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C_1 bacteria in the water column of Chesapeake Bay, USA. III. Immunologic relationships of the type species of marine monomethylamine- and methane-oxidizing bacteria to wild estuarine and oceanic cultures

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ABSTRACT: C_1 bacteria oxidizing methane and monomethylamine (MMA) are readily enriched from seawater and associated marine materials, but only *Methylomonas pelagica* oxidizing methane and *Methylophaga marina* oxidizing MMA have been adequately characterized. Antisera with good specificity were prepared against these taxonomically and trophically different oceanic methylotrophs as well as against methanol dehydrogenase, a key enzyme presumably present in all aerobic methylotrophs. These antisera were used in an indirect immunofluorescence procedure to compare the relationship of these type oceanic species to wild-type cultures from the estuarine waters of Chesapeake Bay (USA) and the open sea. Of the 32 methanotrophic enrichments attempted from the Sargasso Sea, 23 (72%) yielded methane-oxidizers. Of the 27 isolates obtained in pure culture, 25 (96%) were identical to *Methlyomonas pelagica*. By contrast, none of the 54 estuarine methane-oxidizers from Chesapeake Bay were identical to *M. pelagica*, 13% were related and 87% were unrelated. The anti-MDH (methanol dehydrogenase) serum reacted to 23% of the wild methanotroph cultures. Of the 18 oceanic methylaminotroph enrichments, 44% were indistinguishable from *Methylophaga marina*, 6% were related, and 50% were unrelated. In contrast, of the 41 Chesapeake Bay MMA-oxidizers, 12% were indistinguishable from *M. marina*, 17% were related, while 71% were unrelated. The anti-MDH antiserum reacted with only 15% of the wild MMA-oxidizing bacteria. The implications of the taxonomic affinities and trophic requirements of the methylotrophs to their estuarine and oceanic distribution and to their anaerobic methanogenic bacterial consorts are discussed.

INTRODUCTION

The aerobic obligate methylotrophs belong to distinct C_1 bacterial taxa that have a unique C_1 metabolism, while the facultative methylotrophs that can also utilize C-C bonded organic matter belong to diverse and unrelated bacterial taxa (Anthony 1982, Large 1983, Green 1992). The methane-oxidizers grow on methane and methanol and have distinctive cytomembranes in thin section (Whittenbury & Dalton 1981, Green 1992). Only 1 oceanic species, *Methylomonas pelagica* (Sieburth et al. 1987), has been recognized (Green 1992). The monomethylamine (MMA)-oxidizers grow on methanol, apparently formaldehyde, and usually dimethylamine and trimethylamine in addition to MMA. They lack the distinctive cytomembranes of the methane- oxidizers and in thin section are indistinguishable from C–C utilizing heterotrophs. Two very similar marine species that are serologically indistinguishable, *Methylophaga marina* and *Methylophaga thallassica* (Janvier et al. 1985), have been recognized (Green 1992). The MMA-oxidizers are ubiquitous, algal associated, and apparently concentrate with the nanoalgae in the pycnocline (Sieburth & Keller 1988/89). The role of these aerobes in creating reduced microniches for their methyl-

otrophic methanogens has been reported in a preliminary way (Sieburth 1987, 1988a) and has been more fully described for Chesapeake Bay, USA, enrichments by Sieburth (1993). Studies on the occurrence of methylotrophs in oceanic waters have been conducted in this laboratory over the past decade. They have resulted in the enrichment and attempted maintenance of hundreds of methane- and MMA-oxidizing cultures. Characterizing the bacteria obtained in these enrichment cultures includes isolation, examination of ultrastructure using electron microscopy, determination of DNA base pair composition and the identification of enzymatic pathways (Janvier et al. 1985, Sieburth et al. 1987, Green 1992). These procedures are tedious and prohibitive when large numbers of cultures are involved. When such large numbers of isolates have been obtained, the tendency has been to leave them minimally characterized and unnamed (Whittenbury et al. 1970). A method which can discriminate between similar and dissimilar species of methylotrophs with a high degree of specificity was required in order to describe the estuarine and oceanic distribution of the type marine species, and to detect unrelated cultures requiring further characterization.

The indirect immunofluorescence procedure (Kawamura 1977), is a proven technique which has been used to investigate the distribution of diverse trophic groups of bacteria (Reed & Dugan 1978, Ward & Perry 1980, Campbell et al. 1983, Xu et al. 1984). Most of these studies, however, have concentrated on enumeration and distribution of these bacteria in natural populations. In order to develop a panel of antibodies that would greatly reduce the effort involved in categorizing our aerobic marine methylotrophic bacteria, 3 different antisera were prepared. One antiserum was to *Methylomonas pelagica,* the first methane-oxidizing bacterium isolated and characterized from the open Sargasso Sea (Sieburth et al. 1987). A second antiserum was also to an open Sargasso Sea culture of ours, B3P, that is indistinguishable from *Methylophaga marina* (Janvier et al. 1985), at least serologically. A third antiserum was against methanol dehydrogenase (MDH), the key enzyme reported to be present in all methylotrophic bacteria (Anthony 1982, Large 1983). A number of different pure cultures of C_1 bacteria that included methane-oxidizers and MMA-oxidizers, as well as C-C utilizing heterotrophs, were first used to test the specificity of the antisera. The main body of this study was a comparison of methane-oxidizing and MMA-oxidizing enrichment cultures from Chesapeake Bay with oceanic forms, and is based on the thesis of Church (1987).

This is a first serological look at both trophic forms of aerobic marine methylotrophs. The MMA-oxidizers appear to be ubiquitous due to the ubiquitous nature of the nanaoalgae whose osmoprotectant glycine betaine hydrolyzes to release the methylated amines. The detection of methane-oxidizers appears to be sporadic since stratification is necessary for pycnocline-unique methanogens (Sieburth 1993, Sieburth et al. 1993) to grow, and accumulate to populations that would produce enough methane to support cultivable populations of methane-oxidizers. Only 30 % of the estuarine enrichments were related to the test oceanic species, indicating that estuarine C_1 bacteria may be quite different and deserve further study. This is in contrast to the oceanic cultures of which 96 % of the methanotrophs were related to *Methylomonas pelagica,* while 44 % of the methylotrophs were related to *Methylophaga marina.*

MATERIALS AND METHODS

Bacterial strains. The species of methylotrophs tested and their culture collection sources and numbers are listed in Table 1. The methanotrophs were cultured in an atmosphere of 50:50 methane in air on NMS (nitrate mineral salts) medium (Whittenbury et al. 1970), except for *Methylomonas pelagica* which was grown on NEM-10 (nitrifying enrichment medium) agarose (Sieburth et al. 1987). The MMA-oxidizers were cultured on 0.1 % methanol with the nutrient supplement NEM-1 (Sieburth et al. 1987).

