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

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13

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15

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18

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22 **Abstract:**

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

37

38

39 **Introduction**

40 The methyl halides (CH₃X) methyl bromide (CH₃Br) and methyl chloride
41 (CH₃Cl) are volatile organic compounds with natural and anthropogenic
42 sources, and are present at trace amounts in the atmosphere (10 and 600
43 parts per trillion by volume [pptv] respectively). Despite their low atmospheric
44 concentration, they have a large impact on atmospheric chemistry, delivering
45 bromine and chlorine atomic radicals arising from the breakdown of methyl
46 halides to the stratosphere where they catalyse ozone destruction. The
47 oceans are both a source and a sink of CH₃Br, but overall are a net sink (for a
48 review of methyl halide biogeochemistry (see Schäfer *et al.*, 2007).

49 King & Saltzman (1997) demonstrated that biological loss rates for CH₃Br in
50 surface ocean waters were significantly higher than chemical loss rates,
51 indicating that biological pathways existed for the removal of CH₃Br from
52 these waters. Examination of CH₃Br loss rates associated with individual size
53 fractions of the marine biomass revealed that loss of CH₃Br was associated
54 with the fraction that encompassed the bacterial size range.

55

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

64 2000), however, only one pathway has been identified that is specific for
65 methyl halide degradation in methylotrophic bacteria that utilise methyl halides
66 as sole source of carbon and energy (Vannelli *et al.*, 1999). The initial reaction
67 of the pathway is catalysed by CmuA, a methyltransferase/corrinoid-binding
68 protein which transfers the methyl group of the methyl halide to the Co atom
69 of a corrinoid group on the same enzyme. The methyl group is next
70 transferred to tetrahydrofolate by another methyltransferase (CmuB) and the
71 methyl tetrahydrofolate is progressively oxidised to formate and CO₂, with
72 carbon assimilation at the level of methylene tetrahydrofolate (Vannelli *et al.*,
73 1999). Several species of bacteria use this methyltransferase-based pathway
74 have been isolated from a range of environments, including soils, plant
75 phyllosphere, and the marine environment (Doronina *et al.*, 1996, Connell-
76 Hancock *et al.*, 1998, Goodwin *et al.*, 1998, Coulter *et al.*, 1999, Hoeft *et al.*,
77 2000, McAnulla *et al.*, 2001, Schaefer *et al.*, 2002, Borodina *et al.*, 2005,
78 Schäfer *et al.*, 2005, Nadalig *et al.*, 2011). The unique structure of CmuA has
79 been exploited to design primers for studying the diversity of methyl halide
80 degrading bacteria in the environment (McDonald *et al.*, 2002, Miller *et al.*,
81 2004, Borodina *et al.*, 2005, Schäfer *et al.*, 2005, Nadalig *et al.*, 2011).

82

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

88

89 **Methods**

90 *Large volume seawater DNA samples*

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

95

96 SAPs were left in place varying times and the sample volume through the
97 293 mm diameter, 0.2 µm pore size filters was calculated using time and flow
98 rate (Table 1). DNA extraction was achieved by rinsing SAP filters in 5 mL
99 filtered seawater, then the filtrate was taken up in 1 mL RNALater (Ambion)
100 and stored at 4°C. 0.5 mL of this was centrifuged (14,000 x g and DNA
101 isolated from the resulting pellet using a Qiagen DNA extraction kit with the
102 DNA eluted in 100 µL sterile deionised water (Mike Wyman, pers. comm.).
103 One µL of this DNA extract, or of a 1:10 diluted extract (typically 5-50ng of
104 DNA), was used as template for PCR amplification of *cmuA*. PCR reaction
105 mixtures were 2.5 mM MgCl₂, 200 µM each dNTP, 25 pmol of primers
106 *cmuAF802/cmuAR1609* (Miller *et al.*, 2004), 1.3 M betaine, 1.3% (vol/vol)
107 DMSO, in 1 x Invitrogen *Taq* DNA Polymerase buffer and 2.5 U of *Taq* DNA
108 Polymerase (Invitrogen, Paisley, UK) in a total volume of 50 µL, made up with
109 sterile deionised water. Thermal cycling was carried out on a Hybaid
110 Touchdown thermal cycler with initial denaturation at 95 °C for 5 min,
111 whereupon the *Taq* DNA Polymerase was added as a hot start. This was
112 followed by 35 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C,

113 followed by final extension step of 72 °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.

