 (39). In *M. maripaludis* strain C5, this plasmid is maintained at three copies per cell. Wood et al. (1985) identified dimeric forms of pURB500 in transmission electron micrographs. These data suggest that pURB500 has a theta structure associated with plasmid replication. pURB500, which is 8,285 base pairs in size with a mole percent G+C content of 27.24%, contains 18 ORFs (37). Fourteen of the ORFs encode a minimum of 83 amino acids. The remaining 4 ORFs encode for <83 amino acids, but a mole percent G+C of 32%. Only ORF2 had low sequence identity (39%) at the amino acid level to a *M. jannaschii* ORF. This *M. jannaschii* ORF has a low sequence identity to an integrase family. See Alonso et al. (1996) for a review of recombinases and integrases utilized for plasmid resolution during replication by a theta mechanism. pURB500 provides the *M. maripaludis* origin of replication for the shuttle vectors pDLT44 and pWLG30+*lacZ* (15; 37). Subcloning experiments suggest that *M. maripaludis* strain JJ requires large portions of pURB500 for replication. In this work, pURB500 was mutagenized to identify essential ORFs and *cis*-acting regions for the eventual goals of understanding pURB500 replication and developing a high copy expression shuttle vector for *M. maripaludis*.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions. *Methanococcus maripaludis* JJ was obtained from W. J. Jones. The plasmids used in this study are listed in Table 1. *M. maripaludis* was grown at 37°C on 275 kPa of H_2:CO_2 (80:20) in McN (mineral medium) and McC (complex medium minus the vitamin solution) (38). The sodium bicarbonate in the medium was reduced from 5 g l^{-1} to 2 g l^{-1} during growth at 100 kPa in 1 L Wheaton bottles or in a fermentor. Transformation of *M. maripaludis* with plasmid DNA used a modified protocol based on Tumbula et al. (1994). See below for further details. For selection of puromycin resistant methanococci, a stock solution of 500 μg ml^{-1} puromycin dihydrochloride (Sigma) in distilled water was filter-sterilized and added to the medium at a final concentration of 2.5 μg ml^{-1}. Strictly anaerobic techniques were used for medium preparation and cultivation.

The *E. coli* strains were grown at 37°C on low salt Luria-Bertani (LB) medium with the NaCl at 50% of the regular concentration (32). Antibiotic concentrations were 60 μg ml^{-1} for ampicillin (Amp) and 50 μg ml^{-1} for kanamycin (Kan) in both liquid and solid LB medium. ß-galactosidase was used as a blue/white screen on LB agar plates with 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) according to Sambrook et al. (1989). Plasmids were transformed into *E. coli* strains by electroporation with a Bio-Rad Gene Pulser (Bio-Rad Laboratories) using the settings of 200 Ω, 2.5 kV, and 25 μF with cuvettes (0.2 cm gap width). Plasmids from *E. coli*, grown with selective conditions, were isolated with the Wizard miniprep kit (Promega).

Growth of *E. coli* and *M. maripaludis* was measured at 600 nm with a spectrophotometer (Spectronic 20).

Electrophoresis of agarose gels. The electrophoresis of all agarose gels was performed in 1X TAE and 0.4 microgram/ml ethidium bromide (Sambrook *et al*., 1989). A 10X TAE stock solution contained 0.4 M tris base, 0.2 M glacial acetic acid, and 0.02 M (ethylenedinitrilo)tetraacetic acid, disodium salt, dihydrate. The agarose was also dissolved in 1X TAE.

Rapid colony screen. A single colony was streaked onto a LB plate containing the appropriate antibiotic(s). After 16 hours of growth at 37ºC, the plates were cooled to 4ºC for one hour. Transformants were collected with a sterile toothpick and placed into 50 µl of resuspension solution (50 mM Tris [pH 7.5], 10 mM EDTA, 100 µg/ml RNase A). Each sample was vortexed before the addition of 50 µl lysis solution (0.2 M NaOH, 1% (w/v) sodium dodecylsulfate) followed by 50 µl neutralization solution (1.32 M potassium acetate). The cell debris was pelleted by centrifugation at 16000 *g* for 5 minutes at room temperature. To screen the circular plasmids by size, electrophoresis was performed using 0.8% (w/v) agarose gel with 17 µl of the supernatant and 3 µl of tracking dye.

Plasmid purification from *M. maripaludis*. Plasmid isolation from *M. maripaludis* was begun by centrifuging a 5 ml culture at 16000 x *g* at 4ºC followed by resuspension of the cells in 100 µl of methanococcal medium. After resuspension, the Wizard miniprep kit (Promega) minus the resuspension step was used as described for the preparation of the plasmid DNA for transformation into *E. coli*.

Deoxyribonucleic acid (DNA) sequencing. All DNA sequencing was performed using ABI sequencers at the Molecular Genetics Facility, University of Georgia. All oligonucleotides were made by Integrated DNA Technologies (Coralville, Iowa).

Sequence analysis was performed with the GCG software package (Genetics Computer Group, Madison, WI).

Transposon mutagenesis of pWLG30+*lacZ*. pWLG30+*lacZ* was mutagenized *in vitro* with Transprimer-1 from the GPS-mutagenesis system (Figure 1; New England Biolabs). The New England Biolab GPS-M mutagenesis manual can be found at the NEB website (http://www.neb.com/). To eliminate the heat inactivation of the transposase, the company's protocol was modified to use 10 µl of phenol/chloroform/isoamyl alcohol (pH=8.0, Ameresco) to terminate the transposition reaction. The sample was vortexed with the phenol solution before centrifugation at 16,000 x *g* for 10 minutes. The aqueous phase was placed into a new microcentrifuge tube. Two volumes of 2-propanol were added to precipitate the plasmid DNA. After vortexing, the sample was centrifuged at 16,000 x *g* for 15 minutes at room temperature. The supernatant was decanted before vacuum drying and resuspension in 10 µl of sterile, distilled water. The reaction mixture was not digested with PI-*Sce* I.

The mutagenized pWLG30+*lacZ* was transformed into *E. coli* XL1-Blue MRF'. The transformed cells were plated on LB agar+Amp+Kan+X-gal. The circular plasmids from colonies with a blue phenotype were sized using the rapid colony screen. *Eco*R I digestions confirmed the general region of insertion by Transprimer-1. Isolates were sequenced with the primers TN7Lseq (5'-GACGCTACACCCAGTTCCCATCT-3') and TN7Rseq (5'-CGACCCCACGCCCCTCTTTA-3') to confirm the location in pWLG30+*lacZ.* See Figure 1.

Modified *M. maripaludis* transformation. The modified procedure allowed transformation of 16 different DNA samples with a single suspension of recipient cells.

Four *M. maripaludis* cultures were grown overnight in McC medium with H_2:CO_2 to an absorbance (600 nm) of 0.75. The cultures were pressurized to 275 kPa with H_2:CO_2 before transfer of the four cultures to two sterile, short Balch tubes via vacutainer (22G1) needles. The short Balch tube (17 ml volume), which was degassed in an anaerobic chamber for at least 24 hours, fit in a Beckman JA-20 rotor. The cultures were pressurized to 275 kPa with N_2:CO_2 (80:20) before centrifugation at 3660 x *g* for 10 minutes at 4°C. After decanting the McC, the cells were resuspended in 5 ml transformation buffer before pressurization to 275 kPa with nitrogen. After five minutes of centrifugation at 3660 x *g*, the transformation buffer was decanted. The cells were resuspended in 2 ml of fresh transformation buffer. The cells were pooled inside an anaerobic chamber. Both tubes were taken into an anaerobic chamber before the cells were pooled and gently stirred with a sterile pipet tip. A pipet transferred 0.2 ml of the cell suspension to each DNA sample, which was in a microcentrifuge tube. Each sample was incubated overnight at room temperature in the anaerobic chamber, and contained 1 µg of plasmid DNA in 52 µl of sterile distilled water and 48 µl of TE buffer (36). After the addition of the cells, 225 µl of transformation buffer+40% (w/v) polyethylene glycol 8000 was stirred into each sample. Incubation of this mixture occurred for 1 hour in a 37°C dry bath within the chamber. The transformed cells were inoculated by a syringe into a tube of McC, which was reduced with 0.1 ml of 2.5% (w/v) sodium sulfide. After this point, the transformation followed the method described by Tumbula et al. (1994).

Assays of ß-galactosidase activity. The ß-galactosidase assay was performed with 2-nitrophenyl-ß-D-galactopyranoside (ONPG) and a temperature of 37°C (28). *M. maripaludis* was grown in McC medium+puromycin unless stated otherwise.

Results

Screening the *E. coli* transformation of the mutagenized pWLG30+*lacZ* identified 31 colonies that grew on LB agar+Amp+Kan+X-gal and had a blue phenotype. pGPS3 and pWLG30+*lacZ*+Transprimer contained the same *E. coli* resistance markers. However, only cells containing active LacZ (β-galactosidase) from the expression shuttle vector were blue. If the *lacZ* in pWLG30+*lacZ* were mutated by the transprimer and formed an inactive enzyme, then the colony would have been discarded as a white colony. The rapid colony screen confirmed that the size of the circular forms of the mutagenized plasmids were larger than pWLG30+*lacZ*. This screen identified two sizes of the mutagenized plasmid. *Eco*R I digestions were used to localize the insertion in four areas of pWLG30+*lacZ*. See Figure 1 for the *Eco*R I positions. Using *Eco*R I digested pWLG30+*lacZ* as the control, the digestions demonstrated that 14 colonies were pWLG30+*lacZ*+Transprimer. Based on linear sizes with the linear lambda/*Hin*d III as the standard, the remaining 17 colonies were pWLG30+*lacZ*+Transprimer+pGPS3. These 17 isolates were discarded to avoid potential recombination problems.

Sequencing with the primers TN7Lseq and TN7Rseq confirmed the position and orientation of the transposon in the shuttle vector. See Table 2 and Figure 1 for the results. Two isolates were identical at the site of insertion and orientation of the transposon. Sequencing also demonstrated that five nucleotides of the expression vector were duplicated at each end of the transposon.

All 14 mutagenized shuttle vectors were transformed into *M. maripaludis*. See Table 3. The modified *M. maripaludis* transformation method used a common cell suspension for the transformation. Therefore, the transformation efficiencies were

expected to be nearly the same. The two identical isolates, which were pWLG30+*lacZ*+Transprimer isolate 2, had the same transformation efficiency. The variation seen in Table 3 was within the range seen in other transformations. pWLG30+*lacZ*+Transprimer isolates 9 and 10 failed to transform *M. maripaludis* after multiple attempts.

As an indirect measure of plasmid stability in *M. maripaludis*, the β-galactosidase specific activity was measured in each of the 13 unique pWLG30+*lacZ*+Transprimer. See Table 3. Three individual colonies per transformation were picked to reduce the potential measuring the β-galactosidase specific activity for a plasmid mutated by the cell. In each group of isolates, the standard deviation for the specific activities were between 5% and 10% of the averaged value. Overall, only a two-fold increase or decrease of β-galactosidase specific activity was measured when compared to the pWLG30+*lacZ* control.

E. coli has problems maintaining ColE1-derived plasmids with cloned DNA containing a low mole percent G+C content (11). pURB500, a cryptic plasmid from *M. maripaludis* strain C5, has a mole percent G+C of 27.2, and provides the replication origin in the shuttle vectors pDLT44 and pWLG30+*lacZ* (15; 37). pDLT44 and many of the pURB500 subclones are unstable in *E. coli* (37). In addition to the proposal that A+T-rich regions are recognized by *E. coli* proteins, Tumbula et al. (1997) suggested that the plasmid instability is potentially influenced by toxic products produced from pURB500. The point mutations with pWLG30+*lacZ*+Transprimer isolates 3 through 6 allowed the testing of two hypothesizes: whether a localized increase in mole percent G+C increased stability in *E. coli* and whether mutagenesis of individual ORFs

influenced stability. The mole percent G+C of the transprimer region was 43.2. After transposition into pURB500, the Transprimer increased the mole percent G+C at the point of insertion in pURB500. pDLT44, pWLG30+*lacZ*, and pWLG30+*lacZ*+Transprimer isolates 3 to 6 were selected to test plasmid stability in *E. coli*. An individual colony from a LB agar+Amp plate was inoculated into 10 ml of LB broth. After 16 hours of growth, each culture was diluted 1:1000 into fresh LB broth before incubation for an additional 16 hours. The culture was plated on LB agar and LB agar+Amp. The ratio of resistant colony forming units (cfu) to total cfu was calculated for each plasmid. pWLG30+*lacZ* had a resistant cfu:total cfu ratio of $3.3x10^{-3}$. The other plasmids also had similar ratios.

Discussion

Little is known about replication mechanisms, copy number control, and partitioning of archaeal plasmids (41). By understanding how these processes apply to pURB500, which is not related to other sequenced archaeal plasmids (37), a fundamental understanding of archaeal plasmid maintenance could be gained. Applications of the knowledge include the construction of high copy number expression shuttle vectors for the expression of heterologous genes.

The GPS-mutagenesis system, which contained the transposon-based Transprimer-1 element, was chosen to mutagenized pWLG30+*lacZ* since the mutation site would be easily identified and random. Transprimer-1 (Figure 2), which provides resistance to kanamycin in *E. coli*, was based on Tn7. Transprimer transposed into pWLG30+*lacZ* in a manner consistent with Tn7 transposition events. The transposition of the Transprimer duplicated five nucleotides at the target site, which were represented

at both ends of the transposon (7). A drawback to the GPS-mutagenesis system, which led to the discarding of over half of the mutagenized plasmids, was the integration of the pGPS3 into pWLG30+*lacZ*+Transprimer.

Prior research by Tumbula et al. (1997) suggested that the majority of pURB500 is required for replication in *M. maripaludis*. In this work, the Transprimer mutated seven of the eighteen ORFs identified by Tumbula et al. (1997). Only the two mutations in ORF 1 failed to produce puromycin resistant *M. maripaludis* colonies after transformation. This result was not unexpected for ORF1 since it may be a replication protein (37). Previous experiments suggest that ORF1 is essential for replication. Ligations into the *Eco*R I site after a partial digestion of pURB500 failed to clone the pUC derivatives during the constructions of pDLT44 and pWLG30 (15, 37). Additional subclones of pURB500, which lacked ORF1, also fail to replicate in *M. maripaludis* (37). Mutations in ORFs 4 and 12 were expected not to be essential for replication since the *Eco*R I site, which was used to clone DNA, was contained in these ORFs. However, the mutations eliminated the possibility that a truncated ORF4 protein was still functional.

Isolate 1 was interesting since the insertion of the transprimer was between the heterologous *Methanococcus voltae* promoter and the puromycin transacetylase (*pac*) gene. However, puromycin resistant colonies were recovered. The neomycin phosphotransferase was orientated for transcription in a counter-clockwise direction so the bacterial promoter could not have contributed to the expression of *pac*. Expression of the *pac* was probably either by the P_{mcr}, fortuitous expression of *pac* from the transprimer region, or a second *M. voltae* promoter region downstream of the transprimer insertion.

Transcription of Isolate 1's *pac* cassette was not investigated to confirm the size of the untranslated region of the upstream of *pac* in the mRNA.

pURB500 is a low copy plasmid in *M. maripaludis* strain C5 with three copies per cell (39). The β-galactosidase assays allowed an indirect measurement of plasmid stability within *M. maripaludis*. If a mutated pURB500 ORF or region were nonessential and influenced the expression shuttle vector copy number, then a difference in the β-galactosidase specific activity may be significant. None of the Transprimer mutations influenced the β-galactosidase specific activity more than 2-fold. These results suggested that the mutated regions were not involved in the control of plasmid copy number. Isolate 2 represented a control for the transposon's insertion in a nonessential region of pWLG30+*lacZ.*

An interesting observation was the relatively normal β-galactosidase levels seen with Isolate 8 and slight decrease in the β-galactosidase specific activity in Isolates 6 and 7. ORFLESS 1 and ORFLESS 2 are thought to be possible origins of replication for pURB500 (37). The physical separation of ORFLESS 1 and ORFLESS 2 by the 1.7 kilobase pair (kb) Transprimer did not stop plasmid replication. This result suggested that if both ORFLESS regions were required for replication, they may operate independently. Also Isolate 8, which contained a mutation at one end of ORFLESS 1, replicated in *M. maripaludis*.

Transformation efficiency could be influenced by the plasmid's ability to replicate in the host. The transformation efficiency was not indicative of the β-galactosidase specific activity, but may represent the inability to quantify accurately the plasmid or the influence of damage received during purification.

The neomycin phosphotransferase, which was encoded by *npt* in the Transprimer, confers resistance to both kanamycin and neomycin. *M. maripaludis* is also sensitive to neomycin so this mutagenesis system could potentially be useful in future experiments (3). *M. maripaludis* transformed with the pWLG30+*lacZ*+transprimer isolates were not tested for neomycin resistance.

References

1. **Adams, R. L. P**. 1991. Initiation of replication-single replicons, p.25-47. *In* D. Rickwood and D. Male (ed.), DNA replication. Oxford University Press, New York.
2. **Alonso, J. C., S. Ayora, I. Canosa, F. Weise, and F. Rojo**. 1996. Site-specific recombination in Gram-positive theta-replicating plasmids. FEMS Microbiol Lett. **142**:1-10.
3. **Argyle, J. L., D. L. Tumbula, and J. A. Leigh**. 1996. Neomycin resistance as a selectable marker in *Methanococcus maripaludis*. Appl. Environ. Microbiol. **62**:42233-4237.
4. **Bokranz, M., A. Klein, and L. Meile**. 1990. Complete nucleotide sequence of plasmid pME2001 of *Methanobacterium thermoautotrophicum* (Marburg). Nucleic Acids Res. **18**:363.
5. **Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, G. G. Sutton, J. A. Blake, L. M. FitzGerald, R. A. Clayton, J. D. Gocayne, A. R. Kerlavage, B. A. Dougerty, J. F. Tomb, M. D. Adams, C. I. Reigh, R. Overbeek, E. F. Kirkness, K. G. Weinstock, J. M. Merrick, A. Glodek, J. L. Scott, N. S. M. Geoghagen, J. F. Weidman, J. L. Fuhrmann, D. Nguyen, T. R. Utterback, J. M. Kelley, J. D. Peterson, P. W. Sadow, M. C. Hanna, M. D. Cotton, K. M. Roberts, M. A. Hurst, B. P. Kaine, M. Borodovsky, H. P. Klenk, C. M. Fraser, H. O. Smith, C. R. Woese, and J. C. Venter**. 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science **273**:1017-1140.
6. **Charlebois, R. L., W. L. Lam, S. W. Cline, and W. F. Doolittle**. 1987. Characterization of pHV2 from *Halobacterium volcanii* and its use in demonstrating transformation of an archaebacterium. Proc. Natl. Acad. Sci. USA. **84**:8530-8534.
7. **Craig, N. L**. 1996. Transposon Tn7. Curr. Top. Microbiol. Immunol. **204**:27-48.
8. **Davison, J**. 1984. Mechanism of control of DNA replication and incompatibility in ColE1-type plasmids- a review. Gene **28**:1-15.
9. **del Solar, G., M. Moscoso, and M. Espinosa**. 1993. Rolling circle-replicating plasmids from Gram-positive and Gram-negative bacteria: a wall falls. Mol. Microbiol. **8**:789-796.
10. **del Solar, G., R. Giraldo, M. J. Ruiz-Echevarría, M. Espinosa, and R. Díaz-Orejas**. 1998. Replication and control of circular bacterial plasmids. Microbiol. Mol. Biol. Rev. **62**:434-464.
11. **Dillard, J. P. and J. Yother**. 1991. Analysis of *Streptococcus pneumoniae* sequences cloned into *Escherichia coli*: effect of promoter strength and transcription terminators. J. Bacteriol. **173**:5105-5109.
12. **Erauso, G., S. Marsin, N. Benbouzid-Rollet, M.-F. Baucher, T. Barbeyron, Y. Zivanoic, D. Prieur, and P. Forterre**. 1996. Sequence of plasmid pGT5 from the archaeon *Pyrococcus abyssi*: Evidence for rolling-circle replication in a hyperthermophile. J. Bacteriol. **178**:3232-3237.
13. **Espinosa, M., G. del Solar, F. Rojo, and J. C. Alonso**. 1995. Plasmid rolling circle replication and its control. FEMS Microbiol. Lett. **130**:111-120.
14. **Frey, J. and M. Bagdasarian**. 1989. The molecular biology of IncQ plasmids, p. 79-94. *In* C. M. Thomas (ed.), Promiscuous plasmids of gram-negative bacteria. Academic Press, London.

