'*Methylobacterium ethanolicum*': a Syntrophic Association of Two Methylotrophic Bacteria

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Two methylotrophic bacteria having similar cell morphologies have been isolated from cultures of '*Methylobacterium ethanolicum*' grown on methane. One of these, strain POC, is an obligate methanotroph containing the serine pathway and Type II intracytoplasmic membranes, which appears to be a '*Methylocystis*' species. The second organism, strain H4-14, can fix N₂ and grows on a variety of substrates, including methanol, formate, ethanol, succinate, fructose and H₂ + CO₂; during growth on methanol, carbon is assimilated via the Calvin-Benson cycle. Strain H4-14 appears to be a *Xanthobacter* species. Mixtures of the two organisms formed stable associations during growth on methane, consisting of approximately 5–20% H4-14 and 80–95% POC. Strain POC excreted biotin, which was required by strain H4-14. Our results suggest that '*Methylobacterium ethanolicum*' cultures grown on methane consist of a syntrophic association of two methylotrophs.

INTRODUCTION

Stable mixed cultures involving methane-oxidizing bacteria have been identified and studied in the past. In one four-membered culture, the methane-oxidizer was unable to grow in liquid culture without a methanol-utilizing *Hyphomicrobium* (Harrison, 1978), and showed increased growth yields when the other two members, both heterotrophs, were present. A second mixed culture contained five organisms, a *Methylococcus* species plus four heterotrophs. Although the methanotroph could grow on methane in pure culture, the growth yield was enhanced in the presence of the heterotrophic bacteria (Harrison, 1978). In many cases, methane-oxidizing bacteria have been shown to be sensitive to low concentrations of methanol, which they often excrete, or organic compounds, which accumulate due to cell lysis (Eccleston & Kelly, 1972, 1973; Eroshin *et al.*, 1968; Malashenko *et al.*, 1974; Wilkinson & Harrison, 1973). The basis of these stable mixed cultures has therefore been proposed to be the utilization of toxic compounds by the methanol-utilizers and the heterotrophs, minor elements of the total population.

'*Methylobacterium ethanolicum*' has been described as a facultative methane-oxidizer, capable of growth on a variety of carbon sources (Lynch *et al.*, 1980). This organism has been maintained in the laboratory for three years and by all routine criteria appeared to be a pure culture. Microscopic examination of cultures grown on a variety of carbon sources always revealed only one morphological type, and cultures streaked on to agar plates with a variety of carbon sources always revealed only one colony type, which contained cells that looked identical to those seen in liquid culture. In addition, cells grown under a variety of conditions exhibited similar antibiotic sensitivities, and it was possible to demonstrate low levels of enzymes involved in the metabolism of C₁ compounds in cells grown on non-C₁ substrates (Lynch *et al.*, 1980). However, these cultures also routinely exhibited some peculiar characteristics. Biotin was required for growth on non-methane substrates, but not for growth on methane. The ability to grow on methane was unstable, being lost after subculture on non-methane substrates. Specifically, when liquid cultures grown on methane were streaked on to plates containing any non-methane

substrate, including methanol, single colonies that grew up could not grow on methane in liquid culture. However, heavy inocula from such plates invariably grew on methane in liquid cultures, after a lag period. When single colonies were transferred from a non-methane plate to another non-methane plate and allowed to grow, even heavy inocula did not grow on methane in liquid culture. These cultures that were unable to grow on methane contained no detectable plasmids, while liquid cultures that had grown on methane contained three apparently separate plasmids (O'Connor, 1981). Methane-negative strains showed significant background (non-substrate-dependent) growth on both agar and agarose plates, as well as on membrane filters placed on pads soaked in sterile medium. This characteristic made definitive determinations of growth on methane difficult, requiring the testing of individual colonies in liquid culture. Since we were unable to provide convincing explanations for all of these unusual properties, we investigated '*M. ethanolicum*' more closely. We now present evidence that this culture consists of a stable co-culture of two methylo-trophs: an obligate methane-oxidizer and a facultative, autotrophic methanol utilizer.

