JOURNAL OF BACTERIOLOGY, June 1991, p. 3414–3418
0021-9193/91/113414-05$02.00/0
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Vol. 173, No. 11

Isolation of a Coenzyme M-Auxotrophic Mutant and Transformation by Electroporation in *Methanococcus voltae*

PAMELA A. MICHELETTI, KAREN A. SMENT, AND JORDAN KONISKY*

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Received 17 December 1990/Accepted 16 March 1991

An auxotrophic mutant of *Methanococcus voltae* was isolated that required coenzyme M (CoM) for growth. With the mutant as a recipient, conditions were developed that allowed the introduction of wild-type CoM^+ DNA into the mutant methanogen via electroporation. This method also allowed the rescue of both a histidine and purine auxotroph as well as the introduction of DNA determining resistance to the CoM analog 2-bromoethanesulfonic acid. Electroporation of the CoM^+- determining DNA was 50- to 80-fold more efficient than natural transformation.

A fuller understanding of methanogen physiology and the underlying biochemistry and molecular biology will require the development of genetic methodologies not yet fully available to study these members of the Archaea (10, 17). Nevertheless, some progress has been made. Low-level natural DNA transformation has been reported in both *Methanococcus voltae* (2) and *Methanobacterium thermoautotrophicum* (18), and most recently a method has been reported that should facilitate the isolation of auxotrophic mutants in some species (11). Furthermore, the recent construction of a promising vector for gene transfer (5) may form the basis for the development of useful shuttle vectors for strain construction, introduction of relevant genes, and genetic complementation studies. Finally, although progress in elucidating the molecular biology of methanogens has been slowed by the technical difficulties in working with these strict anaerobes, sufficient information is available (for a review, see reference 3) to begin to address significant questions dealing with the mechanisms of regulation of individual genes, complex metabolic schemes, and other regulatory aspects of methanogen growth.

We have been studying various biochemical and molecular aspects of *M. voltae*. We recently reported that this marine methanogen is capable of taking up 2-mercaptoethanesulfonic acid (HS-CoM, coenzyme M) via an energy-dependent, carrier-mediated uptake system (4, 12). HS-CoM is an intermediate in methanogenesis and carries a methyl moiety in the form of methyl-*S*-CoM, the terminal carrier of C_1 in the process of methane formation (6, 14). *M. voltae* also harbors a less efficient HS-CoM transport system that functions to transport methyl-*S*-CoM and 2-bromoethanesulfonic acid (BES), an analog of HS-CoM (4). Wild-type *M. voltae* is also able to synthesize HS-CoM, and a pathway for HS-CoM biosynthesis has been proposed (15). *M. voltae* does not require exogenous HS-CoM for growth.

To begin to examine possible regulatory features of the HS-CoM biosynthetic pathway, we sought mutants which were dependent on exogenously-added HS-CoM for growth. Such auxotrophs may be exploited to not only verify the proposed biosynthetic pathway but also to study the structure and regulation of the genes encoding the biosynthetic enzymes. In this report, we describe the isolation of an HS-CoM auxotroph and its repair by wild-type DNA introduced via electroporation.

* Corresponding author.

MATERIALS AND METHODS

Organisms and growth conditions. Liquid cultures of *M. voltae* PS(DSM 1537) (16) and strain BES^r-2 (4, 12) were grown in defined medium (16) at 30°C under a pressurized atmosphere of H_2-CO_2 (80:20). The *M. voltae* auxotrophic CoM-1 strain was grown in medium supplemented with 60 μM CoM (Sigma Chemical Co., St. Louis, Mo.), and the *M. voltae* auxotrophs PS-3(his-12) and PS-6(pur-1) (2) were grown in media supplemented with 100 μM histidine and 150 μM hypoxanthine, respectively. Plating techniques were similar to those described by Jones et al. (8). The methanogens were plated on defined medium containing 1.8% Noble agar with appropriate supplements. The plates were incubated at 37°C for 5 to 7 days in brass-stainless steel cannisters under a pressurized atmosphere of H_2-CO_2 (80:20) to which H_2S was added to a final concentration of 0.2% (vol/vol).

