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MORPHOLOGY AND PHYSIOLOGY OF *METHANOMONAS METHANOOXIDANS*

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ABSTRACT

STOCKS, PETER K. (Louisiana State University, Baton Rouge), AND C. S. McCLESKEY. Morphology and physiology of *Methanomonas methanooxidans*. J. Bacteriol. **88**:1071-1077. 1964.—Pure cultures of methane-oxidizing bacteria isolated from soil, from the rumen of a fistulated cow, and from coal-mine water were found to be identical in morphological, cultural, and physiological characteristics with *Methanomonas methanooxidans* of Brown and Strawinski. Two of the isolates were serologically related to the organism of Brown and Strawinski. All the strains required methane for good growth, but a delayed moderate growth occurred on methanol. No other substances were utilized as carbon and energy source. Nitrogen requirements were satisfied by nitrates, ammonium salts, peptone, or certain amino acids. The taxonomic position of the species is discussed.

Since the first description of methane-oxidizing bacteria by Söhngen (1906), numerous investigators have reported studies dealing with microbial oxidation of methane and other gaseous hydrocarbons. Among the more recent reports are those of Hutton and ZoBell (1949), Dworkin and Foster (1956), Strawinski and Brown (1957), Brown and Strawinski (1957, 1958), and Leadbetter and Foster (1958). Recent reviews of hydrocarbon oxidation by microorganisms have been provided by Fuhs (1961) and Foster (1962).

In most reports dealing with methane-oxidizing bacteria, the descriptions of the organisms involved have been inadequate to afford detailed comparison with those described by other investigators, and the cultures are not available for comparative studies. This report describes the methods employed in the isolation of three additional strains of methane-dependent organisms, and compares them with the organism named *Methanomonas methanooxidans* by Brown and Strawinski (1958).

MATERIALS AND METHODS

Cultures. The cultures employed in this study included that of Brown (1958), and Brown and Strawinski (1958) as described by Strawinski and Brown (1957), and three isolates of our own obtained from widely different sources. The Brown strain was isolated from oil field soil near Baton Rouge, La., and was supplied to us by L. R. Brown. Our isolates were obtained from the following sources: strain PBG, coal-mine water from West Germany; strain Rumen, rumen of cow; and strain soil, oil field soil near Baton Rouge, La.

Media. Cultures were routinely propagated on the basal salts medium of Jayasuriya (1955), with methane as the carbon source. Tests to determine the suitability of various carbon and nitrogen compounds for growth were carried out with this basal medium. For comparative purposes, some studies were made with the media of Kaserer (1906), Brown (1958), and Mevius (1953), and also with the usual bacteriological media.

Agar media were prepared by the addition of 2% agar (Difco) to the appropriate mineral salts (MS) solutions.

The hydrocarbon gases employed (Matheson Co., East Rutherford, N.J.) were of the highest purity commercially available: methane, chemically pure grade, 99.0% pure; ethane, 95.0%; *n*-propane and *n*-butane, instrument grade, 99.9% pure; oxygen, extra-dry grade, 99.6%; and carbon dioxide, bone-dry grade, 99.9% pure. The gases were stored in separate containers and were mixed as required in a large graduated cylinder by liquid displacement (Fig. 1). The methane was mixed with oxygen and carbon dioxide in a ratio of 65:30:5, respectively, unless otherwise indicated.