METHODS

Media and growth conditions. All cultures were grown at 30 °C on NMS, the minimal medium described previously (Lynch *et al.*, 1980), or MS, a version of NMS lacking potassium nitrate. Cultures grown on gaseous substrates were incubated under a substrate/air atmosphere of 80:20 (v/v). In liquid cultures, the proportion of air was gradually increased to 50% (v/v) as the turbidity increased. The atmosphere over the cultures was replaced daily. Strain H4-14 was grown under a helium/air atmosphere (70:30) with a biotin supplement (20 µg l⁻¹). Methanol, ethanol, propanol and butanol were filter-sterilized and added at a final concentration of 0.2% (v/v). All other substrates were added as sterile stocks to a concentration of 0.1% (w/v).

Substrate oxidation rates. Substrate-dependent oxygen uptake was determined using a Rank oxygen electrode (Rank Bros., Bottisham, U.K.) as previously described (Lynch *et al.*, 1980).

Electron microscopy. For thin sections, preparations were fixed in cold 3% (v/v) glutaraldehyde (Taab Co., Reading, U.K.) in 0.1 M-sodium cacodylate buffer, dehydrated in a graded ethanol series and embedded in Epon using standard techniques.

Preparation of crude extracts and enzyme assays. Crude extracts were prepared by disruption of bacteria in a French pressure cell as described previously (Lynch *et al.*, 1980). Enzymes were assayed by published methods: hydroxypyruvate reductase (D-glycerate:oxidized nicotinamide adenine dinucleotide oxidoreductase, EC 1.1.1.29) (Large & Quayle, 1963); serine-glyoxylate aminotransferase (L-serine:glyoxylate aminotransferase, EC 2.6.1.45) (Blackmore & Quayle, 1970); methanol dehydrogenase (EC 1.1.99.8) (O'Connor & Hanson, 1977); hexulose phosphate synthase (Dahl *et al.*, 1972); ribulosebiphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] (Tabita *et al.*, 1978).

Plasmid DNA preparation. Cultures were grown to mid-exponential phase (A_{600} 0.5–0.7), harvested and washed in TE buffer (Tris, 0.05 M; EDTA, 0.01 M; pH 8.0). Plasmid DNA was then prepared using the alkaline lysis technique described by Portnoy *et al.* (1981). These preparations were further purified by CsCl/ethidium bromide ultracentrifugation. All plasmid preparations were subjected to agarose gel electrophoresis and visualized by staining the gels with ethidium bromide.

Restriction digests. The restriction endonuclease *Hind*III was incubated with DNA preparations under the conditions recommended by the supplier (Bethesda Research Laboratory, Rockville, MD, U.S.A.). Approximately 0.2 µg of restricted DNA was subjected to agarose gel electrophoresis.

Utilization of ¹⁴C-labelled substrates. Five ml cultures of strain H4-14 grown on 50% CH₄/5% H₂/5% CO₂ were evacuated to remove residual gases, and then incubated with 1 µCi (37 kBq) ¹⁴CH₄ (Amersham Searle, Arlington Heights, IL, U.S.A.; 3.5 mCi mmol⁻¹) under an atmosphere of 20% air/7% CH₄/73% N₂ or with 1 µCi [¹⁴C]bicarbonate (New England Nuclear, Boston, MA, U.S.A.; 9.2 mCi mmol⁻¹) under an atmosphere of 20% air/5% CO₂/75% N₂ for 4 h. For the ¹⁴CH₄ cultures, the reaction was terminated with 0.5 ml 10 M-NaOH to absorb any CO₂ from the atmosphere. The culture was then sparged with N₂ gas for 5 min to remove residual methane, and 0.5 ml was removed for counting. Concentrated HCl (1 ml) was then added to release the CO₂ from solution, and the culture was sparged again with N₂ gas for 5 min to remove residual CO₂. A second 0.5 ml sample was then removed for counting. The difference between the basic and acidic samples was used to compute the amount of ¹⁴CO₂. The remaining portion of the culture was filtered using a 0.45 µm membrane filter to determine the amount of radioactivity in cell material. For the [¹⁴C]bicarbonate cultures, the reaction was terminated with 0.5 ml concentrated HCl, and the culture was sparged with N₂ gas for 5 min and filtered as above. All samples were counted in 10 ml Aquasol II (New England Nuclear). Controls which were killed with NaOH or HCl at zero time were included with each sample and were subtracted from the test sample values. For the ¹⁴CH₄ method, controls using a known quantity of ¹⁴CO₂ produced greater than 90% recovery of counts.

