Antonie van Leeuwenhoek 54: 189–199 (1988)

Isolation and characterization of marine methanotrophs

MARY E. LIDSTROM
Keck Laboratories 138-78, California Institute of Technology, Pasadena, CA 91125, USA

Received 3 December 1987; accepted 21 January 1988

Key words: marine methanotroph, Type I methanotroph

Abstract. Four new methane-oxidizing bacteria have been isolated from marine samples taken at the Hyperion sewage outfall, near Los Angeles, CA. These bacteria require NaCl for growth. All exhibit characteristics typical of Type I methanotrophs, except they contain enzyme activities of both the ribulose monophosphate pathway and the serine cycle. All four strains are characterized by rapid growth in liquid culture and on agar plates, and all have temperature optima above 35° C. One strain, chosen for further study, has been shown to maintain broadhost range cloning vectors and is currently being used for genetic studies.

Introduction

Methane-oxidizing bacteria (methanotrophs) are widespread in natural habitats and apparently play an important role in carbon cycling (Hanson 1980; Rudd & Taylor 1980). In addition, they are capable of oxidizing a variety of hydrocarbons, which makes them of interest for biocatalysis applications and detoxification processes (Higgins et al. 1980). However, in order to understand and manipulate their capabilities it is necessary to develop genetic techniques for methanotrophs and carry out studies of gene organization and expression. Genetic studies are difficult in methanotrophs partly due to their slow growth in liquid culture and on agar plates. Therefore, we have sought strains with better growth characteristics.

The Hyperion sewage outfall near Los Angeles, California is an attractive environment for the development of fast-growing methanotrophs. The high organic input supports a vigorous anaerobic community that produces large volumes of methane (Felbeck, pers. comm.). This substrate diffuses through the anaerobic sediment and mixes with oxygenated seawater, providing a constant supply of methane and oxygen at the sediment-water interface. We report here the isolation from this environment of four strains of marine methanotrophs that appear amenable to genetic analysis. These strains are somewhat unusual in that they have characteristics of both Type I and Type II methanotrophs.

Materials and methods

Media and cultural conditions

All enrichments and isolates were grown in the nitrate mineral salts (NMS) medium described by Whittenbury & Dalton (1981) with NaCl additions as noted in the text. Routinely, the NaCl concentration used was either 1.5% (w/v) (NMS-high salt), 0.5% (w/v) (NMS-med salt) or 0.15% (w/v) (NMS-low salt). For enrichments and subsequent culture a vitamin mixture was added containing (per l, final concentration) biotin, 20 μg; folic acid, 20 μg; thiamine-HCl, 50 μg; calcium pantothenate, 50 μg; B_{12}, 1 μg; riboflavin, 50 μg; nicotinamide, 50 μg. Methanol and ethanol were filter-sterilized and added at a final concentration of 0.2% (v/v). All other substrates were added as sterile stocks to a final concentration of 0.1% (w/v). Nutrient agar and nutrient broth were purchased from Difco, and Luria broth and Luria agar were as described (Miller 1972). Cultures grown on methane were incubated under a mixture of methane : air (1 : 1). Unless otherwise noted, cultures were routinely grown at 37° C.

Enrichment and isolation

Samples of Hyperion outfall sediment were obtained from three different sites by H. Felbeck, Scripps Institution of Oceanography. These samples were kept on ice and were received within five days of sampling. One-gram subsamples from each site were incubated in 20 ml NMS-hi salt medium under a 1 : 1 atmosphere of methane and air at 16° C or 23° C with shaking. Two enrichments (A and C) showed noticeable turbidity after 5–7 days of incubation at both temperatures, although the 16° C samples showed slower growth. Dilutions of these enrichments were plated onto NMS-high salt and -low salt plates containing agarose (SeaKem, Research Organics Inc., Cleveland OH, USA) as the gelling agent and incubated under methane and air at the same temperature as the original enrichment. Colonies were picked and restreaked to duplicate plates, which were incubated under methane and air or nitrogen and air. Colonies that grew only on the plates in the presence of methane were used for further study. These were streaked to the appropriate NMS-salt plates containing Bacto-agar (Difco Laboratories, Detroit MI, USA) as the gelling agent, then to NMS-salt plates containing agarose, and then were inoculated into liquid medium and replated. At each transfer, colonies were picked that showed no ring of secondary growth, as determined using a stereoscopic microscope. Putative pure colonies were tested on nutrient agar plates containing the same NaCl concentration as that used for isolation. This procedure was successful in the isolation of four pure cultures of methanotrophs.

