THE OCCURRENCE AND CHARACTERISTICS OF METHANE-OXIDIZING BACTERIA IN MARINE SEDIMENTS[1]

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The chemical stability of methane, its common occurrence in the biosphere, and its importance in the petroleum industry have aroused considerable interest in the microorganisms that decompose this gas. There have been very few contributions to the study of such microorganisms since Kaserer (1905) and Söhngen (1906, 1910) almost simultaneously reported the bacterial oxidation of methane, and Münz (1915) investigated the physiology of a methane-oxidizing bacterium.

The widespread occurrence of methane-oxidizing bacteria in Italian soils was reported by Giglioli and Masoni (1914). These workers, like Harrison and Aiyer (1914, 1916*a*,*b*) and Aiyer (1920), who investigated methane oxidizers in Indian swamp rice soils, emphasized the importance of such bacteria in the conservation of carbon and in the formation of organic matter. From soil, Hasemann (1927) isolated bacteria which oxidized illuminating gas. Tausz and Donath (1930) isolated from soil a bacterium that was able to oxidize methane and also hydrogen, ethane, propane, butane, and higher hydrocarbons.

Based upon the presence or activities of bacteria that oxidize methane in surface soil overburdening subterranean gas or oil deposits are the so-called geomicrobiological prospecting methods (ZoBell, 1946*b*). Yurovskii *et al.* (1939) reported that in preliminary field tests up to 96 per cent of the methane in the atmosphere of coal mines was destroyed by strategically located cultures of *Methanomonas methanica*. Peroxidase formation by methane-oxidizing bacteria has been reported by Slavnina (1947), who also noted that, unlike allied species that oxidize higher hydrocarbons, *M. methanica* exhibited no fluorescence (Slavnina, 1948).

Although apparently occurring quite commonly in soil, particularly in regions where much methane is formed or found, methane-oxidizing bacteria are still regarded more or less as biological curiosities. These investigations were undertaken in view of the restricted body of data available on the physiology and morphology of methane-oxidizing bacteria and an almost complete lack of knowledge regarding their occurrence in marine materials.

MATERIALS AND METHODS

A modified Söhngen culture apparatus (figure 1) was employed for demonstrating and following the activity of methane-oxidizing bacteria. All-glass appa-

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ratus proved to be no better than 180-ml prescription bottles fitted with two-hole rubber stoppers. Although there are several ways of manipulating the apparatus, one of the best was to introduce the inoculum and the gas mixture in the culture bottle (A, figure 1) and fill the reservoir bottle (B) with sterile medium. Then by releasing the clamps (C, C′) 20 ml of medium were permitted to flow from the reservoir bottle into the culture bottle, after which the clamp on the culture bottle was securely closed and that on the reservoir bottle left open. As methane and oxygen were consumed in the culture bottle more medium was sucked over

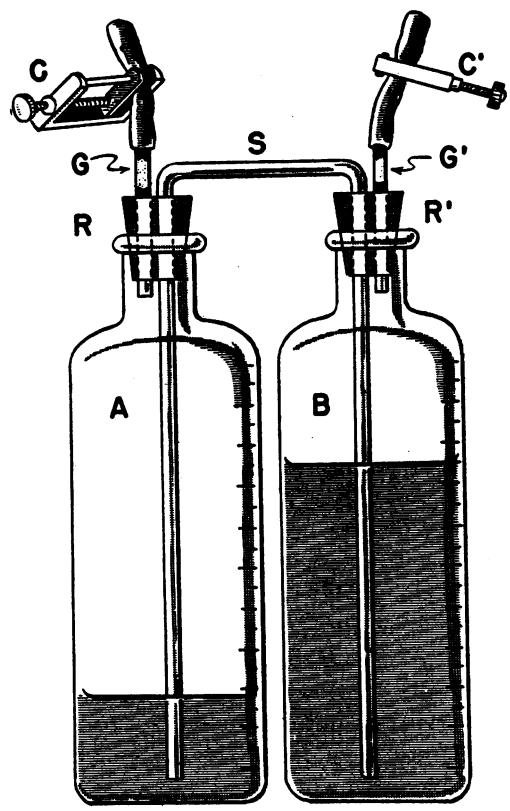

Figure 1. Modified Söhngen apparatus employed for studying respiration of methane-oxidizing bacteria. A, culture bottle; B, reservoir bottle; C, clamps on rubber tubing; G, glass inlet tubes plugged with cotton; R, two-hole rubber stoppers, S, glass siphon.

from the reservoir bottle. Graduation marks on the bottles provided for reading, at constant temperature and corrected atmospheric pressure, the volumes to ±0.5 ml. In this way gas uptake could be followed from day to day without interrupting the experiment.