15. **Gardner, W. L. and W. B. Whitman**. 1999. Expression vectors for *Methanococcus maripaludis*: overexpression of acetohydroxyacid synthase and β-galactosidase. Genetics **152**:1439-1447.
16. **Hackett, N. R., M. P. Krebs, S. DasSarma, W. Goebel, U. L. RajBhandary, and H.G. Khorana**. 1990. Nucleotide sequence of a high copy number plasmid from *Halobacterium* strain GRB. Nucleic Acids Res. **18**:3408.
17. **Hall, M. J. and N. R. Hackett**. 1989. DNA sequence of a small plasmid from *Halobacterium* strain GN101. Nucleic Acids Res. **17**:10501.
18. **Haring, V. and E. Scherzinger**. 1989. Replication proteins of the IncQ plasmid RSF1010, p. 95-124. *In* C. M. Thomas (ed.), Promiscuous plasmids of gram-negative bacteria. Academic Press, London.
19. **Hinnebusch, J. and K. Tilly**. 1993. Linear plasmids and chromosomes in bacteria. Mol. Microbiol. **10**:917-922.
20. **Holmes, M. L. and M. L. Dyall-Smith**. 1990. A plasmid vector with a selectable marker for halophilic archaebacteria. J. Bacteriol. **172**:756-761.
21. **Holmes, M. L., F. Pfeifer, and M. L. Dyall-Smith**. 1995. Analysis of the halobacterial plasmid pHK2 minimal replicon. Gene. **153**:117-121.
22. **Kagramanova, V. K., N. I. Derckacheva, and A. S. Mankin**. 1988. The complete nucleotide sequence of the arcaebacterial plasmid pHSB from *Halobacterium*, strain SB3. Nucleic Acids Res. **16**:4158.
23. **Keeling, P., H.-P. Klenk, R. K. Singh, O. Feeley, C. Schleper, W. Zillig, W. F. Doolittle, and C. W. Sensen**. 1996. Complete nucleotide sequence of the *Sulfolobus islandicus* multicopy plasmid pRN1. Plasmid **35**:141-144.
24. **Keeling, P. J., H.-P. Klenk, R. K. Singh, M. E. Schenk, C. W. Sensen, W. Zillig, and W. F. Doolittle**. 1998. *Sulfolobus islandicus* plasmids pRN1 and pRN2 share distant but common evolutionary ancestry. Extremophiles **2**:391-393.
25. **Krebs, M. P., T. Hauss, M. P. Heyn, U. L. Rajbhandary, and H. G. Khorana**. 1991. Expression of the bacterioopsin gene in *Halobacterium halobium* using a multicopy plasmid. Proc. Natl. Acad. Sci. USA **88**:859-863.
26. **Meile, L., A. Kiener, and T. Leisinger**. 1983. A plasmid in the archaebacterium *Methanobacterium thermoautotrophicum*. Mol. Gen. Genet. **191**:480-484.
27. **Metcalf, W. W., J. K. Zhang, E. Apolinario, K. R. Sowers, and R. S. Wolfe**. 1997. A genetic system for Archaea of the genus *Methanosarcina*: Liposome-mediated transformation and construction of shuttle vectors. Proc. Natl. Acad. Sci. USA **94**:2626-2631.
28. **Miller, J. H**. 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, New York.
29. **Ng, W.-L. and S. DasSarma**. 1993. Minimal replication origin of the 200-kilobase *Halobacterium* plasmid pNRC100. J. Bacteriol. **175**:4584-4596.
30. **Nölling, J., F. J. M. van Eeden, R. I. L. Eggen, and W. M. de Vos**. 1992. Modular organization of related archaeal plasmids encoding different restriction-modification systems in *Methanobacterium thermoformicicum*. Nucleic Acids Res. **20**:6501-6507.
31. **Pfeifer, F. and P. Ghahraman**. 1993. Plasmid pHH1 of *Halobacterium salinarium*: characterization of the replicon region, the gas vesicle gene cluster and insertion elements. Mol. Gen. Genet. **238**:193-200.

32. **Sambrook, J., E. F. Fritsch, and T. Maniatis**. 1989. Molecular cloning: a laboratory manual (2nd edition). Cold Spring Harbor Laboratory Press, New York.
33. **Schleper, C., I. Holz, D. Janekovic, J. Murphy, and W. Zillig**. 1995. A multicopy plasmid of the extremely thermophilic archaeon *Sulfolobus* effects its transfer to recipients by mating. J. Bacteriol. **177**:4417-4426.
34. **She, Q., H. Phan, R. A. Garrett, S.-V. Albers, K. M. Stedman, and W. Zillig**. 1998. Genetic profile of pNOB8 from *Sulfolobus*: the first conjugative plasmid from an archaeon. Extremophiles **2**:417-425.
35. **Sioud, M., G. Baldacci, P. Forterre, and A.-M. de Recondo**. 1988. Novobiocin induces accumulation of a single strand of plasmid pGRB-1 in the archaebacterium *Halobacterium* GRB. Nucleic Acids Res. **16**:7833-7842.
36. **Tumbula, D. L., R. A. Makula, and W. B. Whitman**. 1994. Transformation of *Methanococcus maripaludis* and identification of a *Pst*I-like restriction system. FEMS Microbiol. Lett. **121**:309-314.
37. **Tumbula, D. L., T. L. Bowen, and W. B. Whitman**. 1997. Characterization of pURB500 from the archaeon *Methanococcus maripaludis* and construction of a shuttle vector. J. Bacteriol. **179**:2976-2986.
38. **Whitman, W. B., J. Shieh, S. Sohn, D. S. Caras, and U. Premachandran**. 1986. Isolation and characterization of 22 mesophilic methanococci. System. Appl. Microbiol. **7**:235-240.
39. **Wood, A. G., W. B. Whitman, and J. Konisky**. 1985. A newly-isolated marine methanogen harbors a small cryptic plasmid. Arch. Microbiol. **142**:259-261.
40. **Zillig, W., A. Kletzin, C. Schleper, I. Holz, D. Janekovic, J. Hain, M. Lanzendörfer, and J. K. Kristjansson**. 1994. Screening for *Sulfolobales*, their plasmids and their viruses in Icelandic solfataras. System. Appl. Microbiol. **16**:609-628.
41. **Zillig, W., D. Prangishvilli, C. Schleper, M. Elferink, I. Holz, S. Albers, D. Janekovic and D. Götz**. 1996. Viruses, plasmids and other genetic elements of thermophilic and hyperthermophilic *Archaea*. FEMS Microbiol. Rev. **18**:225-236.

TABLE 1. Plasmids used in this study[a]

Plasmids	Properties	Reference
pGPS-3	contains Transprimer-1, ampicillin resistance (amp^r), kanamycin resistance (kan^r)	New England Biolabs
pWLG30+*lacZ*	expression shuttle vector for *M. maripaludis* which contains *lacZ* for *E. coli* blue/white screen amp^r, puromycin resistance (pur^r)	15
pWLG30+*lacZ*+Transprimer		
Isolate 1	expression shuttle vector mutagenized with Tn7 amp^r, kan^r, pur^r	This work
Isolate 2	expression shuttle vector mutagenized with Tn7 amp^r, kan^r, pur^r	This work
Isolate 3	expression shuttle vector mutagenized with Tn7 amp^r, kan^r, pur^r	This work
Isolate 4	expression shuttle vector mutagenized with Tn7 amp^r, kan^r, pur^r	This work
Isolate 5	expression shuttle vector mutagenized with Tn7 amp^r, kan^r, pur^r	This work
Isolate 6	expression shuttle vector mutagenized with Tn7 amp^r, kan^r, pur^r	This work
Isolate 7	expression shuttle vector mutagenized with Tn7 amp^r, kan^r, pur^r	This work

Isolate 8	expression shuttle vector mutagenized with Tn7 amp^r, kan^r, pur^r	This work
Isolate 9	expression shuttle vector mutagenized with Tn7 amp^r, kan^r, pur^r	This work
Isolate 10	expression shuttle vector mutagenized with Tn7 amp^r, kan^r, pur^r	This work
Isolate 11	expression shuttle vector mutagenized with Tn7 amp^r, kan^r, pur^r	This work
Isolate 12	expression shuttle vector mutagenized with Tn7 amp^r, kan^r, pur^r	This work
Isolate 13	expression shuttle vector mutagenized with Tn7 amp^r, kan^r, pur^r	This work

[a]Ampicillin (amp) and kanamycin (kan) are for selection in *E. coli.* Puromycin (pur) is for selection in *M. maripaludis*.

TABLE 2. Transposon insertions into pWLG30+*lacZ*

pWLG30+*lacZ*+Transprimer Isolate	Position[a] (bp)	Orientation[b]	Feature
1	3602	-	Inserted between P_{hmvA} and *pac*
2	7365	+	
3	7878	+	Interrupts ORF10
4	9342	-	
5	9696	+	
6	10336	-	Interrupts ORF3 and ORF13
7	10876	-	Interrupts ORF3
8	11071	+	Interrupts ORFLESS 1
9	12886	-	Interrupts ORF1
10	13742	-	Interrupts ORF1
11	15163	-	Interrupts ORF5
12	15439	-	Interrupts ORF4
13	15623	-	Interrupts ORF4 and ORF12’

[a]The position of the first nucleotide of the transposon. Number is relative to the first nucleotide of pWLG30+*lacZ* in Figure 1.

[b]When the neomycin phosphotransferase gene, which was within the flanking regions of Transprimer-1, was transcribed in a clockwise manner, a plus symbol was scored. See Figure 2. A minus symbol indicated transcription in a counter-clockwise manner. As an example, *lacZ* had an orientation of "+".

TABLE 3. *Methanococcus maripaludis* transformation efficiencies and β-galactosidase activities for the pWLG30+*lacZ*+Transprimer isolates

Plasmid	Relative percent of transformation efficiency[a]	Relative percent of β-galactosidase specific activity[b]
pWLG30+*lacZ*	(100)	100
pWLG30+*lacZ*+Transprimer		
Isolate 1	150	180
Isolate 2	380	90
Isolate 3	50	96
Isolate 4	290	210
Isolate 5	370	120
Isolate 6	170	70
Isolate 7	38	62
Isolate 8	150	94
Isolate 9	NT[c]	
Isolate 10	NT	
Isolate 11	440	95
Isolate 12	430	88
Isolate 13	770	120

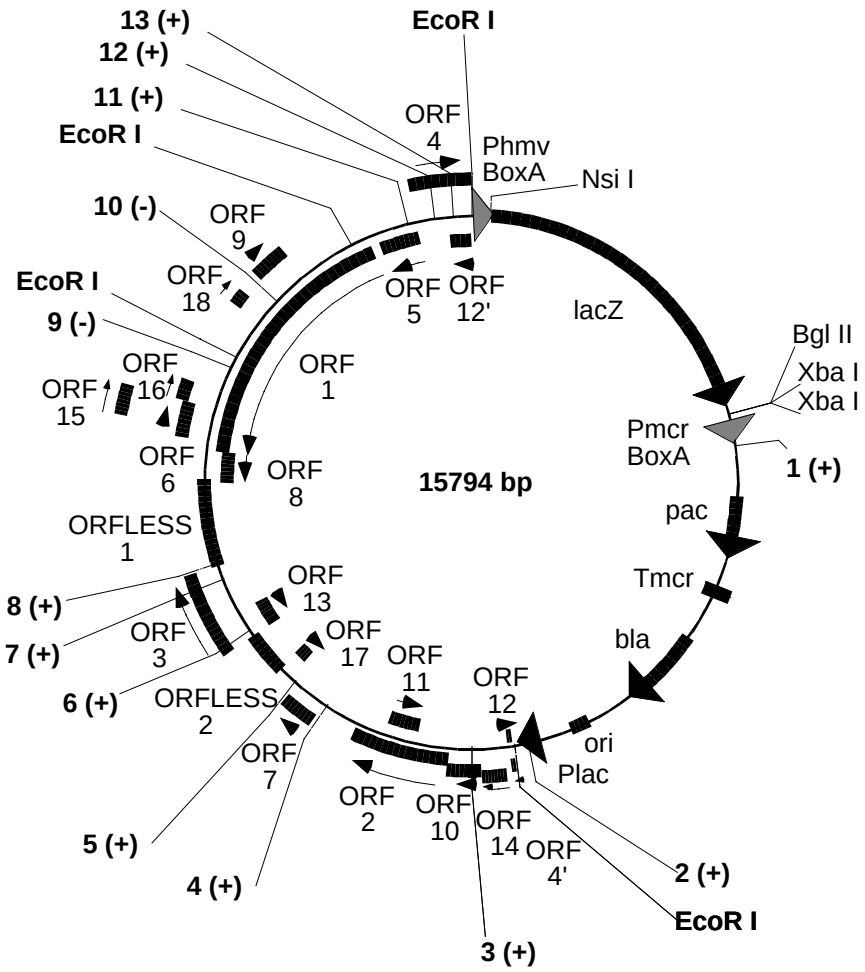
[a]The transformation efficiency of pWLG30+*lacZ* was 8.2×10^3 transformants/μg of plasmid DNA.

[b]The specific activity was μmol of ONPG transformed min^{-1} (mg of protein)$^{-1}$. Excluding pWLG30+*lacZ*, each value represented the average of β-galactosidase specific activities for three independent isolates. Triplicate assays were performed, then averaged for each isolate and *M. maripaludis* transformed with pWLG30+*lacZ*. The standard deviation was between 5-10% of the averaged value for three isolates. An one-way ANOVA calculation on the relative percentage of β-galactosidase specific activities for each isolate was significant at a level of significance of 0.01.

[c]No transformants were observed. The experiment was performed twice.

Figure 1: Position of transposon insertion into *pWLG30+lacZ*.

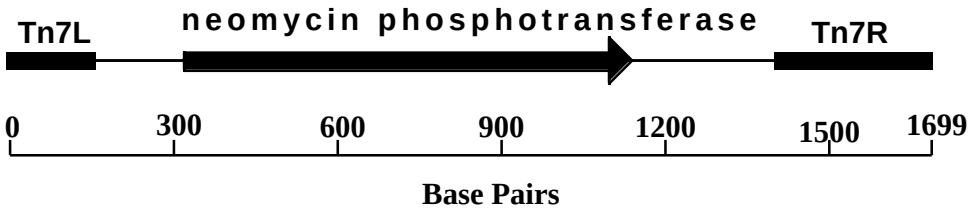
pWLG30+*lacZ* was randomly mutagenized *in vitro* with the GPS-M mutagenesis system, which was based on the Tn7 transposon. The transposon, known as Transprimer-1, contained neomycin phosphotransferase for kanamycin resistance. The numbers, which increase in a clock-wise direction, represented the Transprimer insertion position in each isolate. The "+" or "-" symbols indicated puromycin resistant colonies or no puromycin resistant colonies were recovered after two transformations into *M. maripaludis*, respectively. pURB500 began at nucleotide 15794 and continued in a counter-clockwise direction to nucleotide 7507.


13 (+)
12 (+)
11 (+)
EcoR I
10 (-)
EcoR I
9 (-)
8 (+)
7 (+)
6 (+)
5 (+)
4 (+)
3 (+)
2 (+)
EcoR I
1 (+)
EcoR I
ORF 4
Phmv BoxA
Nsi I
ORF 9
ORF 18
ORF 5
ORF 12'
lacZ
Bgl II
Xba I
Xba I
ORF 16
ORF 15
ORF 1
Pmcr BoxA
ORF 6
ORF 8
15794 bp
pac
ORFLESS 1
Tmcr
ORF 13
bla
ORF 3
ORF 17
ORF 11
ORF 12
ORFLESS 2
ori Plac
ORF 7
ORF 2
ORF 10
ORF 14
ORF 4'

pWLG30+*lacZ*

Figure 2: Transprimer-1.

This 1.7 kb fragment was based on the Tn7 transposon. The Tn7L and Tn7R represented the left and right end regions, respectively. These regions were required for transposition. During transposition, the transprimer was donated from pGPS3. pGPS3, which was 4293 base pairs, contained the transprimer, an *E. coli* origin of replication, and the antibiotic resistant marker for ampicillin. The sequencing primers Tn7Lseq and Tn7Rseq were orientated to sequence a small section of either the left or right end region before reaching the pWLG30+*lacZ* sequence. The neomycin resistance was encoded by the neomycin phosphotransferase (*npt*).


Tn7L
neomycin phosphotransferase
Tn7R
0
300
600
900
1200
1500
1699
Base Pairs

CHAPTER V

CLONING OF HETEROLOGOUS ARCHAEAL GENES INTO THE *METHANOCOCCUS MARIPALUDIS* EXPRESSION SHUTTLE VECTOR[4]

[4] Gardner, W. L. and W. B. Whitman. To be submitted.

Keywords: heterologous, expression, archaea, hyperthermophile, thermophilic, anaerobic, shuttle vector, translation, regulation, methanogen

Abstract

The *Methanococcus maripaludis* expression shuttle vector was used to express heterologous genes from different thermophilic or hyperthermophilic *Euryarchaeota.* Each gene was modified to accommodate a *Nsi* I site at the 5'-end of the coding region and a *Xba* I site downstream of the coding region by the polymerase chain reaction (PCR). Downstream of the stop codon, a second nucleotide region was modified to contain a restriction site represented in the multicloning site (MCS) of either pWLG30 or pWLG30+*lacZ.* After the modified gene was ligated into the expression vector, the *Methanococcus voltae* histone promoter (P_{hmv}) controlled the transcription in *M. maripaludis*. The genes cloned into this system encoded the proteins known as monomethylamine methyltransferase from *Methanosarcina barkeri,* delta subunit from pyruvate ferredoxin oxidoreductase of *Pyrococcus furiosus,* carbon monoxide dehydrogenase beta subunit from *Methanosarcina thermophila*, and alpha subunit from the F_{420}-reducing hydrogenase of *Methanococcus jannaschii*.

Introduction

Collectively, the archaea represent a group which is adapted to some of the most extreme environments on earth. However, a problem hampering studies of archaeal proteins is the inability to harvest or purify sufficient quantities of cells or cellular components. Bacterial expression systems are advantageous in many instances, but these expression systems can lack additional cofactors, metals, and cellular machinery required for production of an active holoenzyme. Therefore, development of archaeal expression systems represents a potential advancement. Archaeal expression systems will have additional benefits such as the elucidation of gene regulation and expression in archaea. Complementation studies of archaeal metabolic pathways are also possible with these

vectors. Altogether, the information gathered from these experiments can begin to address essential differences and similarities between the Archaea, Bacteria, and Eukarya.

Archaeal expression systems are under development for both the *Crenarchaeota* and *Euryarchaeota.* The broad host range plasmid, pAG21, represents the most recent advance for shuttle vectors for the *Crenarchaeota.* This vector is able to replicate in *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, and *Escherichia coli* (3). pGT5, the archaeal portion of the chimeric pAG21, is a completely sequenced plasmid isolated from the hyperthermophile *Pyrococcus abyssi* strain GE5 (16, 17). This plasmid, which uses a rolling-circle method for replication, is found in 25 to 30 copies per *P. abyssi* GE5 cell (17).

Initial obstacles for thermophilic expression systems are the identification of thermostable reporters and thermostable selectable markers. pAG21 utilizes the alcohol dehydrogenase from *Sulfolobus solfataricus* as the selectable marker in *P. furiosus* and *S. acidocaldarius.* To date, the only *Crenarchaeota* thermostable marker is the *S. solfataricus* beta-galactosidase (15). However, the gene for this marker has not been expressed from pAG21.

Many shuttle vectors were constructed for the *Euryarchaeota.* Three families of vectors were constructed in attempts to identify the minimal amount of archaeal plasmid DNA required for replication. pUBP3, pNG100, and pMLH3, which are able to replicate in *E. coli* and *Haloferax volcanii*, contain the replicons from pHH1, pNRC100, and pHK2, respectively (23, 34, 36). pWL102 and pUBP2 have the broadest host range of the halophile shuttle vectors. These vectors are able to replicate in *Halobacterium halobium*, *Haloarcula vallismortis*, *Haloarcula hispanica*, and some *Haloferax* species (7, 11, 27). For selectable markers in halophiles, three different genes were tested in the shuttle vectors. The DNA gyrase B subunit, which has a mutation within its gene, confers resistance to mevinolin (28). The increased levels of 3-hydroxy-3-methylglutaryl-coenzyme A reductase confers resistance to novobiocin (22). The last selectable marker, which confers resistance

to bleomycin in *Haloferax volcanii*, is the *Streptoalloteichus hindustanus* bleomycin resistance protein (ShBle, 35).

A few shuttle vectors have been constructed for the methanogens. pWM307, which uses the methanogen origin of replication from the cryptic plasmid pC2A from *Methanosarcina acetivorans* strain C2A, has a broad host range of 9 *Methanosarcina* species (33). Other methanogen shuttle vectors are pDLT44 and pWLG30. Both vectors use the pURB500 origin for replication in *Methanococcus maripaludis* strains (18, 47). Additionally, pWLG30 is an expression shuttle vector (18). The puromycin transacetylase provides the selective marker in all of these vectors.

There are many characteristics that make *M. maripaludis* a logical choice for hosting an expression system for heterologous genes. *M. maripaludis* is a strictly anaerobic archaeon that produces methane from carbon dioxide and hydrogen or formate (25). It has a relatively rapid doubling time of 2.3 hours at the optimum growth range of 35-37^{o}C while growing under autotroph conditions. As a methanogenic archaeon, *M. maripaludis* contains many unusual coenzymes and cellular components that may be important for producing heterologous proteins. The genetic tools for the methanococci are also increasing in sophistication. *M. maripaludis* has resistance markers for the antibiotics puromycin and neomycin (2, 19). Beta-galactosidase, beta-glucuronidase, and trehalase are the three reporter enzymes known to work in *M. maripaludis* or the related organism *Methanococcus voltae* (5, 12, 18, 43). PEG-mediated transformation of *M. maripaludis* yields 10^7 transformants per microgram of plasmid DNA, making the organism amenable to a wide range of genetic studies (46). In addition to a high transformation frequency, an expression system needs an organism that can be grown to high densities in large volumes. *M. maripaludis* can be grown at the 400 L scale in a minimal medium plus formate (unpublished results). Future developments for the expression system will rely on new nucleotide sequences. The *Methanococcus jannaschii* and *Methanobacterium*

thermoautotrophicum strain ΔH genomic sequences provide new promoter and terminator sequences for vector construction (9, 42).

