Nitrogen-fixing methane-utilizing bacteria

Dit proefschrift met stellingen van

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landbouwkundig ingenieur, geboren te Waalwijk op 6 maart 1947, is goedgekeurd door de promotor, Dr. Ir. E. G. Mulder, hoogleraar in de algemene microbiologie en de microbiologie van bodem en water.

De rector magnificus van de Landbouwhogeschool,
Dr. Ir. J. P. H. van der Want

Wageningen, 5 oktober 1976

J. A. M. de Bont

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Proefschrift
ter verkrijging van de graad van doctor in de
landbouwwetenschappen, op gezag van de rector magnificus,
dr. ir. J. P. H. van der Want, hoogleraar in de virologie,
in het openbaar te verdedigen op vrijdag 26 november 1976
des namiddags te vier uur in de aula van de Landbouwhogeschool
te Wageningen

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STELLINGEN

I

Het vermogen tot stikstofbinding is een algemeen bij methaanoxyderende bakteriën voorkomende eigenschap.

Dit proefschrift.

II

Acetyleen heeft een sterk remmende invloed op de oxydatie van methaan door bakteriën.

Dit proefschrift.

III

De acetyleen-ethyleen test ter bepaling van nitrogenase-aktiviteit kan niet toegepast worden op bakteriën die op lagere alkanen groeien.

Dit proefschrift.

IV

Vissterfte in meren tengevolge van zuurstofgebrek dat ontstaat tijdens langdurige bedekking van het water met ijs kan wellicht tegengegaan worden door geringe hoeveelheden calciumcarbide onder het ijs te brengen.

J.W.M. RUDD, A. FURUTANI, R.J. FLETT and R.D. HAMILTON (1976) Limnology and Oceanography 21, 357–364.
Dit proefschrift.

V

Toevoeging van acetyleen aan aardgas zal de afbraak van methaan in de grond rondom een gaslek slechts gedurende korte tijd verhinderen.

J.A.M. de BONT (1975) Gas 95, 367–374.

VI

Tijdens de acetyleen-ethyleen test vindt er geen mikrobiologische omzetting van ethyleen plaats.

J.A.M. DE BONT (1976) Canadian Journal of Microbiology, 22, 1060–1062.

VII

Het is niet aannemelijk dat waterstof de acetyleenreduktie bij waterstofoxyderende bakteriën remt zoals aangegeven door Gogotov en Schlegel.

J.N. GOGOTOV en H.G. SCHLEGEL (1974) Archiv für Mikrobiologie 97, 359–362.
J.A.M. DE BONT en M.W.M. LEIJTEN (1976) Archives of Microbiology 107, 235–240.

VIII

De vrijwel algemeen gedeelde mening van Stanier, Adelberg en Ingraham dat Caulobacters in hun levenscyclus een beweeglijk stadium doormaken is in zijn algemeenheid niet juist.

R.Y. STANIER, E.A. ADELBERG en J.L. INGRAHAM (1976) The Microbial World. Englewood Cliffs: Prentice Hall, Inc.
J.A.M. DE BONT, J.T. STALEY en H.S. PANKRATZ (1970) Antonie van Leeuwenhoek **36**, 397–407.
J.T. STALEY, J.A.M. DE BONT en K. DE JONG (1976) Antonie van Leeuwenhoek **42**, 333-342.

IX

Smith heeft zijn opvatting dat bakteriën in grond slechts onder anaërobe omstandigheden ethyleen kunnen vormen onvoldoende gefundeerd.

A.M. SMITH (1976) Soil Biology and Biochemistry **8**, 293–298.
J.A.M. DE BONT (1975) Annals of Applied Biology **81**, 119–121.
J.A.M. DE BONT (1976) Antonie van Leeuwenhoek **42**, 59–71.

X

Abeles, Cracker, Forrence en Leather hebben zich ernstig vergist in hun berekening, gebaseerd op hun eigen laboratoriumexperimenten, dat per jaar 7×10^6 ton ethyleen uit de lucht boven de Verenigde Staten van Amerika verwijderd zou kunnen worden door mikrobiologische oxydatie van dit gas in de grond. De uitkomst van hun berekening is 7×10^6 ton per jaar te hoog uitgevallen.

F.B. ABELES, L.E. CRACKER, L.E. FORRENCE en G.R. LEATHER (1971) Science **173**, 914–916.

XI

Acetylcoenzym A is een intermediair in de afbraak van ethyleen door bepaalde bakteriën.

J.A.M. DE BONT en R.A.J.M. ALBERS (1976) Antonie van Leeuwenhoek **42**, 73–80.

Proefschrift van J.A.M. DE BONT
Nitrogen-fixing methane-utilizing bacteria
Wageningen, 26 november 1976.

Promotor: Prof. Dr. Ir. E. G. Mulder

This study was carried out at the Laboratory of Microbiology, Agricultural University, Wageningen, The Netherlands.

Dit proefschrift is voortgekomen uit een onderzoek dat dankzij de geldelijke steun van het VEG Gasinstituut en de N.V. Nederlandse Gasunie in het Laboratorium voor Mikrobiologie van de Landbouwhogeschool uitgevoerd kon worden. Van harte dank ik de vele personen die betrokken zijn geweest bij de totstandkoming ervan. Ik ben bijzonder erkentelijk voor de begeleiding bij het in de uiteindelijke vorm brengen van de manuskripten en voor de hulp die ik op vele andere gebieden gekregen heb. Met name wil ik graag het tekenen van de grafieken aanhalen. Diskussies met lotgenoten, evenals de plezierige samenwerking met doktoraalstudenten, heb ik zeer gewaardeerd. Evenwel, mijn dank gaat speciaal uit naar die mensen die mijn humeur in tijden van algehele tegenspoed weer wat opvijzelden.

PREFACE

After the changeover in the Netherlands from manufactured gas to natural gas, a strong increase in the death rate of trees in urban areas was observed. To study this undesirable phenomenon, a committee was set up by the Netherlands Association of Municipal Park Superintendents. This Committee for the Study of the Influence of Natural Gas on Vegetation was instructed to investigate the cause of the death of trees by natural gas and, if possible, to devise measures to prevent damage. The results of the committee's work have been published in eight reports (SIAB reports, P.O. Box 1240, The Hague).

For this committee, Hoeks investigated the death of vegetations brought about by natural gas (Hoeks (1972), Thesis, Agricultural University, Wageningen; Hoeks (1972), Soil Science 113, 46–54). He found that leakage of natural gas from the gas distribution system affects the physical, chemical and biological processes in the soil, the most important of these processes being the microbial oxidation of methane. In this oxidation process oxygen was consumed and carbon dioxide was produced. When the temperature was not too low, the rate of oxygen consumption in soil around gas leaks was so intensive that the supplied oxygen was fully consumed. Absence of oxygen from the soil atmosphere was seen as the main cause of damage to the vegetation.

Thus it was decided to examine more closely the organisms involved in the microbial oxidation of natural gas. A program of research was initiated by Prof. Mulder and Dr Adamse, both advisory members of the Committee for the Study of the Influence of Natural Gas on Vegetation. As a result, a publication on the microbiology of processes in soil near gas leaks appeared in 1972 (Adamse, Hoeks, de Bont and van Kessel (1972), Archiv für Mikrobiologie 83, 32–51). Methane-oxidizing bacteria were found to be mainly responsible for the increased oxygen consumption in soil around leaks.

Since then, attention has been focussed on the methane-oxidizing bacteria. It was observed that these bacteria have the capacity to utilize atmospheric nitrogen gas for synthesis of cell material. Nitrogen fixation by these organisms was thought to be important in explaining some of the processes occurring in soil around a gas leak (de Bont (1975), Gas 95, 367–374). Consequently, this important property was investigated in more detail. The results obtained on nitrogen-fixation by methane-utilizing bacteria are presented in this thesis.

CONTENTS

GENERAL INTRODUCTION

In nature, methane is continuously produced by bacteria as an end product of anaerobic mineralization of organic matter. Methane-producing bacteria may be active in sediments of lakes, ditches and rivers, submerged soils, anaerobic digesters used in sewage purification and also in ruminant digestion. Methane also occurs in coal and oil deposits; natural gas contains mainly methane.

In view of the natural occurrence of the gas it is not surprising that bacteria have developed the ability to utilize methane as carbon and energy source. Söhngen (1906) demonstrated for the first time that methane is susceptible to bacterial action. From experiments with water plants he concluded that bacteria associated with the plants rather than the plants themselves were responsible for the uptake of methane. He isolated *Bacillus methanica*, a Gram-negative, short, thick rod as well as other bacteria capable of oxidizing methane. Fifty years elapsed after the isolation and description of these methane-oxidizing bacteria by Söhngen, before Foster and co-workers reported their studies on methane oxidation (Dworkin and Foster, 1956; Leadbetter and Foster, 1958; Leadbetter and Foster, 1960; Foster and Davis, 1966). *Bacillus methanica*, originally isolated by Söhngen, was reisolated along with other strains resembling this bacterium. Also, a coccus capable of oxidizing methane was isolated. These workers found that only methane and methanol served as growth substrates for their isolates. The inability of methane-oxidizing bacteria to grow on a variety of substrates other than methane or methanol has since been confirmed in many studies (Stocks and McClesky, 1964; Brown, Strawinski and McClesky, 1964; Whittenbury, Phillips and Wilkinson, 1970; Hazeu and Steennis, 1970; Adamse, Hoeks, de Bont and van Kessel, 1972; Namsaraev and Zavarzin, 1972; Hazeu, 1975). From these studies it also became evident that morphologically the methane-oxidizing bacteria form a heterogeneous group. Non-motile cocci, motile rods, vibrios and pear-shaped organisms and pleomorphic strains were isolated. Often these bacteria were capable of existing in forms resistant to adverse environmental conditions. Exospores and different types of cysts have been observed (Whittenbury, Davies and Davey, 1970). Although the methane-oxidizing bacteria show much variation in morphology, their internal anatomy is more uniform. The presence of a system of internal membranes in these bacteria has been described by Procter, Norris and Ribbons (1969) and later by others (Davies and Whittenbury, 1970; Smith and Ribbons, 1970; Smith, Ribbons and Smith, 1970; de Boer and Hazeu, 1972). These membranes may either be distributed throughout the cytoplasm of the cell (type I) or peripheral (type II). The type I membranes have been found in the rod and coccoid organisms and type II membranes in the vibrioid organisms.

The occurrence of these intracytoplasmatic membrane systems in methane-oxidizing bacteria suggests that they support physiological and biochemical activities unique for the growth of these bacteria. These membranes may be involved in the oxidation of methane to the level of formate. The first step in the oxidation of methane is probably catalysed

by a monooxygenase, yielding methanol as the reaction product. This oxidation activity dependent on NADH and O_2 (Ribbons and Michelover, 1970; Ferenci, 1974) is associated with particulate cell-free fractions that contains membranous material (Ribbons, 1975). Further oxidation of methanol through formaldehyde to formate has also been associated with the internal membranes (Wadzinkski and Ribbons, 1975), although soluble dehydrogenase activity for these two substrates has been demonstrated as well (Patel and Hoare, 1971; Patel, Bose, Mandy and Hoare, 1972). The final step in the oxidation of methane to carbon dioxide is catalysed by a soluble formate dehydrogenase (Patel and Hoare, 1971; Wadzinkski and Ribbons, 1975).

The synthesis of cell constituents by methane-oxidizing bacteria necessarily involves the condensation of C_1-carbon units derived from methane. This requires special biosynthetic capabilities enabling the formation of C_3 units. Once a C_3 unit has been formed, the more complex molecules may be synthesized along metabolic routes that are identical to those encountered in other bacteria. Two completely different pathways for the formation of C_3 units from C_1 units have been found in methane-utilizing bacteria. The ribose phosphate cycle of formaldehyde fixation was first discovered in *Pseudomonas methanica*. The first enzyme operative in this carbon-assimilating pathway is hexulose-phosphate synthetase catalysing the formation of D-arabinose-3-hexulose-6-phosphate from ribulose-5-phosphate and formaldehyde originating from the methane molecule (Kemp, 1974). A cycle of reactions then regenerates the ribulose-5-phosphate and yields C_3 compounds in amounts equivalent to the amount of formaldehyde that has been fixed (Quayle, 1972). Alternatively, via the serine pathway both formaldehyde and carbon dioxide originating from methane may be incorporated into a C_3 compound. Serine transhydroxymethylase catalyses the condensation of formaldehyde with glycine yielding serine, while phosphoenol pyruvate carboxylase catalyses the incorporation of carbon dioxide into phosphoenol pyruvate giving rise to oxaloacetate. Glycine and oxaloacetate are regenerated in a cycle of reactions and the net result is the formation of 3-phosphoglycerate from 2 molecules of formaldehyde and 1 molecule of carbon dioxide (Quayle, 1972).

A provisional classification of the methane-oxidizing bacteria into groups and subgroups, based on morphology, fine structure and type of resting stage formed, has been

Table 1. Division of methane-oxidizing bacteria into groups according to Whittenbury, Phillips and Wilkinson (1970).

Group	Resting stage	Membrane type	Morphology
Methylosinus	Exospore	II	Rod or pear-shaped
Methylocystis	Lipid cyst	II	Rod or vibrio
Methylomonas	Immature Azotobacter-type cyst	I	Rod
Methylobacter	Azotobacter-type cyst	I	Rod
Methylococcus	Immature Azotobacter-type cyst	I	Coccus

proposed by Whittenbury, Phillips and Wilkinson (1970). Table 1 shows this classification into groups. Later Lawrence and Quayle (1971) found that a correlation exists between the membrane type of the bacteria and the assimilatory pathway. Organisms possessing a type I membrane structure use the ribose phosphate cycle of formaldehyde fixation, whereas those possessing a type II membrane structure use the serine pathway.

Nitrogen fixation

A wide range of procaryotic microorganisms, including many types of heterotrophic and photoautotrophic bacteria and species of blue-green algae, have the ability to fix atmospheric nitrogen (Quispel, 1974). The N_2 fixation in all these organisms is accomplished by the catalytic action of the nitrogenase enzyme system. This enzyme system consists of a protein containing Fe and a protein containing Fe and Mo. Cell-free preparations capable of fixing N_2 have been prepared from a number of organisms. From such preparations it has been established that the nitrogenase reaction is catalysed by a combination of the two proteins in which ATP hydrolysis is coupled to electron transfer for the reduction of N_2 to NH_3. Nitrogenase reduces many substrates other than N_2. Reduction of acetylene to ethylene is particularly important because a convenient and sensitive assay system for nitrogenase activity has been based on this reaction. This acetylene-ethylene assay (Hardy, Burns and Holsten, 1973) can be employed for studying activity of both enzyme preparations and intact organisms. It enables not only a ready estimation of the nitrogen-fixing capacity of pure cultures under laboratory conditions, but it can also be used for measuring nitrogen-fixing activities in natural habitats.

Free-living bacteria capable of fixing nitrogen are mainly found amongst the obligate and facultative anaerobes. Obligate aerobic nitrogen-fixing bacteria are much less frequently encountered, presumably because of the reductive character of the nitrogen-fixing process. The family Azotobacteraceae is the best known group of obligate aerobic free-living nitrogen-fixing bacteria (Mulder and Brotonegoro, 1974). Bacteria of the type of *Mycobacterium flavum* (Federov and Kalininskaya, 1961; de Bont and Leyten, 1976) form a second group of aerobic nitrogen fixers.

There is much confusion in the literature about the ability of the obligate aerobic methane-oxidizing bacteria to fix atmospheric nitrogen. Indirect evidence for nitrogen fixation by these bacteria has been available ever since Schollenberger (1930) and Harper (1939) made their observations on the effect of natural gas on soil. They noticed that soil exposed for a prolonged period of time to natural gas escaping from leaking pipe lines had a considerably higher nitrogen content than unexposed control soil. Comparable observations were made by Hoeks (1972) in a study on the effect of natural gas leaking from mains on the composition of soil air. Since methane is the main component of natural gas, and since this hydrocarbon had a similar effect on the nitrogen content of soil (Davis, Coty and Stanley, 1964; Coty, 1967), methane-oxidizing bacteria capable of fixing nitrogen were thought to be responsible. Coty also performed an experiment with

$^{15}N_2$ using an isolated culture instead of soil, but the purity of this culture may be questioned as it also grew on nutrient agar. Employing the acetylene-ethylene assay, Whittenbury, Phillips and Wilkinson (1970) obtained inconclusive results while Adamse, Hoeks, de Bont and van Kessel (1972) concluded that their strain did not fix elementary nitrogen, although growth was obtained in a nitrogen-free medium.

The purpose of the work for this thesis was to obtain more information about possible nitrogen fixation by the methane-oxidizing bacteria.

Outline of the investigations

In this thesis the investigations on nitrogen fixation by methane-oxidizing bacteria are reported in four papers.

Paper I deals with the ability of a pure culture of the methane-oxidizing bacterium strain 41 to fix atmospheric nitrogen. With $^{15}N_2$, it was demonstrated that this *Methylosinus* type bacterium converted the gas into cell material. However, nitrogenase activity could not be assayed by acetylene reduction when the bacterium was growing on methane. The organism was sensitive to oxygen when dependent on N_2 as nitrogen source.

Paper II comprises a study of the erratic behaviour of the acetylene-ethylene assay with strain 41. This study included other strains of methane-oxidizing bacteria and bacteria growing on higher alkanes.

Paper III gives the results of a survey of the nitrogen-fixing capacity amongst methane-oxidizing bacteria. This study could be undertaken with the results obtained in paper I (oxygen sensitivity) and paper II (erratic behaviour of the acetylene-ethylene assay).

Paper IV draws attention to hydrogenase activity in strain 41. This activity was associated with nitrogenase activity.

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NITROGEN FIXATION AND CO-OXIDATION OF ETHYLENE BY A METHANE-UTILIZING BACTERIUM

By J. A. M. DE BONT and E. G. MULDER

Journal of General Microbiology 83 (1974) 113–121

A methane-oxidizing bacterium, isolated from soil, was capable of fixing nitrogen. Nitrogenase activity could be assayed by acetylene reduction when the bacterium was growing on methanol but not when growing on methane, although $^{15}N_2$ was fixed. Bacteria growing on methane co-oxidized ethylene but methanol-growing cells did not. The organism was extremely sensitive to oxygen when dependent on N_2 as nitrogen source, a consequence of the sensitivity of its nitrogenase towards oxygen.