Enrichment and isolation procedures. The enrichment procedure for the methane-oxidizing bacteria has been described previously (Sieburth et al. 1987). MMA-oxidizing enrichments were supplemented with 0.1 % (w/v) MMA (Sigma Chemical Co., St. Louis, MO, USA), and 2nd spike, a nutrient supplement identical to NEM-1 but lacking ammonia, then tightly capped. Pure cultures of methane-oxidizers were obtained by repeated streaking onto NEM-10 plates gelled with 1.2 % agarose (Type I, Sigma Chemical Co.) and incubation in an atmosphere of 50:50 methane in air, until only 1 morphological type of colony was observed. MMA-oxidizers were isolated on 0.1 % MMA/2nd spike agarose plates incubated in air. Pure cultures of heterotrophs utilizing C-C bonded organic matter were isolated from methane- and MMA-oxidizing liquid enrichment cultures on Oppenheimer-Zobell agar plates with reduced peptone content (the OZR agar of Sieburth 1967). All cultures were incubated at room temperature.

Sources of enrichments. Xenic algal cultures used as an inoculum during the fall of 1984 were from the sources noted. The CB cultures were obtained from samples taken in upper Chesapeake Bay in May 1986, and along the length of Chesapeake Bay in May 1987, as described in Sieburth (1993). Stratified tank No. 9 at the Marine Ecosystems Research Laboratory (MERL,

Table 1. Observed reactions of type cultures of methane- and monomethylamine (MMA)-oxidizers to the 3 test antisera. *M.p. Methylomonas pelagica*; MDH: methanol dehydrogenase; B3P: an open Sargasso Sea culture which is indistinguishable from *Methylophaga marina*

Species	Immunofluorescence reaction			
	Anti-*M.p.*	Anti-MDH	Anti-B3P	Normal
Methanotrophs[a]				
Methylococcus capsulatus Strain Bath, IB #11132	–	+	–	–
Methylocystus parvus Strain OBBP, IB #11129	–	–	–	–
Methylomonas albus Strain BG8, IB #11123	–	+	–	–
Methylomonas agile Strain AZO, IB #11124	–	–	–	–
Methylomonas methanica Strain S1, IB #11130	+	++	–	–
Methylomonas pelagica Strain AA-23, MB #2265	+++	+	–	–
Methylosinus sporium Strain 5, IB #11126	–	–	–	–
Methylaminotrophs[b]				
Methylophaga marina ATTC #35842	–	++	+++	–
Methylophaga thalassica ATTC #33146	–	–	+++	–

[a] Cultures from the National Collection of Industrial and Marine Bacteria, Torey Research Station, Aberdeen, Scotland AB9 8DG, UK
[b] Cultures from the American Type Culture Collection, Rockville, Maryland 20852, USA

Univ. of Rhode Island, Narragansett, RI, USA) was the source of isolates enriched from copepod faecal pellets collected in sediment traps in November 1984.

The oceanic enrichments used in this study were inoculated at sea (by colleagues shortly before the end of their cruise) by adding 100 ml portions of freshly collected seawater to prelabeled sterile polycarbonate flasks containing the appropriate nutrient supplements. The flasks were sealed air tight, and upon debarkation were air-expressed to our laboratory. The nature and sources of these and other samples are described below in the order of their appearance in the tables. The water samples from the Sargasso Sea (AA and #T# cultures) were obtained as previously described (Sieburth et al. 1987). The sanitary holding tank inoculum from the RV 'Columbus Iselin' was obtained in the fall of 1985. The RV 'Endeavor' inocula (En133) obtained in August 1985 were diver-collected gelatinous zooplankton from the North Atlantic. The Ber cultures were inoculated from a vertical cast offshore from Bermuda in August 1984. The SL cultures were inoculated on 15 October 1983 from water samples obtained with the submersible Sea-Link in transects through the pycnocline of the Sargasso Sea (24° 43' N, 75° 37' W). The CL cultures were from water obtained with vertical profiles during the VERTEX 5 sedimentation experiments in the northeast Pacific in June 1984 (Martin et al. 1987). In the distribution data shown later in Fig. 3, Stn 1 was at pit site A furthest offshore (33° 06' N, 139° 34' W), Stn 3 was at pit site B (35° 03' N, 128° 05' W), and Stn 5 was at pit site C closest to shore (35° 50' N, 122° 31' W). Stns 2 and 3 were at intermediate distances between Stns 1, 3 and 5. Wish and Al materials were obtained and inoculated in the Santa Catalina Channel, California, USA (33° 19.75' N, 118° 37.32' W) from the mother ship and the submersible Alvin, respectively, on 5 December 1984. The Skan Bay, Alaska, USA, cultures were inoculated on 27 September 1984 and arrived from Dutch Harbor on 3 October. All enrichments were transferred at monthly or bimonthly intervals until financial support and technical assistance were terminated in 1990, making further characterization impossible.

Preparation of antisera. Rabbit antisera produced against the type species of the oceanic methane-oxidizing bacterium *Methylomonas pelagica* (Sieburth et al. 1987) was termed 'anti-*M.p.*'. Antisera produced against isolate B3P, a pure culture of an oceanic MMA-oxidizing bacterium isolated from the first MMA enrichments obtained from Sea Link, Stn III, which was serologically indistinguishable from *Methylophaga marina,* was termed 'anti-B3P'. A purified fraction of methanol dehydrogenase (MDH), allegedly a key enzyme common to both methane and MMA oxidizers, was termed anti-MDH. A non-immune serum was also taken from an uninoculated rabbit as a control. The preparation of the anti-*M.p.* antisera has been previously described (Sieburth et al. 1987). Anti-B3P antisera was prepared in a similar manner. The resulting antisera were absorbed twice with suspensions of *Escherichia coli* and pooled to give the final antiserum. The methane- and MMA-oxidizing bacteria have their process-specific enzymes that produce the common intermediate methanol, which is then oxidized to formaldehyde via methanol dehydrogenase (Large 1983, p. 41). This is shown in Fig. 1, as is the antisera produced against the 3 antigens.

