APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1987, p. 2978–2982
0099-2240/87/122978-05$02.00/0

Vol. 53, No. 12

Factors Affecting the Methanogenic Activity of *Methanothrix soehngenii* VNBF†

BABU Z. FATHEPURE‡

Microbiology and Cell Biology Laboratory, Indian Institute of Science, Bangalore 560012, India

Received 18 August 1987/Accepted 14 September 1987

Methane production by *Methanothrix soehngenii* VNBF grown on acetate (50 mM) as the sole carbon and energy source was influenced by the addition of Fe, trace elements, and pesticides. The addition of Fe and trace elements significantly enhanced the rate of CH_4 production. The addition of pesticides in the early growth phase caused complete inhibition. However, less inhibition was noted when pesticides were added during early exponential growth phase. Addition to culture tubes of Co, Ni, or Mo at 2 μM produced 64, 41, or 17%, respectively, more CH_4 than that produced in tubes lacking the corresponding trace element. A concentration of more than 5 μM of these trace elements in the medium resulted in decreased CH_4 production, presumably because of toxic effects.

Acetate is the major precursor of CH_4 production in anaerobic digesters (22), and its utilization may be the rate-limiting step in the anaerobic degradation of organic matter (33). Therefore, understanding the factors affecting the methanogenic activity of aceticlastic methanogens is important. There have been a few reports on the stimulation of methanogenesis from acetate by the addition of Fe and trace metals to pure and acetate-enriched cultures of aceticlastic methanogens (8, 20, 23, 24, 27, 28, 32). Studies by Scherer and Sahm (27) have shown that the growth of *Methanosarcina barkeri* was dependent on Co and Mo. Addition of Ni and Se stimulated growth, but addition of iron salts neither stimulated nor inhibited growth or gas production of *Methanosarcina barkeri* (27, 28). Patel (25) showed that the presence or absence of Fe, Se, or Ni in the growth medium of *Methanothrix concilii* made no difference in methane production and concluded that, perhaps, trace amounts of these nutrients present as contaminants may be sufficient for the growth of the methanogen. Similar studies with *Methanococcoides methylutens* (30) showed a requirement for Fe, Ni, and Co for growth on trimethylamine.

Several pesticides are being used in crop protection, and considerable quantities of pesticides may enter sewage and may also be found in excreta of humans and animals. It is known that pesticides may persist for a long time in the environment, polluting the ecosphere (18, 19). Anaerobic microorganisms have been implicated in the disappearance of pesticides from anoxic environments. Studies have shown that DDT (dichlorodiphenyltrichloroethane), γ-BHC (benzene hexachloride), and other persistent chlorinated pesticides can be degraded more rapidly in anaerobic ecosystems such as sewage sludge, lake sediments, and flooded soils (1, 18, 31).

I have previously reported the isolation and characterization of a fast-growing aceticlastic methanogen, *Methanothrix soehngenii* VNBF, from a biogas digester (5). In this paper, I discuss the effects of Fe, trace metals, and pesticides on the growth of and CH_4 production by *M. soehngenii* VNBF.

† Dedicated to the memory of the late V. N. Vasantharajan, under whose guidance this work was done.

‡ Present address: Department of Crop and Soil Sciences, Michigan State University, East Lansing, MI 48824.

The methanogen was grown and maintained on prereduced enriched methanogenic medium (PREM medium), the composition of which was essentially that described by Van den Berg et al. (33), except for a few modifications. All cations added as sulfates were added as their respective chlorides on an equimolar basis. PREM medium contained the following (in milligrams per liter of distilled water): NH_4HCO_3 (3,000), K_2HPO_4 (450), KH_2PO_4 (450), NH_4Cl (180), $MgCl_2 \cdot 6H_2O$ (170), $CaCl_2 \cdot 2H_2O$ (120), NaCl (900), $FeCl_3 \cdot 6H_2O$ (20), $MnCl_2 \cdot 5H_2O$ (6), $CoCl_2 \cdot 6H_2O$ (1), $ZnCl_2$ (0.08), $CuCl_2 \cdot 2H_2O$ (0.08), H_3BO_3 (0.1), $Na_2MoO_4 \cdot 2H_2O$ (0.1), pyridoxine hydrochloride (0.1), thiamine hydrochloride (0.05), riboflavin (0.05), nicotinic acid (0.05), biotin (0.05), folic acid (0.02), cobalamin (0.005), and *para*-aminobenzoic acid (0.05). The medium was supplemented with 4,100 mg of CH_3COONa (50 mM) and 0.1% yeast extract. Cysteine hydrochloride and $Na_2S \cdot H_2O$ (0.03% each) and resazurin (2 mg/liter; an oxidation-reduction indicator) were added. The pH of the medium was adjusted to 7.0. All experiments were performed by dispensing 10-ml samples of PREM medium into 25-ml culture tubes. The tubes were flushed with deoxygenated N_2 and closed with a rubber stopper. The effect of Fe^{2+} was studied by omitting the cation from the medium and supplementing it at various concentrations. The culture grown in iron-depleted medium served as an inoculum. Similarly, the PREM medium used for the experiments with trace elements was prepared with deionized double-distilled water and was devoid of the trace element whose effect was being studied. A 5-day-old culture grown in the medium lacking the test trace metal served as the inoculum (5%, vol/vol) and was incubated at 35°C.