The current *M. maripaludis* expression shuttle vector uses the *M. voltae* histone promoter (P_{hmvA}) for the *in vitro* expression of heterologous genes in *M. maripaludis* (18). Figure 1 contains the modified P_{hmvA} sequence found in pWLG14 and pWLG30. This promoter contains *Cla* I and *Nsi* I restriction sites flanking the P_{hmvA} Box A, Box B and ribosome binding site (RBS). The last two nucleotides in the *Nsi* I recognition sequence (nucleotides 167 and 168) are used to form part of the start codon for the heterologous genes cloned in pWLG30. PCR (polymerase chain reaction) modifies the heterologous genes to contain a *Nsi* I site at the 5' end of the gene for ligation into the expression shuttle vector. It is important to note that the *Nsi* I restriction site contains an ATG codon (nucleotides 163-165). However, this codon is adjacent to the predicted P_{hmvA} RBS. In Archaea, start codons are usually separated by a few nucleotides from the putative RBS (8), excluding 'leaderless' mRNAs that lack a 5' untranslated region (13). *M. jannaschii* also has an under-representation of ATG triplets prior to the putative ATG codons (38). Additionally, many of the *M. jannaschii* start codons are predicted to utilize either TTG or GTG instead of ATG (9). With this expression system, these start codons will be mutated during PCR to form an ATG start codon. A potential concern is whether or not TTG or GTG start codons produce a protein with a methioninyl residue at the N-terminus. In one experiment, the *Sulfolobus solfataricus* putative NUSA-like termination/antitermination transcription factor protein, which has a GTG start codon, was shown to contain a methioninyl residue after translation in an *in vitro Sulfolobus* cell-free system (13). The first amino acid residue has not been identified in any archaeal proteins containing a TTG as the start codon.

Cloning PCR modified genes is routine in many laboratories. To increase the efficiency of identifying the correct construction, the *E. coli* beta-galactosidase gene (*lacZ*) provides a blue/white screen during the cloning of modified genes into the expression

shuttle vector. The *lacZ* is excised by *Nsi* I and either *Bgl* II or *Xba* I. After treatment by the restriction endonucleases, the mixture of pWLG30 and *lacZ* fragments are dephosphorylated by calf intestine alkaline phosphatase. The PCR modified heterologous gene is subsequently ligated into the expression vector. An additional benefit in the pWLG30+*lacZ* construct is the ability to use *Bgl* II to lower the background of undigested vector after the initial restriction digestion with *Nsi* I and *Xba* I. Using multiple restriction endonucleases, which have noncomplementary overhangs, allows for the directed ligation of the modified gene into the vector.

Present applications of the *M. maripaludis* expression system focus on two distinct challenges: translation in *M. maripaludis* and expression of an individual subunit. Two collaborations concentrate on studying translational events with *M. maripaludis*. The remaining experiments represent the expression of an individual subunit from a multisubunit complex.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions. *Methanococcus maripaludis* JJ was obtained from W. J. Jones. The plasmids used in this study are listed in Table 1. *M. maripaludis* was grown at 37°C on 275 kPa of H_2:CO_2 (80:20) in McN (mineral medium) and McC (complex medium minus the vitamin solution) (49). The sodium bicarbonate in the medium was reduced from 5 g l^{-1} to 2 g l^{-1} during growth at 100 kPa in 1 L Wheaton bottles. Transformation of *M. maripaludis* with plasmid DNA was described previously (Tumbula *et al.* 1994). For selection of puromycin-resistant methanococci, a stock solution of 500 μg ml^{-1} puromycin dihydrochloride (Sigma) in distilled water was filter-sterilized and added to the medium at a final concentration of 2.5 μg ml^{-1}. Strictly anaerobic techniques were used for medium preparation and cultivation.

The *E. coli* strains were grown at 37°C on low salt Luria-Bertani (LB) medium with the NaCl at 50% of the regular concentration (29). Antibiotic concentrations were 60 μg ml^{-1} for ampicillin (Amp) and 50 μg ml^{-1} for kanamycin (Kan) in both liquid and solid LB

medium. ß-galactosidase was used as a blue/white screen on LB agar plates with 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) according to Sambrook *et al.* (1989). Plasmids were transformed into *E. coli* strains by electroporation with a Bio-Rad Gene Pulser (Bio-Rad Laboratories) using the settings of 200 Ω, 2.5 kV, and 25 μF with cuvettes (0.2 cm gap width). Plasmids from *E. coli*, grown with selective conditions, were isolated with the Wizard miniprep kit (Promega).

Growth of *E. coli* and *M. maripaludis* was measured at 600 nm with a spectrophotometer (Spectronic 20).

Electrophoresis of agarose gels. The electrophoresis of all agarose gels occurred in 1X TAE and 0.4 microgram/ml ethidium bromide (39). A 10X TAE stock solution contained 0.4 M tris base, 0.2 M glacial acetic acid, and 0.02 M (ethylenedinitrilo)tetraacetic acid, disodium salt, dihydrate. The agarose was also dissolved in 1X TAE.

Deoxyribonucleic acid (DNA) sequencing. All DNA sequencing was performed using ABI sequencers at the Molecular Genetics Facility, University of Georgia. All oligonucleotides were made by Integrated DNA Technologies (Coralville, Iowa). Sequence analysis was performed with the GCG software package (Genetics Computer Group, Madison, WI). FRAMES was used to identify open reading frames (ORFs) using ATG, GTG, or TTG as the start codons.

Methanosarcina barkeri monomethylamine methyltransferase

Expression of the *Methanosarcina barkeri* strain MS monomethylamine methyltransferase (MMAMT) and the *M. jannaschii* F_{420}-reducing hydrogenase subunit A (FruA) represents the investigations into translational events with *M. maripaludis* as the host system. The production of methane from monomethylamine in *M. barkeri* involves MMAMT (10). The MMAMT, which is encoded by *mtmB*, contains an in-frame UAG stop codon at nucleotide positions 603-605 in *M. barkeri* strains MS and NIH (10). If translation terminated at this codon, a 23 kilodalton (kDa) protein would be produced. However, the MMAMT has a mass of 52 kDa and termination actually occurs at a second

stop codon 770 nucleotides downstream of the in-frame UAG codon. Expression of the *M. barkeri mtmB* in *M. maripaludis* will test the hypothesis of an equivalent translational mechanism in *M. maripaludis*. *M. maripaludis* is expected to lack a MtmB homolog since it does not produce methane from methylamines (25). In addition, a homolog of *mtmB* was not detected in the genome sequence of *M. jannaschii*, a related methanogen with a similar physiology (9).

Cloning of *M. barkeri* MS *mtmB* into pWLG30+*lacZ*. pWLG30+*lacZ* was purified from *Escherichia coli* XL1-Blue MRF' (Stratagene) before digestion with *Nsi* I and *Xba* I (Figure 2). Following the digestion, the plasmid was dephosphorylated with CIAP (calf intestine alkaline phosphatase).

pLPMB2 (From Ligi Pottenplackel and Joseph Krzycki) contained a PCR modified *M. barkeri mtmB*. The modifications to the *mtmB* nucleotide sequence allowed for the directed ligation of *mtmB* into pWLG30+*lacZ*. pLPMB2, purified from *E. coli* DH5αF', was digested with *Nsi* I and *Xba* I. The 1.4 kilobase pair (kbp) *Nsi* I-*Xba* I fragment containing *mtmB* was gel purified before directed ligation into pWLG30+*lacZ*/*Nsi* I/*Xba* I/CIAP by T4 DNA ligase. The ligated plasmids were transformed into *E. coli* XL1-Blue MRF', and the cells were plated on LB-Amp, X-Gal plates. The plasmids from individual white colonies were screened by size using electrophoresis with a 0.8% (w/v) agarose gel. A *Nsi* I and *Xba* I digestion of the plasmids confirmed the removal of *lacZ* and the addition of the *mtmB* containing fragment to pWLG30+*lacZ*. Sequencing with the previously published primers pWLG30seq1 and pMEB.2seq2 confirmed the construction of pWLG30+*mtmB* (18). pWLG30+*mtmB* was transformed into *M. maripaludis*.

Growth of recombinant *M. maripaludis* containing pWLG30 and pWLG30+*mtmB*. Balch tubes, which contained 5 ml of McN+puromycin, were inoculated with 0.2 ml of *M. maripaludis* transformed with either pWLG30 or pWLG30+*mtmB*. After an overnight incubation, *M. maripaludis* grew to an $O.D._{600}$ of 0.46-0.50 in three tubes and 0.43-0.50 in seven tubes for pWLG30 and pWLG30+*mtmB* respectively. Each

tube was inoculated into 150 ml of McC+puromycin in a modified Wheaton bottle after the addition of 3 ml 2.5% (w/v) sodium sulfide. After 24 hours of growth, the cultures were anaerobically harvested by centrifugation at 4400 *g* and 10°C for 15 minutes in a Beckman JA-21 rotor. The cell pellets were transferred to degassed aluminum seal vials and stoppered. The vials were flushed with nitrogen gas for two minutes while on ice. After flushing, the vials were pressurized to 80 kPa with nitrogen before storage at -20°C. The *M. maripaludis* plus pWLG30 or pWLG30+*mtmB* yielded 0.43 g and 1.05 g of wet cell paste for the respective cultures.

Results and discussion of the *M. maripaludis* transformed with pWLG30+*mtmB* experiment

A Western blot performed on cell extracts of *M. maripaludis* transformed with pWLG30+*mtmB* performed by James and Krzycki (2000) showed a 23 kDa protein that reacted with antibodies against MtmB (24). The control cell extract of *M. maripaludis* transformed with pWLG30 did not react with the antibodies against the *M. barkeri* MtmB. From these results, the recombinant *M. maripaludis* appears to recognize the UAG codon as a stop codon, and the cells are unable to produce the 52 kDa MtmB *in vivo*.

***Pyrococcus furiosus* pyruvate ferredoxin oxidoreductase**

The *P. furiosus* pyruvate ferredoxin oxidoreductase (POR) has four heterologous subunits: alpha, beta, gamma, and delta (6). To characterize the *P. furiosus* POR delta subunit, Menon *et al.* (1998) expressed the subunit in *E. coli*. The native *P. furiosus* POR contains two [4Fe-4S] clusters. However, recombinant PorD produced in *E. coli* was an apoprotein lacking Fe atoms in greater than 90% of the available sites. Reconstitution of the PorD apoprotein results in only a 15% to 20% conversion to the holoenzyme. To facilitate future *P. furiosus* POR studies, *porD* expression in *M. maripaludis* would try to utilize this host's cellular machinery to produce the holoenzyme. *M. maripaludis* contains its own anabolic POR that catalyzes the carboxylation of acetyl-CoA to pyruvate (40). The purified *M. maripaludis* POR subunits are similar sizes to the *P. furiosus* POR (50). At the amino

acid level, the *P. furiosus* and *M. maripaludis* POR gamma units are 75% identical over 28 residues at the N-terminus (50).

Cloning of *P. furiosus porD* into pWLG30+*lacZ*. For directed ligation of the *P. furiosus porD* into pWLG30+*lacZ*, the *porD* was PCR modified to contain a *Nsi* I site at the 5'-end and a *Xba* I site at the 3'-end. pHH4-4 (From Angeli Menon and Michael Adams), purified from *E. coli* XL1-Blue MRF', contained the *P. furiosus porD* template for the PCR (Figure 3). The *porD* was amplified by PCR using the primers 5'-CCAATGCATGGCTGAAAGTCCGTTTAAGGC-3' and 5'-TGCTCTAGATCACTTAACTTCTCTAACCATTTCAATGGC-3' at a 0.5 micromolar concentration. These 5'-phosphorylated primers were designed to add a *Nsi* I site and a *Xba* I site at the flanking 5' and 3' ends of the PCR product respectively. The PCR contained 2.5 units of *Pfu* DNA polymerase (Stratagene). After an initial dwell of 5 minutes at 95°C to denature pHH4-4, PCR was performed for 25 cycles. Each cycle had a denaturing temperature of 94°C for 1 minute, an annealing temperature of 51.2°C for 1 minute, and an extension time of 1 minute at 74°C. The PCR product, *Nsi* I-*porD*-*Xba* I, was ligated into the *Eco*R V site of pZErO-2. The ligated plasmids were drop dialyzed against distilled water with VSWP membranes (Millipore) for 2 hours before transformation into *E. coli* Top10. Colonies, which grew on LB-Kan agar plates, were streaked to isolate an individual transformant. Isolates were grown under selective pressure in a LB-Kan broth before purification of the plasmids. Digestions with *Nsi* I and *Xba* I identified pZErO-2+*porD*. Sequencing the construct also confirmed the addition of *Nsi* I and *Xba* I restriction sites at the respective 5'- and 3'-ends. Digestion of pZErO-2+*porD* with *Nsi* I and *Xba* I was followed by electrophoresis in a 2% (w/v) agarose gel. After staining the DNA within the gel with ethidium bromide, the DNA was viewed during a brief exposure with a transilluminator. The 0.3 kb DNA fragment (*Nsi* I/*porD*/*Xba* I) was excised from the gel. After grinding the agarose matrix with a glass rod, 400 microliters ((l) of pH 8.0 phenol:chloroform:isoamyl alcohol (25:24:1) with 0.1% (w/v) hydroxyquinoline

was introduced and vortexed with the sample before incubation at -20°C for 30 minutes. The sample was centrifuged at 16000 *g* for 12 minutes, and the aqueous phase added to a new centrifuge tube. A half volume of 7.5 M ammonium acetate followed by 2 volumes of cold 100% ethanol were mixed with the sample by vortexing. After incubation at -20°C for 1.5 hours, the sample was centrifugated at 16000 *g* for 10 minutes before decanting the aqueous phase. The tube was dried under vacuum for 5 minutes; then *Nsi* I/*porD*/*Xba* I was rehydrated with sterile, distilled water for ligation with the expression shuttle vector.

pWLG30+*lacZ* was purified from *E. coli* XL1-Blue MRF' (Stratagene) before digestion with *Nsi* I and *Xba* I. The Wizard clean-up kit (Promega) removed the enzymes after the restriction digestion. The plasmid was then dephosphorylated with CIAP (calf intestine alkaline phosphatase). The CIAP was removed with the Wizard clean-up kit. The ligation between the digested and dephosphorylated expression shuttle vector and the *Nsi* I/*porD*/*Xba* I DNA fragment occurred with 1 Weiss unit of T4 DNA ligase (Promega) for 17.5 hours at 14°C. The ligation mixture was drop dialyzed using a 0.025 micron VSWP02500 membrane (Millipore) before transformation into XL1-Blue MRF' *E. coli*. The transformed cells were plated on LB-Amp+X-Gal medium. After overnight growth, individual white colonies were streaked onto a LB-Amp plates. After 16 hours of growth, the plates were cooled to 4°C for one hour. Transformants were collected with a sterile toothpick, and placed into 50 µl of resuspension solution (50 mM Tris [pH 7.5], 10 mM EDTA, 100 µg/ml RNase A). Each sample was vortexed before the addition of 50 µl lysis solution (0.2 M NaOH, 1% (w/v) sodium dodecylsulfate). After 1 minute, 50 µl neutralization solution (1.32 M potassium acetate) was added. The cell debris was pelleted by centrifugation at 16000 *g* for 5 minutes at room temperature. To screen the circular plasmids by size for pWLG30+*porD*, electrophoresis was performed using 0.8% (w/v) agarose gel with 17 µl of the supernatant.

Since the *porD* represents a small portion of pWLG30+*porD*, PCR was chosen to confirm the removal of *lacZ* and the addition of *porD* to the pWLG30 vector. Purified

plasmids of individual isolates provided the template for the PCR reaction. The reaction utilized the primers 30gene1 (5'-GCCTAAATTAATTAAAAAAGACTCTCCAG-3') and 30gene2 (5'-TTTGAATAGTATATTACGAATAGGGCG-3') at a 0.5 micromolar concentration. The PCR contained 2.5 units of *Pfu* DNA polymerase (Stratagene). After an initial dwell of 5 minutes at 95°C to denature pWLG30+*porD*, PCR was performed for 25 cycles. Each cycle had a denaturing temperature of 94°C for 1 minute, an annealing temperature of 48.5°C for 1 minute, and an extension time of 1.5 minutes at 74°C. After the PCR was complete, the PCR products were separated on a 2% (w/v) agarose gel. The product sizes were 0.36 kbp and 0.69 kbp for pWLG30 and pWLG30+*porD*, respectively. The isolate identified with pWLG30+*porD* was also sequenced using the previously published primer pMEB.2seq2 (18). pWLG30+*porD* was transformed into *M. maripaludis*.

PCR was applied to confirm the stability of pWLG30+*porD* after transformation into *M. maripaludis*. *M. maripaludis* with pWLG30+*porD* was grown overnight in a medium of McN+puromycin to an $O.D._{600}$ of 0.5. An aliquot of this culure was diluted 1:10 with sterile distilled water. Using 0.5 microliter of diluted culture to provide the template, a PCR reaction was performed using the conditions described above with the primers 30gene1 and 30gene2.

Growth of recombinant *M. maripaludis* containing pWLG30 or pWLG30+*porD*. *M. maripaludis*, transformed with either pWLG30 or pWLG30+*porD*, was inoculated into 5 ml of McN+puromycin using a 0.3 ml inoculum. One culture of pWLG30 and two cultures of pWLG30+*porD* was grown. After 12 hours of growth at 37°C, the culture containing pWLG30 was moved to room temperature. After 24 hours, the cultures containing pWLG30 and pWLG30+*porD* had an $O.D._{600}$ of 0.45 and 0.27 or 0.28, respectively. Each 5 ml culture was used to inoculate a 150 ml bottle of McN+puromycin (3.3 microgram/ml). After 12 hours of growth at 37°C, the bottles were pressurized with H_2:CO_2. After seven additional hours of growth, the cultures' $O.D._{600}$

were 0.50 and 0.42 for pWLG30 and pWLG30+*porD* respectively. The cells were harvested and analyzed by Mike W. W. Adams's group in the Department of Biochemistry at the University of Georgia.

Results and discussion of the expression of *P. furiosus porD* in *M. maripaludis*

A Western blot, which used the antibodies against the *P. furiosus* POR, was performed on the *M. maripaludis* cell extracts (31). The antibodies reacted to proteins in cell extracts of both the control and cells transformed with pWLG30+*porD*. Since the antibodies crossreacted with the *M. maripaludis* POR in both samples, it was not possible to conclude that *P. furiosus* PorD is expressed by *M. maripaludis*. Future experiments would heat-denature the cell extracts to remove many of the *M. maripaludis* proteins before performing the Western blot.

Introduction for *Methanosarcina* species carbon monoxide dehydrogenase/acetyl-coenzyme synthase

Acetate is disproportionated into carbon dioxide and methane in *M. thermophila* strain TM-1 (52, 53). Acetate utilization is an important source of energy and carbon for *M. thermophila.* The *Methanosarcina thermophila* carbon monoxide dehydrogenase/acetyl-coenzyme synthase (CODH/ACS), which is also known as the acetyl-CoA decarbonylase/synthase (ACDS), represents 20% to 25% of the soluble cellular protein during growth on acetate (26). The CODH/ACS catalyzes two physiological reactions. The first reaction is the splitting of acetyl-Coenzyme A to Coenzyme A, carbon monoxide, and a methyl moiety (37). The methyl group can then be transferred to tetrahydrosarcinapterin to form N^5-methyltetrahydrosarcinapterin, an intermediate in methanogenesis. The carbon monoxide dehydrogenase activity of the same enzyme also oxides carbon monoxide to carbon dioxide and reduced ferredoxin in the second reaction. See Deppenmeier *et al.* (1996) for the relationship of the CODH/ACS to the other enzymes in the *Methanosarcina thermophila* aceticlastic pathway.

Purification of the *M. thermophila* CODH/ACS to homogeneity demonstrates five distinct subunits in the enzyme complex (45). Dodecyltrimethylammonium bromide (DTAB) and Triton X-100 treatment disassociates the *M. thermophila* CODH/ACS into two components before resolution by anion-exchange chromatography (1). The components are the $\alpha_2\varepsilon_x$ nickel/iron-sulfur protein and the $\gamma_1\delta_1$ corrinoid/iron-sulfur protein. The β subunit is not associated with either of the resolved complexes. The purified Ni/Fe-S proteins are able to oxidize carbon monoxide with the reduction of a *M. thermophila* ferredoxin, methyl-viologen, or the corrinoid/Fe-S complex. After reduction, the corrinoid/Fe-S complex can be methylated by methyl iodide. Abbanat and Ferry (1991) suggests that each protein group catalyzes separate reactions in the CODH/ACS complex.

Like *M. thermophila,* the *Methanosarcina barkeri* CODH/ACS contains five different subunits (20). Studies by Grahame and DeMoll (1996) have described the catalytic activities of the CODH/ACS subunits after chymotrypsin proteolysis. Chymotrypsin digestion disrupts the tertiary CODH/ACS structure and allows resolution of the three products by an anion-exchange column. The first peak from the column contains the gamma subunit, the delta subunit, and the delta subunit's degraded forms. The gamma and delta subunits form the corrinoid/iron-sulfur component of the CODH/ACS (20). The second peak contains the alpha and epsilon subunits. The subunits catalyze the reaction associated with carbon monoxide dehydrogenase. The last peak contains the beta subunit (CdhB). Chymotrypsin treatment rapidly digests CdhB to form a truncated CdhB. The truncated CdhB still catalyzes the acetyl exchange/transfer reaction, but its removal from the holoenzyme complex correlates with a decrease the acetyl-CoA synthesis activity. To study the individual beta subunit, a methanosarcinal gene is required.

Using an oligonucleotide probe based on the N-terminal region of the *M. thermophila* delta subunit and a *M. thermophila* genomic library, the *cdhC* (beta subunit) was shown to be present in an operon for the CODH/ACS complex (30). *M. thermophila*

cdhC expression in *M. maripaludis* may improve the understanding of the *M. thermophila* beta subunit and its catalytic properties.