137 100 μL of the filtrate suspension was added to each of twelve pre-prepared
138 25 mL, crimp-sealed, gas-tight, enrichment vials containing 5 mL of 0.1 x
139 Ammonium Nitrate Mineral Salts (ANMS) medium (Whittenbury *et al.*, 1970)
140 with 3.5% (w/v) NaCl, trace element solution SL-10 (Widdel *et al.*, 1983) and
141 0.02 mg L⁻¹ folic acid, 1 mg L⁻¹ *p*-aminobenzoic acid and 1mg L⁻¹
142 cyanocobalamine. Twelve different carbon sources were added to the vials in
143 different combinations and concentrations: 86 μM (0.1% vol/vol) CH₃Br; 430
144 μM (0.5% vol/vol) CH₃Br; 860 μM (1% vol/vol) CH₃Br; 50 mM "Aristar"
145 methanol; 430 μM CH₃Br plus 50 mM methanol; 10 mM methylamine; 430 μM
146 CH₃Br plus 10 mM methylamine; 430 μM (0.5%) CH₃Br plus 10 mM formate;
147 140 μM (10% vol/vol) methane; 1540 μM (2% vol/vol) CH₃Cl; 430 μM CH₃Br
148 plus 10 mM L-methionine. Aqueous phase concentrations of gases were
149 calculated using the Henry's Law constants (DeBruyn and Saltzman 1997).
150 Enrichment cultures were incubated at 20 °C in the dark to prevent growth of
151 photosynthetic organisms, for approximately 2 months.

152 After incubation, the cultures were scored qualitatively for turbidity. The
153 presence or absence of headspace methyl halides (CH₃X) was tested using
154 gas chromatography with flame ionisation detection as described previously
155 (Schäfer *et al.*, 2005). Two mL of each enrichment was centrifuged for 5 min
156 at 14,000 x *g*, the supernatant removed and the pellet resuspended in 10 μL
157 of sterile deionised water. This was then boiled for 10 min in a water bath and
158 1 μL was used as template in PCR reactions.

159

160 *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 CH₃Br added (142 μM CH₃Br). L4.1
167 consumed 313 μmol CH₃Br 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 *EcoRI* and *RsaI* or *EcoRI* and *DdeI* (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 °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 *EcoRI/DdeI* and

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

216

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

223

224 *Arabian Sea Enrichment Cultures*

225 Enrichment cultures with different concentrations of CH₃Br and CH₃Cl, either
226 alone or together with a range of one carbon (C1) compounds (see methods
227 for details) were set up during the AMBITION cruise. Removal of the CH₃X
228 was confirmed by gas chromatography of headspace gas in comparison to
229 sterile chemical controls set up at the same time. Although a number of
230 enrichment vials were depleted of methyl halides even after as little as two
231 weeks of incubation, many of these cultures failed to degrade a second pulse
232 of methyl halide addition to the headspace. This, accompanied by an optical
233 density (560nm) of at least 0.4 was used to determine that there had
234 potentially been enrichment of methyl halide-degrading microorganisms.