INTRODUCTION

Nitrogen accumulates in soil exposed to natural gas (Schollenberger, 1930; Harper, 1939). Since methane is the main component of natural gas, and since this hydrocarbon had a similar effect on the nitrogen content of the soil (Davis, Coty & Stanley, 1964; Coty, 1967), methane-oxidizing bacteria, capable of fixing nitrogen, were thought to be responsible. Methane-oxidizing bacteria capable of growing in nitrogen-free medium were isolated. Nitrogen fixation was measured by Kjeldahl analyses of cultures incubated for periods of up to four months (Davis *et al.* 1964), whilst Coty (1967) performed an experiment with $^{15}N_2$. As the isolated cultures also grew on nutrient agar, the purity of the cultures may be questioned. Whittenbury, Phillips & Wilkinson (1970) isolated a strain of *Methylosinus trichosporium* from Coty's culture, which was able to reduce 25% of the acetylene present during an incubation period of 7 to 14 days. All of the other strains of methane-oxidizing bacteria tested reduced very little acetylene. We report here atmospheric nitrogen fixation by a recently isolated methane-oxidizing bacterium.

METHODS

Medium. The mineral-salts solution (MS medium), used throughout the investigation, contained the following salts in 1 l deionized water: $NaNO_3$, 2·0 g; K_2HPO_4, 0·5 g; KH_2PO_4, 0·5 g; $MgSO_4 \cdot 7H_2O$, 0·2 g; $CaCl_2$, 0·015 g; $FeSO_4 \cdot 7H_2O$, 0·001 g; $CuSO_4 \cdot 5H_2O$, 5 μg; H_3BO_3, 10 μg; $ZnSO_4 \cdot 7H_2O$, 70 μg; $MnSO_4 \cdot 5H_2O$, 10 μg; $Na_2MoO_4 \cdot$

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NITROGEN FIXATION AND CO-OXIDATION OF ETHYLENE BY A METHANE-UTILIZING BACTERIUM

By J. A. M. DE BONT and E. G. MULDER

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A methane-oxidizing bacterium, isolated from soil, was capable of fixing nitrogen. Nitrogenase activity could be assayed by acetylene reduction when the bacterium was growing on methanol but not when growing on methane, although $^{15}N_2$ was fixed. Bacteria growing on methane co-oxidized ethylene but methanol-growing cells did not. The organism was extremely sensitive to oxygen when dependent on N_2 as nitrogen source, a consequence of the sensitivity of its nitrogenase towards oxygen.

INTRODUCTION

Nitrogen accumulates in soil exposed to natural gas (Schollenberger, 1930; Harper, 1939). Since methane is the main component of natural gas, and since this hydrocarbon had a similar effect on the nitrogen content of the soil (Davis, Coty & Stanley, 1964; Coty, 1967), methane-oxidizing bacteria, capable of fixing nitrogen, were thought to be responsible. Methane-oxidizing bacteria capable of growing in nitrogen-free medium were isolated. Nitrogen fixation was measured by Kjeldahl analyses of cultures incubated for periods of up to four months (Davis *et al.* 1964), whilst Coty (1967) performed an experiment with $^{15}N_2$. As the isolated cultures also grew on nutrient agar, the purity of the cultures may be questioned. Whittenbury, Phillips & Wilkinson (1970) isolated a strain of *Methylosinus trichosporium* from Coty's culture, which was able to reduce 25% of the acetylene present during an incubation period of 7 to 14 days. All of the other strains of methane-oxidizing bacteria tested reduced very little acetylene. We report here atmospheric nitrogen fixation by a recently isolated methane-oxidizing bacterium.

METHODS

Medium. The mineral-salts solution (MS medium), used throughout the investigation, contained the following salts in 1 l deionized water: $NaNO_3$, 2·0 g; K_2HPO_4, 0·5 g; KH_2PO_4, 0·5 g; $MgSO_4 \cdot 7H_2O$, 0·2 g; $CaCl_2$, 0·015 g; $FeSO_4 \cdot 7H_2O$, 0·001 g; $CuSO_4 \cdot 5H_2O$, 5 μg; H_3BO_3, 10 μg; $ZnSO_4 \cdot 7H_2O$, 70 μg; $MnSO_4 \cdot 5H_2O$, 10 μg; $Na_2MoO_4 \cdot$

$2H_2O$, 100 μg; final pH, 6·8. For solid media 1·5% agar was added to this nutrient solution.

Chemicals. Methane, ultra pure (99·97%), and CH_3OCH_3, said to be 99·87% pure (see later), were obtained from the Matheson Co., and lithium hydroxypyruvate, 98% pure, from Sigma.

Isolation and growth of the bacterium. Garden soil was incubated at 28 ± 2 °C under 10% methane in air. After 25 days, serial dilutions were made in MS medium, and after another 4 weeks under 10% methane, material of the highest dilution showing growth was streaked on plates of MS agar. Well-developed colonies had grown on these plates after 3 weeks of incubation under 10% methane. Pure cultures, obtained after restreaking on the same medium, were then tested for methane-oxidizing capacity by growing the bacterium on slants of MS with and without methane. Weekly subcultures on slants of MS and slants of MS without nitrate were maintained in a desiccator in which the oxygen tension was lowered by flushing with nitrogen. Methane was injected through a Suba-seal mounted in a rubber stopper. Growth rate was measured in stirred 350 ml side-arm flasks sealed with rubber stoppers, fitted with Suba-seal caps. Cell density was measured with an EEL nephelometer. During assays of acetylene reduction the cultures were stirred on a New Brunswick Scientific G 10 gyrotory shaker at 150 rev./min.

Preparation of cell-free extract. Washed cells, suspended in 0·03 M-sodium phosphate buffer, were disrupted by ultrasonic disintegration. The extract was centrifuged and the supernatant fluid used for assay.

Enzyme assays. Hydroxypyruvate reductase (EC. 1.1.1.29) and glyoxylate reductase (EC. 1.1.1.26) were assayed in cuvettes (3 ml, light path 1 cm) containing 100 μmol phosphate buffer, pH 6·8, 0·4 μmol NADH and extract in a total volume of 3 ml. Lithium hydroxypyruvate (2 μmol) or sodium glyoxylate (2 μmol) were added and the decrease in extinction at 340 nm was measured against a blank containing buffer, NADH and extract.

Determination of protein. The Folin-Ciocalteu method was used (Herbert, Phipps & Strange, 1971).

Gas-chromatographic analyses. Analyses were carried out at 50 °C with a Becker Multigraph type 407 gas chromatograph. Oxygen and nitrogen were measured by thermal conductivity using a 200 cm × 4 mm column containing a 13X molecular sieve (60–80 mesh). Hydrogen was used as carrier gas. Methane, dimethylether, and reduction of acetylene were assayed with a flame-ionization detector, using a 110 cm × 4 mm Porapak R column with nitrogen as carrier gas.

^{15}N analyses. Sample preparation was a modification (Akkermans, 1971) of the method of Faust (1967). Optical N-analyses were made with a ^{15}N analyser (Statron NOI-4). The method of Ferraris & Proksch (1972) was used for calculating the ^{15}N content.

RESULTS

Description of the bacterium. The isolate used, strain 41, is a curved rod, motile in young cultures (Fig. 1). On ageing of the culture, motility is lost and exospores are formed. In old cultures, only exospores and lysed cells were found (Fig. 2). Morphologically, the organism differs slightly from the isolates of Adamse, Hoeks, de Bont & van Kessel (1971) and of Namsareav & Zavarzin (1972), but resembles one of the types described by Whittenbury *et al.* (1970) (*Methylosinus sporium*), and by Hazeu & Steennis (1970) (*Methylovibrio söhngenii*). Purity of the strain was confirmed by microscopic examination and by streaking the culture onto various media for heterotrophs without obtaining growth. On plates of MS, uniform colony development was seen under methane. On slants of MS, without methane added, growth was just visible macroscopically and the bacteria present resembled strain 41 morphologically. A second transfer did not grow. Attempts to promote growth without methane by the addition of small quantities of yeast extract or peptone were unsuccessful. Higher concentrations of these and other

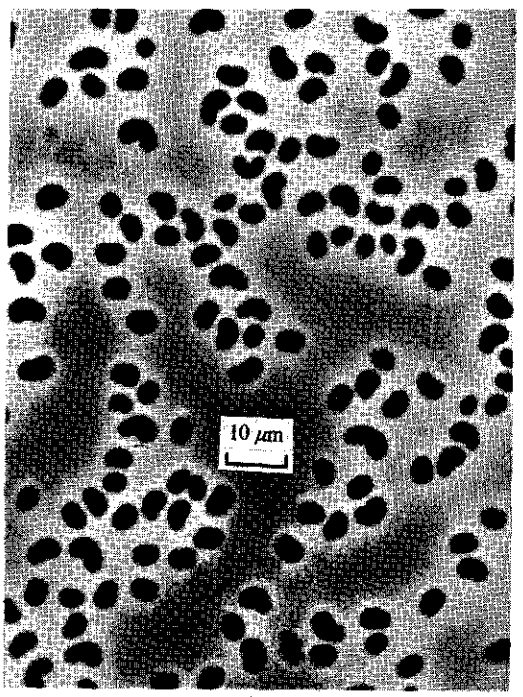

Fig. 1

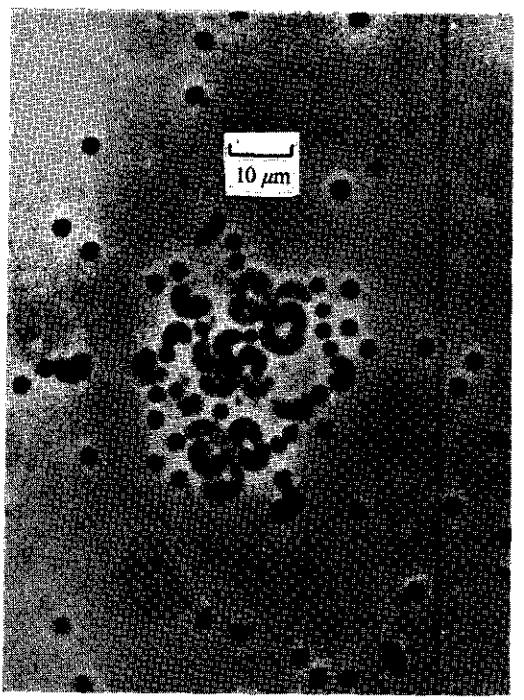

Fig. 2

Fig. 1. Strain 41 from a five-day culture, grown on slants of MS under 10% CH_4.

Fig. 2. Strain 41, exospores and lysed cells, from a three-week culture, grown on slants of MS under 10% CH_4.

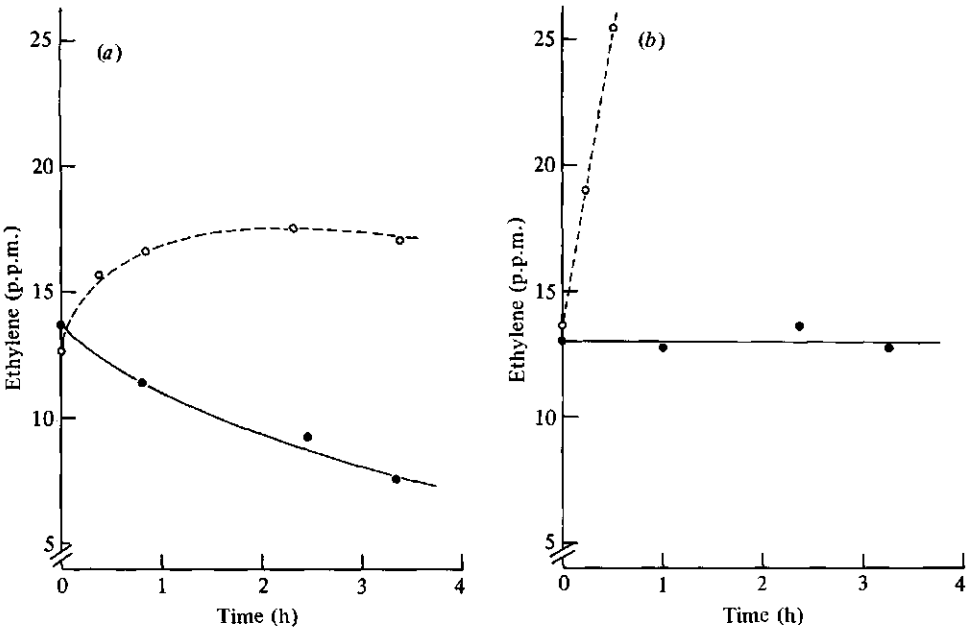

Fig. 3. Effect of strain 41 culture, growing in nitrogen-free MS with 10% CH_4 (*a*) and 0·1% CH_3OH (*b*), on ethylene (13 p.p.m.) in the absence (•) and presence (○) of 10% C_2H_2. Ethylene concentrations were measured in 50 ml flasks with 10 ml growing cultures. The gas phase in the flasks consisted of 5% O_2, 10% CH_4 and 85% N_2 (*a*) and 5% O_2 and 95% N_2 (*b*) before the introduction of ethylene and acetylene.

substrates completely prevented growth of the first transfer. Strain 41 also utilized CH_3OH for its development. No growth occurred with C_2H_2, C_2H_4, C_2H_6, CH_3OCH_3, C_2H_5OH, glucose or acetate when added as potential substrates. No definite conclusions can be drawn for CH_3OCH_3. Our gas-chromatographic analysis of the contents of the cylinder containing CH_3OCH_3 (99·87% pure according to the supplier) indicated the presence of about 5% methane and approximately 0·5% of an unidentified compound among other minor impurities. Utilization of CH_3OCH_3 by methane-oxidizing bacteria has been reported and its role in the biological breakdown of methane discussed (Quayle, 1972).

Hydroxypyruvate reductase, the key enzyme of the serine pathway (Large & Quayle, 1962), was present in strain 41. The activities were 21 μmol NADH oxidized/h/mg protein for hydroxypyruvate and 1·5 for glyoxylate.

Growth in nitrogen-free medium. If incubated without shaking, strain 41 grew slowly in MS medium under methane when nitrate was omitted from the medium. Acetylene-reduction assays for nitrogenase activity gave negative results. Growth in nitrogen-free

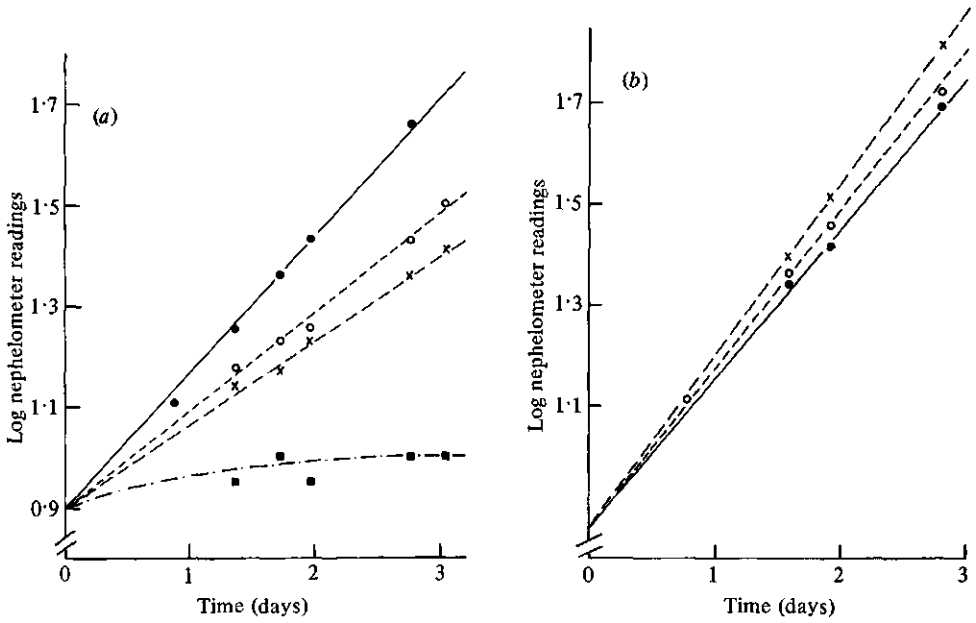

Fig. 4. Growth of strain 41 on CH_4 as influenced by O_2. Portions (10 ml) of a culture growing logarithmically were injected into 350 ml side-arm flasks containing 10% CH_4 and varying concentrations of O_2 and N_2. O_2 consumption during the experiment was less than 2%. (*a*) Growth in nitrate-free medium: •, 4% O_2; ○, 6% O_2; ×, 8% O_2; ▪, 11% O_2. (*b*) Growth with nitrate: •, 6% O_2; ○, 11% O_2; ×, 15% O_2.

medium was strongly promoted by lowering the oxygen tension, although no acetylene reduction was detected. When growing the organism in the same medium with methanol as substrate, ethylene production was clearly demonstrated after 10% acetylene had been introduced. The amount of ethylene formed could quantitatively account for the growth utilizing nitrogen fixation. The apparent difference in acetylene reduction may be due to the ability of CH_4-grown cells to remove ethylene from the gas phase. After introduction of acetylene into such a gas phase, the ethylene concentration remained at the same level (Fig. 3*a*). Cells grown on methanol did not affect ethylene (Fig. 3*b*).

Effect of oxygen. Growth of strain 41 in liquid MS medium with methane was little influenced by oxygen tensions of between 4 and 20%. On nitrogen-free medium, however, increasing the oxygen tension severely reduced growth (Fig. 4*a*, *b*). Similar phenomena were observed when the bacterium was grown on agar plates of MS under methane. Colony size after three weeks of incubation was not influenced by varying the oxygen tension. But when nitrogen-free MS was used, the effect of oxygen became apparent. Under 20% oxygen only meagre growth developed. Growth was better in restricted areas of such a plate (Fig. 5). Incubation in 5% oxygen allowed normal development on nitrogen-free medium (Fig. 6). That the sensitvity towards oxygen is directly related to the nitrogenase activity was shown by measuring acetylene reduction by methanol-grown

Fig. 5

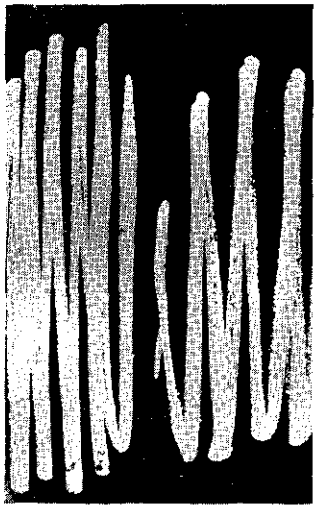

Fig. 6

Fig. 5. Growth of strain 41 on nitrogen-free MS agar medium under 20% O_2, 10% CH_4 and 70% N_2 after 3 weeks of incubation, showing sparse growth except at the edges of the streaks.

Fig. 6. Growth of strain 41 on nitrogen-free MS agar medium under 5% O_2, 10% CH_4 and 85% N_2 after 3 weeks of incubation.

cells exposed to varying oxygen tensions (Fig. 7). In a subsequent experiment, nitrogenase was inactivated by exposure to 28% oxygen in the atmosphere and activity restored after lowering the oxygen tension (Fig. 8).