A pure extract of MDH, prepared from a culture of *Methylococcus capsulatus* (Bath) as described in Woodland & Dalton (1984), was obtained from the University of Warwick, England. Anti-MDH was prepared by inoculating Dutch belted rabbits intramuscularly and subcutaneously with 50 µg of the MDH extract emulsified in complete Freund's adjuvant. Each

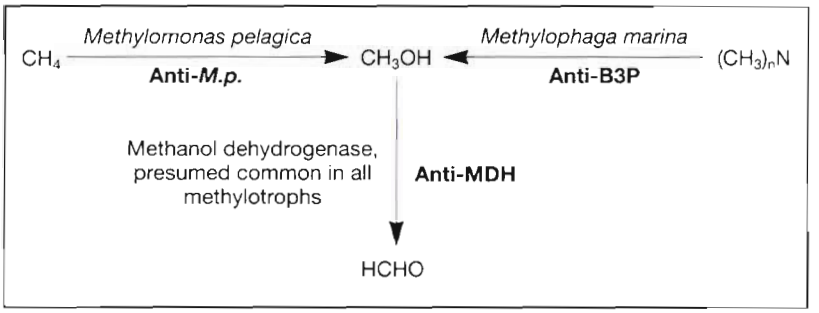

Fig. 1. Three test antisera used in this study shown in context with simplified C_1 pathways to the key intermediate, formaldehyde (adapted from Large 1983, p. 41). Anti-*M.p.*: antisera prepared against an oceanic methane-oxidizer *Methylomonas pelagica* able to grow on methane and methanol; Anti-B3P: antisera against an oceanic methylamine-oxidizer, B3P, a species of *Methylophaga* able to grow on the methylated amines TMA, DMA, and MMA, as well as on methanol and formaldehyde; Anti-MDH: antisera against the common key enzyme methanol dehydrogenase

rabbit received a booster of 100 µg MDH extract in incomplete Freund's adjuvant given intramuscularly and subcutaneously 1 and 2 months after the original inoculation. The rabbits were bled 2 wk after the final inoculation, absorbed with *Escherichia coli*, and pooled to produce the final serum.

Fluorescent antibody technique. For the indirect immuno-fluorescence procedure, cells from agarose plates or liquid enrichment cultures were fixed in either glutaraldehyde or formaldehyde (1% final conc.), and then immersed in histological grade acetone for 10 min to fix the cells to the glass slide, and alter the permeability of the cell membrane. Air-dried slides were stored in a sealed slide box at 4 °C for up to 4 d with no adverse effects. Just enough of each of the 3 antisera and the control serum necessary to cover their respective areas of each slide was applied. The anti-*M.p.* and non-immune sera were used at 1:100 dilution while the anti-MDH and anti-B3P antisera were diluted 1:50. After incubation for 1 h, the slides were washed off with phosphate buffered saline (pH 7.4) for 3 consecutive, 5 min washing periods. A goat anti-rabbit phycoerythrin conjugated antibody (phycoprobe™ PE, Biomeda Corp, Foster City, CA, USA) diluted 1:25, was applied to all sections of all slides in the moist chamber. After a final wash as above, the slides were air dried and coverslips mounted with paraffin oil (J. T. Baker Chem. Co., Phillipsburg, NJ, USA), and were examined using an Olympus Vanox epifluorescence microscope with blue light excitation (400 nm) and a 510 nm barrier filter, a Zeiss 100× Neofluar objective lens and a 10× eyepiece. The intensity of the antigenic reaction was scored using an arbitrary, but consistent, scale based on brightness of –, +, ++ and +++. Reactions designated ++ were considered to suggest an organism with antigens related to those in the immunization inoculum. A reaction of +++ was the brightest observed and was considered to suggest that the test culture contained bacteria very similar or indistinguishable from the inoculum used to produce the antiserum.

RESULTS

Antiserum specificity to described species

The bacterial cells, when specifically labeled with either anti-*M.p.* or anti-B3P sera, appeared bright yellow due to the fluorescence of phycoerythrin against a black background. The differences in morphology between *Methylomonas* (plump cocci) and *Methylophaga* (medium rods) were also useful when screening the enrichment cultures. Cells with a much different morphology usually gave enigmatic results. The less intense brightness of the anti-MDH preparations, as compared to that of the anti-*M.p.* or anti-B3P sera, may be due to fewer binding sites for the enzyme than for the whole cells per se. A slide prepared with non-immune serum would show no specific yellow fluorescence of the phycoerythrin conjugated antibody, although some nonspecific green autofluorescence of the cells due to gluteraldehyde fixation was sometimes visible. Typical reactions are shown in Fig. 2.

The specificity of the 3 test antisera to type cultures of methane- and MMA-oxidizing bacteria are summarized in Table 1. *Methylomonas pelagica* reacted strongly with its specific antiserum. The 2 species of methylaminotrophs, *Methylophaga marina* and *Methylophaga thalassica* both reacted strongly against the antiserum to the Sargasso Sea isolate B3P and show that the original isolate used to prepare the antiserum, as well as other cultures reacting strongly with the anti-B3P serum, are likely to be indistinguishable from *Methylophaga marina*.