Dialysis chamber experiments. A two-chambered dialysis vessel (Bellco, Vineland, NJ, U.S.A.) was fitted with a dialysis membrane and inoculated with strain POC on one side and strain H4-14 on the other. A gas phase consisting of 80% methane/20% air was sparged through both sides of the vessel. The vessel was incubated at 30 °C for 2–3 weeks.

Reconstitution experiments. Strains POC and H4-14 were grown to late-exponential phase on methane and methanol, respectively. Cell numbers were determined using a Petroff-Hausser counting chamber (C. A. Hausser & Son, Philadelphia, PA, U.S.A.), and flasks were inoculated containing each strain in approximately the following proportions of POC to H4-14: 1:1000, 1000:1, 1:1. These cultures were grown on methane in the absence of biotin to stationary phase and transferred with a 1% inoculum through two more growth cycles. At the end of each growth cycle, the cultures were diluted and plated on to NMS/methane and nutrient agar plates, and stained with India ink for microscopic counting.

RESULTS

Isolation of two methylo-trophic bacteria from cultures of 'M. ethanolicum'. Cultures of '*M. ethanolicum*' were unusually unstable with respect to growth on methane. While investigating the possibility of plasmid-encoded methane oxidation in this culture, we demonstrated that three distinctly different plasmids were always present in our methane-grown cultures, but all three were always missing from our cultures unable to grow on methane (A. E. Wopat & M. E. Lidstrom-O'Connor, unpublished). Since we could not explain this by any known mechanism, our suspicions were aroused that although '*M. ethanolicum*' appeared to be a pure culture by several criteria, it might in reality consist of more than one organism. We therefore examined our cultures more carefully.

As mentioned earlier, the colonies that grew on NMS plates incubated under methane did not show methane-dependent growth. However, it was possible to inoculate liquid cultures from these plates and obtain growth on methane. Therefore, we routinely maintained stock cultures on methane plates, but only incubated them under methane for 10–15 d, as this was sufficient to produce large, yellow colonies, and longer incubations often resulted in mould contamination. However, when we re-examined our methane liquid cultures, we discovered that if the cultures were streaked on to NMS plates and incubated under methane for 25–30 d, small, light-pink pigmented colonies were seen at the top of the streak, growing through the yellow background. All viable cultures maintained in our laboratory, including stock cultures, contained the pink-pigmented organism, in varying proportions. The pink colony type, called strain POC, was purified on methane plates and the yellow colony type, called strain H4-14, was purified on nutrient agar plates.

Characteristics of strains POC and H4-14. The characteristics of the two strains isolated from '*M. ethanolicum*' cultures are shown in Table 1. The organisms had similar cell morphologies (irregular rods), although older cells of strain H4-14 tended to be less regular than those of strain POC. Thin sections showed that strain POC contained a Type II intracytoplasmic membrane system while strain H4-14 contained no detectable internal membranes under any growth condition (Fig. 1).

Strain POC was an obligate methanotroph, showing no growth in either liquid or plate cultures on other substrates including methanol. Attempts to 'train' strain POC to grow on methanol by gradually increasing the concentration of methanol in the presence of methane have not been successful. Strain H4-14 was a microaerophilic, facultative methylo-troph, showing growth on a variety of substrates including methanol, formate, $H_2 + CO_2$, and succinate. Very slight growth was observed on all substrates under a 100% air atmosphere, but good growth was achieved under a 50% air atmosphere. Slight growth was observed on methane, ethane and butane.

Both organisms could grow in liquid culture in the absence of a fixed nitrogen source, although strain POC required an increased concentration of molybdenum in the medium under these conditions. Older cultures of strain POC produced cells which appeared similar to the lipid cyst resting stage described by Whittenbury & Dalton (1981).

