A comparison of some methane-oxidizing bacteria
by John LeRoy Johnson

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology
Montana State University
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Abstract:
Three methane-oxidizing bacteria were isolated during the study. Cultures 105 and 106, isolated from local soil, were acid-fast, gram-positive rods which appeared to be similar to Mycobacterium phlei. Cultures 105 and 106 showed weak utilization of methane, ethane, propane, and butane. Culture 107, isolated from a film on the surface of the water in a carboy containing methane, was a gram-negative rod, similar to Pseudomonas methanica (Dworkin and Foster, J. Bacteriol., 72, 646-659, 1956). Culture 107 exhibited rapid oxidation of methane but was unable to utilize ethane, propane, or butane.

A culture of P. methanica was obtained from Dr. J. W. Foster of the University of Texas and compared with culture 107. The organisms were similar in their cell morphology, pigmentation and the fact that they utilized only methane and methanol as sources of carbon. The organisms differed in some cultural characteristics and accessory growth factor requirements. Culture 107 grew in high oxygen concentrations which were inhibitory to P. methanica.

Culture 107 was capable of using (NH4)2SO4, NaNO3, glutamic acid and tryptone as sources of nitrogen but was not able to use NaNO2 or glycine.

The optimum pH range for the growth of culture 107 was from 6.5-8.5 when the pH values were determined at the time of inoculation. During incubation the pH dropped approximately 0.8 pH units in each flask.

Results obtained from a gas mixture experiment on culture 107 indicated the presence of a complex system in which the maximum growth of the culture was dependent upon the actions and interactions of the component gases, oxygen, methane and carbon dioxide. Results of this experiment also indicated that by using the proper gas mixture an appreciable amount of carbon dioxide was utilized by the organism.
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The author wishes to thank Dr. J. W. Foster (University of Texas) for supplying a culture of Pseudomonas methanica.
ABSTRACT

Three methane-oxidizing bacteria were isolated during the study. Cultures 105 and 106, isolated from local soil, were acid-fast, gram-positive rods which appeared to be similar to Mycobacterium phlei. Cultures 105 and 106 showed weak utilization of methane, ethane, propane, and butane. Culture 107, isolated from a film on the surface of the water in a carboy containing methane, was a gram-negative rod, similar to Pseudomonas methanica (Dworkin and Foster, J. Bacteriol., 72, 646-659, 1956). Culture 107 exhibited rapid oxidation of methane but was unable to utilize ethane, propane, or butane.

A culture of P. methanica was obtained from Dr. J. W. Foster of the University of Texas and compared with culture 107. The organisms were similar in their cell morphology, pigmentation and the fact that they utilized only methane and methanol as sources of carbon. The organisms differed in some cultural characteristics and accessory growth factor requirements. Culture 107 grew in high oxygen concentrations which were inhibitory to P. methanica.

Culture 107 was capable of using (NH_4)_2SO_4, NaNO_3, glutamic acid and tryptone as sources of nitrogen but was not able to use NaNO_2 or glycine.

The optimum pH range for the growth of culture 107 was from 6.5-8.5 when the pH values were determined at the time of inoculation. During incubation the pH dropped approximately 0.8 pH units in each flask.

Results obtained from a gas mixture experiment on culture 107 indicated the presence of a complex system in which the maximum growth of the culture was dependent upon the actions and interactions of the component gases, oxygen, methane and carbon dioxide. Results of this experiment also indicated that by using the proper gas mixture an appreciable amount of carbon dioxide was utilized by the organism.
INTRODUCTION

Early interest in the oxidation of methane was prompted by the observation that methane, produced during the fermentation of organic matter, failed to accumulate in the atmosphere. Because large quantities of methane were produced in marshes and stagnant water, it was postulated that water plants were responsible for methane disappearance. Bacteria were found to be the organisms actually responsible.

The first methane-oxidizing bacterium to be isolated was named Bacillus methanicus. The name was later changed to Methanomonas methanica and recently the name Pseudomonas methanica has been proposed.

One of the present interests in methane-oxidizing bacteria centers on their use in petroleum prospecting.

The purpose of this study was to obtain preliminary information about methane-oxidizing bacteria. This information will be used in studying the oxidation of methane, as a biochemical system, in soil research studies under way at Montana State College.
REVIEW OF LITERATURE

Methane-Oxidizing Bacteria

One of the earliest observations of biological hydrocarbon utilization was made by Miyoshi (1895) in studies on the fungi Botrytis cinerea and Penicillium glaucum. The fungi were capable of utilizing paraffin. Since Miyoshi's observation, bacteria capable of utilizing hydrocarbons ranging from methane to paraffinic waxes have been observed and described.

Kaserer (1906) observed the oxidation of methane by microorganisms, which he obtained by inoculating a sterile mineral salts medium with soil. He did not give descriptions of any organisms. Sohngen (1906b) investigating Kaserer's reports, hypothesized that since the methane forming process is anaerobic, occurring in stagnant water, methane was oxidized by plants found in stagnant water. While testing the various water plants, he observed that methane was utilized only when a reddish-brown film, composed of microorganisms, formed on the surface of the water in the experimental flasks. Sohngen described the methane-oxidizing bacteria as having the forms of short, thick gram-negative rods, 2-3μ by 4-5μ in crude cultures and 1.5-2μ by 2-3μ in pure cultures. In older cultures the organisms were shorter, becoming coccoid. The bacteria were motile only in very young cultures and were supplied with a single flagellum fixed in the slime layer. He named the bacterium Bacillus methanicus. Orla-Jensen (1909) renamed Sohngen's Bacillus methanicus as Methanomonas methanica.
Münz (1915) isolated a methane-oxidizing bacterium which he named *Bacterium methanicum*. It formed a film of grayish-white hue on mineral salts-methane medium. The bacterium was rod-shaped, being 0.3-0.4μ by 0.9-2.2μ in size. Chains frequently occurred and motility could not be observed.

Giglioli and Masoni (1917) observed the presence of "the methane organisms of Kaserer and Söhngen" (Kaserer, 1906; Söhngen, 1906b) in field and meadow soils, river mud, manures, and sewer contents.

Tausz and Donath (1930) isolated a methane bacterium which attacked the higher as well as the lower aliphatic hydrocarbons, ranging from methane to paraffin oil. It could also attack the unsaturated hydrocarbons propylene, butylene and possibly ethylene, but not the cyclic hydrocarbons benzene and cyclohexane.

Slavnina (1948) classified as *Methanomonas methanica*, bacteria which could utilize ethane and propane in addition to methane. Slavnina applied a fluorescence method to the study of hydrocarbon-utilizing bacteria. *Methanomonas methanica* showed no fluorescence.

Bokova et al., (1947) classified as *Methanomonas methanica*, methane-oxidizing bacteria which failed to utilize ethane or propane, but were capable of utilizing pentane, hexane and heptane.

Nechaeva (1949) described two methane-oxidizing bacteria, *Mycobacterium flavum* var. *methanicum* and *Mycobacterium methanicum* n. sp. *M. flavum* var. *methanicum* oxidized methane, propane and heptane and was pigmented yellow.
M. methanicum oxidized methane (more readily than M. flavum var. methanicum) and propane and produced no pigment. Both bacteria showed no fluorescence in ultraviolet light.

