# Some cultural and physiological aspects of methane-utilizing bacteria

W. HAZEU

*Laboratory of Microbiology, Delft University of Technology, Julianalaan 67A, Delft, the Netherlands*

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A number of different methane-utilizing bacteria are described and compared with isolates of other investigators. The strains can be divided into three groups based on pigmentation, cell morphology and internal membrane structures.

The oxidation of hydrocarbons, alcohols, aldehydes, fatty acids, methyl ethers and sugar phosphates by these bacteria was studied. There was much similarity between strains within the same group. Differences between groups as regards oxidative properties could be detected, but these were mainly quantitative and could not be used as taxonomical criteria.

In addition, the inhibition of methane oxidation by metabolites and enzyme inhibitors was investigated. Formaldehyde proved to be the most active of the organic compounds tested. Iodoacetic acid inhibited both methane and methanol oxidation at concentrations of 0.03 M or above. Of the inorganic compounds, KCN completely suppressed methane oxidation at $5 \times 10^{-4}$ M and to more than 90% at $5 \times 10^{-5}$ M.

## INTRODUCTION

The number of more or less well characterized methane-utilizing bacteria has increased considerably during the last few years. Before 1970 only a few pure cultures had been described. Dworkin and Foster (1956) isolated *Pseudomonas methanica*; *Methanomonas methanooxidans* was characterized by Brown, Strawinski and McCleskey (1964) and *Methylococcus capsulatus* by Foster and Davis (1966). Many other publications on methane-oxidizing bacteria dealt with mixed cultures or less well identified isolates. In 1970 Whittenbury, Phillips and Wilkinson reported over 100 new isolates belonging to different genera and species. Hazeu and Steennis (1970) described *Methylovibrio soehngenii*. New *Methylococcus* spp. were mentioned in a taxonomical study by Malashenko, Romanovskaya and Kvasnikov (1972). Isolation methods for

methane-utilizing bacteria were recently reviewed by Wake, Rickard and Ralph (1973).

The physiology of methane-oxidizing bacteria and other microorganisms metabolizing C1-compounds has been reviewed by Ribbons, Harrison and Wadzinski (1970) and more recently by Quayle (1972). A peculiar aspect of methane-utilizing bacteria is the correlation found between the carbon assimilation pathway and the internal membrane structure of the cell (Lawrence and Quayle, 1970). The type I membrane system as defined by Davies and Whittenbury (1970) is correlated with the ribulose phosphate pathway of formaldehyde fixation, while in organisms with a type II membrane system the serine pathway is found. Other differences in the enzyme systems of both groups of organisms have been investigated by Davey, Whittenbury and Wilkinson (1972).

In this paper a number of strains isolated in pure culture will be described and compared with those of other investigators. The carbon and nitrogen requirements for growth of our isolates have been examined and their physiological properties investigated by manometric techniques. The aim of these studies was to obtain additional criteria for a taxonomical identification by comparing both physiological properties and the morphology of the cells.

## MATERIALS AND METHODS

The cultures were obtained from enrichments carried out in the mineral salts medium of Leadbetter and Foster (1958). The media were solidified by adding 1.5% Bacto agar (Difco). The isolates were maintained by transfer on agar slants every 10 days, and were incubated in a methane–air mixture (1:3) in jars. More details are to be found in a previous paper (Hazeu and Steennis, 1970).

The assimilation of different nitrogen compounds was tested in mineral salts medium by replacing $NaNO_3$ with 1 g of the test compound per litre. The experiments were carried out in 50-ml flasks containing 20 ml medium, which was inoculated with 0.1 ml cell suspension prepared from agar slants. The turbidity was estimated visually after 1, 2 and 3 weeks incubation in a methane–air atmosphere and compared with that of a nitrogen-free medium. A nitrate-containing medium was used as a reference for growth. The assimilation of a number of carbon compounds was tested as a sole source of carbon and energy at a concentration of 0.1% in mineral salts medium.

