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Methods for Isolation of Auxotrophic Mutants of *Methanobacterium ivanovii* and Initial Characterization of Acetate Auxotrophs

MAHENDRA K. JAIN AND J. GREGORY ZEIKUS*

Michigan Biotechnology Institute and the Departments of Biochemistry and Microbiology, Michigan State University, East Lansing, Michigan 48824

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To develop a biochemical genetic approach to understanding cell carbon synthesis or metabolic pathways in methanogens, *Methanobacterium ivanovii* was selected as a model organism for genetic manipulation studies. The organism displayed a colony size of 3 to 6 mm in less than 2 weeks and had a plating efficiency of about 90%, which made it suitable for replica plating. Mutagenesis and selection techniques were developed for selection of acetate auxotrophs. Chemical mutagenesis with ethyl methanesulfonate, followed by enrichment with bacitracin as a selective agent, resulted in stable acetate auxotrophs. *M. ivanovii* was very sensitive to UV, but UV-induced acetate auxotrophs were unstable and reverted within two to four transfers. The acetate auxotrophs were analyzed in relation to wild type for carbon monoxide dehydrogenase enzyme activity.

Although methanogens are nutritionally diverse, most described *Methanobacterium* species have minimal nutrient requirements and grow autotrophically under H_2-CO_2 with sulfide and ammonium as the sole sulfur and nitrogen sources, respectively (19). *Methanobacterium* species (2, 9, 21) do not have obligate requirements for acetate, whereas *Methanococcus* and *Methanobrevibacter* species (1, 5) require acetate and can also require amino acids (17).

To develop strategies for selection of auxotrophic mutants in *Methanobacterium* species, we have used *Methanobacterium ivanovii* as a model organism for genetic manipulation because it significantly assimilates amino acids and acetate and rapidly forms large colonies on agar at 37°C, which facilitates replica plating (3, 9).

Although several studies on the genetic manipulation of methanogenic bacteria have appeared during the last few years (11, 15, 16), not much work has been reported on the development of techniques suitable for the isolation of auxotrophic metabolic mutants. The purpose of this paper is to describe techniques useful for the isolation of *Methanobacterium* mutants, with acetate-requiring strains as the target auxotroph.

All chemicals used were analytical grade and were obtained from either Mallinckrodt, Inc., Paris, Ky., or Sigma Chemical Co., St. Louis, Mo. Purified agar was obtained from Difco Laboratories, Detroit, Mich. All gases were obtained from Matheson Scientific, Inc., Joliet, Ill., and were rendered free of oxygen by passage over heated (370°C) copper filings.

M. ivanovii (2, 9) was grown under H_2-CO_2 (80:20, vol/vol) at 37°C in phosphate-buffered basal (PBB) medium as described elsewhere (14). Culture bottles were inoculated and pressurized daily with 202.65 kN of H_2-CO_2 (80:20, vol/vol) per m^2. This medium will be referred to as minimal medium, while minimal medium with 20 mM sodium acetate will be referred to as complete medium. All media were reduced with 2.6 mM sodium sulfide, and to make the media solid, purified agar (2.3%) was added. Solutions of heat-labile substances were filter sterilized.

Growth was measured by light scattering at 660 nm either in a Spectronic 20 colorimeter or in a Gilford 250 spectrophotometer. CH_4 was analyzed with a 600D Aerograph gas chromatograph (Varian Associates, Palo Alto, Calif.) equipped with a Poropak R column (Anspec Co., Inc., Warrenville, Ill.) and a flame ionization detector by injecting 0.4 ml of gas from culture headspace.