Growth was monitored by measuring optical density at 540 nm with a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.). Growth curves obtained by measuring optical density at 540 nm were identical in shape to curves of CH_4 production. The amount of CH_4 in the headspace was determined by gas chromatography. Gas samples were removed from the headspaces of culture tubes with an airtight 100-μl-capacity microsyringe (Top Syringe Company, Bombay, India) and injected into the gas chromatograph (model RL04; Toshiniwal, Bombay, India). Methane gas was separated in a steel column (150 by 0.3 cm) packed with Porapak-R and measured with a flame ionization detector. The

column, injection port, and detector temperatures were 100, 120, and 110°C, respectively. The flow rates of both N_2 (carrier) and H_2 gases were 40 ml/min.

The effect of Fe, particularly at lower concentrations, on the growth of *M. soehngenii* VNBF was examined. The addition of Fe stimulated CH_4 production significantly (Table 1). Almost all the added acetate (50 mM) was converted to CH_4 at concentrations of $FeSO_4$ as low as 0.1 mM within 12 days of incubation. In cultures without added iron, methane production was around 50% of the total expected CH_4 yield. Furthermore, turbidity (A_{540}) increased with increased amounts of added $FeSO_4$.

The results clearly showed that the minimum amount of Fe needed for the complete conversion of acetate to CH_4 was 0.1 mM, and addition of Fe at a concentration as low as 0.01 mM caused a significant increase in CH_4 production compared with the control. It has been shown with an anaerobic digester and acetate enrichment cultures (8, 13, 32) that the addition of Fe stimulates the conversion of acetate to CH_4 gas. Iron is an important micronutrient for many microorganisms and is essential for various enzymatic systems. Studies have suggested that factor F_{420} becomes degraded 10 times faster in *Methanobacterium thermoautotrophicum* cells grown under conditions of Fe deficiency (29). This finding suggested the presence of an iron-containing enzyme, probably superoxide dismutase, which can protect factor F_{420} from degradation by oxygen (14). Iron may also be required in the synthesis of various iron-sulfur proteins by methanogens. Cytochromes have been detected only in methylotrophic and acetotrophic methanogens (9, 12, 15–17).

The effects of Co, Ni, and Mo on CH_4 production by *M. soehngenii* VNBF were studied (Fig. 1). The results indicated that the addition of Co, Ni, or Mo at a concentration of 2 μM markedly stimulated CH_4 production and resulted in the complete conversion of the added acetate to CH_4. Tubes depleted of Co, Ni, or Mo produced 36, 59, or 83%, respectively, of the total CH_4 produced at the optimal concentration (~2 μM) of the corresponding trace element at the end of the growth phase. It is clear that molybdate increased CH_4 production by only 20% at the end of the growth period (Fig. 1C). However, its effect was more pronounced in younger cultures. The addition of higher concentrations (>5 μM) of Co or Mo resulted in decreased CH_4 production, possibly because of toxic effects (Fig. 1A and C). However, no inhibition was noted for Ni even at concentrations of up to 10 μM (Fig. 1B).

The Co dependence of methanogenesis in *M. soehngenii* VNBF may be due to the fact that Co is required for the synthesis of corrinoids (34). Methanogens contain corrinoids at high concentrations (27), but the complete role of corrinoids in methanogenesis is still unclear. Recent studies

TABLE 1. Effect of ferrous sulfate on methane production by and growth of *M. soehngenii* VNBF

Concn (mM)	A_{540}[a]	Total methane[b]
0	0.07	265.3 ± 12.3
0.01	0.09	361.6 ± 6.7
0.05	0.12	435.3 ± 20.6
0.08	0.15	450.4 ± 18.5
0.10	0.20	552.5 ± 0.3
0.20	0.24	560.0 ± 0.0

[a] After 12 days.