Isolation of a CoM-auxotrophic mutant strain. *M. voltae* PS was grown overnight in defined medium to an optical density at 660 nm of 0.4 (~3.5×10^7 cells per ml). The cells were then diluted to 3.5×10^2 cells per ml, and 100 μl was plated anaerobically on plates containing basal medium (16) supplemented with 60 μM CoM. The plates also contained the frameshift mutagen ICR191 {6-chloro-9-[3-(2-chloro-ethylamino)propylamino]-2-methoxyacridine; Sigma}, which was applied (100 μl) to the center of each plate as an 11 mM solution. The plates were incubated in cannisters for 5 days. Individual colonies were transferred to two roll tubes (7), one containing basal medium and the other containing basal medium supplemented with 60 μM HS-CoM. Roll tubes were prepared by melting 5 ml of basal agar in Balch tubes (1) and spinning the tubes so that a thin layer of agar formed along the inside surface. Two hundred individual colonies were screened by this method, yielding 10 isolates that grew in medium supplemented with CoM but did not grow in unsupplemented medium. Of these, one proved stable after repeated subculturing and was designated CoM-1.

To mark the CoM-requiring mutant with an additional genetic marker, we isolated a mutant that was resistant to 5-methyl tryptophan by inoculating the CoM-1 mutant into medium containing 92 mM 5-methyl tryptophan and picking survivors.

We found that mutants could be maintained stably by adding glycerol (final concentration, 20%) to log-phase cells, quickly freezing the mixture for 5 min in an ethanol-dry ice bath, and then storing samples at −20°C.

Preparation of DNA. A 5-ml log-phase culture of *M. voltae* grown in defined medium was pelleted by centrifugation; the pellet was suspended in 500 μl of 10 mM Tris-Cl–1 mM EDTA (pH 8.0) containing proteinase K (100 μg/ml), and the mixture was incubated for 5 min at 37°C. Sodium dodecyl sulfate (25 μl of a 25% solution in water) was added, and incubation was continued at room temperature until the lysis was complete (5 to 10 min). RNase A (5 μl of a 5-μg/ml solution) was added, and incubation was continued for 20 min at 37°C. The lysate was extracted with phenol-chloroform, and the DNA was recovered from the aqueous phase by ethanol precipitation. The ethanol precipitate was suspended in sterile, anaerobic water and stored frozen at −20°C until used.

Natural DNA transformation. The procedure used for DNA transformation was based on that of Bertani and Baresi (2). All manipulations of cells were performed anaerobically. Log-phase cultures were centrifuged and suspended in 0.1 volume of defined medium. DNA (1 to 10 μl, depending on the amount of DNA and concentration of the DNA preparation used) was next added to 650 μl of this cell suspension contained in a Balch tube. The tube was then pressurized to 2 atm (ca. 202.58 kPa) with H_2-CO_2 (80:20) and incubated for 2 h at 30°C. The culture was then centrifuged and suspended in 200 μl of defined medium; 10 μl was removed and serially diluted to determine the initial plate count. The remainder was added to 800 μl of defined medium, pressurized as above, and incubated at 30°C. Initial experiments with incubation times that varied from 0 (immediate plating) to 3 days demonstrated that a period of incubation of 1 day was optimal; the data presented here represent experiments in which 1-day incubations were used. After incubation the culture was pelleted by centrifugation, suspended in 200 μl of defined medium, and plated on defined medium with supplements to determine the total number of cells and on selection media to determine the number of transformants.