Hutton and ZoBell (1949) isolated methane-oxidizing bacteria from marine sediments. The bacteria were gram-negative, non-sporeforming rods, motile in early stages by one or more polar flagella. Hutton and ZoBell (1953) found that some of the bacterial cultures isolated from marine and soil samples oxidized ethane, ethylene and propane as readily as methane. A few oxidized butane more readily than any other gaseous hydrocarbons, but others failed to attack any except methane. The cells were all gram-negative, non-sporeforming rods, and a few had a single polar flagellum.

Strawinski and Tartorich (1955) isolated methane-oxidizing bacteria which grew well on nutrient agar when in the presence of methane.

Strawinski and Brown (1957) isolated methane-oxidizing bacteria producing macrocolonies which upon closer examination, were found to be contaminated with a microcolonizing methane oxidizer. The latter was a non-sporeforming, motile rod which stained unevenly gram-positive and was non-acid-fast.

Dworkin and Foster (1956) isolated a methane-oxidizing bacterium by methods similar to those used by Sohngen (1906b) and obtained a pink isolate similar to his. This they assumed to be of the same species as Methanomonas methanica (Sohngen) and changed the name to Pseudomonas methanica (Sohngen) nov. comb.

Leadbetter and Foster (1957) isolated methane-oxidizing bacteria, which
they recognized as at least four new species, distinguishable by characteristic pigmentation.

**Physiology of Methane-Oxidizing Bacteria**

Ever since the observation of hydrocarbon utilization, the metabolic mechanism by which organisms utilize hydrocarbons has been of interest to workers in the field. ZoBell (1945) gives the following generalizations:

"(1) aliphatic or paraffinic compounds are oxidized more readily than corresponding aromatic or naphthenic compounds, (2) long chain hydrocarbons are more susceptible than those of shorter chain length, (3) branched chain or iso-compounds are oxidized more readily than straight chain or normal hydrocarbons, (4) unsaturated hydrocarbons are more readily oxidized than saturated, however cyclohexane is more easily oxidized than benzene."

Methane has been found to be one of the more difficult of the hydrocarbons to utilize and its utilization is limited to oxidation by a much more restricted group of bacteria.

Söhnge (1906b and 1910) postulated the mechanism by which methane is utilized by *Bacillus methanicus* as follows:

\[
\text{CH}_4 + 2 \text{O}_2 \rightarrow \text{CO}_2 + 2 \text{H}_2\text{O}
\]

Apparently because of this mechanism he classified the organism as an autotroph. However Hutton (1948) reports that at the turn of the century, methane was considered by some as an inorganic compound.

Slavnina (1947) reported the existence of a peroxidase in *Methanomonas methanica* and this was found to be the only hydrocarbon-utilizing organism, among those he tested, to possess this enzyme.
Brown and Strawinski (1957) found that resting cells of methane-oxidizing bacteria were unable to oxidize any of a number of organic substrates except methanol, formaldehyde, formate and ethanol (slightly). By the use of sulfite as a trapping agent, they were able to recover formaldehyde from methanol oxidation. They postulated that the production of CO₂ from methane occurs according to the following scheme:

\[
\text{CH}_4 \rightarrow \text{CH}_3\text{OH} \rightarrow \text{HCHO} \rightarrow \text{HCOOH} \rightarrow \text{CO}_2
\]

Leadbetter and Foster (1958) recognized two modes by which methane-oxidizing bacteria may convert methane-carbon to cell-carbon: "(1) dehydrogenative oxidation to CO₂ followed by autotrophic assimilation of the CO₂ by means of the 'active' hydrogen produced during the oxidation, (2) oxidation of methane to one or more oxidation levels short of CO₂ and direct heterotrophic assimilation of carbon at those levels." Four strains of Pseudomonas methanica were cultivated in a closed atmosphere containing unlabeled CH₄ and C¹⁴O₂. At maximal growth, the specific radioactivities of the cell-carbon and of the CO₂-carbon were measured. In every case the specific radioactivity of the cell-carbon was considerably less than the specific radioactivity of the CO₂-carbon, indicating that in all probability the cell-carbon did not originate via CO₂-carbon exclusively, and therefore came by direct assimilation of methane-carbon at an oxidation level below CO₂. From this study they concluded that methane-oxidizing bacteria are heterotrophic.

Brown and Strawinski (1958) found that during methane metabolism by resting cell suspensions of Methanomonas methanooxidans in the presence of
iodoacetate, a considerable quantity of methanol was produced. By employing sodium sulfite as a trapping agent, the major portion of the methane consumed was shown to be present terminally as formaldehyde. In the absence of a blocking agent, a significant quantity of formic acid accumulated in the test solution.

Leadbetter and Foster (1959) showed that resting cells of *Pseudomonas methanica* could oxidize ethane to ethanol and acetic acid. When added to methane growth cultures, propane was oxidized to n-propanol and propionic acid, butane was oxidized to n-butanol and n-butyric acid. The use of deuterated ethane established that a methyl group of ethane remained intact during the conversion of ethane to acetate. Thus, a dehydrogenation of ethane to ethylene is eliminated as an intermediate step in the oxidation.

Harrington and Kallio (1960) showed that *Pseudomonas methanica* was capable of utilizing methanol for growth, but not formaldehyde and formate. Resting cells of *P. methanica* were capable of oxidizing all three. Manometric experiments, using crude enzyme preparations, failed to demonstrate either methanol oxidation or methanol dehydrogenase activity. Methanol oxidation, by crude enzyme preparations, was demonstrated when hydrogen peroxide was present in the system, indicating an alcohol peroxidase enzyme system.

\[
\text{CH}_3\text{OH} + \text{H}_2\text{O}_2 \xrightarrow{\text{alcohol peroxidase}} \text{HCHO} + 2 \text{H}_2\text{O}
\]

Formaldehyde oxidation experiments, on *P. methanica*, indicated that oxidation was via a dehydrogenase enzyme system. Both intact cells and the crude extract decolorized methylene blue in the presence of formaldehyde. Extracts,
partially purified by centrifugation, reduced diphosphopyridine nucleotide (DPN) in the presence of formaldehyde when glutathione (GSH) was also present.

\[
\text{HCHO} \xrightarrow{\text{DPN}} \text{HCOOH} \text{, } \text{DPNH} \xrightarrow{\text{H}^+} \text{(GSH)}
\]

The ability of methane-utilizing bacteria to utilize as carbon sources, carbohydrates, alcohols, proteins, and salts of organic acids has been reported while other workers have not been able to substantiate it. Sohngen (1906a) reported that *Bacillus methanicus* grew on malt extract, nutrient broth, asparagine, malate, lactate, citrate, succinate, glucose, sucrose, and lactose. Münz (1915) reported that *Bacterium methanicum* utilized a number of salts of organic acids, alcohols and carbohydrates. Nechaeva (1949) reported *Mycobacterium flavum* var. *methanica* and *M. methanicum* as being heterotrophic. Hutton and ZoBell (1953) found that although their methane-oxidizing bacteria could not utilize as a sole source of carbon, a number of alcohols, organic acids and carbohydrates, some of these substances seemed to stimulate pure cultures when grown in methane. But in crude cultures the normal heterotrophs would take over. Five micro-grams per liter of thiamin, pyridoxine, riboflavin and nicotinamide showed a stimulating effect, especially when small inocula were used. Dworkin and Foster (1956) reported *Pseudomonas methanica* as not being capable of utilizing any organic compounds other than methane and methanol, although agar extract and calcium pantothenate were needed as growth factors. Leadbetter and Foster (1957) isolated a methane-oxidizing bacterium which could grow on nutrient agar, when in the presence of methane.
Cultural Conditions

The cultural conditions to which methane-oxidizing bacteria have been subjected in the laboratory have been varied as to medium, content of methane, oxygen and carbon dioxide in the gaseous atmosphere, pH, temperature, and aeration.