Many attempts to grow *M. ivanovii* on plates had failed initially. The following protocol, developed after many trials, has facilitated the plating of *M. ivanovii* on agar medium. Minimal or complete medium was made as described earlier with 2.3% Difco purified agar to which potassium phosphate (pH 7.8) and vitamin solution were added at 0.2 and 0.1 ml/10 ml, respectively. The medium was reduced with either 2.06 mM cysteine sulfide or 2.6 mM sodium sulfide. Reduced medium (20 to 25 ml per plate) was dispensed on plastic plates in an anaerobic glove box (M/S Coy Manufacturing Co., Ann Arbor, Mich.). Individual plates were again reduced by spreading 0.1 to 0.2 ml of reducing agent 2 to 4 h before use. After the culture was spread with a glass hockey stick, the agar plates were put in an inverted position in a paint tank (W. R. Brown Intermatic, Spring Grove, Ill.) which already contained paper towels soaked with 5 ml of 15% sodium sulfide. After the lid was secured, the paint tank was flushed with H_2-CO_2 (80:20, vol/vol) at least twice by overpressurizing it to 20 lb/in^2 and releasing the gas. The paint tank was finally pressurized to 202.65 kN/m^2, and any entry of oxygen during this manipulation was avoided. The paint tank was incubated at 37°C, during which time production of CH_4 was monitored as a measure of growth of *M. ivanovii* on the plates. The paint tank was repressurized with H_2-CO_2 when necessary. After 14 days, the paint tank was opened in the glove box, and the colonies were examined. *M. ivanovii* failed to form colonies on the same medium until the Difco Bacto-Agar was replaced by Difco purified agar. Also, putting a paper towel soaked with 5 ml of 15% sodium sulfide in the paint tank helped create a favorable environment for *M. ivanovii* to grow and make colonies.

Antibiotic solutions were prepared in distilled water, ethanol-water (1:4, vol/vol), or methanol-water (1:4, vol/vol). Cultures of *M. ivanovii* were spread onto minimal medium agar plates. After 20 to 30 min, five sterile paper disks (6.35-mm diameter; no. 740-E; Schleicher & Schuell, Inc., Keene, N.H.) were placed on each plate. About 20 to

* Corresponding author.

25 μl of each antibiotic (1 mg/ml) was put onto each filter paper, with water as a control on the central disk. The plates were put in the upright position and incubated at 37°C in paint tanks. After 2 weeks, the inhibition zones were measured.

Direct cell counts were made with a Petroff-Hausser counting chamber and a microscope, whereas viable cell counts (CFU) were made by plating appropriate dilutions of the culture onto PBB agar medium and allowing growth of the plates under H_2-CO_2 (80:20, vol/vol) gas phase in a paint tank at 37°C. Plating efficiency is defined as (viable cell count/direct cell count) × 100. The diameters of well-separated colonies were measured. A minimum of 10 colonies were measured for each sample.

Mid-exponential-phase cells of *M. ivanovii* were centrifuged at 3,000 × *g* for 10 min, and the pellet was suspended in 2.5 ml of PBB medium. To this medium was added 2.5 ml of ethyl methanesulfonate solution (0.1 ml of ethyl methanesulfonate per 2.5 ml of reduced minimal medium), and the culture was incubated at 37°C for 1 h after the tubes were pressurized with H_2-CO_2 (6). The cells were then centrifuged and washed twice with minimal medium. The pellet was suspended in 10 ml of complete medium. The culture was allowed to grow at 37°C for 3 days under H_2-CO_2, after which the cells were centrifuged, and the pellet was resuspended in 10 ml of complete medium and incubated under H_2-CO_2. After 1 week, the cells were centrifuged and washed twice with minimal medium. The pellet was then suspended in 10 ml of minimal medium containing bacitracin (1 mg/ml), and the culture was incubated at 37°C under H_2-CO_2. After 40 h, the culture was centrifuged and washed twice with complete medium, and the pellet was suspended in 10 ml of complete medium and incubated at 37°C under H_2-CO_2. After growth, the culture was plated onto complete agar medium, and after 15 days of incubation in paint tanks under H_2-CO_2 at 37°C, colonies were picked by sterile toothpicks and placed onto master plates (complete medium) in an anaerobic glove box. After 15 days of growth, master plates were replica plated in order on minimal and complete medium plates.

