Diversity of Methyl Halide-Degrading Microorganisms in Oceanic and Coastal Waters

Michael J. Cox[1], Hendrik Schäfer[1]*, Phillip D. Nightingale[2], Ian R. McDonald[3], and J. Colin Murrell[1]¶

[1] School of Life Sciences, University of Warwick, Gibbet Hill, Coventry, CV4 7AL, United Kingdom

[2]Plymouth Marine Laboratory, West Hoe, Plymouth, PL1 2DH, United Kingdom

[3]Department of Biological Sciences, University of Waikato, Private Bag 3105, Hamilton, 3240, New Zealand.

*corresponding author: H.Schaefer@warwick.ac.uk

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¶ Present address:

School of Environmental Sciences, University of East Anglia, Norwich, NR4 7TJ, United Kingdom

Abstract:

Methyl halides have a significant impact on atmospheric chemistry, particularly in the degradation of stratospheric ozone. Bacteria are known to contribute to degradation of methyl halides in the oceans and marine bacteria capable of using methyl bromide and methyl chloride as sole carbon and energy source have been isolated. A genetic marker for microbial degradation of methyl bromide (*cmuA*) was used to examine the distribution and diversity of these organisms in the marine environment. Three novel marine clades of *cmuA* were identified in unamended seawater and in marine enrichment cultures degrading methyl halides. Two of these *cmuA* clades are not represented in extant bacteria, demonstrating the utility of this molecular marker in identifying uncultivated marine methyl halide degrading bacteria. The detection of populations of marine bacteria containing *cmuA* genes suggests that marine bacteria employing the CmuA enzyme contribute to methyl halide cycling in the ocean.

Introduction

The methyl halides (CH_3X) methyl bromide (CH_3Br) and methyl chloride (CH_3Cl) are volatile organic compounds with natural and anthropogenic sources, and are present at trace amounts in the atmosphere (10 and 600 parts per trillion by volume [pptv] respectively). Despite their low atmospheric concentration, they have a large impact on atmospheric chemistry, delivering bromine and chlorine atomic radicals arising from the breakdown of methyl halides to the stratosphere where they catalyse ozone destruction. The oceans are both a source and a sink of CH_3Br, but overall are a net sink (for a review of methyl halide biogeochemistry (see Schäfer *et al.*, 2007).

King & Saltzman (1997) demonstrated that biological loss rates for CH_3Br in surface ocean waters were significantly higher than chemical loss rates, indicating that biological pathways existed for the removal of CH_3Br from these waters. Examination of CH_3Br loss rates associated with individual size fractions of the marine biomass revealed that loss of CH_3Br was associated with the fraction that encompassed the bacterial size range.

Microbial degradation of methyl halides by several metabolic pathways has been demonstrated in a range of microorganisms. Methyl halides can be co-oxidised by three different classes of mono-oxygenases; methane monooxygenase (Stirling *et al.*, 1979, Stirling & Dalton, 1979), ammonia monooxygenase (Rasche *et al.*, 1990), and toluene monooxygenase (Goodwin *et al.*, 2005). In the methanotroph *Methylomicrobium album* BG8, assimilation of carbon from methyl chloride and its use as a supplementary energy source (alongside methane) has been demonstrated (Han & Semrau,

2000), however, only one pathway has been identified that is specific for methyl halide degradation in methylotrophic bacteria that utilise methyl halides as sole source of carbon and energy (Vannelli *et al.*, 1999). The initial reaction of the pathway is catalysed by CmuA, a methyltransferase/corrinoid-binding protein which transfers the methyl group of the methyl halide to the Co atom of a corrinoid group on the same enzyme. The methyl group is next transferred to tetrahydrofolate by another methyltransferase (CmuB) and the methyl tetrahydrofolate is progressively oxidised to formate and CO_2, with carbon assimilation at the level of methylene tetrahydrofolate (Vannelli *et al.*, 1999). Several species of bacteria use this methyltransferase-based pathway have been isolated from a range of environments, including soils, plant phyllosphere, and the marine environment (Doronina *et al.*, 1996, Connell-Hancock *et al.*, 1998, Goodwin *et al.*, 1998, Coulter *et al.*, 1999, Hoeft *et al.*, 2000, McAnulla *et al.*, 2001, Schaefer *et al.*, 2002, Borodina *et al.*, 2005, Schäfer *et al.*, 2005, Nadalig *et al.*, 2011). The unique structure of CmuA has been exploited to design primers for studying the diversity of methyl halide degrading bacteria in the environment (McDonald *et al.*, 2002, Miller *et al.*, 2004, Borodina *et al.*, 2005, Schäfer *et al.*, 2005, Nadalig *et al.*, 2011).