235 Enrichment cultures that showed successful enrichment of methyl halide-
236 degrading microorganisms are reported in Table 2. Enrichment numbers 165,
237 165.2, 189, 249 and 273, all cultures initially supplied with formate (10mM)
238 and methyl bromide (430 μ M), degraded between 89 and 268 μ mol of methyl
239 bromide. These cultures were subcultured at least twice into fresh 0.1 x
240 ANMS medium with 0.2% (v/v) CH₃Br in the headspace. GC monitoring of
241 these enrichment cultures was carried out at intervals of approximately one to
242 two weeks meaning that it was not possible to accurately determine the time
243 of depletion of substrate. Generally, initial degradation of methyl halides of
244 these enrichments required at least one month, and the time it took to
245 degrade the total amount of methyl halide shown in Table 2 was between two
246 and four months. Enrichment cultures initially supplied with methanol,
247 methylamine, formate and methane as enrichment substrates were pooled,
248 amended with an additional 0.2% (v/v) headspace CH₃Br and subcultured
249 again. This pooled enrichment culture (PE2) also degraded methyl bromide
250 (580 μ mol in total) over the course of four months.. PCR products generated
251 using the *cmuA* primer pair from two of these enrichment cultures, the station
252 8 enrichment (189) and the pooled enrichments (PE2) which had consumed
253 89 and 580 μ mol of CH₃Br, respectively) were cloned as before.

254

255 *English Channel Enrichment Cultures*

256 An alternative enrichment strategy was used with samples of seawater from
257 L4, a sampling station off the coast of Plymouth. Larger volumes of water
258 unamended with media were incubated with 0.2% (v/v) CH₃Br and the amount
259 of CH₃Br consumed was recorded (Table 2). PCR products were obtained

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

262

263 The four clone libraries were dereplicated by RFLP, as for the SAP sample
264 libraries, and representative clones were sequenced. Phylogenetic trees of
265 *cmuA* sequences from all seven libraries were constructed (Fig 2), and
266 indicated that sequences fell into three major clades with strong nearest
267 neighbour interchange value support. Two of these clades (1 and 3) are
268 novel, with no similar CmuA sequences from extant bacteria. The closest
269 relatives of clade 1 members were cloned *cmuA* genes from soils and
270 *Hyphomicrobium chloromethanicum* CM2 *cmuA* with pairwise identities of
271 approximately 91% and 90% at the protein level, respectively, while the
272 closest relatives of clade 3 were *cmuA* genes obtained from soil CH₃Br and
273 CH₃Cl stable isotope probing experiments (approximately 85% similarity) and
274 marine strains 179 and 198 of the Rhodobacteraceae (similarity of
275 approximately 83%). The third clade had not been found in marine samples
276 previously and shared high similarity (95-99%) with *cmuA* sequences from
277 *Aminobacter* spp., a genus previously identified in terrestrial, rather than
278 marine environments.

279

280 **Discussion**

281 This study has revealed the presence in two distinct marine environments of
282 genes encoding the methyltransferase/corrinoid binding protein CmuA, which
283 carries out the first step in the methyl halide degradation pathway of
284 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.

