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ATMOSPHERIC NITROGEN FIXATION BY METHANE-OXIDIZING BACTERIA

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ABSTRACT

DAVIS, J. B. (Socony Mobil Oil Co., Inc., Dallas, Tex.), V. F. COTY, AND J. P. STANLEY. Atmospheric nitrogen fixation by methane-oxidizing bacteria. J. Bacteriol. **88**:468–472. 1964.—Methane-oxidizing bacteria capable of fixing atmospheric nitrogen were isolated from garden soil, pond mud, oil field soil, and soil exposed to natural gas, indicating a rather wide prevalence in nature. This may explain the high concentration of organic nitrogen commonly found in soils exposed to gas leakage from pipelines or natural-gas seeps. Added molybdenum was a requirement for growth in a nitrogen-free mineral salts medium. All nitrogen-fixing, methane-oxidizing bacteria isolated were gram-negative, nonsporeforming, usually motile rods. Colonies were light yellow, yellow, or white. The most common isolate, which formed light-yellow colonies, is referred to as *Pseudomonas methanitrificans* sp. n., and is distinguished from *Pseudomonas* (*Methanomonas*) *methanica* by nitrogen-fixing ability and a preponderance of poly-β-hydroxybutyrate in the cellular lipid fraction.

Schollenberger (1930) observed a peculiar effect on soil of natural-gas leaks from pipe lines; the soil exposed to the gas was dark in color and emitted what he described as an acrid odor. He pointed out the higher nitrogen content of this soil as compared with normal soil. Harper (1939) specifically studied the effect of natural-gas leaks on the growth of microorganisms and the accumulation of nitrogen and organic matter in soil. Organic nitrogen concentrations of ten soils exposed to natural-gas leaks averaged 0.26%, as compared with only 0.098% for normal soils. Corresponding organic matter values, based on organic carbon analyses, were 4.2 and 1.52%, respectively.

Neither of these workers isolated or identified hydrocarbon-oxidizing microbes from the soil samples. Harper (1939), in particular, attributed the increased soil nitrogen content to the nitrogen-fixing activities of clostridia. Davis (1952), while studying soil samples taken from a natural-gas seep ("paraffin dirt" bed), isolated bacteria capable of utilizing methane and other gaseous hydrocarbons. The soil contained a very high organic nitrogen content (1.2%) and a correspondingly high organic carbon content (17.6%). The hydrocarbon-oxidizing bacteria isolated were not tested for their nitrogen-fixing ability.

Two mechanisms are probably involved in the accumulation of nitrogen and carbon in soils exposed to natural gas. (i) The hydrocarbon is utilized by bacteria capable of fixing atmospheric nitrogen, the result being an ultimate increase in organic carbon and nitrogen of the soil. (ii) The hydrocarbon is utilized by microbes incapable of fixing nitrogen, but the hydrocarbon thus converted into microbial cells ultimately becomes available to other soil microorganisms, some of which are capable of fixing nitrogen.

The end result of both mechanisms is the same, and either could explain the observed phenomenon. This report presents data in direct support of the first mechanism cited.

MATERIALS AND METHODS

Isolation. Portions (0.1 g) of air-dried oil-field soil, soil from a natural-gas seep, pond mud, garden soil, and soil through which methane and air was flowed in the laboratory were added to 25 ml of mineral-salts medium of the following composition (in g per liter of nitrogen-free water): Na_2HPO_4, 0.3; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.005; and $Na_2MoO_4 \cdot 2H_2O$, 0.002. The enrichment culture systems, 100-ml bottles fitted with stopcocks, were evacuated and charged with 30% pure-grade methane (Phillips Petroleum Co., Bartlesville, Okla.) in air, followed by incubation at 30 C. After turbidity developed, bacterial growth was streaked on plates of the above mineral-salts medium containing 1.5% washed agar, and was reincubated under 30% pure-grade methane in air. Purification of colonies was achieved after several restreakings and culture upon the nitrogen-free mineral-salts washed agar medium.

Growth of the common methane-oxidizing bacterium, *P.* (*Methanomonas*) *methanica* and of one hydrocarbon-oxidizing nocardia was also tested on the mineral-salts medium with and without added nitrogen salts for comparative purposes.

