Original citation:
Cox, M et al. Diversity of methyl halide-degrading microorganisms in oceanic and coastal waters. FEMS Microbiology Letters, 334(2), pp. 111-118

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

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Running title: Marine methyl halide degrading microorganisms

Keywords: methyl halide, functional genetic marker, Arabian Sea, English Channel, cmuA, functional diversity

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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$_3$X) methyl bromide (CH$_3$Br) and methyl chloride (CH$_3$Cl) 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$_3$Br, 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$_3$Br in surface ocean waters were significantly higher than chemical loss rates, indicating that biological pathways existed for the removal of CH$_3$Br from these waters. Examination of CH$_3$Br loss rates associated with individual size fractions of the marine biomass revealed that loss of CH$_3$Br 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 Methylothermus 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 CO₂, 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 MgCl₂, 200 µM each dNTP, 25 pmol of primers cmuAF802/cmuAR1609 (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 °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 °C, 1 min at 55 °C, and 1 min at 72 °C,
followed by final extension step of 72 °C for 10 min. Genomic DNA from *Hyphomicrobium chloromethanicum* strain CM2 was used as a positive control.

*Arabian Sea Enrichment Cultures*

Enrichment cultures were set up with seawater on a range of substrates during a research cruise on board the *RRS Charles Darwin* in 2001 (Cruise CD132). Water samples were taken at eleven stations (Fig. 1A) using a SeaBird rosette sampler equipped with 24 x 30 L Niskin bottles and CTD (conductivity, temperature and depth) devices. The exact system configuration can be found in the AMBITION Cruise report, from the Biological Oceanographic Data Centre website ([www.bodc.ac.uk/projects/m&fmb.html](http://www.bodc.ac.uk/projects/m&fmb.html)).

The Niskin bottles were sub-sampled using their integral taps and a short length of Tygon tubing into 2 L polycarbonate bottles rinsed three times with seawater sample.

2 L of water from 5 m depth (surface) and the chlorophyll maximum for each station (as determined by the CTD profile) were vacuum-filtered through 47 mm, 0.2 µm polyethersulfone Supor-200 filters (Pall-Gelman, Port Washington, NY) and the filtrate was then resuspended in ~3 mL of sample water. At station 6 (15°12.0′N 67°00.0′E) an additional set of enrichment cultures were set up with water sampled from a deep cast of 2501 m. An additional set was also taken at 250 m, station 8 (20°55.0′N 63°40.0′E), together with a final additional set at station 11 (26°00.0′N 56°35.0′E) at the 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 1 mg L⁻¹ cyanocobalamine. Twelve different carbon sources were added to the vials in different combinations and concentrations: 86 µM (0.1% vol/vol) CH₃Br; 430 µM (0.5% vol/vol) CH₃Br; 860 µM (1% vol/vol) CH₃Br; 50 mM "Aristar" methanol; 430 µM CH₃Br plus 50 mM methanol; 10 mM methylamine; 430 µM CH₃Br plus 10 mM methylamine; 430 µM (0.5%) CH₃Br plus 10 mM formate; 140 µM (10% vol/vol) methane; 1540 µM (2% vol/vol) CH₃Cl; 430 µM CH₃Br 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 °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 (CH₃X) 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*
Seawater was collected from the Western Channel Observatory site L4 (Fig 1) in the English Channel during routine sampling on the 18th April (L4.1), 20th June (L4.2), and 30th July (L4.3) 2002 using 5 L manually operated Niskin bottles from surface waters at approximately 1 m depth. On each date, 300 mL of seawater was transferred to 1.15 L crimp-seal flasks with butyl-rubber stoppers and 0.2% (v/v) headspace CH$_3$Br added (142 µM CH$_3$Br). L4.1 consumed 313 µmol CH$_3$Br in total; L4.2 and L4.3 consumed 188 µmol each.

PCR template from enrichment culture L4.1 was prepared as for the Arabian Sea enrichment cultures.

**Clone library construction**

PCR products were cloned using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Plasmid mini-preps were carried out from 2 mL of overnight culture using the alkaline lysis mini-prep procedure (Sambrook & Russell, 2001). Plasmid DNA was resuspended in 50 µL of sterile deionised water. Different RFLP types were assigned manually, and the first clone representing each type selected for sequencing, thus dereplicating the library. Double restriction digests were carried out using restriction enzymes EcoRI and Rsal or EcoRI and Ddel (Fermentas, Vilnius, Lithuania). 2 µL of 100 µg/mL RNase (Promega, Madison, WI) was added to each reaction. Restriction digests were incubated at 37 °C for 16 hours and analysed by agarose gel electrophoresis. Gels were stained with ethidium bromide to enable visualisation of the DNA fragments using UV-transillumination. Different RFLP types were assigned manually.
**Phylogenetic tree construction**

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

**Results**

*Detection of cmuA in large volume seawater DNA samples*

PCR products of the expected size (~807 bp) were only obtained from three of the nine cruise stations where stand alone pumps (SAPs) had been used to concentrate large volumes of seawater for DNA extraction; Faint PCR products were obtained from stations 1, 4 and 9. Libraries of 50 cmuA clones were produced from each of the PCR products. Clones were assigned to different RFLP pattern types by RFLP analysis with EcoRI/DdeI and
EcoRI/Rsal 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$_3$Br and CH$_3$Cl, 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$_3$X 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) CH₃Br 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 CH₃Br 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 CH₃Br, 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) CH₃Br and the amount of CH₃Br 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 \textit{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$_3$Br and CH$_3$Cl 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 \textit{Aminobacter} spp., a genus previously identified in terrestrial, rather than marine environments.