310

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

333

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

346

347 The ecology and diversity of marine methyl halide degrading microorganisms
348 and their role in the biogeochemical cycling of methyl halides remains a
349 challenging field of biological oceanography. Further work is required to
350 determine the extent to which methyl bromide is oxidised to CO₂ or
351 assimilated into microbial biomass in seawater. The diversity and activity of
352 methyl halide-utilising bacteria in these environments should also be studied
353 in more detail. Stable isotope probing with ¹³C-methyl bromide is a potential
354 approach for detecting active methyl halide degrading bacteria based on
355 assimilation of methyl halide carbon during growth linked catabolism and has
356 been used to detect bacteria related to *Roseobacter* and *Methylophaga* in
357 samples from the English Channel (Neufeld *et al.*, 2008). The primer set for
358 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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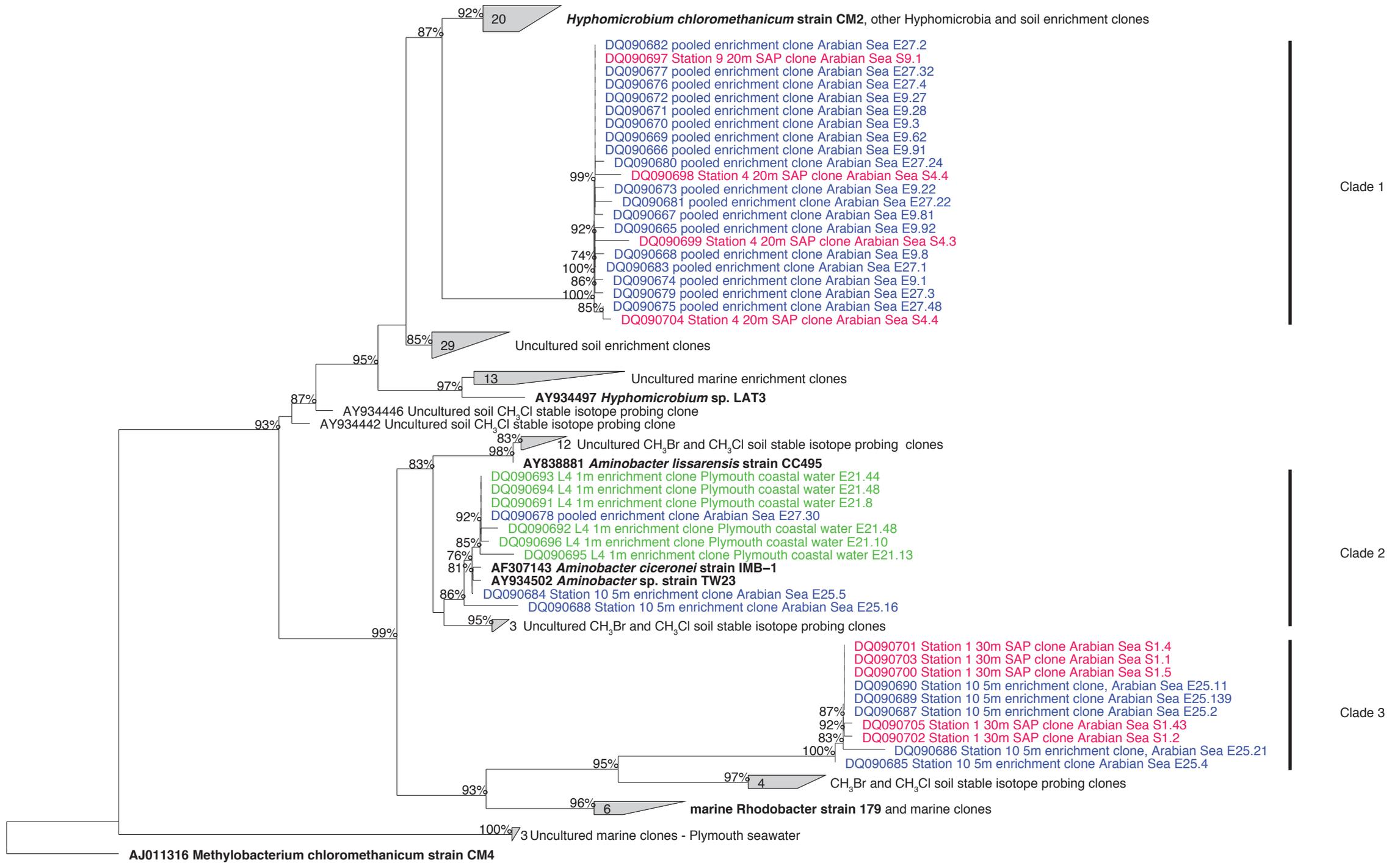
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470 **Fig 1. Maps of sampling locations.** A, Arabian Sea AMBITION cruise track,
471 traversed from South to North, crosses indicate sampling stations 1 (the
472 southernmost) to 11 (northernmost); B, English channel sampling station L4
473 off the coast of Plymouth, UK. Latitude and longitude of each station are
474 indicated.