[b] In micromoles per culture tube, after 12 days. Values are means of three replicates ± standard deviations.

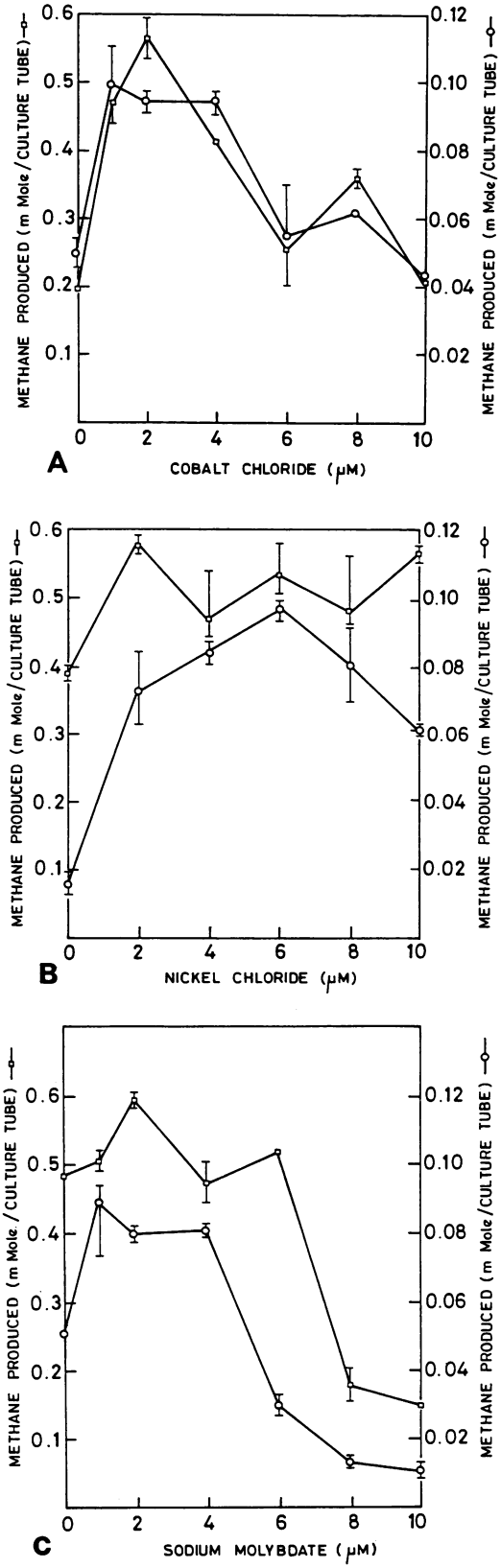

FIG. 1. Effect of the addition of trace element to PREM medium devoid of the added metal on CH_4 production by *M. soehngenii* VNBF. Total CH_4 at the end of 9 (○) and 18 (□) days. Values are the means of three replicates ± standard deviations.

have presented evidence for the role of corrinoids in CH_4 formation from acetate by *Methanosarcina barkeri* (3). In the present study, the omission of Ni slightly affected the total conversion of acetate to CH_4. However, Ni addition was stimulatory, as shown by increased CH_4 formation during the log phase. This suggests that the low levels of Ni present in the medium as contaminants may support the growth of methanogens in tubes lacking this element. Nickel is an essential growth factor for many methanogens (4, 10, 27, 30) and is known to be associated with the hydrogenase activity of *Methanobacterium thermoautotrophicum* (6). It is a component of factor F_{430}, which is a prosthetic group of methyl coenzyme M reductase (2, 4). Nickel is also a component of the carbon monoxide dehydrogenase in *Methanosarcina barkeri* (15). Nickel was shown to be essential in the growth medium of *Methanobacterium bryantii* to prevent rapid cell lysis (10). Acetate was almost completely converted to CH_4 in PREM medium devoid of Mo, but the addition of Mo enhanced the rate of CH_4 production during the early growth phase. Additions of Ni and Mo to the medium may not be necessary because low levels of these metals in the medium as contaminants may be sufficient for the growth of *M. soehngenii* VNBF. The stimulation of CH_4 production by Mo agrees with earlier observations with *Methanosarcina barkeri* grown on acetate (27) and with aceticlastic methanogens from a fixed-film reactor (24), indicating the requirement for this trace metal in systems other than the CO_2 reductase system (27).