Cells for manometric experiments were grown in 5-litre Erlenmeyer flasks containing 1 litre of medium with 1 g/litre $NaNO_3$, closed with a rubber stopper and gassed daily with a mixture of natural gas — deodorized over activated

carbon — and air. The flasks were incubated at 30 or 37 C on a rotary or a reciprocating shaker. After 3–4 days the cells were harvested by centrifugation, washed and resuspended in 0.01 M phosphate buffer pH 6.8. The suspensions were diluted to 300 Klett units (filter 66), unless insufficient cell material was obtained. This turbidity corresponds to 1.1–1.2 mg/ml dry weight for different strains.

In media with methanol as source of carbon and energy the phosphate buffer was increased to 0.01 M, instead of 0.002 M in the original mineral salts medium.

The manometric experiments were carried out according to Umbreit, Burris and Stauffer (1964). The Warburg vessel contained 2 ml cell suspension in the main compartment, 0.2 ml substrate solution or buffer in the side arm, and 0.1 ml 20% KOH on a filter paper in the center well.

The results of all manometric experiments have been expressed as the volume of gas consumed during a time interval of 60 min after addition of the substrate, or after the start of the experiment. All values have been corrected for endogenous respiration and calculated for a suspension of 300 Klett units. In some manometric experiments the cells were after appropriate time intervals removed by centrifugation, and the supernatant liquid was analysed for oxidation products by gas chromatography. These tests were conducted with a Varian gas chromatograph, model 1520. The column material was Porapak Q which was used at a temperature of 150 or 175 C. The carrier gas was nitrogen.

All chemicals were of analytical grade if required by the particular type of experiment. The methane used was 99.6% pure, with 0.25% ethylene and traces of $H_2$, $O_2$ and $N_2$. Ethane, propane and butane were of laboratory grade without further specification.

## RESULTS

From a variety of inocula different pure cultures were obtained. Two of these, viz. N3C and WIT, were described earlier as strains of *Methylovibrio soehngenii* (Hazeu and Steennis, 1970). The isolates were divided into three groups, which could be distinguished rather easily on the basis of morphology and pigmentation of the cells. Within the groups variations occasionally occurred.

For the investigations reported in this paper a number of isolates were selected. Their characteristics are summarized in Table 1. In cultures belonging to the white and brown groups, abnormal cell shapes were sometimes observed (Figs. 3–9). It is not clear whether these cells are cysts or cyst-like bodies. Especially in the brown-pigmented strains rather large spherical, non-motile, cells of low refractivity were present, which could be partly separated from the