In this study we examined *cmuA* sequences obtained from seawater samples, and methyl halide enrichment cultures, from the Arabian Sea and English Channel to determine the presence and diversity of marine methyl halide degrading bacteria that utilise the methyl halide degradation pathway involving the enzyme CmuA.

Methods

Large volume seawater DNA samples

Stand-alone pumps (SAPs; Challenger mark 2 SAP, Challenger Oceanic, UK) were used to obtain large volume samples from the deep-chlorophyll maximum at stations of the NERC AMBITION research cruise in the Arabian Sea on board the *RRS Charles Darwin* in 2001 (Cruise CD132; Fig. 1).

SAPs were left in place varying times and the sample volume through the 293 mm diameter, 0.2 µm pore size filters was calculated using time and flow rate (Table 1). DNA extraction was achieved by rinsing SAP filters in 5 mL filtered seawater, then the filtrate was taken up in 1 mL RNALater (Ambion) and stored at 4°C. 0.5 mL of this was centrifuged (14,000 x *g* and DNA isolated from the resulting pellet using a Qiagen DNA extraction kit with the DNA eluted in 100 µL sterile deionised water (Mike Wyman, pers. comm.). One µL of this DNA extract, or of a 1:10 diluted extract (typically 5-50ng of DNA), was used as template for PCR amplification of *cmuA*. PCR reaction mixtures were 2.5 mM $MgCl_2$, 200 µM each dNTP, 25 pmol of primers *cmuA*F802/*cmuA*R1609 (Miller *et al.*, 2004),1.3 M betaine, 1.3% (vol/vol) DMSO, in 1 x Invitrogen *Taq* DNA Polymerase buffer and 2.5 U of Taq DNA Polymerase (Invitrogen, Paisley, UK) in a total volume of 50 µL, made up with sterile deionised water. Thermal cycling was carried out on a Hybaid Touchdown thermal cycler with initial denaturation at 95 ^{o}C for 5 min, whereupon the *Taq* DNA Polymerase was added as a hot start. This was followed by 35 cycles of 1 min at 95 ^{o}C, 1 min at 55 ^{o}C, and 1 min at 72 ^{o}C,

113 followed by final extension step of 72 ^{o}C for 10 min. Genomic DNA from
114 *Hyphomicrobium chloromethanicum* strain CM2 was used as a positive
115 control.
116
117 *Arabian Sea Enrichment Cultures*
118 Enrichment cultures were set up with seawater on a range of substrates
119 during a research cruise on board the *RRS Charles Darwin* in 2001 (Cruise
120 CD132). Water samples were taken at eleven stations (Fig. 1A) using a
121 SeaBird rosette sampler equipped with 24 x 30 L Niskin bottles and CTD
122 (conductivity, temperature and depth) devices. The exact system
123 configuration can be found in the AMBITION Cruise report, from the Biological
124 Oceanographic Data Centre website (www.bodc.ac.uk/projects/m&fmb.html).
125 The Niskin bottles were sub-sampled using their integral taps and a short
126 length of Tygon tubing into 2 L polycarbonate bottles rinsed three times with
127 seawater sample.
128 2 L of water from 5 m depth (surface) and the chlorophyll maximum for each
129 station (as determined by the CTD profile) were vacuum-filtered through 47
130 mm, 0.2 µm polyethersulfone Supor-200 filters (Pall-Gelman, Port
131 Washington, NY) and the filtrate was then resuspended in ~3 mL of sample
132 water. At station 6 (15°12.0'N 67°00.0'E) an additional set of enrichment
133 cultures were set up with water sampled from a deep cast of 2501 m. An
134 additional set was also taken at 250 m, station 8 (20°55.0'N 63°40.0'E),
135 together with a final additional set at station 11 (26°00.0'N 56°35.0'E) at the
136 salinity maximum.

100 µL of the filtrate suspension was added to each of twelve pre-prepared 25 mL, crimp-sealed, gas-tight, enrichment vials containing 5 mL of 0.1 x Ammonium Nitrate Mineral Salts (ANMS) medium (Whittenbury *et al.*, 1970) with 3.5% (w/v) NaCl, trace element solution SL-10 (Widdel *et al.*, 1983) and 0.02 mg L^{-1} folic acid, 1 mg L^{-1} *p*-aminobenzoic acid and 1mg L^{-1} cyanocobalamine. Twelve different carbon sources were added to the vials in different combinations and concentrations: 86 µM (0.1% vol/vol) CH_3Br; 430 µM (0.5% vol/vol) CH_3Br; 860 µM (1% vol/vol) CH_3Br; 50 mM "Aristar" methanol; 430 µM CH_3Br plus 50 mM methanol; 10 mM methylamine; 430 µM CH_3Br plus 10 mM methylamine; 430 µM (0.5%) CH_3Br plus 10 mM formate; 140 µM (10% vol/vol) methane; 1540 µM (2% vol/vol) CH_3Cl; 430 µM CH_3Br plus 10 mM L-methionine. Aqueous phase concentrations of gases were calculated using the Henry's Law constants (DeBruyn and Saltzman 1997). Enrichment cultures were incubated at 20 ^{o}C in the dark to prevent growth of photosynthetic organisms, for approximately 2 months.