Enrichment and isolation procedures. The presence of methane-oxidizing organisms was initially determined with modified Söhngen units, by the use of two graduated prescription bottles (6 oz) as described by Hutton and ZoBell (1949), but

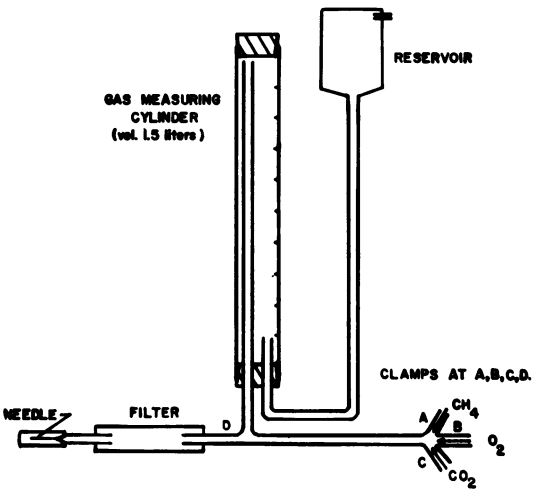

FIG. 1. *Apparatus for mixing and introducing the gases into the culture bottles.*

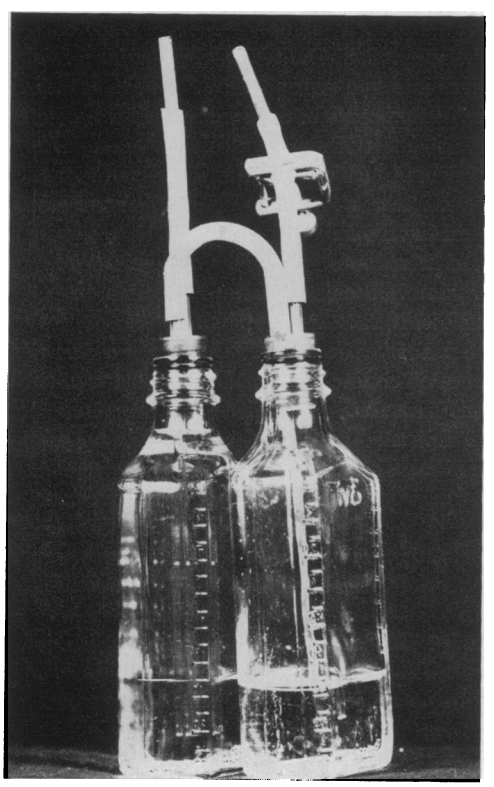

FIG. 2. *Modified Söhngen culture unit. Left, reservoir bottle; right, reactor bottle.*

with some modifications in procedures. The bottles of the Söhngen units, hereafter referred to as the reactor or culture bottle, and the reservoir, were both filled almost to the 110-ml mark with the appropriate medium, fitted with connections (Fig. 2), and autoclaved at 121 C for 20 min. The openings leading to the air were protected from contamination by cotton filters.

After cooling, vacuum was applied to the reactor bottle until the medium from the reservoir filled the reactor; the outlet of the reactor was then closed with a clamp. The gas mixture was introduced through a sterile cotton filter (Fig. 1) into the reactor bottle by a needle inserted through the rubber hose just below the clamp. The gas was allowed to displace the medium to the 20-ml graduation mark on the reactor bottle, and the units were then allowed to stand at least 1 day to insure that there was no leakage.

The inoculum (2.5 ml) was injected into the reactor with a hypodermic syringe. The rubber tube below the clamp was sterilized with Merthiolate, and the needle was inserted through the rubber and down into the inlet tube of the reactor bottle. To insure the removal of all the inoculum from the inlet tube down into the medium, vacuum was applied to the reservoir bottle, and the clamp on the reactor was momentarily released. The vacuum was then disconnected from the reservoir, and the clamp on the reactor was again released until the medium in the reactor reached the 50-ml mark. At this point, the reactor was sealed by placing the clamp on the inlet tube below the needle punctures. All the manipulations were carried out in an inoculating hood. Incubation was at room temperature (28 C) on New Brunswick shakers adjusted to rotate at approximately 180 rev/min.