The most common type of basal medium which has been used has been composed of mineral-salts and distilled water (Kaserer, 1906; Söhngen, 1906b; Hutton and ZoBell, 1953). Dworkin and Foster (1956) used a complex medium composed of mineral salts and distilled water supplemented with the growth factors agar extract and calcium pantothenate.

The gaseous atmosphere percentages of methane and carbon dioxide have not been reported to be critical, but the percentage of oxygen, at times, has been reported as critical. Söhngen (1906b) cultivated *Bacillus methaniconus* in the presence of an atmosphere composed of 1/3 methane and 2/3 air. Münz (1915) obtained optimal growth when the gas mixture consisted of 90 per cent methane and 10 per cent air or 98 per cent methane and 2 per cent oxygen. Hutton and ZoBell (1949) reported optimal growth when the initial gas mixture consisted of from 40-70 per cent methane, 30-40 per cent oxygen and 5-10 per cent carbon dioxide. Dworkin and Foster (1956) reported equally good growth when the methane content of the gas atmosphere varied from 10-90 per cent. They found oxygen to be toxic when at a concentration of 21 per cent with its optimal concentration being 15 per cent. Optimum growth occurred when the initial carbon dioxide content of the atmosphere was 0.3 per cent.
The pH optima, for methane utilizing bacteria, have not been reported within a precise range. Beerstecher (1954) in reviewing the literature, found that the optimal pH for hydrocarbon consumption, might not be the optimal pH in terms of product yield. ZoBell (1945) reported an optimal pH for methane-oxidizers in marine sediments ranging from 6.4-9.5. Hutton and ZoBell (1949) reported the optimal pH of 6.5 for a methane-oxidizer. Strawinski and Brown (1957) reported a pH optimum of 6.1 for methane oxidation. Dworkin and Foster (1956) found that with Pseudomonas methanica, using (NH₄)₂SO₄ as a nitrogen source, the pH range was 6.0-6.6, whereas when NaNO₃ was used as the nitrogen source, the pH range was from 6.6-8.0.

A temperature of 23-37 C has been used in most studies on methane utilizing bacteria (Söhngen, 1906b; Yurovskii et al., 1939; Strawinski and Brown, 1957; Giglioli and Masoni, 1917; and Harrington and Kallio, 1960). Hutton and ZoBell (1949) demonstrated oxidation at 3-5 C but the organism was most active from 15-30 C.

Dworkin and Foster (1956) found stationary cultures superior to those incubated on a shaker for primary enrichment cultures. The stationary cultures allowed the surface pellicle, characteristic of their methane oxidizing organism, to form. Harrington and Kallio (1960) incubated their organism on a gyratory shaker.

Economic Importance

Methane oxidation by bacteria has been implicated as the mechanism by which methane, produced by anaerobic degradation of organic matter in soil, is conserved as organic matter in the soil. (Giglioli and Masoni, 1917 and
Beerstecher, 1954)

Yurovskii et al., (1939) applied *Bacillus methanicus*, in the form of a special paste, onto the rocks of a coal mine as a method of destroying methane in the mine.

Methane-oxidizing bacteria have been used to some extent in biological prospecting for oil. The method is based on the theory that the more volatile hydrocarbons, and especially methane, are capable of seeping from the crude oil and gas deposits through the substrata to the surface soil layers. The presence of methane-utilizing bacteria may therefore be an indication of oil and gas deposits. Mogilevskii (1940) found that the use of methane-oxidizers as indicators for gas and oil deposits may be complicated by the fact that cellulose-decomposing bacteria produce methane during the decomposition of cellulose. Thus the methane may be from other sources than oil and gas deposits. Mogilevskii, in trying to eliminate this problem, simultaneously ran determinations for both cellulose-decomposing and methane-oxidizing bacteria. The samples then considered as indicating possible petroleum deposits were the ones positive for methane-oxidizing bacteria and negative for cellulose-decomposing bacteria. This method met with some success, according to Mogilevskii.

Bokova et al., (1947) inoculated samples of soil, obtained at a depth of 2 meters from a gas survey bore hole, with *Methanomonas methanica* and passed subsoil air obtained from the bore hole through the inoculated soil samples. At the beginning and at the end of the experiment, the concentration of light fractions in the subsoil air and the content of the bacteria
which oxidize methane were determined.

Several patents have been issued in the United States dealing with methods for the biological prospecting for oil. Sanderson (1942) determined the amount of hydrocarbons in soil gases by exposing the gases to pure cultures of hydrocarbon utilizing bacteria and measuring the bacterial growth. Blau (1942 and 1943) obtained patents for prospecting methods involving the determinations of products, in the soil, of certain hydrocarbon-consuming bacteria. Strawinski (1954) incubated aliquots of soil samples with a gas mixture comprising a gaseous aliphatic hydrocarbon and oxygen. A mineral salts medium was added in such an amount as to eliminate variables in soil moisture, nutrients and hydrogen ion concentration.

Subbota (1947) showed that seasonal climatic variations in a subtropical arid climate greatly affected the population of methane-oxidizing bacteria and that the results of bacterial analysis may therefore be inconclusive for oil prospecting.

Pape and Hansen (1950) reported obstruction of filters due to the presence of methane in water and the formation of slimes due to protozoa and such bacteria as *Methanomonas methanica*. The slime formation could be prevented by aerating the water to remove methane.
MATERIALS AND METHODS

Hydrocarbon Source and Purification

The hydrocarbons used in the study were methane, ethane, propane, and butane. The methane and ethane were obtained from the natural gas which is piped into the laboratory. The propane and butane were supplied by the Gallatin Farmers Company, Bozeman, Montana.

The hydrocarbons were purified in a low temperature distillation apparatus which is shown in figure 1. The hydrocarbons were condensed in a 48 by 200 mm side-arm test tube which was suspended in a Dewar flask containing a condensing agent (table 1) cold enough to liquefy the desired hydrocarbon. After enough hydrocarbon condensate had collected, the side-arm test tube and both purifying traps were suspended in Dewar flasks containing a distilling agent (table 1) which was warm enough to allow the boiling of the desired hydrocarbon but not its next higher homologue. The vaporized hydrocarbon passed over the mercury safety valve, through the first and second purifying traps, through the water trap and was collected over water in an inverted carboy.