After incubation, the cultures were scored qualitatively for turbidity. The presence or absence of headspace methyl halides (CH_3X) was tested using gas chromatography with flame ionisation detection as described previously (Schäfer *et al.*, 2005). Two mL of each enrichment was centrifuged for 5 min at 14,000 x *g*, the supernatant removed and the pellet resuspended in 10 µL of sterile deionised water. This was then boiled for 10 min in a water bath and 1 µL was used as template in PCR reactions.

English Channel seawater enrichment cultures

161 Seawater was collected from the Western Channel Observatory site L4 (Fig 1)
162 in the English Channel during routine sampling on the 18th April (L4.1), 20th
163 June (L4.2), and 30th July (L4.3) 2002 using 5 L manually operated Niskin
164 bottles from surface waters at approximately 1 m depth. On each date, 300
165 mL of seawater was transferred to 1.15 L crimp-seal flasks with butyl-rubber
166 stoppers and 0.2% (v/v) headspace CH_3Br added (142 µM CH_3Br). L4.1
167 consumed 313 µmol CH_3Br in total; L4.2 and L4.3 consumed 188 µmol each.
168 PCR template from enrichment culture L4.1 was prepared as for the Arabian
169 Sea enrichment cultures.

170

171 *Clone library construction*

172 PCR products were cloned using the TOPO TA cloning kit (Invitrogen)
173 according to the manufacturer's instructions. Plasmid mini-preps were carried
174 out from 2 mL of overnight culture using the alkaline lysis mini-prep procedure
175 (Sambrook & Russell, 2001). Plasmid DNA was resuspended in 50 µL of
176 sterile deionised water. Different RFLP types were assigned manually, and
177 the first clone representing each type selected for sequencing, thus
178 dereplicating the library.. Double restriction digests were carried out using
179 restriction enzymes *Eco*RI and *Rsa*I or *Eco*RI and *Dde*I (Fermentas, Vilnius,
180 Lithuania). 2 µL of 100 µg/mL RNase (Promega, Madison, WI) was added to
181 each reaction. Restriction digests were incubated at 37 ^{o}C for 16 hours and
182 analysed by agarose gel electrophoresis. Gels were stained with ethidium
183 bromide to enable visualisation of the DNA fragments using UV-
184 transillumination. Different RFLP types were assigned manually.
185

186 *Phylogenetic tree construction*

187 DNA sequences obtained in this study were aligned in ARB (Ludwig, *et al.,*
188 2004) together with all other available *cmuA* sequences using the integrated
189 aligner and the resulting alignments were edited manually. Primer sequences
190 were removed and the remaining sequence translated to amino acid
191 sequence, resulting in a 252 residue alignment (min 251, max 252, mean
192 251.99) presence of conserved functional amino acid residues was confirmed,
193 before export of the alignment from ARB. FastTree [version 2.1.3,(Price *et al.*,
194 2010)] was used to construct nearest neighbour interchange neighbour-joining
195 trees rapidly with the parameters -spr 4, -mlacc 2 and -slownni, increasing the
196 number of rounds of minimum-evolution SPR (subtree pruning and regrafting)
197 moves and making the maximum likelihood nearest neighbour interchanges
198 more exhaustive in order to increase the accuracy of the tree.. The tree was
199 imported into ARB, where it was annotated and rooted with reference to
200 AJ011316 *Methylobacterium chloromethanicum* strain CM4.

201

202 **Results**

203 *Detection of cmuA in large volume seawater DNA samples*
204 PCR products of the expected size (~807 bp) were only obtained from three of
205 the nine cruise stations where stand alone pumps (SAPs) had been used to
206 concentrate large volumes of seawater for DNA extraction; Faint PCR
207 products were obtained from stations 1, 4 and 9. Libraries of 50 *cmuA* clones
208 were produced from each of the PCR products. Clones were assigned to
209 different RFLP pattern types by RFLP analysis with *Eco*RI/*Dde*I and

*Eco*RI/*Rsa*I double digests. The station 1 *cmuA* library was shown to contain only two OTUs; 70% of clones belonged to OTU 1 and 30% to OTU 2. The station 4 clone library was dominated by OTU 3 (98%) with a single clone designated OTU 4. Station 9 was similarly dominated, with 100% of clones affiliated by RFLP to OTU 3. Representatives of each OTU were sequenced and deposited in Genbank with the accessions DQ090698 to DQ090705.