In order to determine whether strain H4-14 was a variant of strain POC, chromosomal DNA was isolated from both strains and digested with *Hind*III, and the restriction fragments were

Table 1. *Characteristics of strains H4-14 and POC*

Property	H4-14	POC
Cell morphology	irregular rod	irregular rod
Capsule	heavy	light
Gram reaction	negative	negative
Colony characteristics	yellow, mucoid	light pink, opaque
Growth factors	biotin	none
Sensitivity to oxygen	+	—
Growth substrates*		
H ₂ + CO ₂	+++	—
Formate	++	—
Methane	+/-	+++
Methanol	++	—
Ethane	+/-	—
Ethanol	++	—
Butane	+/-	—
Butanol	++	—
Glycerol	+++	—
Succinate	+++	—
Sucrose	++	—
Fructose	+++	—
Growth in absence of fixed nitrogen	+++	+++†

* All tests were made using broth cultures incubated up to 6 weeks; no growth was observed for either culture on glucose, lactate, malate, glycollate, acetate or hexanol; +++, heavy growth; ++, good growth; +/-, slight growth; —, no growth.

† Addition of molybdate (20 µM) was required.

Fig. 1. Electron micrographs of strains H4-14 and POC. (a) Strain H4-14 grown on H₂ + CO₂; (b) strain POC grown on methane. The bar marker represents 0.5 µm.

separated by agarose gel electrophoresis. The patterns obtained were quite different, indicating that these were indeed two different strains, rather than variants of the same strain.

Growth of strain H4-14 on short-chain alkanes and alcohols. The ability of strain H4-14 to grow on short-chain alkanes and alcohols was studied in more detail (Table 2). Although growth on methane, ethane and butane was extremely slow, it was reproducible. No growth was observed in the absence of an added substrate. It was not possible to obtain reliable oxidation and incorporation data from these cultures, due to the small amounts of cell material produced. However, H4-14 cells grown on limiting amounts of H₂ + CO₂ in the presence of excess methane were able to oxidize and incorporate small amounts of methane when incubated in the presence of ¹⁴CH₄ (Table 3). The total amount of methane utilized was about 10% of the CO₂ incorporation rate. However, the amount of growth and incorporation was considered to be too

Table 2. Growth rate of strain H4-14 on short-chain alkanes and alcohols

Substrate added	Generation time* (h)
None	NG
Methane†	204
Methanol	12
Ethane	50
Ethanol	15
Butane	195
Butanol	16

* NG, no growth detectable after three weeks. In all cases, the atmosphere contained 30% air; where necessary, the difference was made up with nitrogen gas.

† The methane used was determined to contain less than 0.1% ethane, by gas chromatography.

Table 3. Oxidation and incorporation of ^{14}C -labelled substrates by strain H4-14 grown on 5% H_2 + 5% CO_2 + 50% CH_4

Culture	Utilization of substrate [nmol h ⁻¹ (mg protein) ⁻¹]		
	$^{14}\text{CH}_4$ *		$^{14}\text{CO}_2$ †
	CO_2	Cell material	Cell material
1	0.45	0.20	4.85
2	0.34	0.12	5.11
3	0.24	0.06	2.55
4	0.44	0.17	4.96

* Cells were incubated with 5% H_2 + 5% CO_2 + 1 μCi $^{14}\text{CH}_4$ (7% CH_4 total).

† Cells were incubated with 5% H_2 + 5% CO_2 + 1 μCi [^{14}C]bicarbonate.

Table 4. Enzyme activities in extracts of strains H4-14 and POC grown on C_1 substrates

All values are the average of two or more experiments, and are expressed as nmol min⁻¹ (mg protein)⁻¹. Values agreed within $\pm 15\%$; 0, not detectable.

Enzyme	POC grown on methane	H4-14 grown on methanol
Methanol dehydrogenase	21	220
Hydroxypyruvate reductase	780	0
Serine-glyoxylate aminotransferase	320	0
Hexulose phosphate synthase	0	0
Ribulosebiphosphate carboxylase	0	120

small to justify terming this organism a methanotroph. The slow growth rate on short-chain alkanes did not appear to be due to a lack of reducing power, since it was not increased by the addition of small amounts of H_2 , over controls lacking the alkanes. Cells incubated in this manner, could oxidize H_2 at high rates.

Enzyme activities. The two methylotrophs were screened for the presence of key enzymes of C_1 metabolism (Table 4). Both organisms contained methanol dehydrogenase activity. This activity was low in extracts of strain POC, but whole cells showed high levels of methanol-dependent oxygen uptake, suggesting that the methanol dehydrogenase activity in this strain was unstable in crude extracts. Two key enzymes of the serine pathway, hydroxypyruvate reductase and serine-glyoxylate aminotransferase were detectable in strain POC but not in strain H4-14. Hexulose phosphate synthase, a key enzyme of the ribulose monophosphate pathway, was not detected in either strain. Ribulosebiphosphate carboxylase, a key enzyme of the Calvin-Benson cycle, was found only in strain H4-14. Further characterization of the autotrophic growth of strain H4-14 is in progress (L. G. Lehmiche & M. E. Lidstrom-O'Connor, unpublished).