At the end of the experiment the residual gas in the culture bottle was transferred over mercury to a Fisher unitized precision model Orsat type gas analyzer (Matuszak, 1934). The apparatus provided for determining methane with an accuracy of ±0.4 per cent and oxygen and carbon dioxide somewhat more pre-

cisely. Gases of highest obtainable purity were employed for preparing the mixtures of methane, oxygen, carbon dioxide, and nitrogen.

Sea water aged to minimize its organic content (ZoBell and Grant, 1943) or a balanced mineral salts solution was employed in the preparation of the media. The nitrogen requirements were satisfied by ammonium salts, nitrate, or certain amino acids.

In the isolation and characterization of pure cultures, culture tubes or petri dishes were incubated in vacuum desiccators filled with the desired gas mixtures. Washed agar or silica gel was employed to prepare solid media.

DISTRIBUTION OF METHANE OXIDIZERS

Sediment samples from numerous regions have been tested for the presence of methane-oxidizing bacteria. In making the ecological survey, approximately one

TABLE 1

Number of samples tested in which methane-oxidizing bacteria were detected in one-gram inocula

DESCRIPTION OF MATERIAL	TOTAL NUMBER OF SAMPLES TESTED	NUMBER SHOWING PRESENCE OF METHANE OXIDIZERS
Surface samples (topmost 10 cm)		
Marine mud	21	18
Brackish water mud	41	36
Paraffin earth	9	9
Oil- or gas-field soil	187	139
Beach sand	9	2
Subsurface samples (below 10 cm)		
Marine mud	17	4
Brackish water mud	12	1
Beach sand	3	0

gram of material (wet weight) was used as an inoculum. Preliminary experiments indicated that, when such bacteria were present in a given environment, nearly all one-gram samples produced positive results, although a longer incubation period might be required than when 10- or 50-gram inocula were used. Actually 0.1-gram samples of marine mud often yielded positive results, and methane oxidizers were demonstrated in some 0.01-gram samples after several weeks' incubation in sea water medium enriched with methane. Such bacteria were not detected in surface sea water even when 100-ml samples were used as inocula.

Since the samples were not collected or examined simultaneously, the results are not strictly comparable. Some samples collected far at sea were stored in a refrigerator at near 0 C until they could be examined in the laboratory. Therefore results are recorded as positive and negative only. Negative results may not establish the complete absence of methane-oxidizing bacteria because they may have perished or failed to multiply under the experimental conditions to which they were subjected, but complete reliance can be placed upon positive results.

Methane-oxidizing bacteria were found in marine sediments collected off the coast of California, in the Gulf of Mexico, in the Atlantic Ocean off the coast of New Jersey and Massachusetts, in brackish water sediments from California and Louisiana, and in surface soil from oil and gas fields in California, Louisiana, Oklahoma, and Texas (table 1).

With very few exceptions methane-oxidizing bacteria were not detected in subsurfaces mud samples collected at core depths exceeding 10 cm, presumably because free oxygen becomes a limiting factor at such depths. Not only is free oxygen nearly always absent below the topmost few millimeters of marine mud, but negative redox potentials generally prevail (ZoBell, 1946*a*). In arid soil penetrated by oxygen methane-oxidizing bacteria have been found at depths of several feet.

Finding methane-oxidizing bacteria in marine mud under 1,170 meters of water where the hydrostatic pressure is approximately 117 atmospheres indicates that such pressures do not limit the activities of these bacteria. In fact, experiments now in progress suggest that much higher hydrostatic pressures may actually enhance the activity of barophilic (ZoBell and Johnson, 1949) methane oxidizers from oceanic deeps.