The behaviour of strain 41 towards oxygen (Fig. 4*a*, *b*) resembles that of certain Azotobacter species (Dalton & Postgate, 1969). Both types of bacteria are highly sensitive to moderate concentrations of oxygen when depending on N_2 as nitrogen source, but insensitive to oxygen in the presence of combined nitrogen. The poor growth of strain 41 under methane on a nitrogen-free agar medium when exposed to air, in contrast to normal growth at decreased oxygen tension (Figs. 5 and 6), agrees with a similar behaviour of the nitrogen-fixing *Derxia gummosa* (Hill, 1971). In both organisms the nitrogenase system does not function when the agar plates are kept in air, but normal nitrogen fixation occurs at reduced oxygen pressure. Sporadically occurring nitrogen-fixing colonies may arise on plates exposed to air as a result of locally occurring clumps of the inoculated organism. Oxygen supply to cells in the interior of the clumps would be lowered, so the development of the nitrogenase system would become possible. Nitrogen-fixing colonies are often seen at the end of an inoculation streak of a nitrogen fixer where accumulation of cells easily occurs.

A response similar to that of nitrogenase of strain 41 towards oxygen (Figs. 7 and 8) has been found for nitrogenase of *Azotobacter chroococcum* (Postgate, 1971; Drozd & Postgate, 1970).

Table I. $^{15}N_2$ *fixation by strain* 41

	Time of incubation (h)	Nephelometer readings	Suspension (ml)	Nitrogen/ sample (μg)	Atom % ^{15}N	
					Measured	Theoretical*
Blank	0	9	70	180	0·37	–
Sample 1	8	13	40	134	1·84	3·45
Sample 2	23	24	30	225	5·13	6·62

* See results.

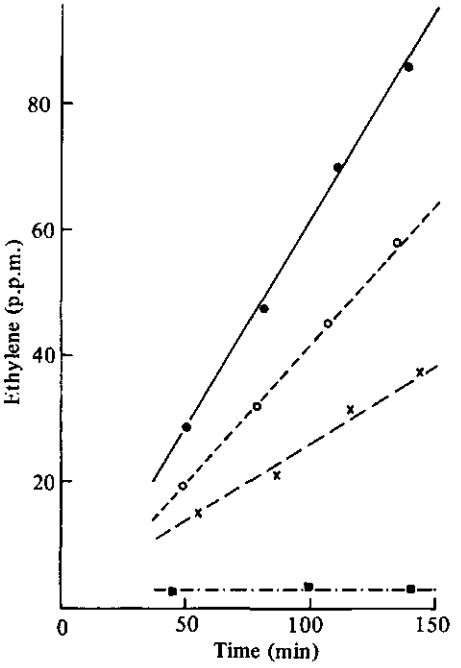

Fig. 7

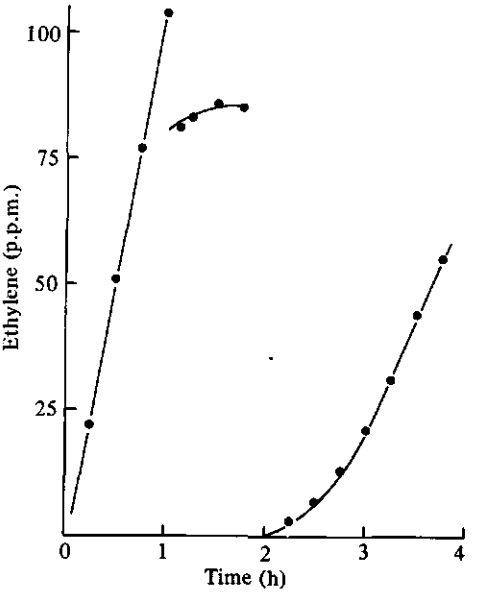

Fig. 8

Fig. 7. Acetylene reduction as influenced by O_2. Portions (10 ml) of a culture growing under reduced O_2 tension on CH_3OH in nitrogen-free MS were injected into 50 ml flasks containing 10% C_2H_2 and different levels of O_2 and N_2. •, 4·4% O_2; ○, 6·3% O_2; ×, 10·2% O_2; ■, 18·0% O_2.

Fig. 8. Reactivation of nitrogenase, inactivated after exposure to high O_2 concentration. Thirty ml of a culture growing under reduced O_2 tension on CH_3OH in nitrogen-free MS was injected into a 100 ml flask containing 10% C_2H_2, 4·5% O_2 and 85·5% N_2, and ethylene production measured for 1 h. Excess O_2 was then injected. After releasing pressure, the O_2 concentration was 28%. After 45 min the flask was opened, flushed with N_2, and C_2H_2 was restored to 10%; O_2 tension was now 3·5%.

Nitrogen fixation on methane. Evidence for nitrogen fixation by strain 41 when growing with methane was obtained with $^{15}N_2$ (Table 1). The bacteria were grown in nitrogen-free medium at reduced oxygen tension. In early logarithmic phase, 70 ml of the culture was injected into a 250 ml flask. Another 70 ml was used as a blank. The gas phase in the flask consisted of 20% methane, 5% oxygen and 75% nitrogen. The nitrogen gas was enriched with 10% ^{15}N. After 8 h, 40 ml of the sample was withdrawn and the gas phase inside the flask was brought back to atmospheric pressure with helium. The second sample was taken after the culture had grown for 23 h.

After correcting for the blank, the excess ^{15}N percentages of 1·47 and 4·76 in the culture were of the same comparative magnitude as values of 3·08 and 6·25, respectively, calculated by using nephelometer readings as a measure for nitrogen assimilation in growing cultures. The low value for the first sample could be due to dissolved nitrogen gas in the culture when it was placed under the gas phase enriched with $^{15}N_2$.

Additional evidence for nitrogen fixation was obtained by growing strain 41 in nitrogen-free medium under, initially, 10% methane and 6% oxygen, with helium/nitrogen gas mixtures to give different nitrogen pressures. Fig. 9 shows that under these conditions, growth increased as the N_2 increased. From this experiment, it was estimated that the K_m value for N_2 will be approximately 16%. This value is in agreement with K_m constants found for crude extracts of other nitrogen-fixing bacteria (Parejko & Wilson, 1971).

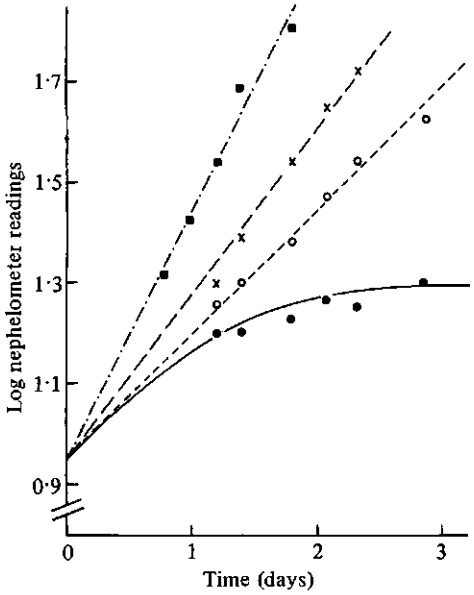

Fig. 9. Effect of N_2 on the growth of strain 41 in nitrogen-free MS medium with methane. Portions (10 ml) of a culture growing in nitrogen-free MS under CH_4 were injected into 350 ml side-arm flasks with 6% O_2, 10% CH_4 and different concentrations of N_2 and He to 1 atm. •, 5·7% N_2; ○, 11·9% N_2; ×, 20·4% N_2; ■, 80·6% N_2.

DISCUSSION

Strain 41 has the physiological characteristics of a methane-oxidizing bacterium. Only methane and methanol support growth. The presence of hydroxypyruvate reductase indicated that synthesis of cellular material proceeded by the serine pathway (Lawrence & Quayle, 1970).

Measurement of nitrogen fixation by methane-oxidizing bacteria during prolonged periods of incubation (Davis *et al.* 1964) is open to criticism. Uptake of $^{15}N_2$ by one of the isolated strains (Coty, 1967) provided more convincing evidence of the nitrogen fixation. The fact that the isolated culture grew on nutrient agar suggests that it was not pure. This was corroborated by the isolation of a strain of *Methylosinus trichosporum* from Coty's culture (Whittenbury *et al.* 1970). Upon exposure to an atmosphere containing 4·4% methane and 1·8% acetylene for 7 or 14 days, this isolate had reduced the acetylene concentration by 25%. It is unknown whether the disappearance of acetylene was due to co-oxidation of acetylene or to reduction of this compound to ethylene; data on ethylene production were not reported. From this result no definite conclusions can be drawn concerning the nitrogen-fixing ability of the organism, because only short-term exposure times should be used in the acetylene-reduction technique for assessing presence of nitrogenase (Hardy, Burns & Holsten, 1973).

Our results demonstrate fixation of atmospheric nitrogen by the methane-oxidizing strain 41. Nitrogenase activity, measured by acetylene reduction, could only be detected when the bacteria were growing on methanol without combined nitrogen. Co-oxidation of ethylene by cultures grown under methane and acetylene may explain the apparent lack of ethylene production from acetylene. Alternatively, acetylene may block methane oxidation and thus prevent the supply of ATP to nitrogenase.

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INVALIDITY OF THE ACETYLENE REDUCTION ASSAY IN ALKANE-UTILIZING, NITROGEN-FIXING BACTERIA

By J. A. M. DE BONT and E. G. MULDER

Applied and Environmental Microbiology, 31 (1976) 640–647

The cause of the failure of the C_2H_2-C_2H_4 assay for nitrogen-fixing bacteria growing on lower alkanes was studied. Acetylene was a strong competitive inhibitor of methane oxidation for methane-utilizing bacteria, as well as for the oxidation of lower alkanes by other bacteria, so that energy and reducing power were no longer available for the reduction of acetylene by nitrogenase. Nitrogen-fixing bacteria grown on alkanes may reduce acetylene when intermediates of alkane-breakdown or other substrates oxidizable in the presence of acetylene are supplied. Ethylene co-oxidation is not responsible for the failure of the test, because acetylene also inhibits this co-oxidation along with methane oxidation.

INTRODUCTION

Because of its sensitivity and simplicity, the acetylene reduction assay is generally applied to measure biological nitrogen fixation (7). In using the C_2H_2-C_2H_4 assay, it is assumed that (i) acetylene does not interfere with other metabolic activities of the system under study and (ii) ethylene is stable during the investigation. These two factors have not been studied thoroughly as yet, although reports have appeared suggesting that they may influence the results of the test. Brouzes and Knowles (1) reported the prevention of the normal growth pattern of a nitrogenase-repressed culture of *Clostridium pasteurianum* in a medium supplemented with $(NH_4)_2SO_4$ with 0.1 atmosphere of acetylene in the gas phase. The acetylene inhibited cell proliferation and prevented an increase in the rate of carbon dioxide production, normally associated with growth.

The C_2H_2-C_2H_4 test failed to show nitrogenase activity in nitrogen-fixing, methane-oxidizing bacteria of strain 41 of the *Methylosinus* type (2). Nitrogenase activity could not be assayed by the test when the bacterium was growing on methane, although $^{15}N_2$ was fixed. Bacteria growing on methane co-oxidized ethylene, but cells growing on methanol did not. This co-oxidation of ethylene by methane-grown cells might have caused the test to fail. Alternatively, acetylene might have blocked methane oxidation, thus preventing a supply of energy and reducing power to the nitrogenase.

This paper gives the results of a more detailed study of acetylene inhibition of cell metabolism in the C_2H_2-C_2H_4 assay in alkane-utilizing bacteria.

MATERIALS AND METHODS

Organisms. Mycobacterium vaccae, originally isolated by Ooyama and Foster (11), was provided by J. J.Perry, North Carolina State University, Raleigh. Strains 41 and E20 have been described previously (2, 3).

Strain Et32 was isolated by incubating 10 g of soil with 25 ml of mineral salts (MS) medium (see below) under 10% ethane in air in a 1-liter Erlenmeyer flask. After 2 weeks of incubation, the enrichment was streaked on plates of MS medium. The bacterium was isolated after incubating under ethane in air, followed by restreaking on the same medium. Strain Et32 is a gram-negative, yellow-pigmented rod.

Strain H2 was similarly isolated from soil, except that hexane replaced ethane. Strain H2 is a gram-positive pleomorphic bacterium.

Strain 3b was isolated from garden soil incubated in an Erlenmeyer flask containing 10% ethane in air. Every 3 weeks the flask was flushed with air, and the ethane concentration was restored. After 4 months, a macroscopically visible, yellowish colony had appeared on the surface of the soil sample. Material from this colony was streaked on plates of nitrate-free MS medium. Colonies had grown on these plates after 6 weeks of incubation in an ethane-containing desiccator at reduced oxygen pressure. A pure culture was obtained after restreaking on the same medium.

Strain H12 was isolated by incubating a mixture of different soil and water samples of approximately 10 g with 25 ml of nitrate-free MS medium in a sealed 2-liter Erlenmeyer flask. Hexane (2 ml) was added, and the oxygen pressure was reduced by flushing with nitrogen. Material from the enrichment was streaked on plates of nitrate-free MS medium after 40 days of incubation. After 4 weeks of incubation in a desiccator containing 5% oxygen and hexane, colonies on these plates were well developed. The pure culture was obtained after restreaking on the same medium.

Medium. The MS medium, used throughout the investigation, containing the following salts in 1 liter of deionized water: $NaNO_3$, 2.0 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.015 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; $CuSO_4 \cdot 5H_2O$, 5 μg; H_3BO_3, 10 μg; $ZnSO_4 \cdot 7H_2O$, 70 μg; $MnSO_4 \cdot 5H_2O$, 10 μg; $Na_2MoO_4 \cdot 2H_2O$, 100 μg; final pH, 6.8. For solid media, 1.5% agar was added to this nutrient solution.

Chemicals. Methane, ultrapure (99.97%), and other gaseous substrates (commercial purity) were obtained from the Matheson Co.

Culture conditions. Weekly subcultures on slants of MS medium or nitrate-free MS medium were kept in desiccators at reduced oxygen pressure (5% or less) with approximately 5% of the desired gas and N_2 present at 30 C. Growth experiments were also carried out at this temperature.

Oxygen uptake. Dissolved oxygen concentrations were measured at 30 C with a Yellow Springs Instruments model 53 oxygen monitor equipped with a polarographic sensor. This electrode fits into a 15-ml cell; it has an access slot for removal of overflowing liquid. The slot can also be used for adding or withdrawing small samples from the cell.

Gas chromatorgraphic analyses. Analyses were carried out with a flame-ionization

detector with a Becker type 409 or type 417 chromatograph. A Porapak R column (110 cm by 4 mm) with nitrogen as carrier gas was used for assaying methane (50 or 150 C when in solution), acetylene reduction (50 C), ethylene and ethane (80 C), and methanol (120 C). Acetylene was assayed with a Porapak T column (120 cm by 4 mm) at 90 C.

RESULTS

Methane-oxidizing bacterium strain 41 of the Methylosinus type. The methane-oxidizing, nitrogen-fixing strain 41 did not accumulate ethylene when exposed to methane in the presence of acetylene. However, ethylene was produced linearly by methane-grown cells when methanol or formate was added during the assay (Fig. 1). Also, compounds other than methanol or formate, which are both intermediates of methane oxidation, supported the reduction of acetylene, e.g., ethanol and butanol.

If co-oxidation of ethylene by methane-grown cells caused the failure of the test, ethylene should not be accumulated by such cells in the presence of methanol or formate unless these compounds specifically suppress the co-oxidation. Addition of methanol to a

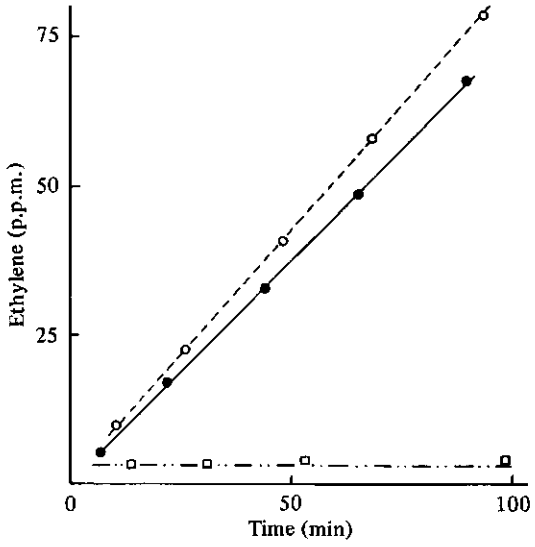


Fig. 1. Effect of methanol and formate on acetylene reduction by strain 41 grown on methane in nitrate-free MS medium. Portions (9 ml) of a 100-ml culture, growing logarithmically in a 1,000-ml Erlenmeyer flask, were injected into 100-ml Erlenmeyer flasks containing 10% CH_4, 5% O_2, 10% C_2H_2, and 75% N_2 with 1 ml of nitrate-free MS medium containing 0.1% of the compounds indicated. Symbols: (●) Methanol; (○) sodium formate; (□) no supplementary compound added.

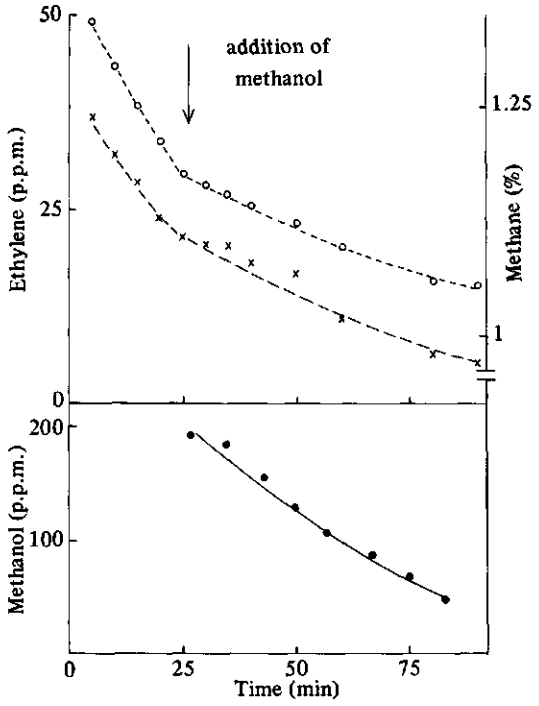


Fig. 2. Inhibition by methanol of methane oxidation and of ethylene co-oxidation. A 100-ml culture of strain 41, grown on methane in MS medium, was centrifuged and suspended in 10 ml of 0.03 M sodium phosphate buffer, pH 6.8. This suspension was injected into a 100-ml Erlenmeyer flask, containing methane and ethylene in air. Methanol was injected at the time indicated. Symbols: (•) Methanol (liquid phase); (○) ethylene (gas phase); (×) methane (gas phase).

cell suspension reduced the rate of ethylene co-oxidation as well as the rate of methane oxidation, but the co-oxidation was not completely stopped by methanol (Fig. 2), suggesting that co-oxidation was not responsible for the failure of the test with methane-grown cells.