Cloning of *M. thermophila* strain TM-1 *cdhC* into pWLG30+*lacZ.* pWLG30+*lacZ* was digested with *Nsi* I and *Xba* I. After removing the restriction endonucleases with the Wizard Clean-up kit, the pWLG30+*lacZ*/*Nsi* I/*Xba* I was dephosphorylated with CIAP. The CIAP was removed by the Wizard Clean-up kit. The digested plasmid was ligated, in a 1:4.5 microgram ratio, with 0.9 microgram of gel purified 1.4 kbp *Nsi* I/*cdhC*/*Xba* I fragment (From David Grahame). See Figure 4. After ligation, the DNA was drop dialyzed using a 0.025 micron VSWP02500 membrane (Millipore) before transformation into XL1-Blue MRF' *E. coli*. The transformed *E. coli* were plated onto LB-Amp containing X-Gal. Individual white isolates were screened by size using a 0.6% (w/v) agarose gel to identify pWLG30+*cdhC.* Restriction digestions followed by the sequencing of pWLG30+*cdhC* confirmed the addition of *cdhC* and the removal of *lacZ.*

Growth and harvesting of *M. maripaludis* and recombinant *M. maripaludis* containing pWLG30 or pWLG30+*cdhC*. To date, *M. maripaludis* transformed with pWLG30+*cdhC* was grown in two types of media for 21 hours. When the cells were grown in 150 ml of McC+puromycin in modified Wheaton bottles, the averaged (n=5) A_{600} was 0.67 at harvesting. When grown in McN+puromycin, the averaged (n=8) A_{600} was 0.52 at harvesting. Using the same harvesting conditions as the pWLG30+*mtmB* experiment, the cell pellet was resuspended in a small amount of medium. The concentrated cells were placed into an aluminum seal vial. The vial was sealed before flushing for 2 minutes with nitrogen. The vial was pressurized at 80 kPa and stored at –20°C. For controls, *M. maripaludis* and *M. maripaludis* transformed with pWLG30 were grown in McC and McN+puromycin respectively.

Results and discussion of the expression of *M. thermophila cdhC* in *M. maripaludis*

The results are from personal communications with D. Grahame. Cell-free extracts from *M. maripaludis* transformed with pWLG30+*cdhC* were separated by gel filtration to isolate the fractions that contained the acetyltransferase activity associated with the *M. thermophila* CODH/ACS beta subunit. Two peaks of acetyltransferase activity were found in the column fractions. The peak, which represented the low molecular weight region, contained a protein that reacted with antibodies against the *M. thermophila* CODH/ACS in Western blots. Control extracts lacked the fraction in this region of the elution profile that cross-reacted with the antibodies. The fraction containing high molecular weight proteins and acetyltransferase activity failed to react with antibodies against the *M. thermophila* CODH/ACS in Western blots. These results demonstrated a protein that has acetyltransferase activity and cross-reactivity to antibodies against *M. thermophila* CODH/ACS in the recombinant *M. maripaludis*. Additionally, *M. maripaludis* contained native acetyltransferase activity, which was probably associated with the *M. maripaludis* CODH/ACS. Previous experiments demonstrated that *M. maripaludis* contained a CODH with specific activities of 4.3 or 0.095 (mol min^{-1} mg of $protein^{-1}$ when grown under autotrophic conditions in McN or with acetate as the carbon source in McNA, respectively (Shieh and Whitman, 1988). Recently, Yu (1997) demonstrated that *M. maripaludis* cell-free extracts contained both the CODH and the ACS activities. Molecular sieve chromatography separated the CODH and ACS activities into two separate protein fractions. The CODH activity was not assayed in the fractions separated from the recombinant *M. maripaludis* cell-free extracts in this work.

Selenocysteine insertion elements

The Archaea, Bacteria, and Eukarya have *cis*-acting, secondary structures in the mRNA that direct selenocysteine insertion during translation of in-frame, internal UGA codons (4). These secondary structures are named selenocysteine insertion sequence

(SECIS) elements (48). The Bacteria contain the SECIS element within the mRNA, while in the Archaea and Eukarya the SECIS element is believed to be located in the 3' untranslated region of the mRNA (48). A possible exception is the *M. jannaschii* formate dehydrogenase. This formate dehydrogenase, which contains a selenium atom, has a putative SECIS element upstream of the start codon (48).

Following analysis of ^{75}Se labelled polypeptides with SDS-PAGE, evidence for six selenoproteins was obtained in *M. jannaschii* (48). One *M. jannaschii* selenoprotein is the F_{420}-reducing hydrogenase subunit A (FruA). The F_{420}-reducing hydrogenase, which is composed of FruABG, catalyzes the oxidation of molecular hydrogen with the deazaflavin coenzyme F_{420} as the electron acceptor (21, 44). The *M. jannaschii fruA* has an internal UGA codon in the mRNA and a putative SECIS element downstream of the coding region (9). *In vivo* experiments could confirm the identity of the SECIS element by site-directed mutagenesis of the element. If successful, these studies would be the first *in vivo* test of the proposed function of the SECIS element in an archaeon.

Transformation and PCR confirmation of pWLGfruAWT and pWLGfruAdel in *M. maripaludis*. One microgram of either pWLGfruAWT or pWLGfruAdel (From Michael Rother and August Böck), which was purified from *E. coli* XL1-Blue MRF', was transformed into *M. maripaludis*. The transformed cells were serially diluted into McC+0.7% (w/v) top agar before plating onto McC+puromycin+1% (w/v) bactoagar. Individual colonies were inoculated into McC+puromycin.

PCR was chosen to confirm the *fruA* region in the expression shuttle vector after transformation into *M. maripaludis*. The transformed *M. maripaludis* isolates were grown in McC+puromycin to an A_{600} of 0.54 to 0.62. Each culture was diluted 1:10 in sterile, distilled water before 0.5 µl was added to the PCR. The reaction utilized the primers 30gene1 and 30gene2 at a 0.5 micromolar concentration. The PCR contained 2.5 units of *Pfu* Turbo DNA polymerase (Stratagene) in a final volume of 100 µl. After an initial dwell of 5 minutes at 95°C to denature the plasmid, PCR was performed for 25 cycles. Each

cycle had a denaturing temperature of 94°C for 1 minute, an annealing temperature of 48.5°C for 1 minute, and an extension time of 1.5 minutes at 74°C. pWLG30, which was purified from *E. coli* XL1-Blue MRF', was the positive PCR control. Electrophoresis through a 2% (w/v) agarose gel was performed on the PCR reactions. A 0.36 kb PCR product was produced for pWLG30, while a 1.6 kb product was formed for pWLGfruAWT and pWLGfruAdel.

Construction of integrative vectors with a modified puromycin transacetylase (*pac*) cassette for use in *M. maripaludis*. One microgram of pJK3 was digested with *Xba* I. See Figure 5. The 1.4 kb *Xba* I/modified *pac*/*Xba* I was gel purified from a 0.8% (w/v) agarose gel. pWLG13 was also digested with *Xba* I. The Wizard clean-up kit (Promega) removed the enzymes after the restriction digestion. The *Xba* I/pWLG13/*Xba* I was dephosphorylated with CIAP (calf intestine alkaline phosphatase). The ligation between the digested and dephosphorylated *Xba* I/pWLG13/*Xba* I and the *Nsi* I/modified *pac*/*Xba* I DNA fragment occurred with 1 Weiss unit of T4 DNA ligase (Promega) for 21 hours at 4°C. The ligation mixture was drop dialyzed using a 0.025 micron VSWP02500 membrane (Millipore) before transformation into *E. coli* MC1061 (39). The transformed cells were plated on LB-Amp medium. After overnight growth, individual white colonies were streaked onto a LB-Amp plates. After 16 hours of growth, the plates were cooled to 4°C for one hour. Transformants were collected with a sterile toothpick, and placed into 50 µl of resuspension solution (50 mM Tris [pH 7.5], 10 mM EDTA, 100 µg/ml RNase A). Each sample was vortexed before the addition of 50 µl lysis solution (0.2 M NaOH, 1% (w/v) sodium dodecylsulfate). After 1 minute, 50 µl neutralization solution (1.32 M potassium acetate) was added. The cell debris was pelleted by centrifugation at 16000 *g* for 5 minutes at room temperature. To screen the circular plasmids by size for pSMK1, electrophoresis was performed using 0.8% (w/v) agarose gel with 17 µl of the supernatant and 3 µl tracking dye. The orientation of the modified *pac* cassette was confirmed by a *Pst* I digestion.

To remove the original *pac* cassette, pSMK1 was dialyzed before digestion with *Spe* I. The Wizard clean-up kit removed the *Spe* I before treatment by *Eco*O109 I. *Eco*O109 I was removed by the Wizard clean-up kit prior to the treatment of the products, which contained *Spe* I/pSMK1/*Eco*O109 I, with CIAP. Additional restrictions sites were added with the phosphorylated Paclink oligonucleotides. Paclink 1 (5'-GCCCTATCGATAGCGCTGCTAGCCTCGAGCACGTGA-3') and paclink 2 (5'-CTAGTCACGTGCTCGAGGCTAGCAGCGCTATCGATAG-3') were mixed in equal μM concentrations then boiled for 10 minutes. After boiling, the Paclink oligonucleotides were allowed to cool slowly to room temperature. The ligation contained 3.6 (M Paclink oligonucleotides, 1 Weiss unit of T4 DNA ligase, and 0.5 micrograms of the dephosporylated mixture containing *Spe* I/pSMK1/*Eco*O109 I. After an overnight incubation at 4^{o}C, the ligation was dialyzed before transformation into *E. coli* XL1-Blue MRF'. The colonies were screened by the rapid colony method. *Spe* I and *Xho* I digestions initially confirmed the identity of pWLG15. Sequencing with the pWLG15 primer (5'-AAGCTGCTGGTGAAAGAGAC-3') confirmed the MCS downstream of the modified *pac* cassette.

References

1. **Abbanat, D. R. and J. G. Ferry**. 1991. Resolution of component proteins in an enzyme compex from *Methanosarcina thermophila* catalyzing the synthesis or cleavage of acetyl-CoA. Proc. Natl. Acad. Sci. **88**:3272-3276.
2. **Argyle, J. L., D. L. Tumbula, and J. A. Leigh**. 1996. Neomycin resistance as a selectable marker in *Methanococcus maripaludis*. Appl. Environ. Microbiol. **62**:4233-4237.
3. **Aravalli, R. N. and R. A. Garrett**. 1997. Shuttle vectors for hyperthermophilic archaea. Extremophiles **1**:183-191.
4. **Bell, S. D. and S. P. Jackson**. 1999. Transcription and translation in Archaea: a mosaic of eukaryal and bacterial features. Trends Microbiol. **6**:222-227.
5. **Beneke, S., H. Bestgen, and A. Klein**. 1995. Use of the *Escherichia coli uidA* gene as a reporter in *Methanococcus voltae* for the analysis of the regulatory function of the intergenic region between the operons encoding selenium-free hydrogenases. Mol. Gen. Genet. **248**:225-228.
6. **Blamey, J. M., and M. W. W. Adams**. 1993 Purification and characterization of pyruvate ferredoxin oxidoreductase from the hyperthermophilic archaeon *Pyrococcus furiosus*. Biochim. Biophys. Acta **1161**:19-27.
7. **Blaseio, U. and F. Pfeifer**. 1990. Transformation of *Halobacterium halobium*: Development of vectors and investigation of gas vesicle synthesis. Proc. Natl. Acad. Sci. USA **87**:6772-6776.
8. **Brown, J. W., C. J. Daniels, and J. N. Reeve**. 1989. Gene structure, organization, and expression in archaebacteria. Crit. Rev. Microbiol. **16**:287-338.
9. **Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, G. G. Sutton, J. A. Blake, L. M. FitzGerald, R. A. Clayton, J. D. Gocayne, A. R. Kerlavage, B. A. Dougerty, J. F. Tomb, M. D. Adams, C. I. Reigh, R. Overbeek, E. F. Kirkness, K. G. Weinstock, J. M. Merrick, A. Glodek, J. L. Scott, N. S. M. Geoghagen, J. F. Weidman, J. L. Fuhrmann, D. Nguyen, T. R. Utterback, J. M. Kelley, J. D. Peterson, P. W. Sadow, M. C. Hanna, M. D. Cotton, K. M. Roberts, M. A. Hurst, B. P. Kaine, M. Borodovsky, H. P. Klenk, C. M. Fraser, H. O. Smith, C. R. Woese, and J. C. Venter**. 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science **273**:1017-1140.
10. **Burke, S. A., S. L. Lo, and J. A. Krzycki**. 1998. Clustered genes encoding the methyltransferases of methanogenesis from monomethylamine. J. Bacteriol. **180**:3432-3440.
11. **Cline, S. W. and W. F. Doolittle**. 1992. Transformation of members of the genus *Haloarcula* with shuttle vectors based on *Halobacterium halobium* and *Haloferax volcanii* plasmid replicons. J. Bacteriol. **174**:1076-1080.
12. **Cohen-Kupiec, R., C. Blank, and J. A. Leigh**. 1997. Transcriptional regulation in Archaea: *In vivo* demonstration of a repressor binding site in a methanogen. Proc. Natl. Acad. Sci. USA **94**:1316-1320.
13. **Condò, I., A. Ciammaruconi, D. Benelli, D. Ruggero, and P. Londei**. 1999. *Cis*-acting signals controlling translational initiation in the thermophilic archaeon *Sulfolobus solfataricus*. Mol. Microbiol. **34**:377-384.
14. **Deppenmeier, U., V. Müller, and G. Gottschalk**. 1996. Pathways of energy conservation in methanogenic archaea. Arch. Microbiol. **165**:149-163.
15. **Elferink, M. G. L., C. Schleper, and W. Zillig**. 1996. Transformation of the extremely thermoacidophilic archaeon *Sulfolobus solfataricus* via a self-spreading vector. FEMS Microbiol. Lett. **137**:31-35.
16. **Erauso, G., F. Charbonnier, T. Barbeyron, P. Forterre, and D. Prieur**. 1992. Preliminary characterization of a hyperthermophilic archaebacterium with a plasmid, isolated from a North Fiji basin hydrothermal vent. C. R. Acad. Sci. **314**:387-393.

17. **Erauso, G., S. Marsin, N. Benbouzid-Rollet, M.-F. Baucher, T. Barbeyron, Y. Zivanoic, D. Prieur, and P. Forterre**. 1996. Sequence of plasmid pGT5 from the archaeon *Pyrococcus abyssi*: Evidence for rolling-circle replication in a hyperthermophile. J. Bacteriol. **178**:3232-3237.
18. **Gardner, W. L. and W. B. Whitman**. 1999. Expression vectors for *Methanococcus maripaludis*: overexpression of acetohydroxyacid synthase and (-galactosidase. Genetics **152**:1439-1447.
19. **Gernhardt, P., O. Possot, M. Foglino, L. Sibold, and A. Klein**. 1990. Construction of an integration vector for use in the archaebacterium *Methanococcus voltae* and expression of a eubacterial resistance gene. Mol. Gen. Genet. **221**:273-279.
20. **Grahame, D. A. and E. DeMoll**. 1996. Partial reactions catalyzed by protein components of the acetyl-CoA decarbonylase synthase enzyme complex from *Methanosarcina barkeri*. J. Biol. Chem. **271**:8352-8358.
21. **Halboth, S. and A. Klein**. 1992. *Methanococcus voltae* harbors four gene clusters potentially encoding two [NiFe] and two [NiFeSe] hydrogenases, each of the cofactor F_{420}-non-reducing types. Mol. Gen. Genet. **233**:217-224.
22. **Holmes, M. L. and M. L. Dyall-Smith**. 1991. Mutations in DNA gyrase result in novobiocin resistance in halophilic archaebacteria. J. Bacteriol. **173**:642-648.
23. **Holmes, M., F. Pfeifer, and M. Dyall-Smith.** 1994. Improved shuttle vectors for *Haloferax volcanii* including a dual-resistance plasmid. Gene **146**:117-121.
24. **James, C. and J. A. Krzycki**. Personal communication.
25. **Jones, W. J., M. J. B. Paynter, and R. Gupta**. 1983. Characterization of *Methanococcus maripaludis* sp. nov., a new methanogen isolated from salt marsh sediment. Arch. Microbiol. **135**:91-97.
26. **Kocsis, E., M. Kessel, E. DeMoll, and D. A. Grahame**. 1999. Structure of the Ni/Fe-S protein subcomponent of the acetyl-CoA decarbonylase/synthase complex from *Methanosarcina thermophila*. J. Struct. Biol. **128**:165-174.
27. **Lam, W. L. and W. F. Doolittle**. 1989. Shuttle vectors for the archaebacterium *Halobacterium volcanii*. Proc. Natl. Acad. Sci. USA **86**:5478-5482.
28. **Lam, W. L. and W. F. Doolittle**, 1992. Mevinolin-resistant mutations identify a promoter and the gene for a eukaryote-like 3-hydroxy-3-methylglutaryl-coenzyme A reductase in the archaebacterium *Haloferax volcanii*. J. Biol. Chem. **267**:5829-5834.
29. **Maniatis, T., E. F. Fritsch, and J. Sambrook**. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.
30. **Maupin-Furlow, J. A., and J. G. Ferry**. 1996. Analysis of the CO dehydrogenase/acetyl-coenzyme A synthase operon of *Methanosarcina thermophila*. J. Bacteriol. **178**:6849-6856.
31. **Menon, A. L.** Personal communication.
32. **Menon, A. L., H. Hendrix, A. Hutchins, M. F. J. M. Verhagen, and M. W. W. Adams**. 1998. The delta-subunit of pyruvate ferredoxin oxidoreductase from *Pyrococcus furiosus* is a redox-active, iron-sulfur protein: evidence for an ancestral relationship with 8Fe-type ferredoxins. Biochemistry **37**:12838-12846.
33. **Metcalf, W. W., J. K. Zhang, E. Apolinario, K. R. Sowers, and R. S. Wolfe**. 1997. A genetic system for Archaea of the genus *Methanosarcina*: Liposome-mediated transformation and construction of shuttle vectors. Proc. Natl. Acad. Sci. USA **94**:2626-2631.
34. **Ng, W.-L. and S. DasSarma**. 1993. Minimal replication origin of the 200-kilobase *Halobacterium* plasmid pNRC100. J. Bacteriol. **175**:4584-4596.
35. **Nuttall, S. D., S. E. Deutschel, R. A. Irving, J. A. Serrano-Gomicia, and M. L. Dyall-Smith**. 2000. The ShBle resistance determinant from *Streptoalloteichus hindustanus* is expressed in *Haloferax volcanii* and confers resistance to bleomycin. Biochem. J. 346:251-254.

36. **Pfeifer, F. and P. Ghahraman**. 1993. Plasmid pHH1 of *Halobacterium salinarium*: characterization of the replicon region, the gas vesicle gene cluster and insertion elements. Mol. Gen. Genet. **238**:193-200.
37. **Ragsdale, S. W., and M. Kumar**. 1996. Nickel-containing carbon monoxide dehydrogenase/acetyl-CoA synthase. Chem. Rev. **96**:2515-2539.
38. **Saito, R. and M. Tomita**. 1999. Computer analyses of complete genomes suggest that some archaebacteria employ both eukaryotic and eubacterial mechanisms in translation initiation. Gene **238**:79-83.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis**. 1989. Molecular cloning: a laboratory manual (2nd edition). Cold Spring Harbor Laboratory Press, New York.
40. **Shieh, J. S., and W. B. Whitman**. 1987. Pathway of acetate assimilation in autotrophic and heterotrophic methanococci. J. Bacteriol. **169**:5327-5329.
41. **Shieh, J. S., and W. B. Whitman**. 1988. Autotrophic acetyl Coenzyme A biosynthesis in *Methanococcus maripaludis*. J. Bacteriol. **170**:3072-3079.
42. **Smith, D. R., L. A. Doucette-Stamm, G. Deloughery, H. Lee, J. Dubois, T. Aldredge, R. Bashirzadeh, D. Blakely, R. Cook, K. Gilbert, D. Harrison, L. Hoang, P. Keagle, W. Lumm, B. Pothier, D. Qiu, R. Spadafora, R. Vicaire, Y. Wang, J. Wierzbowski, R. Gibson, N. Jiwani, A. Caruso, D. Bush, H. Safer, D. Patwell, S. Prabhakar, S. McDougall, G. Shimer, A. Goyal, S. Pietrokovski, G. M. Church, C. J. Daniels, J.-I. Mao, P. Rice, J. Nölling, and J. N. Reeve**. 1997. Complete genome sequence of *Methanobacterium thermoautotrophicum* ΔH: functional analysis and comparative genomics. J. Bacteriol. **179**:7135-7155.
43. **Sniezko, I., C. Dobson-Stone, and A. Klein**. 1998. The *treA* gene of *Bacillus subtilis* is a suitable reporter for the archaeon *Methanococcus voltae*. FEMS Microbiol. Lett. **164**:237-242.
44. **Sorgenfrei, O., S. Müller, M. Pfeiffer, I. Sniezko, and A. Klein**. 1997. The [NiFe] hydrogenases of *Methanococcus voltae*: genes, enzymes, and regulation. Arch. Microbiol. **167**:189-195.
45. **Terlesky, K. C., M. J. K. Nelson, and J. G. Ferry**. 1986. Isolation of an enzyme complex with carbon monoxide dehydrogenase activity containing corrinoid and nickel from acetate-grown *Methanosarcina thermophila*. J. Bacteriol. **168**:1053-1058.
46. **Tumbula, D. L., R. A. Makula, and W. B. Whitman**. 1994. Transformation of *Methanococcus maripaludis* and identification of a *Pst*I-like restriction system. FEMS Microbiol. Lett. **121**:309-314.
47. **Tumbula, D. L., T. L. Bowen, and W. B. Whitman**. 1997 Characterization of pURB500 from the archaeon *Methanococcus maripaludis* and construction of a shuttle vector. J. Bacteriol. **179**:2976-2986.
48. **Wilting, R., S. Schorling, B. C. Persson, and A. Böck**. 1997. Selenoprotein synthesis in Archaea: identification of an mRNA element of *Methanococcus jannaschii* probably directing selenocysteine insertion. J. Mol. Biol. **266**: 637-641.
49. **Whitman, W. B., J. Shieh, S. Sohn, D. S. Caras, and U. Premachandran**. 1986. Isolation and characterization of 22 mesophilic methanococci. System. Appl. Microbiol. **7**:235-240.
50. **Yang, Y.-L**. 1994. Ph.D. thesis. University of Georgia, Athens. Investigation of pyruvate oxidation regulation of *Methanococcus*.
51. **Yu, J.-P**. 1997. Ph.D. thesis. University of Georgia, Athens.
52. **Zinder, S. H. and R. A. Mah**. 1979. Isolation and characterization of a thermophilic strain of *Methanosarcina* unable to use H_2-CO_2 for methanogenesis. Appl. Environ. Microbiol. **38**:996-1008.
53. **Zinder, S. H., K. R. Sowers, and J. G. Ferry**. 1985. *Methanosarcina thermophila* sp. nov., a thermophilic, acetotrophic, methane-producing bacterium. Int. J. Syst. Bacteriol. **34**:444-450.