Inhibition of CH_4 production at elevated concentrations of trace elements may be due to nonspecific binding of trace elements with the carrier proteins that are involved in their uptake and incorporation (11). An excess of a particular element may saturate the carrier molecules and thereby restrict the uptake of other essential metal ions. A metal ion in excess may also replace the essential metal of an enzyme. This would result in decreased methanogenesis, as was observed at higher levels of trace elements in this study.

For the experiment on the effect of pesticides, stock solutions (100×) of various pesticides were prepared in distilled acetone. Different volumes (10, 50, and 100 μl) of the stock solutions were directly injected into the culture tubes (in duplicate) to obtain the desired final concentrations. The tubes were kept uninoculated for 1 day to equilibrate the pesticide with the aqueous medium. To one set of tubes, additions were made at the time of inoculation (Table 2, experiment 1), while to the other set, pesticides were added in the early log phase, i.e., 5 days after inoculation (Table 2, experiment 2). In the latter set, approximately 40 μmol of CH_4 had already been produced before the addition of pesticides. Addition of hinosan, fenitrothion, parathion, methyl parathion, and quinolphos completely inhibited CH_4 formation at the lowest concentration tested (10 μg/ml). Fenthion, dichlorvos (DDVP), and carbofuran were less effective (Table 2, experiment 1). However, addition of pesticides to the actively growing cells (at the end of 5 days) showed slightly decreased inhibition, and this decrease was pronounced in the case of fenitrothion and quinolphos. Conversely, the addition of carbofuran to the 5-day-old

TABLE 2. Effect of pesticides on production of methane from acetate by *M. soehngenii* VNBF

Pesticide[a] or control	Chemical name	Amt (μg/ml)	Methane inhibition[b] in:	
			Expt 1[c]	Expt 2[d]
No addition			—	—
Acetone		100 μl	—	—
Hinosan	*O*-Ethyl *S*,*S*-diphenyl phosphorodithioate	10	93	97.00
		50	100	98.05
		100	100	ND[e]
Fenitrothion	*O*,*O*-Dimethyl *O*-4-nitro-*m*-tolyl phosphorothioate	10	89	ND
		50	100	44.55
		100	100	73.82
Fenthion	*O*,*O*-Dimethyl *O*-[4-(methylthio)-*m*-tolyl] phosphorothioate	10	36	41.47
		50	92	70.74
		100	100	83.00
Parathion	*O*,*O*-Diethyl *O*-*p*-nitrophenyl phosphorothioate	10	100	92.30
		50	100	92.30
		100	100	ND
Methyl parathion	*O*-*O*-Dimethyl *O*-*p*-nitrophenyl phosphorothioate	10	100	ND
		50	100	95.38
		100	100	98.46
Quinolphos	*O*,*O*-Diethyl *O*-2-quinoxalinyl phosphorothioate	10	100	46.1
		50	100	69.2
		100	100	95.4
Dichlorvos (DDVP)	*O*,*O*-Dimethyl *O*-(2,2-dichlorovinyl) phosphate	10	57	ND
		50	90	84.6
		100	100	84.6
Carbofuran	2,3-Dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate	10	22	ND
		50	79	95.38
		100	92	96.38

[a] Hinosan, fenitrothion, fenthion, parathion, methyl parathion, and quinolphos are organophosphates; dichlorvos is an organochloride; and carbofuran is a carbamate. Stock solutions were prepared in acetone, and final concentrations of 10, 50, or 100 μg/ml were obtained by injecting 10, 50, or 100 μl, respectively, of stock solution into each culture tube (10 ml).

[b] Calculated on the basis of methane formed in unamended bottles. —, No inhibition. In experiment 1, 437 μmol of methane per culture tube was produced in 12 days, while in experiment 2, 400 μmol of methane per culture tube was produced between days 6 and 12 of incubation.

[c] Pesticide added at the time of inoculation.

[d] Pesticide added 5 days after inoculation.

[e] ND, Not determined.

culture resulted in increased inhibition, and the exact reason for this inhibition is not known.