Fig. 2. Agarose gel electrophoresis of plasmid DNA from strain POC. (A) Molecular mass standards (MDal); (B) strain POC. LF, linear DNA fragments.

Presence of plasmids. Both strains were tested for the presence of plasmid DNA. Using a variety of techniques, no plasmids have been detected in strain H4-14, although it has been possible to detect plasmids that have been conjugated into this strain (G. Fulton & M. Lidstrom-O'Connor, unpublished). However, three plasmids were routinely detectable in strain POC. These have been identified by agarose gel electrophoresis (Fig. 2). The molecular mass of each plasmid has been estimated from the agarose gels using whole plasmids and from summing the molecular masses of restriction fragments (data not shown). The average of several measurements was 50 (range ± 5), 102 (± 7) and 118 (± 4) Mdal, respectively. The restriction digests of the plasmid preparations obtained from strain POC appeared identical to those observed for methane-grown cultures of '*M. ethanolicum*'. Each of the plasmids has been isolated separately using low melting point agarose gels, and restriction digests of the separated plasmids have shown that the three were indeed unique plasmids rather than different forms of a single plasmid (A. E. Wopat & M. E. Lidstrom-O'Connor, unpublished).

Characteristics of the co-culture. The relative proportions of the two methylotrophs were determined in a number of separate co-cultures present in the laboratory. Strain H4-14 could be easily enumerated on nutrient agar plates. However, the enumeration of strain POC was more difficult. A comparison of microscopic counts with 4-week plate counts for pure cultures revealed a plating efficiency on methane of 1–20% indicating the unreliability of plate counts. Since newly divided cells of the two strains were morphologically indistinguishable, it was not possible to determine numbers by direct phase-contrast microscopy. However, strain H4-14 produces a copious capsule under most growth conditions, and strain POC produces little or no capsule during growth on methane. Therefore, it was possible to determine the proportions of each strain by staining cultures with India ink and microscopically counting the relative numbers of heavily and lightly encapsulated cells. The H4-14 counts made in this fashion correlated well with counts made using nutrient agar plates.

All viable cultures of '*M. ethanolicum*' in the laboratory that were able to grow on methane in the laboratory were found to be composed of 80–95% strain POC and 5–20% strain H4-14. Our initial estimates showing low percentages of strain POC were due to the low plating efficiency of this methanotroph. The constancy of the ratio of the two strains prompted further investigations into the stability of the co-culture.

Table 5. *Proportion of strain H4-14 in artificially prepared co-cultures of different initial compositions*

All values are the average of two or more determinations, and are expressed as the percentage of strain H4-14 in the culture. Values agreed within $\pm 20\%$. Mixture 2 showed a 3 d lag before growth was noticeable.

Transfer	Mixture 1	Mixture 2	Mixture 3
Initial	0.1	99.9	44.4
1	9.7	12.2	23.2
2	12.8	7.0	11.6
3	15.1	17.5	9.8

Table 6. *Growth of strains POC and H4-14, and the co-culture, on various substrates*

All values are the average of two or more experiments. Values agreed within $\pm 15\%$. All cultures were incubated with an atmosphere containing 30% (v/v) air.

Culture	Substrate	Generation time (h)	Maximum absorbance (Klett units)
Co-culture	Methane	10	170
H4-14	Methanol	12	95
	Succinate	10	98
POC	Methane	7	150

Strain mixing experiments. In order to test the persistence of the two methyilotrophs in the co-culture, flasks were inoculated with three different ratios of strain POC to strain H4-14 (approximately 1:1000, 1000:1 and 1:1), and grown to stationary phase on methane in the absence of biotin. Each culture was transferred with a 1% inoculum three times. Samples were withdrawn from each culture initially and at stationary phase for determinations of the relative proportions of each strain. By the first outgrowth of the mixtures to stationary phase, all three had stabilized to a proportion of H4-14 that eventually varied between 7 and 23% of the total (Table 5). This number correlated well with the determinations made from existing co-cultures in the laboratory.