The faint PCR products and low diversity of *cmuA* sequences obtained from the large volume SAP DNA samples indicated that these organisms were probably a small component of the microbial community. We attempted enrichment of methyl halide-utilising bacteria in seawater samples from both the Arabian Sea and the English Channel near Plymouth, UK in order to increase their relative abundance.

Arabian Sea Enrichment Cultures

Enrichment cultures with different concentrations of CH_3Br and CH_3Cl, either alone or together with a range of one carbon (C1) compounds (see methods for details) were set up during the AMBITION cruise. Removal of the CH_3X was confirmed by gas chromatography of headspace gas in comparison to sterile chemical controls set up at the same time. Although a number of enrichment vials were depleted of methyl halides even after as little as two weeks of incubation, many of these cultures failed to degrade a second pulse of methyl halide addition to the headspace. This, accompanied by an optical density (560nm) of at least 0.4 was used to determine that there had potentially been enrichment of methyl halide-degrading microorganisms.

Enrichment cultures that showed successful enrichment of methyl halide-degrading microorganisms are reported in Table 2. Enrichment numbers 165, 165.2, 189, 249 and 273, all cultures initially supplied with formate (10mM) and methyl bromide (430μM), degraded between 89 and 268 μmol of methyl bromide. These cultures were subcultured at least twice into fresh 0.1 x ANMS medium with 0.2% (v/v) CH_3Br in the headspace. GC monitoring of these enrichment cultures was carried out at intervals of approximately one to two weeks meaning that it was not possible to accurately determine the time of depletion of substrate. Generally, initial degradation of methyl halides of these enrichments required at least one month, and the time it took to degrade the total amount of methyl halide shown in Table 2 was between two and four months. Enrichment cultures initially supplied with methanol, methylamine, formate and methane as enrichment substrates were pooled, amended with an additional 0.2% (v/v) headspace CH_3Br and subcultured again. This pooled enrichment culture (PE2) also degraded methyl bromide (580μmol in total) over the course of four months.. PCR products generated using the *cmuA* primer pair from two of these enrichment cultures, the station 8 enrichment (189) and the pooled enrichments (PE2) which had consumed 89 and 580 μmol of CH_3Br, respectively) were cloned as before.

English Channel Enrichment Cultures

An alternative enrichment strategy was used with samples of seawater from L4, a sampling station off the coast of Plymouth. Larger volumes of water unamended with media were incubated with 0.2% (v/v) CH_3Br and the amount of CH_3Br consumed was recorded (Table 2). PCR products were obtained

from all three enrichment cultures and one of these, enrichment L4.1, was selected for clone library analysis.

The four clone libraries were dereplicated by RFLP, as for the SAP sample libraries, and representative clones were sequenced. Phylogenetic trees of *cmuA* sequences from all seven libraries were constructed (Fig 2), and indicated that sequences fell into three major clades with strong nearest neighbour interchange value support. Two of these clades (1 and 3) are novel, with no similar CmuA sequences from extant bacteria. The closest relatives of clade 1 members were cloned *cmuA* genes from soils and *Hyphomicrobium chloromethanicum* CM2 *cmuA* with pairwise identities of approximately 91% and 90% at the protein level, respectively, while the closest relatives of clade 3 were *cmuA* genes obtained from soil CH_3Br and CH_3Cl stable isotope probing experiments (approximately 85% similarity) and marine strains 179 and 198 of the Rhodobacteraceae (similarity of approximately 83%). The third clade had not been found in marine samples previously and shared high similarity (95-99%) with *cmuA* sequences from *Aminobacter* spp., a genus previously identified in terrestrial, rather than marine environments.

Discussion

This study has revealed the presence in two distinct marine environments of genes encoding the methyltransferase/corrinoid binding protein CmuA, which carries out the first step in the methyl halide degradation pathway of methylotropic bacteria. In a marine context, investigation of the diversity of

