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

Abstract:
Three me thane-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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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. Söhngen (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. Söhngen 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 Söhngen'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 Söhngen (1906b) and obtained a pink isolate similar to his. This they assumed to be of the same species as Methanomonas methanica (Söhngen) and changed the name to Pseudomonas methanica (Söhngen) 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.

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

$$CH_4 + 2\ O_2 \longrightarrow CO_2 + 2\ H_2O$$

Apparently because of this mechanism he classifed 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 CO_2 from methane occurs according to the following scheme:

$$CH_4 \longrightarrow CH_3OH \longrightarrow HCHO \longrightarrow HCOOH \longrightarrow CO_2$$

Leadbetter and Foster (1958) recognized two modes by which methane-oxidizing bacteria may convert methane-carbon to cell-carbon: "(1) dehydrogenative oxidation to CO_2 followed by autotrophic assimilation of the CO_2 by means of the 'active' hydrogen produced during the oxidation, (2) oxidation of methane to one or more oxidation levels short of CO_2 and direct heterotrophic assimilation of carbon at those levels." Four strains of Pseudomonas methanica were cultivated in a closed atmosphere containing unlabeled CH_4 and $C^{14}O_2$. At maximal growth, the specific radioactivities of the cell-carbon and of the CO_2-carbon were measured. In every case the specific radioactivity of the cell-carbon was considerably less than the specific radioactivity of the CO_2-carbon, indicating that in all probability the cell-carbon did not originate via CO_2-carbon exclusively, and therefore came by direct assimilation of methane-carbon at an oxidation level below CO_2. 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.

$$CH_3OH + H_2O_2 \xrightarrow[\text{peroxidase}]{\text{alcohol}} HCHO + 2\ H_2O$$

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.

$$HCHO \xrightarrow[H_2O]{DPN} HCOOH + DPNH + H^{+}$$
(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. Söhngen (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. methanicum 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 methanicus 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 $(NH_4)_2SO_4$ as a nitrogen source, the pH range was 6.0-6.6, whereas when $NaNO_3$ 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

Hydrocarbon		Condensing or retaining agent		Distilling agent	
Name	Boiling point(C)	Name	Temp.(C)	Name	Temp.(C)
Methane	-161.50	Liquid air	ca -190	Freezing n-propyl alcohol	-127
Ethane	-88.30	Freezing n-propyl alcohol	-127	Dry ice in acetone	ca -78
Propane	-42.17	Dry ice in acetone	ca -78	54.9% $CaCl_2 \cdot 6H_2O$ in water	-39.9
Butane	-0.6 to -0.3	None		NaCl in ice	-16

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.

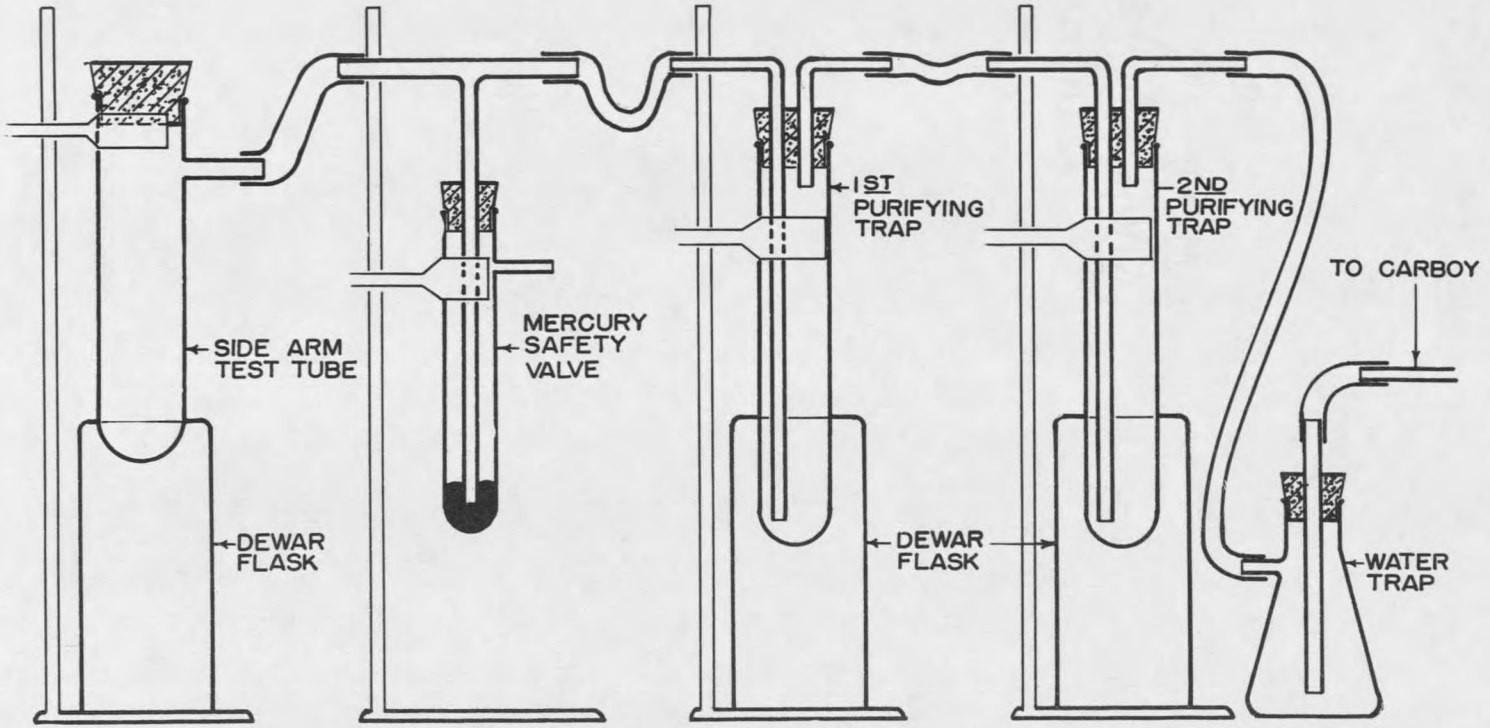

Figure 1. Low temperature distillation apparatus.

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