The purifying traps were designed to condense traces of the hydrocarbon's higher homologues which might have vaporized with it. Because some of the distilling agents were freezing mixtures, which had to be kept cold by the addition of liquid air, there was the possibility of a temporary negative pressure in the system after such an addition. The water trap was needed to prevent the water in the carboy from entering the second purifying trap under negative pressure conditions.
### TABLE 1

Hydrocarbon condensing and distilling agents

<table>
<thead>
<tr>
<th>Hydrocarbon</th>
<th>Condensing or retaining agent</th>
<th>Distilling agent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Boiling Name point (°C)</td>
<td>Name Temp. (°C)</td>
</tr>
<tr>
<td>Methane</td>
<td>-161.50</td>
<td>Liquid air ca -190</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Freezing n-propyl alcohol -127</td>
</tr>
<tr>
<td>Ethane</td>
<td>-88.30</td>
<td>Dry ice in acetone ca -78</td>
</tr>
<tr>
<td>Propane</td>
<td>-42.17</td>
<td>None</td>
</tr>
<tr>
<td>Butane</td>
<td>-0.6 to -0.3</td>
<td></td>
</tr>
</tbody>
</table>

Pipe line natural gas, containing methane and ethane, was condensed in the side-arm test tube, using liquid air as the condensing agent. Placing the side-arm test tube in a Dewar flask containing freezing n-propyl alcohol allowed the methane to vaporize but retained the ethane. After the methane had boiled off, the side-arm test tube was placed in a Dewar flask containing a mixture of dry ice and acetone which allowed the ethane to vaporize.

Commercial household grade propane was purified by the same general procedure.

The commercial household grade butane was poured directly from the cylinder into the side-arm test tube, which was suspended in a Dewar flask containing the distilling agent.
Figure 1. Low temperature distillation apparatus.
Culture Apparatus

Four types of culture apparatus were used in the study. Diagrams of the apparatuses are shown in figures 2 and 3. The flasks were 125 ml Erlenmeyer flasks, selected because of their convenient size and ability to fit on the rotary shaker. They were fitted with number 5 rubber stoppers.

Routine culturing and the determination of an optimum pH were carried out in culture flasks such as is shown in figure 2A. The culture flask's stopper assembly was also used as the means by which the culture flasks in figures 2B and 2C were filled with gas. The culture flask was fitted with a rubber stopper, through which passed a 6.5 cm section of 4 mm bore glass tubing. A constriction was placed in the glass tubing to prevent the cotton plug, placed above the constriction, from being drawn into the flask while it was being filled with gas. The flask was closed by placing either a vaccine bottle cap or a short piece of rubber tubing, closed with a pinch clamp, over the open end of the glass tubing.

The manometer flask (figure 2B) was used for the detection of methane-oxidizing bacteria in crude cultures. The flask was fitted with a rubber stopper through which passed a manometer. The water-filled manometer shaft was an 18-20 cm section of 3 mm bore glass tubing. The air-filled manometer bulb was made from a 5 cm section of 6 mm bore glass tubing and was connected to the manometer shaft with a short piece of rubber tubing. The manometer shaft was filled with distilled water by removing the bulb, placing the stopper end into a beaker of distilled water and drawing the water into the manometer shaft as one would draw water into a pipette. When the manometer
Figure 2. A. Culture flask. B. Manometer flask. C. Modified two-flask Söhngen apparatus.
Figure 3. Modified three-flask Söhngen apparatus.
shaft was full, the water was prevented from running out by placing a finger over the stopper end of the shaft, until the manometer bulb was put in place.

For obtaining higher concentrations of the methane-oxidizing bacteria from the primary enrichment cultures, and for quantitatively measuring the amount of the gas mixture utilized, a two-flask modification of Söhngen's (1906b) apparatus, shown in figure 2C, was used. The culture flask was connected to the reservoir flask by 4 mm bore glass and rubber tubing. Air entered the reservoir flask through a drying tube which was filled with cotton. As gas was utilized, sterile medium was drawn from the reservoir flask into the culture flask. The culture flask was filled with the gas mixture as described for figure 2A.

A three-flask modification of Söhngen's apparatus, figure 3, was used for studying cultural characteristics and for determining gas utilization. The advantage of this apparatus was that the culture was not continually being diluted with sterile culture medium as was the case with Söhngen's apparatus (1906b). The apparatus consisted of a culture flask, a gas reservoir flask and a water reservoir flask, connected by means of glass and rubber tubing. The stopper of the culture flask contained a section of cotton-plugged glass tubing, identical to that in figure 2A, and was used to fill the system with the gas mixture. The culture flask was connected to the gas reservoir flask by a shallow inverted U tube constructed from two sections of 4 mm bore glass tubing and a section of rubber tubing. The glass section of the U tube entering the culture flask contained a cotton
plug which filtered the gas as it entered from the gas reservoir flask. The culture flask was the only sterilized flask in the system. The gas and water reservoir flasks were connected by an inverted U tube, constructed in the same manner as the first, but extended to the bottoms of the flasks. As the gas mixture was utilized, water was drawn from the water reservoir flask into the gas reservoir flask. Air entered the water reservoir flask through a section of 4 mm bore glass tubing.

Methane-oxidizing bacteria were also cultivated in petri dishes and in test tubes, placed in vacuum desiccators which were then filled with a gas mixture.

Culture Gassing Apparatus

The culture flasks, up to 10 at a time, were filled with a gas mixture from a glass manifold (figure 4). The culture flasks were attached at the manifold outlets. With the gas outlet of the gas supply flask closed with a pinch clamp, a vacuum was pulled with a water aspirator. The pressure was lowered by about 610 mm of mercury as measured with an open end, U shaped, mercury manometer. When the proper vacuum had been obtained, the rubber tubing leading to the aspirator was clamped and the pinch clamp closing the gas supply outlet was released so that the gas mixture would slowly flow through the manifold into the culture flasks. As the gas mixture left the gas supply flask, it was replaced with water from the water supply bottle. The water supply bottle was situated higher than the gas supply flask, so that after the gas had been admitted, the water would
Figure 4. Culture gassing apparatus.
exert a positive pressure of about 30 mm of mercury on the manifold system. The positive pressure was maintained for 10 minutes to insure that all of the flasks would be filled to the same pressure. While under positive pressure, the manifold outlet to each flask was closed with a pinch clamp. When the culture flasks were removed from the manifold, to be closed with a vaccine bottle cap, the pressure of the culture flasks was reduced to that of the atmosphere by gas escaping from the flasks. This also reduced the possibility of air entering the flasks.

The vacuum desiccators were filled with gas by attaching the desiccator outlet to the manometer and aspirator in the same manner as was the manifold. When the proper vacuum was reached, the desiccator outlet was closed, disconnected from the manometer and aspirator and connected, by way of a cotton filter, to the gas outlet of the gas supply flask. The desiccator outlet was opened and the pinch clamp on the gas outlet opened, releasing the gas mixture into the desiccator.

Media

The mineral-salts media used in the study were those recommended by Dworkin and Foster (1956) and Temple (1960). Temple's medium was designated as no. 5 medium and had the following composition:

\[
\begin{align*}
K_2HPO_4 & : 1.0 \text{ g} \\
MgSO_4\cdot7H_2O & : 0.5 \text{ g} \\
NaCl & : 0.1 \text{ g} \\
CaCl_2 & : 0.1 \text{ g} \\
(NH_4)_2SO_4 & : 1.0 \text{ g} \\
FeSO_4 & : \text{trace} \\
\text{Distilled water} & : 1 \text{ liter} \\
\text{Agar (for solid medium)} & : 15 \text{ g}
\end{align*}
\]
The sugars and the salts of organic acids were sterilized, in 10 per cent solutions, by autoclaving them at 10 pounds pressure for 20 minutes. The anhydrous methanol, 95 per cent ethanol and n-propanol were not sterilized but were removed from their containers aseptically. The sugars, salts of organic acids and alcohols were added to the mineral-salts medium to give a final concentration of 1 per cent.