Antiserum specificity to uncharacterized pure cultures

The 3 antisera and non-immune serum control were tested against pure cultures of uncharacterized methane- and MMA-oxidizing bacteria, and pure cultures of bacteria utilizing C–C bonded substrates that were isolated from both types of enrichment cultures (Table 2). The pure cultures of methane-oxidizing bacteria include the 'AA' series from which

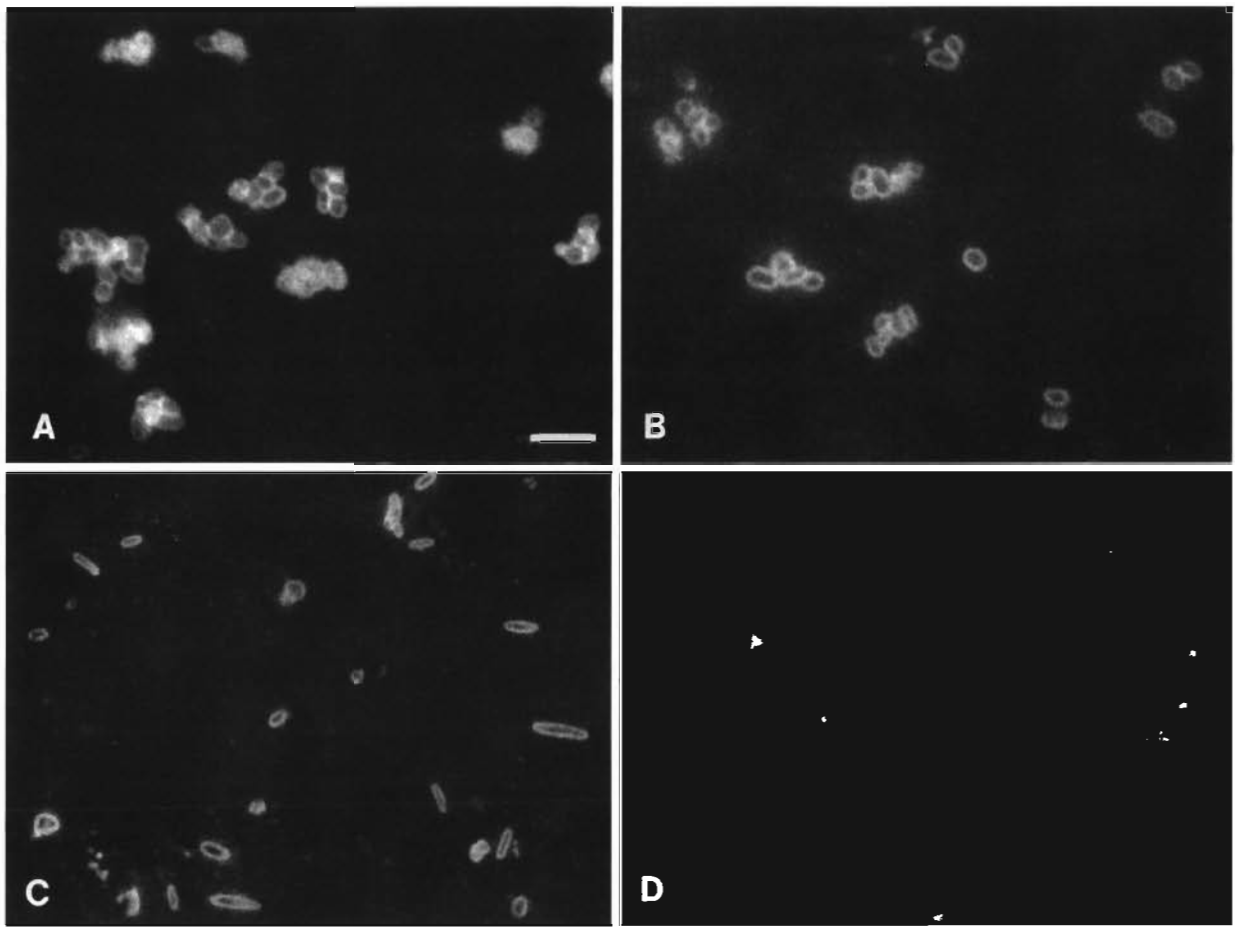

Fig. 2. Representative reactions of the 3 immunofluorescent antisera against pure cultures of a methane- and a MMA-oxidizer when viewed with epifluorescence microscopy. (A) Anti-*M.p.* serum against a culture of *Methylomonas pelagica* (AA-3). (B) Anti-MDH serum against the same culture. (C) Anti-B3P serum against a culture of a species of *Methylophaga* (B3P). (D) Anti-MDH against the same culture. Scale bar = 5.0 µm

the type strain of *Methylomonas pelagica* was originally selected. Out of the original 27 cultures isolated from the Sargasso Sea that were grown on methanol, 25 reacted strongly against the anti-*M.p.* serum with little or no cross reactivity against anti-B3P or the non-immune serum control. The only other methanotrophs tested in this study that appeared similar to *M. pelagica* were obtained from copepod faecal pellets in the MERL tanks. The scoring of anti-MDH reactivity was generally lower than that for the other antisera. In 2 cultures, Merl,S,B and U.M.(II)#2, there was some cross reactivity with the anti-B3P serum. This could be attributable to the possible presence of antibodies to periplasmic MDH in the anti-B3P serum.

Of the 6 pure cultures of MMA-oxidizers only CL-5-20 was able to grow on OZR agar plates containing peptone and yeast extract, indicating that it was a facultative methylotroph. There was no cross reactivity to anti-*M.p.* or to the non-immune serum in any of the 6 isolates tested. There was strong reactivity of +++ suggesting strains very similar to the original inoculum in 4 out of the 6 oceanic cultures. Virtually no reactivity against CL-2-60 and CL-5-20 suggests that these are entirely different species or serotypes. None of these cultures reacted against anti-MDH.

Heterotrophs utilizing C–C bonded substrates were isolated from 6 MMA-oxidizing and 6 methane-oxidizing enrichments from various locations. These bacteria were serially transferred a number of times on OZR agar and then plated onto the C_1 medium corresponding to the enrichment from which they were originally isolated. Het-Wish C was capable of growing on MMA plates, while HET U.M. III was capable of growing on NEM-10 plates in an atmosphere of 50 : 50 methane in air, at least transiently. These isolates may be facultative methylotrophs. Despite this, the immunofluorescence results showed no reactivity against any of the test antisera or the non-immune control with one exception. Het-U.M. III, isolated from the RV 'Columbus Iselin' sanitary system, showed some reactivity against

Table 2. Observed reactions of uncharacterized pure cultures of methanotrophs, methylaminotrophs and C–C utilizing heterotrophs against the 3 test antisera