Acetylene, always present during the assay, suppressed the oxidation of methane as well as the co-oxidation of ethylene (Fig. 3). This observation definitely rules out co-oxidation of ethylene as the possible cause of the failure of the C_2H_2-C_2H_4 assay.

Mechanism of inhibition of methane oxidation by acetylene. When strain 41 was cultivated on methane in MS medium, the growth of the bacterium was completely inhibited in the presence of 10% acetylene, whereas growth on methanol in MS medium was not affected. Apparently, the first step in the degradation route of methane, the oxidation of methane to methanol, was blocked by acetylene. This was also shown by the course of the oxygen uptake of whole-cell suspensions of strain 41 grown on methane

(Fig. 4). Acetylene suppressed methane-dependent oxygen uptake, whereas it did not influence methanol-dependent oxygen uptake.

The first step in the breakdown of methane by strain 41 is performed by an adaptive enzyme system. Methane did not increase oxygen uptake of whole-cell suspensions of methanol-grown bacteria (Table 1). The enzyme system involved in methane oxidation is presumably analogous to the methane hydroxylase described for *Methylococcus capsulatus* (12) and for *Pseudomonas methanica* (5). Methane hydroxylase is also capable of oxidizing ethane (12) and carbon monoxide (5), explaining the adaptive nature of the co-oxidation of these compounds by strain 41. The co-oxidation of ethylene was also adaptive, demonstrating that methane hydroxylase probably oxidized this compound. Oxidation of methane and co-oxidation of methane hydroxylase-dependent cosubstrates

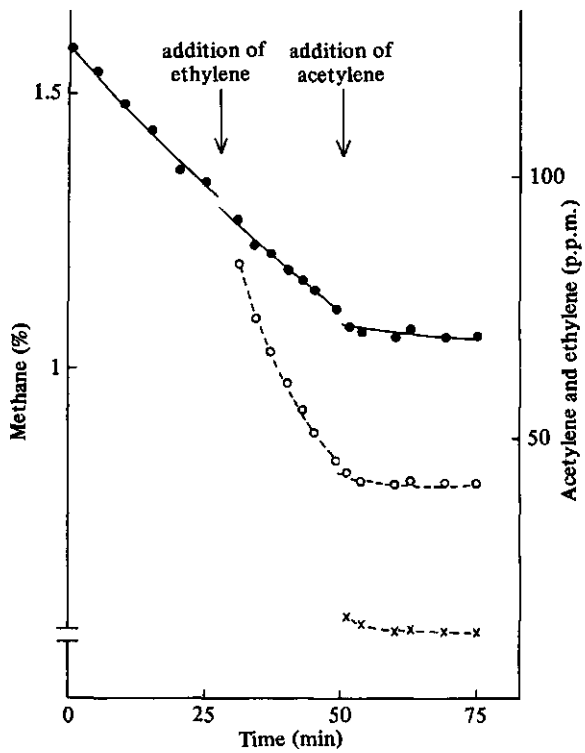


Fig. 3. Inhibition by acetylene of methane oxidation and of ethylene co-oxidation. A 100-ml culture of strain 41, grown on methane in MS medium, was centrifuged and suspended in 10 ml of 0.03 M sodium phosphate buffer, pH 6.8. This suspension was injected into a 100-ml Erlenmeyer flask, containing methane in air. At the time indicated, 1.5 ml of approximately 5,000 µl of C_2H_4 per liter in air and 2 ml of approximately 750 µl of C_2H_2 per liter in air were injected into the flask. Symbols: (•) Methane; (o) ethylene; (x) acetylene.

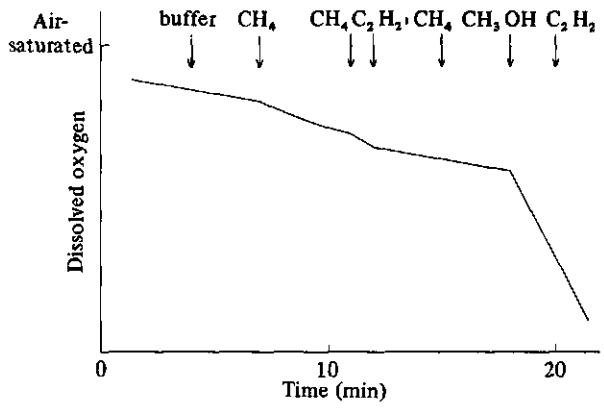


Fig. 4. Recorder trace of the uptake of dissolved oxygen by a whole-cell suspension of strain 41. A culture growing logarithmically on CH_4 in MS medium was centrifuged and suspended in an equal volume of 0.03 M sodium phosphate buffer, pH 6.8. At the times indicated by the arrows, 0.05 ml of the same buffer, 0.05 ml of buffer saturated with CH_4 or with C_2H_2, or 0.05 ml of buffer with 0.1% CH_3OH was injected into the suspension.

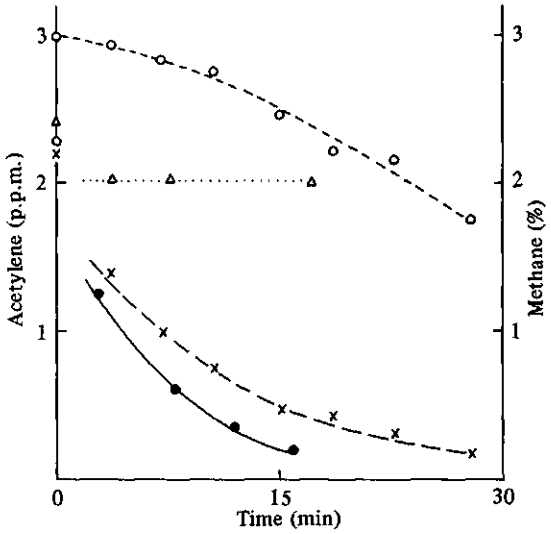


Fig. 5. Uptake of acetylene by strain 41 grown on CH_4 or on CH_3OH. Cultures of 250 ml were centrifuged and suspended in 10 ml of 0.03 M sodium phosphate buffer, pH 6.8. The suspensions were injected into 100-ml Erlenmeyer flasks containing C_2H_2 in air. Methane-grown cells in the presence (x) or absence (•) of CH_4; concentration of CH_4 (○); methanol-grown cells (△).

Table 1. Rates of oxygen uptake by cell suspensions of strain 41[a]

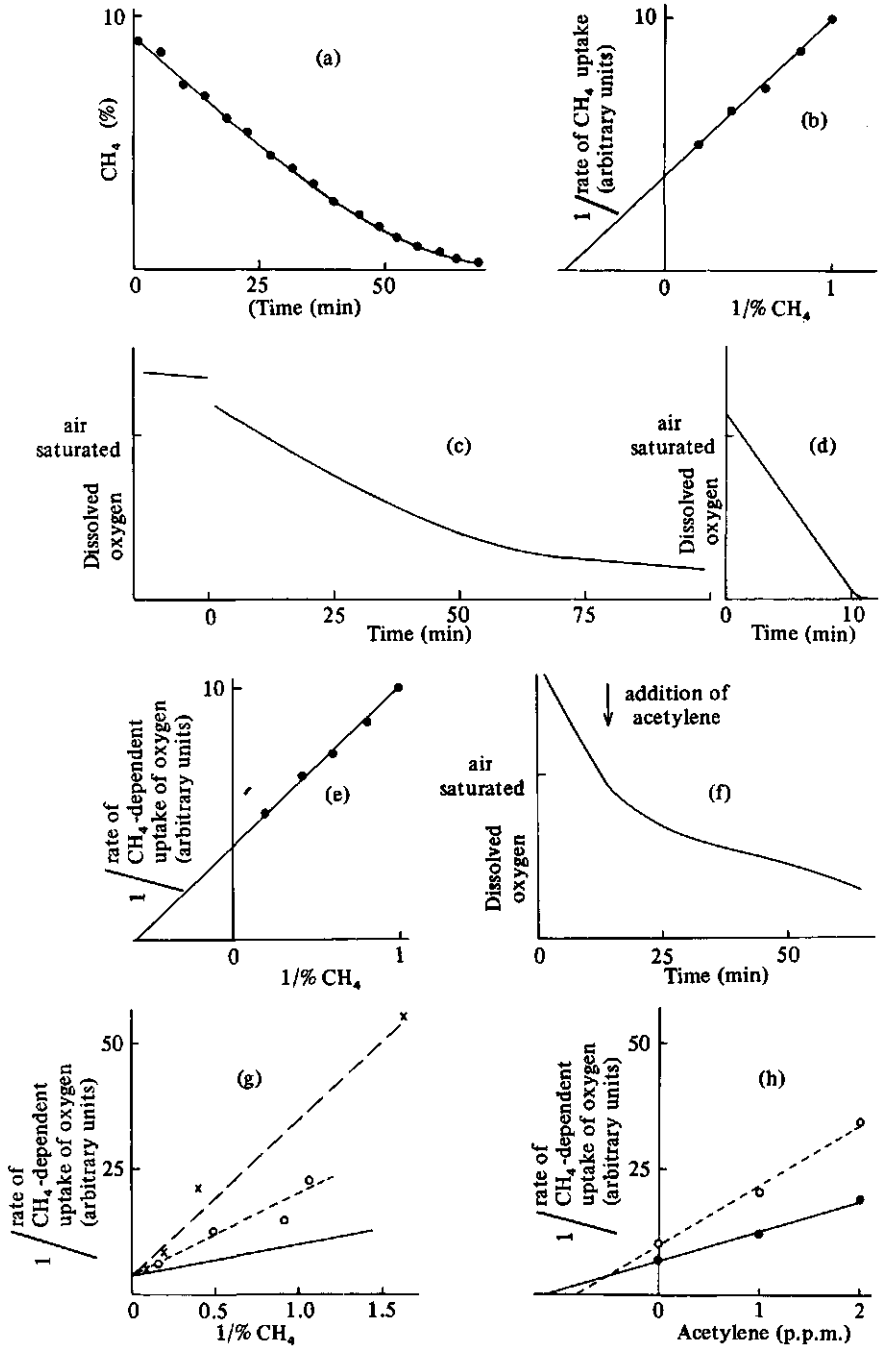
Substrate	CH_4 grown		CH_3OH grown	
	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$
Methane	100	0	0	
Ethane	100	0	0	
Ethylene	100	0	0	
Acetylene		0		0
Carbon monoxide	50	0	0	
Methanol	1,300	1,300	500	500
Ethanol	800	800	400	400
Formate	250	250	100	100
Formaldehyde	600	600	500	500

a Cultures of strain 41 pregrown on methane (CH_4 grown) or methanol (CH_3OH grown) were centrifuged and suspended in 0.03 M sodium phosphate buffer, pH 6.8. The course of the oxygen uptake was recorded for 5 min with an oxygen electrode with ($+C_2H_2$) and without ($-C_2H_2$) acetylene at a final concentration of 1%. Substrates were added by injection into the suspension phosphate buffer with 1% of substrate dissolved (liquid and solid substrates) or saturated with 100% of the gases. Rates of oxygen uptake are expressed as percentages of increase in oxygen uptake over the endogenous rate.

were inhibited by acetylene. Oxidation of intermediates of the methane breakdown (e.g., methanol), as well as that of cosubstrates not dependent upon the hydroxylase for their breakdown (e.g., ethanol), was not influenced by acetylene (Table 1). Acetylene itself was apparently not co-oxidized by the hydroxylase, as judged by the oxygen uptake data. However, by applying lower concentrations of acetylene, its disappearance from the gas phase could be directly measured, and then the effect of methane-grown cells on acetylene was assessed. Methanol-grown cells did not show uptake of acetylene, indicating that methane hydroxylase was involved in the removal of acetylene from the gas phase (Fig. 5).

Activity of methane hydroxylase is obtained only with difficulty in cell-free extracts and, moreover, it does not remain stable for long (5, 12). Therefore, experiments with whole-cell suspensions were undertaken to study the inhibition of methane hydroxylase activity by acetylene. Uptake of methane and of dissolved oxygen, both dependent on methane concentration, by wholecell suspensions of strain 41 was measured with an oxygen-electrode cell.

The rate of oxygen uptake, after correcting for endogenous respiration, was directly proportional to the rate of methane uptake but independent of the actual concentration of methane. An apparent K_m of approximately 1.7% for methane in the gas phase at 30 C was obtained both by measuring the uptake of methane and by recording the rate of oxygen uptake as a function of the actual methane concentration (Fig. 6a, c, and e). The rate of oxygen uptake was independent of the oxygen concentration (Fig. 6d). The inhibition of oxygen uptake after the addition of acetylene was taken as a measure of the inhibition of methane oxidation. Due to the uptake of acetylene, only the deviation


10
(a)
CH_4 (%)
0
25
50
(Time (min)
10
1/rate of CH_4 uptake (arbitrary units)
(b)
0
1
1/% CH_4
air saturated
Dissolved oxygen
(c)
0
25
50
75
Time (min)
air saturated
Dissolved oxygen
(d)
0
10
Time (min)
10
1/rate of CH_4-dependent uptake of oxygen (arbitrary units)
(e)
0
1
1/% CH_4
addition of acetylene
air saturated
Dissolved oxygen
(f)
0
25
50
Time (min)
50
25
1/rate of CH_4-dependent uptake of oxygen (arbitrary units)
(g)
0
0.5
1.0
1.5
1/% CH_4
50
25
1/rate of CH_4-dependent uptake of oxygen (arbitrary units)
(h)
0
1
2
Acetylene (p.p.m.)

during the first 3 min after acetylene addition could be used for the purpose of this experiment (Fig. 6f). The result of eight measurements of inhibition of methane-dependent oxygen uptake by two different concentrations of acetylene at varying methane concentrations is shown in Fig. 6g. The graph obtained is consistent with a competitive pattern of inhibition. An apparent K_m of 0.5 μl/liter for acetylene in the gas phase at 30 C can be decuded from Fig. 6h.

Other alkane-utilizing bacteria. Inhibition of growth on methane by acetylene was not restricted to strain 41. Fifteen other strains of methane-oxidizing bacteria isolated from soil and water, including *Methylosinus* as well as *Methylomonas* types, were cultivated on slants of MS medium with 10% methane in desiccators with and without 50 μl of acetylene per liter. The strains grew only in the absence of acetylene.

The study of the inhibition of cell metabolism by acetylene has been extended to bacteria utilizing straight-chain hydrocarbons. Five such bacteria were isolated from soil. Two of them (strain 3b and H12) were capable of fixing atmospheric nitrogen. Except strain H12, growth at the expense of lower hydrocarbons was prevented by acetylene. With hexadecane and decane, or with nonhydrocarbon substrates, acetylene did not influence growth (Table 2).

Because the oxidation of ethane by strain 3b was inhibited by acetylene, no appreciable ethylene formation from acetylene was found with ethane as substrate. After the addition of acetaldehyde, presumably an intermediary product in the breakdown of ethane, ethylene was formed (Fig. 7). Strain H12 actively reduced acetylene with butane as its energy source, as could be anticipated from its capacity to grow on this substrate in the presence of acetylene.

Fig. 6. Inhibition of methane oxidation by acetylene. A culture of strain 41 pregrown on methane in MS medium was centrifuged and suspended in 0.03 M sodium phosphate buffer, pH 6.8. Approximately 0.5 ml of the suspension was placed in the oxygen-electrode cell together with appropriate amounts of buffer solution saturated with CH_4 or O_2 to give the desired concentrations of the gases in the solution. The final volume was brought to 10 ml with buffer solution. Concentrations of CH_4 and C_2H_2 in solution are expressed as concentrations in the atmosphere in equilibrium with the solution. Methane concentration in the liquid phase was followed by periodically taking 2.5-μl samples from the cell for gas chromatographic analyses (a). From this progress curve, tangents at different CH_4 concentrations were used to obtain a Lineweaver-Burk reciprocal plot (b). The same procedure was used to find the apparent K_m for methane using the oxygen-electrode trace after correcting for endogenous respiration (c). The actual CH_4 concentrations needed for the reciprocal plot (e) were known from the methane progress curve (a). Independence of CH_4 oxidation of O_2 concentration was demonstrated with a suspension that was not limited in methane (d). Inhibition by C_2H_2 of CH_4-dependent O_2 uptake was found by injecting buffer solution in equilibrium with 1,000 μl of C_2H_2 per liter into the cell. (f) shows the oxygen trace as affected by 30 μl of C_2H_2-containing buffer; the arrow indicates the addition of C_2H_2. The solid line in (g) represents the result obtained in (e). Taking this line as a standard, points have been calculated by measuring the slope of the oxygen trace before and after the addition of 10 μl (○) or 20 μl (×) of C_2H_2-containing buffer at different CH_4 concentrations. Replotting values of (g) for 1% CH_4 (○) and 2% CH_4 (●) produces (h).

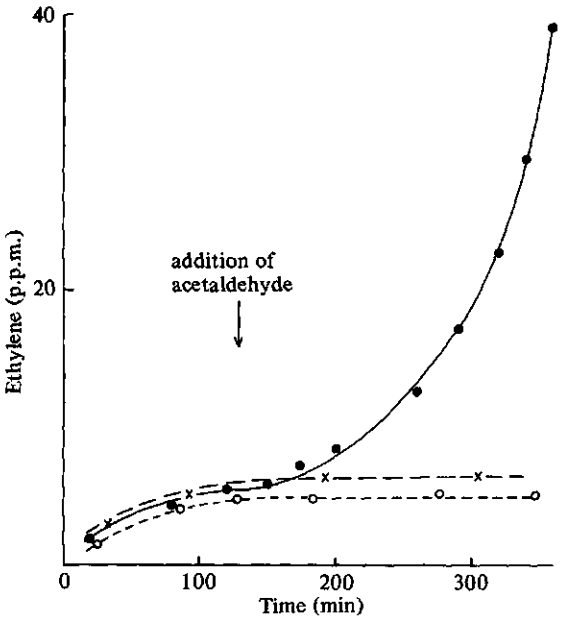


Fig. 7. Acetylene reduction by strain 3b as affected by ethane and acetaldehyde. Portions (10 ml) of a culture growing on ethane in nitrate-free MS medium were injected into 100-ml Erlenmeyer flasks containing 10% C_2H_2, 2% O_2, 10% C_2H_6, and 78% N_2 (○); (×) C_2H_6 omitted, 88% N_2; (●) 5 µl of acetaldehyde injected as indicated by the arrow.