Gases, in addition to the hydrocarbons, used in the study were oxygen, carbon dioxide and nitrogen, which were obtained in cylinders. The nitrogen contained 5 per cent carbon dioxide which was removed by passing the gas through a column containing sodium hydroxide solution. The component gases of each gas mixture, used for culturing the bacteria, were collected over water in calibrated 2 liter Erlenmeyer flasks. A gas mixture of 45 per cent methane, 45 per cent oxygen and 10 per cent carbon dioxide was used for the isolation and culturing of the methane-oxidizing bacteria, unless otherwise stated.

**Chemical Determinations**

Determinations of pH were made with a Beckman model GS pH meter.

Growth was measured by a wet-combustion carbon analysis, using a Van Slyke blood gas apparatus (Van Slyke and Folch, 1940; and Van Slyke et al., 1951). In the Van Slyke procedure, organic material is oxidized by an acid mixture and the resulting carbon dioxide is measured manometrically. The entire contents of a culture flask were concentrated by centrifugation, transferred to a combustion tube and dried before analysis.
Carbon dioxide production in the culture flasks was measured by connecting these flasks to the Van Slyke apparatus. The carbon dioxide could then be absorbed and measured directly.

Isolation of Methane-Oxidizing Bacteria

Attempts to isolate methane-oxidizing bacteria from soil were carried out by inoculating 10 gram soil samples into manometer flasks containing 40 ml of no. 5 mineral-salts medium. The manometer flasks were filled with the gas mixture and incubated at room temperature on a rotary shaker. Methane oxidation was indicated by the lowering of the water meniscus in the manometer shaft. Mineral-salts agar plates, of no. 5 medium, were then streaked from manometer flask cultures showing methane oxidation. Although methane oxidation was obtained in the manometer flask cultures, the author failed to isolate any methane-oxidizing bacteria. However, two gram-negative bacteria were isolated and for a time were thought to be methane-oxidizers. This view was abandoned when inoculated control plates were incubated in the absence of methane. The control cultures grew as well as the cultures grown in the presence of methane.

Dr. Temple furnished some mineral-salts agar plates which had been inoculated with soil and incubated, for a long period of time, in an atmosphere containing methane. Growth was washed off the plates with sterile distilled water and inoculated into manometer flasks containing 40 ml of no. 5 medium. The manometer flasks were filled with the gas mixture and incubated at room temperature on the rotary shaker. From the manometer-
flask cultures showing methane utilization, 2 ml volumes were inoculated into culture flasks of the modified two-flask Schüngen apparatuses (figure 2C) containing 40 ml of no. 5 medium. Each reservoir flask contained 100 ml of sterile no. 5 medium. The culture flasks were filled with the gas mixture and the apparatuses incubated at room temperature on the rotary shaker. Gas utilization ceased when approximately 80 ml of the sterile medium had been drawn into a culture flask. The culture medium was then decanted until about 40 ml remained in the flask. The reservoir flask was again filled with sterile medium. The culture flask was filled with the gas mixture and the apparatus again incubated on the rotary shaker. This procedure was repeated several times with the idea of obtaining populations in which methane bacteria were predominant. No. 5 mineral-salts agar and nutrient agar plates were then streaked from the above cultures. The mineral-salts agar plates were incubated in the gas mixture and the nutrient agar plates were incubated in air, in a 30° C incubator. The author was again unable to isolate any methane-oxidizing bacteria from the mineral-salts agar plates but was able to pick predominant colony types from the nutrient agar plates. By restreaking these colonies, he was able to isolate two gram-positive, acid-fast bacteria which gave indications of weak methane utilization.

Material from a whitish film, which was on the surface of the water in a carboy containing methane, was inoculated into manometer flasks. Growth in the manometer flasks was of a pink color and upon inoculating no. 5 mineral-salts agar plates from the manometer flask cultures, a gram-negative
methane-oxidizing bacterium was isolated which formed pink colonies.

**Purification of Methane-Oxidizing Bacteria**

Two methods were used for the purification of the methane-oxidizing bacteria. The methods were picking and restreaking isolated colonies and streaking from serial dilutions.

Isolated colonies of the gram-positive, acid-fast bacteria were picked from nutrient agar plates and restreaked on nutrient agar plates. Nutrient broth cultures were diluted by serial dilutions and streaked on nutrient agar plates.

Isolated colonies of the methane-oxidizing bacterium which formed pink colonies were picked from no. 5 mineral-salts agar plates which had been incubated in the presence of methane and restreaked on mineral-salts agar plates and again incubated in the presence of methane. Isolated colonies were suspended in sterile water and after serial dilution streaked on mineral-salts agar plates which were incubated in the presence of methane.

**Pure Culture Criteria**

The general criteria which were used in the determination of the purity of cultures were the homogeneity of colonies on nutrient and mineral-salts agar plates and the finding of microscopically homogeneous gram-stains and cellular morphology.

The acid-fast, gram-positive isolates were considered pure when they met these general criteria.

The absence of growth on nutrient agar plates, when inoculated from
cultures grown in the presence of methane, was used as an additional criterion of purity for the gram-negative methane-oxidizer.
EXPERIMENTS AND RESULTS

Descriptions of Methane-Oxidizing Bacteria

The organisms isolated were designated 105, 106 and 107. Only isolate 107 utilized methane at a rate fast enough to allow more detailed experiments on this substrate.

Isolate 107, the methane-oxidizing bacterium which produced pink growth, was a gram-negative rod which did not form spores and was not encapsulated. The average size of the cells was 0.6μ by 1.6μ. The majority of the cells occurred singly but occasionally pairs and short chains were found. The cells were actively motile by means of a single polar flagellum. When the bacterium was grown on no. 5 mineral-salts agar slants, incubated in the presence of methane, smooth, pink, filiform growth occurred. Colonies from mineral-salts agar streak plates, grown in the presence of methane, were circular, entire and convex. The average size of colonies in 7 day old cultures was 1 mm in diameter. Although the colonies were smooth and looked as if they might be mucoid, they proved to be hard when touched with a needle. Liquid cultures, incubated on the rotary shaker, produced pink flocculent growth which settled to the bottom of the culture flask as soon as shaking was stopped. Growth in stationary flasks consisted of a pink film growing on the surface of the medium.

The acid-fast, gram-positive isolates 105 and 106 were very similar to each other. Morphological differences in the type of growth could be detected; 105 produced dry wrinkled growth and 106 produced smooth mucoid colonies. The cellular morphology was identical.
Both isolates, 105 and 106, were acid-fast, gram-positive rods which did not produce spores and were not encapsulated. The cells were irregular in length, with an average size of 1.0μ by 2.5μ. The majority of the cells occurred singly but occasionally pairs and short chains were found. Motility could not be detected by the hanging drop method. The organisms formed a membrane on the surface when grown in liquid medium. Gelatin was not liquefied and litmus milk was not visibly altered, although both supported growth. The organisms were capable of reducing nitrates to nitrites. Young nutrient agar cultures were light orange in color becoming salmon pink in older cultures. Mineral-salts agar plates, incubated in the presence of methane, produced punctiform colonies which were colorless in young cultures and became orange in older cultures.