Culture no.	Nature/Source	Immunofluorescence reaction			
		Anti-*M.p.*	Anti-MDH	Anti-B3P	Normal
Obligate methane oxidizers					
AA-3[a]	Seawater/Sargasso Sea	+++	+++	–	–
Merl,S,A	Copepod faecal pellets/MERL	+++	++	–	–
Merl,S,B	Copepod faecal pellets/MERL	+++	++	+	–
U.M.(II)#2	Sanitary tank/RV 'Iselin'	++	+	++	–
Obligate MMA oxidizers					
En133 1A	Pteropod faecel pelllets/N Atlantic	–	–	+++	–
Ber 100	Seawater/Bermuda	–	–	+++	–
SL-I-1	Seawater/Off Bahamas	–	–	+++	–
SL-II-6	Seawater/Off Bahamas	–	–	+++	–
CL-2-60	Seawater/VERTEX-Pacfic	–	–	+	–
CL-5-20[b]	Seawater/VERTEX-Pacfic	–	–	–	–
C–C heterotrophs from methane enrichment cultures:					
Het-1T2	Seawater/Sargasso Sea	–	–	–	–
Het-3T3	Seawater/Sargasso Sea	–	–	–	–
Het-CB17	Seawater/Chesapeake Bay	–	–	–	–
Het-CB19	Seawater/Chesapeake Bay	–	–	–	–
Het-Merl SC	Copepod faecal pellet/MERL	–	–	–	–
Het-U.M. III[c]	Sanitary tank/RV 'Iselin'	++	++	+	–
C–C heterotrophs from MMA enrichment cultures:					
Het-Wish C[b]	Benthic nephloid/Pacific	–	–	–	–
Het-IV 3	Prasinophyte algal culture	–	–	–	–
Het-Bac 983	Diatom algal culture	–	–	–	–
Het-858D13h	Sediment trap/Chesapeake Bay	–	–	–	–
Het-CL5-20	Seawater/Pacific	–	–	–	–
Het-SL II-6	Seawater/Bahamas	–	–	–	–

[a] Type culture for *Methlyomonas pelagica* used to prepare anti-*M.p.* serum was AA-23. Note: 24 other uncharacterized AA isolates also gave 3+ with anti-*M.p.*; reactions of + to 3+ with anti-MDH; and – to normal serum

[b] Growth on both OZR and MMA media suggests that these bacteria could be facultative methylaminotrophs, although they apparently lack MDH

[c] Growth on both OZR and on NEM-10 in a methane atmosphere suggests that this bacterium may be a facultative methanotroph

both the anti-*M.p.* and anti-MDH antisera and may be the only true facultative methanotroph isolated, and thus not the source of the *Methylomonas pelagica* cultures.

Antiserum specificity of methane-oxidizing enrichment cultures

The results against 66 active methane-oxidizing enrichment cultures are shown in Table 3. The '1T', '2T' and '3T' enrichment series were from the original enrichment series used as the source of the isolates for the type species of *Methylomonas pelagica* (Sieburth et al. 1987) which

Table 3. Testing of 3 methylotrophic antisera for the sorting of 66 enrichment cultures of methane-oxidizing bacteria[a]. Sorting categories: A: reactions indicative of *Methylomonas pelagica*; B: reactions suggesting an antigenic relation to *M. pelagica*; C: reactions indicate no relationship to *M. pelagica*, may be an, as yet, undescribed methanotroph; D: unexplained cross reactivity

Enrichment culture	Immunofluorescence reaction				Sorting category
	Anti-*M.p.*	Anti-MDH	Anti-B3P	Normal	
Seawater/Sargasso Sea[b]					
1T2	+++	+++	–	–	A
1T5	+++	+	+	++	A,D
2T1	++	+	–	–	B
2T3	++	++	–	–	B
3T2	+++	–	–	–	A
3T3	+++	++	–	–	A
Copepod faecal pellets					
MERL-SA	+	+	–	–	C
MERL-SB	++	+	–	–	B
MERL-SC	++	++	–	–	B

Table 3 (continued)

Enrichment culture	Immunofluorescence reaction Anti-*M.p.*	Anti-MDH	Anti-B3P	Normal	Sorting category
Sanitary tank RV 'Columbus Iselin'					
UM-I	+	+	–	–	C
UM-II	+	++	–	–	C
UM-III	++	++	–	–	B
Bay water/Chesapeake 1986					
CB-1	+	+	–	–	C
CB-2	–	–	–	–	C
CB-3	–	–	–	–	C
CB-4	–	++	–	–	C
CB-5	+	+	–	–	C
CB-6	–	–	–	–	C
CB-7	++	++	–	–	B
CB-8	–	+	–	–	C
CB-9	+	+	–	–	C
CB-10	–	+	–	–	C
CB-11	–	+	+	–	C
CB-12	–	+	–	–	C
CB-13	+	+	–	–	C
CB-14	–	+	–	+	C
CB-15	+	+	–	–	C
CB-16	++	–	–	–	B
CB-17	++	++	–	–	B
CB-18	+	+	–	–	C
CB-19	++	+	–	–	B
CB-20	–	–	–	–	C
CB-21	+	–	–	–	C
CB-23	–	–	–	–	C
CB-24	–	+	–	–	C
CB-25	–	–	–	–	C
CB-26	+	++	–	–	C
Bay water/Chesapeake 1987					
1B,Whole100#1	–	++	–	–	C
1B>3.0,100#1	–	++	–	–	C
2B,Whole100#1	+	+	–	–	C
2B>3.0,500#1	+	+	–	–	C
2M,Whole100#1	++	++	–	–	B
2M>3.0,500#1	–	–	–	–	C
2T,Whole100#1	–	++	–	–	C
2T>3.0,500#1	–	–	–	–	C
5b,Whole100#2	–	–	–	–	C
5b>3.0,500#1	–	+	++	–	D
5b,Whole100#1	–	–	++	–	
D5bD>3.0,500#1	–	–	–	–	C
5b,Whole,100#1	–	–	–	–	C
5b>3.0,500#1	–	–	–	–	C
9aB,Whole100#1	–	+	++	–	D
9aB>3.0,500#1	–	+	–	–	C
9aM,Whole100#1	++	+	+	–	B
9aM>3.0,500#1	–	–	–	–	C
9aT,500>3.0#1	–	–	+	–	C
9aT,Whole100#1	–	–	–	–	C
15B,>3.0,200#1	+	–	–	–	C
15B,Whole100#1	–	++	–	–	C
15M>3.0,200#1	–	–	–	–	C
15T,Whole100#1	–	–	–	–	C
15T>3.0,200#1	+	+	–	–	C
Shelf water/Adjacent Chesapeake Bay					
11B,500>3.0#2	++	–	+	–	B
11M,500>3.0#1	–	–	+	–	C
11M,Whole,100#2	–	–	+	–	C
11T,500>3.0#1	–	+	+	–	C