Table 1. Characteristics of different strains of methane-utilizing bacteria

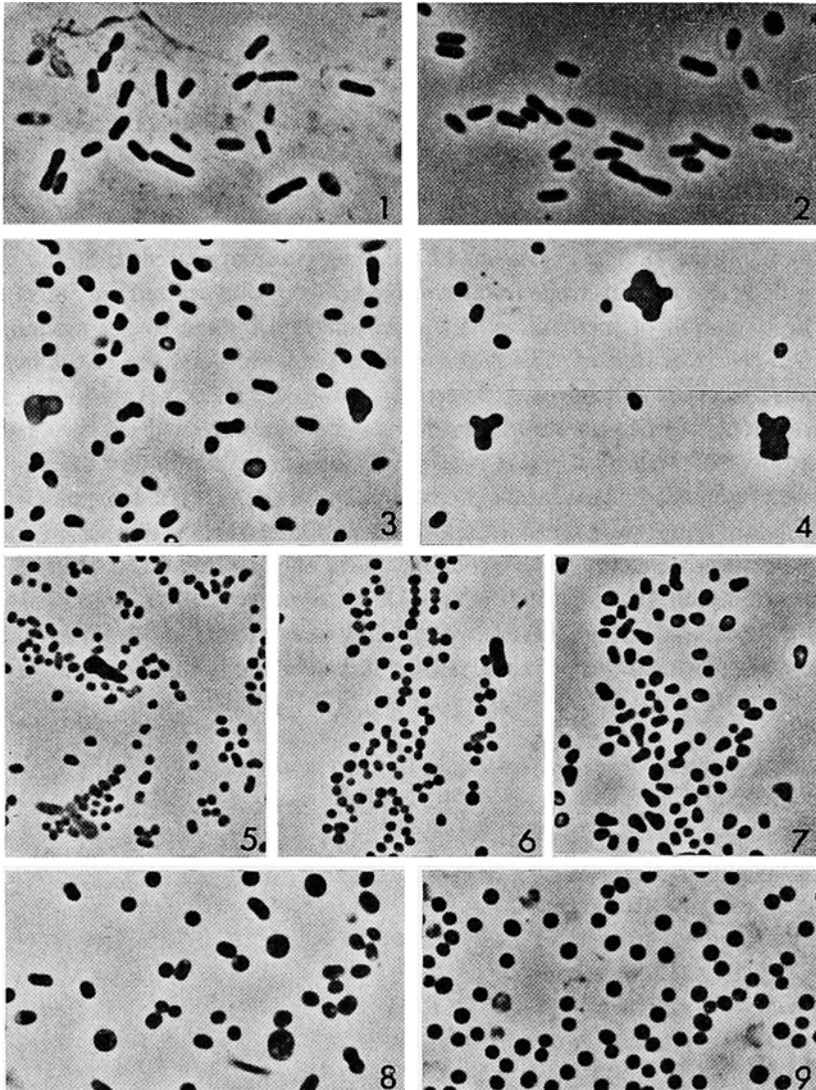
| Strain | Morphology | Motility | Membrane type | Pigment | Growth temp. | Inoculum from | Enrichment temp. |
|---|---|---|---|---|---|---|---|
| *Pink* | | | | | | | |
| N1.1 | rod | + | I | pink | 20–37 | water[1] | 30 |
| N1D1 | rod | + | I | pink | 20–37 | water[1] | 30 |
| N1A | rod | + | I | pink | 20–37 | water[1] | 37 |
| ROSE | rod | + | I | pink | 20–37 | water[2] | 30 |
| *White* | | | | | | | |
| N3C | coccus, vibrio | – | II | white-cream | 25–40 | soil[4] | 30 |
| WIT | coccus, vibrio | +[6] | II | white | 22–40 | water[1] | 30 |
| D2 | coccus, pear | – | II | white-cream | 20–37 | water[1] | 30 |
| 1 | coccus | – | II | white-pink | 20–37 | water[3] | 30 |
| NIG 3.1 | coccus, pear | – | II | white | 20–40 | soil[5] | 37 |
| *Brown* | | | | | | | |
| N3A | short rod | + | I | brown | 30–40 | soil[4] | 37 |
| D37 | short rod | + | I | light brown | 30–40 | water[1] | 37 |
| 3.2B2 | short rod | + | I | light brown | 25–40 | soil[4] | 37 |
| NIG 3A | short rod | + | I | brown | 30–40 | soil[5] | 37 |

[1] Ditch water.
[2] Weed from water.
[3] Ditch water kept in plastic bottle for one year in the refrigerator. The same sample was used for isolation of strain N1.1.
[4] Garden soil.
[5] Dry powdery soil from Nigeria.
[6] Motile cells preferably found in cultures grown at 22–25 C.

other cells by centrifugation. In the white group, exospore formation as described by Whittenbury et al. (1970) was never observed. The organisms of this group were more desiccation-resistant than the pink strains, and survived after lyophilization for at least 4 years. Strain 1 was isolated from a sample of water kept in the refrigerator in a plastic bottle for one year. The same sample also yielded strain N1.1.

All isolates were gram-negative, catalase-positive and oxidase-positive. Strain WIT formerly assumed to be oxidase-negative (*Methylovibrio soehngenii* strain B) (Hazeu and Steennis, 1970) was in later experiments found to become positive after a few hours.