TABLE 1. Plasmids and DNA fragments used in this study[a]

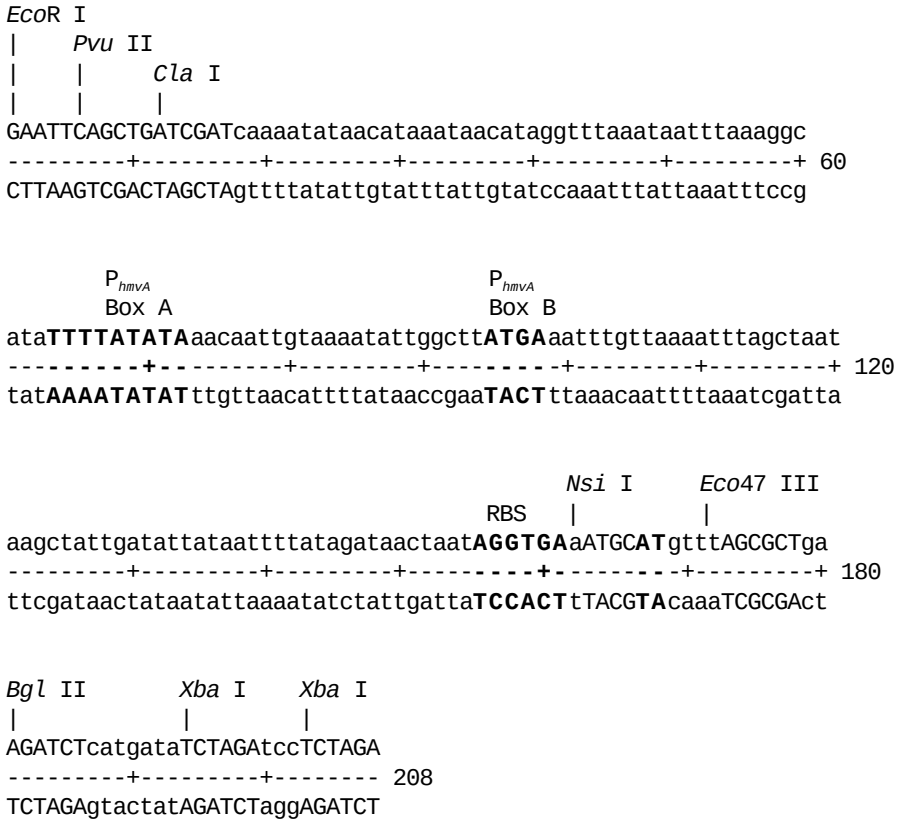
Plasmids	Properties	Reference
Nsi I/*cdhC*/*Xba* I	gel purified DNA fragment containing the *M. thermophila* strain TM-1 *cdhC*	From David Grahame
pHH4-4	pCR-Script™ SK(+) (Stratagene) derivative with *porD* from *P. furiosus*, ampicillin resistance (amp^r)	From Angeli Menon and Michael Adams
pJK3	pBluescript derivative, contains a *pac* cassette (puromycin resistance, pur^r) modified with the *pac* immediately downstream of the *M. voltae* P*mcr* while introducing flanking restriction sites	Metcalf et al., 1997
pLPMB2	pGEM-T (Promega) derivative with *mtmB* from *M. barkeri* strain MS, amp^r	From Ligi Pottenplackel and Joseph Krzycki
pSMK1	contains two pac cassettes and two multiple cloning sites, amp^r, pur^r	This work
pWLG13	integrative expression vector with *Nde* I site downstream of *M. voltae* histone promoter, amp^r, pur^r	Gardner and Whitman, 1999
pWLG15	contains the modified *pac* cassette and two multiple cloning sites, amp^r, pur^r	This work
pWLG30	expression shuttle vector for *M. maripaludis*, amp^r, pur^r	Gardner and Whitman, 1999
pWLG30+*cdhC*	contains *M. thermophila* strain TM-1 *cdhC*	This work

pWLGfruAdel	expression shuttle vector with the *M. jannaschii fruA* with the putative SECIS element deleted, amp^r, pur^r	From Michael Rother and August Böck
pWLGfruAWT	expression shuttle vector with the *M. jannaschii fruA* with the wild type putative SECIS element, amp^r, pur^r	From Michael Rother and August Böck
pWLG30+*lacZ*	contains *lacZ* for *E. coli* blue/white screen amp^r, pur^r	Gardner and Whitman, 1999
pWLG30+*mtmB*	contains *M.barkeri* strain MS *mtmB*, amp^r, pur^r	This work
pWLG30+*porD*	contains *P. furiosus porD*, amp^r, pur^r	This work
pZErO-2	requires *E. coli* Top10, kanamycin resistance (kan^r)	Invitrogen
pZErO-2+*porD*	contains the modified *P. furiosus porD*, kan^r	This work

[a]Ampicillin (amp) and kanamycin (kan) are for selection in *E. coli*. Puromycin (pur) is for selection in *M. maripaludis*.

Figure 1: The engineered *Methanococcus voltae* histone promoter (P_{hmvA}).

The PCR-modified P_{hmvA} contained the unique *Cla* I and *Nsi* I sites flanking the promoter in pWLG14 and pWLG30 (Gardner and Whitman, 1999). The *Cla* I site was 5' of the P_{hmvA}, and the *Nsi* I site was at the 3' end of P_{hmvA}. The last two nucleotides of the *Nsi* I site (bold) formed the start codon of genes ligated into these expression vectors. The restriction sites between and including the *Nsi* I and *Xba* I composed the multicloning site (MCS). This MCS was designed for multiple digestions that utilized the same restriction buffer. Introducing a third endonuclease could lower the background level of undigested vector. The third restriction site was required to be between the endonuclease sites in the initial digestion.


EcoR I
Pvu II
Cla I
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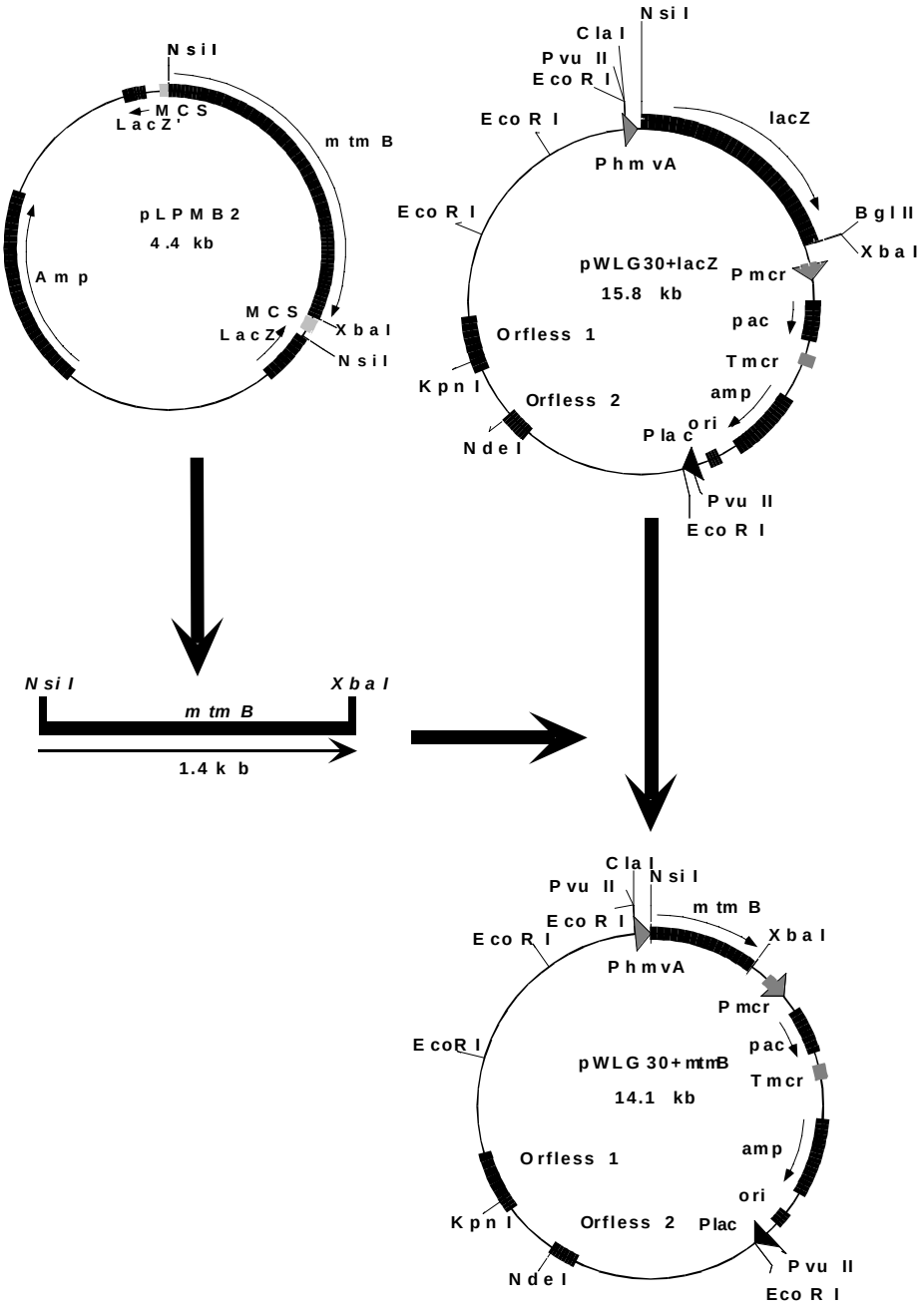
1 --------+---------+---------+---------+---------+---------+ 60
CTTAAGTCGACTAGCTAgttttatattgtatttattgtatccaaatttattaaatttccg
P hmvA
Box A
P hmvA
Box B
ataTTTTATATAaacaattgtaaaatattggcttATGAaatttgttaaaatttagctaat
61 --------+--------+---------+---------+---------+---------+ 120
tatAAAATATATttgttaacattttataaccgaaTACTttaaacaattttaaatcgatta
Nsi I
Eco47 III
RBS
aagctattgatattataattttatagataactaatAGGTGAaATGCATgtttAGCGCTga
121 --------+---------+---------+---------+---------+---------+ 180
ttcgataactataatattaaaatatctattgattaTCCACTtTACGTAcaaaTCGCGAct
Bgl II
Xba I
Xba I
AGATCTcatgataTCTAGAtccTCTAGA
181 --------+---------+-------- 208
TCTAGAgtactatAGATCTaggAGATCT

Figure 2: Cloning of *Methanosarcina barkeri mtmB* into pWLG30+*lacZ.*

A complete digest of pLPMB2 by *Nsi* I and *Xba* I released the *Nsi* I/*mtmB*/*Xba* I from the vector. At the same time, *Nsi* I and *Xba* I digested pWLG30+*lacZ.* The mixture of *Nsi* I/pWLG30/*Xba* I and *Nsi* I/*lacZ*/*Xba* I fragments was dephosphorylated by calf intestine alkaline phosphatase. Ligation of the gel-purified *Nsi* I/*mtmB*/*Xba* I occurred in the dephosphorylated mixture of *Nsi* I/pWLG30/*Xba* I and *Nsi* I/*lacZ*/*Xba* I. The ligation yielded pWLG30+*mtmB*.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LacZ'
mtmB
pLPMB2
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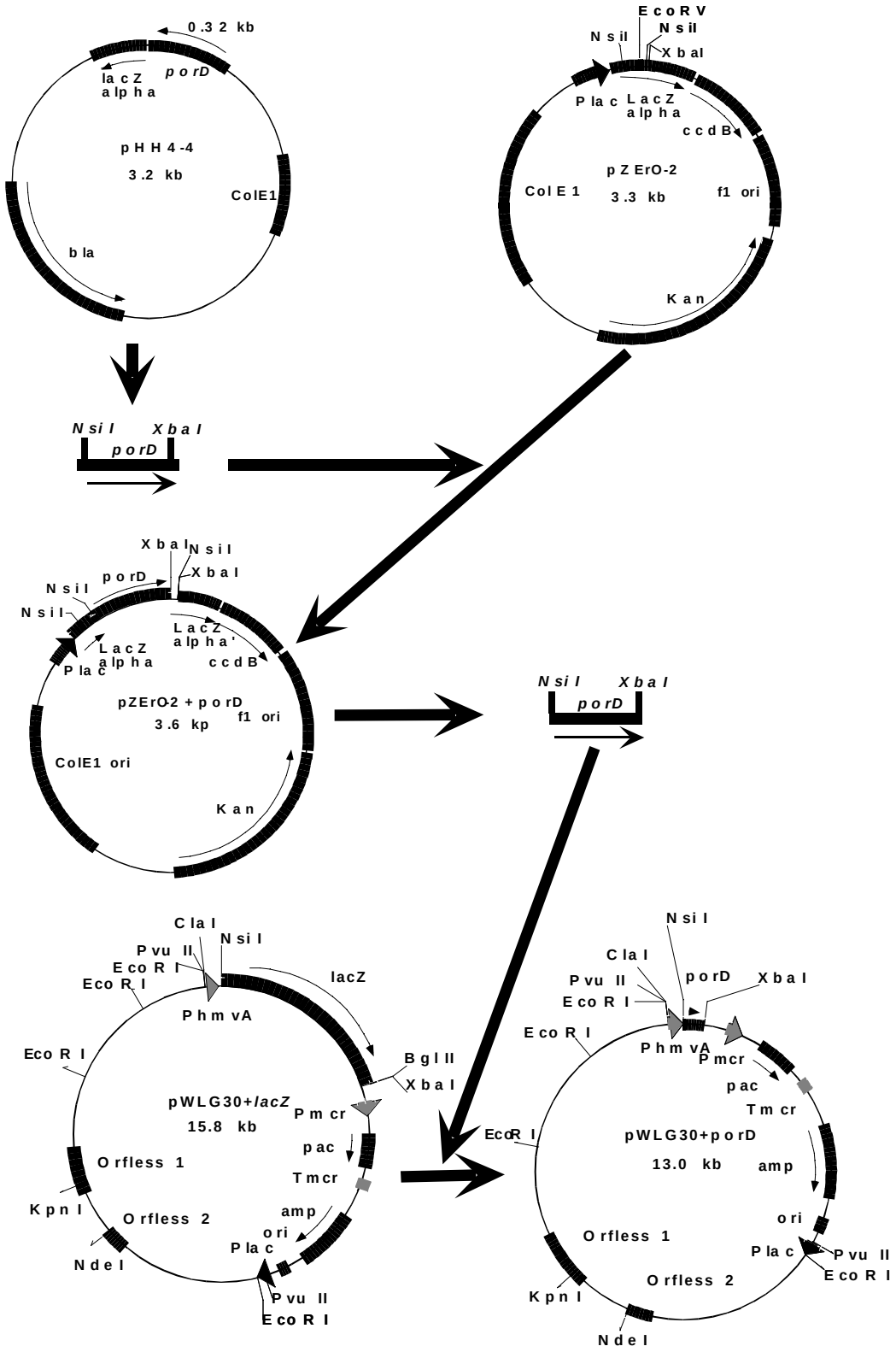
Amp
MCS
LacZ
XbaI
NsiI
NsiI
ClaI
PvuII
EcoRI
EcoRI
lacZ
PhmvA
EcoRI
BglII
XbaI
pWLG30+lacZ
15.8 kb
Pmcr
pac
Orfless 1
Tmcr
KpnI
amp
Orfless 2
ori
Plac
NdeI
PvuII
EcoRI
NsiI
XbaI
mtmB
1.4 kb
ClaI
NsiI
PvuII
mtmB
EcoRI
XbaI
EcoRI
PhmvA
Pmcr
EcoRI
pac
pWLG30+mtmB
Tmcr
14.1 kb
amp
Orfless 1
ori
KpnI
Orfless 2
Plac
PvuII
NdeI
EcoRI

Figure 3: Cloning of *Pyrococcus furiosus porD* into pWLG30+*lacZ.*

PCR modifications of the *P. furiosus porD* in pHH4-4 added a *Nsi* I and a *Xba* I site at the 5'-end and 3'-end of the gene, respectively. This modified *porD* was cloned into the EcoR V site of pZErO-2 yielding pZErO-2+*porD*. Digestion of pZErO-2+*porD* by *Nsi* I and *Xba* I allowed for the *Nsi* I/*porD*/*Xba* I fragment to be gel purified. *Nsi* I and *Xba* I digestion of pWLG30+*lacZ* yielded *Nsi* I/pWLG30/*Xba* I and *Nsi* I/*lacZ*/*Xba* I. Calf intestine alkaline phosphatase dephosphorylated both products before the ligation with the *Nsi* I/*porD*/*Xba* I. This ligation produced the pWLG30+*porD*.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lacZ alpha
porD
pHH4-4
3.2 kb
ColE1
bla
EcoRV
NsiI
NsiI
XbaI
Plac LacZ alpha
ccdB
pZErO-2
ColE1
3.3 kb
f1 ori
Kan
NsiI XbaI
porD
XbaI
NsiI
porD
NsiI
NsiI
LacZ alpha
Plac
LacZ alpha'
ccdB
pZErO-2 + porD
3.6 kp
f1 ori
ColE1 ori
Kan
NsiI XbaI
porD
ClaI
NsiI
PvuII
EcoRI
EcoRI
lacZ
PhmvA
EcoRI
BglII
XbaI
pWLG30+lacZ
15.8 kb
Pmcr
pac
Tmcr
Orfless 1
amp
KpnI
Orfless 2
ori
Plac
NdeI
PvuII
EcoRI
NsiI
ClaI
PvuII
EcoRI
porD
XbaI
EcoRI
PhmvA
Pmcr
pac
Tmcr
EcoRI
pWLG30+porD
13.0 kb
amp
ori
Orfless 1
Plac
PvuII
EcoRI
Orfless 2
KpnI
NdeI

Figure 4: Cloning of *Methanosarcina thermophila cdhC* into pWLG30+*lacZ.*

Nsi I and *Xba* I digestion of pWLG30+*lacZ* yielded *Nsi* I/pWLG30/*Xba* I and *Nsi* I/*lacZ*/*Xba* I. Calf intestine alkaline phosphatase dephosphorylated both products before the ligation with the *Nsi* I/*cdhC/Xba* I. This ligation produced the pWLG30+*cdhC.*