285 this functional genetic marker has previously been limited to detection in
286 marine methyl halide-degrading isolates and enrichment cultures (McAnulla *et*
287 *al.*, 2001, Schäfer *et al.*, 2005), in this study *cmuA* genes from marine
288 organisms have also been detected using direct amplification from
289 environmental DNA. The discovery of three new clades of marine *cmuA*
290 sequences in the relatively small number of samples investigated, indicates
291 that the diversity of bacterial populations utilising this pathway of methyl halide
292 degradation is higher than previously realised. Enrichment of methyl halide
293 degrading bacteria was successful from oligotrophic and meso/eutrophic
294 marine samples using methyl halides as sole carbon source. Interestingly,
295 subcultivation on methyl halides of pooled enrichments of methylotrophic
296 microorganisms using a range of C1 compounds also resulted in methyl
297 halide degrading cultures, suggesting that some of the methyl halide
298 degrading populations detected here may be representative of methylotrophs
299 that are not restricted to use of methyl halides alone. Methyl halide degrading
300 isolates of the *Roseobacter* clade obtained previously (Schaefer *et al.*, 2002,
301 Schäfer *et al.*, 2005) were all facultative methylotrophs, with some using more
302 than one C1 compound as carbon source, while for others, methyl halides
303 were the only C1 compounds (of those tested) supporting growth. Sequences
304 in clade 1 may represent populations degrading more than one C1 compound,
305 as this clade was entirely composed of sequences obtained from pooled
306 methylotrophic enrichments and from clones obtained directly from large
307 volume seawater DNA samples of stations 4 and 9 from the Arabian Sea.
308 Interestingly, clade 3 was only detected in enrichments on methyl halides
309 alone and in large volume seawater samples from the oligotrophic station 1.

Given the low concentrations of methyl halides present in seawater which are in the pM range (Baker *et al.*, 1999, Yang *et al.*, 2010), it has been suggested that methyl halides may not be physiologically relevant carbon sources *in situ*, and that a specialised enzyme system for methyl bromide degradation is unlikely to exist (Hoeft *et al.*, 2000). Other studies have shown that some marine bacteria are capable of growth on methyl halides, albeit exhibiting relatively poor growth compared to their terrestrial counterparts (Schaefer *et al.*, 2002, Schäfer *et al.*, 2005). The fact that some marine methyl halide degrading bacteria do employ an enzyme system such as CmuA, which is specific for degradation of the related compounds methyl chloride and methyl bromide, suggests that methyl halide degradation in the marine environment is not just a case of co-metabolism or detoxification of these compounds. On a scale relevant to microorganisms, and considering the vicinity of methyl halide producing phytoplankton as potential hotspots of higher local concentrations, these trace gases may potentially be of selective advantage for specialised bacterial populations that could utilise methyl halides as energy and/or carbon source. Recent work by Halsey et al suggests that degradation of C1 compounds including methyl chloride by the methylotrophic bacterium HTCC2181 may indeed be primarily linked to energy gain rather than carbon assimilation (Halsey *et al.*, 2012). The enzymatic basis of methyl chloride degradation in strain HTCC2181 is as yet unidentified, the genome sequence of strain HTCC2181 does not contain a gene encoding CmuA.

Also of interest is the wide geographic and environmental distribution of some highly similar *cmuA* sequences. Clade 2 was detected in the Arabian Sea, Plymouth coastal waters and *Aminobacter* spp. isolated from soils. Given the enrichment methods used, it is not possible to associate particular sequences or clades of *cmuA* with biogeochemical data from the research cruise in the Arabian Sea. The Arabian Sea, at the time of sampling, had a gradient of nutrient levels, from oligotrophic waters in the South to strongly eutrophic waters in the North. It is interesting to note that all station 1 (oligotrophic) clones grouped in clade 3 whereas clones from station 4 and 9 (higher nutrient levels) fell into clade 1. Further work with a higher resolution of *cmuA* diversity would be required to investigate whether this might indicate distinct ecological niches for these *cmuA* clades.

The ecology and diversity of marine methyl halide degrading microorganisms and their role in the biogeochemical cycling of methyl halides remains a challenging field of biological oceanography. Further work is required to determine the extent to which methyl bromide is oxidised to CO_2 or assimilated into microbial biomass in seawater. The diversity and activity of methyl halide-utilising bacteria in these environments should also be studied in more detail. Stable isotope probing with ^{13}C-methyl bromide is a potential approach for detecting active methyl halide degrading bacteria based on assimilation of methyl halide carbon during growth linked catabolism and has been used to detect bacteria related to *Roseobacter* and *Methylophaga* in samples from the English Channel (Neufeld *et al.*, 2008). The primer set for amplifying *cmuA* genes has demonstrated that it is useful in the discovery of

359 novel clades, thus proving its worth for further environmental studies, but
360 future studies should also utilise other primer sets developed more recently
361 (Nadalig *et al.*, 2011), and be coupled to quantitative PCR approaches and *in*
362 *situ* measurements of methyl halides using sensitive gas chromatographic
363 techniques such as electron capture detection.