Table 2. *Inhibition of growth of hydrocarbon-utilizing bacteria by acetylene*[a]

Organism	Growth substrates																			
	Methane		Ethane		Propane		Butane		Hexane		Decane		Hexadecane		Methanol		Ethanol		YEG	
	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$
41	+	−	−												+	+			−	
M. vaccae	−		+	−	+	−			+	−	+	+	+	+						
Et32			+	−			+	−	+	−			+	+			+	+	+	+
H2			−		−				+	−	+	+	+	+			−		+	+
E20			+	−	+	−	+	−	+	−	+	+	+	+			+	+	+	+
3b	−		+	−			+	−	−				−				+	+	+	+
H12			−				+	+	+	+	+	+	−						+	+

a Organisms were grown on the respective substrates and streaked onto slants of MS medium and slants of a yeast extract-glucose medium (YEG). The slants were placed in sealed 1-liter Erlenmeyer flasks in the absence ($-C_2H_2$) or presence ($+C_2H_2$) of 10% acetylene. Substrates were given to the MS medium slants at concentrations of 10% (gases), as 10 ml of a 1% solution in water in the flasks (alcohols), or by putting a drop of the substrate onto the slants (higher alkanes). Growth (+) or no growth (−) was recorded after 3 weeks of incubation.

DISCUSSION

Co-oxidation of ethylene does not cause the failure of the C_2H_2-C_2H_4 test with the nitrogen-fixing strain 41 of the *Methylosinus* type when acetylene is supplied with methane as the only carbon and energy source. Co-oxidation of ethylene is completely prevented in the presence of acetylene. Acetylene not only completely prevents the co-oxidation of ethylene, but it also inhibits very strongly the oxidation of methane. Thus the supply of energy and reducing power to the nitrogenase, needed for the reduction of acetylene, is impeded. Moreover, if no substrate is available to the bacterium, the nitrogenase presumably is insufficiently protected from oxygen. The bacterium may overcome these obstacles when it is given methanol or other substrates that are oxidized in the presence of acetylene. Results supporting these conclusions have recently been reported by Whittenbury et al. (14) who also found that the use of methane as an electron donor in the C_2H_2-C_2H_4 test led to negative results.

Experiments with whole-cell suspensions showed that acetylene is a competitive inhibitor for methane. However, results of experiments of this type should be treated with caution. The physiological response of the bacterium towards different levels of methane and acetylene was recorded. This response does not need to coincide with the action of methane hydroxylase since, apart from methane, the enzyme depends upon oxygen and presumably nicotinamide adenine dinucleotide, reduced form (NADH), for its catalytic activity. The level of oxygen does not interfere with the physiological response to methane oxidation. The actual NADH level in the bacterial cell during the experiments is unknown but will probably vary with varying methane concentrations or after the addition of acetylene. It is uncertain what influence this varying NADH level exerts on methane hydroxylase activity, but the straight line in the Lineweaver-Burk plot, obtained from the progress curve for methane oxidation, is encouraging. Oxygen uptake, dependent on the concentration of the substrate, has been employed previously for establishing the apparent K_m for methane (8). The value obtained was approximately 1%, which is of the same order as the apparent K_m of 1.7% found in the present investigation. If this method of assessing an apparent K_m value is acceptable, then the measurement of an apparent K_i performed in this way should be possible.

Acetylene is an extremely effective inhibitor of methane oxidation. The K_i value of 0.5 μl/liter in the gas phase represents a concentration as low as approximately 0.02 μM C_2H_2 in solution (6). Specific inhibitors of methane hydroxylase have been searched for by Hubley et al. (9), but acetylene or substrates co-oxidizable by the hydroxylase (except carbon monoxide) were not considered as likely candidates. These co-oxidizable substrates would presumably also act as competitive inhibitors for methane, albeit with a much higher K_i than acetylene. Ethane, for instance, may inhibit the growth of methane-oxidizing bacteria (13). The fate of acetylene was not studied in the present investigation. The situation with strain 41 and the other methane-oxidizing bacteria tested was also met with the bacteria growing on lower hydrocarbons. Only the enzyme system involved in the first attack on the hydrocarbon was inhibited by acetylene. Growth upon long-chain

hydrocarbons was not inhibited by acetylene, suggesting that the inhibition of the alkane hydroxylase by acetylene depends on the size of the substrate molecule. This indicates the existence of a direct relation between substrate and inhibitor with respect to the hydroxylase. The type of inhibition is probably the same as that found for the methane-oxidizing bacteria. Strain H12 was an exception, because it was not inhibited by acetylene, even when it was growing on butane.

The experiments show that the C_2H_2-C_2H_4 test cannot be employed for measuring nitrogenase activity in methane-utilizing bacteria or in bacteria utilizing lower hydrocarbons when alkanes are the sole energy source. Nitrogenase activity can be measured by growing these bacteria on carbon sources other than the alkanes. However, this may raise a problem with methane-oxidizing bacteria since methanol, the only alternative carbon source, is toxic to some strains when supplied at substrate-level concentrations (13). Nitrogenase activity of methane-grown cells can only be measured by supplying the organism during the test with a different energy source, but the linear rate of ethylene production was not always reproducible. Furthermore, in this way it is impossible to measure nitrogenase activity quantitatively.

In natural habitats, the contribution to the fixation of atmospheric nitrogen by methane-oxidizing bacteria and bacteria using lower hydrocarbons is overlooked when using the C_2H_2-C_2H_4 test. Preliminary experiments in which small amounts of methanol or formate were added to samples from various habitats to reveal possible nitrogen fixation by methane-oxidizing bacteria were not successful. Scaling up the activity of these organisms by incubating water samples under methane and then applying the test with methanol or formate added has given inconsistent results. As a consequence, nitrogenase activity should not be measured with the C_2H_2-C_2H_4 test in natural habitats where bacteria utilizing methane or lower hydrocarbons are present. Nitrogenase activity could be measured with $^{15}N_2$.

The strong effect of acetylene upon the metabolic activities of the bacteria studied calls for a careful approach when employing the C_2H_2-C_2H_4 test. Other metabolic activities already found to be affected by acetylene include the production of methane (4, 10) and the growth of *C. pasteurianum* (1).

ACKNOWLEDGMENT

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NITROGEN FIXATION BY METHANE-UTILIZING BACTERIA

By J. A. M. DE BONT

Antonie van Leeuwenhoek 42 (1976) 245–254

Several pure cultures of methane-utilizing bacteria, including types I and II membrane representatives, were found to be capable of fixing nitrogen. One nitrogen-fixing isolate grew in liquid medium, but not on a solid agar medium. Apparently, the ability to fix nitrogen is common in methane-oxidizing bacteria.

INTRODUCTION

The fixation of nitrogen by a pure culture of the methane-oxidizing bacterium strain 41 of the *Methylosinus* type has been demonstrated with $^{15}N_2$ by de Bont and Mulder (1974). The bacterium was very sensitive to oxygen when dependent upon N_2 as the source of nitrogen, a consequence of the nitrogenase's sensitivity towards oxygen.

When measured by the acetylene-reduction assay, the nitrogenase activity of the bacterium was erratic. Ethylene was formed from acetylene when the bacterium was grown in a nitrogen-free medium and methanol served as the substrate. There was no appreciable reduction of acetylene when methane was the source of carbon and energy, probably because the acetylene competitively inhibited the oxidation of methane in this bacterium (de Bont and Mulder, 1976). However, when substrates were available that were oxidizable regardless of whether acetylene was present, the activity of the nitrogenase in methane-grown cells was not affected by acetylene.

The present study surveyed the nitrogen-fixing capacity of methane-oxidizing bacteria.

MATERIALS AND METHODS

Culture medium. The mineral-salts solution (MS medium) used in this study contained the following salts per liter of deionized water: $NaNO_3$, 2.0 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.015 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; $CuSO_4 \cdot 5H_2O$, 5 μg; H_3BO_3, 10 μg; $ZnSO_4 \cdot 7H_2O$, 70 μg; $MnSO_4 \cdot 5H_2O$, 10 μg; $Na_2MoO_4 \cdot 2H_2O$, 100 μg; the final pH was 6.8. For solid media 1.5% agar was added to this nutrient solution.

Chemicals. Ultra pure (99.97%) methane (Matheson Co., East Rutherford, N. J., USA) was used.

Bacteria. Strain XX (Patt et al., 1974), strain 1 G (Adamse et al., 1972), strain 41 (de Bont and Mulder, 1974), and strains N3A, 3.2B2, D2, 1, NIG 3A, NIDI, and ROSE (Hazeu, 1975) were used.

Culture conditions. Weekly subcultures on slants of MS medium or nitrate-free MS medium were kept at 30 C in desiccators with lowered oxygen pressure with about 5% methane. Cultures growing in 25-ml tubes with 10 ml of liquid medium were also kept in desiccators.

Gas chromatography. A Becker model 409 or model 417 chromatograph with a flame ionization detector was used with a 110 cm x 4 mm Porapak R column with nitrogen as the carrier gas at 50 C to assay for methane and acetylene reduction. Oxygen and methane were measured by thermal conductivity using a 200 cm x 4 mm column with a 13X molecular sieve (60–80 mesh) and nitrogen as the carrier gas.

Acetylene reduction. The reduction of acetylene was assayed after quickly replacing with a suba seal the cotton plugs of the 25-ml tubes in which cultures were grown on slants or in liquid methane-containing desiccators. A methanol or formate solution was injected prior to the addition of 2 ml of acetylene.

Electron microscopy. Strain 41 and strain 3 cells collected from mid-exponential phase cultures by centrifugation and washed in Sørensen buffer pH 7.0 were prefixed in 0.5% OsO_4 for 15 min, fixed overnight in 1% OsO_4 and washed in Kellenberger buffer. The material was transferred into agar blocks, stained with uranyl acetate for 1 hour, and embedded in Epon-812.

Strain K cells, embedded in agar microcapsules, were treated with Palade's fixative for 4 hours at pH 7.4 at 4 C, dehydrated in acetone, and embedded in Epon-812. Sections were stained with uranyl acetate for 30 min and with lead citrate for 1 min.

RESULTS

Enrichment and isolation. About 1 g of inoculum was incubated in 10 ml of a nitrogen-free MS medium to which 50 mg/liter of yeast extract was added. Nine soil samples from various locations and two soil samples that had been exposed to natural gas escaping from pipe lines, seven water samples from ditches and rivers, saliva of a cow, decaying leaves, and decaying straw from a dung hill were used. The inoculated medium was kept in a 100-ml Erlenmeyer flask with an atmosphere of 5% oxygen, 5% methane, and 90% nitrogen gas. The presence of oxygen and methane was monitored weekly, and whenever needed, these gases were refurnished. Growth appeared in the flasks after 2 to 6 weeks of incubation. Then, a drop of the turbid medium was transferred to fresh medium and after incubating this under the same conditions, the material was streaked onto plates of nitrogen-free MS medium; some plates contained medium to which 50 mg/liter yeast extract had been added.

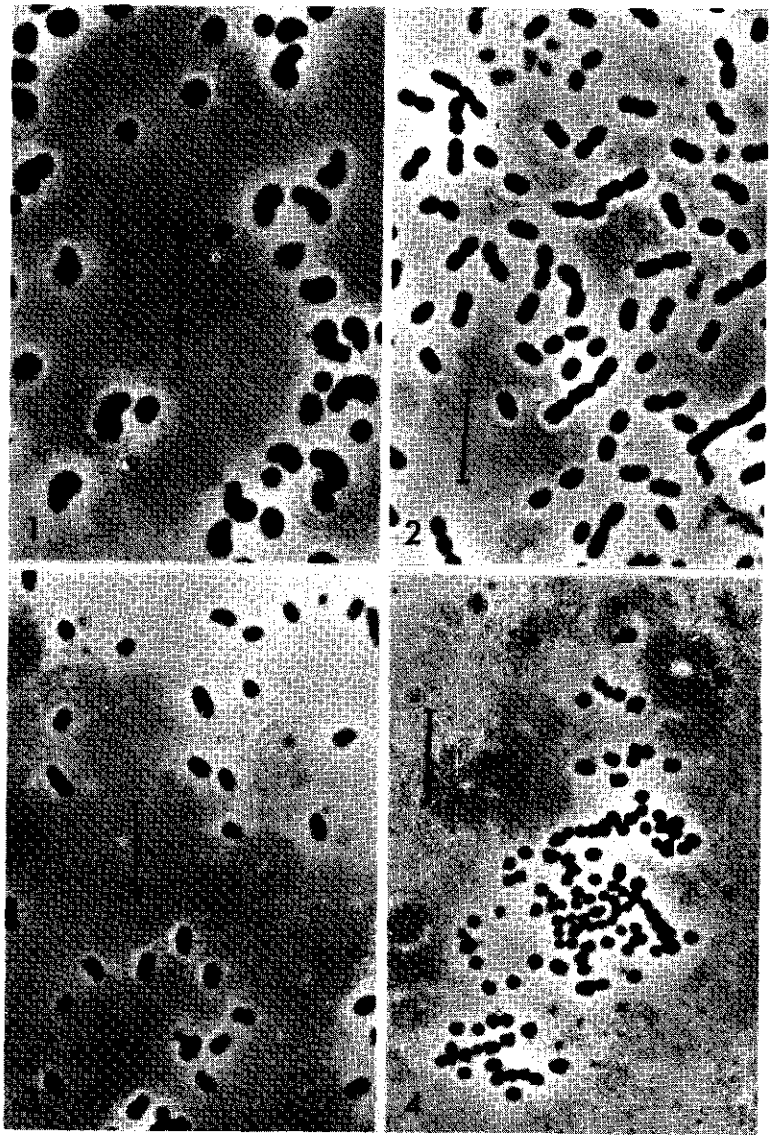

Fig. 1. Strain 41 of a *Methylosinus* sp. from a 10-day culture grown on slants of MS medium with methane. The bar represents 10 μm.
Fig. 2. Strain 2 of a *Methylomonas* sp. from a 3-day culture grown on slants of MS medium with methane. The bar represents 10 μm.
Fig. 3. Strain 16 from a 5-day culture grown on slants of MS medium with methane. The bar represents 10 μm.
Fig. 4. Strain K from a 10-day culture grown in liquid MS medium on methane. The bar represents 10 μm.

The plates were incubated for up to 2 months in desiccators with an atmosphere of about 5% oxygen, 5% methane, and 90% nitrogen. Colonies were selected at different time intervals and streaked to purity.

Although good growth appeared in the liquid medium with all samples, isolation of the nitrogen-fixing methane-oxidizing bacteria was difficult. Colonies of methane-oxidizing bacteria readily appeared on plates, but the bacteria were often lost on further purification by restreaking.

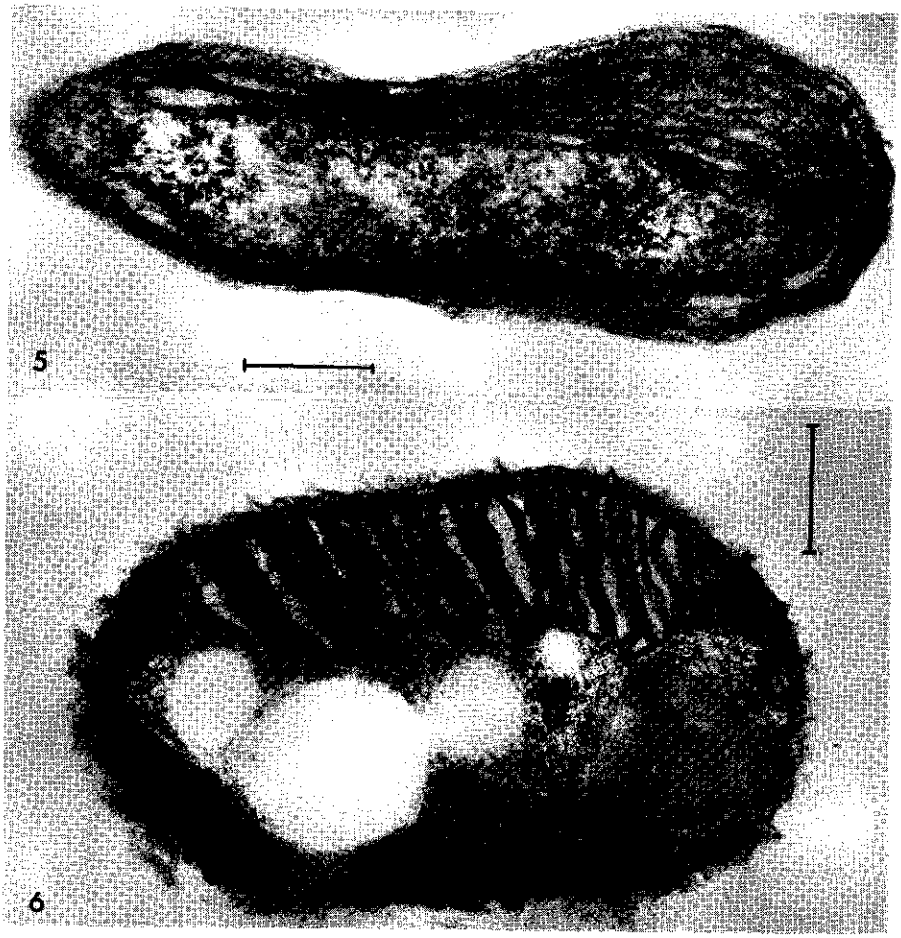

Fig. 5. Thin section of strain 41 of a *Methylosinus* sp. grown in nitrogen-free MS medium on methane, showing the type II membrane system. The bar represents 0.25 μm.
Fig. 6. Thin section of strain 2 of a *Methylomonas* sp. grown in nitrogen-free MS medium on methane, showing the type 1 membrane system. The bar represents 0.25 μm.

Table 1. Methane-oxidizing bacteria counted in different sources of inoculum.

Source of inoculum	Most probable number	
	Nitrate-free medium	Nitrate-containing medium
Ditch water (a)	350	700
Mud of ditch (a)	130 000	110 000
Ditch water (b)	240	540
Mud of ditch (b)	70 000	13 000
Sandy soil	13 000	13 000
Clay	11 000	17 000

Samples taken in winter time from ditch water, the aerobic upper mud layers of ditches, and soil, were serially diluted (1:10) in nitrogen-free MS medium. Tubes (5 per dilution) containing 9 ml of nitrogen-free MS medium and tubes containing 9 ml of MS medium were incubated in desiccators containing an atmosphere of approximately 5% oxygen, 5% methane, and 90% nitrogen. The desiccators were opened weekly to remove tubes showing growth. The number of positive tubes did not increase after seven weeks of incubation. Numbers are calculated per g.