The inoculated Söhngen units were incubated until gas consumption was nearly complete, as indicated by the volume of medium drawn back into the reactor vessel from the reservoir. Further enrichment was accomplished by several successive transfers into small volumes of medium in 125-ml rectangular bottles, in the following manner. From the enriched cultures in Söhngen units, 5-ml portions were withdrawn, washed three times, and resuspended in 5 ml of sterile phosphate buffer. The small culture bottles which contained 10 ml of sterile MS medium were inoculated with 0.2 ml of the washed-cell suspension. The bottles were closed with serum stoppers and flushed with the gas mixture, with one needle used to admit the gas and another to allow the gas to escape. The bottles were incubated at 28 C

on a New Brunswick shaker and were observed daily.

As soon as slight turbidity appeared, a portion of the culture was withdrawn, the cells were washed and resuspended as before, and fresh cultures were started. After the fifth transfer showed the slightest turbidity, a portion of the culture was withdrawn; the cells were washed, resuspended in MS solution, and serial dilutions were carried out to 10^{-10}. A 0.1-ml portion of each dilution was transferred to individual bottles containing 10 ml of sterile medium, to which the gas mixture was added. Tryptone Glucose Extract Agar (TGE) and MS agar plates were streaked from each dilution, the former for incubation in air, the latter for incubation under the methane gas mixture in a desiccator. Bottles which showed growth under methane, and colonies which appeared on the MS agar plates, were used to inoculate Söhngen units to confirm the presence of methane-oxidizing bacteria. Söhngen units showing consumption of methane were tested for purity by again streaking TGE agar plates for incubation in air and MS agar plates for incubation under methane.

Serological studies. Cells for immunological studies were grown in the MS medium in Söhngen units until gas consumption was completed. The cultures were stored at 5 C until their purity was determined. To test purity, a loopful of culture from each Söhngen unit was streaked on TGE plates which were incubated in air at room temperature for 48 hr. The cultures which contained no organisms capable of growth on TGE agar without methane were centrifuged, washed twice in physiological saline, and resuspended in formollized saline. Cell suspensions corresponding to the McFarland no. 3 standard were injected intraperitoneally into rabbits (1 ml of antigen every 2 days for 2 weeks). Sera were collected 7 days after the last injection. Tube agglutination tests were incubated at 50 C in a water bath for 24 hr; readings were made at 2, 24, and finally after an additional 24 hr at 5 C.

RESULTS AND DISCUSSION

Isolation of methane-oxidizing organisms. Two types of colonies were noted on plates of MS agar which had been inoculated from a completed Söhngen unit and incubated in the methane gas mixture. One type was a small discrete colony, often pink, varying from 0.1 to 1.0 mm in diameter after 10 days of incubation at 28 C. The other type of colony was not visible with the unaided eye, and was apparently identical to those described by Strawinski and Brown (1957) and Brown (1958; Fig. 3a). The pink-pigmented organism in pure culture did not utilize methane, and grew on the usual bacteriological media. The organism forming minute colonies (microcolonies) invariably consumed methane, and did not grow on ordinary media. The criterion of purity of these cultures was, therefore, the absence of organisms capable of growth on ordinary media without methane.

With the use of these procedures, pure cultures of methane-oxidizing bacteria were obtained from soil, water obtained from a coal mine in West Germany, and from the rumen of a fistulated cow. Brown (1958) reported the isolation of apparently identical strains from various types of soil, drainage ditch mud, swamp mud, and a fermenting hay pile.

Morphology. All the isolates from whatever the source are morphologically indistinguishable. The methane-oxidizing organism is a nonsporeforming rod, 1.0 by 1.5 to 4 μ, and motile by means of a single polar flagellum (Fig. 3b). It is gram-negative, but stains unevenly because of vacuolation of the cells; it is not acid-fast. The frequent occurrence of vacuolated and club-shaped cells is an interesting characteristic of these organisms. The vacuoles do not take the usual stains (Fig. 3c), and are apparently not fat bodies (do not stain with Sudan black). Some of the cells are much enlarged at one end, causing the other end to appear as a budlike projection, suggestive of the genus *Hyphomicrobium* Stutzer and Hartleb (Fig. 3d). However, the long slender filaments observed in *H. vulgare* by Kingma-Boltjes (1936) were not seen in our cultures.

