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Author(s): Boden, Rich; Thomas, Elizabeth; Savani, Parita; Kelly, Donovan P.; Wood, Ann P

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3 **Novel methylotrophic bacteria isolated from the River Thames**
4 **(London, UK)**

5

6 **Rich Boden,^{1,2} Elizabeth Thomas,¹ Parita Savani,¹ Donovan P. Kelly^{2*} and Ann P.**
7 **Wood³**

8

9 ¹ *Department of Life Sciences, King's College London, Franklin Wilkins Building, 150*
10 *Stamford Street, London SE1 9NH, UK.*

11 ² *Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK.*

12 ³ *Department of Microbiology, King's College London Dental Institute, Floor 17 Tower*
13 *Wing, Guy's Campus, London SE1 9RT, UK*

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17 Running title: Methylotrophs from the River Thames.

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20 * For correspondence. E-mail D.P.Kelly@warwick.ac.uk; Tel. (+44) (0)24 7657 2907; Fax
21 (+44) (0)24 7652 3701.

22 **Summary**

23

24 **Enrichment and elective culture for methylotrophs from sediment of the River Thames**
25 **in central London yielded a diversity of pure cultures representing several genera of**
26 **Gram-negative and Gram-positive bacteria, which were mainly of organisms not**
27 **generally regarded as typically methylotrophic. Substrates leading to successful**
28 **isolations included methanol, monomethylamine, dimethylamine, trimethylamine,**
29 **methanesulfonate and dimethylsulfone. Several isolates were studied in detail and**
30 **shown by their biochemical and morphological properties and 16S rRNA gene**
31 **sequencing to be *Sphingomonas melonis* strain ET35, *Mycobacterium fluoranthenvorans***
32 **strain DSQ3, *Rhodococcus erythropolis* strain DSQ4, *Brevibacterium casei* strain MSQ5,**
33 ***Klebsiella oxytoca* strains MMA/F and MMA/1, *Pseudomonas mendocina* strain TSQ4,**
34 **and *Flavobacterium* sp. strains MSA/1 and MMA/2. The results show that facultative**
35 **methylotrophy is present across a wide range of *Bacteria*, suggesting that turnover of**
36 **diverse C₁-compounds is of much greater microbiological and environmental**
37 **significance than is generally thought. The origins of the genes encoding the enzymes of**
38 **methylotrophy in diverse heterotrophs need further study, and could further our**
39 **understanding of the phylogeny and antiquity of methylotrophic systems.**

40

41 **Introduction**

42 Bacteria with the ability to grow on one-carbon compounds as their sole source of energy and
43 carbon have been known since the late 19th century (Loew, 1892). One of the earliest
44 organisms isolated was the Gram-positive bacterium, *Bacillus methylicus* (later renamed
45 *Bacterium methylicum*, but no longer available in culture): an aerobic, non-spore-forming,
46 facultative methylotroph producing red pigmentation when grown on formate or methanol,
47 and which also grew on formaldehyde (Loew, 1892; Migula, 1900; Bergey *et al.*, 1939).
48 During the 20th century, numerous specialized methylotrophs were described, including a
49 great diversity of methanotrophs, some of which were obligate methane-users, many also
50 using methanol, and a few being capable of growth on multicarbon compounds (Whittenbury
51 *et al.*, 1970; Dedysh *et al.*, 2005a). Methylotrophy came to be recognized as a property of
52 specialized bacteria capable of growth on C₁-compounds (and some of which would only
53 grow on C₁-compounds), with the names of numerous genera and species reflecting this
54 concept (e.g. *Methylobacterium*, *Methylomonas*, *Methylosinus*, *Methyloversatilis*, *Bacillus*
55 *methanolicus*; Whittenbury *et al.*, 1970; Arfman *et al.*, 1992; Kalyuzhnaya *et al.*, 2006;
56 Lidstrom, 2006). The occurrence of methylotrophy in prokaryotes was subsequently realized
57 not to correlate with traditional bacterial classification (Brautaset *et al.*, 2004), as facultative
58 methylotrophy was progressively shown to be a property of diverse typically heterotrophic
59 genera including *Paracoccus*, *Hyphomicrobium*, *Micrococcus*, *Arthrobacter*, *Brevibacterium*,
60 *Beijerinckia*, *Bacillus*, *Klebsiella*, *Afipia*, *Variovorax*, *Amycolatopsis*, *Mycobacterium*, and
61 *Acinetobacter* (Nishio *et al.*, 1975; Bamforth and Quayle, 1978; Duménil *et al.*, 1979;
62 Levering *et al.*, 1981; Nazina, 1981; Dijkhuizen *et al.*, 1988; Kato *et al.*, 1988; Nesvera *et al.*,
63 1991; Cercel, 1999; Mitsui *et al.*, 2000; Alves *et al.*, 2001; Borodina *et al.*, 2002; Anesti *et*
64 *al.*, 2005; Moosvi *et al.*, 2005a, b; Dedysh *et al.*, 2005b; Kelly *et al.*, 2006; Lidstrom, 2006;
65 Ghosh *et al.*, 2007). The ribulose monophosphate (RuMP) cycle and its key enzymes (3-