364

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373

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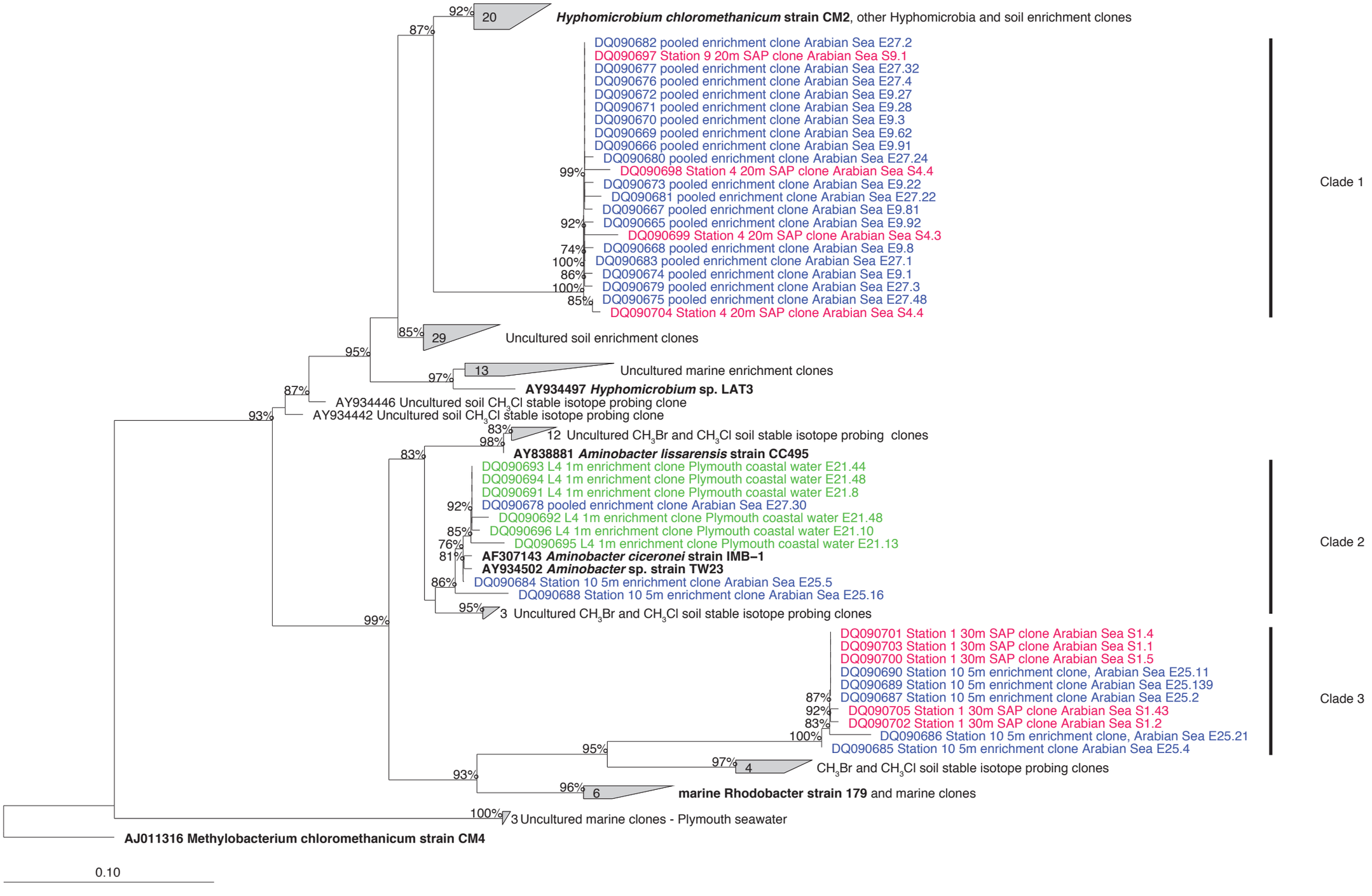
Fig 1. Maps of sampling locations. A, Arabian Sea AMBITION cruise track, traversed from South to North, crosses indicate sampling stations 1 (the southernmost) to 11 (northernmost); B, English channel sampling station L4 off the coast of Plymouth, UK. Latitude and longitude of each station are indicated.

Fig 2. Neighbour-joining with nearest neighbour interchange tree of CmuA amino acid sequences. Sequences from isolated methyl halide degrading organisms are highlighted in bold. Sequences obtained in this study are coloured: Arabian Sea SAP sequences in red, Arabian Sea enrichment culture sequences in blue and Plymouth seawater enrichment culture sequences in green. The tree is displayed with *Methylobacterium chloromethanicum* strain CM4 as root and the scale bar indicates 0.1 amino acid substitutions. Nearest neighbour interchange values from FastTree greater than 70% are displayed on the tree.