ISOLATION OF PURE CULTURES

After 5 to 14 days' incubation in an atmosphere containing methane, oxygen, and carbon dioxide, enrichment cultures were streaked on solid media. Although many colonies developed, most of them failed to utilize methane when transferred back to Söhngen culture bottles containing methane, oxygen, and carbon dioxide. Serial transfers of the enrichment cultures yielded on solid media colonies that definitely utilized methane when transplanted to appropriate liquid media.

Out of 82 colonies picked from washed agar medium only two proved to be methane oxidizers. Many more colonies developed on nutrient agar (0.5 per cent peptone and 0.3 per cent yeast extract), but none of them consumed methane when transplanted to Söhngen culture bottles. Growth was rather sparse on silica gel plates even when the inoculum was large, but out of 68 colonies picked from this purely mineral medium 12 showed ability to consume methane.

Cultures of methane oxidizers, which proved to be pure according to all standard criteria, including both cultural and microscopic tests, formed discrete colonies 0.1 to 0.5 mm in diameter. The colonies were pearly or bronzy in appearance, convex, and circular with an entire margin. Other colonies of methane oxidizers subsequently isolated proved to have similar characteristics, although two other colonial types, which did not utilize methane, often developed from enrichment cultures.

CHARACTERISTICS OF PURE CULTURES

Morphologically all methane-oxidizing bacteria isolated from marine mud or soil were similar. The organisms are gram-negative, nonsporeforming rods with rounded ends, ranging in diameter from 0.6 to 1.0 micron and in length from 2.0 to 3.3 microns. The rods frequently showed lightly stained areas when stained with 3 per cent nigrosine, suggesting vacuolation.

Establishing that in the early stages of their life cycle the methane oxidizers are motile by means of one or more polar flagella required much patience and perseverance. The difficulty was in finding young cells for examination because from 5 to 20 days' incubation are required for the cultures to develop turbidity in liquid media or macroscopically visible growth on solid media. By this time most of the cells are nonmotile.

In older cultures the bacteria produced a melanoid pigment, eventually forming a chocolate-colored growth, echinulate at the bottom of the stroke and becoming beaded on top of potassium nitrate agar slants in the presence of an atmosphere of methane, oxygen, and carbon dioxide. When streaked on plates of such medium the bacteria formed colonies of the type described above. After 5 days' incubation at 27 C the colonies were fluid and, with further incubation, gradually became sticky and brownish in color. Deep colonies were biconvex, lens-shaped, and brown in color after 20 days. They failed to grow on nutrient agar, broth, or gelatin in the absence of gaseous hydrocarbons. (Either methane, ethane, or propane supported the growth of the bacteria in the presence of oxygen and carbon dioxide.)

In the presence of appropriate mixtures of methane, oxygen, and carbon dioxide the methane oxidizers started to render liquid medium cloudy after 4 to 10 days' incubation at 27 C. A delicate pellicle sometimes appeared in the later stages of growth. After 2 or 3 weeks the medium became less turbid and a clumpy sediment formed on the bottom of the culture vessel. In active cultures gas uptake became apparent within a day or two and continued for 2 or 3 weeks.

Ammonium salts, nitrate, glutamic acid, or peptone served as nitrogen sources. Glycine was not utilized. Certain strains of methane-oxidizing bacteria oxidized ammonia to nitrite, an observation of special significance which will be elaborated elsewhere.

The methane-oxidizing bacteria studied by Söhngen (1906), Münz (1915), Aiyer (1920), Hasemann (1927), and Tausz and Donath (1930) are too inadequately described to permit detailed comparison with our pure cultures. The latter differ from *Methanomonas methanica* (Söhngen) Orla-Jensen (Breed *et al.*, 1948) in at least two respects: the growth of *M. methanica* is described as membranous whereas all of our methane oxidizers produced a turbid growth in liquid medium and, unlike our cultures, *M. methanica* required for its growth the initial presence of no more carbon dioxide than is normally present in the atmosphere. Also unlike our methane-oxidizing bacteria the culture of Münz (1915) readily utilized as energy sources acetate, butyrate, tartrate, glycerol, mannitol, glucose, sucrose, and peptone. These and other minor differences suggest the likelihood of two or more different species being involved, a subject which will be treated separately.