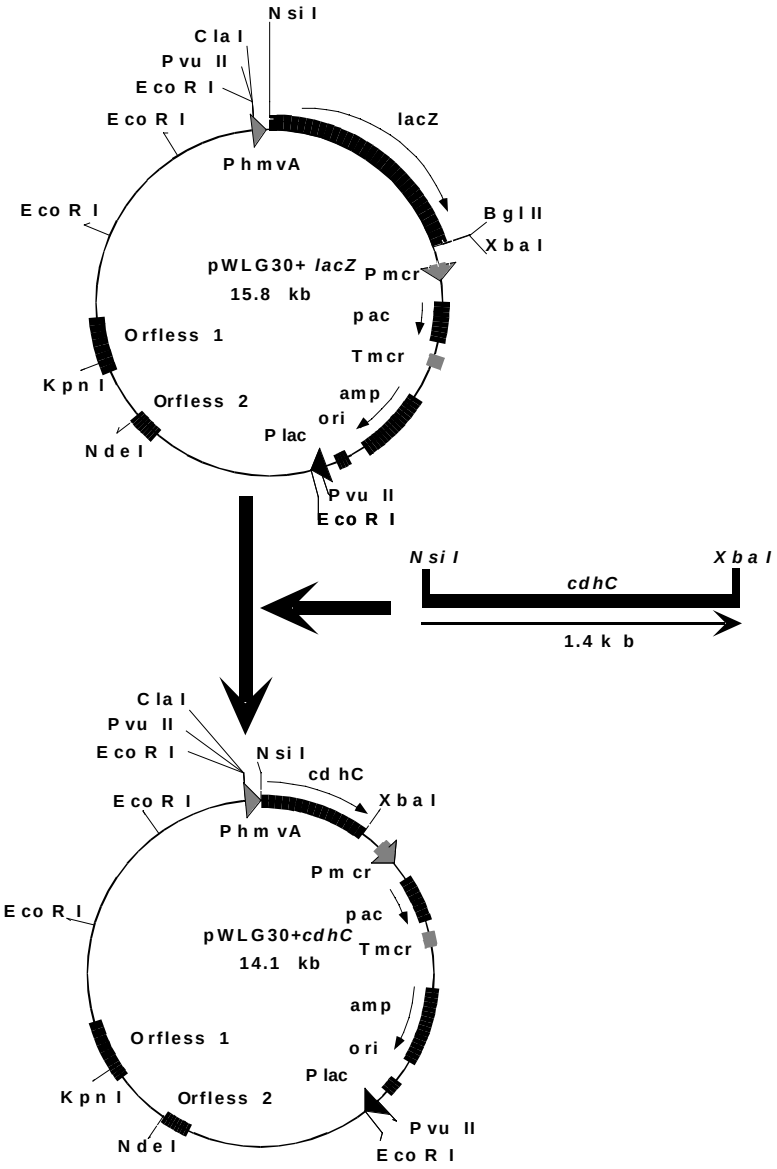
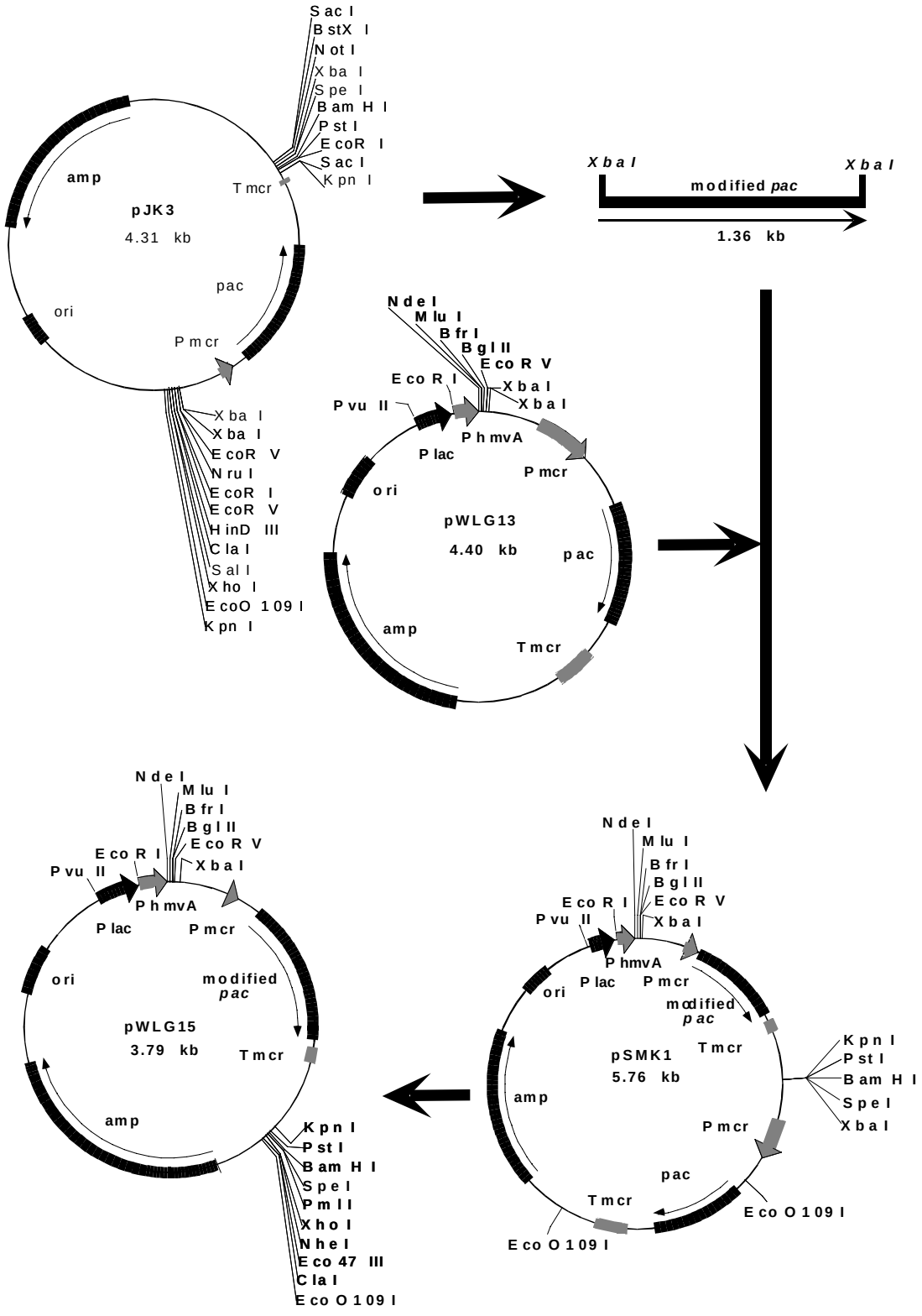

Nsi I
Cla I
Pvu II
EcoR I
EcoR I
lacZ
PhmvA
EcoR I
BglII
Xba I
pWLG30+ lacZ
15.8 kb
Pmcr
pac
Orfless 1
Tmcr
Kpn I
amp
Orfless 2
ori
Plac
Nde I
Pvu II
EcoR I
Nsi I
Xba I
cdhC
1.4 kb
Cla I
Pvu II
EcoR I
Nsi I
cd hC
EcoR I
Xba I
PhmvA
Pmcr
EcoR I
pac
pWLG30+cdhC
Tmcr
14.1 kb
amp
Orfless 1
ori
Plac
Kpn I
Orfless 2
Pvu II
Nde I
EcoR I

Figure 5: Cloning the modified puromycin transacetylase cassette into pWLG13.

Xba I digested pJK3 before the gel purification of the modified *pac* cassette. pWLG13 was digested with *Xba* I then treated with calf intestine alkaline phosphatase. pSMK1 was formed by ligation of the modified *pac* cassette into the digested, dephosphorylated pWLG13. An *Eco*O109 I and *Spe* I digestion removed the original *pac* cassette. Synthetic oligonucleotides added new restriction sites to form pWLG15.


Sac I
BstX I
Not I
Xba I
Spe I
Bam H I
Pst I
EcoR I
Sac I
Kpn I
amp
Tmcr
pJK3
4.31 kb
pac
ori
Pmcr
Xba I
Xba I
EcoR V
Nru I
EcoR I
EcoR V
HinD III
Cla I
Sal I
Xho I
EcoO 109 I
Kpn I
XbaI
XbaI
modified pac
1.36 kb
NdeI
MluI
BfrI
BglII
EcoR V
EcoR I
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Plac
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ori
pWLG13
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pac
amp
Tmcr
NdeI
MluI
BfrI
BglII
EcoR V
EcoR I
XbaI
Pvu II
PhmvA
Plac
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ori
modified pac
pSMK1
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Tmcr
amp
Pmcr
pac
Tmcr
EcoO 109 I
EcoO 109 I
KpnI
PstI
Bam H I
SpeI
XbaI
NdeI
MluI
BfrI
BglII
EcoR V
EcoR I
XbaI
Pvu II
PhmvA
Plac
Pmcr
ori
modified pac
pWLG15
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amp
KpnI
PstI
Bam H I
SpeI
PmlI
XhoI
NheI
Eco47 III
ClaI
EcoO 109 I

CHAPTER VI

LARGE SCALE GROWTH OF *METHANOCOCCUS MARIPALUDIS* WITH FORMATE AND *IN VIVO* EXPRESSION OF THE *MOORELLA THERMOACETICA* CARBON MONOXIDE DEHYDROGENASE GENES IN *METHANOCOCCUS MARIPALUDIS*[5]

[5] Gardner, W. L. and W. B. Whitman. To be submitted.

Keywords: Clostridium, CODH, *acs*, expression, archaea, thermophile, thermophilic, anaerobic, shuttle vector, translation, methanogen, and bacterium

Abstract

The ability to grow and harvest large quantities of a recombinant organism represents a significant feature for an expression system. In this work, a mineral medium with sodium formate was developed for large scale growth of *M. maripaludis*. *M. maripaludis* does not require H_2:CO_2 (80:20) for growth in this medium. A recombinant *M. maripaludis*, which was harvested at an $O.D._{600}$ of 0.75, yielded about 1 g of wet cell paste of per liter of medium.

Polymerase chain reaction (PCR) modification of the *Moorella thermoacetica acsAB*, which encoded the carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS), allowed directed ligation into pWLG30+*lacZ* to yield pWLG30+*acsAB*. pWLG30+*acsAB*, a *Methanococcus maripaludis* expression shuttle vector with *M. thermoacetica acsAB*, was transformed into *M. maripaludis*. After transformation, CODH assays evaluated the CODH specific activity within the recombinant *M. maripaludis*.

Introduction

Moorella thermoacetica, which was formerly known as *Clostridium thermoaceticum*, is a thermophilic bacterium (3). This saccharolytic sporeformer grows at temperatures of 45°C to 65°C (12). During growth, *M. thermoacetica* only produces acetate from the fermentations of fructose, glucose, and xylose (12; See Figure 1). See Ragsdale (1991) and Ferry (1996) for a review of the enzymes involved in this process. Thus, *M. thermoacetica* is an acetogen. An acetogen is an organism that reductively produces acetyl-coenzyme A (acetyl-CoA) from carbon dioxide (4). In addition to heterotrophic growth, *M. thermoacetica* grows autotrophically with H_2:CO_2 (80:20) or 100% CO (10).

Carbon dioxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) is a central enzyme in the Ljungdahl-Wood pathway, which is also known as the acetyl-CoA pathway.

As the name implies, the CODH/ACS has two general activities. One activity is the conversion of carbon dioxide to carbon monoxide. The second activity requires a methylated corrinoid iron-sulfur protein (CFeS protein), carbon monoxide, and Coenzyme A (CoA) to form acetyl-CoA.

The CODH/ACS is encoded by *acsAB*, which respectively encodes the β and α subunits (16). Expression of the *acsAB* produces an oxygen-sensitive enzyme with a quaternary structure of $\alpha_3\beta_3$ (19). Ragsdale and Wood (1985) first demonstrated that the CODH/ACS contains ACS activity but not the CFeS protein. Later, the α subunit was identified as containing the acetyl-CoA synthase activity (11; See Figure 2). The β subunit contains the carbon monoxide dehydrogenase activity (30). Each subunit contains a Ni/Fe/S cluster involved in the respective reactions for the subunit (6). The β subunit also has a Fe/S cluster involved with electron transfer for the CODH activity (6). See Fontecilla-Camps and Ragsdale (1999), Ragsdale and Kumar (1996), and Ferry (1995) for recent reviews on CODH/ACS.

Many of the reaction intermediates associated with CODH/ACS are bound to metals within the holoenzyme (6). Thus, the CODH/ACS represents an opportunity to study novel bioorganometallic reactions. An expression system that can produce the AcsAB holoenzyme will allow the development of site-directed mutagenesis experiments. These experiments could focus on an individual subunit or interactions between subunits in reconstitution studies. When *E. coli* expresses *M. thermoacetica acsAB*, the recombinant *E. coli* lacks CODH activity (22). Additionally, the recombinant AcsAB subunits from *E. coli* are not thermostable and fail to associate together (22). Preliminary work by Loke and Lindahl (1999) suggests that a recombinant expression system for an active CODH/ACS complex is possible.

Methanococcus maripaludis is a strictly anaerobic archaeon that produces methane from carbon dioxide and hydrogen or formate (9). There are many characteristics that make

M. maripaludis a logical choice for hosting an expression system for the *M. thermoacetica* CODH/ACS. *M. maripaludis* utilizes a modified Wood-Ljungdahl pathway with its CODH/ACS (32). Instead of using tetrahydrofolate to carry the reduced forms of CO_2, methanogens use methanofuran and tetrahydromethanopterin from the methanogenesis pathway (8). The methyl group from methyltetrahydromethanopterin is transferred to the CODH/ACS corrinoid Fe/S protein in *M. maripaludis* (27). See Yu (1997) and Fontecilla-Camps and Ragsdale (1999) for the reviews of *M. maripaludis* CODH/ACS and other methanogenic CODH/ACS, respectively. The necessary pathways for the assembly of an active CODH/ACS in *M. maripaludis* are already present, so these pathways may allow the production of an active *M. thermoacetica* CODH/ACS.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions. *Methanococcus maripaludis* JJ was obtained from W. J. Jones. The plasmids used in this study are listed in Table 1. *M. maripaludis* was grown at 37°C on 275 kPa of H_2:CO_2 (80:20) in McN (mineral medium) and McC (complex medium minus the vitamin solution) (29). The sodium bicarbonate in the medium was reduced from 5 g l^{-1} to 2 g l^{-1} during growth at 100 kPa in 1 L Wheaton bottles or in a fermentor. Transformation of *M. maripaludis* with plasmid DNA was described previously (28). For selection of puromycin-resistant methanococci, a stock solution of 500 µg ml^{-1} puromycin dihydrochloride (Sigma) in distilled water was filter-sterilized and added to the medium at a final concentration of 2.5 µg ml^{-1}. Strictly anaerobic techniques were used for medium preparation and cultivation.

The *E. coli* strains were grown at 37°C on low salt Luria-Bertani (LB) medium with the NaCl at 50% of the regular concentration (15). Antibiotic concentrations were 60 µg ml^{-1} for ampicillin (Amp) and 50 µg ml^{-1} for kanamycin (Kan) in both liquid and solid LB medium. ß-galactosidase was used as a blue/white screen on LB agar plates with 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) according to Sambrook et al. (1989).

Plasmids were transformed into *E. coli* strains by electroporation with a Bio-Rad Gene Pulser (Bio-Rad Laboratories) using the settings of 200 Ω, 2.5 kV, and 25 μF with cuvettes (0.2 cm gap width). Plasmids from *E. coli*, grown with selective conditions, were isolated with the Wizard miniprep kit (Promega).

Growth of *E. coli* and *M. maripaludis* was measured at 600 nm with a spectrophotometer (Spectronic 20).

Electrophoresis of agarose gels. The electrophoresis of all agarose gels occurred in 1X TAE and 0.4 microgram/ml ethidium bromide (23). A 10X TAE stock solution contained 0.4 M tris base, 0.2 M glacial acetic acid, and 0.02 M (ethylenedinitrilo)tetraacetic acid, disodium salt, dihydrate. The agarose was also dissolved in 1X TAE.

Plasmid purification from *M. maripaludis*. Plasmid isolation from *M. maripaludis* was begun by centrifuging a 5 ml culture at 16000 x *g* at 4^{o} followed by resuspension of the cells in 100 μl of methanococcal medium. After resuspension, the Wizard miniprep kit (Promega) minus the resuspension step was used for preparation of the plasmid DNA for transformation into *E. coli*.

Deoxyribonucleic acid (DNA) sequencing. All DNA sequencing was performed using API sequencers at the Molecular Genetics Facility, University of Georgia. All oligonucleotides were made by Integrated DNA Technologies (Coralville, Iowa). Sequence analysis was performed with the GCG software package (Genetics Computer Group, Madison, WI). FRAMES was used to identify open reading frames (ORFs) using ATG as the start codon.

Cloning of the *M. thermoacetica acsAB* into pWLG30+*lacZ*. For directed ligation of the *M. thermoacetica acsAB* into pWLG30+*lacZ*, the *acsAB* was PCR modified to contain a *Nsi* I site at the 5'-end and a *Xba* I site at the 3'-end. pCt946A, purified from *E. coli* JM109, contained the *M. thermoacetica acsAB* template for the polymerase chain

reaction (PCR). See Figure 3. The *acsAB* was amplified by PCR using the primers 5'-CCAATGCATGCCCAGGTTCCGCGATCTC-3' and 5'-TGCTCTAGACGGTTCCGGGGCCAATCC-3' at a 0.5 micromolar concentration. These 5'-phosphorylated primers were designed to add a *Nsi* I site and a *Xba* I site at the flanking 5' and 3' ends of the PCR product respectively. The PCR contained 2.5 units of *Pfu* DNA polymerase (Stratagene). After an initial dwell of 5 minutes at 95°C to denature pCt946A, PCR was performed for 25 cycles. Each cycle had a denaturing temperature of 94°C for 1 minute, an annealing temperature of 54.9°C for 1 minute, and an extension time of 8.5 minutes at 74°C. The PCR product mixture was separated by gel electrophoresis in a 0.8% (w/v) agarose gel (23). The band, which corresponded to the 4.4 kilobase (kb) pair *Nsi* I-*acsAB*-*Xba* I product, was excised and then homogenized with a glass rod in a microcentrifuge tube. After adding 200 microliters of pH 8.0 phenol:chloroform:isoamyl alcohol (25:24:1) with 0.1% (w/v) hydroxyquinoline, the sample was vortexed and then frozen at –20°C for 1.5 hours. The frozen agarose/phenol aggregate was centrifuged at 16,000 x *g* for 15 minutes. The aqueous phase (top layer), which contained the PCR product, was recovered. To remove any remaining phenol, the *Nsi* I-*acsAB*-*Xba* I PCR product was treated with the Wizard Clean-Up Kit (Promega). This purified PCR product was ligated into an *Eco*R V digested pZErO-2 in a reaction volume of 10 microliters (µl) with 1 Weiss unit of T4 DNA ligase. The ligated plasmids were drop dialyzed against distilled water with VSWP membranes (Millipore) for 2 hours before transformation into *E. coli* Top10. Colonies, which grew on the LB-Kan agar plates, were streaked to isolate an individual transformant.

The rapid colony screen was utilized to confirm the addition of *Nsi* I-*acsAB*-*Xba* I to pZErO-2. A single colony was streaked onto a new LB-Kan plate. After 16 hours of growth at 37°C, the plates were cooled to 4°C for one hour. Transformants were collected with a sterile toothpick and placed into 50 µl of resuspension solution (50 mM Tris [pH 7.5], 10 mM EDTA, 100 µg/ml RNase A). Each sample was vortexed before the addition

of 50 µl of lysis solution (0.2 M NaOH, 1% (w/v) sodium dodecylsulfate) and then 50 µl of neutralization solution (1.32 M potassium acetate). The cell debris was pelleted by centrifugation at 16000 *g* for 5 minutes at room temperature. To screen the circular plasmids by size for pZErO-2+*Nsi* I-*acsAB*-*Xba* I, electrophoresis was performed using 0.8% (w/v) agarose gel with 17 µl of the supernatant and 3 µl of tracking dye. An isolate that matched the expected size of pZErO-2+*Nsi* I-*acsAB*-*Xba* I was digested with either *Nsi* I or *Xba* I to confirm the addition and orientation of the *Nsi* I-*acsAB*-*Xba* I product. pZErO-2+*Nsi* I-*acsAB*-*Xba* I was digested with *Nsi* I and *Xba* I to yield *Nsi* I/*acsAB*/*Xba* I and *Nsi* I/pZErO-2/*Xba* I. The *Nsi* I/*acsAB*/*Xba* I DNA fragment was gel purified as described above.

pWLG30+*lacZ* was purified from *E. coli* XL1-Blue MRF' (Stratagene) before digestion with *Nsi* I and *Xba* I. The enzymes in the reactions were removed using the Wizard clean-up kit. Following the digestion, the plasmid was dephosphorylated with CIAP (calf intestine alkaline phosphatase). The *Nsi* I/*acsAB*/*Xba* I DNA fragment was ligated to the digested and dephosphorylated expression shuttle vector. The ligated plasmids were drop dialyzed against distilled water with VSWP membranes (Millipore) for 2 hours before transformation into *E. coli* XL1-Blue MRF'. The transformed cells were plated on LB-Amp, X-Gal. Individual white colonies were screened with the rapid colony preparatory method described above. An isolate containing pWLG30+*acsAB* was identified by the rapid colony screen. *Nsi* I or *Xba* I restriction digestions initially confirmed the construction of pWLG30+*acsAB* before sequencing with the previously published primers pMEB.2seq2 and pWLG30seq1 (Gardner and Whitman, 1999).

Isolation of pWLG30+*acsAB* from *M. maripaludis* to confirm stability of the construction. *M. maripaludis* was transformed with pWLG30+*acsAB* and plated on McC+1% (w/v) bactoagar+2.5 µg/ml puromycin. Two individual isolates were inoculated into McN+puromycin liquid medium. After three transfers, the plasmids were isolated from the recombinant *M. maripaludis* for transformation into *E. coli* XL1-Blue MRF'. The

transformed *E. coli* cells were plated on LB-Amp plates. Individual isolates were grown in LB-Amp broth overnight. The plasmids were harvested before a double digestion with *Nsi* I and *Xba* I. Electrophoresis of the restriction digestions was performed using 0.8% (w/v) agarose gel. The restriction pattern from both *M. maripaludis* isolates and the control plasmid were identical. The control plasmid was pWLG30+*acsAB* maintained in *E. coli* XL1-Blue MRF'.

Growth of recombinant *M. maripaludis* in a 20 liter stainless steel fermentor with sodium formate as the carbon and electron source. Balch tubes, which contained 5 ml of McN+puromycin, were inoculated with 0.2 ml of *M. maripaludis* transformed with pWLG30+*acsAB.* After an overnight incubation, the recombinant *M. maripaludis* grew to an $O.D._{600}$ of 0.45-0.48 in five tubes. Each tube was inoculated into 150 ml of McN+puromycin in a modified Wheaton bottle after the addition of 3 ml 2.5% (w/v) sodium sulfide. After 24 hours of growth, the cultures grew to an $O.D._{600}$ of 0.52-0.54. These cultures were inoculated into a 20 L fermentor that contained 16 L of McN+0.4 M sodium formate+1.25 μg/ml puromycin at a pH of 7.5. The medium was agitated at 200 rpm during growth at 37°C. The pH was maintained by the addition of 11.7 M formic acid, which was sparged at 60 bubbles/minute with nitrogen, from a reservoir.

The 20 L fermentor (W.B. Moore Inc., PA) was prepared in the following manner on the day of inoculation. Table 2 contains the recipe for the McN+0.4 M sodium formate medium. Polyoxypropylene glycol with an average molecular weight of 2000 (PPG-2000; Lambent Technologies, IL) was the antifoam agent. The trace mineral solution was described by Whitman *et al.* (1986). The chemicals, excluding the cysteine hydrochloride and the sodium sulfide solution, were dissolved in 16 L of deionized water in the fermentor. The propellers agitated the medium at 200 revolutions per minute (rpm). The medium was sparged with 2.0 standard liters per minutes (slpm) of N_2:CO_2 (80:20) for 0.5 minutes. After sparging, the cysteine hydrochloride was added to the medium, and the pH adjusted to 7.5 with 50% (w/v) sodium hydroxide. The medium, which was agitated at 200 rpm, was

autoclaved *in situ* for 1.5 hours. After sterilization, the medium was sparged with N_2:CO_2 (80:20) at 2.0 slpm as the solution was rapidly cooled to 47°C. The temperature control, which was set to maintain the temperature at 37°C, was engaged at 47°C, and the N_2:CO_2 gas was turned off. The vessel remained at atmospheric pressure during the growth of *M. maripaludis*. After 1 hour, the 20% (w/v) sodium sulfide was added to reduce the medium. The pH was adjusted to 7.5 with 11.7 M formic acid two hours after the sodium sulfide addition. At this point, both the puromycin and the 750 ml inoculum were added to the fermentor. The puromycin (1.25 mg) was dissolved in 50 ml of distilled water then sparged with nitrogen gas for 1 hour. After 1 hour, the puromycin solution was filter sterilized inside an anaerobic chamber with a Millipore 100 ml stericup (0.22 microns). The sterile puromycin solution was placed into sterile Balch tubes and sealed before pressurization to 275 kPa with H_2:CO_2 (80:20).

