JOURNAL OF BACTERIOLOGY, June 1977, p. 1404-1406

Vol. 130, No. 3
Printed in U.S.A.

Methanococcus vannielii: Culture and Effects of Selenium and Tungsten on Growth

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Received for publication 25 March 1977

The reisolation, culture, and method of preservation of the methane-producing organism *Methanococcus vannielii* are described. Growth of the organism on formate is markedly stimulated by selenium and tungsten.

Methanococcus vannielii, a strictly anaerobic, motile coccus that produces methane, was isolated from black mud from San Francisco Bay (10). Among a number of low-molecular-weight alcohols and organic acids tested as fermentable substrates (T. C. Stadtman, Ph.D. thesis, University of California, 1949), the only compound found to support growth of *M. vannielii* was formate, which is fermented to methane and carbon dioxide as shown: $4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$. If the pH of the growth medium is maintained in the optimal range (about 8.0 to 8.5), the stoichiometry of the equation is obeyed, but the continued fermentation of sodium formate in poorly buffered media results in a marked increase in pH due to the accumulation of sodium carbonate. At pH 8.8 and above considerable hydrogen is produced, and the resulting increased rate of gas evolution is due to the fact that the yield of water-insoluble gas now approaches 1 mol per mol of formate decomposed, whereas when methane is formed 4 mol of the substrate must be fermented to produce 1 mol of water-insoluble gas. At pH 9 the energy made available to the organism by the decomposition of formate is very low, and growth virtually ceases. Although *M. vannielii*, like other methane-producing bacteria, actively reduces carbon dioxide to methane with hydrogen when the pH of the medium is 6.5 to 7.5, this series of reactions is not catalyzed to any appreciable extent under alkaline conditions.

For several years all viable cultures of *M. vannielii* were lost, and numerous attempts to reisolate the organism both from the original site (H. A. Barker, personal communication) and from mud samples from many other countries were unsuccessful. Recently, however, the organism was recovered from a single remaining tube of frozen cell paste that had been stored at −10°C for 18 years. This tube of cells was thawed under strictly anaerobic conditions in the nitrogen atmosphere laboratory at the National Institutes of Health (8), and the center portion was used to inoculate a 6-liter flask of mineral salts medium containing 1.5% sodium formate (10) supplemented with 0.05% sterile potassium pyruvate. After 8 days at 30°C, methane was produced, and microscopic examination of the culture fluid revealed the presence of numerous typical motile cocci. Active cultures of *M. vannielii* eventually developed from portions of the frozen cell paste added to flasks of formate-mineral salts medium lacking the pyruvate supplement, but these were much slower in starting. After several successive subcultures, a pure culture of *M. vannielii* was isolated by the agar shake culture technique as described previously (3, 10).

Unless transfers are made at weekly intervals, it is difficult to maintain viable pure cultures of *M. vannielii* in liquid media or in solid or semisolid agars because of the limited buffering capacity of the media and continued gas evolution. However, if active cultures are supplemented with sterile sucrose and then placed in liquid nitrogen, they can be maintained in a viable state for prolonged periods of storage. Addition of sucrose to a final concentration of 10% (0.29 M) or 20% (0.58 M) prevented rupture of the extremely fragile cells of *M. vannielii* during freezing, and these cultures were uniformly turbid after thawing and provided active inocula for transfer to fresh media. Supplementation with sterile glycerol (5 to 20% final concentration) was less effective.

A mineral salts-formate medium, similar to that originally described (10), is used for routine large-scale culture of *M. vannielii* in 50-gallon (about 190-liter) stainless-steel drums or in a 400-liter fermentor. In the 1950s, media were prepared in tap water to supply additional trace elements that might be needed; but now the water in Bethesda is no longer suitable, and distilled water must be employed. Currently the organism fails to grow in 50% local tap water-50% distilled water media or even in me-

dia prepared in deionized local tap water. It is presumed that nonionic detergents are the toxic compounds present in the deionized tap water, since foaming frequently is observed. In some locations the normal tap water may still be pure enough to be used for culture of the methane organism. The ingredients per liter of medium are: NH_4Cl, 1 g; $MgSO_4 \cdot 7H_2O$, 200 mg; $CaCl_2 \cdot 2H_2O$, 10 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; $CoCl_2 \cdot 6H_2O$, 2.38 mg; $MnSO_4 \cdot H_2O$, 0.75 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.75 mg; Na_2SeO_3, 0.173 mg; K_2HPO_4, 3.48 g; HCOONa, 15 g; L-cysteine-hydrochloride, 300 mg (neutralized with KOH to pH 7); and $Na_2S \cdot 9H_2O$, 150 mg. For growth of bacteria in nonsterile media, all of the ingredients except the cysteine and sodium sulfide are dissolved in the appropriate volume of distilled water, and the solution is mixed and deaerated by vigorous passage of a stream of argon through the liquid for 20 to 30 min. Immediately before inoculation, the cysteine and the sodium sulfide solutions are added. Then, after addition of an actively fermenting inoculum (5 to 10% by volume) prepared in the same medium, the pH of the culture is adjusted to about 8, if necessary, by the addition of HCl or Na_2CO_3. Connection of the culture vessel to a water trap, after flushing the head space with argon, protects the bacteria from oxygen and allows gas to escape once fermentation starts.