In general, the concentration-dependent inhibitory effect was less when pesticides were added to the cells in early exponential phase. For example, after 5 days of growth, methanogenic activity was not completely (<100%) inhibited even at a pesticide concentration of 50 or 100 μg/ml. The decreased inhibition by pesticides in actively growing cultures may be because of a larger cell population and a higher degree of resistance of organisms to such synthetic chemicals. Pesticides influence the density and composition of microbial populations in natural environments as well as in pure cultures (18). Methanogenic bacteria constitute an important class of nontarget organisms likely to be affected by pesticides. McBride and Wolfe (21) have shown the inhibition of CH_4 formation in cell extracts and whole cells of *Methanobacterium* sp. strain M · o · H by DDT and its analogs. Gunsalus and Wolfe (7) have suggested that the conversion of CH_3-S-coenzyme M to CH_4 is the step susceptible to pesticide action. In addition, pesticides inhibit various metabolic and respiratory enzymes in many nonmethanogens (18, 19). In conclusion, studies of nutritional requirements and the factors affecting methanogenic activity in pure cultures could help in devising a suitable medium for obtaining high cell mass and increased methane production (26). The data could be extended to mixed culture for enhanced CH_4 production. In view of the rapid growth of *M. soehngenii* VNBF and its resistance to pesticides, *M. soehngenii* VNBF merits attention in the development of a microbial consortium for the anaerobic degradation of the above-mentioned pesticides. However, the performance of *M. soehngenii* VNBF under mixed-culture conditions remains to be tested.

I thank G. Ramananda Rao and P. K. Yadava for helpful discussions and for commenting on the manuscript and I. Lalithakumari and N. A. Prakash for their help.

I was supported by a fellowship from the Indian Council of Medical Research, New Delhi.