92%
20
Hyphomicrobium chloromethanicum strain CM2, other Hyphomicrobia and soil enrichment clones
87%
DQ090682 pooled enrichment clone Arabian Sea E27.2
DQ090697 Station 9 20m SAP clone Arabian Sea S9.1
DQ090677 pooled enrichment clone Arabian Sea E27.32
DQ090676 pooled enrichment clone Arabian Sea E27.4
DQ090672 pooled enrichment clone Arabian Sea E9.27
DQ090671 pooled enrichment clone Arabian Sea E9.28
DQ090670 pooled enrichment clone Arabian Sea E9.3
DQ090669 pooled enrichment clone Arabian Sea E9.62
DQ090666 pooled enrichment clone Arabian Sea E9.91
DQ090680 pooled enrichment clone Arabian Sea E27.24
99%
DQ090698 Station 4 20m SAP clone Arabian Sea S4.4
DQ090673 pooled enrichment clone Arabian Sea E9.22
DQ090681 pooled enrichment clone Arabian Sea E27.22
DQ090667 pooled enrichment clone Arabian Sea E9.81
92%
DQ090665 pooled enrichment clone Arabian Sea E9.92
DQ090699 Station 4 20m SAP clone Arabian Sea S4.3
74%
DQ090668 pooled enrichment clone Arabian Sea E9.8
100%
DQ090683 pooled enrichment clone Arabian Sea E27.1
86%
DQ090674 pooled enrichment clone Arabian Sea E9.1
100%
DQ090679 pooled enrichment clone Arabian Sea E27.3
85%
DQ090675 pooled enrichment clone Arabian Sea E27.48
DQ090704 Station 4 20m SAP clone Arabian Sea S4.4
Clade 1
85%
29
Uncultured soil enrichment clones
95%
13
Uncultured marine enrichment clones
97%
AY934497 Hyphomicrobium sp. LAT3
87%
AY934446 Uncultured soil CH3Cl stable isotope probing clone
93%
AY934442 Uncultured soil CH3Cl stable isotope probing clone
83%
12 Uncultured CH3Br and CH3Cl soil stable isotope probing clones
98%
AY838881 Aminobacter lissarensis strain CC495
83%
DQ090693 L4 1m enrichment clone Plymouth coastal water E21.44
DQ090694 L4 1m enrichment clone Plymouth coastal water E21.48
DQ090691 L4 1m enrichment clone Plymouth coastal water E21.8
92%
DQ090678 pooled enrichment clone Arabian Sea E27.30
DQ090692 L4 1m enrichment clone Plymouth coastal water E21.48
85%
DQ090696 L4 1m enrichment clone Plymouth coastal water E21.10
76%
DQ090695 L4 1m enrichment clone Plymouth coastal water E21.13
81%
AF307143 Aminobacter ciceronei strain IMB–1
AY934502 Aminobacter sp. strain TW23
86%
DQ090684 Station 10 5m enrichment clone Arabian Sea E25.5
DQ090688 Station 10 5m enrichment clone Arabian Sea E25.16
Clade 2
95%
3 Uncultured CH3Br and CH3Cl soil stable isotope probing clones
99%
DQ090701 Station 1 30m SAP clone Arabian Sea S1.4
DQ090703 Station 1 30m SAP clone Arabian Sea S1.1
DQ090700 Station 1 30m SAP clone Arabian Sea S1.5
DQ090690 Station 10 5m enrichment clone, Arabian Sea E25.11
DQ090689 Station 10 5m enrichment clone Arabian Sea E25.139
87%
DQ090687 Station 10 5m enrichment clone Arabian Sea E25.2
92%
DQ090705 Station 1 30m SAP clone Arabian Sea S1.43
83%
DQ090702 Station 1 30m SAP clone Arabian Sea S1.2
100%
DQ090686 Station 10 5m enrichment clone, Arabian Sea E25.21
DQ090685 Station 10 5m enrichment clone Arabian Sea E25.4
Clade 3
95%
97%
4
CH3Br and CH3Cl soil stable isotope probing clones
93%
96%
6
marine Rhodobacter strain 179 and marine clones
100%
3 Uncultured marine clones - Plymouth seawater
AJ011316 Methylobacterium chloromethanicum strain CM4
0.10