Culture 105, when grown on nutrient agar plates, produced erose rough colonies. Grown on nutrient agar slants, it produced rough filiform to echinulate growth. The average colony size was approximately 1 mm in diameter.

Culture 106 produced circular, entire, convex to pulvinate, smooth mucoid colonies when grown on nutrient agar plates. The approximate colony size was 1 mm in diameter.

**Carbon Sources of Methane-Oxidizing Bacteria**

Numerous organic compounds were tested for their ability to serve as carbon sources for the methane-oxidizing bacteria.

Bacterial growth, as well as the production of acid and gas, was checked
on the various sugars, salts of organic acids, and alcohols. Growth was determined by visual observation. Acid production was determined by the addition of brom cresol purple indicator to the culture tubes at the end of the incubation period. Gas production was determined by the use of Durham fermentation tubes. The results are given in table 2.

**TABLE 2**

Sugars, salts of organic acids and alcohols tested as carbon sources

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cultures</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>105</td>
<td>106</td>
<td>107</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>Acid</td>
<td>Gas</td>
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<td>Glucose</td>
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<td>Galactose</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sorbitol</td>
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<td>-</td>
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<td>-</td>
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<tr>
<td>Sodium formate</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methanol</td>
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<td>-</td>
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<td>-</td>
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<tr>
<td>n-Propanol</td>
<td>/</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Cultures 105 and 106 were almost identical in their utilization of these carbon sources. Culture 105 grew on sucrose whereas no. 106 failed to do so and culture 105 produced acid on both sucrose and propanol in contrast to culture 106. Culture 107 grew only on methanol, the carbon source most closely related to methane.

The utilization, by the methane-oxidizing bacteria, of the hydrocarbons ethane, propane and butane was determined by incubating cultures in the
culture flasks (figure 2A) and in modified three-flask Söhngen apparatuses (figure 3). Each culture flask contained 40 ml of no. 5 mineral-salts medium and was inoculated with 1 ml of culture. Two gas mixtures were used in the experiment: (1) 45 per cent oxygen, 45 per cent hydrocarbon and 10 per cent carbon dioxide and (2) 15 per cent oxygen, 80 per cent hydrocarbon and 5 per cent carbon dioxide. Growth was determined by visual observation of the turbidity of the culture, microscopic observation of the culture and hydrocarbon oxidation. Hydrocarbon oxidation was evidenced by the drawing of water from the water reservoir flask into the gas reservoir flask of the modified three-flask Söhngen apparatus.

Cultures 105 and 106 showed weak utilization of ethane, propane and butane. Culture 107 was not capable of utilizing ethane, propane or butane as sources of carbon. These results are presented in table 3.

TABLE 3
Ethane, propane and butane tested as carbon sources

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cultures</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>105</td>
<td>106</td>
<td>107</td>
</tr>
<tr>
<td>Ethane</td>
<td>poor growth</td>
<td>poor growth</td>
<td>no growth</td>
</tr>
<tr>
<td>Propane</td>
<td>poor growth</td>
<td>poor growth</td>
<td>no growth</td>
</tr>
<tr>
<td>Butane</td>
<td>poor growth</td>
<td>poor growth</td>
<td>no growth</td>
</tr>
</tbody>
</table>

Nitrogen Sources for Methane-Oxidizing Bacteria

Various nitrogen sources were substituted for the \((\text{NH}_4\text{)}_2\text{SO}_4\) in the no. 5 medium to determine if they could serve as nitrogen sources for the methane-oxidizing bacterium 107. The nitrogen sources NaNO₂, NaNO₃, glycine,
glutamic acid and tryptone were added to ammonium sulfate-free no. 5 medium in the concentration of one gram per liter. The cells of a liquid culture of the methane-oxidizer 107 were washed three times with sterile distilled water by centrifugation, after which the cell suspension was negative when tested for NH$_4^+$, NO$_2^-$ and NO$_3^-$ ions. The culture flasks each contained 40 ml of medium and each one was inoculated with 1 ml of the washed cell suspension. The flasks were filled with the gas mixture and incubated at room temperature in a stationary position. There were duplicate flasks of each nitrogen source plus an inoculated control which was not filled with the gas mixture.

Growth was determined by visual observation.

Sodium nitrate, glutamic acid and tryptone were utilized as nitrogen sources by the methane-oxidizing bacterium. Sodium nitrite and glycine were not utilized. Table 4 shows these results.

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Growth</th>
</tr>
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<tbody>
<tr>
<td>Sodium nitrite</td>
<td>-</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>+</td>
</tr>
<tr>
<td>Glycine</td>
<td>-</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>+</td>
</tr>
<tr>
<td>Tryptone</td>
<td>+</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>+</td>
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</tbody>
</table>

Optimum pH of Methane-Oxidizing Bacteria

The pH optimum for growth was determined for the methane-oxidizing bacterium 107. No. 5 mineral-salts medium was used. The pH of the medium was adjusted by the addition of either NaOH or H$_2$SO$_4$. The pH was determined at the time of pH adjustment, after sterilization, after inoculation and at
the end of the incubation period. Each culture flask contained 40 ml of mineral-salts medium and was inoculated with 1 ml of culture. Duplicates and one inoculated control containing no methane were run at each pH level. After an incubation period of 4 days, the cultures were analyzed for net cell-carbon production. The results are shown in table 5 and figure 5.

**TABLE 5**

<table>
<thead>
<tr>
<th>Adjusted pH</th>
<th>pH after sterilizing</th>
<th>pH after inoculation</th>
<th>pH after incubation</th>
<th>Total cell-carbon in mg</th>
<th>Net cell-carbon in mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.00</td>
<td>5.94</td>
<td>5.90</td>
<td>5.46</td>
<td>1.21134</td>
<td>1.08866</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1.23200</td>
<td>1.10932</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.12268*</td>
<td></td>
</tr>
<tr>
<td>6.60</td>
<td>6.40</td>
<td>6.40</td>
<td>5.30</td>
<td>4.58920</td>
<td>4.43585</td>
</tr>
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<td>4.55840</td>
<td>4.40505</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.15335*</td>
<td></td>
</tr>
<tr>
<td>7.64</td>
<td>7.10</td>
<td>7.10</td>
<td>6.50</td>
<td>4.55840</td>
<td>4.34455</td>
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<tr>
<td>(no pH adjustment of the medium)</td>
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<td>5.02988</td>
<td>4.81603</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>0.21385*</td>
<td></td>
</tr>
<tr>
<td>8.00</td>
<td>7.30</td>
<td>7.27</td>
<td>6.70</td>
<td>6.22601</td>
<td>6.04271</td>
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<td>4.23741</td>
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<td></td>
<td>0.18330*</td>
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<td>8.70</td>
<td>8.05</td>
<td>7.85</td>
<td>7.10</td>
<td>4.57764</td>
<td>4.33324</td>
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<td>4.83760</td>
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<td>0.24440*</td>
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<td>9.40</td>
<td>8.75</td>
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<td>5.56831</td>
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<td>3.86442</td>
<td>3.64882</td>
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</tr>
<tr>
<td>10.00</td>
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<td>9.00</td>
<td>8.10</td>
<td>0.15464</td>
<td>0.03244</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.06134</td>
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<tr>
<td>10.60</td>
<td>9.80</td>
<td>9.20</td>
<td>8.35</td>
<td>0.27720</td>
<td>0.12445</td>
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<td>0.12268</td>
<td>-0.03007</td>
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<tr>
<td>11.00</td>
<td>10.10</td>
<td>9.40</td>
<td>8.55</td>
<td>0.12320</td>
<td>-0.02955</td>
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<td>0.15400</td>
<td>0.00125</td>
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<td></td>
<td></td>
<td></td>
<td>0.15275*</td>
<td></td>
</tr>
</tbody>
</table>