After 15 days of incubation, many colonies showing no growth or leaky growth on minimal plates were picked and transferred to tubes containing complete medium. After two transfers, the cultures were plated again onto complete medium plates. After 2 weeks of incubation, colonies were picked on master plates. The master plates, after growth, were replica plated onto minimal and complete medium plates. After 2 weeks of incubation at 37°C under H_2-CO_2, plates were examined for growth. Colonies on complete medium plates which had had no growth on minimal medium plates were picked and transferred to tubes containing complete medium. After growth, these cultures were inoculated into tubes containing minimal or complete medium for rechecking the growth response in minimal medium. Strains that did not display growth in minimal medium were called mutants and were examined further.

The requirement for acetate in auxotrophic mutants was checked as follows. After growth (optical density at 660 nm, >0.2) in acetate-supplemented medium, auxotrophic cells were centrifuged and washed twice with minimal medium. The cultures were suspended in 5 ml of minimal medium. The experimental tubes containing 10 ml of minimal medium were inoculated with a 2% inoculum of auxotrophic mutants. To tubes 1 and 2, acetate (20 mM) was added at zero time and 7 days, respectively. Tube 3 served as a control in which no acetate was present. Growth and CH_4 production were monitored in these tubes for 15 days of incubation under H_2-CO_2 at 37°C.

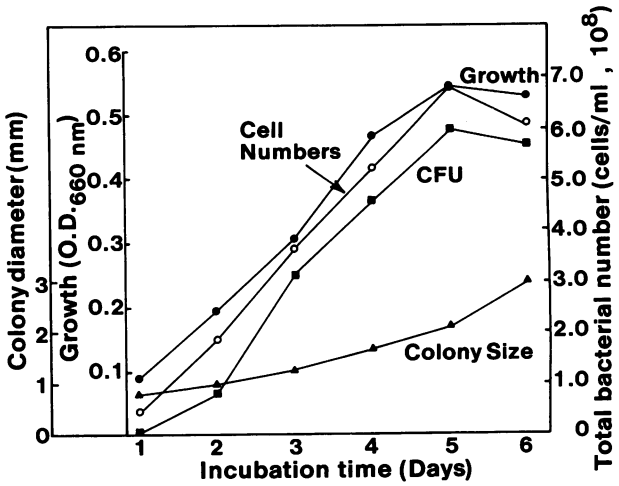

FIG. 1. Growth and plating efficiency of *M. ivanovii*. The organism was grown under H_2-CO_2 in culture bottles that contained 50 ml of PBB medium. At intervals, total cells were counted with a Petroff-Hausser counting chamber, and the CFU were obtained by plating 1- to 6-day-old cultures on PBB agar medium and incubating at 37°C for 15 days in a paint tank pressurized to 202.65 kN of H_2-CO_2 per m^2. $O.D._{660\ nm}$, Optical density at 660 nm.

For assay of carbon monoxide dehydrogenase, the wild-type and mutant cultures of *M. ivanovii* were grown in 158-ml serum bottles (Bellco Glass, Inc., Vineland, N.J.) containing 20 ml of PBB medium under H_2-CO_2. The medium was reduced with sodium sulfide. When necessary, acetate was added to 20 mM final concentration. After 1 week, 10 ml from each culture was placed into an empty anaerobic pressure tube. The tube had already been evacuated, flushed extensively with N_2, and rinsed with low-phosphate basal medium (20) which had been reduced with 4.1 mM Na_2S and titanium citrate (0.1 ml/10 ml). The culture was centrifuged at 3,000 × *g* for 20 min at 4°C. The clear supernatant was removed via syringe, and the pellet was suspended in 2 ml of cold reduced buffer (0.1 M Tris hydrochloride [pH 7.0] plus 5.0 mM dithiothreitol). Toluene (0.1 ml) was added to these tubes, and they were vortexed for 15 s. The tubes were then placed on ice for 10 min before being tested for carbon monoxide dehydrogenase activity. The enzyme assay was performed at 37°C in anoxic cuvettes containing 0.8 ml of 55 mM phosphate buffer (pH 7.2 at 37°C) and 5 mM methyl viologen. Carbon monoxide (100%; 0.8 ml) was added by gas-tight syringe to the headspace of the bunged cuvettes. The reaction was measured by dye reduction at 578 nm with an Eppendorf spectrophotometer.