DNA transformation by electroporation. All manipulations of the cells were carried out under anaerobic conditions. A midlogarithmic culture was pelleted by centrifugation, and the cell pellet was washed once in an equal volume of electroporation buffer [0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 6.5), 50 mM KCl, 50 mM $MgCl_2$, 50 mM NaCl, 0.4 M sucrose, 0.0001% resasurin, and 1 mM titanium(III) citrate]. The cells were pelleted again and suspended in 0.1 volume of electroporation buffer. Aliquots (650 μl) of the cell suspension were placed in plastic cuvettes with an electrode with a 1.9-mm gap, and various amounts of DNA were added as anaerobic water solutions. Each sample was prepared in triplicate. The cuvettes were covered with sterile rubber serum stoppers, and the stoppered cuvettes were removed from the anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.) and placed on ice for 10 min. Electroporation was performed with a BTX Transfector 300. To estimate the conditions optimal for transformation, electric field strengths ranging from 1.59 to 2.25 kV/cm at various time constants were tested for transformation of *M. voltae* PS-3(his-12) to prototrophy with total chromosomal DNA (data not shown). Optimal electroporation transformation occurred at an electric field strength of 1.99 kV/cm with a time constant of 1.47 ms, and these conditions were chosen for further electroporation experiments. After electroporation, the cells were injected immediately into Balch tubes containing 2 ml of defined medium. The cells were next pelleted by centrifugation and returned to the anaerobic chamber. After removal of the supernatant, the cells were suspended in 200 μl of reduced defined medium. A 10-μl aliquot was removed for serial dilutions and plating to determine the initial plate count. The remaining cells were transferred to a Balch tube containing 800 μl of reduced defined medium with appropriate additions, and the tubes were pressurized with H_2-CO_2 and incubated overnight at 30°C to allow segregation. Immediate plating of the cells on selective media yielded no electrotransformants. The cells were then centrifuged and washed in reduced defined medium, the final cell pellet was suspended in 200 μl of reduced defined medium, and the total cell number and the number of CoM^+ electrotransformants were determined by plating on the appropriate media.

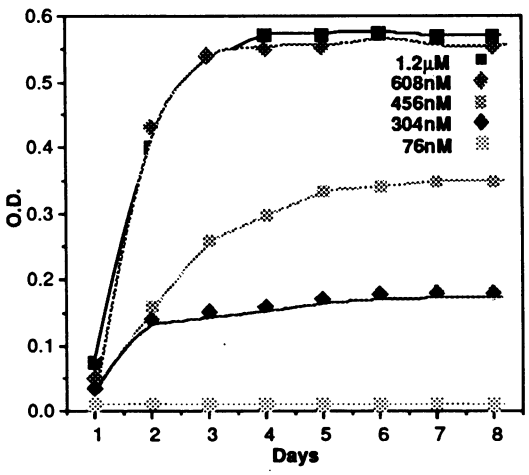

FIG. 1. CoM-dependent growth of *M. voltae* CoM-1. The concentrations given take into account the CoM carryover in the inoculum. A log-phase culture of the mutant grown in defined medium supplemented with 60 μM CoM was diluted 10,000-fold into defined medium containing the indicated concentration of added CoM.

RESULTS

Characterization of the CoM-auxotrophic mutant. Of 200 colonies picked from plates containing both ICR191 and CoM, 10 were found to require exogenous CoM for growth in defined medium. Of these, only one retained this growth requirement upon repeated subculturing. The magnitude of the dependency for growth of the mutant in defined medium was determined in medium supplemented with various amounts of CoM (Fig. 1). Growth was absolutely dependent on the addition of the coenzyme, and the addition of 0.456 μM CoM led to an approximately half-maximal growth rate and a half-maximal growth yield. The response of the mutant to added CoM was similar to that of a CoM-requiring strain of *Methanobacterium ruminantium* that grew when 0.6 μM CoM was added to the medium (13).