Growth requirements of strain H4-14 in the co-culture. The persistence of both strains in the co-culture suggested a syntrophic relationship between the two methyilotrophs during growth on methane. Attempts were made to determine the nature of this relationship. Table 6 outlines the generation times of the two strains as well as the co-culture under various culture conditions. The generation time of the co-culture in batch culture was approximately 10 h. Since strain H4-14 was unable to grow on methane at this growth rate (see Table 2), it seemed clear that strain POC must be providing the growth substrate to support strain H4-14 under these conditions. Two candidates for substrates that might be excreted by strain POC were methanol and multicarbon compounds. Ribulosebiphosphate carboxylase activity was detected in the co-culture at levels about 10% of that detected in the pure culture of strain H4-14. This activity was not detectable in the pure culture of strain POC grown under identical conditions. Since the co-cultures contained about 10% strain H4-14, these data suggest strain H4-14 might have been growing on methanol excreted by strain POC. This did not rule out the possibility that strain H4-14 might also be utilizing organic compounds from strain POC. Since the growth rate of the co-culture was faster than that of strain H4-14 grown on methanol (see Table 2), it seems likely that other substrates were involved in the interaction.

Strain H4-14 required biotin for growth on all substrates, which suggested that strain POC might excrete biotin. In order to test this possibility, filter-sterilized growth medium from methane-grown POC cultures was substituted for biotin in H4-14 cultures. Strain H4-14 grew equally well on methanol in the presence of biotin or 2% (v/v) POC culture supernatant, but showed no growth in the absence of supplementation.

Growth requirements of strain POC. Strain POC grew in a minimal salts medium in the presence of methane with a generation time of 7 h (Table 6). The maximum absorbance reached was reproducibly slightly less than that of the co-culture, and significantly greater than that reached by strain H4-14. Growth was not inhibited by the presence of 0.1% succinate or 0.2% ethanol, but was slowed considerably by the presence of 0.5% methanol.

Dialysis chamber experiments. The results described above suggested that the basis of the interaction between strains POC and H4-14 was the provision of dialysable components. Therefore, it should have been possible to grow both strains on methane in a vessel containing two chambers separated by a dialysis membrane. When such a vessel was inoculated with strain POC on one side and strain H4-14 on the other, then sparged with a methane/air mixture, only the side containing strain POC grew initially. After about 2 weeks, the side containing strain H4-14 also became turbid. However, both strains were found in this culture, suggesting that the membrane had leaked. In two different experiments, no growth of strain H4-14 was observed until strain POC was found in the chamber.

DISCUSSION

Our results suggest that methane-grown '*Methylobacterium ethanolicum*' consists of a stable co-culture of two methylotrophic bacteria. The two bacteria were difficult to separate during growth on methane, and only after prolonged incubation on plates was it possible to detect the presence of two colony types and to isolate the individual pure cultures. The apparent instability of the culture for growth on methane and the correlation of this instability with the loss of three plasmids now has a satisfactory explanation: strain POC, an obligate methanotroph, contained three plasmids but strain H4-14, a facultatively autotrophic methanol utilizer, contained none. An explanation also exists for the persistence of characteristics of strain POC in biochemical and molecular determinations. Strain H4-14 is difficult to harvest by centrifugation, due to its heavy capsule. Therefore, cell pellets from cultures containing both organisms would be enriched in strain POC, simply due to the different efficiencies of harvesting. Strain POC also lyses more readily than strain H4-14, so that cultures containing even small proportions of strain POC would have resulted in enzyme and DNA preparations highly enriched in POC components.

Based on published classification schemes (Whittenbury & Dalton, 1981; Romanovskaya *et al.*, 1978) in which non-motile Type II methanotrophs that form lipid cyst type resting stages are considered to be in the genus '*Methylocystis*', strain POC appears to be a '*Methylocystis*' species. The formal naming of this strain awaits further taxonomic studies, however, as it has characteristics that distinguish it from other described '*Methylocystis*' species (Galchenko *et al.*, 1980): for example, it does not grow on methanol, it is pink-pigmented, and the cells are irregular rods.