M. ivanovii was plated on PBB agar medium and grown under H_2-CO_2 (80:20, vol/vol) with a consistent plating efficiency between 87 and 93%. Figure 1 shows a typical growth curve and a comparison of CFU and total cell numbers throughout the phase of growth. Interestingly, the diameter of the colonies formed at 15 days increased in relation to those of inocula sampled late in the growth phase. Colony size increased very rapidly between days 10 and 15 of incubation, and at day 12 of incubation, the colonies were large enough for replica plating.

We previously reported the qualitative sensitivity of *M. ivanovii* in liquid medium to bacitracin, chloramphenicol, monensin, neomycin, gramicidin S, novobiocin, and

TABLE 1. Comparison of selective antibiotics as inhibitors of *M. ivanovii*[a]

Antibiotic	Concn (amt/disk)	Growth inhibition zone diam (mm)
None	0	0
Bacitracin	1.4 U	25
Monensin	25 μg	19
Penicillin	41 U	0
Streptomycin	19 U	0

[a] *M. ivanovii* was grown to mid-exponential phase and was plated on PBB agar medium. Five filter paper disks were put on each plate. One disk received only 25 μl of sterile distilled water, while the others received one of the antibiotics. The growth inhibition zone was measured after 15 days of incubation.

TABLE 2. Comparison of carbon monoxide dehydrogenase activity in wild type versus acetate auxotrophs of *M. ivanovii*[a]

Strain	Sp act[b]
Wild type	
Minus acetate	0.0062
Plus acetate	0.0023
Auxotroph, B-2	0.0023
Auxotroph, B-7	0.0021
Auxotroph, B-9	0.0042

[a] A reaction mixture (0.8 ml per anaerobic cuvette) contained 55 mM anaerobic phosphate buffer (pH 7.2, 37°C), 5 mM methyl viologen, and 0.8 ml of 100% CO (ca. 1 atm [101.29 kPa] of CO) per cuvette. The reaction was initiated by injecting 20 μl of cell suspension at 37°C, and CO-dependent dye reduction was measured at 578 nm with an Eppendorf spectrophotometer.
[b] Micromoles of CO oxidized per minute per milligram of protein.

aphidicolin (9). Table 1 gives the growth inhibition zone sizes on agar medium that were caused by bacitracin, monensin, penicillin, and streptomycin. Bacitracin was selected as the selective agent for enrichment of auxotrophs after mutagenesis because higher concentrations of monensin were required to get equivalent inhibition zones.

When ethyl methanesulfonate (185 mM), however, was used to mutagenize *M. ivanovii*, with bacitracin used in the enrichment step, auxotrophs for acetate were obtained. Three acetate auxotrophs were streaked, purified, and transferred at least 10 to 12 times in PBB medium supplemented with acetate; they tested negative on minimal medium before they were assumed to be stable auxotrophs.

Figure 2 shows a typical growth response curve for an acetate auxotroph in minimal medium supplemented with acetate at various times. Growth of these auxotrophic strains was totally dependent on the presence of acetate. The inoculum used for these experiments was centrifuged cells of an acetate auxotroph washed twice with acetate-free PBB medium. An attempt was made to see if these acetate auxotrophs were defective in carbon monoxide dehydrogenase because this enzyme activity is required in the synthesis of a two-carbon cellular intermediate from H_2 and CO_2 (18). Hence, these mutants may require acetate to form a two-carbon cell carbon precursor from CO_2 and H_2 because