Natural transformation of the CoM-1 mutant strain. Since our goal was to examine the possibility of using electroporation for the introduction of DNA into the *M. voltae* CoM-1 mutant, we first ascertained the base level of natural transformation of this marker by using the general procedures of Bertani and Baresi (2). The mutant strain CoM-1 MTP^r (5-methyl tryptophan resistance) was transformed to pro-

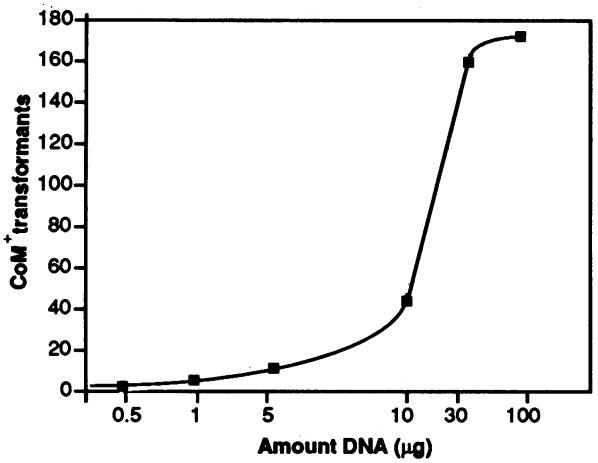

FIG. 2. Natural transformation of the CoM$^+$ gene as a function of added DNA. The reversion frequency in this experiment was 1.3 $\times$ 10^{-9} revertants per plated bacterium. The number of transformants was normalized to take into account cell doubling and background (Table 1).

totrophy (CoM independence) with chromosomal DNA isolated from the wild-type *M. voltae* strain. Selection was on plates containing 92 mM 5-methyl tryptophan but lacking CoM. The number of transformants increased with increasing DNA concentration, and an average of 5 transformants per μg of DNA was observed (Fig. 2). No transformants were observed with either *Escherichia coli* DNA or DNA isolated from the *M. voltae* CoM-1 mutant strain. In a similar set of experiments in which cells were plated on medium lacking both CoM and 5-methyl tryptophan, a random screening of 20 CoM$^+$ prototrophic transformants showed that all were resistant to 5-methyl tryptophan. This rules out the possibility that viable CoM$^+$ cells were carried over in the DNA preparation. The efficiency of natural transformation that we observed was similar to that observed by Bertani and Baresi (2 to 100 transformants per μg of DNA) (2).

Electroporation of the CoM-1 mutant strain. The CoM-1 auxotrophic strain was transformed by electroporation with conditions which in preliminary experiments were determined to be most effective. In the experiment shown, a field strength of 1.99 kV/cm was applied, and a range of time constants was tested. The optimal electroporation was observed at a time constant of 1.47 ms (Table 1). For the purpose of calculation, the number of cells considered to have successfully incorporated CoM$^+$ DNA via electroporation was considered to be the total number of CoM$^+$ colonies growing on the selection plates minus the number of colonies growing on such plates under control conditions. The electrotransformants were stable, as indicated by the fact that they retained the CoM$^+$ phenotype upon subculturing.

The control conditions shown in Table 1 were cells placed in the electroporation apparatus in the absence of exogenous DNA and not subjected to further treatment (i.e., a condition of no electroporation). However, a similar background level of spontaneous revertants was obtained in experiments in which cells were subjected to a field strength of 1.99 kV/cm for 1.47 ms in the absence of added DNA or when either *E. coli* or DNA isolated from the *M. voltae* CoM-1 mutant strain replaced DNA isolated from the *M. voltae* CoM$^+$ strain (data not shown). Under all of these conditions the numbers of CoM$^+$ colonies that grew on the selection plates were quite similar and ranged from approximately 1 $\times$ 10^{-9} to 2.5 $\times$ 10^{-9} CoM$^+$ colonies per bacterium plated. Under conditions in which cells were incubated in the electroporation appararus with DNA isolated from the CoM$^+$ wild-type strain in the absence of electroporation treatment and then carried through the same manipulations as for electroporated cells, the number of CoM$^+$ colonies that grew on the selection plates was similar to that obtained in natural transformation experiments. Finally, DNase-treated CoM$^+$ DNA was not effective in electroporation (Table 1, experiment 2).