When stained with Sudan black the cells of all strains showed small blue granules or diffuse blue areas (Burdon, 1946). Slime formation was occasionally found in strains of the pink group, but capsules were not detectable by negative staining with nigrosin. Threads of slime were sometimes present in older cultures, especially in those grown on methanol.




Photomicrographs of a few representative methane-utilizing bacteria. Phase contrast, 1600 ×.
Fig. 1. N1.1; some slime threads can be observed.
Fig. 2. ROSE.
Fig. 3. N3C; vegetative and enlarged cells.
Fig. 4. WIT; branched and normal cells.
Fig. 5. D2; normal cells and enlarged specimen.
Fig. 6. 1; vegetative and enlarged cells.
Fig. 7. NIG 3.1; vegetative spherical and pear-shaped cells.
Fig. 8. 3.2B2; cyst-like bodies and vegetative cells.
Fig. 9. NIG 3A; vegetative cells and probably cyst-like bodies.

A number of different carbon compounds, including sugars, sugar phosphates, methylamines, dimethyl ether, hydrocarbons and fatty acids were tested as sole source of carbon and energy. Growth was exclusively found on methane and methanol. With organisms of the pink group, growth on methane was enhanced by adding 0.1% glucose.

Methane-oxidizing bacteria were less limited as regards the source of nitrogen. Both organic and inorganic nitrogen compounds sustained good growth on methane (Table 2). The organisms of the pink group appeared to be the most versatile in this respect, while isolates of the brown group utilized only nitrate.

The oxidation of methane, ethane, propane and butane by suspensions of washed cells was studied. The flasks were gassed with a mixture of the hydrocarbon concerned and air (ca. 1:5 v/v). The results are given in Table 3. Because the differences between strains from the same group were within experimental error, only the results for one or two representative strains for each group will be presented. The oxidation products found by gas chromatography of the supernatant liquid were alcohols, aldehydes and fatty acids with the same number of carbon atoms as the hydrocarbon used as substrate. Minor peaks, the nature of which has not yet been elucidated, were sometimes present. In all tests the final and main oxidation product formed was the corresponding fatty acid, which lowered the pH considerably in inadequately buffered cell suspen-

Table 2. Assimilation of inorganic and organic nitrogen compounds as sole source of nitrogen by methane-utilizing bacteria

| Strain | $NaNO_3$ | $NH_4Cl$ | Urea | Peptone | Yeast extract | Glutamate | Asparagin | Aspartic acid | Lysine |
|---|---|---|---|---|---|---|---|---|---|
| *Pink* | | | | | | | | | |
| N1.1 | + | + | + | ± | — | + | + | + | — |
| N1D1 | + | + | + | + | ± | + | + | + | — |
| N1A | + | + | + | ± | + | + | + | + | — |
| ROSE | + | ± | + | ± | ± | ± | — | — | — |
| *White* | | | | | | | | | |
| N3C | + | + | — | + | + | — | + | — | — |
| WIT | + | + | ± | + | + | — | — | — | — |
| D2 | + | ± | ± | + | — | — | — | — | ± |
| 1 | + | + | — | + | + | — | — | — | ± |
| NIG 3.1 | + | ± | ± | + | + | — | — | — | — |
| *Brown* | | | | | | | | | |
| N3A | + | — | — | — | — | — | — | — | — |
| D37 | + | — | — | — | — | — | — | — | — |
| 3.2B2 | + | — | — | — | — | — | — | — | — |
| NIG 3A | + | — | — | — | — | — | — | — | — |

+ good growth ± slight growth — no growth

Table 3. Oxidation of methane, ethane, propane, butane and dimethyl ether by resting cells of methane-oxidizing bacteria. Gas consumption (μl) after 60 min.