Only three strains were isolated from the nine soil samples while no pure culture was obtained from the dung hill sample. The bacteria isolated from the leaves, from the cow saliva, from one of the two soil samples that had been exposed to natural gas, and seven isolates from the water samples all closely resembled the three soil isolates. All these strains were motile in young cultures and formed exospores upon aging, but the cell size and cell shape varied. Both straight rods and vibrioid forms were identified. They were similar to strain 41 (Fig. 1) and were classified as *Methylosinus*-type bacteria according to Whittenbury, Phillips and Wilkinson (1970). No clear resting stage was found for the pink *Methylomonas* type of bacteria (strains 2, 3, and 21) from three of the water samples (Fig. 2). Strain 41 had a type II membrane system (Fig. 5) as compared with the type I membrane system of strain 2 (Fig. 6).

One isolate (strain 16), obtained from soil exposed to natural gas, formed no resting stages and its growth was stimulated by the presence of yeast extract in the medium (Fig. 3).

Methane-oxidizing bacteria capable of fixing nitrogen. The occurrence of methane-oxidizing bacteria with the capacity to fix nitrogen was investigated by incubating serial dilutions of samples in both nitrogen-free MS medium and in nitrate-containing MS medium. The results of the most probable number (MPN) counts are given in Table 1. Microscopic examination of the tubes used for the MPN counts revealed the presence of *Methylosinus* type bacteria and the pink *Methylomonas*-like organisms in the lower dilutions. The presence of nitrate in the medium did not affect the morphological characteristics observed microscopically.

The higher dilutions of soil and mud samples contained bacteria that differed morphologically from the two forementioned groups. Efforts to isolate these bacteria revealed large colonies on plates of nitrogen-free MS medium streaked with material from the higher dilutions when incubated in an atmosphere containing approximately 5% oxygen,

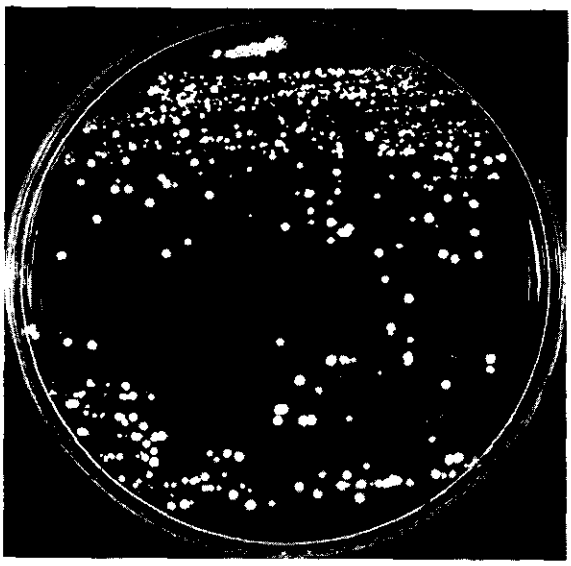

Fig. 7. Colonies of strain K grown for 6 weeks under methane on plates of nitrogen-free MS medium after the first transfer from the liquid enrichment culture to the solid medium. Subsequent transfers from these colonies grew only in liquid medium.

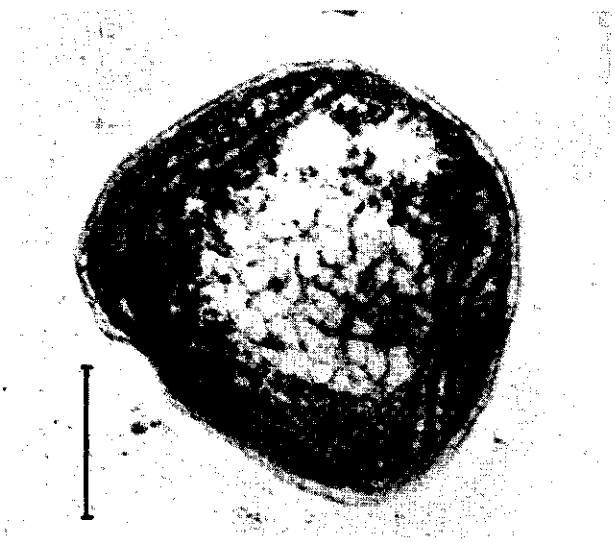

Fig. 8. Thin section of strain K grown in nitrogen-free MS medium on methane, showing the type II membrane system. The bar represents 0.25 μm.

5% methane, and 90% nitrogen (Fig. 7). To avoid losing the bacteria when material from the colonies was restreaked, a large number of colonies at an immature stage was transferred directly to liquid medium. Good growth was obtained. One isolate, designated strain K (Fig. 4), isolated from a 10^{-6} dilution of soil, was found to be an immotile, coccoid bacterium that had the type II membrane system (Fig. 8). This nitrogen-fixing obligate methylotrophic bacterium grew only in liquid medium. Streaks on agar slants did not grow. No resting stage was observed.

Strain 1G, reported by Adamse et al. (1972) to be incapable of fixing atmospheric nitrogen, was studied and was found to reduce acetylene when grown on methanol in nitrogen-free MS medium under reduced oxygen pressure. Of the seven strains of Hazeu (1975) studied, type II strains (D2 and 1) were found to fix nitrogen as did type I strains N3A and 3.2B2. No acetylene reduction could be demonstrated with type I strains NIG 3A, N1D1, and ROSE, or with the facultative methylotrophic methane-oxidizing bacterium strain XX of Patt et al. (1974).

DISCUSSION

Apparently, the capacity to fix atmospheric nitrogen is widespread among methane-oxidizing bacteria. *Methylosinus* type strains were without difficulty isolated from enrichments, and although growth was good in the liquid enrichments, other types of methane oxidizers could not easily be isolated. This was also the case with material from higher dilutions of soil and mud samples. The coccoid strains that were finally isolated did not fit into the classification scheme of Whittenbury et al. (1970). They resembled the coccoid strain 1 of Hazeu (1975).

Although *Methylomonas* type strains were difficult to isolate and grow, three strains were isolated from water. A number of other enrichments also contained these bacteria. The pink *Methylomonas* type bacteria were difficult to keep alive in pure culture. Only strain 3 survived one year of subculturing. The growth of strain 3 was enhanced in a mixed culture with a small motile rod that frequently appeared as a contaminant of pure cultures. The addition of traces of yeast extract to the nitrogen-free MS medium had no appreciable effect on the isolation of methane-oxidizing bacteria from enrichment cultures. All pure cultures, except strain 16, were isolated from plates with yeast extract, as well as from plates without yeast extract. The effect of yeast extract on promoting the growth of strain 16 has not yet been studied.

Methane-oxidizing bacteria are known to be difficult to isolate and grow, and cultivating methane-oxidizing bacteria that fix nitrogen is even more difficult because they are sensitive to oxygen when grown without combined nitrogen. Thus, in this study only strains that are relatively easy to isolate and to handle emerged from the enrichments. The types which are difficult to culture in the laboratory may, nevertheless, play a more important role in nature than might be expected from the random isolations from enrichments. Only very few *Methylosinus* type bacteria were present in the mud samples whereas the coccoid forms were predominant from microscopic observations.

Apparently, many types of methane-oxidizing bacteria can fix nitrogen under laboratory conditions and in nature. Although nitrate is generally accepted as the best source of nitrogen for growing methane-oxidizing bacteria (Hazeu, 1975), in this study it was not found to be a significantly better source than N_2 when serving as the sole source of nitrogen. Bacteria isolated from the highest dilutions of the nitrate-containing tubes were also able to fix nitrogen. Four of the seven strains of Hazeu (1975) showed nitrogenase activity. Whittenbury et al. (1975) demonstrated that most strains of methane-oxidizing bacteria, previously reported to be unable to fix nitrogen (Whittenbury et al. 1970), showed nitrogenase activity when the acetylene reduction test was properly used.

The results of the present study suggest that in the soil and in the water of the ditches investigated, the fixation of nitrogen by these bacteria is not quantitatively significant. However, in the aerobic mud layers, where oxygen and methane come together, the amount of nitrogen fixed by these organisms may be substantial. Particularly high activities of methane-oxidizing bacteria have been recorded in the thermocline of lakes (Patt et al., 1974; Rudd, Hamilton and Cole, 1974), where it is likely that high rates of nitrogen fixation accompanied the rapid oxidation of methane.

Nitrogenase activity was not obtained with the facultative methylotrophic methane-oxidizing bacterium of Patt et al. (1974). All nitrogen-fixing strains isolated in the present investigation were obligately methylotrophic. Rather than taking this result as an indication of the failure of facultative methylotrophic methane-oxidizing bacteria to fix dinitrogen, one might ascribe it to the isolation procedure used.

I would like to thank Miss Th. E. Werdmuller von Elgg for conducting the electron microscopy at the Technical and Physical Engineering Research Service at Wageningen, Professor R. S. Hanson of the University of Wisconsin (Madison, U.S.A.) for providing strain XX, and Mr. W. Hazeu of the University of Technology of Delft (The Netherlands) for supplying strains N3A, 3.2B2, D2, 1,NIG 3A, NIDI, and ROSE. Support for the research by grants of the VEG Gasinstituut and the N.V. Nederlandse Gasunie is gratefully acknowledged.

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HYDROGENASE ACTIVITY IN NITROGEN-FIXING METHANE-OXIDIZING BACTERIA

By J. A. M. DE BONT

Antonie van Leeuwenhoek 42 (1976) 255–260

Hydrogenase activity in cells of the nitrogen-fixing methane-oxidizing bacterium strain 41 of the *Methylosinus* type increased markedly when growth was dependent upon the fixation of gaseous nitrogen. A direct relationship may exist between hydrogenase and nitrogenase in this bacterium. Acetylene reduction was supported by the presence of hydrogen gas.

INTRODUCTION

Hydrogen gas is associated in different ways with the fixation of nitrogen by bacteria. Some nitrogen-fixing bacteria produce hydrogen gas, probably because of the activity of the nitrogen-fixing enzyme complex (nitrogenase). This was shown in work with cell-free extracts of *Azotobacter vinelandii* supplied with ATP, Mg^{2+}, and a reductant (Bulen, Burns and LeComte, 1965). This system is not inhibited by CO and is generally described as the ATP-dependent H_2 evolution to differentiate this reaction from the so-called classical hydrogenase-catalyzed reaction which does not require ATP and which is inhibited by CO. *Azotobacter chroococcum* produced hydrogen when the cells were exposed to air containing 10% actylene and 1% CO, but no hydrogen was produced without acetylene in the atmosphere (Brotonegoro, 1974).

A number of coryneform strains were shown to fix nitrogen and use hydrogen for autotrophic growth (Gogotov and Schlegel, 1974; de Bont and Leijten, 1976.) Hydrogenase supplies energy and reductant in these strains.

The function of hydrogenase in heterotrophic nitrogen-fixing bacteria is less clear. In *Azotobacter* species an association between hydrogenase and nitrogenase may exist (Green and Wilson, 1953). A hydrogenase of *Mycobacterium flavum* 301 was reported by Biggins and Postgate (1969) to resemble that of *Azotobacter* which does not evolve hydrogen gas.

Competitive inhibition of N_2 fixation by H_2 was assessed both in intact cells (Bradbeer and Wilson, 1963) and in cell-free extracts (Hwang, Chen and Burris, 1973) of *Azotobacter vinelandii*.

This communication reports on the presence of hydrogenase activity in the recently described nitrogen-fixing methane-oxidizing bacteria of the *Methylosinus* type (de Bont and Mulder, 1974).

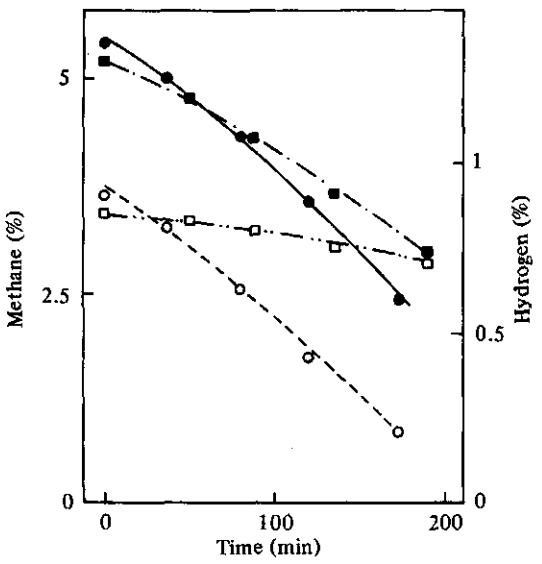

Fig. 1. Effect of the source of nitrogen on the oxidation of hydrogen by strain 41 growing on methane. Suspensions (4 ml) of cultures growing logarithmically under reduced oxygen pressure in the nitrate-containing MS medium (30 μg protein/ml) or in the nitrate-free MS medium (35 μg protein/ml) were injected into silicon rubber-sealed vaccine bottles (16 ml) containing 5% O_2, 5% CH_4, 1% H_2, and 89% N_2. After equalizing the pressure, 0.1 ml samples were periodically withdrawn from the gas phase for gas-chromatographic analyses of hydrogen and methane. The bottles were incubated at 30 C while shaking. Hydrogen concentration above nitrate-containing (□) and nitrate-free (○) MS medium; methane concentration above nitrate-containing (■) and nitrate-free (●) MS medium.

MATERIALS AND METHODS

Bacterium and culture conditions. Strain 41 of the *Methylosinus* type, described previously by de Bont and Mulder (1974) was used. The mineral salts solution (MS medium) contained per 1 liter of deionized water: $NaNO_3$, 2.0 g: K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.015 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; $CuSO_4 \cdot 5H_2O$, 5 μg; H_3BO_3, 10 μg; $ZnSO_4 \cdot 7H_2O$, 70 μg; $MnSO_4 \cdot 5H_2O$, 10 μg; $Na_2MoO_4 \cdot 2H_2O$, 100 μg; final pH 6.8. Cells were grown at 30 C while shaking in Erlenmeyer flasks containing an atmosphere of about 5% O_2, 10% CH_4, and 85% N_2.

Gas chromatography. A Becker model 409 chromatograph equipped with a flame-ionization detector was used to assay actylene reduction at 50 C, using a 110 cm x 4 mm Porapak R column with nitrogen as the carrier gas. Methane was measured on a Becker model 406 chromatograph; oxygen and hydrogen were determined by thermal conductivity using a 300 cm x 2 mm Porapak Q column with nitrogen as the carrier gas at 50 C.

Protein assay. The Folin-Ciocalteu method was used to measure protein (Herbert, Phipps and Strange, 1971).

RESULTS AND DISCUSSION

Attempts to cultivate the nitrogen-fixing obligate methylotroph strain 41 of the *Methylosinus* type on H_2 in the presence of CO_2 have not been successful. However, hydrogenase activity in methane-growing cells could be assessed in growing cultures by measuring the uptake of H_2. There was some hydrogenase activity when the bacterium was grown in nitrate-containing medium, but the enzymic activity increased markedly when bacterial growth was dependent on the fixation of gaseous nitrogen (Fig. 1.). Apparently, the function of hydrogenase in this strain is similar to that in *Azotobacter* species and is associated with the fixation of nitrogen by the bacterium. Azotobacters grown on N_2 have a higher hydrogenase activity than ammonia-grown cells (Green and Wilson, 1953). Three possible functions of the hydrogenase in the oxidation of H_2 (presumably formed by ATP-dependent nitrogenase activity) in nitrogen-fixing organisms were considered by Dixon (1972). (1) The oxidation of hydrogen uses excess oxygen and helps to maintain the nitrogenase in an anaerobic environment within the cell. This could

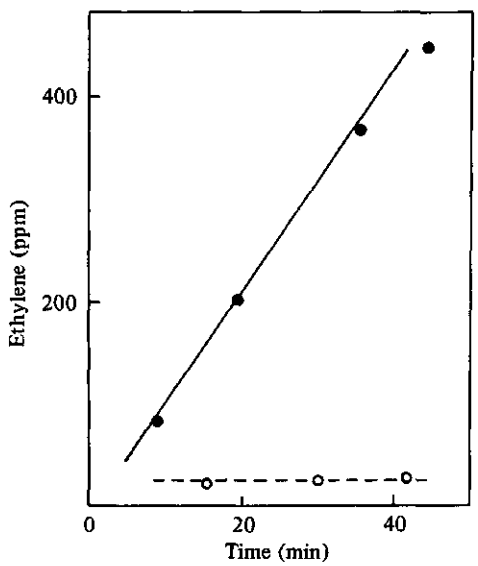

Fig. 2. Acetylene reduction by strain 41 with (●) and without (○) 5% hydrogen in the atmosphere. Portions, 10 ml, of a culture growing on methane in a nitrate-free medium were injected into 100-ml Erlenmeyer flasks containing 10% actylene, 5% oxygen, and 85% nitrogen before hydrogen gas was introduced.

be vital in the methane-oxidizing bacteria since oxygen was reported to inhibit the nitrogen-fixing reaction (de Bont and Mulder, 1974). (2) The oxidation of hydrogen prevents nitrogenase activity from being inhibited by hydrogen. (3) Reductant and ATP are formed upon the oxidation of hydrogen, thereby increasing the efficiency of the fixation of nitrogen.

Because the *Azotobacter* hydrogenase catalyzes only the reduction of electron acceptors with H_2 but does not evolve H_2 (Postgate, 1974), it is unlikely that hydrogen ions are excluded from the site of nitrogenase.

In *Methylosinus* strain 41, the function of nitrogenase-associated hydrogenase is unclear. Hydrogen gas supported the reduction of acetylene, indicating that its oxidation by hydrogenase supplies reductant and energy to the nitrogenase (Fig. 2). A functional operation of hydrogenase under these conditions is obvious, but it remains unclear whether this mechanism explains the observed hydrogenase-nitrogenase relationship in methane-oxidizing bacteria because the presence of hydrogen or of nitrogenase-catalyzed, ATP-dependent, hydrogen formation was not demonstrated during growth in nitrogen-free medium.

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GENERAL DISCUSSION

The results presented in paper I show that the methane-oxidizing bacterium strain 41 of the *Methylosinus*-type is capable of fixing atmospheric nitrogen. This is the first report on nitrogen fixation by a pure culture of a methane-oxidizing bacterium. However, in earlier literature there are indications that these bacteria have the capacity to fix nitrogen.

The effect of leaking natural gas upon the soil was studied by Schollenberger (1930). A chemical analysis showed that the affected soil was higher in replaceable ammonia than was the soil from an adjacent area which had not been exposed to natural gas. Schollenberger did not consider bacterial oxidation of natural gas. He thought the effects of the gas to be due to displacement of air from soil resulting in anaerobic conditions. The notably higher concentration of exchangeable ammonia would then be the result of biological action under these anaerobic conditions.