TABLE 1. Introduction of CoM$^+$ DNA by electroporation

Expt	DNA added (μg)	Time constant (ms)	N_o (10^8)	N_f (10^8)	CoM$^+$ colonies	CoM$^+$ electrotransformants[a] per μg of DNA
1	0	0	6.3	9.2	6	
			6.1	7.3	9	
			5.8	6.9	11	
	0.5	1.26	1.2	10.3	156	22
			2.5	12.6	121	34
			2.2	11.5	101	24
	0.5	1.47	1.1	2.7	416	318
			0.9	1.8	422	408
			2.6	3.0	452	740
	0.5	1.68	2.3	2.6	38	56
			1.7	1.9	73	118
			1.8	2.1	92	140
2	0	0	6.3	8.4	3	
			5.2	5.6	7	
			7.1	9.0	10	
	0.5 (DNase treated)	1.47	4.1	5.2	5	8
			3.7	4.5	0	0
			6.1	1.6	12	3

[a] Each sample was corrected to the number of viable cells present immediately after the treatment. This was calculated by dividing the number of CoM$^+$ colonies by the factor 2^n, where n was the number of cell doublings occurring between the time of initial plating (N_o) and final plating (N_f). After the normalization, the number of CoM$^+$ electrotransformants per μg of DNA was calculated by subtracting the number of CoM$^+$ colonies arising under the control conditions from the number of CoM$^+$ colonies derived from the samples after electroporation. For the control, no DNA was added to the electroporation cuvette.

To rule out the possibility that the CoM$^+$ colonies that grew on the selection plates derived from CoM$^+$ cells that were carried over in the DNA isolation procedure, we screened 50 colonies arising on selective plates for the presence of the unselected MTP^r marker. All 50 were resistant to 5-methyl tryptophan, indicating that they derived from the CoM-1 MTP^r strain added to the electroporation cuvette. Furthermore, the DNA preparations used in these experiments contained no viable cells. Taken together, these data convincingly demonstrate the introduction of DNA into *M. voltae* by electroporation.

An examination of the data contained in Table 1 shows that we observe a significant degree of variance in the plating efficiency of cells presumably subjected to identical treatment. Although we do not fully understand the basis for such variability, it probably reflects the extreme dependence of

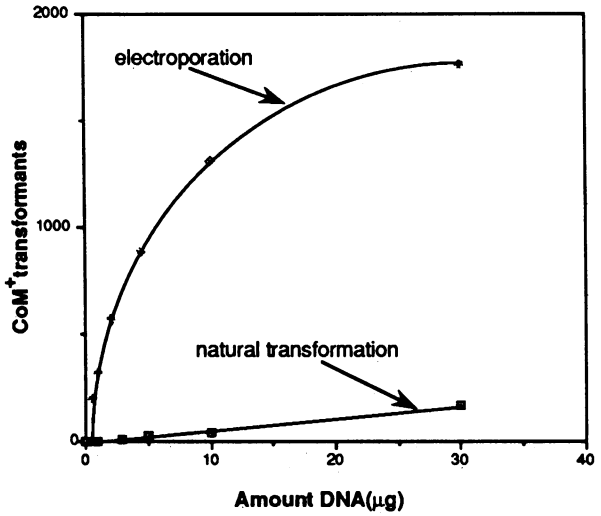

FIG. 3. Transformation by electroporation of the CoM^+ gene as a function of added DNA. The reversion frequency in this experiment was 1.4×10^{-9} revertants per bacterium. The number of transformants was normalized to take into account cell doubling and background (Table 1).

cell viability on maintenance of an anaerobic condition of low redox potential as well as the general toxic effect of the electroporation procedure. As a result, our calculations should be considered an approximate estimate of the frequency of electroporation.

When the DNA concentration dependence of electroporation was compared with the DNA dependence of natural transformation, electroporation is approximately 50- to 80-fold more efficient at low DNA concentrations (Fig. 3). In the experiment shown in Fig. 3, electroporation led to 384 CoM^+ colonies per μg of DNA, whereas natural transformation yielded 5 colonies per μg of DNA.