| Strain | Methane | Ethane | Propane | Butane | Dimethyl ether |
|---|---|---|---|---|---|
| *Pink* | | | | | |
| N1D1 | 1275 | 58 | 3 | 9 | 83 |
| ROSE | 598 | 210 | 29 | 25 | 96 |
| *White* | | | | | |
| N3C | 849 | 302 | 54 | 58 | 108 |
| 1 | 395 | 254 | 52 | 80 | 15 |
| *Brown* | | | | | |
| N3A | 273 | 12 | 6 | 8 | 44 |

sions. Special attention was given to dimethyl ether, a hypothetical intermediate of methane oxidation (Wilkinson, 1971). Other compounds tested were dimethyl carbonate, dimethoxymethane and dimethoxyethane. The oxidation rate of dimethyl ether was not always reproducible in different experiments with the same organism, but all strains were able to oxidize this substrate as shown in Table 3. No oxygen consumption above the endogenous respiration was found with dimethyl carbonate and with dimethoxyethane. With dimethoxymethane the gas consumption never exceeded the endogenous respiration by more than 25–35 μl, and stopped after 20–30 min indicating that some impurity might have been present.

The oxidation of alcohols, aldehydes and fatty acids was studied using methanol, ethanol, *n*-propanol, *n*-butanol and the corresponding aldehydes and fatty acids. Ethylene glycol and ethylene glycol monomethyl ether were also included. The results are summarized in Table 4. The presence of aldehydes and fatty acids as oxidation products of alcohols was confirmed by gas chromatography. Oxidation products of ethylene glycol and of its monomethyl ether have not been identified, but a drop in pH after the test indicated that acids had been produced. A few experiments with *n*-alcohols from $C_5$ to $C_{10}$ revealed that strains of different groups oxidized most of these alcohols up to $C_8$ or $C_9$. This upper limit may be due primarily to the poor solubility of the higher alcohols in water.

There was no oxidation of D-glucose, D-glucose-6-phosphate, D-fructose, D-fructose-6-phosphate, D-ribose, D-ribose-5-phosphate and glycerol.

In previous experiments we found that unknown substances inhibiting methane oxidation were excreted into the medium during growth. A comparable phenomenon was observed by Harwood and Pirt (1972), who added Amberlite CG120 to a growing culture of *Methylococcus capsulatus* in order to remove inhibiting substances from the medium.

Table 4. Oxidation of alcohols, aldehydes and fatty acids by methane-grown cell suspensions of methane is given.

| Substrate | Methanol | Formaldehyde | Formate | Ethanol | Acetaldehyde | Acetate | *n*-Propanol |
|---|---|---|---|---|---|---|---|
| μMoles added | 5 | 5 | 5 | 25 | 25 | 25 | 25 |
| *Pink* | | | | | | | |
| N1D1 | 95 | 75 | 33 | 275 | 65 | 3 | 290 |
| ROSE | 63 | 30 | 7 | 198 | 25 | 0 | 122 |
| *White* | | | | | | | |
| N3C | 73 | 38 | 9 | 159 | 48 | 3 | 158 |
| 1 | 63 | 35 | 7 | 105 | 36 | 9 | 74 |
| *Brown* | | | | | | | |
| N3A | 103 | 54 | 12 | 61 | 22 | 0 | 53 |

The influence of metabolites and inhibitors on methane oxidation was examined with a few strains. A part of the results expressed as the percentage inhibition of gas uptake during a 60 min interval after addition of the test compound, are summarized in Table 5. The inhibition due to 25 μmoles of ethanol, *n*-propanol, *n*-butanol, the corresponding aldehydes, glucose, ribose, acetate and pyruvate varied between 0 and 30%. An exception was found with butanol and butyraldehyde which inhibited gas uptake in het strain N3C to 56 and 48%, respectively.