Another striking morphological feature of these organisms is the common occurrence of the cells in rosettes, resembling the spokes of a wheel (Fig. 3e). These structures are similar to those observed in *Agrobacterium*, *Phyllobacterium*, *Rhizobium*, and *Chromobacterium* by various workers (*see* Knösel, 1962, 1963). The characteristic of rosette or star formation is most obvious when there are only three to six radially arranged cells, but the rosettes may attain sufficient size to cause large clumps and the appearance of a fine-grained sandy precipitate in MS methane broth. The presence of the rosette masses, and their entanglement with methanol-oxidizing bacteria in enrichments, no doubt in-

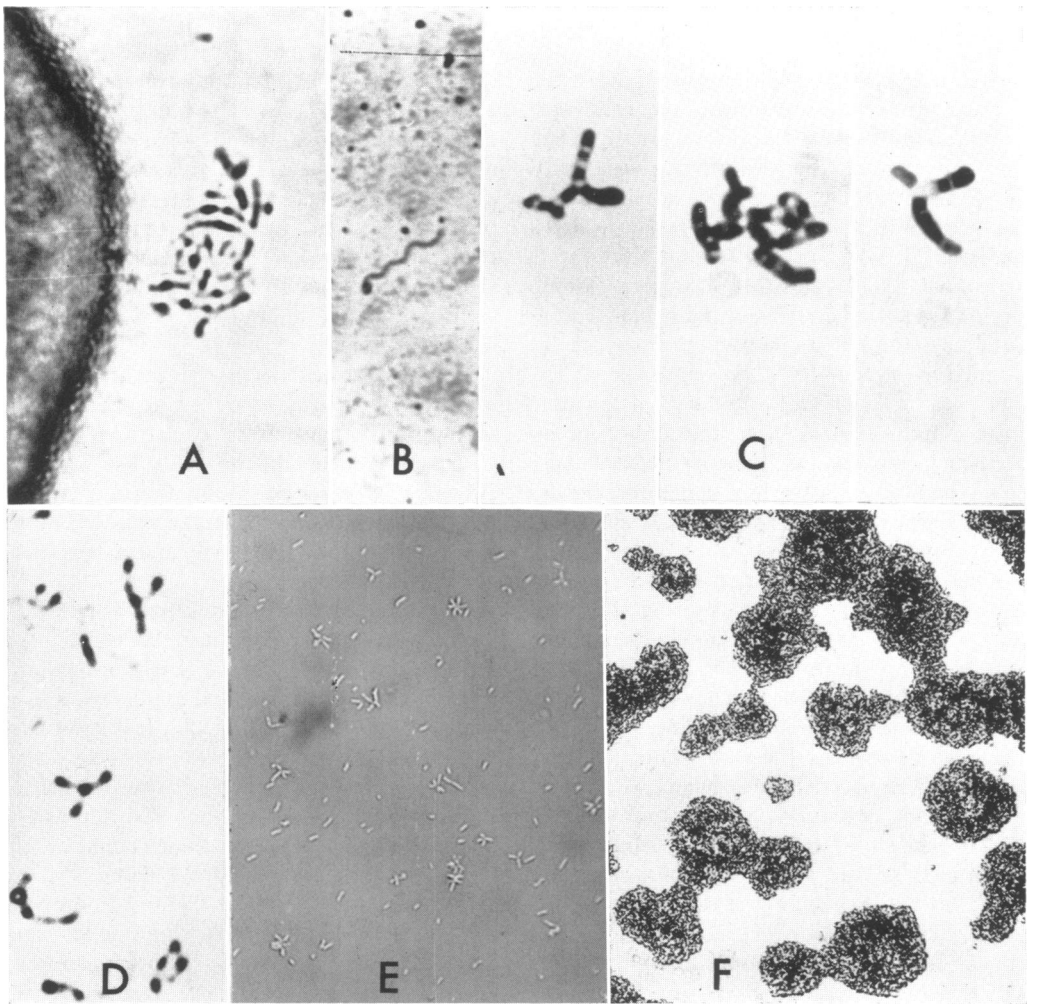

FIG. 3. *Morphological characteristics of Methanomonas methanooxidans. (A) Microcolony of strain Rumen near a larger colony of a pink-pigmented methanol oxidizer. Mineral salts agar under methane gas mixture, 14 days at 28 C. × 3,000. (B) Strain PBG stained by the method of Bailey as modified by Conn and Fisher (Conn et al., 1957). × 2,000. (C) Strain Brown stained with crystal violet. × 4,000. (D) Unstained cells of strain Brown on mineral salts agar under methane. × 3,000. (E) Rosettes in mineral salts methane broth. Negative stain with congo red. × 1,000. (F) Colonies of strain Brown on mineral salts-methane-agar incubated at 25 to 27 C for 8 weeks. × 450.*

terferes with ready isolation of these organisms in pure culture. Strawinski and Tortorich (1955) referred to the "characteristic clumping" of their cultures, and attempted to prevent this condition by the use of Tween 80.

Cultural characteristics. The methane oxidizers develop only minute colonies under the most favorable conditions thus far devised for their cultivation. On MS agar in an atmosphere of methane, oxygen, and carbon dioxide (65:30:5), the colonies are about 0.05 to 0.1 mm in diameter after incubation for 3 weeks at 28 C, and their size is not increased appreciably with longer incubation. There is no pigmentation. Changes in the composition of the gas mixture, and the addition to the medium of various organic adjuncts (vitamins, amino acids, peptones, and carbohydrates), did not appreciably increase colony size. This is in agreement with the experience of Brown (1958). Our attempts to improve colonial develop-