[a] All cultures formed a pellicle or were turbid
[b] Enrichment cultures which yielded the original AA isolates of *Methylomonas pelagica*

had been subcultured regularly for 3 yr. There were no cultures containing bacteria closely related to *M. pelagica*, except for the enrichments from which it was originally obtained. There was a total of 12 category B enrichments (18%) showing some relatedness to *M. pelagica*. There were 47 category C enrichments (71%) that are candidates for undescribed species and genera of marine methane oxidizers. Only 4 of the 66 cultures tested contained cells that showed any significant cross reactivity with the anti-B3P or normal antisera and were placed in category D. Reactivity against anti-MDH was generally low. Only 1 enrichment out of the 66 (2%) showed a +++ reactivity; 14 (21%) reacted moderately, ++; and 51 (77%) showed little or no reactivity.

Comparison of pure cultures of methane-oxidizers with the original enrichments from which they were obtained

In Table 4, the T-series of enrichments are compared with pure cultures obtained from them 3 yr earlier. All the pure cultures showed +++ reactions with no reactivity with anti-B3P or normal serum. In contrast, 2 of the 6 enrichments were ++ while another enrichment reacted with B3P and the normal serum.

Antiserum specificity of MMA-oxidizing enrichments

A total of 69 MMA enrichment cultures from Chesapeake Bay and a variety of waters and materials from both the Atlantic and Pacific oceans were tested against the 3 antisera, and the results are shown in Table 5. For the non-Chesapeake Bay water sample locations, there was a vertical profile, sometimes at several stations.

Table 4. Comparison of 6 of the Sargasso Sea T-series methane-oxidizing enrichments with 23 of the AA series of pure cultures, obtained from them 3 yr earlier, using the 3 methylotrophic antisera

Type of culture	Immunofluorescence reaction			
	Anti-*M.p.*	Anti-MDH	Anti-B3P	Normal
Enrichment[a]				
1T2	+++	+++	–	–
1T5	+++	+	+	++
2T1	++	+	–	–
2T3	++	++	–	–
3T2	+++	–	–	–
3T3	+++	++	–	–
Pure culture				
AA-1	+++	+	–	–
AA-3	+++	+++	–	–
AA-4	+++	++	–	–
AA-5	+++	++	–	–
AA-6	+++	++	–	–
AA-7	+++	++	–	–
AA-7P	+++	++	–	–
AA-9	+++	+	–	–
AA-10	+++	++	–	–
AA-12	+++	++	–	–
AA-13P	+++	+	–	–
AA-14	+++	+++	–	–
AA-15	+++	+	–	–
AA-17	+++	++	–	–
AA-18	+++	++	–	–
AA-20P	+++	+	–	–
AA-22	+++	+	–	–
AA-23[b,c]	+++	+	–	–
AA-23P	+++	++	–	–
AA-25	+++	++	–	–
AA-27	+++	–	–	–
AA-27P	+++	++	–	–
AA-28	+++	+	–	–

[a] Some of the surviving enrichment cultures that yielded the original AA series of isolates of *Methylomonas pelagica*
[b] Type culture for *Methylomonas pelagica*, National Collection of Industrial and Marine Bacteria # 226
[c] Used to prepare anti-*M.p.* serum

The distribution of the positive MMA enrichments in the vertical profiles taken during VERTEX 5, 1984, is shown in Fig. 3. The MMA-oxidizers had a wide vertical and spatial distribution. There was a trend for vertical stratification with apparent accumulations at depths where algae and their particulates might accumulate, the mixed layer, the seasonal pycnocline and the oxygen minimum layer. The samples from the pycnocline at each station developed before the others, indicating a larger population at this depth. The MMA enrichment cultures were also divided into 4 categories similar to those used for the methane-oxidizers. A total of 14 enrichments were indistinguishable from *Methylophaga marina* and show the ubiquity of this algal-associated species. Although 10 enrichments showed a lesser antigenic relationship, 46 enrichments displayed little or no reactivity, and are good candidates for new species or genera of MMA-oxidizing bacteria. Only 1 enrichment, IV 3, gave enigmatic results by reacting against both anti-B3P (strong) and anti-M.p (moderate). This spiral shaped bacterium came from a culture of an oceanic algal nanoflagellate isolated from the Sargasso Sea. Of the 69 enrichments tested against anti-MDH, only 1 showed a brightness of +++.

DISCUSSION

The application of the indirect immunofluorescence technique to the screening and sorting of enrichment cultures was quite successful. The sorting scheme was more than adequate for differentiating the enrichment cultures into distinct categories. The intensity of the antigenic reaction decreased slightly with time, while cross reactivity tended to increase with time. Pure cultures of *Methylomonas pelagica* gave unequivocal results, while the original enrichments that had been transferred at 1 to 2 mo intervals for 3 yr gave results that were not as clearcut. It is probably a good idea to make agar isolations from liquid enrichments as early as possible to avoid selection through species succession as discussed by Whittenbury et al. (1970). Fixation of the test bacteria with glutaraldehyde resulted in an increase of non-specific green autofluorescence which made scoring difficult, but cultures fixed with formaldehyde displayed no adverse effects. The choice of a phycoerythrin probe, although very useful for the detection of methylotrophs in pure and enrichment cultures, was unsatisfactory for the detection of methylotrophs in natural seawater samples (Church 1987) due to the ubiquitous nature of phycoerytherin containing chroococcoid cyanobacteria in the genus *Synechococcus* (Johnson & Sieburth 1979, Waterbury et al. 1987).