Electron microscopy
Cultures were prepared as described (Johnson & Sieburth 1982) and examined using a Zeiss EM9S-2 transmission electron microscope. We thank Paul Johnson and John Sieburth for preparing the thin sections.

Preparation of crude extracts
Mid-log phase cells (OD_{600} = 0.5–0.7) were harvested by centrifugation at 10,000 × g for 10 min. The cells were washed once with 20 mM phosphate buffer containing 1.5% NaCl (pH 7.5) and the pellets were stored at −20° C for 1–10 days. Crude extracts were prepared by resuspending the frozen cell pellets in ice-cold 20 mM phosphate buffer, pH 7.5 (4 ml buffer per g wet wt.) and vortexing 4 times for 30 sec each at high speed. Marked lysis occurred under these conditions. The preparation was centrifuged at 15,000 × g for 15 min, and the supernatant was used for enzyme assays.

Enzyme assays
The following enzymes were assayed by published methods: hydroxypyruvate reductase, (Large & Quayle 1963); serine-glyoxylate aminotransferase (Blackmore & Quayle 1970); hexulose phosphate synthase (Dahl et al. 1972); serine transhydroxymethylase (glyoxylate-activating), (O'Connor & Hanson 1975); ribulosebisphosphate carboxylase (Tabita et al. 1978); alpha-ketoglutarate dehydrogenase (Zhao & Hanson 1984). Ribulose phosphate-dependent formaldehyde disappearance was measured by incubating extract with 0.25 mM formaldehyde, 100 mM $NaPO_4$ buffer pH 7.0 and 5 mM $MgCl_2$ in the presence or absence of 1 mM ribulose 5–phosphate. 0.2 ml subsamples were taken at 10 min intervals and the reaction was stopped by addition to 1 ml 5% trichloroacetic acid (TCA). These were assayed for formaldehyde using the Nash reagent and measuring the absorbance at 412 nm (Nash 1953). Controls were carried out with no cells and with cells incubated with TCA at zero time.

^{14}C-acetate conversion to $^{14}CO_2$
Acetate oxidation was measured by incubating duplicate samples of 5 ml whole cells (mid-log phase cultures) with 1 uCi U-^{14}C-acetic acid (sodium salt), 5 mCi/mmol (Amersham, Inc. Arlington Heights IL, USA) for 4 h in the presence of methane : air (1 : 1) at 37° C in serum vials capped with grey butyl stoppers and aluminum crimp seals. After four hours, the stoppers were replaced with stoppers containing filter paper rings soaked in phenethylamine and recrimped. 0.1 ml 10 N HCl was added to acidify the liquid, and the vials were shaken gently for 4 h to allow CO_2 absorption. Filters were counted in a Beckmen liquid scintillation counter using Aquasol II (New England Nuclear, Boston MA, USA) as a fluor. Controls were carried out using no cells and using autoclaved cells.

DNA isolations and %G + C determinations
DNA was isolated by the method of Marmur (1961), scaled down to 1.5 ml volume. The %G + C ratio was determined from thermal melting curves as described (Johnson 1981), using *E. coli* HB101 as a standard.

Antibiotic sensitivities and plating frequencies
Sensitivity to antibiotics was determined by spreading cells onto an agar plate containing NMS + 0.5% NaCl (NMS-med salt plates) and placing a filter disc containing 100 μg of antibiotic in the center. Growth under methane was assessed after 1, 3, 5, and 7 days. Efficiency of plating was determined by comparing direct counts made using a Petroff-Hauser counting chamber to plate counts obtained by plating dilutions onto NMS-med salt plates.