\textbf{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 methylothrophic bacteria. In a marine context, investigation of the diversity of
this functional genetic marker has previously been limited to detection in
marine methyl halide-degrading isolates and enrichment cultures (McAnulla et
al., 2001, Schäfer et al., 2005), in this study cmuA genes from marine
organisms have also been detected using direct amplification from
environmental DNA. The discovery of three new clades of marine cmuA
sequences in the relatively small number of samples investigated, indicates
that the diversity of bacterial populations utilising this pathway of methyl halide
degradation is higher than previously realised. Enrichment of methyl halide
degrading bacteria was successful from oligotrophic and meso/eutrophic
marine samples using methyl halides as sole carbon source. Interestingly,
subcultivation on methyl halides of pooled enrichments of methylotrophic
microorganisms using a range of C1 compounds also resulted in methyl
halide degrading cultures, suggesting that some of the methyl halide
degrading populations detected here may be representative of methylotrophs
that are not restricted to use of methyl halides alone. Methyl halide degrading
isolates of the Roseobacter clade obtained previously (Schaefer et al., 2002,
Schäfer et al., 2005) were all facultative methylotrophs, with some using more
than one C1 compound as carbon source, while for others, methyl halides
were the only C1 compounds (of those tested) supporting growth. Sequences
in clade 1 may represent populations degrading more than one C1 compound,
as this clade was entirely composed of sequences obtained from pooled
methylotrophic enrichments and from clones obtained directly from large
volume seawater DNA samples of stations 4 and 9 from the Arabian Sea.
Interestingly, clade 3 was only detected in enrichments on methyl halides
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 \textit{cmuA} sequences. Clade 2 was detected in the Arabian Sea, Plymouth coastal waters and \textit{Aminobacter} spp. isolated from soils. Given the enrichment methods used, it is not possible to associate particular sequences or clades of \textit{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 \textit{cmuA} diversity would be required to investigate whether this might indicate distinct ecological niches for these \textit{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 \textit{Roseobacter} and \textit{Methylophaga} in samples from the English Channel (Neufeld \textit{et al.}, 2008). The primer set for amplifying \textit{cmuA} genes has demonstrated that it is useful in the discovery of
novel clades, thus proving its worth for further environmental studies, but
future studies should also utilise other primer sets developed more recently
(Nadalig et al., 2011), and be coupled to quantitative PCR approaches and in
situ measurements of methyl halides using sensitive gas chromatographic
techniques such as electron capture detection.

Acknowledgements

This work was funded under the NERC Marine and Freshwater Microbial
Biodiversity thematic programme, grant number NE/C001/923/1. We thank
the officers and crew of RVS Sepia, Squilla and Plymouth Quest, RRS
Charles Darwin and the AMBITION cruise participants for their assistance in
obtaining samples. We thank Clare Bird and Mike Wyman (University of
Stirling) for supplying stand alone pump DNA samples and Gez Chapman
(University of Warwick) for technical assistance.
References


Goodwin KD, Tokarczyk R, Stephens FC & Saltzman ES (2005) Description of toluene inhibition of methyl bromide biodegradation in seawater and...
isolation of a marine toluene oxidizer that degrades methyl bromide.


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.
Hyphomicrobium chloromethanicum strain CM2, other Hyphomicrobia and soil enrichment clones

Clade 1

- DQ090682 pooled enrichment clone Arabian Sea E27.2
- DQ090687 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 E9.81
- DQ090668 pooled enrichment clone Arabian Sea E27.1
- DQ090665 pooled enrichment clone Arabian Sea E9.92
- DQ090689 Station 4 20m SAP clone Arabian Sea S4.4
- DQ090681 pooled enrichment clone Arabian Sea E27.24
- DQ090698 Station 4 20m SAP clone Arabian Sea S4.4