It should be noted that although a number of C–C utilizing heterotrophs initially appeared to grow on MMA or methane, only one of each persisted to utilize C_1 substrates. This suggests that facultative forms (Anthony 1982, Large 1983) are in the minority. Those that transiently utilized both C–C and C_1 substrates may be obligate forms such as those described by Zhao & Hanson (1984) which grow poorly on C_1 substrates but are stimulated by C–C compounds. Some of our transient C_1 forms were also of faecal origin.

Table 5. Test results of 3 methylotrophic antisera against 69 enrichment cultures of MMA-oxidizing bacteria. Sorting category: A: reactions similar to *Methylophaga marina* isolate; B: reactions suggesting an antigenic relation to *M. marine* isolate; C: reactions indicate no relationship to *M. marina* isolate, may be an, as yet, undescribed methylaminotroph; D: spiral bacterium unrelated to *M. marina*, except serologically

Enrichment culture	Nature/Source	Immunofluorescence reaction				Sorting category
		Anti-*M.p.*	Anti-MDH	Anti-B3P	Normal	
Xenic algal cultures						
995/20[a]	*Prymnesium parvum*	–	–	–	–	C
Bac983[a]	*Bacteriastrum hyalinum*	–	–	–	–	C
IG-3[b]	Chrysophyte	–	++	+	–	C
IV 3[b]	*Micromonas pusilla*	++	+	+++	–	D
IV P16 ax[b]	Chlorophyte	–	+	++	–	B
LB 913-1[a]	*Cyclotella pelagica*	–	–	–	–	C
Gelatinous zooplankton						
En133(1A)	Pteropod/N Atlantic	–	+	+++	–	A
En133(3)	Ctenophore/N Atlantic	–	+	+++	–	A
En133(6)	Colonial radiolarian/N Atlantic	–	++	+	–	C
Particulates						
Al Sest 2	Benthic nephloid/Santa Catalina	–	–	–	–	C
CB858ST16m	#1 Sediment trap/Chesapeake Bay	–	++	++	–	B
Sea water						
Ber-100	Bermuda	–	+	+++	–	A
Ber-25	Bermuda	–	–	+++	–	A
CL-2-60	VERTEX-Pacfic	–	–	+++	–	A
CL-4-150	VERTEX-Pacfic	–	–	+++	–	A
CL-5-20	VERTEX-Pacfic	–	++	–	–	C
MERL (5) DMA	MERL tank	–	+	–	–	C
MERL (5) TMA	MERL tank	–	+	+	–	C
MERL-2	MERL tank	–	–	++	–	B
MERL-4	MERL tank	–	+	+	–	C
Skan Bay-40	Alaska	–	–	+	–	C
Skan Bay-5	Alaska	–	++	+	–	C
SL-I-1	Bahamas	–	+	–	–	C
SL-I-3	Bahamas	–	–	–	–	C
SL-II-6	Bahamas	–	+++	+++	–	A
SL-III-10	Bahamas	–	+	+	–	C
SL-III-3	Bahamas	–	+	++	–	B
SL-III-6	Bahamas	–	++	+++	–	A
Wish-C	Santa Catalina	–	+	+	–	C
Bay water/Chesapeake Bay 1986						
CB858A,1,100,1		–	+	–	–	C
CB858A,2,100,1		–	–	–	–	C
CB858A,3,100,1		–	–	–	–	C
CB858A,4,100,2		–	–	–	–	C
CB858A,5,100,1		–	–	–	–	C
CB858ST2m#1		–	–	–	–	C
CB904A,1,100,1		–	–	+	–	C
CB904A,2,100,1		–	++	–	–	C
CB904A,3,100,1		–	–	–	–	C
Bay water/Chesapeake Bay 1987						
1B,Whole100#1		–	–	+++	–	A
1B>3.0,100#1		+	+	–	–	C
2B,Whole,100#1		–	–	–	–	C
2B>3.0,500#1		+	+	+	–	C
2M,Whole100#1		–	–	–	–	C
2M>3.0,500#1		–	++	++	–	B

(Table continued on next page)

Table 5 (continued)

Enrichment culture Nature/Source	Immunofluorescence reaction				Sorting category
	Anti-*M.p.*	Anti-MDH	Anti-B3P	Normal	
Shelf water/Chesapeake Bay 1987, continued					
2T,500>3.0#1	–	+	+	–	C
2T,Whole100#1	–	+	++	–	B
5bB,Whole,100#1	–	+	++	–	B
5bB>3.0,500#2	–	+	++	–	B
5bM,>3.0,500#1	–	–	+	–	C
5bT,Whole,100#1	–	–	++	–	B
5bT>3.0,500#1	–	–	–	–	C
9aB,Whole,100#1	–	–	–	–	C
9aB>3.0,500#2	–	–	–	–	C
9aM,Whole,100#1	–	–	–	–	C
9aM>3.0,500#2	–	–	–	–	C
9aT,Whole,100#1	–	–	–	–	C
9aT(>3.0,500#1	–	–	+++	–	A
15B,Whole100#1	–	++	+++	–	A
15B>3.0,200#1	–	+	–	–	C
15M,Whole100#1	–	–	+++	–	A
15M>3.0,200#1	–	–	+++	–	A
15T,Whole100#1	–	–	+	–	C
15T>3.0,200#1	–	–	–	–	C
Shelf water/Adjacent to Chesapeake Bay					
11B,Whole100#1	–	–	–	–	C
11BA>3.0,500#1	–	–	–	–	C
11M,Whole100#1	–	–	–	–	C
11M>3.0,500#1	–	–	++	–	B
11T(,Whole100#2	–	–	–	–	C
11T>3.0,500#1	–	–	+	–	C

[a] Cultures from Paul E. Hargraves, Univ. of Rhode Island, RI, USA
[b] Cultures from P-GCCMP, Bigelow Laboratory for Ocean Sciences, Boothbay Harbor, ME, USA

Z_m	VERTEX Station number, 1984				
	1	2	3	4	5
20	++++		–	–	++++
30		+++	–	–	+++
40	–	+++	+++	+++	–
50	–	–	–	–	+++
60	+++	++++	–	++++	+++
70	+++	+		–	++++
80	–	–	++++	–	+++
90					–
100	+++	+++	+++	–	
110		–		–	–
120	+				
130					
140	++				
150			+++		++
160	+++				