It is still problematical how many bacterial species are able to oxidize methane, though certainly more than one. In this connection it is of interest to point out that considerably more than 100 microbial species representing nearly 40 genera have been shown to be able to attack higher hydrocarbons (ZoBell, 1946*b*). The methane oxidizer of Münz (1915) was said to utilize neither ethane nor ethylene, but the "methane bacterium" of Tausz and Donath (1930) allegedly oxidized

hydrogen, ethane, propane, butane, and higher paraffin hydrocarbons as well as methane. Slavnina's (1948) culture, like some of ours, utilized methane, ethane, and propane. Störmer's (1908) *Bacillus hexacarbovorum*, which utilized toluene and xylene, could also assimilate methane.

FACTORS AFFECTING METHANE OXIDATION

Oxygen tension. In order to evaluate the influence of oxygen tension on the growth and activity of methane-oxidizing bacteria, gas mixtures were prepared consisting of 20 per cent methane, 10 per cent carbon dioxide, and from 0 to 70 per cent oxygen. Nitrogen was employed as an inert diluent, when necessary, to bring the partial pressure up to 100 per cent, after having established that it was neither fixed nor evolved. Söhngen culture bottles inoculated with 1.0 ml of strain 37:146-1 were filled with the various gas mixtures and 20 ml of NH_4Cl mineral

TABLE 2

Average amount of methane consumed per day by methane-oxidizing bacteria during 6 days at 32 C in an atmosphere initially containing 20 per cent methane, 10 per cent carbon dioxide, and varying partial pressures of oxygen and nitrogen

PARTIAL PRESSURE OF		METHANE CONSUMED PER DAY BY	
Oxygen	Nitrogen	A	B
%	%	*ml*	*ml*
0	70	0.00	0.00
10	60	1.05	0.94
20	50	0.88	0.88
30	40	1.05	1.05
40	30	—	0.94
60	10	0.35	0.52
70	0	0.23	0.29

solution, thereby leaving approximately 150 ml of gas in each bottle at the beginning of the experiment. The cultures were incubated for 6 days at 32 C after which the gas volumes were measured and analyzed. Representative data from one experiment are summarized in table 2.

From such experiments the dependence of methane oxidizers upon free oxygen was established. As the oxygen partial pressure increased from 0 to 10 per cent the rate of methane consumption increased. Within the limits of experimental error methane consumption was about the same between oxygen partial pressures of 10 and 40 per cent. Increasing the oxygen partial pressure above 40 per cent resulted in a decreased rate of methane consumption. This latter observation is in agreement with findings reported by ZoBell (1946*b*), but Münz (1915) obtained the best results with only 2 per cent oxygen.

Below 10 per cent oxygen partial pressure the rate of methane consumption appears to be independent of oxygen tension until reduced to such a low level that oxygen is consumed by bacteria faster than it can be replaced by diffusion. Shoup (1929) and ZoBell (1940) have observed a similar relationship between

oxygen concentration and oxygen consumption by other bacteria. It should be obvious, however, that in spite of the influence of oxygen tension on microbial activity, oxidations dependent upon oxygen will stop when oxygen is depleted. Therefore, in order to provide for maximum activity over extended periods of time it is desirable to start with gas mixtures having the highest allowable oxygen partial pressure in closed systems. The same applies to methane partial pressures.

Methane partial pressure. The rate of methane oxidation was found to increase as the methane partial pressure was increased from 0 to 40 per cent, above which the rate was approximately the same within the limits of experimental error (table 3). In supplementary experiments it was found that methane partial pressures up to 95 per cent were well tolerated, but under these conditions oxygen and carbon dioxide become limiting factors. Similar observations were made by Münz (1915).