Conjugative transfer of broad host range plasmids
Three-way filter matings were carried out as described previously (Toukdarian & Lidstrom 1984) using ratios of mobilizer : donor : recipient of 1 : 1 : 5 and incubating on NMS agar + 0.5% NaCl + 20% (v/v) Luria broth. The mobilizer plasmid (pRK2013, Figurski & Helinski 1979) was carried in *E. coli* strain CSR603 (Sancar & Rupert 1978) and the broad host range cosmid vector pVK100 (Knauf & Nester 1982) was carried in *E. coli* strain HB101 (Maniatis et al. 1982).

Results

Isolation of four marine methanotrophs
Enrichments were carried out in NMS medium containing NaCl as described in "Methods" using sediment samples from three different sites at the Hyperion sewage outfall. Two of the enrichments (A and C) developed turbidity, and two pure cultures were isolated from each enrichment, which were termed isolates A1, A4, C1 and C2, respectively. These cultures appeared to be pure by the following criteria:
- No secondary growth rings were observed around the colonies after 4 weeks incubation, on either agarose or Bacto-agar plates.
- No growth was observed on either nutrient agar + NaCl or Luria Broth plates.
- Microscopic analysis revealed only one morphological type from both plate and liquid cultures.

These cultures were used for further studies.

Characterization
Table 1 summarizes the characteristics of these strains. All were gram negative

motile rods and showed typical Type I (Whittenbury & Dalton 1981) internal membranes (Fig. 1). All grew well on methane, with doubling times on the order of 3–4 h in liquid culture and none were capable of growth on any substrates tested that contained C-C bonds (see Table 1). None of the strains were capable of growth on formate, methylamine, or methanol, although isolate A4 could be permanently trained to grow on methanol by successive transfer to medium containing increasing methanol concentrations and decreasing methane concentrations (A. DiSpirito, unpubl. data). All strains showed marked lysis on plates and in liquid culture after 2–3 weeks, with viability decreasing to undetectable levels after 4 weeks. Strain A1 required nicotinic acid, while the other strains had no growth factor requirements.

All strains were unable to grow in the absence of added NaCl. Strains A1, A4 and C2 had broad NaCl optima, while strain C1 had a narrow optimum peaking at 0.15% (w/v) (Fig. 2*a*). All strains showed a growth temperature optimum of 37–40° C (Fig. 2*b*). All strains initially produced what appeared to be cyst-type resting stages typical of other Type I methanotrophs (Whittenbury & Dalton 1981), but the ability to produce these was lost with subsequent culture in strains A1 and A4. Cyst-forming ability appeared to be stable in strain C1. Cultures of strain C2 (brown-pigmented) showed white colonies on plates at a frequency of a few percent of the total. These were purified and were found to have identical characteristics to the brown-pigmented strain, except that cysts were no longer produced. All strains had a G + C ratio of 54–56%.

Table 1. Characteristics of four marine methanotrophs.

	A1	A4	C1	C2
Morphology	Gr⁻ motile rod	Gr⁻ motile rod	Gr⁻ motile rod	Gr⁻ motile rod
Colony	mucoid, creamy	creamy	brown	brown
Membranes	Type I	Type I	Type I	Type I
Maximum growth rate (h/generation)	3	3.5	4	3.5
(Conditions)	37C, 0.6% NaCl	37C, 0.6% NaCl	40C, 0.15% NaCl	37C, 0.5% NaCl
Alternate substrates[1]	none	methanol[2]	none	none
Growth factors	nicotinic acid	none	none	none
Resting stage	cyst	cyst	cyst	cyst
NaCl optimum	broad; 0.5–1.5%	broad; 0.5–1.5%	narrow; 0.15–0.2%	broad; 0.05–0.5%
Temperature optimum (° C)	37	37	40	37
Assimilation pathway[3]	RuMP; serine	RuMP; serine	RuMP; serine	RuMP; serine

[1] None of the strains grew on fructose, glucose, maltose, glycerol, ethanol, succinate, aspartate, asparagine, glutamate, glutamine, serine, casamino acids, nutrient broth + 0.5% NaCl or Luria broth. [2] Grew on methanol only after adaptation. [3] RuMP = ribulose monophosphate.