Hydrocarbons. The composition of pure-grade methane employed was as follows (mol per cent): methane, 99.00 to 99.20; ethane, 0.26 to 0.32; carbon dioxide, 0.12 to 0.16; nitrogen, 0.26 to 0.32.

Bacterial isolates from methane cultures were tested for their ability to utilize ethane, propane, *n*-butane, and *n*-tetradecane. The bacteria streaked on nitrogen-free, mineral-salts, washed agar medium were incubated with the gas or vapor of the respective hydrocarbons and air in closed systems for a period of at least 30 days.

Atmospheric nitrogen fixation. Nitrogen fixation by the isolated methane-oxidizing bacteria was first tested on a small scale by inoculating triplicate 10-ml portions of nitrogen-free mineral-salts medium (<2 μg of Kjeldahl N per ml) in 100-ml bottles. These systems were evacuated and filled with 30% pure-grade methane in air. One culture system was placed in the cold at 5 C to inhibit growth; the other two systems were incubated at 30 C. All systems were analyzed for fixed nitrogen content after an incubation period of 2 weeks. This analysis was performed by adding 3 ml of Kjeldahl reagent to each of the systems, thus acidifying the contents and trapping any ammonia that might be in the atmosphere or in the liquid. The contents were quantitatively transferred from each bottle to a Kjeldahl digestion flask with washings of nitrogen-free distilled water. Larger scale tests of nitrogen fixation by the methane-oxidizing bacteria were performed by adding a small inoculum to 1- to 2-liter quantities of the nitrogen-free mineral-salts medium. A 50% solution of H_2SO_4 (200 ml) was employed in the gas-flow line preceding the culture system (Fig. 1). This was for the purpose of absorbing ammonia that might possibly be in the methane-air mixture (1:9) which was bubbled at about 50 ml per min through the culture system. Turbidity due to bacterial growth was definite in these large systems in about 2 weeks and gradually increased. After an incubation period of 2 to 4 months, the growth systems were acidified to pH 2.0 with H_2SO_4, and the bacterial cells were harvested by centrifugation prior to analysis of their nitrogen content. The cell-free culture liquor was reduced in volume by evaporation to about 50 ml prior to Kjeldahl analysis. The H_2SO_4 solution was also tested for nitrogen content.

Lipid analysis. The harvested, dried bacterial cells were extracted with chloroform-methanol (2:1), and the lipid extract was divided into its ethyl ether soluble and insoluble fractions. The ether-soluble fraction consisting of triglycerides was hydrolyzed, and the fatty acids were converted to methyl esters by using boron trifluoride as catalyst according to the method of Metcalfe and Schmitz (1961). The methyl esters were chromatographed with linear programmed vapor chromatography. The ether-insoluble chloroform-soluble lipid fraction was analyzed by infrared spectroscopy of a thin film of the material on a silver chloride disc.

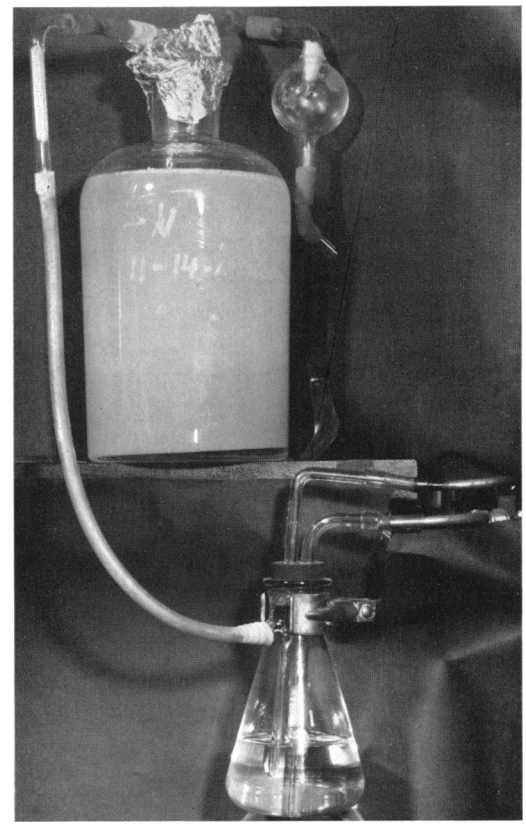

FIG. 1. *Growth of Pseudomonas methanitrificans in N-free mineral-salts (molybdate-containing) solution through which a H_2SO_4-scrubbed methane and air mixture was slowly bubbled.*