TABLE 3

Average amount of methane consumed per day by methane-oxidizing bacteria during 6 days at 32 C in an atmosphere initially containing 20 per cent oxygen, 10 per cent carbon dioxide, and varying partial pressures of methane and nitrogen

PARTIAL PRESSURE OF		METHANE CONSUMED PER DAY BY	
Methane	Nitrogen	A	B
%	%	*ml*	*ml*
0	70	0.00	0.00
10	60	0.33	0.40
20	50	1.00	1.00
30	40	—	0.94
40	30	1.80	1.80
60	10	1.74	—
70	0	1.86	2.04

Carbon dioxide requirements. Although there is enough carbon dioxide in ordinary air (0.03 per cent) to provide for the multiplication of some methane-oxidizing bacteria, the presence of from 5 to 10 per cent carbon dioxide in the initial gas mixture materially enhances multiplication and methane oxidation. Neither the carbonate nor the bicarbonate ion proved to be as satisfactory as free carbon dioxide for initiating the growth of methane oxidizers. Once they start to grow, from 40 to 90 per cent of the methane oxidized may be converted into carbon dioxide (table 4). Since the bacteria require carbon dioxide and utilize no carbon compound except gaseous hydrocarbons, carbon dioxide is believed to be converted into bacterial cell substances. A slight decrease in the rate of methane consumption occurred as the carbon dioxide partial pressure was increased from 20 to 30 per cent.

Oxidation-reduction potential. Experiments designed to determine the effect of initial oxidation-reduction potential on the activity of methane-oxidizing bacteria were complicated by the incompatibility of free oxygen, which is required by the bacteria, with reducing agents required to obtain low potentials. Sodium thio-

glycolate proved to be the most satisfactory reducing agent for reducing the redox potential in the presence of free oxygen. With it a series of mineral media were prepared having redox potentials ranging from E_h −46 to +322 mv at pH 6.6. The media were inoculated uniformly with culture 37:146-1 and incubated at 32 C in an atmosphere consisting initially by volume of 30 per cent methane, 60 per cent oxygen, and 10 per cent carbon dioxide. The E_h was determined with a platinum electrode and the pH with a glass electrode using a saturated calomel half cell with a Beckman meter. The values at the beginning of the experiment and after 10 days' incubation for a representative experiment are recorded in table 5 together with data on the amount of methane consumed.

TABLE 4

Methane and oxygen consumed and carbon dioxide produced by six different cultures of methane-oxidizing bacteria in 10 days at 30 C

CULTURE NUMBER	METHANE CONSUMED	OXYGEN CONSUMED	CARBON DIOXIDE PRODUCED
	ml	*ml*	*ml*
37:206- 1	14.0	24.1	8.9
2	19.7	39.6	8.2
3	18.8	39.3	7.9
6	4.1	8.0	3.0
9	7.0	12.8	5.7
10	13.8	29.3	8.1

TABLE 5

Effect of oxidation-reduction potential, expressed as E_h values at pH 6.6, of medium on the amount of methane consumed by methane-oxidizing bacteria during 10 days' incubation at 32 C

INITIAL E_h OF CULTURE		FINAL E_h OF CULTURE		METHANE CONSUMED BY CULTURE	
A	B	A	B	A	B
mv	*mv*	*mv*	*mv*	*ml*	*ml*
−46	−44	+75	+76	5	—
+72	+70	+297	+297	10	6
+95	+94	+281	+252	8	7
+226	+213	+278	+254	15	22
+322	+322	+335	+361	45	46

Although certain anomalies appeared in this and similar experiments, it is clear that methane oxidation is favored by higher potentials. It is doubtful that methane-oxidizing bacteria are active in media having negative E_h values at pH 6 to 8.

Effect of pH. The effect of pH on the activity of methane-oxidizing bacteria is illustrated by the data in figure 2. Balanced mineral salts solutions enriched with KNO_3 as a nitrogen source and 0.3 M phosphate buffers were prepared having initial pH values ranging from 4.5 to 8.3 when in equilibrium with an atmosphere containing by volume 10 per cent carbon dioxide, 30 per cent methane, and 60 per cent oxygen. The media were incubated at 27 C after being inoculated with 1.0 ml of an active liquid culture of methane-oxidizing bacteria.

Although culture 37:146-1 exhibited maximum methane consumption at around pH 6.5 and no activity above pH 8.0 under these experimental conditions, methane oxidation by other cultures has been demonstrated in medium as alkaline as pH 8.8. Large numbers of methane-oxidizing bacteria have been found in marine sediments, the pH of which ranges from 6.4 to 9.5 (ZoBell, 1946*a*).