Harper (1930) observed a strong increase in total nitrogen and organic matter in soil that had been exposed to natural gas. He collected samples of soils exposed to natural gas from leaking pipe lines and samples of normal soil taken from the same locality. In every soil that had been exposed to gas for a considerable length of time, the total nitrogen content increased. The average nitrogen content in the soils affected by the gas was nearly three times that in normal soils. Oats was planted in soils collected near the gas leaks and in similar soil that had not been affected by the escaping gas. The oat leaves were darker and more forage was produced by the plants in the soil collected near the gas leak than by those in normal soil. These observations indicated that nitrogen accumulating in soils exposed to natural gas is available to plants. Harper realized that microorganisms were involved in the processes he observed. He expected that hydrocarbons in the natural gas would supply the necessary energy, either directly or indirectly, for the growth of the microorganisms capable of fixing nitrogen.

Comparable results were reported by Hoeks (1972). His analysis of soil showed that the nitrogen content in soil that had been exposed to natural gas was higher than it was in normal soil.

The two possible mechanisms for accumulation of nitrogen already suggested by Harper (1939) were also considered by Davis, Coty and Stanley (1964). (i) The hydrocarbon is utilized by bacteria capable of fixing atmospheric nitrogen, the result being an ultimate increase in organic carbon and nitrogen of the soil. (ii) The hydrocarbon is utilized by microbes incapable of fixing nitrogen, but the hydrocarbon thus converted into microbial cells ultimately becomes available to other soil microorganisms, some of which are capable of fixing nitrogen. Davis, Coty and Stanley isolated methane-oxidizing bacteria that were able to grow in nitrogen-free mineral salts medium. The isolated bacteria were Gram-negative motile rods. Nitrogen fixation was measured by Kjeldahl analysis of cultures incubated for two weeks or for two or four months. Later work (Coty, 1967) employing $^{15}N_2$ corroborated the Kjeldahl results. From the experimental data it was concluded that the increase in nitrogen content of soils exposed to natural gas

was probably directly due to the hydrocarbon-utilizing bacteria. However, the isolates used in the experiments grew on nutrient agar, indicating that the cultures were not pure.

Two attempts to demonstrate nitrogen fixation by methane-oxidizing bacteria using the acetylene-ethylene test have been reported. Whittenbury, Phillips and Wilkinson (1970) provided puzzling results. The authors isolated a *Methylosinus trichosporium* from Coty's culture and the organism was exposed to an atmosphere containing 4.4% methane and 1.8% acetylene for 7 or 14 days. Reduction of 25% of the acetylene present was reported, but it was not stated whether ethylene was the product formed. This is no evidence of nitrogen fixation as the acetylene may have disappeared by co-oxidation or by simply leaking from the incubation system. These authors also tried to grow *Methylobacter* strains in nitrogen-free medium because of the morphological similarity between these strains and *Azotobacter* species. None, however, grew in nitrogen-free medium. Adamse, Hoeks, de Bont and van Kessel (1972) obtained negative results with the acetylene-ethylene test even though the organism under investigation grew in a nitrogen-free medium.

Failure of the acetylene-ethylene assay

In papers I and II an explanation is given for the erratic results obtained with the acetylene-ethylene assay for nitrogen-fixing methane-oxidizing bacteria. When strain 41 was grown in a nitrogen-free medium with methane as substrate, the acetylene-ethylene assay gave negative results. But when the organism was grown in the same medium with methanol as substrate, ethylene production from acetylene was demonstrated. The apparent difference in acetylene reduction might have been due to the ability of methane-grown cells to co-oxidize ethylene. In this way, the product formed by the nitrogenase-catalysed reaction would be removed, thus masking the acetylene reduction by the enzyme. This hypothesis was supported by the observation that ethylene was not co-oxidized by cells grown on methanol. However, the hypothesis had to be given up when it was found that co-oxidation of ethylene by methane-grown cells was completely inhibited by acetylene. Acetylene not only prevented the co-oxidation of ethylene, but it also inhibited very strongly the oxidation of methane. This result explains why the bacteria cannot reduce acetylene when methane is given as oxidizable substrate; reduction of acetylene by nitrogenase is dependent on energy and reducing power. Blockage by acetylene of the first step of dissimilation of methane prevents the generation of energy and reducing power. This explanation was further corroborated by the observation that methanol or other substrates that are oxidizable in the presence of acetylene, support acetylene reduction by methane-grown cells.

Other pure cultures of methane-oxidizing bacteria also exhibited similar behaviour to strain 41. Bacteria growing on lower hydrocarbons responded similarly to acetylene. Only the enzyme involved in the first attack on the hydrocarbon was inhibited by acetylene. Thus the acetylene-ethylene test cannot be employed for measuring nitrogenase activity

in methane-utilizing bacteria or in bacteria utilizing lower hydrocarbons when alkanes are the sole energy source.

Alternatives in assaying nitrogenase activity of methane-oxidizing bacteria have been considered. Acrylonitrile reduction by nitrogenase yields propane, propylene and ammonia as products. However, the predominant hydrocarbon product propylene was co-oxidized while acrylonitrile inhibited methane oxidation and growth of the methane-oxidizing bacteria (de Bont, unpublished results). Dalton (personal communication) used N_2O as artificial substrate for nitrogenase. This assay may be of value for pure culture studies, but in ecological studies the method is not applicable since the reduction product is N_2. Consequently, nitrogenase activity should be measured with $^{15}N_2$.

Oxygen sensitivity

In paper I attention is drawn to the oxygen sensitivity of methane-oxidizing bacteria. Growth of strain 41 in nitrate-containing medium was little influenced by oxygen pressure. But the bacterium was extremely sensitive to oxygen when dependent on N_2 as nitrogen source because of the sensitivity of its nitrogenase towards oxygen. This was demonstrated by acetylene reduction under different levels of oxygen. A similar behaviour has been observed for other obligate aerobic nitrogen-fixing bacteria. Dalton and Postgate (1969) reported that *Azotobacter chroococcum* showed no unusual sensitivity to oxygen when growing in NH_4^+-containing media. But N_2-growing cells were inhibited by high aeration rates even at atmospheric oxygen pressure. Bacteria of the type of *Mycobacterium flavum* are also sensitive to oxygen when fixing N_2 (Biggins and Postgate, 1969; de Bont and Leijten, 1976). The sensitivity of methane-oxidizing bacteria to oxygen when fixing N_2 was confirmed by Whittenbury, Dalton, Eccleston and Reed (1975) and Rudd, Furutani, Flett and Hamilton (1976).

Incidence of nitrogen fixation among methane-utilizing bacteria

The results presented in paper III show that the capacity to fix atmospheric nitrogen is common among methane-oxidizing bacteria. Growth appeared readily in enrichments of nitrogen-fixing methane-oxidizing bacteria that had been set up using inocula from various habitats. *Methylosinus*-type strains were isolated without difficulty from such enrichments. All these strains resembled strain 41 described in paper I. A number of enrichments also contained *Methylomonas*-type bacteria. But these organisms were much more difficult to grow and to isolate than the *Methylosinus*-type strains. However, the isolation of these strains was important in demonstrating that both type I and type II methane-oxidizing bacteria may fix nitrogen. *Methylomonas*-type strain 2 possesses the type I membrane system as opposed to the type II membrane system of strain 41 showing that nitrogen fixation is not restricted to the type II organisms. Pure cultures of Hazeu (1975)

including type I as well as type II representatives were also able to fix nitrogen. Although *Methylosinus* and *Methylomonas* type bacteria appeared in enrichments, inoculated with soil, mud and ditch water, it was concluded from dilution series that in the samples used as inoculum, coccoid bacteria outnumbered these bacteria. The coccoid bacteria, resembling strain 1 of Hazeu (1975), were very difficult to isolate. They would not grow on solid agar media. In the enrichments they were overgrown by the fast growing *Methylosinus* and *Methylomonas*-type bacteria.

The occurrence in nature of methane-oxidizing bacteria with the capacity to fix nitrogen was investigated by incubating serial dilutions of samples in both nitrogen-free and nitrate-containing medium. Although nitrate is generally accepted as the best nitrogen source for methane-oxidizing bacteria, in this experiment it was not found to be a significantly better source than N_2. This result indicates that in nature most methane-oxidizing bacteria have the capacity to fix nitrogen.

Ecology of methane-oxidizing bacteria

In nature, methane-oxidizing bacteria are active when methane and oxygen are simultaneously present. Methane is produced in an anaerobic environment by methanogenic bacteria. The methane thus formed has to diffuse away from the anaerobic zone to an aerobic environment where it can be oxidized.

Examples of such situations include the aerobic top layer of soil at the mud-water interface in ditches and ponds that contain oxygenated water. In the underlaying anaerobic mud, methane is produced continuously and may diffuse upwards. In the aerobic mud-water interfacial region it then may be metabolized by the methane-oxidizing bacteria using oxygen diffusing from the overstanding water. Rates of methane oxidation in such systems were estimated employing acetylene as an inhibitor of methane oxidation (de Bont, unpublished results). Submerged paddy soil was kept in pots and rates of escape of methane from the pots were measured in the presence and absence of 25 ppm acetylene in the atmosphere. There was no increase in the methane content of the atmosphere above pots without acetylene. Presumably all of the methane diffusing into the aerobic zone had been oxidized at the soil surface. In the presence of acetylene, this oxidation was inhibited and methane accumulated in the atmosphere at a rate of about 100 nmoles CH_4/cm^2 soil surface/day. These observations suggest that oxygen is not the rate-limiting factor in such a system. Methane oxidation is limited by the diffusion of methane from the anaerobic to the aerobic environment. It is not known whether the methane-oxidizing bacteria actually fixed nitrogen under these circumstances.

Oxidation of methane may be a very important process in lakes. In the sediments methane is formed as one of the most important end products of the anaerobic decomposition of organic matter. In stratified lakes, the methane diffuses through the hypolimnion till it reaches the thermocline and epilimnion. Because oxygen is available here, the bacteria can oxidize methane. However, during summer stratification the oxidation of

methane was absent from the epilimnion despite adequate methane concentrations. Oxidation was confined to a narrow zone of activity within the thermocline where oxygen concentrations were low (Rudd, Hamilton and Campbell, 1974; Rudd and Hamilton, 1975). These workers further observed that during autumn and spring overturn, and in winter, methane oxidation also continued at high oxygen concentrations throughout the water column. These observations could be explained by considering the oxygen sensitivity of the methane-oxidizing bacteria when dependent on nitrogen fixation. During summer stratification the low concentration of nitrogen compounds limits the activity of the methane-oxidizing bacteria. Because of the low oxygen concentration in a zone in the thermocline, nitrogen fixation by the methane-oxidizing bacteria can provide the nitrogen. But in the epilimnion the oxygen tension is too high to allow nitrogen fixation. During overturn of the lake, ammonia from the hypolimnion becomes available throughout the water column, so that the methane-oxidizing bacteria can grow without fixing N_2 (Rudd, Furutani, Flett and Hamilton, 1976). Attempts to detect nitrogen-fixation in the thermocline within the zone of methane-oxidizing activity using $^{15}N_2$ were unsuccessful. The rate of nitrogen fixation was probably below the limit of detection with this method (Rudd, Furutani, Flett and Hamilton, 1976).

The aerobic root-soil interfacial region around the roots of aquatic plants may form a third ecological niche where methane oxidation may be an important reaction. Water plants possess internal gas-filled spaces that form an avenue for diffusion of oxygen from the phyllosphere to the root system. Part of this oxygen is used to support respiration of root tissue and possibly some is lost from the root system and is used in the root-soil interfacial region for oxidation of reduced substances and microbial respiration. Since methane is a constituent of flooded soils, the bacteria may use this excess oxygen to oxidize methane in the root-soil interfacial region. However, in experiments with rice plants it was found, that the bacterial oxidation of methane in this region was of minor importance in terms of the amount of methane available, even though there were more nitrogen-fixing methane-oxidizing bacteria in the rhizosphere soil than in the surrounding anaerobic soil (de Bont, unpublished results).

Co-oxidation

Co-oxidation is the concomitant oxidation of a non-growth substrate during growth of a microorganism on a utilizable carbon and energy source. It is generally assumed that a microorganism co-oxidizing a substance is not able to obtain energy from this oxidation (Horvath, 1972). Oxidation of non-utilizable substrates by resting cell suspension grown at the expense of substances capable of supporting growth is also termed co-oxidation.

The phenomenon of co-oxidation was first reported by Leadbetter and Foster (1958, 1960). They noted that washed cell suspensions of *Pseudomonas methanica* oxidized ethane even though the bacterium was unable to utilize ethane as a growth substrate. The products of oxidation of ethane were ethanol, acetaldehyde and acetic acid. Propane and

n-butane were not oxidized by washed cells, but they were oxidized when present in cultures growing on methane. Non-hydrocarbon compounds that dit not support growth but caused an increase in oxygen uptake by washed cell suspensions included ethanol and 1-propanol. The observations of Leadbetter and Foster (1960) showed that incorporation of ethane carbon in cell material by *Pseudomonas methanica* was of minor importance. The major portion of the ethane carbon, after oxidation of ^{14}C ethane, was recovered as volatile C_2 compounds. Minor amounts were found as carbon dioxide or cell material.

Later work by Davey, Whittenbury and Wilkinson (1972) and Patel, Hoare, Hoare and Taylor (1975) with other type I methane-oxidizing bacteria indicated that the inability of these organisms to incorporate substantial amounts of C_2-carbon units was due to the absence of α-ketoglutarate dehydrogenase. The tricarboxylic acid cycle thus cannot be operative in these bacteria. As a consequence, carbon dioxide was not produced from acetate and cells incorporated acetate-carbon only in a few amino acids (Patel, Hoare, Hoare and Taylor, 1975). This situation contrasts with that observed in the type II methane-oxidizing bacteria. In *Methanomonas methanooxidans* the tricarboxylic acid cycle is functional during growth on methane in the presence of acetate. When acetate was incorporated into the growth medium, the cell yield was greatly enhanced for the same amount of methane supplied. Utilization of acetate for cellular synthesis was confirmed by showing that both 1- ^{14}C and 2- ^{14}C acetate were incorporated. $^{14}CO_2$ was also released from both radiochemical species of ^{14}C acetate (Wadzinski and Ribbons, 1975b).

Methane hydroxylase catalyses the co-oxidation of hydrocarbon substrates by methane-oxidizing bacteria. Ribbons and Michelover (1970) demonstrated that crude extracts of *Methylococcus capsulatus* readily oxidized ethane in a similar way to the oxidation of methane. Both oxygen consumption and NADH oxidation accompanied the oxidation of ethane by methane hydroxylase. The results in papers I and II indicate that methane hydroxylase oxidizes ethylene and acetylene as well. Co-oxidation of these substrates was adaptive as was the oxidation of methane.

Non-hydrocarbon substrates may be oxidized because of the unspecific activity of the enzymes involved in the metabolism of methanol to carbon dioxide. For instance, methanol dehydrogenase from *Methylococcus capsulates* (Patel, Bose, Mandy and Hoare, 1972) or methanol oxidase from this organism (Wadzinski and Ribbons, 1975a) not only catalyse the oxidation of methanol. Other primary alcohols are degraded as well by these enzymes.

However from such observations it remains unclear whether the microorganism actually benefits from the oxidation of the co-oxidizable alcohol. The bacterium might utilize the energy obtained from such an oxidation or it might incorporate the product of the co-oxidized alcohol into its cell material. From the results given in paper II it can be concluded that co-oxidation of, for instance, ethanol is beneficial to strain 41. This conclusion contradicts the general opinion that co-oxidation is of no use to an organism. Ethanol apparently provides energy and reducing power to the nitrogenase as manifested by acetylene reduction in the presence of this substrate. Because inhibition by acetylene

prevents co-oxidation of hydrocarbon compounds by the hydroxylase, it was not possible to determine in this way whether such co-oxidation yielded energy.

Hydrogenase activity

Paper IV describes a special case of oxidation of a non-growth substrate by methane-oxidizing bacteria. Strain 41 showed hydrogenase activity when the bacterium was grown in a medium containing nitrate, but the enzymic activity increased markedly when bacterial growth was dependent on the fixation of gaseous nitrogen. Apparently, hydrogenase in this strain is associated with the fixation of nitrogen by the bacterium. Oxidation of hydrogen gas supported the reduction of acetylene, indicating that its oxidation by hydrogenase supplies reductant and energy to the nitrogenase. Recently, it was demonstrated that acetylene also inhibited hydrogenase activity by *Azotobacter chroococcum* (Smith, Hill and Yates, 1976). Hydrogenase activity of whole cells of this organism was inhibited for 75% in the presence of 10% acetylene. The influence of acetylene on hydrogen oxidation by strain 41 was not investigated in the present study.

The acetylene-ethylene assay method

Although the acetylene-ethylene assay is a very sensitive and convenient method for estimating nitrogenase activity, it does have its drawbacks. Some of these disadvantages have received attention in the past.

Theoretically the stoichiometric relationship between acetylene reduced and N_2 fixed is 3 : 1. But many investigators observed conversion factors that deviated from this ratio. The proportions found ranged from 2.3 : 1 to 6.9 : 1 and even higher (Hardy, Burns and Holsten, 1973). This stoichiometric relationship should be known for the system under investigation when absolute values for nitrogen fixation are required.

The diffusion rates of acetylene and ethylene in water may also seriously influence the test results, especially in water-saturated soil systems that are assayed under undisturbed conditions, such as in rice fields. Many reports give estimates on the *in situ* N input by nitrogen fixation in rice paddies, but it should be realized that only nitrogen-fixing activities in the top few centimetres of the field can be recorded as acetylene penetrates only slowly into the mud. Hindrance of gas diffusion was experimentally demonstrated by Balandreau, Rinaudo, Ibtissam, Fares-Hamad and Dommergues (1975); Rice and Paul (1971) considered this theoretically.

Until very recently, the influence of acetylene on the biological system under investigation had not been considered. The first report providing evidence for the influence of acetylene on metabolic activities was by Brouzes and Knowles (1971). They demonstrated that acetylene at a partial pressure of 0.05 atmosphere inhibited growth of *Clostridium pasteurianum* on a medium supplemented with sufficient ammonium nitrogen to

completely repress the formation of nitrogenase. Acetylene caused inhibition of cell proliferation and of cell nitrogen accumulation and prevented the increase in rate of carbon dioxide evolution normally associated with growth. These authors concluded that it was not clear yet what implications an acetylene inhibition might have for nitrogen fixation assays. Furthermore they said the results of long-term assays of low-activity materials should be interpreted with caution until more information is available on the systems and effects involved. Other indications that acetylene may interfere with the test results are available. Elleway, Sabine and Nicholas (1971) studied acetylene reduction by filtrates of rumen contents. They observed that acetylene inhibited methane production. But they did not consider the possibility that the test results were influenced by this inhibition. Raimbault (1975) showed that acetylene at a partial pressure of 0.05 atmosphere stopped all the methane evolution when paddy soil was incubated anaerobically. Inhibition of methanogenesis in sediments by acetylene was very recently reported by Oremland and Taylor (1975). These authors discussed the effects of acetylene during the nitrogenase assay. They suspected that these effects cause either an over or an under estimation of sediment nitrogen fixation rates because methanogenic bacteria probably play a symbiotic role in nitrogen fixation by anaerobic microbial communities. These results become even more important in view of the observations of Pine and Barker (1954). They demonstrated $^{15}N_2$ incorporation by *Methanobacterium omelianskii*. This culture was later found to be a symbiotic association of two species of bacteria (Bryant, Wolin, Wolin and Wolfe, 1967), but none of them seems to reduce acetylene (Oremland and Taylor, 1975). Inhibition of cell metabolism by acetylene may perhaps explain this contradiction. Another example of interference by acetylene in cell metabolism was provided by Lloyd Balderston, Sherr and Payne (1976). These authors showed that reduction of nitrate and nitrite to N_2 by *Pseudomonas perfectomarinus* was inhibited by acetylene. Reduction of nitrous oxide was also inhibited by acetylene.