Table 5. The inhibition (%) of the rate of gas consumption by metabolites and inhibitors added to cell suspensions of methane oxidizers gassed with methane–air

| Compound tested | Conc. $10^{-3}$M | N1D1 | N3C |
|---|---|---|---|
| Methanol | 2.3 | 6 | 0 |
| | 11.4 | 11 | st |
| | 22.7 | 26 | st |
| Formaldehyde | 2.3 | st | 0 |
| | 4.5 | 9 | 38 |
| | 11.4 | 39 | 90 |
| | 22.7 | 73 | 95 |
| Formate | 22.7 | 15 | 14 |
| KCN | 0.05 | 93 | 61 |
| | 0.5 | 100 | 100 |
| $NaN_3$ | 0.05 | 24 | 4 |
| E.D.T.A. | 2.3 | 80 | 9 |

st: slight stimulation

oxidizing bacteria. The volume of $O_2$ (μl) consumed during a 60 min interval after addition of the substrate

| Propion-aldehyde | Propionate | *n*-Butanol | Butyr-aldehyde | Butyrate | Ethylene glycol | Ethylene glycol monomethyl ether |
|---|---|---|---|---|---|---|
| 25 | 25 | 25 | 25 | 25 | 10 | 10 |
| | | | | | | |
| 110 | 0 | 241 | 55 | 0 | 50 | 140 |
| 44 | 0 | 100 | 66 | 0 | 25 | 75 |
| | | | | | | |
| 48 | 3 | 172 | 47 | 3 | 36 | 70 |
| 45 | 5 | 168 | 32 | 0 | 18 | 53 |
| | | | | | | |
| 32 | 0 | 35 | 21 | 0 | 6 | 25 |

The influence of monoiodoacetic acid on oxidation of methane, methanol and formaldehyde is given in Table 6 as percentage inhibition.

Table 6. Inhibition (%) of the oxidation of methane, methanol and formaldehyde by monoiodoacetic acid. The cells were suspended in 0.1 M phosphate buffer, pH 6.8.

| Strain | Iodoacetic acid $10^{-3}$ M | Methane–air | Methanol $2.3 \times 10^{-3}$ M | Formaldehyde $2.3 \times 10^{-3}$ M |
|---|---|---|---|---|
| *Pink* | | | | |
| N1D1 | 1 | st | – | – |
| | 3 | st | – | – |
| | 10 | 40 | 15 | 18 |
| | 30 | 86 | 71 | 57 |
| *White* | | | | |
| N3C | 1 | 0 | – | – |
| | 3 | 10 | – | – |
| | 10 | 28 | 37 | st |
| | 30 | 99 | 95 | 22 |
| 1 | 1 | st | – | – |
| | 3 | st | – | – |
| | 10 | 41 | 6 | 7 |
| | 30 | 100 | 74 | 38 |

st: slight stimulation

## DISCUSSION

*Morphology and general properties.* Methane-oxidizing bacteria have been found in almost any sample of surface soil or water. This is not surprising, in

view of the enormous amount of methane produced from decaying organic matter under anaerobic conditions. The organisms responsible for methane oxidation form a distinct physiological and morphological group. Our isolation procedures showed (Table 1) that the pink group of organisms, probably closely related to *Pseudomonas methanica* (Dworkin and Foster, 1956), was only obtained from water samples or from weeds growing in water. This confirms the observations of Leadbetter and Foster (1958). According to the scheme of Whittenbury et al. (1970) our pink isolates resemble *Methylomonas methanica* or *Methylomonas rubrum*.

The taxonomic position of the organisms belonging to the white group is not very clear. All strains possessed a type II membrane system, as was revealed by electron microscopy (unpublished results). The vibrioid cells found in a number of these isolates might suggests that they are related to *Methylosinus* or *Methylocystis*. Our strain N3C, formerly described as *Methylovibrio soehngenii* A, and the strain D2 were both non-motile, did not produce exospores and correspond with *Methylocystis*. Strain WIT, earlier designated as *Methylovibrio soehngenii* B, might be related to *Methylosinus*. The strains 1 and NIG 3.1 showed a strong tendency to grow in aggregates of closely packed, more or less spherical cells in liquid media. In older cultures the colonies of strain 1 turned from white to pink. Both strains are non-motile, but they are distinct from *Methylocystis parvus* by absence of rosette formation and by the ability to grow on methanol.