LITERATURE CITED

1. **Adhiya, T. E., B. Sudhakar, and N. Sethunathan.** 1980. Fate of fenitrothion, methyl-parathion, and parathion in anoxic sulfur-containing soil system. Pestic. Biochem. Physiol. **16**:14–20.
2. **Diekert, G., B. Klee, and R. K. Thauer.** 1980. Nickel, a component of factor F_{430} from *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **124**:103–106.
3. **Eikmanns, B., and R. K. Thauer.** 1985. Evidence for the involvement and role of a corrinoid enzyme in methane formation from acetate in *Methanosarcina barkeri*. Arch. Microbiol. **142**:175–179.
4. **Ellefson, W. L., and W. B. Whitman.** 1982. The role of nickel in methanogenic bacteria, p. 403–414. *In* A. Hollaender, R. D. DeMoss, S. Kaplan, J. Konisky, D. Savage, and R. S. Wolfe (ed.), Genetic engineering of microorganisms for chemicals. Plenum Publishing Corp., New York.
5. **Fathepure, B. Z.** 1983. Isolation and characterization of an aceticlastic methanogen from a biogas digester. FEMS Microbiol. Lett. **19**:151–156.
6. **Graf, E.-G., and R. K. Thauer.** 1981. Hydrogenase from *Methanobacterium thermoautotrophicum*, a nickel containing enzyme. FEBS Lett. **136**:165–169.
7. **Gunsalus, R. P., and R. S. Wolfe.** 1978. ATP activation and properties of the methyl coenzyme M reductase system in *Methanobacterium thermoautotrophicum*. J. Bacteriol. **135**: 851–857.
8. **Hoban, D. J., and L. van den Berg.** 1979. Effect of iron on conversion of acetic acid to methane during methanogenic fermentations. J. Appl. Bacteriol. **47**:153–159.
9. **Huser, B. A., K. Wuhrmann, and A. J. B. Zehnder.** 1982. *Methanothrix soehngenii*, gen. nov. sp. nov., a new acetotrophic non-hydrogen-oxidizing methane bacterium. Arch. Microbiol. **132**:1–9.
10. **Jarrell, K. F., J. R. Colvin, and G. D. Sprott.** 1982. Spontaneous protoplast formation in *Methanobacterium bryantii*. J. Bacteriol. **149**:346–353.
11. **Jarrell, K. F., and G. D. Sprott.** 1982. Nickel transport in *Methanobacterium bryantii*. J. Bacteriol. **151**:1195–1203.
12. **Jussofie, A., and G. Gottschalk.** 1986. Further studies on the distribution of cytochromes in methanogenic bacteria. FEMS Microbiol. Lett. **37**:15–18.
13. **Kelly, C. R., and M. S. Switzenbaum.** 1984. Anaerobic treatment: temperature and nutrient effects. Agric. Wastes **10**:135–154.
14. **Kirby, T. W., J. R. Lancaster, Jr., and I. Fridovich.** 1981. Isolation and characterization of the iron containing superoxide-dismutase of *Methanobacterium bryantii*. Arch. Biochem. Biophys. **210**:140–148.
15. **Krzycki, J. A., and J. G. Zeikus.** 1984. Characterization and purification of carbon monoxide dehydrogenase from *Methanosarcina barkeri*. J. Bacteriol. **158**:231–237.
16. **Kuhn, W., K. Fiebig, and G. Gottschalk.** 1979. Presence of a cytochrome b_{559} in *Methanosarcina barkeri*. FEBS Lett. **105**:271–274.
17. **Kuhn, W., K. Fiebig, H. Hippe, R. A. Mah, B. A. Huser, and G. Gottschalk.** 1983. Distribution of cytochromes in methanogenic bacteria. FEMS Microbiol. Lett. **20**:407–410.
18. **Lal, R.** 1982. Accumulation, metabolism and effect of organophosphorus insecticides on microorganisms. Adv. Appl. Microbiol. **28**:149–200.
19. **Lal, R., and D. M. Saxena.** 1982. Accumulation, metabolism, and effects of organochlorine insecticides on microorganisms. Microbiol. Rev. **46**:95–127.
20. **Mah, R. A., M. R. Smith, and L. Baresi.** 1978. Studies on an acetate-fermenting strain of *Methanosarcina*. Appl. Environ. Microbiol. **35**:1174–1184.
21. **McBride, B. C., and R. S. Wolfe.** 1971. Inhibition of methanogenesis by DDT. Nature (London) **234**:551–552.
22. **Mountfort, D. O., and R. A. Asher.** 1978. Changes in proportions of acetate and carbon dioxide used as methane precursors during the anaerobic digestion of bovine waste. Appl. Environ. Microbiol. **35**:648–654.
23. **Murray, W. D., and L. van den Berg.** 1981. Effect of support material on the development of microbial fixed films converting acetic acid to methane. J. Appl. Bacteriol. **51**:257–265.
24. **Murray, W. D., and L. van den Berg.** 1981. Effects of nickel, cobalt, and molybdenum on performance of methanogenic fixed-film reactors. Appl. Environ. Microbiol. **42**:502–505.
25. **Patel, G. B.** 1984. Characterization and nutritional properties of *Methanothrix concilii* sp. nov., a mesophilic, aceticlastic methanogen. Can. J. Microbiol. **30**:1383–1396.
26. **Patel, G. B., A. W. Khan, and L. A. Roth.** 1978. Optimum levels of sulfate and iron for the cultivation of pure cultures of methanogens in synthetic media. J. Appl. Bacteriol. **45**:347–356.
27. **Scherer, P., and H. Sahm.** 1981. Effect of trace elements and vitamins on the growth of *Methanosarcina barkeri*. Acta Biotechnol. **1**:57–65.
28. **Scherer, P., and H. Sahm.** 1981. Influence of sulfur-containing compounds on the growth of *Methanosarcina barkeri* in a defined medium. Eur. J. Appl. Microbiol. Biotechnol. **12**:28–35.
29. **Schonheit, P., H. Kewloh, and R. K. Thauer.** 1981. Factor F_{420} degradation in *Methanobacterium thermoautotrophicum*. FEMS Microbiol. Lett. **12**:347–349.
30. **Sowers, K. R., and J. G. Ferry.** 1985. Trace metal and vitamin requirements of *Methanococcoides methylutens* grown with trimethylamine. Arch. Microbiol. **142**:148–151.
31. **Tiedje, J. M., S. A. Boyd, and B. Z. Fathepure.** 1987. Anaerobic degradation of chlorinated aromatic hydrocarbons. Dev. Ind. Microbiol. **27**:117–127.

32. **van den Berg, L., N. A. Lamb, W. D. Murray, and D. W. Armstrong.** 1980. Effects of sulfate, iron and hydrogen on the microbiological conversion of acetic acid to methane. J. Appl. Bacteriol. **48**:437–447.
33. **van den Berg, L., G. B. Patel, D. S. Clark, and C. P. Lentz.** 1976. Factors affecting rate of methane formation from acetic acid by enriched methanogenic cultures. Can. J. Microbiol. **22**:1312–1319.
34. **Wood, J. M., I. Moura, J. J. G. Moura, M. H. Santos, A. V. Xavier, J. Le Gall, and M. Scandellari.** 1982. Role of vitamin B_{12} in methyl transfer for methane biosynthesis by *Methanosarcina barkeri*. Science **216**:303–305.