Clade 2

- DQ090674 pooled enrichment clone Arabian Sea E9.1
- DQ090679 pooled enrichment clone Arabian Sea E27.3
- DQ090675 pooled enrichment clone Arabian Sea E27.48
- DQ090673 pooled enrichment clone Arabian Sea E9.22
- DQ090678 pooled enrichment clone Arabian Sea E27.1
- DQ090667 pooled enrichment clone Arabian Sea E9.8
- DQ090666 pooled enrichment clone Arabian Sea E9.91
- DQ090665 pooled enrichment clone Arabian Sea E9.92
- DQ090668 pooled enrichment clone Arabian Sea E9.8
- DQ090667 pooled enrichment clone Arabian Sea E9.81
- AY934446 Uncultured soil CH₃Cl stable isotope probing clone
- AY934442 Uncultured soil CH₃Cl stable isotope probing clone
- AY934452 Uncultured soil CH₃Br and CH₃Cl stable isotope probing clone

Clade 3

- DQ090693 L4 1m enrichment clone Plymouth coastal water E21.44
- DQ090694 L4 1m enrichment clone Plymouth coastal water E21.48
- DQ090696 L4 1m enrichment clone Plymouth coastal water E21.8
- DQ090676 L4 1m enrichment clone Plymouth coastal water E21.30
- DQ090692 L4 1m enrichment clone Plymouth coastal water E21.10
- DQ090691 L4 1m enrichment clone Plymouth coastal water E21.13
- DQ090688 Station 10 5m enrichment clone Arabian Sea E25.5
- DQ090689 Station 10 5m enrichment clone Arabian Sea E25.139
- DQ090690 Station 10 5m enrichment clone Arabian Sea E25.16
- DQ090687 Station 10 5m enrichment clone Arabian Sea E25.2
- DQ090686 Station 10 5m enrichment clone Arabian Sea E25.21
- Clade 3 Uncultured CH₃Br and CH₃Cl soil stable isotope probing clones

Uncultured soil enrichment clones

- AJ011316 Methylobacterium chloromethanicum strain CM4
- Clade 1 Uncultured soil enrichment clones
- Clade 2 Uncultured soil enrichment clones
- Clade 3 Uncultured soil enrichment clones

Uncultured marine enrichment clones

- AY934446 Uncultured soil CH₃CI stable isotope probing clone
- AY934442 Uncultured soil CH₃CI stable isotope probing clone
- AY938881 Aminobacter lissarensis strain CC495
- AY934502 Aminobacter sp. strain TW23
- AY934503 Aminobacter ciceronei strain IMB−1
- AY934502 Aminobacter sp. strain TW23
- DQ090688 Station 10 5m enrichment clone Arabian Sea E25.5
- DQ090689 Station 10 5m enrichment clone Arabian Sea E25.139
- DQ090687 Station 10 5m enrichment clone Arabian Sea E25.16
- DQ090686 Station 10 5m enrichment clone Arabian Sea E25.2
- DQ090685 Station 10 5m enrichment clone Arabian Sea E25.21
- Aminobacter ciceronei strain IMB−1
- Aminobacter sp. strain TW23
- DQ090688 Station 10 5m enrichment clone Arabian Sea E25.5
- DQ090689 Station 10 5m enrichment clone Arabian Sea E25.139
- DQ090687 Station 10 5m enrichment clone Arabian Sea E25.16
- DQ090686 Station 10 5m enrichment clone Arabian Sea E25.2
- DQ090685 Station 10 5m enrichment clone Arabian Sea E25.21
- Clade 3 Uncultured CH₃Br and CH₃Cl soil stable isotope probing clones
- marine Rhodobacter strain 179 and marine clones
- marine Rhodobacter strain 179 and marine clones
- 3 Uncultured marine clones - Plymouth seawater

92% 74% 99% 100% 92% 74% 100% 99% 86% 100% 86% 100% 99% 100% 97% 92% 100% 97% 92% 100% 97% 100% 92% 87% 4 100% 92% 95% 6 100% 92% 95% 6 100%
Table 1. Volumes of seawater sampled at sampling stations in the Arabian Sea using stand-alone pumps for which cmuA PCR products were obtained

<table>
<thead>
<tr>
<th>Station</th>
<th>Sampling depth (m)</th>
<th>Volume filtered (L)</th>
<th>Effective volume sampled in PCR (mL)</th>
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<tr>
<td>1</td>
<td>30</td>
<td>96</td>
<td>80</td>
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<td>4</td>
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<td>9</td>
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Table 2. \( \text{CH}_3\text{Br} \) consumption by enrichment cultures

<table>
<thead>
<tr>
<th>Enrichment source and number</th>
<th>Sampling date</th>
<th>( \text{CH}_3\text{Br} ) consumed [( \mu\text{mol} )]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabian Sea, station 7 (165)</td>
<td>17/09/2001</td>
<td>223</td>
</tr>
<tr>
<td>Arabian Sea, station 7 (165.2)</td>
<td>18/09/2001</td>
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</tr>
<tr>
<td>Arabian Sea, station 8 (189)</td>
<td>20/09/2001</td>
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</tr>
<tr>
<td>Arabian Sea, station 10 (249)</td>
<td>25/09/2001</td>
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<td>Arabian Sea, station 11 (273)</td>
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<td>313</td>
</tr>
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<td>English Channel, enrichment L4.2</td>
<td>20/06/2002</td>
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