The isolates of the brown group showed similarities in cell shape (Figs. 8 and 9), consistency of the colonies, motility and temperature for growth. These strains may be related to *Methylobacter vinelandii*. Occasionally non-pigmented colonies were found on plates of the brown strains. A comparable phenomenon was observed by Leadbetter and Foster (1958) with methane-oxidizing bacteria isolated at 37 C. Capsule formation was rarely observed with any of our strains. This is contrary to the findings of Whittenbury et al. (1970), but agrees with those of Malashenko et al. (1972). These investigators isolated strains of *Methylomonas*, *Methylobacter* and *Methylococcus* and were unable to demonstrate capsules.

The spectrum of carbon compounds supporting growth of our isolates agrees with that found by other investigators. Methane proved to be superior to methanol as a source of carbon and energy. The majority of the methane-oxidizing bacteria can assimilate methanol to some extent, but concentrations of this alcohol above 0.1 % are inhibitory. Wilkinson (1971) noted that methane-utilizing bacteria could grow on dimethyl ether. In none of our numerous experiments any of our isolates ever grew in an atmosphere containing 20% (v/v) of dimethyl ether in air. No isolate would grow on glucose-6-phosphate, which according to Amemiya (1972) sustained good growth of *Methanomonas*

*methanooxidans*. These observations stress the unique taxonomical position of *Methanomonas methanooxidans*, which possesses a type II membrane system and does not seem to be related to any other strain known at this moment.

It is not surprising that nitrate, which was used in the enrichment medium, is preferred as a nitrogen source by most isolates. Ammonium could not always replace nitrate, contrary to the results of Whittenbury et al. (1970). In a few strains, organic nitrogen compounds produced equal or even better growth (Table 2). The results with peptone and yeast extract were not always reproducible, and partly differed from results obtained a few years ago. These variations may be due to strain variability or to fluctuations in the composition of the organic compounds. Nitrogen assimilation in methane-utilizing bacteria has been studied mainly in *Methylococcus capsulatus*. These studies (Eroshin, Harwood and Pirt, 1968; Eccleston and Kelly, 1972) indicated that amino acids differed markedly in their effect on the growth of this organism. Many amino acids stimulated growth at lower, but completely suppressed it at higher concentrations. Therefore results of tests with complex organic nitrogen compounds should be interpreted with care.

*Oxidation of hydrocarbons*. The organisms of the pink group oxidized methane faster than any other (Table 3). In methanol-grown cells the rate of methane oxidation was considerably lower. Electron microscopy revealed that in these cells the internal membrane structure was less developed than in methane-grown cells. This suggests a correlation between methane oxidation and membranes.

The oxidation of higher hydrocarbons by methane-utilizing bacteria has been reported by several investigators. In our experiments the capacity of resting cells to oxidize hydrocarbons varied considerably between strains and groups. The organisms of the white group were best adapted to oxidation of hydrocarbons (Table 3). No indications were found that the presence of small amounts of ethane (2.71% v/v), propane (0.38%) and butane (0.13%) in natural gas stimulated the organism's capacity to oxidize these hydrocarbons when compared with methane-grown cells. The end products of hydrocarbon oxidation are the corresponding fatty acids, but intermediates could be demonstrated by gas chromatography. Oxidation products found were the alcohols and aldehydes in addition to the fatty acids. No special tests for ketones have been carried out in our investigations. According to Leadbetter and Foster (1960) acetone is formed from propane, and butanone from butane by *Pseudomonas methanica*.

Dimethyl ether was oxidized by all strains, but at a moderate rate. Though there is some correlation between oxidation of methane and of dimethyl ether, the latter compound does not seem to be an intermediate of methane oxidation. Other methyl ethers were not oxidized. It seems therefore unlikely that the oxidation of dimethyl ether was performed by splitting of the C–O–C bond.