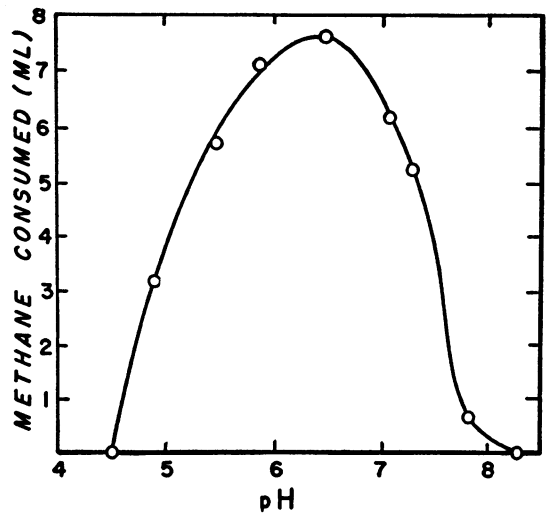

Figure 2. Amounts of methane consumed in 42 days at 27 C by methane-oxidizing bacteria in media of different initial pH values.

TABLE 6

Average amount of methane consumed by cultures of strain 37:146-1 during different periods of incubation at various temperatures

INCUBATION TEMPERATURE	METHANE CONSUMED AFTER			
	5 days	10 days	15 days	20 days
	ml	*ml*	*ml*	*ml*
5 C	0	0	0	0
15 C	0	0	1	2
22 C	2	6	21	34
27 C	3	10	28	59
32 C	3	19	59	100
37 C	2	9	23	42
40 C	0	0	0	0

Temperature. The occurrence of methane-oxidizing bacteria in sediments on the floor of the ocean where the temperature may be perpetually as low as 3 C suggests that such bacteria are active at low temperatures. Methane oxidation in the laboratory by bacteria from the sea has been demonstrated at 3 to 5 C, although the rate was extremely slow. In one experiment an incubation period of nearly 9 months was required to obtain conclusive results. All cultures examined, including those from the deep sea where low temperatures prevail, were most active at temperatures ranging from 15 to 35 C.

Culture 37:146-1 consumed methane more rapidly at 32 C than at higher or lower temperatures, as shown by the data in table 6. The data are based upon duplicate determination. Since the solubilities of gases vary with the temperature, uninoculated controls were incubated at each temperature to correct for this factor. Münz (1915) observed maximum activity of methane-oxidizing bacteria at 30 to 36 C, the limits being 18 and 40 C.

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SUMMARY AND CONCLUSIONS

Methane-oxidizing bacteria appear to occur quite commonly in the topmost layers of marine sedimentary materials and in soil, being particularly abundant where methane and free oxygen are present. Although methane is attacked less readily and by fewer microbial species than higher hydrocarbons, presumably because the latter have more vulnerable points and are thermodynamically less stable than methane, *Methanomonas methanica* (Söhngen) Orla-Jensen (Breed *et al.* 1948) is not the only species endowed with the ability to oxidize methane.

Pure cultures of methane-oxidizing bacteria, isolated from marine materials, are described that failed to utilize peptone, glucose, sucrose, starch, or glycerol as carbon sources. In purely mineral medium, ethane and propane as well as methane were assimilated by some cultures as the sole source of energy. Carbon dioxide was required for the initiation of growth. Ammonium salts, nitrate, peptone, or glutamic acid served as nitrogen sources. Some strains formed nitrite from the oxidation of ammonia.

The growth of the bacteria was accompanied by the disappearance of methane, the consumption of oxygen, and the formation of carbon dioxide. The medium became turbid and it is calculated from the number of cells present that an equivalent of from 10 to 40 per cent of the carbon from the methane oxidized was converted into bacterial cell substances.

Partial pressures producing the most rapid utilization of methane were oxygen 10 to 40 per cent, methane up to 70 per cent, and carbon dioxide 5 to 20 per cent. An atmosphere consisting by volume of 10 per cent carbon dioxide, 40 per cent oxygen, and 50 per cent methane is recommended for cultivating methane-oxidizing bacteria.

The optimum temperature, pH, redox potential, and osmotic pressure seem to be partly a function of the habitat and history of the organisms. Those studied in this investigation were most active at 32 C, pH 6 to 7, and $E_h > +30$ mv.

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