475

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



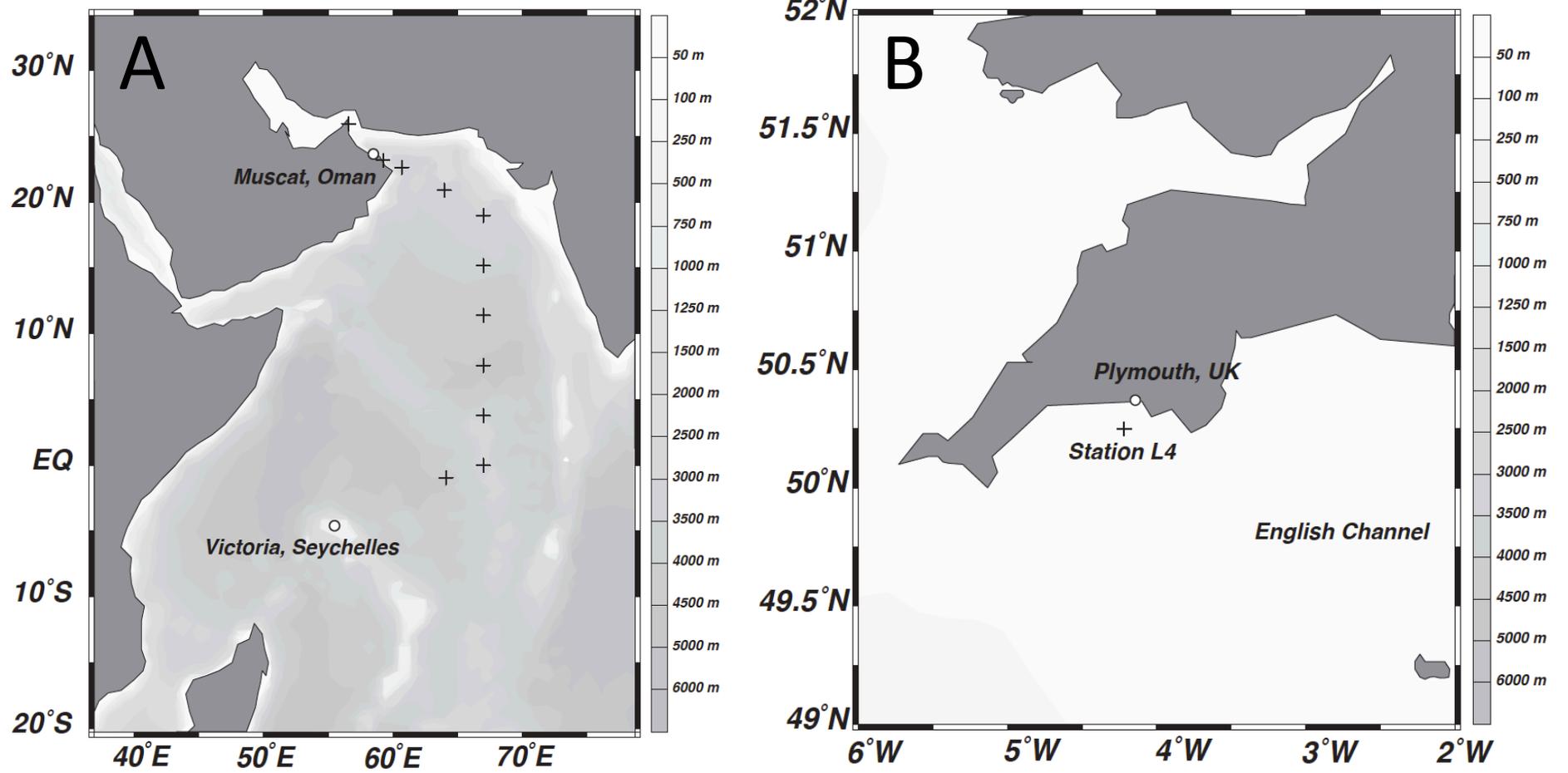


Figure 1

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. CH₃Br consumption by enrichment cultures

Enrichment source and number	Sampling date	CH ₃ Br 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