The other partner of the co-culture appears to be a *Xanthobacter* species. *Xanthobacter* is a genus that includes nitrogen-fixing hydrogen autotrophs other than those that form symbioses with legumes (Wiegel *et al.*, 1978). All of the strains described to date are similar to strain H4-14 in that they are yellow-pigmented, heavily encapsulated irregular rods (DeBont & Leijten, 1976; Malik & Claus, 1979; Wiegel *et al.*, 1978). Some are capable of slow growth on short-chain alkanes (DeBont & Leijten, 1976), and all strains that have been tested can grow on methanol (DeBont & Leijten, 1976; Wiegel *et al.*, 1978). Strain H4-14 has some of the characteristics of one described strain, *X. flavus* (Malik & Claus, 1979), in that it is microaerophilic and requires biotin, but it exhibits different substrate utilization patterns, in that it does not grow on glucose, but it grows quite well on fructose. The formal naming of this strain will therefore also await further taxonomic studies.

The ability of strain H4-14 to oxidize methane at low rates may reflect the presence of a non-specific short-chain alkane hydroxylase. It has not yet been possible to obtain a sufficient quantity of either ethane- or methane-grown cells to investigate this possibility further.

The details of the interaction between the two methylotrophs during growth on methane are only partially understood. The *Xanthobacter* species clearly benefited from the association, since

it grew only very slightly on methane in pure culture. It would appear that the '*Methylocystis*' supplied biotin as well as a carbon and energy source to the *Xanthobacter*. Since a low level of ribulosebiphosphate carboxylase activity was detectable in the co-culture, it seems likely that methanol or another C₁ compound supported the growth of the *Xanthobacter*. In addition, since the growth rate of the co-culture was close to the maximum growth rate of the *Xanthobacter* in minimal medium, the *Methylocystis* may have provided organic substrates via excretion or cell lysis.

It is not apparent how the '*Methylocystis*' benefited from this association. The pure culture grew quite well on methane in minimal medium, and the growth rate of the co-culture on methane was actually less than that of the pure culture. The persistence of the *Xanthobacter* in the co-culture may well be due to its ability to scavenge low concentrations of organic compounds. Since liquid cultures were invariably transferred after stationary phase had been reached, it might be expected that once the '*Methylocystis*' stopped growing, cell lysis could result in increased growth of the *Xanthobacter*. Therefore, further transfers of liquid cultures would have resulted in maintenance of both strains. A similar argument could be applied to the mixing experiments in this study, since numbers were determined only after cultures reached stationary phase. Concerning inocula from non-methane plates that had been streaked from methane liquid cultures, or from methane plates, these would have always contained small amounts of the '*Methylocystis*' growing underneath the *Xanthobacter* colonies, and this type of inoculum would have been strongly enriched for the *Xanthobacter* due to the more rapid growth of this bacterium on these plates. It is possible, therefore, that the persistence of *Xanthobacter* in this co-culture may have been strictly fortuitous. However, the rapid achievement of steady-state proportions of the two organisms in the mixing experiments might suggest the relationship was more complex than it appeared. Perhaps the '*Methylocystis*' benefited from the co-culture, even though the growth rate was slowed. Evidence from batch culture experiments suggested the overall yield was slightly higher in the co-culture.

The '*Methylocystis*' strain contained three separate plasmids, all 50 Mdal or greater. At present, these plasmids are entirely cryptic. '*Methylocystis*' POC is sensitive to a variety of antibiotics, and therefore the plasmids do not appear to encode antibiotic resistance.

Our experience with this culture confirms other findings (Whittenbury & Dalton, 1981) that ordinary methods for determining culture purity are not sufficient in the case of methanotrophs, as well as other bacteria characterized by slow growth rates, limited substrate range, and poor growth on agar plates. It is important to note that, in certain cases, results suggesting the existence of plasmid-mediated characteristics can be explained by stable associations such as we have described here.

