Nitrogen Fixation in Obligate Methanotrophs

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A number of representative species of obligate methane-oxidizing bacteria were surveyed for their ability to fix N_2 by growth experiments and the acetylene reduction test. Although all strains exhibited growth on nitrogen-free plates, only type II organisms and the type X methanotroph *Methylococcus capsulatus* (Bath) grew well in nitrogen-free liquid medium and were capable of active acetylene reduction. N_2 -fixation in type II methanotrophs was less sensitive to O_2 than in the type X methanotroph *Methylococcus capsulatus* (Bath) and batch cultures of type II organisms could be established at pO_2 values of up to 0.2 bar. N_2 -fixation in *Methylococcus capsulatus* (Bath) was inhibited at pO_2 values above 0.15 bar and the 'switch-off' of nitrogenase activity by ammonia was also observed in this organism.

INTRODUCTION

Although N_2 -fixing methane oxidizing bacteria have been known to exist for many years (Davis *et al.*, 1964), initial attempts to measure N_2 -fixation by the classical acetylene reduction test (Dilworth, 1966; Schöllhorn & Burris, 1967) were unsuccessful (Whittenbury *et al.*, 1970). It was subsequently observed that acetylene was a potent inhibitor of methane oxidation (Dalton & Whittenbury, 1976; de Bont & Mulder, 1974) but that whole-cell nitrogenase activity could be measured if a suitable electron donor such as methanol or formate was included in this system (Dalton & Whittenbury, 1976). Using this technique, de Bont (1976) reported that several 'Methylomonas-type' and 'Methylosinus-type' methane oxidizers (Whittenbury *et al.*, 1970) were capable of fixing N_2 although he found that the facultative organism *Methylobacterium organophilum* strain XX did not reduce acetylene.

In the few methanotrophs that have been shown to fix N_2 , diazotrophic growth was sensitive to O_2 due to the intrinsic sensitivity of their nitrogenase proteins (de Bont & Mulder, 1974; Dalton & Whittenbury, 1976; Dalton, 1980). This sensitivity was manifest in the characteristic 'bell-shaped' curves for nitrogenase activity versus applied pO_2 values (Dalton & Postgate, 1969) or by the 'switch-off' phenomenon observed by Drozd & Postgate (1970) when aerobic cultures were subjected to a sudden increase in dissolved oxygen tension (DOT).

In addition to O_2 , the fixed nitrogen source is also a regulator of synthesis and activity of nitrogenase in many diazotrophs. In a few organisms such as *Azospirillum lipoferum* (Ludden *et al.*, 1978) and some of the *Rhodospirillaceae* (Zumft *et al.*, 1981; Falk *et al.*, 1982; Haaker *et al.*, 1982), NH_4^+ ions inhibit N_2 -fixation immediately, whereas in others such as *Clostridium pasteurianum* (Daesch & Mortenson, 1972) and *Klebsiella pneumoniae* (Gordon *et al.*, 1981), they only affect biosynthesis of the enzyme. Several mechanisms for the short-term regulation of nitrogenase activity *in vivo* have been discussed (see Eady, 1981). In phototrophic bacteria it appears that the rapid inactivation of nitrogenase by ammonia is not due to changes in the membrane potential or the ATP/ADP ratio of the cell. It has been postulated that in the purple

Abbreviation: DOT, dissolved oxygen tension.

bacteria, the response to ammonia is due to the covalent modification of the Fe protein to an inactive form (discussed by Zumft *et al.*, 1981).

This paper reports on the distribution of the N₂-fixing character of all the representative strains of obligate methanotrophs in the University of Warwick Culture Collection and investigates the effects of O₂ and ammonium ions on the activity of nitrogenase in these organisms.

METHODS

Organisms. Only obligate methane-oxidizing organisms were considered in these studies and their isolation and properties have been described previously (Whittenbury *et al.*, 1970). The type I organisms were: '*Methylomonas albus*' BG8; '*Methylomonas agile*' A20; '*Methylomonas methanica*' S1, A4 and PM; and '*Methylobacter capsulatus*' Y. The type II organisms were: '*Methylosinus sporium*' 5 and 12; '*Methylosinus trichosporium*' PG, OB3b, OB5b and OB4; and '*Methylocystis parvus*' OBBP. The type X strain (Whittenbury & Dalton, 1980) was *Methylococcus capsulatus* (Bath).

Media. The basic mineral salts medium (MS) of Dalton & Whittenbury (1976) was used throughout these studies. The medium was either supplemented with 1 g ammonium chloride l⁻¹ to give AMS medium or 1 g potassium nitrate l⁻¹ to give NMS medium. All organisms were grown at 30 °C with the exception of *Methylococcus capsulatus* (Bath) which was grown at 45 °C. Batch cultures of methanotrophs were grown in 250 ml quickfit glass flasks fitted with Suba seals. The required pO₂ for growth was established by gassing flasks with N₂ after inoculation and then introducing O₂ into the flask via a syringe. Continuous cultures of methanotrophs were set up as described previously (Murrell & Dalton, 1983) with the growth-limiting substrate being O₂.