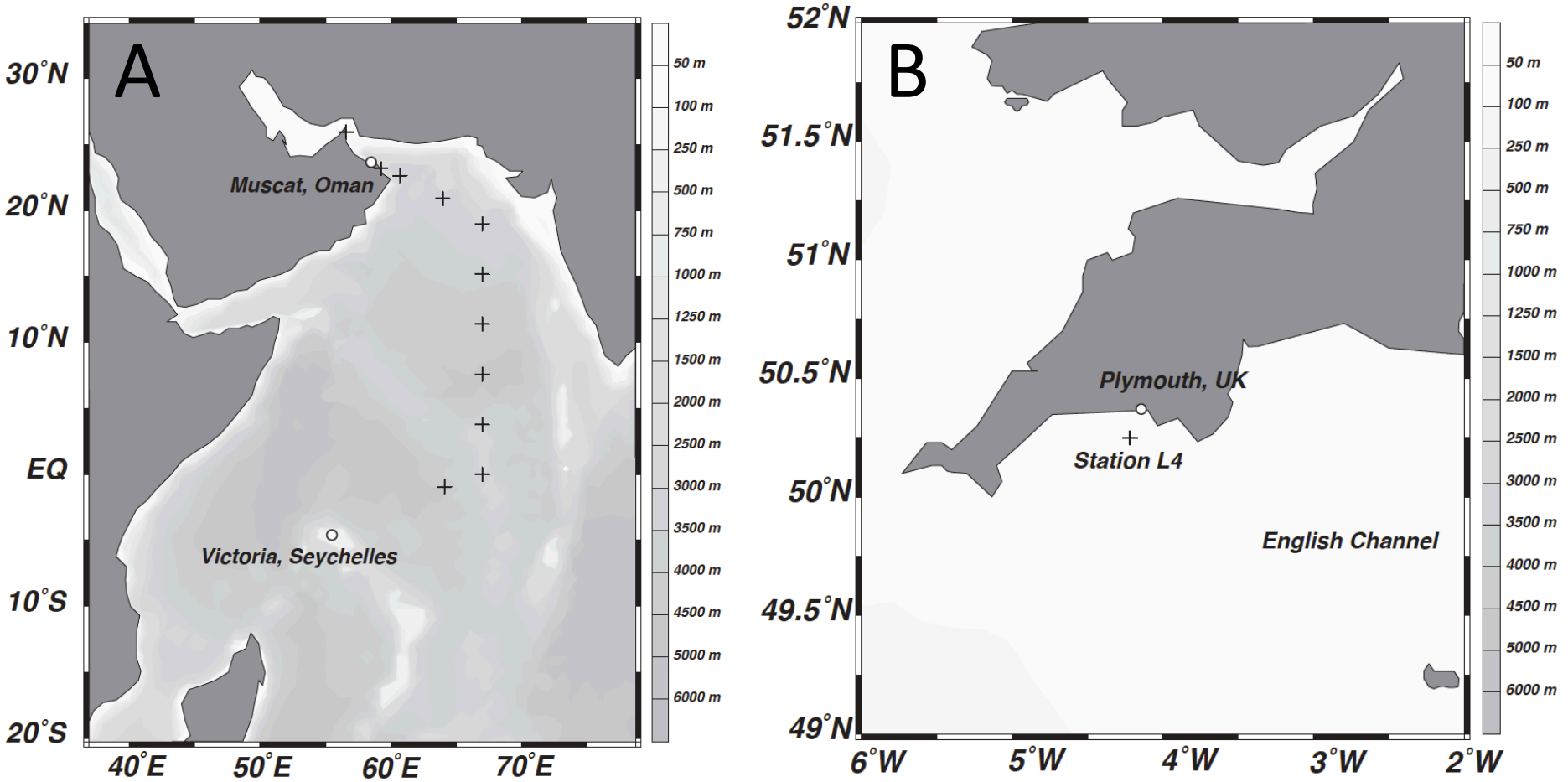

A
30°N
20°N
10°N
EQ
10°S
20°S
40°E
50°E
60°E
70°E
Muscat, Oman
Victoria, Seychelles
50 m
100 m
250 m
500 m
750 m
1000 m
1250 m
1500 m
2000 m
2500 m
3000 m
3500 m
4000 m
4500 m
5000 m
6000 m
B
52°N
51.5°N
51°N
50.5°N
50°N
49.5°N
49°N
6°W
5°W
4°W
3°W
2°W
Plymouth, UK
Station L4
English Channel

Figure 1

Table 1. Volumes of seawater sampled at sampling stations in the Arabian Sea using stand-alone pumps for which *cmuA* PCR products were obtained

Station	Sampling depth (m)	Volume filtered (L)	Effective volume sampled in PCR (mL)
1	30	96	80
4	20	200	167
9	20	36	30

Table 2. CH_3Br consumption by enrichment cultures

Enrichment source and number	Sampling date	CH_3Br consumed [µmol]
Arabian Sea, station 7 (165)	17/09/2001	223
Arabian Sea, station 7 (165.2)	18/09/2001	89
Pooled Arabian Sea enrichments (PE2)	3/09/2001-27/09/2001	580
Arabian Sea, station 8 (189)	20/09/2001	89
Arabian Sea, station 10 (249)	25/09/2001	223
Arabian Sea, station 11 (273)	26/09/2001	268
English Channel, enrichment L4.1	18/04/2002	313
English Channel, enrichment L4.2	20/06/2002	188
English Channel, enrichment L4.3	30/07/2002	188