*Oxidation of other carbon compounds.* Oxidation of alcohols, aldehydes and fatty acids seems to be similar for different strains. Several methane-oxidizing bacteria have been reported to behave differently. *Methylococcus capsulatus* did not oxidize aldehydes to the corresponding fatty acids (Patel and Hoare, 1971), whereas *Methanomonas methanooxidans* was unable to oxidize any alcohol, aldehyde or fatty acid other than methanol, formaldehyde and formate. According to Wilkinson (1971) the majority of strains isolated by Whittenbury et al. (1970) oxidized alcohols only to the corresponding aldehydes. Our results are in agreement with those of Leadbetter and Foster (1958) who found that *Pseudomonas methanica* produced fatty acids from higher alcohols.

The oxidation rate of ethylene glycol was significantly lower than that of the monoalcohols. Its monomethyl ether was oxidized at a higher rate, about half that for ethanol. These differences may reflect either the permeability of the cell wall and cytoplasmic membrane, or the enzyme's affinity for the substrate.

None of our isolates oxidized the sugars or sugar phosphates mentioned above. This is in contrast with the results of Amemiya (1972) who found both oxidation and assimilation of glucose-6-phosphate, fructose-6-phosphate and to some extent also of ribose-5-phosphate by *Methanomonas methanooxidans*, and oxidation of these compounds by *Methylococcus capsulatus*. As stated above, these species are different from our isolates in several respects.

In the course of these investigations it was noted that the reproducibility of the manometric experiments was rather poor. This made a quantitative comparison between the various oxidation rates of the 3 groups difficult. The poor reproducibility almost certainly resulted from variations in growth rate and growth yield in various batches of the same cells, despite the fact that test organisms were always grown under the same conditions.

*Inhibition of methane oxidation.* Methane oxidation can be partly or completely inhibited by organic metabolites. Whittenbury et al. (1970) found that methane oxidation was partly suppressed by methanol, ethanol and ethane. Eccleston and Kelly (1973) showed that the growth of *Methylococcus capsulatus* was inhibited by methanol (0.1% and above) and by ethanol, propanol and butanol (0.01% and above). Our findings partly confirm these results (Table 5). The strongest inhibition of methane oxidation in resting cell suspensions was obtained with formaldehyde, a key intermediate in the pathway of methane metabolism. Its inhibitory action was more pronounced in the white group than in the pink. The alcohols, aldehydes, fatty acids, sugars and pyruvate, when applied at the same concentration, gave no or only moderate inhibition.

KCN was the most active of a few known inhibitors tested. Comparable concentrations of KCN were found to inhibit methanol and methane oxidation by cell suspensions of *Methylococcus capsulatus* (Patel and Hoare, 1971).

The inhibitory action of iodoacetic acid on methane, methanol and formaldehyde oxidation was also tested (Table 6). The results show that methanol oxidation was partly inhibited by 0.01 and 0.03 M iodoacetic acid. No inhibition was found at lower concentrations (0.003 M), which is in agreement with the results of Higgins and Quayle (1970). Methane oxidation was equally or more sensitive to iodoacetic acid than methanol oxidation. At lower concentrations of this acid a slight stimulation was observed. The nature of an inhibitory compound produced in cultures of strain N1D1 grown on methane could not be elucidated. Intra-or extracellular formaldehyde may have been formed, but the presence of a yet unknown ionic compound can not be excluded since Harwood and Pirt (1972) by adding an ion exchange resin, removed an inhibitory factor from a culture of *Methylococcus capsulatus*.

From the physiological studies discussed in this paper it is apparent that they have little significance in taxonomy. The identification of methane-oxidizing bacteria should therefore mainly be based on careful morphological observations, combined with electron-microscopical studies of the internal membrane structures, and possibly analysis of the GC content of the DNA.

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