Electroporation of other genes. Using a histidine or purine auxotroph, we were able to demonstrate electroporation-mediated transfer of other *M. voltae* genes (Table 2). In each case, electroporation was optimal at a field strength of 1.99 kV/cm and a pulse length (time constant) of 1.47 ms. The incorporation of chromosomal DNA was gene specific. There are many factors that might be responsible for such variation, ranging from the specific nature of the mutation to the physical state of the DNA used in the experiment. In the experiments shown, the chromosomal DNA used for electroporation of the *his-12* auxotroph was partially digested with the restriction enzyme *Eco*RI; however, partial digestion with *Hin*dIII gave similar levels of electroporation as did undigested DNA. For electroporation of the *pur-1* auxotroph, undigested DNA or DNA partially digested with *Sal*I or with *Pst*I was equally effective.

TABLE 2. Transformation and electroporation of additional markers

Marker	Prototrophic colonies per μg of DNA		Ratio
	Natural	Electroporation	
CoM-100	5	384	77
His-12	23	122	5.3
Pur-1	11	39	3.5
BES^r	0	477	

Wild-type *M. voltae* was subjected to electroporation with DNA partially digested with *Eco*RI or *Hin*dIII isolated from a BES^r strain (12). BES, a methyl-CoM analog, is an inhibitor of methyl reductase (5), an enzyme complex responsible for the final steps of methanogenesis. In the experiment shown in Table 2, the spontaneous rate of mutation to BES resistance was quite high (1.1×10^{-5} per bacterium plated) which was not significantly increased by natural transformation. Nevertheless, we were able to demonstrate successful electroporation of BES^r. These results emphasize the utility of electroporation for introduction of chromosomal DNA into *M. voltae*.

In contrast, we have not yet been able to demonstrate electroporation of plasmid DNA. In these attempts, we utilized the Mip2 plasmid vector, which carries a selectable puromycin transacetylase marker derived from *Streptomyces alboniger* and which has been used to introduce the puromycin resistance marker into *M. voltae* by natural transformation (5). Although we were able to demonstrate introduction of the marker gene into *M. voltae* by natural transformation, we observed no enhancement of gene transfer with several conditions of electroporation.

DISCUSSION

We have succeeded in isolating a CoM-requiring mutant of *M. voltae* PS. Although the mutant is entirely dependent on exogenously added CoM for growth, we have no information on the biochemical basis for this requirement. Although the most obvious explanation would be some block in the CoM biosynthetic pathway, alternative explanations are possible. For example, a mutational alteration in a CoM-specific regulatory pathway is certainly possible, as is a nonspecific alteration in CoM production due to an alteration in a more general metabolic pathway. Biochemical characterization of the mutant is clearly required.

The success of the methods described here indicates that it should be possible to isolate additional CoM-requiring mutants defective in a variety of genes influencing CoM production. Characterization of these mutants will require the application of both genetics and molecular biology, and the use of electroporation should prove invaluable.

Although we have convincingly demonstrated that electroporation can be used to introduce DNA into *M. voltae*, it has been difficult to quantitate the efficiency of the process and to compare our results with those obtained for other microorganisms. We observed significant variation in cell viability under the various conditions used, as a result, we have had to normalize the data. Without knowing the basis for the variation in our data, it is difficult to derive reliable estimates of the absolute frequency of electroporation. Furthermore, we have no information on the efficiencies of the intermediate steps in the process, such as DNA uptake, possible restriction, integration of DNA into the chromosome, delayed expression, etc. However, it is clear that electroporation is at least 1 order of magnitude more efficient than the natural transformation system described for this methanogen by Bertani and Baresi (2). Most strikingly, although the rate of spontaneous mutation to BES resistance was too high to allow a demonstration of natural transformation of the BES^r marker by the natural transformation system, it was easily demonstrable by electroporation.