Acetylene has also strong effects on plants. It acts as an ethylene analogue and half maximum effects of acetylene may occur at a concentration of 280 ppm. The plant hormone ethylene is involved in many biological activities of the plant. It plays an important role in many metabolic and physiological systems of the plant. The effects can be rapid and may occur within 2 hours (Abeles, 1973). Consequently, especially when long-term acetylene-ethylene assays are carried out the test results obtained with intact plants may be influenced.

From the results reported in paper II, together with the information cited from literature, it is obvious that the effects of acetylene on the biological system under study can no longer be ignored. Acetylene should not be considered as an inert gas except for its interaction with nitrogenase. Especially when absolute values of N_2 fixation are required, the acetylene-ethylene assay should be calibrated with a N-based method.

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SUMMARY

Methane occurs abundantly in nature. In the presence of oxygen this gas may be metabolized by bacteria that are able to use it as carbon and energy source. Several types of bacteria involved in the oxidation of methane have been described in literature. Methane-utilizing bacteria have in common that they can only grow on methane or methanol and not on other carbon compounds. There is much confusion in the literature about the ability of these bacteria to fix atmospheric nitrogen. The object of the investigations presented in this thesis was to obtain more information about possible nitrogen fixation by methane-utilizing bacteria.

Paper I is concerned with the isolation and description of the methane-oxidizing bacterium strain 41 of the *Methylosinus* type. This isolate is a curved rod, motile in young cultures, but on ageing of the culture motility is lost and exospores are formed. Only methane or methanol serves as growth substrate for this bacterium. Attempts to demonstrate nitrogen fixation by this organism were only successful after it was recognized that (i) the organism was sensitive to oxygen when dependent on N_2 as nitrogen source (ii) nitrogenase activity could not be assayed by acetylene reduction when the bacterium was growing on methane.

Growth of strain 41 in nitrate-containing medium with methane was little influenced by varying the oxygen pressure. But increasing the oxygen pressure when growing the bacterium in nitrogen-free medium severely reduced growth. Similar phenomena were observed when the bacterium was grown on agar plates. Colony size on nitrate-containing plates was not affected by varying the oxygen pressure but on nitrogen-free plates the effect of oxygen became apparant. Incubation in an atmosphere containing 5% oxygen allowed normal growth on nitrogen-free medium while with 20% oxygen growth was only meagre. The nitrogen-fixing colonies that developed sporadically probably were a result of locally occurring clumps of the inoculated organism.

Evidence of nitrogen fixation by strain 41 when growing with methane was obtained by using $^{15}N_2$. Excess ^{15}N percentages of the culture were measured after the bacterium was grown at reduced oxygen pressure in nitrogen-free medium with methane and $^{15}N_2$.

Although strain 41 fixed N_2 it did not reduce acetylene when growing on methane. In papers I and II two possible explanations were considered for this erratic behaviour. It was observed that nitrogenase activity could be assayed by acetylene reduction when the bacterium was growing on methanol in nitrogen-free medium and that ethylene was co-oxidized by methane-grown cells and not by methanol-grown cells. In spite of this observation, the hypothesis that failure of the acetylene-ethylene assay was due to co-oxida-

tion of ethylene by the methane-oxidizing bacterium was incorrect because co-oxidation of ethylene by methane-grown cells was completely prevented by acetylene.

Acetylene not only completely prevented the co-oxidation of ethylene, but it also inhibited very strongly the oxidation of methane. Thus, the supply of energy and reducing power to nitrogenase, needed for the reduction of acetylene, was impeded. This explanation for the failure of the acetylene-ethylene assay with methane-grown bacteria was corroborated by the observation that methanol or other substrates oxidizable in the presence of acetylene supported acetylene reduction by methane-grown cells.

In paper II, experiments are reported that were undertaken to study the mechanism of inhibition of methane oxidation by acetylene. Growth of the bacterium on methanol in a nitrate-containing medium was not affected by acetylene whereas growth on methane was completely prevented in the presence of acetylene. Apparently, only the first step in the degradation route of methane, the oxidation of methane to methanol, was blocked by acetylene. This was also shown by the course of the oxygen uptake of whole-cell suspensions of strain 41. Acetylene suppressed methane-dependent oxygen uptake, whereas it did not influence methanol-dependent oxygen uptake. Interaction of acetylene with methane hydroxylase, the enzyme involved in the oxidation of methane to methanol, was further demonstrated by showing that acetylene also inhibited co-oxidation of methane hydroxylase-dependent co-substrates. Acetylene itself was only co-oxidized by methane-grown cells. Methanol-grown cells, lacking methane hydroxylase, did not co-oxidize acetylene. Experiments with whole-cell suspensions were undertaken to study the mechanism of inhibition of methane hydroxylase by acetylene. The uptake by such suspensions of methane and of dissolved oxygen, both dependent on methane concentration, was measured. From these experiments it was tentatively concluded that acetylene inhibited methane oxidation competitively. Other strains of methane-oxidizing bacteria behaved similarly to strain 41 in that growth on methane was inhibited by acetylene. Furthermore, bacteria other than the methane-oxidizing bacteria that could grow on lower hydrocarbons were inhibited by acetylene as well when growing on the alkane but not when growing on non-hydrocarbon substrates. Thus the acetylene-ethylene assay likewise could not be employed for measuring nitrogenase activity in these bacteria when the alkane was the sole energy source.

The study presented in paper III surveyed the nitrogen-fixing capacity among methane-oxidizing bacteria. Nitrogen-fixing methane-oxidizing bacteria grew readily in enrichment cultures that had been inoculated with material from various habitats. *Methylosinus*-type bacteria were most abundant in such enrichments but *Methylomonas*-type organisms occurred as well. The *Methylosinus*-type bacteria were isolated without difficulty from

the enrichments. These strains all resembled strain 41. They were motile in young cultures and formed exospores upon ageing, but the cell size and shape varied. Both straight rods and vibrioid forms were identified. The *Methylomonas*-type bacteria were much more difficult to isolate and to cultivate than the *Methylosinus*-type strains. Growth of these bacteria was enhanced in mixed culture with a small motile rod that frequently appeared as a contaminant of pure cultures. The nitrogen-fixing *Methylomonas*-type strain 2 possessed the type I membrane system as opposed to the type II membrane system of strain 41. This result shows that nitrogen fixation is not restricted to type II methane-oxidizing bacteria.

The occurrence in nature of methane-oxidizing bacteria with the capacity to fix nitrogen was investigated by incubating dilution series of samples in nitrate-containing and nitrogen-free media. Nitrate was not found to be a significantly better nitrogen source than N_2, indicating that the capacity to fix N_2 is common among methane-oxidizing bacteria in nature. The higher dilutions of the samples contained coccoid bacteria that differed morphologically from the isolated *Methylosinus* and *Methylomonas*-type bacteria. Apparantly, these coccoid bacteria were more abundant in the mud and soil samples investigated than were the *Methylosinus* and *Methylomonas*-type bacteria. But in enrichments they were overgrown by these faster growing organisms. Isolation of the coccoid bacteria was difficult. The first transfer from the liquid enrichment cultures to plates with nitrogen-free medium yielded colonies, but subsequent transfers from these colonies only grew in a liquid medium. One isolated coccoid organism was found to possess the type II membrane system.

Paper IV comprises a study of the hydrogenase activity of strain 41. This activity was assayed by measuring the uptake of H_2 by growing cultures. There was some hydrogenase activity when the bacterium was growing in nitrate-containing medium, but the enzymic activity increased markedly when bacterial growth was dependent on the fixation of N_2. The function of the hydrogenase-nitrogenase association was not clear. Hydrogen gas supported the reduction of acetylene, indicating that its oxidation by hydrogenase supplied reductant and energy to the nitrogenase.

SAMENVATTING

Methaan is ruim voorhanden in de natuur. Bij aanwezigheid van zuurstof kan het omgezet worden door bakteriën die in staat zijn het als koolstof- en energiebron te gebruiken. In de literatuur zijn verscheidene typen van bakteriën beschreven die bij de oxydatie van methaan betrokken zijn. Methaanoxyderende bakteriën hebben gemeen dat ze alleen maar op methaan of methanol kunnen groeien. Er heerst in de literatuur verwarring omtrent het vermogen van deze bakteriën om stikstof uit de lucht te binden. De onderzoekingen die in dit proefschrift vermeld zijn, hadden tot doel informatie te verkrijgen betreffende eventuele stikstofbinding door methaanoxyderende bakteriën.

In hoofdstuk I zijn de isolatie en de karakterisering van de methaanoxyderende bakterie stam 41 van het *Methylosinus*-type beschreven. Deze bakterie is een gebogen staafje dat beweeglijk is in jonge kulturen, maar als de kultuur ouder wordt gaat de beweeglijkheid verloren en worden exosporen gevormd. De bakterie groeit alleen maar op methaan of methanol. Stikstofbinding door deze reinkultuur kon pas aangetoond worden nadat het duidelijk geworden was dat (i) de bakterie gevoelig is voor zuurstof als hij stikstof bindt (ii) de aktiviteit van nitrogenase niet geregistreerd kan worden met behulp van de acetyleenreduktie-methode wanneer de bakterie op methaan groeit.

Variatie van de zuurstofspanning had weinig invloed op de groei van stam 41 in nitraathoudend medium. Maar in stikstofvrij medium werd de groei sterk geremd door hoge zuurstofspanningen. Dergelijke verschijnselen deden zich ook voor wanneer de bakterie op platen gekweekt werd. De afmeting van de kolonies op platen met nitraat werd niet beinvloed door de zuurstofspanning. Maar wanneer de bakterie op stikstofvrije platen gekweekt werd ontstond er normale groei indien de platen onder 5% zuurstof werden geïnkubeerd, terwijl de groei onder 20% zuurstof zeer gering was. De stikstofbindende kolonies die hier en daar op zulke platen verschenen waren waarschijnlijk ontstaan als gevolg van plaatselijke ophopingen van de afgestreken bakteriën.

Het vermogen tot stikstofbinding door stam 41 bij groei op methaan werd aangetoond met behulp van $^{15}N_2$. Nadat de bakterie op methaan gekweekt was in stikstofvrij medium onder verlaagde zuurstofspanning konden verhoogde ^{15}N-gehalten van de kulturen gemeten worden.

Stam 41 kan geen acetyleen reduceren als hij op methaan groeit hoewel hij dan wel stikstof kan binden. In de hoofdstukken I en II werden twee mogelijkheden in beschouwing genomen om dit merkwaardige gedrag te kunnen verklaren. Het falen van de acetyleenreduktiemethode was niet het gevolg van

de ko-oxydatie van ethyleen. Dit ondanks het feit dat bakteriën die in stikstofvrij medium op methanol groeiden wel acetyleen konden reduceren terwijl ze geen ethyleen ko-oxydeerden. Bacteriën die op methaan groeiden ko-oxydeerden weliswaar ethyleen, maar het bleek, dat deze ko-oxydatie volledig verhinderd werd door aanwezigheid van acetyleen.

Niet alleen de ko-oxydatie van ethyleen werd sterk door acetyleen geremd. Ook de oxydatie van methaan werd er door tot stilstand gebracht. Hierdoor kon nitrogenase niet langer beschikken over de energie en de reduktie-equivalenten die het nodig heeft om acetyleen te reduceren. Deze verklaring voor het falen van de acetyleenreduktiemethode bij bakteriën die op methaan groeiden werd nog ondersteund door de waarneming dat deze bakteriën wel acetyleen reduceerden indien ze de beschikking hadden over methanol of een ander substraat dat wel in de aanwezigheid van acetyleen geoxydeerd kon worden.

In hoofdstuk II zijn verder experimenten beschreven die tot doel hadden het mechanisme van de remming van de methaanoxydatie door acetyleen te bestuderen. De groei van stam 41 in nitraathoudend medium werd niet beïnvloed door acetyleen indien methanol als substraat was gegeven, maar groei op methaan werd volledig geremd door acetyleen. Kennelijk werd de oxydatie van methaan tot methanol, de eerste stap in de afbraak van methaan tot koolzuur, onmogelijk gemaakt door acetyleen. Dit kon eveneens worden aangetoond door de zuurstofopname door gewassen cellen van stam 41 te meten. Zuurstofopname, veroorzaakt door de oxydatie van methaan, werd door acetyleen verhinderd, maar zuurstofopname gekoppeld aan methanoloxydatie werd niet door acetyleen geremd. Een interaktie van acetyleen met methaanhydroxylase, het enzymsysteem dat de oxydatie van methaan tot methanol katalyseert, werd ook nog op andere manieren gedemonstreerd. Het bleek dat de ko-oxydatie van verbindingen die voor hun oxydatie van methaan-hydroxylase afhankelijk waren eveneens door acetyleen verhinderd werd. Verder werd nog gevonden dat acetyleen zelf ook geko-oxydeerd werd door cellen die op methaan gekweekt waren. Maar cellen die op methanol gegroeid waren, en het enzym methaan-hydroxylase niet bezaten, ko-oxydeerden acetyleen niet. Het mechanisme van de remming van methaan-hydroxylase door acetyleen werd bestudeerd met behulp van gewassen cellen die op methaan waren voorgekweekt. De zuurstofopname en de methaanopname door deze cellen werden gelijktijdig gemeten. Uit deze experimenten werd onder enig voorbehoud gekonkludeerd dat acetyleen de methaanoxydatie kompetitief remt. Andere methaanoxyderende bakteriën gedroegen zich op dezelfde manier als stam 41. Ook hier werd groei op methaan verhinderd door acetyleen. Bakteriën die op lagere alkanen groeiden vertoonden een overeenkomstig gedrag. De groei werd door acetyleen verhinderd, maar deze verbinding oefende geen remmende invloed uit op de ontwikkeling van de bakteriën indien andere

substraten gegeven werden. Als gevolg hiervan kon de acetyleenreduktiemethode voor deze bakteriën eveneens niet gebruikt worden indien het alkaan als energiebron dienst deed.

De onderzoekingen vermeld in hoofdstuk III waren erop gericht het voorkomen van de eigenschap stikstof te kunnen binden bij methaanoxyderende bakteriën na te gaan. Stikstofbindende methaanoxyderende bakteriën konden gemakkelijk worden opgehoopt onder gebruikmaking van entmateriaal van verschillende herkomst. Bakteriën van het *Methylosinus*-type traden op de voorgrond in deze ophopingen, maar organismen van het *Methylomonas*-type kwamen ook voor. Bakteriën van het *Methylosinus*-type konden zonder moeite geïsoleerd worden. Zij leken alle op stam 41. In jonge kulturen waren ze beweeglijk en in oude kulturen werden exosporen gevormd. De vorm en de afmetingen van de cellen varieerden. Er werden zowel stammen met rechte staafjes als met vibrio-achtige cellen geïsoleerd. Bakteriën van het *Methylomonas*-type waren moeilijker te isoleren en te kweken dan die van het *Methylosinus*-type. De groei van bakteriën van de eerstgenoemde groep werd sterk bevorderd in mengkultuur met een klein beweeglijk staafje dat vaak als infektie in reinkulturen terecht kwam. De onderzochte stikstofbindende methaanoxyderende stam 2 van het *Methylomonas*-type had het type I membraansysteem in tegenstelling tot het type II membraansysteem van stam 41. Hieruit blijkt dat het vermogen om stikstof te binden niet tot de type II methaanoxyderende bakteriën beperkt is.

Het voorkomen in de natuur van methaanoxyderende bakteriën die in staat zijn om stikstof te binden werd onderzocht door verdunningsreeksen van grond- en watermonsters te inkuberen in zowel nitraathoudend als stikstofvrij medium. Nitraat was geen betere stikstofbron dan N_2, wat er op wees dat het vermogen tot stikstofbinding een veel voorkomende eigenschap is van methaanoxyderende bakteriën. In de hoogste verdunningen van deze monsters kwamen coccen voor die zich duidelijk onderscheidden van de geïsoleerde bakteriën van de *Methylosinus*- en *Methylomonas*-typen. In de modder- en grondmonsters kwamen deze coccen dus kennelijk in grotere aantallen voor dan bakteriën van de beide andere typen. Maar in de ophopingskulturen werden de coccen overgroeid door sneller groeiende typen. Isolatie van de coccen was moeilijk. Op platen met stikstofvrij medium groeiden kolonies na een eerste overenting uit de vloeibare ophopingskultuur, maar volgende overentingen groeiden slechts in vloeibaar medium. Van een geïsoleerde stam van het coccen-type werd vastgesteld dat het type II membraansysteem voorkwam.

Hoofdstuk IV bevat gegevens over hydrogenase-aktiviteit van stam 41. Deze aktiviteit werd bepaald in groeiende kulturen door de opname van H_2 te meten. Bij groei van de bakterie in nitraathoudend medium was de hydrogenase-aktiviteit gering, maar bij stikstofbindende bakteriën was deze aktivi-

teit veel groter. De betekenis van het verband tussen hydrogenase en nitrogenase voor deze bakterie is niet duidelijk. Gevonden werd dat acetyleen gereduceerd kon worden met behulp van waterstof. Hieruit valt af te leiden, dat nitrogenase van energie en reduktie-equivalenten werd voorzien door de oxydatie van H_2 door hydrogenase.

CURRICULUM VITAE

Op verzoek van het College van Dekanen van de Landbouwhogeschool volgt hier een korte levensbeschrijving. De auteur van dit proefschrift werd geboren op 6 maart 1947 te Waalwijk. Hij behaalde het H.B.S.-B diploma in 1964. In datzelfde jaar liet hij zich inschrijven als student aan de Landbouwhogeschool. In september 1972 studeerde hij af in de richting waterzuivering waarna hij wetenschappelijk assistent werd op de afdeling mikrobiologie van de eerder genoemde hogeschool.