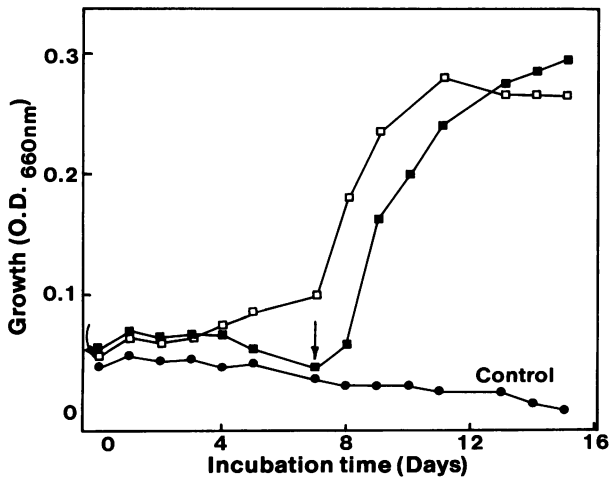

FIG. 2. Growth response for *M. ivanovii* acetate auxotroph B-2. The organism was grown under H_2-CO_2 in pressure tubes that contained 10 ml of PBB medium. Acetate (20 mM) was added either at time zero (□) or after 7 days of incubation (■) (arrows). Controls lacked acetate. $O.D._{660nm}$, Optical density at 660 nm.

they possess an altered carbon monoxide dehydrogenase activity. Table 2 compares the carbon monoxide-oxidizing activity of the wild type with that of the acetate auxotrophs. All strains displayed significant carbon monoxide-oxidizing activity. Interestingly, higher carbon monoxide dehydrogenase activity was found in the wild-type cells grown in acetate-free medium than in cells grown in acetate-supplemented medium, implying enzyme regulation by substrate. This observation supports the implied importance of the enzyme in autotrophic carbon metabolism.

In general, the present data provide methods useful for obtaining classic auxotrophic mutants of *Methanobacterium* species with an acetate auxotroph as the target. With the procedures described here, *M. ivanovii* is consistently plated with a plating efficiency of ≥90%. This plating efficiency is comparable to those reported for other methanogens used as models for genetic studies (7, 10, 12). We have subsequently used these same methods to obtain *M. ivanovii* auxotrophs that require glutamine (4).

Penicillin has been used over the years to enrich the mutants of other bacteria, but methanogens are not sensitive to many common antibiotics (8). Bacitracin, which was found to inhibit the growth of *M. ivanovii* wild type (9), was used successfully in our work for enrichment of *M. ivanovii* auxotrophic mutants. A generalized procedure for selective enrichment of methanogen metabolic mutants has been reported in the literature. 2-Bromoethane sulfonate has been successfully used by Smith and Mah (16) as a selective agent for isolating strains of *Methanosarcina* sp. strain 227 resistant to this agent.

Analysis of these acetate auxotrophs is in progress to ascertain the exact biochemical basis for these auxotrophic mutants. We report here that *M. ivanovii* has carbon monoxide dehydrogenase activity as expected for autotrophic methanogens. Carbon monoxide dehydrogenase presumably catalyzes the reaction involving carbonylation of a methyl group during synthesis of acetyl-coenzyme A or acetate from one-carbon compounds. It is likely that these acetate auxotrophs have a mutation in the region responsible for this enzyme. Although all the auxotrophs displayed carbon monoxide-oxidizing activity, this does not eliminate the fact that the carbon monoxide dehydrogenase enzyme complex is not altered in these mutant strains. The catabolic function of carbon monoxide dehydrogenase on methanogens grown on acetate, however, is in the cleavage of acetate (13).

In conclusion, it is hoped that the methods developed here will be a useful starting point in obtaining auxotrophic mutants in various species of methanogens. Furthermore, the acetate auxotrophs isolated here may prove useful in

understanding the biochemical mechanism of autotrophy in *Methanobacterium* species.