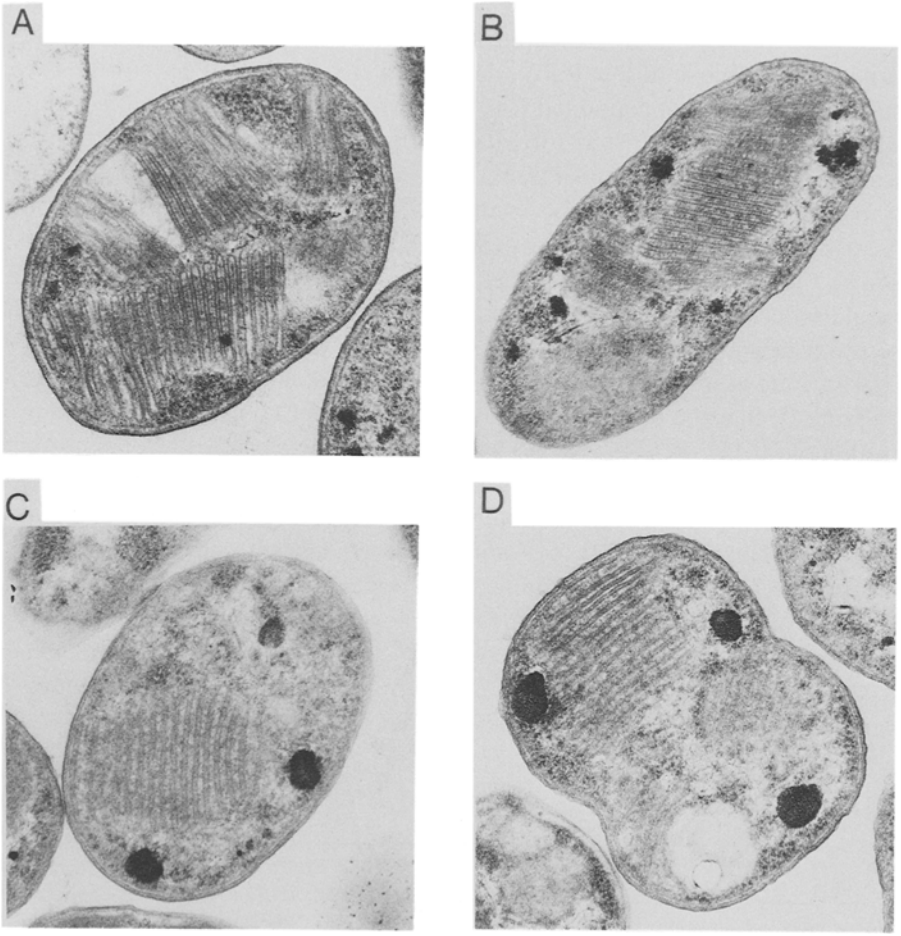

Fig. 1. Ultrastructure of the four isolates described in the text. (*A*) strain A1, (*B*) strain A4, (*C*) strain C1, (*D*) strain C2. The bars represent 0.5 μm in each case.

All strains contained enzyme activities typical of both the ribulose monophosphate and serine pathways for formaldehyde assimilation (Table 2) but no activity was detectable for ribulose bisphosphate carboxylase. The apparent presence of the serine pathway suggested that these organisms might contain a complete TCA cycle (Anthony 1982). Alpha-ketoglutarate activities were low (1–4 nmol/min/mg protein) and not reproducible. ^{14}C-acetate was oxidized to $^{14}CO_2$ in all four strains, suggesting that these methanotrophs may contain a functional TCA cycle. The ^{14}C-acetate oxidation rates were similar to the measured alpha-ketoglutarate dehydrogenase activities, but were well above background rates measured in controls.