We routinely obtained an approximate efficiency of electroporation in the range of 250 to 800 CoM^+ electrotransfor-

mants per μg of added chromosomal DNA. Assuming a genome size of 1.8×10^9 Da for *M. voltae* (9), we estimate that this organism harbors approximately 1,500 genes. Thus, we estimate an efficiency of electroporation of approximately 3.75×10^{-5} to 1×10^{-6} per gene. Assuming that we can maintain such a level of efficiency for cloned genes, we are optimistic that electroporation will prove very useful as a routine method to introduce DNA into *M. voltae* and perhaps other methanogens. The development of shuttle vectors would greatly expedite this approach.

ACKNOWLEDGMENT

This work was supported by Department of Energy research grant DOE DEFG 02-84ER13241.

REFERENCES

1. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260–296.
2. **Bertani, G., and L. Baresi.** 1987. Genetic transformation in the methanogen *Methanococcus voltae* PS. J. Bacteriol. **169**:2730–2738.
3. **Brown, J. W., C. J. Daniels, and J. N. Reeve.** 1989. Gene structure, organization, and expression in archaebacteria. Crit. Rev. Microbiol. **16**:287–338.
4. **Dybas, M., and J. Konisky.** 1989. Transport of coenzyme M (2-mercaptoethanesulfonic acid) and methylcoenzyme M [(2-methylthio)ethanesulfonic acid] in *Methanococcus voltae*: identification of specific and general uptake systems. J. Bacteriol. **171**:5866–5871.
5. **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 gene. Mol. Gen. Genet. **221**:273–279.
6. **Gunsalus, R. P., D. Eirich, J. Romesser, W. Balch, S. Shapiro, and R. S. Wolfe.** 1976. Methyl transfer and methane formation, p. 191–198. *In* H. G. Schlegel, G. Gottschalk, and N. Pfennig (ed.), Microbial production and utilization of gases (H_2, CH_4, CO). E. Goltze, K.G., Gottingen, Germany.
7. **Hungate, R. E.** 1969. A roll tube method for cultivation of strict anaerobes, p. 117–132. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 3B. Academic Press, Inc., New York.
8. **Jones, W. J., W. B. Whitman, R. D. Fields, and R. S. Wolfe.** 1983. Growth and plating efficiency of methanococci on agar media. J. Bacteriol. **46**:220–226.
9. **Klein, A., and M. Schnorr.** 1984. Genome complexity of methanogenic bacteria. J. Bacteriol. **158**:628–631.
10. **Konisky, J.** 1989. Methanogens for biotechnology: application of genetics and molecular biology. Trends Biotechnol. **7**:88–92.
11. **Ladapo, J., and W. B. Whitman.** 1990. Method for the isolation of auxotrophs in the methanogenic archaebacteria: role of the acetyl-CoA pathway of autotrophic CO_2 fixation in *Methanococcus maripaludis*. Proc. Natl. Acad. Sci. USA **87**:5598–5602.
12. **Santoro, N., and J. Konisky.** 1987. Characterization of bromoethanesulfonate-resistant mutants of *Methanococcus voltae*: evidence of a coenzyme M transport system. J. Bacteriol. **169**:660–665.
13. **Taylor, C. D., B. C. McBride, R. S. Wolfe, and M. P. Bryant.** 1974. Coenzyme M, essential for growth of a rumen strain of *Methanobacterium ruminatium*. J. Bacteriol. **120**:974–975.
14. **Taylor, C. D., and R. S. Wolfe.** 1974. Structure and methylation of coenzyme M ($2\text{-}HSCH_2CH_2SO^{3-}$). J. Biol. Chem. **249**:4879–4885.
15. **White, R. H.** 1986. Intermediates in the biosynthesis of coenzyme M (2-mercaptoethanesulfonic acid). Biochemistry **25**: 5304–5308.
16. **Whitman, W. B., E. Ankwanda, and R. S. Wolfe.** 1982. Nutrition and carbon metabolism of *Methanococcus voltae*. J. Bacteriol. **149**:852–863.
17. **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.
18. **Worrell, V. E., D. P. Nagle, Jr., D. McCarthy, and A. Eisenbraun.** 1988. Genetic transformation system in the archaebacterium *Methanobacterium thermoautotrophicum* Marburg. J. Bacteriol. **170**:653–656.