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REFERENCES

- BLACKMORE, M. A. & QUAYLE, J. R. (1970). Microbial growth on oxalate by a route not involving glyoxylate carboxylase. *Biochemical Journal* **118**, 53-59.
- DAHL, J. S., MEHTA, R. J. & HOARE, D. S. (1972). New obligate methylotroph. *Journal of Bacteriology* **109**, 916-921.
- DEBONT, J. A. M. & LEIJTEN, M. W. M. (1976). Nitrogen fixation by hydrogen-utilizing bacteria. *Archives of Microbiology* **107**, 235-240.
- ECCLESTON, M. & KELLY, D. P. (1972). Assimilation and toxicity of exogenous amino acids in the methane-oxidizing bacterium *Methylococcus capsulatus*. *Journal of General Microbiology* **71**, 541-554.
- ECCLESTON, M. & KELLY, D. P. (1973). Assimilation and toxicity of some exogenous C₁ compounds, alcohols, sugars and acetate in the methane-oxidizing bacterium, *Methylococcus capsulatus*. *Journal of General Microbiology* **75**, 211-221.
- EROSHIN, V. K., HARWOOD, J. H. & PIRT, S. J. (1968). Influence of amino acids, carboxylic acids and sugars on the growth of *Methylococcus capsulatus* on methane. *Journal of Applied Bacteriology* **31**, 560-567.
- GALCHENKO, V. F., NESTEROV, A. I., ANDREEV, L. V. & TROTSENKO, YU. A. (1980). New species of methanotrophic bacteria *Methylocystis*. Publication of the U.S.S.R. Academy of Sciences, Biological Research Centre, Pushchino, U.S.S.R., pp. 3-25.

- HARRISON, D. E. F. (1978). Mixed cultures in industrial fermentation processes. *Advances in Applied Microbiology* **24**, 129–164.
- LARGE, P. J. & QUAYLE, J. R. (1963). Microbial growth on C_1 compounds. 5. Enzyme activities in extracts of *Pseudomonas* AM1 grown on C_1 compounds or succinate. *Biochemical Journal* **87**, 386–396.
- LYNCH, M. J., WOPAT, A. E. & O'CONNOR, M. L. (1980). Characterization of two new facultative methanotrophs. *Applied and Environmental Microbiology* **40**, 400–407.
- MALASHENKO, Y. R., ROMANOVSKAYA, V. A., BOGACHENKO, V. N. & KRYSHYAV, T. P. (1974). Influence of organic substances on the assimilation of methane by obligate methanotrophs. *Mikrobiologiya* **43**, 290–294.
- MALIK, K. A. & CLAUS, D. (1979). *Xanthobacter flavus*, a new species of nitrogen-fixing hydrogen bacteria. *International Journal of Systematic Bacteriology* **29**, 283–287.
- O'CONNOR, M. L. (1981). Regulation and genetics in facultative methylotrophic bacteria. In *Microbial Growth on C_1 Compounds*, pp. 294–300. Edited by H. Dalton. London: Heyden.
- O'CONNOR, M. L. & HANSON, R. S. (1977). Enzyme regulation in *Methylobacterium organophilum*. *Journal of General Microbiology* **101**, 327–332.
- PORTNOY, D. A., MOSELEY, S. L. & FALKOW, S. (1981). Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. *Infection and Immunity* **31**, 775–782.
- ROMANOVSKAYA, V. A., MALASHENKO, Y. R. & BOGACHENKO, V. N. (1978). Corrected diagnosis of the genera and species of methane oxidizing bacteria. *Mikrobiologiya* **47**, 120–130.
- TABITA, F. R., CARUSO, P. & WHITMAN, W. (1978). Facile assay of enzymes unique to the Calvin cycle in intact cells with special reference to ribulose-1,5-bisphosphate carboxylase. *Analytical Biochemistry* **84**, 462–472.
- WHITTENBURY, R. & DALTON, H. (1981). The methylotrophic bacteria. In *The Prokaryotes*, pp. 894–902. Edited by M. P. Starr, H. Stolp, H. G. Truper, A. Balows & H. G. Schlegel. Heidelberg: Springer Verlag.
- WIEGEL, J., WILKE, D., BAUMGARTEN, J., OPITZ, R. & SCHLEGEL, H. G. (1978). Transfer of the nitrogen-fixing hydrogen bacteria *Corynebacterium autotrophicum* Baumgarten *et al.* to *Xanthobacter* gen. nov. *International Journal of Systematic Bacteriology* **28**, 573–581.
- WILKINSON, T. G. & HARRISON, D. E. F. (1973). The affinity for methane and methanol of mixed cultures grown on methane in continuous culture. *Journal of Applied Bacteriology* **36**, 309–313.