After 18 hours of growth, the cells were harvested at an absorbance (600 nm) of 0.75 with a water-cooled (13°C) Alfa Laval Sharples (Model T-1P) continuous flow centrifuge at 23,000 rpm. After centrifugation of about 10 L, the cell pellet was transferred to a degassed aluminum seal vial while being flushed with nitrogen. This vial was also flushed with nitrogen during the transfer of the cell pellet to the vial. The container was weighed, sealed, then flushed with hydrogen gas for two minutes. The 10.9 g of wet cell paste was stored at -20°C.

Growth of recombinant *M. maripaludis* containing pWLG30+*acsAB* in a 12 liter glass fermentor using H_2:CO_2. Balch tubes, which contained 5 ml of McN+puromycin, were inoculated with 0.2 ml of *M. maripaludis* transformed with pWLG30+*acsAB.* After an overnight incubation, the recombinant *M. maripaludis* grew to an $O.D._{600}$ of 0.54-0.56 in three tubes. Each tube was inoculated into 150 ml of McN+puromycin in a modified Wheaton bottle after the addition of 3 ml of 2.5% (w/v) sodium sulfide, which was added 2 hours before the culture. After 24 hours of growth, the

cultures grew to an $O.D._{600}$ of 0.80-0.90. These cultures were inoculated into a 12 L fermentor that contained 10 L of McN+ 2.5 μg/ml puromycin.

To prepare the fermentor, 10 L of McN minus cysteine hydrochloride and sodium sulfide were added to the fermentor. The medium was sparged with H_2:CO_2 (80:20) for 0.5 minutes while being agitated at 240 rpm. At the same time, the medium was heated to autoclaving conditions. The cysteine hydrochloride was added to the fermentor at 70°C then sealed. The medium was autoclaved at 121°C for 1 hour with agitation. After autoclaving, the medium was cooled. Upon reaching 80°C, the medium was sparged with H_2:CO_2. After the medium was at 37°C for 1 hour, 10 ml of 20% (w/v) sodium sulfide reduced the McN. The puromycin solution and 450 ml of inoculum were inoculated two hours after the sodium sulfide addition. The fermentor was pressurized to 150 kPa with H_2:CO_2 and then sparged at 50 ml/min with the gas mixture.

The puromycin solution was prepared by dissolving 2.5 mg in 50 ml of distilled water. This solution was sparged with nitrogen gas for 1 hour. After 1 hour, the solution was filter sterilized inside an anaerobic chamber with a Millipore 100 ml stericup, 0.22 microns. The sterile puromycin solution was placed into sterile Balch tubes and sealed before pressurization to 275 kPa with H_2:CO_2 (80:20).

The recombinant *M. maripaludis* was grown twice in McN+puromycin with H_2:CO_2. After 55 and 74 hours of growth, the cells were harvested at an absorbance (600 nm) of 0.75 and 0.70 with a water-cooled (13°C) Alfa Laval Sharples (Model T-1P) continuous flow centrifuge as described above. The wet cell paste, which was 6.6 g and 6.9 g for the respective runs, was stored at -20°C.

CODH assays with *M. maripaludis* cell-free extracts. *M. maripaludis* and recombinant *M. maripaludis* were grown in modified Wheaton bottles with 150 ml of McN and McN+puromycin, respectively. The *M. maripaludis* cells were anaerobically harvested at 10°C by centrifugation at 4420 x *g* in a Beckman JA-10 rotor. After decanting the supernatant, the cell pellet was resuspended in 2 ml of the resuspension solution per 150 ml

of culture. The resuspension solution, which was modified from Shieh and Whitman (1988), contained 25 mM potassium PIPES [piperazine-*N*, *N*'-bis(2-ethanesulfonic acid)] (pH 6.8), 1 mM cysteine dihydrochloride, 1 mM dithiothreitol, and 1 mg of bovine pancreatic DNase (Boehringer Mannheim)/100 ml solution. Hydrogen gas sparged the resuspension solution for a minimum of 1 hour. Following resuspension, the cell suspension was transferred to an aluminum seal vial and then sealed before flushing with nitrogen for 2 minutes while on ice. The cells were frozen at –20°C. Prior to the assay, the headspace of each vial of cell-free extract was flushed for two minutes with hydrogen gas while the sample thawed on ice.

The carbon monoxide assay solution was modified from Ragsdale et al. (1983). The solution contained 50 mM TRIS base (pH 7.6), 10 mM methyl viologen, and 2 mM dithiothreitol. For two hours, nitrogen gas sparged the solution. All assays were 1 ml final volume. The assay solution was added to degassed cuvettes in an anaerobic chamber before a red stopper sealed the top. Each cuvette was flushed with nitrogen gas for two minutes to remove any hydrogen gas from the anaerobic chamber. Carbon monoxide (100%), which was bubbled through a 25 mM methyl viologen solution reduced with 10 mM sodium dithionite to remove oxygen, was bubbled for 30 seconds into the assay solution in each cuvette (Shieh and Whitman, 1988). Preincubation at the assay temperature lasted for at least five minutes for each cuvette. A Hamilton syringe, which had been flushed with nitrogen-sparged distilled water, was used to add a small grain of solid sodium dithionite to reduce the methyl viologen in the assay solution to a slight blue color. The assay was initiated with cell-free extract. The absorbance was monitored at 603 nm by a Beckman DV 640B spectrophotometer with a water-regulated single cell holder. The methyl viologen extinction coefficient was 11.3×10^3 cm^{-1} M^{-1} (26). For protein quantification by the bicinchoninic acid protein assay (Pierce), the cell-free extract was first diluted 1:50 in 0.1 M sodium hydroxide, heated in a boiling water bath for 10 minutes, and cooled on ice.

Results and Discussion

The mesophilic methanococci utilize either hydrogen or formate as electron donors during methanogenesis (1). In Balch tubes with formic acid reservoirs, *Methanococcus voltae*, *M. maripaludis*, *Methanococcus vannielii*, and "*Methanococcus aeolicus*" grow to absorbances (600 nm) between 0.70 and 1.15 in McC+59 mM sodium formate (25). Additionally, large scale cultures (400L) of "*M. aeolicus*" in McN supplemented with 74 mM sodium formate are also possible (31). In this work, the recombinant *M. maripaludis* grew to normal optical densities in a 20L fermentor containing a mineral medium with formate and puromycin. See Figure 4. This experiment is significant because large amounts of *M. maripaludis* cells can be obtained without using hydrogen gas, which is dangerous and expensive. *M. maripaludis* also grew in this medium, minus the puromycin, at the 400 L scale (results not shown).

The recombinant *M. maripaludis* was harvested from a 20 L fermentor at an absorbance (600 nm) of 0.75 to limit the amount of autolysis in the culture. Autolysis occurred around an absorbance (600 nm) of 0.9 (Waters and Whitman, unpublished data). The recombinant *M. maripaludis* yielded about 1 g wet cell paste per liter of McN+0.4 M sodium formate+1.25 μg/ml puromycin. Initially, *M. maripaludis* was not expected to grow with 400 mM sodium formate since *M. voltae* was significantly inhibited by 88 mM sodium formate (25). Recently, Ciulla and Roberts (1999) reported that *Methanococcus thermolithotrophicus* grew in a medium containing 200 mM sodium formate.

Increasing sodium chloride concentrations in the medium from 0.48 M to 1.2 M increases the doubling time by 8-fold for *M. thermolithotrophicus* (2). *M. maripaludis* grows optimally in a medium that contains between 0.2 M and 0.4 M sodium chloride (29). The McN medium contains a 0.47 M sodium concentration when *M. maripaludis* grows at 100 kPa of H_2:CO_2 (29). However, the McN+0.4 M formate medium used the sodium salt of formic acid. Removing the sodium chloride from McN+0.4 M formate medium avoided

a 0.87 M sodium concentration and thus, potential osmolarity problems. The McN+0.4 M formate medium contained a 0.42 M sodium concentration.

The rate of formic acid addition increased during growth in the fermentor. Formate oxidization by a methanogen produces sodium hydroxide according to the following equation: $4HCOONa + 5H_2O \rightarrow 3HCO_3^- + 3H^+ + CH_4 + 4NaOH$ (24). Therefore, by titrating the pH, the formate concentration of the medium is maintained at a high level.

To determine the puromycin concentration for a fermentor run, 9.9×10^4 and 7.0×10^7 *M. maripaludis* cells were inoculated in duplicate series of 2-fold puromycin dilutions in McN. Culture tubes were pressurized with H_2:CO_2 to 275 kPa before incubation at 37°C for six days. A 1.2 μg/ml puromycin concentration inhibited growth for both inoculum sizes for at least five days. Measurable growth at six days was observed with the larger inoculum (results not shown). Two-fold lower puromycin concentrations inhibited growth for only two days with the high inoculum. The lower inoculum size reduced the chance of a spontaneous resistant cell being present. To test the lower level of puromycin required for inhibition of the cells, puromycin concentrations of 0.29 μg/ml and 0.58 μg/ml were tested with a small inoculum. *M. maripaludis* growth was inhibited for one day and four days in puromycin concentrations of 0.29 μg/ml and 0.58 μg/ml, respectively. In *M. voltae*, the minimum inhibitory concentration of puromycin was 2 μg/ml after 24 hours and a spontaneous resistance frequency was $<10^{-7}$ (17).

The methyl viologen dependent CODH assays demonstrated CODH activity from *M. maripaludis* cell-free extracts when the cells were grown in modified Wheaton bottles (Tables 3 and 4). The temperatures of 25°C and 55°C for the CODH assays represented previous assay conditions for *M. maripaludis* and the optimum temperature for *M. thermoacetica* growth, respectively (26; 22). *M. maripaludis* CODH specific activity appears to be repressed by 45-fold when grown in a McN supplemented with acetate (26). However, since *M. maripaludis* may produce enzymes and/or cofactors necessary for the holo-form of AcsAB, *M. maripaludis* was grown in McN or McN+puromycin for these

experiments. These cofactors/enzymes may include proteins involved in metal insertion or folding of the CODH/ACS. The *M. maripaludis* control and both recombinants were within the reported range for the CODH specific activity at 25°C. The CODH specific activities at 55°C were not different in Table 3 since there was less than a 2-fold difference. Preincubation of the cell-free extracts (Table 4) at 55°C was an attempt to lower the background of the native *M. maripaludis* CODH. In both recombinant *M. maripaludis* strains, the specific activities were not different before or after the heat treatment.

CODH assays failed to indicate an increase of CODH specific activity in the cell-free extracts from *M. maripaludis* transformed with pWLG30+*acsAB*. Western blots of *M. maripaludis* transformed with pWLG30+*acsAB* showed a positive reaction with antibodies against *M. thermoacetica* CODH (J. Seravalli, personal communication). CODH assays discovered two fractions of CODH activity following purification with a Superose 6 column (J. Seravalli, personal communication). One fraction was unreactive to antibodies against the *M. thermoacetica* CODH while the second region showed a positive reaction (J. Seravalli, personal communication). These results suggest that the *M. thermoacetica* CODH is expressed in *M. maripaludis*. However, the fraction containing the *M. thermoacetica* CODH had a low CODH specific activity.

References

1. **Boone, D. R., W. B. Whitman, and P. Rouvière**. 1993. Diversity and taxonomy, p. 35-80. *In* James G. Ferry (ed.), Methanogenesis: ecology, physiology, biochemistry and genetics. Chapman & Hall, New York
2. **Ciulla, R. A. and M. F. Roberts**. 1999. Effects of osmotic stress on *Methanococcus thermolithotrophicus*: ^{13}C-edited ^{1}H-NMR studies of osmolyte turnover. Biochim. Biophys. Acta **1427**:193-204.
3. **Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. E. Farrow**. 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. **44**:812-826.
4. **Drake, H. L**. 1994. Acetogenesis, acetogenic bacteria, and the acetyl-CoA "Wood/Ljungdahl" pathway: past and current perspectives. *In* H. L. Drake (ed.), Acetogenesis, Chapman and Hall, New York, NY.
5. **Ferry, J. G**. 1995. CO dehydrogenase. Annu. Rev. Microbiol. **49**:305-333.
6. **Fontecilla-Camps, J. C. and S. W. Ragsdale**. 1999. Nickel-Iron-Sulfur active sites: hydrogenase and CO dehydrogenase. Adv. Inorg. Chem. **47**:283-333.
7. **Gardner, W. L. and W. B. Whitman**. 1999. Expression vectors for *Methanococcus maripaludis*: overexpression of acetohydroxyacid synthase and β-galactosidase. Genetics **152**:1439-1447.
8. **Jones, W. J., D. P. Nagel, and W. B. Whitman**. 1987. Methanogens and the diversity of archaebacteria. Microbiol. Rev. **51**:135-177.
9. **Jones, W. J., M. J. B. Paynter, and R. Gupta**. 1983. Characterization of *Methanococcus maripaludis* sp. nov., a new methanogen isolated from salt marsh sediment. Arch. Microbiol. **135**:91-97.
10. **Kerby, R. and J. G. Zeikus**. 1983. Growth of *Clostridium thermoaceticum* on H_2/CO_2 or CO as energy source. Curr. Microbiol. **8**:27-30.
11. **Kumar, M., W.-P. Lu, L. Liu, and S. W. Ragsdale**. 1993. Kinetic evidence that carbon monoxide dehydrogenase catalyzes the oxidation of carbon monoxide and the synthesis of acetyl-CoA at separate metal centers. J. Am. Chem. Soc. **115**:11646-11647.
12. **Ljungdahl, L. G.** 1979. Physiology of thermophilic bacteria. Adv. Microb. Physiol. **19**:149-243.
13. **Ljungdahl, L. G.** 1986. The autotrophic pathway of acetate synthesis in acetogenic bacteria. Ann. Rev. Microbiol. **40**:415-450.
14. **Loke, H.-K. and P. A. Lindahl**. 1999. Carbon monoxide dehydrogenase: purification and characterization of active recombinant protein. J. Inorg. Biochem. **74**:210.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook**. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.
16. **Morton, T. A., J. A. Runquist, S. W. Ragsdale, T. Shanmugasundaram, H. G. Wood, and L. G. Ljungdahl**. 1991. The primary structure of the subunits of carbon monoxide dehydrogenase/acetyl-CoA synthase from *Clostridium thermoaceticum*. J. Biol. Chem. **266**:23824-23828.
17. **Possot, O., P. Gernhardt, A. Klein, and L. Sibold**. 1988. Analysis of drug resistance in the archaebacterium *Methanococcus voltae* with respect to potential use in genetic engineering. Appl. Environ. Microbiol. **54**:734-740.
18. **Ragsdale, S. W**. 1991. Enzymology of the acetyl-CoA pathway of CO_2 fixation. Crit. Rev. Biochem. Mol. Biol. **26**:261-300.

19. **Ragsdale, S. W., J. E. Clark, L. G. Ljungdahl, L. L. Lundie, and H. L. Drake**. 1983. Properties of purified carbon monoxide dehydrogenase from *Clostridium thermoaceticum*, a nickel, iron-sulfur protein. J. Biol. Chem. **258**:2364-2369.
20. **Ragsdale, S. W. and M. Kumar**. 1996. Nickel-containing carbon monoxide dehydrogenase/acetyl-CoA synthase. Chem. Rev. **96**:2515-2539.
21. **Ragsdale, S. W. and H. G. Wood**. 1985. Acetate biosynthesis by acetogenic bacteria: evidence that carbon monoxide dehydrogenase is the condensing enzyme that catalyzes the final steps of the synthesis. J. Biol. Chem. **260**:3970-3977.
22. **Roberts, D. L., J. E. James-Hagstrom, D. K. Garvin, C. M. Gorst, J. A. Runquist, J. R. Baur, F. C. Haase, and S. W. Ragsdale**. 1989. Cloning and expression of the gene cluster encoding key proteins involved in acetyl-CoA synthesis in *Clostridium thermoaceticum*: CO dehydrogenase, the corrinoid/Fe-S protein, and methyltransferase. Proc. Natl. Acad. Sci. **86**:32-36.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis**. 1989. Molecular cloning: a laboratory manual (2nd edition). Cold Spring Harbor Laboratory Press, New York.
24. **Schauer, N. L. and J. G. Ferry**. 1980. Metabolism of formate in *Methanobacterium formicicum.* J. Bacteriol. **142**:800-807.
25. **Schauer, N. L. and W. B. Whitman**. 1989. Formate growth and pH control by volatile formic and acetic acids in batch cultures of methanococci. J. Microbiol. Methods **10**:1-7.
26. **Shieh, J**. **and W. B. Whitman**. 1988. Autotrophic acetyl Coenzyme A biosynthesis in *Methanococcus maripaludis*. J. Bacteriol. **170**:3072-3079.
27. **Simpson, P. G. and W. B. Whitman**. 1993. Anabolic pathways in methanogens, p. 445-472. *In* James G. Ferry (ed.), Methanogenesis: ecology, physiology, biochemistry and genetics. Chapman & Hall, New York.
28. **Tumbula, D. L., R. A. Makula, and W. B. Whitman**. 1994. Transformation of *Methanococcus maripaludis* and identification of a *Pst*I-like restriction system. FEMS Microbiol. Lett. **121**:309-314.
29. **Whitman, W. B., J. Shieh, S. Sohn, D. S. Caras, and U. Premachandran**. 1986. Isolation and characterization of 22 mesophilic methanococci. System. Appl. Microbiol. **7**:235-240.
30. **Xia, J. and P. A. Lindahl**. 1995. Decomposition of carbon monoxide dehydrogenase into α metallosubunits and a catalytically-active form consisting primarily of α metallosubunits. Biochemistry **34**:6037-6042.
31. **Xing, R. and W. B. Whitman**. 1992. Characterization of amino acid aminotransferases of *Methanococcus aeolicus*. J. Bacteriol. **174**:541-548.
32. **Yu, J.-P**. 1997. Ph.D. thesis. University of Georgia, Athens.

TABLE 1. Plasmids and DNA fragments used in this study[a]

Plasmids	Properties	Reference
pCt946A	contains the *M. thermoacetica acsAB*, ampicillin resistance (amp^r)	22
pWLG30	expression shuttle vector for *M. maripaludis*, amp^r, puromycin resistance (pur^r)	7
pWLG30+*acsAB*	expression shuttle vector with modified *M. thermoacetica acsAB*, amp^r, pur^r	This work
pWLG30+*lacZ*	expression shuttle vector for *M. maripaludis* which contains *lacZ* for *E. coli* blue/white screen, amp^r, pur^r	7
pZErO-2	requires *E. coli* Top10, kanamycin resistance (kan^r)	Invitrogen
pZErO-2+*acsAB*	contains the modified *M. thermoacetica acsAB*, kan^r	This work

[a]Ampicillin (amp) and kanamycin (kan) are for selection in *E. coli*. Puromycin (pur) is for selection in *M. maripaludis*.

TABLE 2. McN+0.4 M sodium formate recipe

	Fermentor working volume		
	16 L	25 L	400 L
Chemical	Amount		
deionized water	final volumes above		
KCl	5.4 g	8.4 g	134 g
$MgCl_2 \cdot 6H_2O$	44.0 g	68.8 g	1100 g
$MgSO_4 \cdot 7H_2O$	55.2 g	86.3 g	1380 g
NH_4Cl	8.0 g	12.5 g	200 g
$CaCl_2 \cdot 2H_2O$	2.24 g	3.5 g	56 g
K_2HPO_4	2.24 g	3.5 g	56 g
Trace Mineral Solution	160 ml	250 ml	4000 ml
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.16 g	0.25 g	4.0 g
0.01%(w/v) Resazurin	16 ml	25 ml	-------
$NaHCO_3$	32 g	50 g	800 g
sodium formate	435.3 g	680.1 g	10.88 kg
PPG-2000[a]	1.6 ml	2.5 ml	10 ml
L-Cysteine hydrochloride hydrate	8 g	12.5 g	200 g
20%(w/v) $Na_2S \cdot 9H_2O$	16 ml	25 ml	400 ml

[a]PPG-2000 (Lambent Technologies, IL) is polyoxypropylene glycol with an average molecular weight of 2000.

TABLE 3. CO dehydrogenase assays of *M. maripaludis* grown in mineral medium[a]

Medium	Plasmid	$O.D._{600}$[b]	Assay Incubation Temperature (^oC)	Specific activity (μmol min^{-1} mg of $protein^{-1}$)[c]
McN	None	0.58	25 55	0.91 1.5[d]
McN+ puromycin	pWLG30	0.52-0.54	25 55	0.47 1.4
McN+ puromycin	pWLG30 +*acsAB*	0.38-0.48	25 55	0.51 0.78

[a]Enzyme assays performed after cell extracts were stored at –20°C for 6 days.
[b]$O.D._{600}$ at harvesting.
[c]Methyl viologen dependent. The average value for assays performed in triplicate.
[d]Individual assay.