*mg carbon found in the control flask
Effects of Various Gas Concentrations

The effects of various gas concentrations were determined on culture 107. The concentrations of each gas, i.e., oxygen, methane and carbon dioxide, were set at two percentage levels and arranged in all possible combinations. In gas mixtures where the percentages of the three gases did not equal 100 per cent, the remainder was made up with nitrogen. The gas percentage levels were as follows:
Each of the culture apparatus units used in the experiment was constructed from two culture flasks (figure 2A) which were connected with a T tube using short pieces of rubber tubing. The open end of the T tube was connected to a short section of cotton-plugged 4 mm bore glass tubing by another short piece of rubber tubing. One of the flasks was the culture flask and contained 40 ml of no. 5 mineral-salts medium; the other flask contained sufficient water so that each apparatus held approximately the same total amount of methane after being filled with a gas mixture. After being filled with a gas mixture, the culture apparatuses were closed by placing a pinch clamp on the rubber tubing between the T tube and the cotton plugged glass tube.

The culture flasks were inoculated in duplicate with 1 ml of culture, filled with a gas mixture, and incubated at room temperature on a rotary shaker.

The combinations of the gas mixtures, the approximate total volumes of culture apparatuses and the approximate volume of each gas in the apparatuses are listed in table 6.
The cultures, after an incubation period of 4 days, were analyzed for cell-carbon and carbon dioxide-carbon production.
The results are shown in table 7.

**TABLE 7**

Cell-carbon and carbon dioxide-carbon production in various gas mixtures

<table>
<thead>
<tr>
<th>Code No.</th>
<th>Cell-carbon production in mg</th>
<th>Carbon dioxide-carbon production in mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.71107</td>
<td>-0.0275</td>
</tr>
<tr>
<td>1</td>
<td>3.49638</td>
<td>0.3680</td>
</tr>
<tr>
<td>2</td>
<td>2.23891</td>
<td>0.0760</td>
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<tr>
<td>2</td>
<td>1.81720</td>
<td>-0.0170</td>
</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>3</td>
<td>3.14160</td>
<td>-6.5376</td>
</tr>
<tr>
<td>4</td>
<td>1.87880</td>
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</tr>
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<td>4</td>
<td>2.02422</td>
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<td>5</td>
<td>4.91787</td>
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<td>5</td>
<td>4.82508</td>
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<td>6</td>
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</tr>
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<tr>
<td>7</td>
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<td>-3.5808</td>
</tr>
<tr>
<td>8</td>
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</tr>
<tr>
<td>8</td>
<td>4.32447</td>
<td>-2.3204</td>
</tr>
</tbody>
</table>

An analysis of variance was made on the data obtained in table 7. There was a slight production of carbon dioxide in units that initially contained a high oxygen and a low carbon dioxide concentration. In the other units carbon dioxide either was not produced or was actually utilized. The results are shown on tables 8 and 9.
Analysis of variance for the cell-carbon production in the gas mixture experiment

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>Variance ratio</th>
<th>Significance probability level</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂</td>
<td>1</td>
<td>1.88791</td>
<td>1.88791</td>
<td>19.3930</td>
<td>**</td>
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<tr>
<td>CH₄</td>
<td>1</td>
<td>7.18636</td>
<td>7.18636</td>
<td>73.8198</td>
<td>**</td>
</tr>
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<td>CO₂</td>
<td>1</td>
<td>4.14110</td>
<td>4.14110</td>
<td>42.5383</td>
<td>**</td>
</tr>
<tr>
<td>O₂ - CH₄</td>
<td>1</td>
<td>0.01812</td>
<td>0.01812</td>
<td>0.1861</td>
<td></td>
</tr>
<tr>
<td>O₂ - CO₂</td>
<td>1</td>
<td>0.70529</td>
<td>0.70529</td>
<td>7.2449</td>
<td>*</td>
</tr>
<tr>
<td>CH₄ - CO₂</td>
<td>1</td>
<td>0.46164</td>
<td>0.46164</td>
<td>4.7421</td>
<td></td>
</tr>
<tr>
<td>O₂ - CH₄ - CO₂</td>
<td>1</td>
<td>14.59351</td>
<td>14.59351</td>
<td>149.9077</td>
<td>**</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>0.09735</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>29.77275</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* significant at the 5 per cent probability level  
** significant at the 1 per cent probability level  

The results of table 8 indicate that all of the gases, i.e., oxygen, methane and carbon dioxide were highly significant in their effect on the production of cellular carbon. The two-factor interaction between oxygen and carbon dioxide was significant, but the interaction between oxygen and methane, and between methane and carbon dioxide did not significantly affect the production of cellular carbon. The three-factor interaction was highly significant.
TABLE 9
Analysis of variance for the CO₂-carbon production in the gas mixture experiment

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>Variance ratio</th>
<th>Significance probability level</th>
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<tbody>
<tr>
<td>O₂</td>
<td>1</td>
<td>10.46410</td>
<td>10.46410</td>
<td>2,326.00</td>
<td>**</td>
</tr>
<tr>
<td>CH₄</td>
<td>1</td>
<td>2.69850</td>
<td>2.69850</td>
<td>909.80</td>
<td>**</td>
</tr>
<tr>
<td>CO₂</td>
<td>1</td>
<td>90.05580</td>
<td>90.05580</td>
<td>30,362.00</td>
<td>**</td>
</tr>
<tr>
<td>O₂ - CH₄</td>
<td>1</td>
<td>0.14570</td>
<td>0.14570</td>
<td>49.10</td>
<td>**</td>
</tr>
<tr>
<td>O₂ - CO₂</td>
<td>1</td>
<td>0.40240</td>
<td>0.40240</td>
<td>135.60</td>
<td>**</td>
</tr>
<tr>
<td>CH₄ - CO₂</td>
<td>1</td>
<td>3.28940</td>
<td>3.28940</td>
<td>1,109.00</td>
<td>**</td>
</tr>
<tr>
<td>O₂ - CH₄ - CO₂</td>
<td>1</td>
<td>0.24230</td>
<td>0.24230</td>
<td>81.69</td>
<td>**</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>0.02373</td>
<td>0.002966</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>107.32193</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** significant at the 1 per cent level

The results in table 9 show that each gas, the two-factor interactions and the three-factor interactions are all highly significant in what appears to be a net utilization of carbon dioxide-carbon.
DISCUSSION

Isolates 105 and 106

The acid-fast, gram-positive cultures 105 and 106 appeared to be similar to *Mycobacterium phlei*. The cellular morphology, cultural characteristics, pigmentation, production of acid from glucose, mannitol and sorbitol, reduction of nitrates to nitrites and lack of motility corresponded closely to the taxonomic features given for *M. phlei* in *Bergey's Manual of Determinative Bacteriology* (Breed et al., 1957). Cultures 105 and 106 did not grow when incubated in a 57 °C incubator. It is stated in *Bergey's Manual* that *M. phlei* is capable of growing at 52 °C but that growth is variable at 55 °C. The increase of the temperature by 5 degrees may have had an inhibitory effect on the growth of the organism. Because these organisms exhibited only a weak utilization of the hydrocarbons methane, ethane, propane and butane, the experiments on nitrogen sources, optimum pH and the effects of various gas concentrations were not made with these cultures.