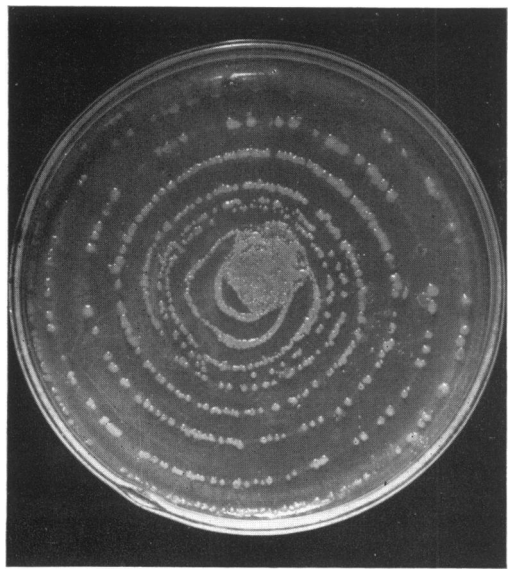

FIG. 2. *Rotary streak of Pseudomonas methanitrificans grown on N-free mineral-salts washed agar medium.*

TABLE 1. *Nitrogen fixed by methane-oxidizing bacteria**

System	Kjeldahl N
	mg
A, growth at 30 C	0.86
B, growth at 30 C	0.66
C, no growth at 5 C	0.03

* Amount of culture used was 10 ml.

TABLE 2. *Kjeldahl analyses for nitrogen in cells, culture liquor, and H_2SO_4 scrubber*

Expt	Sample	Kjeldahl N
		mg
1-liter culture, incubated 2 months	Bacterial cells, 0.6 g (dry wt)	42.4
	Culture liquor	3.7
	H_2SO_4 scrubber*	0.2
2-liter culture, incubated 4 months	Bacterial cells, 3.23 g (dry wt)	204.0
	Culture liquor	53.0†

* See Fig. 1.

† High concentration probably due to autolysis of cells during longer incubation period.

RESULTS

Bacterial isolates. Isolated methane-oxidizing, nitrogen-fixing bacteria grown on mineral-salts medium formed colonies which vary from white to yellow, and dull to glistening. No diffusible, water-soluble pigment was produced. The colony morphology varied from smooth to rough, but the colonies were usually smooth, glistening, and entire. Growth on nutrient agar was meager and often did not retain the ability to fix nitrogen. All bacteria isolated were gram-negative, short to medium nonsporeforming rods, 2 to 4 μ in length, that were usually motile. Molybdenum ion was a requirement for growth in nitrogen-free mineral salts media. The most common isolate formed light-yellow, smooth, glistening, entire colonies (Fig. 2). The cells were about 1 by 2 μ and, while motile, were not actively motile. Homogeneous, diffuse growth was obtained in liquid nitrogen-free mineral-salts medium under methane and air; a pellicle was not formed even under static conditions. The cells contained granules, usually two per cell, which had an affinity for Sudan black B.

The methane-oxidizing, nitrogen-fixing bacterial isolates did not utilize ethane, propane, *n*-butane, or *n*-tetradecane when these hydrocarbons were used as sole carbon sources. The data concerning nitrogen fixation and bacterial cellular composition given in this report were obtained with the common isolate that formed light-yellow colonies.

Nitrogen fixation. The results of a small-scale test for nitrogen fixation are given in Table 1. Fixation of nitrogen averaged over 70 μg/ml, which is sufficient to establish nitrogen fixation by the Kjeldahl method (Wilson, 1951). In larger scale experiments (Table 2), the maximal nitrogen fixed was about 0.13 mg/ml. Nitrogen fixation of this magnitude obviated experimental N^{15} uptake to establish nitrogen fixation by the bacteria.

Consistent, profuse, growth of methane-oxidizing nitrogen-fixing bacteria was obtained under pure-grade methane and air in nitrogen-free mineral salts liquid and washed agar media. These media without added nitrogen compounds did not support the growth of *P.* (*Methanomonas*) *methanica* (Dworkin and Foster, 1956) or a *Nocardia* species (Davis and Raymond, 1961) capable of utilizing ethane, propane, *n*-butane, and *n*-tetradecane.