TABLE 4. CO dehydrogenase assays with recombinant *M. maripaludis* grown in mineral medium with puromycin[a]

Plasmid	$O.D._{600}$[b]	Heat Treated[c]	Assay Incubation Temperature (^oC)	Specific activity ($\mu mol\ min^{-1}\ mg\ of\ protein^{-1}$)[d]
pWLG30	0.50	No	55	0.51
		Yes		0.27
pWLG30 +*acsAB*	0.43-0.50	No	55	0.58
		Yes		0.16

[a]Enzyme assays performed after cell extracts were stored at –20°C overnight.
[b]$O.D._{600}$ at time of harvesting.
[c]Cell extracts were heated to 55°C for 1.5 hours under hydrogen atmosphere before cooling to room temperature for 5 minutes and placing on ice.
[d]Methyl viologen dependent. The average value for assays performed in triplicate.

Figure 1: Fermentation of glucose to acetic acid.

Two moles of pyruvate are formed from glucose by using the glycolytic pathway. Pyruvate oxidoreductase (1) catalyzes the formation of acetyl-Coenzyme A (acetyl-CoA), carbon dioxide and reduced ferredoxin from pyruvate. The carbon dioxide is reduced to a methyl group attached to tetrahydrofolate (H_4Folate) by the following enzymes: formate dehydrogenase (2), formyl-H_4folate synthetase (3), methylene-H_4folate cyclohydrolase (4), and methylene-H_4Folate reductase (6). The methyl group is transferred by methyltransferase (7) to a corrinoid-containing protein (CoE) to form the methylated corrinoid protein (CH_3-CoE). The carbon dioxide is reduced with electrons from a ferredoxin (9) to a carbon monoxide bound to a Ni atom in the CODH/ACS (8). The methyl group from CH_3-CoE is transferred to the CODH/ACS to yield acetyl-CoA. Phosphotransacetylase (10) phosphorylated acetyl-CoA to form acetyl phosphate. The acetyl phosphate is converted to acetic acid with the generation of ATP from ADP. The enzymes forming the Ljungdahl-Wood pathway were 2, 3, 4, 5, 6, 7, and 8. This figure was modified from Ljungdahl (1986) and Ragsdale and Kumar (1996).

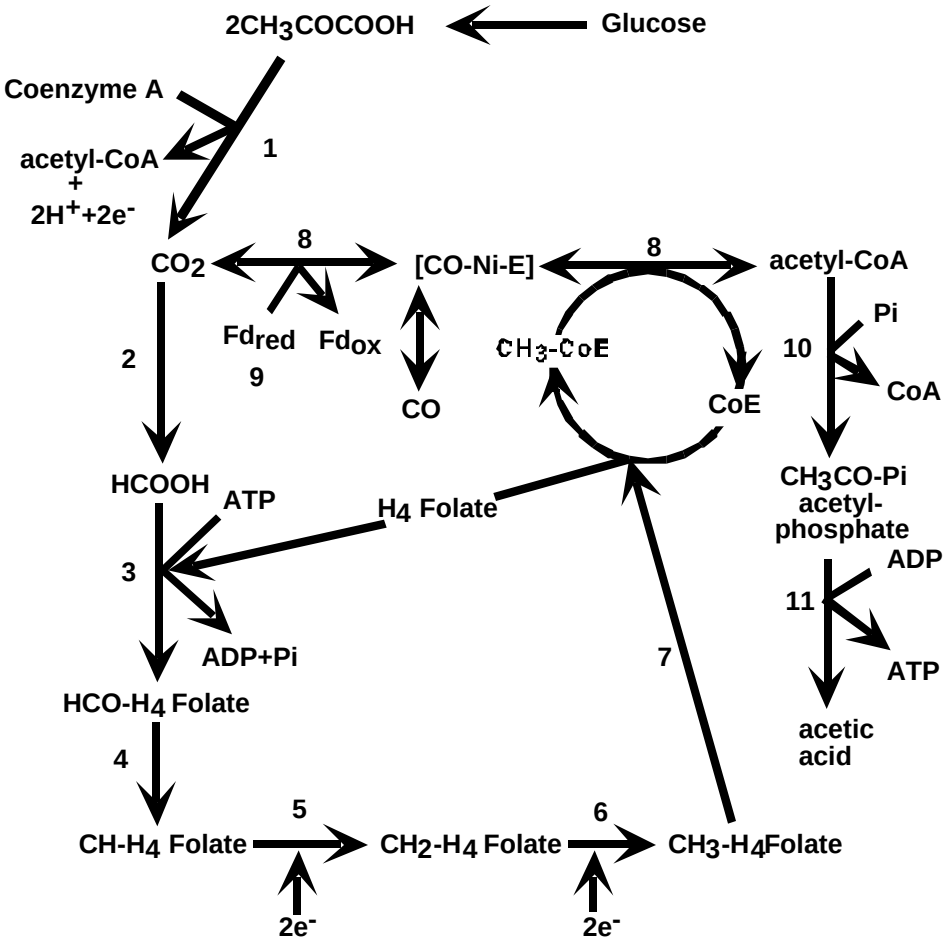

2CH3COCOOH
Glucose
Coenzyme A
acetyl-CoA
+
2H+ +2e-
1
CO2
8
[CO-Ni-E]
8
acetyl-CoA
Fdred
Fdox
9
2
CO
CH3-CoE
CoE
10
Pi
CoA
HCOOH
ATP
H4 Folate
CH3CO-Pi
acetyl-
phosphate
3
ADP
11
ADP+Pi
7
ATP
HCO-H4 Folate
acetic
acid
4
5
6
CH-H4 Folate
CH2-H4 Folate
CH3-H4Folate
2e-
2e-

Figure 2: Reactions catalyzed by the carbon monoxide dehydrogenase/acetyl-CoA synthase.

The α subunit catalyzes the formation of acetyl-CoA from carbon monoxide, coenzyme A (CoA), and a methylated corrinoid iron/sulfur protein (CFeS protein) and α contains one Fe-S cluster, which is named Cluster A. The β subunit containsed the carbon monoxide dehydrogenase (CODH) activity. The CODH used two electrons for the reduction of carbon dioxide to carbon monoxide and water. The β subunit had two Fe-S clusters, which are named Cluster B and Cluster C. This figure was modified from Fontecilla-Camps and Ragsdale (1999).

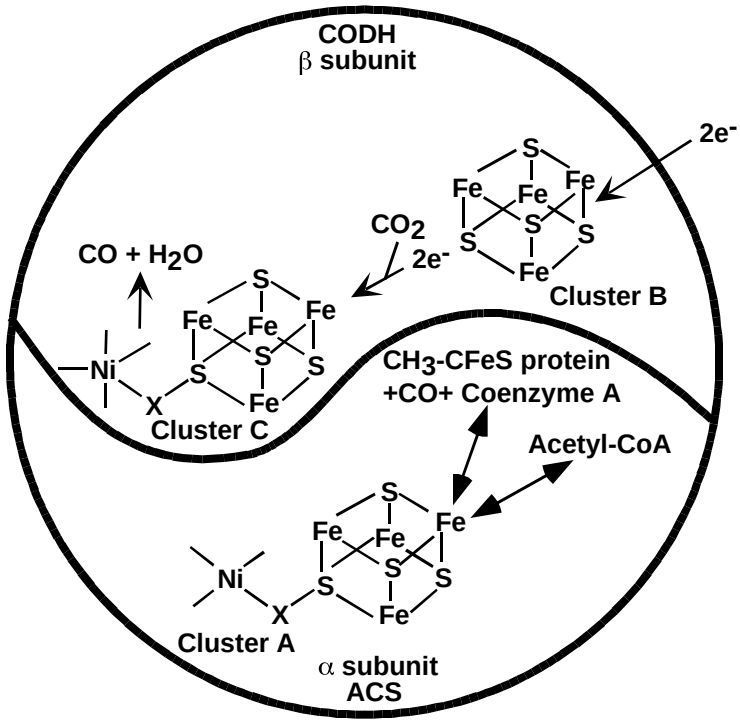

CODH
β subunit
2e-
CO2
2e-
CO + H2O
Cluster B
Cluster C
CH3-CFeS protein
+CO+ Coenzyme A
Acetyl-CoA
Cluster A
α subunit
ACS

Figure 3: Construction of pWLG30+*acsAB*.

Polymerase chain reaction (PCR) was used to modify the *Moorella thermoacetica acsAB*, which encoded the carbon monoxide/acetyl-CoA synthase, to contain a *Nsi* I site and a *Xba* I site at the 5' and 3' ends, respectively. The PCR product was cloned into the *Eco*R V site of pZErO-2 to yield pZErO-2+*acsAB*. pZErO-2+*acsAB* was digested with *Nsi* I and *Xba* I to release the modified *acsAB*. This modified DNA was gel purified before ligation. The ligation reaction contained pWLG30+*lac*, which was digested with *Nsi* I and *Xba* I before treatment with calf intestine alkaline phosphatase, and the *Nsi* I/*acsAB*/*Xba* I.

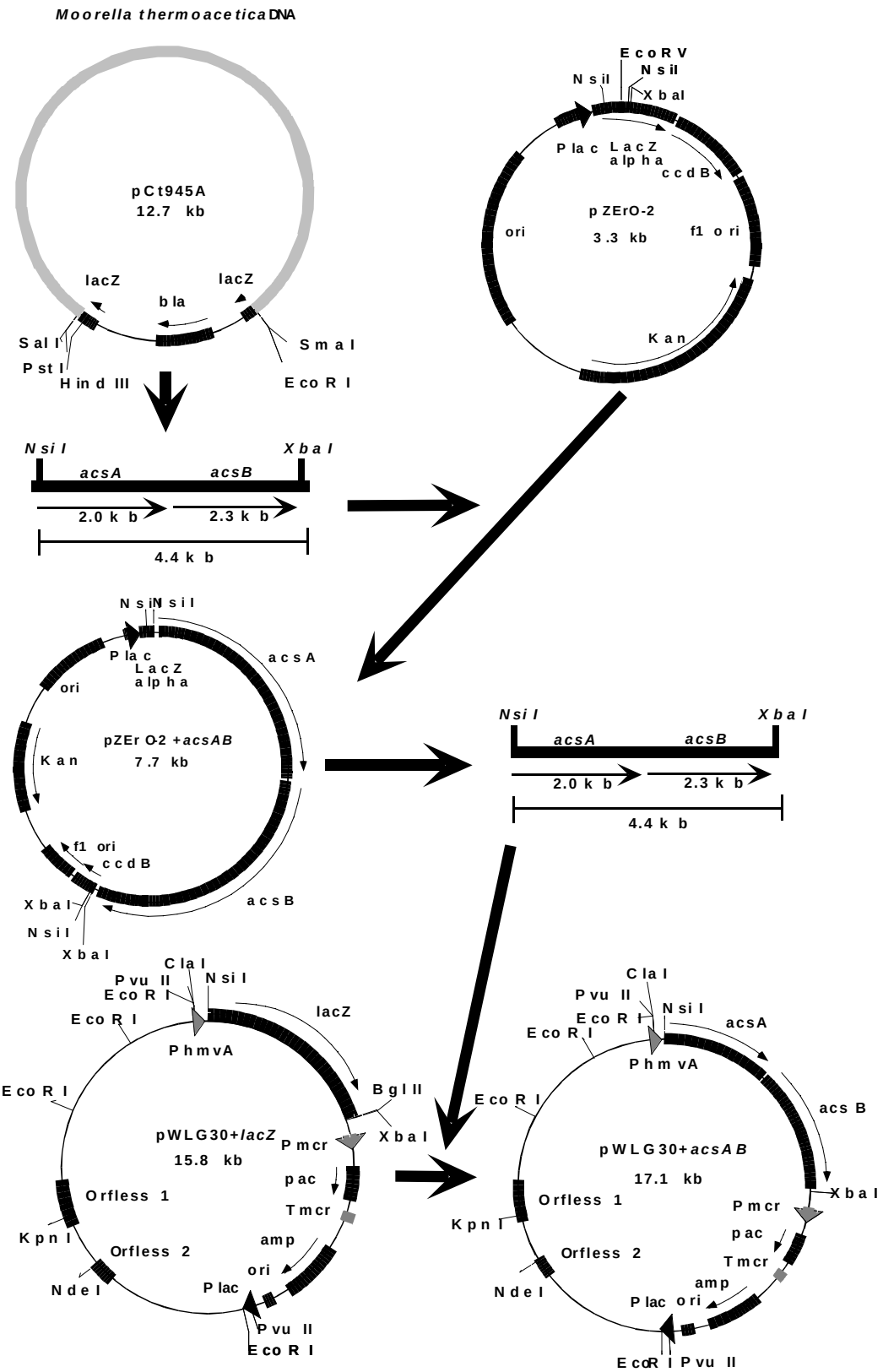
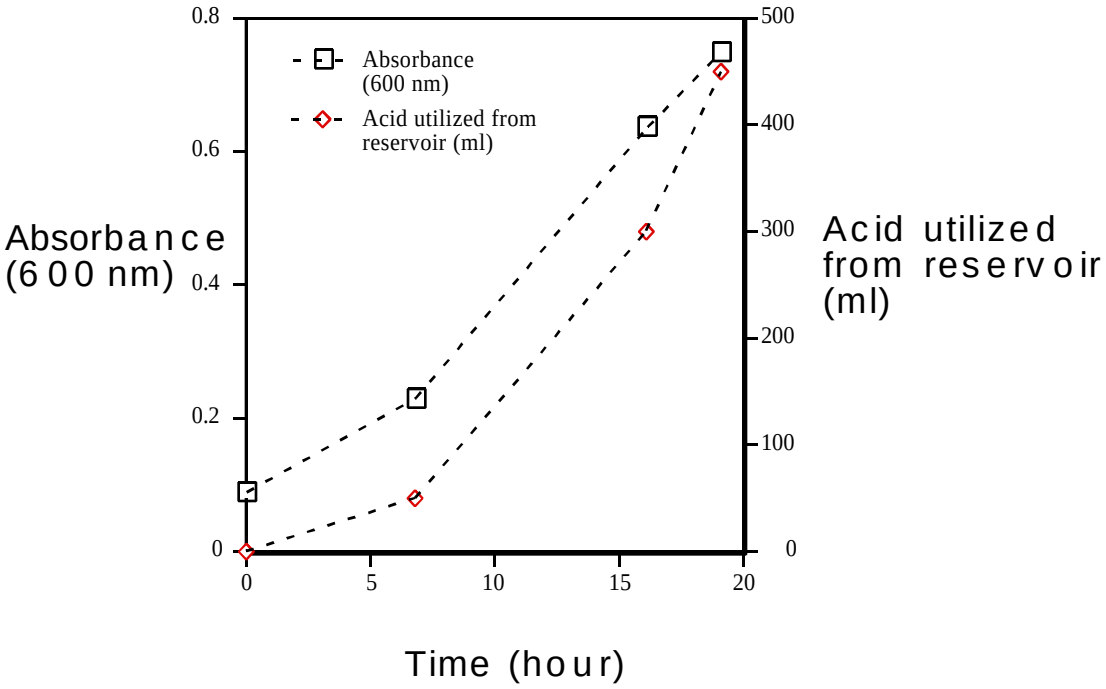

Moorella thermoacetica DNA
pCt945A
12.7 kb
lacZ
lacZ
bla
Sal I
Pst I
Hind III
Sma I
EcoR I
Nsi I
Xba I
acsA
acsB
2.0 kb
2.3 kb
4.4 kb
EcoR V
Nsi I
Nsi I
Xba I
Plac
LacZ alpha
ccdB
pZErO-2
3.3 kb
ori
f1 ori
Kan
Nsi I
Nsi I
Plac
LacZ alpha
acsA
ori
pZErO-2 +acsAB
7.7 kb
Kan
f1 ori
ccdB
acsB
Xba I
Nsi I
Xba I
Nsi I
acsA
acsB
2.0 kb
2.3 kb
4.4 kb
Cla I
Pvu II
EcoR I
Nsi I
lacZ
EcoR I
PhmvA
EcoR I
BglII
pWLG30+lacZ
15.8 kb
Pmcr
Xba I
pac
Orfless 1
Tmcr
Kpn I
amp
Orfless 2
ori
Nde I
Plac
Pvu II
EcoR I
Cla I
Pvu II
EcoR I
Nsi I
acsA
EcoR I
PhmvA
EcoR I
acs B
pWLG30+acsAB
17.1 kb
Xba I
Orfless 1
Pmcr
Kpn I
pac
Orfless 2
Tmcr
Nde I
amp
Plac ori
EcoR I Pvu II

Figure 4: Growth of *M. maripaludis* transformed with pWLG30+*acsAB* in a 20 L fermentor.

The recombinant *M. maripaludis* grew at 37°C in 16 L of McN+0.4 M sodium formate+1.25 μg/ml puromycin at a pH of 7.5. As the pH increased, 11.7 M formic acid was injected from a reservoir and into the medium. The rate of formic acid addition to the vessel increased as the culture's density increased.


Absorbance (600 nm)
Acid utilized from reservoir (ml)
0.8
0.6
0.4
0.2
0
500
400
300
200
100
0
0
5
10
15
20
Absorbance (600 nm)
Acid utilized from reservoir (ml)
Time (hour)

CHAPTER VII

CONCLUSIONS

Development of an expression vector requires multiple steps. After the initial construction of the *Methanococcus maripaludis* shuttle vector, the identification a strong heterologous promoter was necessary for the expression of recombinant genes from the vector. In this work, the *Methanococcus voltae* histone promoter (P_{hmvA}) was tested for its ability to express genes in *M. maripaludis* after insertion into the *M. maripaludis* genome. After confirming the P_{hmvA} level of expression, the promoter was added to the shuttle vector to form an expression shuttle vector. The *Escherichia coli* β-galactosidase gene was ligated downstream of P_{hmvA} to evaluate the expression level of this gene from the shuttle vector. After confirming the expression of the β-galactosidase gene, other heterologous genes were cloned into the expression shuttle vector. The expression of these heterologous genes allowed hypotheses involving translational events to be tested in *M. maripaludis*. Additionally, genes encoding oxygen-sensitive metalloenzymes were also expressed in *M. maripaludis*, a strict anaerobe. Some of these recombinant proteins represented <1% of the cellular protein after expression. In an effort to increase the quantity of recombinant protein, a medium was developed for large scale growth of *M. maripaludis*, and modifications of the expression vector were tested.

Heterologous promoters are essential for the construction of integrative and shuttle expression vectors. By using a heterologous promoter, integration into the genomic homolog of the promoter is significantly decreased. Additionally, the level of expression should remain constant when expressing different genes. The expression level the *Methanococcus voltae* histone promoter (P_{hmvA}) was tested by overexpressing the *M. maripaludis* acetohydroxyacid synthase after insertion into the genome (Chapter 3). These experimental results demonstrated a simple and direct method for testing the

strength of a heterologous promoter. They also identified a promoter suitable for the expression of heterologous genes in an expression shuttle vector.

pWLG30 was the first expression shuttle vector developed for the methanogens (Chapter 3). This vector allows the expression of heterologous genes from other archaea and bacteria. The *Escherichia coli lacZ*, which encodes for β-galactosidase, was the first heterologous gene expressed from the expression shuttle vector in *M. maripaludis* (Chapter 3). The recombinant LacZ represented about 1% of the cellular protein in *M. maripaludis*.

In this work, some archaeal genes were expressed in *M. maripaludis* to test hypotheses about translation in methanogens (Chapter 5). The *Methanococcus jannaschii* F_{420}-reducing hydrogenase subunit A (FruA) was expressed to aid in the identification of the archaeal *cis*-acting element for the incorporation of selenocysteine. A second gene, which was the *Methanosarcina barkeri* monomethylamine methyltransferase (*mtmB*), was expressed to investigate translation of this gene. *mtmB* contains an UAG stop codon within its coding region. However, *M. barkeri* cells do not recognize this UAG stop codon and insert a presently unidentified amino acid at this position instead. Heterologous expression of *mtmB* in *M. maripaludis* was performed to determine if methanococci also possess the ability to read through this stop codon. *M. maripaludis* produced at truncated product at the UAG codon (C. James and J. Krzycki, personal communication).

Some archaeal and bacterial proteins may require cofactors and maturation proteins found in methanogens and genes encoding this protein are candidates for heterologous expression in *M. maripaludis*. Oxygen-sensitive metalloenzymes are

especially relevant since *M. maripaludis* is a strict anaerobe. *Moorella thermoacetica* and *Methanosarcina thermophila* carbon monoxide dehydrogenase/acetyl-coenzyme A synthase (CODH/ACS) genes and *Pyrococcus furiosus* pyruvate oxidoreductase (POR) subunit D gene encode oxygen-sensitive enzymes, and expression of their genes in *E. coli* yield inactive proteins. Because *M. maripaludis* also contains its own CODH/ACS and POR, the maturation enzymes required for biosynthesis of functional enzyme may be present. For example, expression of the *M. thermophila* CODH/ACS gene yielded an active, recombinant protein that represented 0.2-0.3% of the *M. maripaludis* cellular protein (D. Grahame, personal communication).

To better utilize the expression system when only small amounts of protein were produced, a medium containing formate was developed for large scale growth (Chapter 6). In this medium, *M. maripaludis* was capable of growing at large scale (400 L) with a recovery of about 0.75 gram wet cell paste per liter of medium. A recombinant *M. maripaludis*, which was grown in a 20 liter fermentor, yielded about 1 gram of wet cell paste per liter of medium.

To increase the yield of a recombinant protein, the expression shuttle vector can be modified. Replacing the P_{hmvA} with a stronger, heterologous promoter is a possibility. Now that the genomes of *M. jannaschii*, *Methanobacterium thermoautotrophicum*, and *Methanosarcina mazei* have been sequenced, a number of strong promoters can be easily identified. In addition to stronger promoters, heterologous terminator regions may be added to the vector at the 3' end of the multiple cloning site to increase mRNA stability. Thus, increasing the half-life of the mRNA may increase the amount of recombinant protein. Alternatively, the copy number of the vector could be increased. To increase the

copy number, a fundamental understanding of the vector is important. By using a transposable element for site-directed mutagenesis of pWLG30+*lacZ*, essential regions of the pURB500 were identified (Chapter 4). Altogether, these modifications may increase the yield of recombinant protein in *M. maripaludis*.