Isolate 107

The gram-negative culture 107 was very similar taxonomically to the organism isolated by Dworkin and Foster (1956) which they classified as *Pseudomonas methanica*. A culture of *P. methanica* was obtained from Dr. J. W. Foster for comparison with culture 107. The colonies of *P. methanica* were of a mucoid consistency in contrast to the hard colonies of culture 107. *P. methanica* produced a turbid growth in liquid cultures in contrast to the flocculent or membranous growth of culture 107. The cultural
differences were not regarded as being important because Dworkin and Foster (1956) reported that: "Although the colonies have a mucoid appearance, they have a tacky consistency and after one or two weeks incubation, become difficult to disrupt with a needle". They also reported that there was a tendency toward dispersed growth after the organism had been maintained in the laboratory for some time. *P. methanica*, obtained from Foster, required a complex mineral-salts medium, containing agar extract and calcium pantothenate. Culture 107 grew well on a simple mineral-salts medium (no. 5) without accessory growth factors. Culture 107 grew at high oxygen concentrations which were inhibitory to Foster's *P. methanica*.

Culture 107 was able to utilize \((NH_4)_2SO_4, NaNO_3,\) glutamic acid and tryptone as nitrogen sources, but not NaNO2 or glycine. These results agreed with the findings of Hutton (1948) in his study on methane-oxidizing bacteria.

The experiment to determine the pH optimum was set up so that the composition of the mineral-salts medium would be altered as little as possible. For that reason a buffer was not used, but the medium was adjusted to the desired pH with a strong acid or a strong base. The pH values as determined directly after inoculation and at the end of the incubation period showed an average drop of about 0.8 pH units. When the pH values at the time of inoculation were considered, the optimum pH range was found to be 6.5 to 8.5.

The results of the experiment on the effects of the various gas concentrations indicate that the percentages of all three gases, oxygen, methane
and carbon dioxide are important for the growth of the organism. Interpretation of this experiment was based upon statistical analysis. In the gas levels tested, oxygen and methane were found to be quite independent of each other in their effect on the growth of culture 107. The carbon dioxide and methane interaction was not significant. Oxygen and carbon dioxide did show significant interaction. The interaction of the three gases was highly significant. Possible interpretations of this are that the oxygen-carbon dioxide interaction was influenced by the methane level, or that the methane-carbon dioxide interaction was strongly influenced by the concentration of oxygen.

All of the gases and combinations of gases were highly significant in the production of carbon dioxide. In 10 out of the 16 culture flasks which were employed, there was a net loss of carbon dioxide. These findings might be explained in two ways. Either the carbon dioxide was not completely recovered by the Van Slyke apparatus or the methane-oxidizing bacterium utilized some of the carbon dioxide.

If the carbon dioxide determinations (table 4) are correct, the findings indicate that with a proper gas mixture, the organism can utilize an appreciable amount of carbon dioxide. Such results would be contrary to the findings of Leadbetter and Foster (1958).

This experiment on the effects of various gas concentrations on carbon dioxide production was a preliminary experiment and more work must be carried out before any definite conclusions can be drawn.
Taxonomic Status of Methane-Oxidizing Bacteria

Søhngen isolated a methane-oxidizing bacterium which he named *Bacillus methanicus*. In his doctoral dissertation Søhngen (1906a) reported that this organism utilized a number of organic compounds for growth. His later publications omitted reference to this fact, and Søhngen considered *Bacillus methanicus* to be an autotroph. Dworkin and Foster (1956) considered their strains of methane-oxidizing bacteria to be of the same species as that isolated by Søhngen. However, Dworkin and Foster were of the opinion that the bacterium was a typical heterotroph and should be placed in the genus *Pseudomonas*. Consequently they have renamed the bacterium *Pseudomonas methanica*.

The results obtained with culture 107, like Dworkin and Foster's results, do not substantiate the report in Søhngen's thesis that numerous organic compounds support growth. While methane is an organic compound, the methane-oxidizing bacteria would be more nearly autotrophic than heterotrophic if methane were the only energy source and carbon dioxide were utilized in any quantity as a carbon source. Preliminary results with culture 107 indicate the uptake of large amounts of carbon dioxide under certain conditions. If this should be confirmed by further experimental work, the methane-oxidation might properly be considered to be an example of chemoautotrophy. This would not necessarily be a cause for removing this organism from the genus *Pseudomonas*, but might be taken as an example of autotrophy by organisms in this genus.
Throughout the course of the study, several types of apparatus were constructed for use in culturing methane-oxidizing bacteria. The apparatuses are shown in figures 1, 2, 3 and 4.

Bacteria which were capable of rapid methane oxidation could not be isolated from local soil samples. Cultures 105 and 106, which were isolated from local soil, showed weak methane utilization. Culture 107, which rapidly utilized methane, was isolated from a film on the surface of the water in a carboy containing methane.

Isolates 105 and 106 were acid-fast, gram-positive rods, and appeared to be similar to *Mycobacterium phlei*. Cultures 105 and 106 exhibited weak utilization of methane, ethane, propane and butane.

Isolate 107 was a gram-negative rod, similar to *Pseudomonas methanica* (Dworkin and Foster, 1956). A culture of *P. methanica* was obtained from Dr. J. W. Foster and compared with culture 107. The organisms were similar in their cell morphology, pigmentation and in that they utilized only methane and methanol as sources of carbon. The organisms differed in that *P. methanica* produced mucoid colonies in contrast to the hard colonies of culture 107. *P. methanica* exhibited a turbid growth in liquid cultures, whereas culture 107 exhibited a membranous growth on liquid medium incubated in a stationary position and a flocculent growth in liquid medium incubated on the shaker. *P. methanica* required a complex mineral-salts medium containing agar extract and calcium pantothenate, but culture 107 grew well on a simple mineral-salts medium. Culture 107 grew well at high oxygen concentrations...
which were inhibitory to *P. methanica*. Culture 107 utilized methane rapidly but was unable to utilize ethane, propane and butane.

Culture 107 was capable of using \((\text{NH}_4)_2\text{SO}_4\), NaNO\(_3\), glutamic acid and tryptone as sources of nitrogen but was not able to use NaNO\(_2\) or glycine.

The optimum pH range for the growth of culture 107 was from 6.5-8.5 when using the pH values obtained at the time of inoculation. During incubation the pH dropped approximately 0.8 pH units in each flask.

Results obtained from the gas mixture experiment on culture 107 indicated the presence of a complex system in which the maximum growth of the culture was dependent upon the actions and interactions of the component gases, oxygen, methane, and carbon dioxide. Results of this experiment also indicated that by using the proper gas mixture an appreciable amount of carbon dioxide was utilized by the organism.
REFERENCES


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ZoBell, C. E. 1945 The role of bacteria in the formation and transformation of petroleum hydrocarbons. Science 102, 364-369.