66 hexulose-6-phosphate synthase and 6-phospho-3-hexulose isomerase) were once regarded as
67 diagnostic characters of some methylotrophs (Dijkhuizen *et al.*, 1992), but are now known to
68 be widespread among bacteria and Archaea, for formaldehyde fixation and detoxification,
69 and ribulose 5-phosphate biosynthesis in Archaea (Reizer *et al.*, 1997; Yasueda *et al.*, 1999;
70 Kato *et al.*, 2006; Orita *et al.*, 2006). The RuMP cycle also seems to function not only in
71 “typical” methylotrophs, but in methylotrophic *Brevibacterium*, *Bacillus brevis* and other
72 Gram-positive methylotrophs (Yurimoto *et al.*, 2002; Anesti *et al.*, 2005). Examination of the
73 GenBank database shows the occurrence of the gene for 3-hexulose-6-phosphate synthase in
74 the genomes of at least 12 species of *Gammaproteobacteria*, and the gene for
75 hydroxypyruvate reductase (a key enzyme in the serine pathway of formaldehyde fixation)
76 occurs in numerous members of the *Alpha*-, *Beta*-, *Gamma*- and *Deltaproteobacteria* (D. P.
77 Kelly, database searches, unpublished). The development and persistence of methylotrophic
78 pathways in phylogenetically diverse bacteria and Archaea is not surprising, given the
79 evidence for the activity for methanotrophic and methylotrophic organisms in the late
80 Archaean (2.8 billion years ago; Brocks *et al.*, 2003).

81 While classical methods of enrichment culture led to the isolation of most currently
82 recognized methylotrophs, more recent studies have used molecular methods for the
83 detection of the organisms in samples of environmental DNA. These have included the
84 detection of enzyme-encoding genes (such as methanol dehydrogenase) and the use of the
85 polymerase chain reaction with primers for the 16S rRNA genes of known methylotrophs
86 (McDonald and Murrell, 1997; Wang *et al.*, 2004; Anesti *et al.*, 2005). The development of
87 stable isotope probing (SIP), in which soil or water samples are incubated with ¹³C-labelled
88 methane or methanol, has enabled the isolation of ¹³C-labelled DNA produced by
89 methylotrophs, and their subsequent identification by 16S rRNA gene analysis (Radajewski
90 *et al.*, 2000, 2002; Ginige *et al.*, 2004; Borodina *et al.*, 2005; McDonald *et al.*, 2005;
91 Nercessian *et al.*, 2005). SIP sometimes reveals ¹³C-labelling in the DNA of organisms not

92 previously regarded as methylotrophs, such as the Flavobacteria, but by direct culture some
93 *Flavobacterium* strains are now known to be methylotrophic (Moosvi *et al.*, 2005b). There is
94 thus a need for the isolation into pure culture of novel strains detected by SIP if they are to be
95 rigorously assessed for methylotrophy.