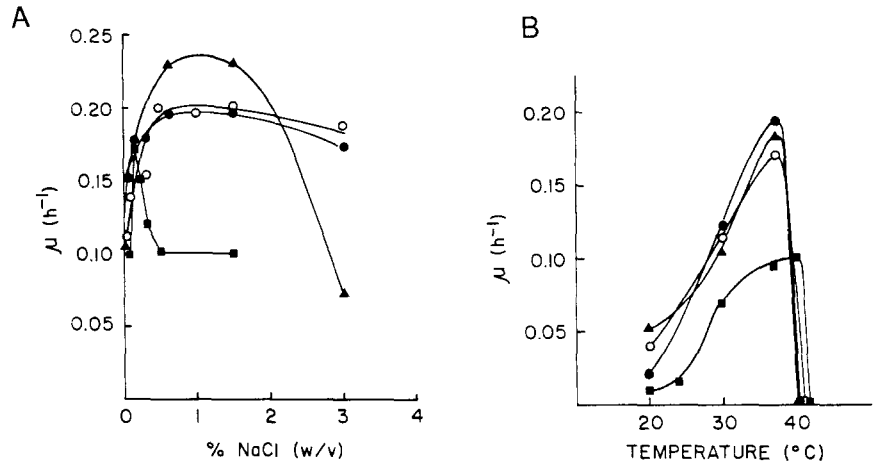

Fig. 2. Salt and temperature profiles for the four isolates. (*A*) salt profile, (*B*) temperature profile. (●) Strain A1, (▲) Strain A4, (■) Strain C1, (○) Strain C2.

All attempts to grow cultures in the absence of an added inorganic nitrogen source failed, even at 10 μM dissolved oxygen concentration. In addition, no hybridization could be detected to Southern blots of genomic digests of DNA from each of the isolates using the *Klebsiella nif* probe pSA30, when carried out as described earlier for *Methylosinus* 6 (Toukdarian & Lidstrom 1984). These data suggest that these cultures were incapable of utilizing dinitrogen as a nitrogen source.

Table 2. Levels of C-1 assimilation enzyme activity in the four marine methanotrophs.

Enzyme	Enzyme activity (nmol/min/mg protein)			
	A1	A4	C1	C2
Ribulose monophosphate pathway				
Hexulose phosphate synthase/ Phosphohexulose isomerase	80	75	33	13
Serine pathway				
Hydroxypyruvate reductase	1360	410	170	120
Serine glyoxylate aminotransferase	135	170	30	32
Serine transhydroxymethylase (glyoxylate-activated)	35	30	22	25
Calvin cycle				
Ribulose bisphosphate carboxylase	ND	ND	ND	ND
TCA cycle				
Alpha-keto glutarate dehydrogenase	+/−	+/−	+/−	+/−

ND = not detectable; +/− = low and variable.

Analysis of genetic capabilities of isolate A4

The rapid growth of these strains suggested they might be amenable to genetic analysis. Isolate A4 showed particulary rapid growth on agar plates, producing colonies in 3 days. Therefore, this strain was chosen for further study. Table 3 summarizes the relevant properties of this isolate. Isolate A4 was sensitive to a variety of antibiotics at levels commonly used for genetic selection. For these experiments the NaCl concentration in the medium was decreased to 0.5%. A strain (A4R) was isolated that was resistant to 20 μg/ml rifamycin, and this strain was used to test potential broad host range cloning vectors. The IncP broad host range vector pVK100 (Knauf & Nester 1982) was transferable to isolate A4 by three-way matings using the mobilizing plasmid pRK2013 (Figurski & Helinski 1979) at frequencies of approximately 10^{-2} per recipient.

Many strains of methanotrophs exhibit poor plating efficiencies, which makes genetic studies difficult. Isolate A4 plated to NMS-med salt plates at a frequency of 0.95–0.98 as judged by direct microscopic counts, with either methane or methanol as the substrate.