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LITERATURE CITED

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. **Belyaev, S. S., R. Wolkin, W. R. Kenealy, M. J. DeNiro, S. Epstein, and J. G. Zeikus.** 1983. Methanogenic bacteria from the Bondyuzhskoe oil field: general characterization and analysis of stable-carbon isotopic fractionation. Appl. Environ. Microbiol. **45**:691–697.
3. **Bhatnagar, L., M. K. Jain, J.-P. Aubert, and J. G. Zeikus.** 1984. Comparison of assimilatory organic nitrogen, sulfur, and carbon sources for growth of *Methanobacterium* species. Appl. Environ. Microbiol. **48**:785–790.
4. **Bhatnagar, L., M. K. Jain, J. G. Zeikus, and J.-P. Aubert.** 1986. Isolation of auxotrophic mutants in support of ammonia assimilation via glutamine synthetase in *Methanobacterium ivanovii*. Arch. Microbiol. **144**:350–354.
5. **Bryant, M. P., S. F. Tzeng, I. M. Robinson, and A. E. Joyner.** 1971. Nutrient requirements of methanogenic bacteria. Adv. Chem. Ser. **104**:23–40.
6. **Carlton, B. C., and B. J. Brown.** 1981. Gene mutation, p. 222–242. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
7. **Edwards, T., and B. C. McBride.** 1975. New method for the isolation and identification of methanogenic bacteria. Appl. Microbiol. **29**:540–545.
8. **Hilpert, H., J. Winter, W. P. Hammes, and O. Kandler.** 1981. The sensitivity of archaebacteria to antibiotics. Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Orig. Reihe C **2**:11–20.
9. **Jain, M. K., T. E. Thompson, E. Conway de Macario, and J. G. Zeikus.** 1987. Speciation of *Methanobacterium* sp. strain Ivanov as *Methanobacterium ivanovii*. Syst. Appl. Microbiol. **9**:77–82.
10. **Jones, W. J., W. B. Whitman, R. D. Fields, and R. S. Wolfe.** 1983. Growth and plating efficiency of methanococci on agar media. Appl. Environ. Microbiol. **46**:220–226.
11. **Kiener, A., C. Holliger, and T. Leisinger.** 1984. Analogue resistant and auxotrophic mutants of *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **139**:87–90.
12. **Kiener, A., and T. Leisinger.** 1982. Plating efficiency of methanogenic bacteria. Experientia **38**:1379.
13. **Krzycki, J. A., and J. G. Zeikus.** 1984. Characterization and purification of carbon monoxide dehydrogenase from *Methanosarcina barkeri*. J. Bacteriol. **158**:231–237.
14. **Moench, T. T., and J. G. Zeikus.** 1983. Nutritional requirements for *Butyribacterium methylotrophicum* on single carbon substrates and glucose. Curr. Microbiol. **9**:151–154.
15. **Reeve, J. N., N. J. Trun, and P. T. Hamilton.** 1982. Beginning genetics with methanogens, p. 233–244. *In* A. Hollander, R. D. DeMoss, S. Kaplan, S. Kenisky, D. Savage, and R. S. Wolfe (ed.), Genetic engineering of microorganisms for chemicals. Plenum Publishing Corp., New York.
16. **Smith, M. R., and R. A. Mah.** 1981. 2-Bromoethane sulfonate: a selective agent for isolating resistant *Methanosarcina* mutants. Curr. Microbiol. **6**:321–326.
17. **Whitman, W. B., E. Ankwanda, and R. S. Wolfe.** 1982. Nutrition and carbon metabolism of *Methanococcus voltae*. J. Bacteriol. **149**:852–863.
18. **Wood, H. G., S. W. Ragsdale, and E. Pezacka.** 1986. The acetyl-CoA pathway of autotrophic growth. FEMS Microbiol. Rev. **39**:345–362.
19. **Zeikus, J. G.** 1977. The biology of methanogenic bacteria. Bacteriol. Rev. **41**:514–541.
20. **Zeikus, J. G., P. W. Hegge, and M. A. Anderson.** 1979. *Thermoanaerobium brockii* gen. nov. and sp. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. Arch. Microbiol. **122**:41–48.
21. **Zeikus, J. G., and R. S. Wolfe.** 1972. *Methanobacterium thermoautotrophicus* sp. n., an anaerobic, autotrophic, extreme thermophile. J. Bacteriol. **109**:707–713.