200			+		++++

400					++++

Fig. 3. Vertical and spatial distribution of oceanic MMA-oxidizers in the northeast Pacific as indicated by relative growth (–, +, ++, +++ and ++++) in 2 to 3 wk incubation time of MMA-enriched 100 ml portions of seawater from 5 hydrocasts during the VERTEX 5 experiment, 1984, on a transect from Monterey Bay, CA, USA, towards the Hawaiian Islands (Martin et al. 1987)

Of 135 enrichments examined, 97 (72%) showed little or no reactivity suggesting that they are candidates for new species or genera of methylotrophic bacteria. Conversely, almost 30% of the enrichments examined contained microorganisms similar to the bacteria used to produce the *Methylomonas pelagica* (anti-*M.p.*) and *Methylophaga marina* (anti-B3P) sera. Thus, it might have been possible to characterize most of the bacteria present in these enrichment cultures with only a few additional antisera. The ability to detect either type of methylotroph with anti-MDH with any degree of precision was disappointing. All aerobic methylotrophs are thought to contain MDH (Large 1983). Although 23% of the methane-oxidizers reacted to anti-MDH, only 15% of the MMA-oxidizers did.

Methylomonas pelagica, when originally enriched from the open North Atlantic, was only obtained from stratified waters in the Sargasso Sea, but not in well-mixed slope waters (Sieburth et al. 1987). This suggested that methane cycle bacteria may require stratification. It is more likely, however, that the methanogenic bacterial consortia discussed in Sieburth (1993) are ubiquitous in the water column, but only accumulate to concentrations detectable in small volume enrichment cultures in the pycnocline of stratified waters. The use of

larger volume enrichment cultures during 1987 in the Chesapeake Bay was more successful than the smaller volumes used in 1986 (Sieburth 1993). The lack of methane-oxidizers in Chesapeake Bay identical to *M. pelagica* is of interest, and indicates that estuarine species may be quite different from oceanic species. The only nearshore enrichments similar to *M. pelagica* were those from copepod faecal pellets in a stratified MERL mesocosm.

In contrast to the methane-oxidizers, 12% of the MMA-oxidizers in Chesapeake Bay were indistinguishable from *Methylophaga marina*. Methylaminotrophs have been found to be associated with algal cells (Janvier et al. 1985, Sieburth & Keller 1988/89), and to be ubiquitous in the open sea (this paper). Some 44% of our oceanic cultures reacted to the B3P antiserum. None of the 6 pure cultures of MMA-oxidizers reacted against anti-MDH. This is an unexpected result given that all methylotrophs are thought to contain MDH (Large 1983).

The aerobic C_1 bacteria dominating methanogenic bacterial consortia (Sieburth 1993) are the methylamine-oxidizers and the methane-oxidizers studied in this work. The key intermediate of these bacteria is formaldehyde which peaks where particulates would be expected to accumulate (Eberhardt & Sieburth 1985). The distribution of the methylamine-oxidizers in the VERTEX samples (Fig. 3) also appeared to be associated with depths where algae and/or particles would accumulate. The high incidence of MMA-oxidizers in xenic algal cultures associated with the pycnocline (Sieburth & Keller 1988/89), the serendipitous coenrichment of H_2S- and methane-producing bacterial consortia with MMA (Sieburth 1987), as well as their presence in xenic algal cultures (Sieburth 1988a), has led to the concept of algal-induced consortia (Sieburth 1988b) *in situ* as well as in culture.

Although the data base of this study is small, it might be sufficient to make a preliminary examination of the factors that might control the C_1 bacteria involved in the production and oxidation of methane. The information from this three part study is summarized in Fig. 4. Enrichments indistinguishable from the type species of marine MMA-oxidizers, *Methylophaga marina*, accounted for 44% of the oceanic cultures and 12% of the Chesapeake Bay cultures, indicating the cosmopolitan nature of this bacterium. The reduced microniches that such bacteria create (Regimens 1 and 2 of Sieburth 1993) accounted for 60% of the more estuarine methanogens and 84% of the more oceanic methanogens from the shelf. While the suphide-reduced methanogens that were 40% in the estuary fell to 16% in the shelf, the H_2S-sensitive methanogen populations of Regimen 1 (13 to 26%) doubled from the estuary to the shelf.

		Estuarine →	Oceanic
MMA-oxidizers	Category A	12%	44%
	B	17%	6%
	C	71%	50%
Methanogens	Regimen 3	40%	16%
	2	47%	58%
	1	13%	26%
Methane-oxidizers	Category A	0%	96%
	B	13%	4%
	C	87%	0%

Fig. 4. Geographic, taxonomic and trophic trends of the C_1 bacteria involved in the induction of reduced microniches (MMA-oxidizers) and the production (methylotrophic methanogens) and consumption (methane-oxidizers) of methane in the oxygenated water column of Chesapeake Bay shown in this 3 part study. The data for the methane- and MMA-oxidizers is from Tables 3 & 5, respectively, with the same A, B and C sorting categories. The data for the sub-populations of methanogens enriched in Regimens 1, 2 and 3 is from Table 2 of Sieburth (1993), in which the more estuarine populations were from Stns 1 to 16 and the more oceanic populations were from Stns 5 to 13. There is a trend for the MMA-oxidizers to be cosmopolitan, for the methanogens that peak in the pycnocline to be selected both vertically (Sieburth et al. 1993) and horizontally (Sieburth 1993), and for the methane-oxidizers to be geographically selected

The methanogens in Regimen 2 are all new species, while those in the particles of the pycnocline that sediment to the bottom (Sieburth et al. 1993) may include new taxa. The novel types of methanogens that dominate the pycnocline of the stratified Chesapeake Bay may also enrich novel types of methane-oxidizers. None were identical to *Methylomonas pelagica*, and only 13% were somewhat related. The oceanic value of 96% may be biased by all the cultures coming from 3 nearby stations in the Sargasso Sea, but it does indicate less diversity. The step-wise dependence of the C_1 bacteria upon each other may dictate the physiological and taxonomic types of methane-oxidizers. The salinity and sulphide gradients of the estuary may cause an increased diversity of methane-oxidizers. The increase in methanogens dependent upon their aerobic bacterial consorts for reduced microniches may lead to a less diverse community of oceanic methane-oxidizers.

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