Discussion

Very few marine methanotrophs have been described. Both Type I and Type II isolates were reported by Heyer et al. (1984), but no characteristics were given. An isolate obtained from Sargasso Sea samples has been recently characterized (Sieburth et al. 1987) and it appears to be a typical Type I *Methylomonas* strain that requires salt for growth. The marine methanotrophs described here are similar to the Sargasso Sea strains in that they contain Type I membranes, have a G + C ratio below 60% and require salt for growth, but they differ in that they contain enzyme activities of both the serine and ribulose monophosphate pathways and they may contain a complete TCA cycle. These

Table 3. Characteristics of isolate A4 relating to the development of genetic systems.

Antibiotic and drug sensitivity[1]	Tetracycline, kanamycin, streptomycin, rifamycin, naladixic acid, spectinomycin, sulfanilamide, chloramphenicol
Antibiotic resistance[1]	trimethoprim, ampicillin
Frequency of mobilization[2] (pVK100)	$2–5 \times 10^{-2}$
Efficiency of plating[3]	95–98%

[1] Sensitive: no growth on a plate containing a disc saturated with 100 μg test substance. Resistant: growth on a plate as above.

[2] By a three-way filter mating using pRK2013 as the mobilizer.

[3] Onto NMS + 0.5% NaCl plates with either methane or methanol as substrate.

characteristics are not unprecedented in freshwater strains. A Type I methanotroph has recently been described that contains both the serine and ribulose monophosphate pathways (Imai et al. 1986) and another Type I strain has been described that contains a complete TCA cycle (Zhao & Hanson 1984). The "Type X" methanotroph (Whittenbury & Dalton 1981), *Methylococcus capsulatus* Bath exhibits Type I membranes and appears to contain both serine and ribulose monophosphate pathways and a complete TCA cycle, but it contains nitrogenase and has a G + C ratio of 65%, characteristics of Type II methanotrophs. It appears that the group of methanotrophic bacteria containing Type I membranes may be more diverse than is commonly thought. This is especially interesting in light of the recently described symbiosis between deep sea and shallow water mussels and what appear to be Type I methanotrophs (Childress et al. 1986; Cavanaugh et al. 1987).

The unusual characteristic of the strains described here that makes them of particular interest is their relatively rapid growth in both liquid and agar culture. Genetic studies in the methanotrophs are difficult and time-consuming due to poor colony development (Toukdarian & Lidstrom 1984). Strain A4 is attractive as a host for genetic studies since it produces colonies on agar plates within 3 days. In addition, mobilization of the broad host range cosmid vector pVK100 occurs at high frequencies, and this strain is sensitive to a variety of antibiotics commonly used for vector selection. It also grows well on methanol in both liquid and agar culture and shows high plating efficiencies with both methane and methanol as substrates. The main disadvantage of this strain is its tendency to lyse in older cultures, which necessitates careful attention to storage and handling of the cultures.

We have now constructed a clone bank of *Methylomonas* A4 DNA in a broad host range vector and have used it to isolate genes involved in methanol oxidation (DiSpirito & Lidstrom 1987). These preliminary studies suggest that this organism will provide a useful system in which to carry out genetic analysis of methanotrophic functions.

Acknowledgement

This work was supported by grant # N00014-85-K-0444-P00001 from the Office of Naval Research.

References

Anthony, C. (1982) Biochemistry of Methylotrophs. Academic Press, London

Blackmore, M.A. & J.R. Quayle (1970) Microbial growth on oxalate by a route not involving glyoxylate carboligase. Biochem. J. 118: 53–59

Cavanaugh, C.M., P.R. Levering, J.S. Maki, R. Mitchell & M. Lidstrom (1987) Symbiosis of methylotrophic bacteria and deep-sea mussels. Nature 325: 346–348

Childress, J.J., C.R. Fisher, J.M. Brooks, M.C. Kennicutt, R. Bidigare & A.E. Anderson (1986) A methanotrophic marine molluscan (Bivalvia, Mytilidae) symbiosis: mussels fueled by gas. Science 233: 1306–1308

Dahl, J.S., R.J. Mehta & D.S. Hoare (1972) New obligate methylotroph. J. Bacteriol. 109: 916–921