Assays. Nitrogenase activity in whole cells was assayed by the method of Dalton & Whittenbury (1976).

Culture cell dry weights were estimated by injecting washed cell suspensions into a Beckmann model 915-B total carbon analyser. The cell carbon content was assumed to be 47% of the dry weight (Van Dijken & Harder, 1975). 3y dilution of chemostat grown cells, a curve of optical density (OD₅₄₀) against dry weight was prepared for *Methylococcus capsulatus* (Bath). This gave average dry weight values at OD₅₄₀ 0.1 of 0.024 mg ml⁻¹.

Measurement of methane, oxygen, nitrogen or argon in culture atmospheres was done using a Pye Unicam series 104 gas chromatograph fitted with a thermal conductivity detector and 2 m × 4 mm columns filled with Molecular Sieve 5A or Porapak R.

Ammonia present in cell supernatants was measured using Nessler's reagent. Protein in cell-free extracts was measured by the Biuret method of Herbert *et al.* (1971).

RESULTS AND DISCUSSION

The N₂-fixing ability of methanotrophs

In a preliminary experiment, single colonies of each methanotroph grown on an AMS agar plate were streaked out on to MS agar plates. Plates were incubated in a 50% methane/50% air atmosphere (v/v) at 30 °C, for 5 d and then examined for growth. All methanotrophs exhibited some growth on these nitrogen-free plates, however, type I methanotrophs showed far less growth than the type II methanotrophs. A single colony of each organism from an AMS agar plate was inoculated into 250 ml flasks containing 100 ml MS medium and gassed with O₂ and N₂ to give a range of pO₂ values from 0.01 to 0.2 bar. Methane (50 ml) was added to each flask which was then incubated for 3 d at the appropriate temperature on a gyratory shaker. Flasks were examined for growth by measuring the OD₅₄₀ of each culture. All type I organisms showed little or no growth on nitrogen-free medium whereas the control flasks containing NMS or AMS medium at pO₂ values of 0.2 bar showed good growth (Table 1). In contrast to the type I organisms, all type II methanotrophs and *Methylococcus capsulatus* (Bath) grew well in nitrogen-free medium. Type II methanotrophs appeared to be less sensitive to O₂ than *Methylococcus capsulatus* (Bath) under high pO₂ values suggesting that their nitrogenase proteins were either less sensitive to O₂ damage than the enzyme from *Methylococcus capsulatus* (Bath) or that these organisms could augment respiratory activity to protect nitrogenase.

To verify that these organisms did indeed possess nitrogenase and that the observed growth was not due to scavenging of trace amounts of fixed nitrogen present in medium (see Hill & Postgate, 1969), the acetylene reduction test was done on each culture. Cells from flasks grown at their optimum pO₂ values were injected into assay flasks and re-gassed to the required pO₂ values. No type I methanotrophs could reduce acetylene (results not shown) although good activities were observed with all type II cultures and *Methylococcus capsulatus* (Bath) (Table 2).

Table 1. *Growth of methanotrophs in nitrogen-free medium at a variety of pO₂ values*

Experimental details were as described in Methods. Growth was determined by measuring the OD₅₄₀ after 3 d growth on a gyratory shaker incubator.

Organism	OD ₅₄₀ at pO ₂ (bar) values:								
	0.01	0.02	0.04	0.06	0.08	0.10	0.15	0.20	Control*
Type I									
BG8	0.02	0.02	0.04	0.04	0	0	0	0.02	0.56
A20	0	0	0	0	0	0.03	0	0.02	0.31
S1	0	0	0	0	0	0.04	0	0.05	0.42
A4	0	0	0.04	0	0	0.01	0	0	0.27
PM	0	0	0	0	0	0.02	0.02	0.03	0.44
Y	0	0	0	0	0	0	0	0	0.62
Type II									
5	0	0.08	0.21	0.27	0.36	0.44	0.42	0.37	0.53
12	0	0.10	0.17	0.26	0.36	0.47	0.45	0.45	0.56
PG	0	0.12	0.26	0.32	0.33	0.47	0.45	0.45	0.49
OB3b	0	0.03	0.14	0.26	0.41	0.49	0.49	0.51	0.57
OB5b	0.04	0.09	0.21	0.36	0.41	0.41	0.41	0.37	0.48
OB4	0	0.08	0.22	0.31	0.41	0.42	0.40	0.36	0.48
OBBP	0.02	0.11	0.27	0.29	0.31	0.30	0.31	0.28	0.62
Type X									
<i>Methylococcus capsulatus</i> (Bath)	0	0.10	0.31	0.40	0.29	0.13	0.04	0	0.48

* The control was NMS medium at pO₂ 0.20 bar.

