Nitrogen Fixation and Co-oxidation of Ethylene by a Methane-utilizing Bacterium

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SUMMARY

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 $^{15}\text{N}_2$ 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 $^{15}\text{N}_2$. 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; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; CaCl_2 , 0.015 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5 μg ; H_3BO_3 , 10 μg ; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 70 μg ; $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 10 μg ; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 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 $28 \pm 2^\circ\text{C}$ 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 μ mol phosphate buffer, pH 6.8, 0.4 μ mol NADH and extract in a total volume of 3 ml. Lithium hydroxypyruvate (2 μ mol) or sodium glyoxylate (2 μ mol) 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 Multi-graph type 407 gas chromatograph. Oxygen and nitrogen were measured by thermal conductivity using a 200 cm $\times$ 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 $\times$ 4 mm Porapak R column with nitrogen as carrier gas.

¹⁵N analyses. Sample preparation was a modification (Akkermans, 1971) of the method of Faust (1967). Optical N-analyses were made with a ¹⁵N analyser (Statron NOI-4). The method of Ferraris & Proksch (1972) was used for calculating the ¹⁵N 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 Namsaraev & Zavarzin (1972), but resembles one of the types described by Whittenbury *et al.* (1970) (*Methylosinus sporium*), and by Hazeu & Steennis (1970) (*Methylovibrio söhngeni*). 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 substrates completely prevented growth of the first transfer. Strain 41 also utilized CH₃OH for its development. No growth occurred with C₂H₂, C₂H₄, C₂H₆, CH₃OCH₃,

Fig. 1

Fig. 1. Strain 41 from a five-day culture, grown on slants of MS under 10% CH₄.

Fig. 2

Fig. 2. Strain 41, exospores and lysed cells, from a three-week culture, grown on slants of MS under 10% CH₄.

C₂H₅OH, glucose or acetate when added as potential substrates. No definite conclusions can be drawn for CH₃OCH₃. Our gas-chromatographic analysis of the contents of the cylinder containing CH₃OCH₃ (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 CH₃OCH₃ 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 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 CH₄-grown cells to remove ethylene from the gas phase. After introduction of acetylene into such a gas phase, the ethylene

Fig. 3. Effect of strain 41 culture, growing in nitrogen-free MS with 10% CH₄ (a) and 0.1% CH₃OH (b), on ethylene (13 p.p.m.) in the absence (●) and presence (○) of 10% C₂H₂. Ethylene concentrations were measured in 50 ml flasks with 10 ml growing cultures. The gas phase in the flasks consisted of 5% O₂, 10% CH₄ and 85% N₂ (a) and 5% O₂ and 95% N₂ (b) before the introduction of ethylene and acetylene.

Fig. 4. Growth of strain 41 on CH₄ as influenced by O₂. Portions (10 ml) of a culture growing logarithmically were injected into 350 ml side-arm flasks containing 10% CH₄ and varying concentrations of O₂ and N₂. O₂ consumption during the experiment was less than 2%. (a) Growth in nitrate-free medium: ●, 4% O₂; ○, 6% O₂; ×, 8% O₂; ■, 11% O₂. (b) Growth with nitrate: ●, 6% O₂; ○, 11% O₂; ×, 15% O₂.

Fig. 5

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

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.

concentration remained at the same level (Fig. 3*a*). Cells grown on methanol did not affect ethylene production (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 sensitivity towards oxygen is directly related to the nitrogenase activity was shown by measuring acetylene reduction by methanol-grown 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

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%.

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).

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

Table 1. $^{15}\text{N}_2$ 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. 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 .

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 $^{15}\text{N}_2$.

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).

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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