DiSpirito, A.A. & M.E. Lidstrom (1987) Identification of methanol oxidation (Mox) genes from the marine methanotroph *Methylomonas* sp. A4. Abstr. Ann. Meeting Am. Soc. Microbiol. I115

Figurski, D. & D.R. Helinski (1979) Replication of an origin-containing derivative of the plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. U.S.A. 76: 1648–1652

Hanson, R.S. (1980) Ecology and diversity of methylotrophic bacteria. Adv. Appl. Microbiol. 26: 3–39

Heyer, J., Yu Malashenko, U. Berger & E. Budkova (1984) Verbreitung methanotropher Bakterien (in German). Zeitschr. fur allg. Mikrobiol. 24: 725–744

Higgins, I.J. D.J. Best & R.C. Hammond (1980) New findings in methane-utilizing bacteria highlight their importance in the biosphere and their commercial potential. Nature 286: 561–564

Imai, T., H. Takigamwa, S. Nakagawa, G-J. Shen, T. Kodama & Y. Minoda (1986) Microbial oxidation of hydrocarbons and related compounds by whole-cell suspensions of the methane-oxidizing bacterium H-2. Appl. Environ. Microbiol. 52: 1403–1406

Johnson, J.L. (1981) Genetic characterizations. In: P. Gerhardt (Ed) Manual of Methods of General Bacteriology. (pp. 450–472). Am Soc. Microbiol. Washington

Johnson, P.W. & J. McN. Sieburth (1982) In-situ morphology and occurrence of eukaryotic phototrophs of bacterial size in the picoplankton of estuarine and oceanic waters. J. Phycol. 18: 318–327

Knauf, V.C. & E.W. Nester (1982) Wide host range cloning vectors: cosmid clone bank of *Agrobacterium* Ti plasmids. Plasmid 8: 45–54

Large, P.J. & J.R. Quayle (1963) Microbial growth on C1 compounds. 5. Enzyme activities in extracts of *Pseudomonas* AM1. Biochem. J. 87: 386–395

Maniatis, T., E.F. Fritsch & J. Sambrook (1982) Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Marmur, J. (1961) A procedure for the isolation of desoxyribonucleic acid from microorganisms. J. Molec. Biol. 3: 208–218

Miller, J. (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Nash, T. (1953) The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochem. J. 55: 416–421

O'Connor, M.L. & R.S. Hanson (1975) Serine transhydroxymethylase isoenzymes from a facultative methylotroph. J. Bacteriol. 124: 985–996

Rudd, J.W.M. & D.D. Taylor (1980) Methane cycling in aquatic environments. Adv. Aquat. Microbiol. 1: 77–150

Sancar, A. & C.S. Rupert (1978) Determination of plasmid molecular weights from ultraviolet sensitivities. Nature (London) 272: 471–472

Sieburth, J.McN., P.W. Johnson, M.A. Eberhardt, M.E. Sieracki M. Lidstrom & D. Laux (1987) The first methane-oxidizing bacterium from the upper mixing layer of the deep ocean: *Methylomonas pelagica* sp. nov. Curr. Microbiol. 14: 285–293

Tabita, F.R., P. Caruso & W. Whitman (1978) Facile assay of enzymes unique to the Calvin Cycle in intact cells, with special reference to ribulose bis- phosphate carboxylase. Anal. Biochem. 84: 462–472

Toukdarian, A.E. & M.E. Lidstrom (1984) Molecular construction and characterization of *nif* mutants of the obligate methylotroph *Methylosinus* sp. strain 6. J. Bacteriol. 157: 979–983

Whittenbury, R. & H. Dalton (1981) The methylotrophic bacteria. In: M.P. Starr, H. Stolp, H.G. Truper, A. Balows & H.G. Schlegel (Eds) The Prokaryotes (pp. 894–902). Springer, Heidelberg

Zhao, S.-J. & R.S. Hanson (1984) Isolate 761-M: a new Type I methanotroph that possesses a complete tricarboxylic acid cycle. Appl. Environ. Microbiol. 48: 1237–1242