Bacterial cellular composition. The bacterial cells cultivated on methane and air in the nitrogen-free mineral-salts medium as described above consisted of 46 to 48% protein based on their nitrogen content. The lipid fraction of the cells determined by extraction with chloroform-methanol was 25% of the weight of the cells. The ether-insoluble portion of the lipid fraction was 72% of the total, and proved to consist of poly-β-hydroxybutyrate, as shown by the infrared spectrum of a thin film of the material on a silver chloride disc (Fig. 3). The remainder of the lipid fraction consisted primarily of triglycerides. Vapor chromatography of the methyl esters showed that the glyceride fatty acids were myristic, palmitic, and stearic, with possibly some oleic.

P. (Methanomonas) methanica isolated from pond mud was cultivated under methane and air in mineral-salts medium containing nitrogen salts. The harvested cells (110 mg, dry weight), extracted with chloroform-methanol, contained no poly-β-hydroxybutyrate in the ether-insoluble, chloroform-soluble lipid fraction.

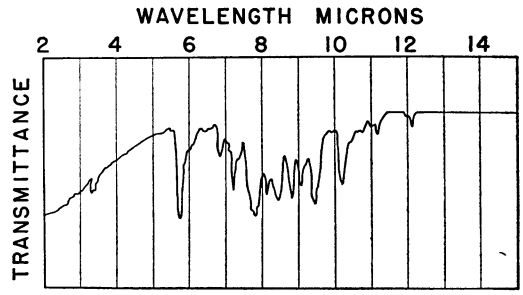

FIG. 3. *Infrared spectrum of chloroform-soluble, ether-insoluble fraction of the cellular lipid of Pseudomonas methanitrificans sp. n., identical to published spectrum of poly-β-hydroxybutyrate (Blackwood and Epp, 1957; Haynes et al., 1958).*

DISCUSSION

The existence in soils and muds of methane-oxidizing bacteria which fix atmospheric nitrogen affords a simple explanation for the observed increase in organic nitrogen of soils associated with natural-gas leakages (Schollenberger, 1930; Harper, 1939) or seepages in nature (Davis, 1952). Although this process is not the only possible means for nitrogen enrichment in such soils, it is the most straightforward. Soil kept moist, through which methane and air are passed, invariably shows an increase in nitrogen content. Methane-oxidizing bacteria, capable of fixing nitrogen, may be isolated from soil treated in this manner.

The most common bacterial isolate encountered produces light-yellow colonies and represents one of several colony types observed. The bacterium has the characteristics of a pseudomonad by virtue of its morphology, staining, and cultural characteristics, and we propose the species name, *Pseudomonas methanitrificans*. Classification may be based on morphology, concomitant methane-oxidizing and nitrogen-fixing ability, and, as a corollary, a preponderance of poly-β-hydroxybutyrate in the chloroform-soluble lipid extract of the cells. *P. methanitrificans* and the common, pink *P. (Methanomonas) methanica* originally isolated by Söhngen (1906) were isolated by us from the same pond mud source. *P. (Methanomonas) methanica* is not readily isolated from certain other sources (e.g., a natural-gas seep). This bacterium does not grow under methane and air on nitrogen-free mineral-salts medium, but, when grown with methane in nitrogen salts-containing media, poly-β-hydroxybutyrate was not observed in the cellular lipid fraction of the bacterial cells.

Bergey's Manual of Determinative Bacteriology (Breed, Murray, and Smith, 1957) designates *Methanomonas methanica* (Söhngen) as the lone methane-oxidizing member of the family Methanomonadaceae in the order Pseudomonadales. The bacterium was originally referred to by Söhngen (1906) as *Bacillus methanicus* but was designated *M. methanica* by Orla-Jensen (1909).

Krasil'nikov (1949) placed methane-oxidizing bacteria, all of which appeared to be pseudomonads, in the family Pseudomonadaceae, genus *Pseudomonas*. The physiology of the bacteria is designated by the species name only, thus *M. methanica* is referred to as *P. methanica*. This designation was suggested also in the United States by Dworkin and Foster (1956). Pigment variation in forms of *P. (Methanomonas) methanica* was reported by Leadbetter and Foster (1958), the morphology of these forms being the same. The species designation, *P. methanitrificans*, follows the rule of relegating assimilatory physiological characteristics to the species name rather than the genus.

Fixation of nitrogen by microorganisms capable of utilizing higher hydrocarbons is presently under investigation.

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