ment by modifications of the mineral composition of the medium failed, although Brown (1958) reported partial success by the addition of 0.02% NaCl to the basal salts medium when prepared with distilled water.

The colonies of the four strains of methane oxidizers on MS agar under methane are indistinguishable; the margins are somewhat irregular and the structure is granular in appearance (Fig. 3f). The colonies bear some resemblance to those of *H. vulgare*, but the latter are considerably larger and have entire margins. The colonies are distinctly different from the commonly associated methanol oxidizers, which are treated in a separate paper (Stocks and McCleskey, 1964).

In agitated liquid MS methane medium, the organisms produce good growth in 4 to 8 days, with moderate turbidity and white sandy sediment. Growth in MS methanol is less abundant than with methane; the type of growth, however, is essentially the same as with methane.

Physiological characteristics. All our strains, regardless of source, utilized methane as the sole carbon source; none of the isolates consumed ethane, *n*-propane, *n*-butane, *n*-hexane, *n*-heptane, or *n*-octane. There was no growth when the organisms were supplied with a mixture of hydrogen and carbon dioxide, or hydrogen, carbon dioxide, and oxygen.

Other compounds tested as carbon sources included methanol, ethanol, *n*-propanol, *n*-butanol, formate, acetate, propionate, butyrate, xylose, glucose, lactose, maltose, sucrose, glycerol, formaldehyde, methylamine, acetone, benzene, acetone, and phenol. All tests were carried out in triplicate, and incubation was at 28 C for 6 weeks. Of the compounds tested, only methanol permitted growth of the methane oxidizers, and growth with this substrate was delayed and less abundant than with methane.

Various substances were tested to determine their availability as nitrogen sources for the methane-oxidizing bacteria when methane was provided as carbon and energy source. Cultures which showed growth through two transfers in a substrate were retested for purity, and were used to inoculate Söhngen units to verify methane oxidation by the culture. Satisfactory nitrogen sources included $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, KNO_3, L(−)leucine, L-glutamate, L-asparagine, L(+)arginine, L-cysteine, and peptone. No growth and no methane consumption occurred with KNO_2, glycine, DL-valine, DL-alanine, DL-aspartate, DL-methionine, L(+)-cystine, DL-phenylalanine, methylamine, and urea.