Table 2. *Nitrogenase activity in type II and type X methanotrophs as assayed by the acetylene reduction test*

The acetylene reduction test was done on cultures grown in MS medium using 150 mM-methanol as electron donor. No acetylene reduction was observed with any of the type I methanotrophs.

Organism	pO ₂ (bar) . . .	Nitrogenase activity [nmol ethylene produced min ⁻¹ (mg dry wt cells) ⁻¹]			
		0.04	0.08	0.12	0.20
Type II					
5		3.5	4.1	4.2	4.0
12		2.6	2.8	2.8	3.1
PG		2.7	3.3	3.4	3.2
OB3b		3.7	4.8	5.1	4.9
OB5b		4.1	4.7	5.1	4.9
OB4		3.6	3.8	3.9	3.8
OBBP		2.1	2.4	2.3	2.0
Type X					
<i>Methylococcus capsulatus</i> (Bath)		2.9	3.7	0.4	0

Of particular interest was the observation that type II methanotrophs were capable of growing on N₂ and reducing acetylene at pO₂ values as high as 0.2 bar because generally, chemoautotrophs only fix N₂ under microaerophilic conditions (see Dalton, 1980). To investigate this further, chemostat cultures of the type II methanotroph '*Methylosinus trichosporium*' OB3b and the more oxygen-sensitive N₂-fixer *Methylococcus capsulatus* (Bath) were established on methane under N₂-fixing conditions with O₂ as the growth-limiting substrate. The oxygen tension to the cultures was then progressively increased and steady-state

Fig. 1. pO_2 profiles of nitrogenase activity in *Methylococcus capsulatus* (Bath) and '*Methylosinus trichosporium*' OB3b.

Fig. 2. Effect of 'ammonia-shock' on nitrogenase activity of a N_2 -fixing culture of *Methylococcus capsulatus* (Bath).

was established at the new DOT. The maximum DOT tolerated by *Methylococcus capsulatus* (Bath) was $6 \mu M$ before the culture density decreased and wash-out occurred, whereas the '*Methylosinus trichosporium*' OB3b culture would tolerate a DOT value of $22 \mu M$ before wash-out occurred. In each case, a steady-state could be established at a DOT value of $1 \mu M$ less than the wash-out value. This higher tolerance to O_2 by the type II organism was also demonstrated by measuring the pO_2 /acetylene reduction activity profiles of samples removed from each O_2 -limited chemostat in which the cell density was $0.9 \text{ mg dry weight ml}^{-1}$ and the dilution rate was 0.05 h^{-1} (Fig. 1). Despite the higher growth temperature of *Methylococcus capsulatus* (Bath), when the solubility of O_2 at $45^\circ C$ is approximately 20% lower than at $30^\circ C$, the nitrogenase from this organism exhibited a much lower pO_2 optimum than the enzyme from '*Methylosinus trichosporium*' OB3b.

Effects of fixed nitrogen on nitrogenase activity

No N_2 -fixation, as assayed by the acetylene reduction test, was observed in any AMS or NMS grown batch cultures of methanotrophs, indicating that fixed nitrogen was repressing nitrogenase. *Methylococcus capsulatus* (Bath) was chosen to investigate how ammonium ions exerted this effect on nitrogenase. NH_4Cl (10 ml, 1 M) was added to an O_2 -limited, N_2 -fixing culture in steady-state at a dilution rate of 0.05 h^{-1} to give an initial concentration of $4 \text{ mM-}NH_4^+$. After 10 min the nitrogenase activity of the culture had decreased to zero from $3.9 \text{ nmol ethylene produced min}^{-1} (\text{mg dry wt})^{-1}$ (Fig. 2), suggesting that initially NH_4^+ inhibited nitrogenase activity and not synthesis, since active nitrogenase would still be present after several hours if only synthesis was affected. The rapid 'switch-off' of nitrogenase activity by NH_4^+ is an unusual phenomenon so far only observed in a few photosynthetic N_2 -fixers (Zumft

et al., 1981) and it will be interesting to learn from future studies with *Methylococcus capsulatus* (Bath) if a similar short-term control mechanism of nitrogenase activity operates in methanotrophs.

From these physiological studies it appears that methanotrophs may be divided into two groups on the basis of their ability to fix N_2 , thus strengthening the classification scheme proposed by Whittenbury & Dalton (1980, 1982). Again the exception to this rule is *Methylococcus capsulatus* (Bath) which appears to exhibit characteristics of both groups (Whittenbury & Dalton, 1982). Recent studies (Murrell *et al.*, 1983), which complement data presented in this paper, have shown that specific chromosomal DNA sequences from a number of N_2 -fixing methanotrophs exhibit homology with the plasmid pSA30 which contains the N_2 -fixation structural genes *K*, *D*, and *H* from *Klebsiella pneumoniae* (Cannon *et al.*, 1979).

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