For growth of the organism in strictly pure culture, all of the ingredients of the medium except the sodium sulfide are heat sterilized in one solution and the sodium sulfide solution is sterilized separately. The cooled solutions are mixed just before addition of the inoculum. For rapid growth of *M. vannielii*, the cultures should be maintained at 37 to 42°C. Large cultures are not stirred mechanically because of fragility of the cells. Once fermentation starts, gas evolution is sufficient to provide mixing and maintain temperature equilibration throughout the culture. The pH is maintained between 8 and 8.5 by addition of HCOOH.

Growth of *M. vannielii* in a formate-mineral salts-cysteine medium prepared in distilled water and unsupplemented with selenite is slow and maximal density is reached only after several days of incubation (Fig. 1). In contrast, similar cultures supplemented with 1 μM selenite grow rapidly and attain a higher cell density within about 3 days. Even better growth is observed when the medium contains tungstate in addition to selenite. In the typical experiments shown in Fig. 1, molybdate (10 μM) was added to all of the media, but little or no effect of omission of this component on growth of *M. vannielii* has been observed. Presumably sufficient Mo is supplied by the other constituents of the medium and from the steel culture vessels to satisfy the growth requirements of the organism. The stimulatory effect of tungstate on growth of *M. vannielii*, even in the presence of 10 μM molybdate, has been observed repeatedly (6). Similar effects of tungstate on growth of other strictly anaerobic bacteria that utilize formate have been reported (1, 2). Partial purification of formate dehydrogenase from *Clostridium thermoaceticum* cultured in the presence of [^{185}W]tungstate resulted in the parallel enrichment of ^{185}W and enzyme activity (7). It is suggested that tungsten substitution for molybdenum in the formate dehydrogenase of these strictly anaerobic bacteria is beneficial and may be the basis of the growth-stimulatory effects. In contrast, growth of *Escherichia coli* in the presence of tungstate results in the formation of an inactive formate dehydrogenase that is converted to the active form only upon the addition of molybdate and an energy source (4, 9). Selenium is also an essential component of formate dehydrogenase from *E. coli* (5), from *C. thermoaceticum* (2), and from *Clostridium sticklandii* and *M. vannielii* (J. B. Jones and T. C. Stadtman, unpublished experiments). With

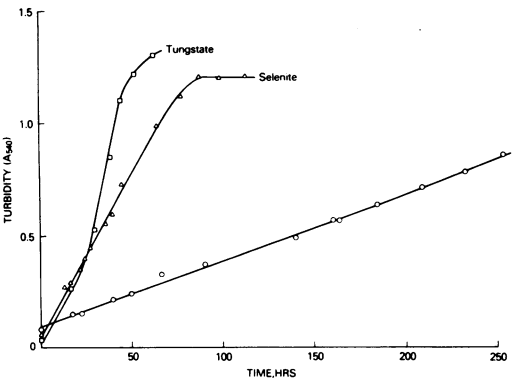

FIG. 1. *Effect of selenite and tungstate on growth of M. vannielii. The culture vessel was a 400-liter fermentor maintained at 37°C. The medium was sterilized, cooled under argon, and inoculated with a 10% (vol/vol) active culture. During the incubation, the liquid was stirred only when HCOOH was added to maintain the pH between 8 and 8.5. Symbols:* (○) *The standard formate-mineral salts medium was prepared as described in the text except that the* $Na_2MoO_4 \cdot 2H_2O$ *concentration was 2.42 mg/liter (10 μM) rather than 0.75 mg/liter, and selenite was omitted.* (△) *Same as* (○) *but plus 1 μM selenite.* (□) *Same as* (○) *but plus 1 μM selenite and 100 μM tungstate. The cell yields (wet weight) in grams per liter were:* (○) *0.76;* (△) *1.0; and* (□) *1.1. Turbidity measured at 540 nm (1-cm light path) is plotted as a linear function of incubation time.*

the latter organism there is a direct relationship between the stimulatory effect of selenite on growth and increased levels of formate dehydrogenase activity of the cells. This suggests that the selenium-dependent, dehydrogenase-mediated oxidation of formate to carbon dioxide and reducing equivalents is more favorable for growth and methane production than alternative pathways (e.g., one involving pyruvate as an intermediate or, possibly, a selenium-independent formate dehydrogenase similar to that of *Vibrio succinogenes* [A. Kröger, personal communication]). In the early studies with *M. vannielii* selenium was supplied in the tap water, and only when the quality of the water supply deteriorated and it became necessary to use distilled water did the requirement for this micronutrient become apparent.

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