TABLE 1. *Serological relationships among methane-oxidizing and morphologically related organisms*

Antigen	Antiserum					
	Brown	PBG	Rumen	Soil	*A. tumefaciens*	*A. radiobacter*
Brown	160	160	160	—	—	—
PBG	320	320	320	—	—	—
Rumen	160	160	320	—	—	—
Soil	—	—	—	320	—	—
Agrobacterium tumefaciens	—	—	—	—	5,120	640
A. radiobacter	—	—	—	—	640	5,120
A. stellulatum	—	—	—	—	—	—
Rhizobium leguminosarum	—	—	—	—	—	—

Serology. Serological studies showed that the strain of Brown and Strawinski (1958) is antigenically related to the strain isolated from the water of a coal mine in West Germany and to the strain isolated from the rumen of a cow. Interestingly enough, it shows no relationship to our "soil" strain, although both were isolated from soil in the same vicinity. No antigenic relationship was found between the methane oxidizers and the star-forming species of *Agrobacterium* and *Rhizobium* (Table 1).

Taxonomy. The methane-oxidizing bacteria described in this paper appear to differ in some important respects from those previously reported. The organism described by Söhngen (*Bacillus methanicus*) produced a pink pigment and grew on common media. The methane oxidizers of Hutton and ZoBell (1949) and of the earlier workers (Münz, 1915; Aiyer, 1920; Haseman, 1927; Tausz and Donath, 1930) are too inadequately described for detailed comparison with our own isolates, but none of them was reported to form only nonpigmented microcolonies, to have irregular cell morphology, and to form rosettes. Some of the previously described cultures were similar to ours in dependence on methane or methanol; others utilized various hydrocarbons besides methane, and also other substrates.

The organisms of Nechaeva (1949), *Mycobacterium methanicum* and *M. flavum* var. *methanicum*, are unlike our methane oxidizers in that they are nonmotile, gram-positive, and utilize various carbon substrates in addition to methane. In these

species, the cells were described as rod-shaped in young cultures and cocci in old cultures; this is suggestive of the genus *Arthrobacter*.

The *Pseudomonas methanica* of Dworkin and Foster (1956) and Leadbetter and Foster (1958) agree in some details with that of Söhngen (1906); perhaps Dworkin and Foster (1956) were correct in attributing the discrepancies to an impurity in the culture of Söhngen. Doubt as to the purity of the Söhngen culture was also expressed by Münz (1915).

The formation of rosettes, as observed in our isolates and in the organism designated *M. methanooxidans* by Brown and Strawinski (1958), has not been reported as a cultural property of other methane oxidizers, except that Nechaeva (1949) noted rosette structure in colonies of *Mycobacterium methanicum*. Rosette or star formation has been reported in all the genera of the family Rhizobiaceae (Conn, Bartholomew, and Jennison, 1957) and also in the genera *Pseudomonas* and *Xanthomonas* of the Pseudomonadaceae, Winslow et al. Morphologically, our isolates are indistinguishable from *A. stellulatum*, *A. tumefaciens*, and *A. radiobacter*. Our organisms resemble these species also in Gram reaction, and in motility by means of one or possibly more flagella. Culturally and physiologically, however, our organisms do not resemble any species of the genus *Agrobacterium*, nor any species of the family Rhizobiaceae. Serological studies did not reveal any antigenic relationship between *Agrobacterium* species and the methane oxidizers.

The organisms used in this study, although apparently distinct from other methane oxidizers, are sufficiently like that of Söhngen (1906) to be considered members of the same genus. The differences are great enough, however, to justify a separate species. We therefore accept the name suggested by Brown and Strawinski (1958), *Methanomonas methanooxidans*. A culture of this species has been deposited with the American Type Culture Collection.

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