96 We have used classical enrichment culture and isolation on various elective C₁-
97 substrates in a study of methylotrophs present in the River Thames, with the aim of obtaining
98 a ‘snapshot’ overview of the diversity of such bacteria in the river, and to recover and
99 identify novel organisms. Microbiological studies of the Thames date back to the 19th
100 century, with numerous bacteria being reported by Frankland (1885, 1899) and Ward (1898,
101 1899). The river is routinely monitored for its bacterial load, especially with respect to faecal
102 contamination, but prior to our studies, which have been conducted over a five-year period
103 (2002-2006), there had been no report of any methylotrophs in the River Thames where it
104 runs through central London.

105

106 **Results and Discussion**

107

108 *Diversity of methylotrophic bacteria recovered from the River Thames, 2002-2006*

109

110 Samples were taken on six separate occasions and enrichments set up with methanol,
111 monomethylamine (MMA), dimethylamine (DMA), trimethylamine (TMA),
112 methanesulfonate (MSA), or dimethylsulfone (DMSO₂) as elective substrates. Numerous
113 colony types were observed on subsequent plating on to agar media, with as many as five
114 different morphologies and colours from one sampling. These ranged in colour from white to
115 orange, but few appeared to give the characteristic pink pigmentation of the facultative
116 methylotroph, *Methylobacterium* (Kelley *et al.*, 2004; Green, 2006; Lidstrom, 2006),
117 suggesting that most methylotrophs recovered were examples of other genera. Of the new

118 isolates studied in detail, most were obtained from liquid shake-flask enrichment cultures, but
119 strains DSQ3 and DSQ4 were obtained from direct plating of sediment suspensions from the
120 Sugar Quay site on to DMA-agar medium. These two strains grew very poorly in liquid
121 DMA shake-flask cultures and were routinely grown and maintained on DMA in static
122 culture or on agar slants. These strains would thus have been unlikely to have been recovered
123 by the shake-flask enrichment method. Only strains positively assigned to specific genera are
124 described below, but additional strains of *Rhodococcus*, *Arthrobacter*, *Xanthobacter*, and
125 *Paracoccus* were also tentatively identified among the numerous other methylotrophic
126 isolates obtained, growing variously on methanol, formate, or DMSO₂.

127 Substrates on which the isolates were obtained, and their growth substrate ranges are
128 summarized in Table 1, and Gram-stain, physiological and morphological properties of the
129 novel isolates are summarized in Tables 2 and 3.

130

131 [TABLE 1]

132

133 *Mycobacterium fluoranthenorans* strain DSQ3

134

135 Strain DSQ3 showed short straight to curved rods during active growth (Fig. 1), but became
136 more pleomorphic with changes from long thin rods to vibrioid and spiral morphology in
137 older cultures. Its partial 16S rRNA gene sequence (944 bp; EU416230) showed 99.6%
138 identity (937/941 nucleotides) to *Mycobacterium fluoranthenorans* (AJ617741; Hormish *et al.*,
139 2004), which is a high G-C, acid-fast, but non-pigmented *Mycobacterium*. The orange
140 pigmentation of strain DSQ3 is, however, common in *Mycobacterium* species (Holt *et al.*,
141 1994). The G+C content of its DNA (73.8 mol%) was slightly above the range reported for
142 *Mycobacterium* (64-71 mol%; Wayne and Gross, 1968; Hartmans *et al.*, 2006). Unlike some
143 *Mycobacterium* species (Holt *et al.*, 1994), strain DSQ3 was catalase negative, and some

144 cells were observed in Gram-stained preparations to have single, central, swellings, typical of
145 endospores. Methanol-using *Mycobacterium* strains have previously been reported (Urakami
146 and Yano, 1989; Galkin *et al.*, 2006), and a facultative methanol-using strain of *M. gastri*
147 was shown to use the RuMP cycle (Kato *et al.*, 1988; Mitsui *et al.*, 2000). Growth was
148 supported by DMA as the sole source of carbon and energy, but little or no growth occurred
149 with MMA, TMA, methanol, formate, MSA, and DMSO₂. Chemoorganotrophic growth was
150 shown on a wide range of multicarbon substrates (Table 2).

151

152 *Rhodococcus erythropolis* strain DSQ4

153

154 Strain DSQ4 showed a filamentous rod-coccus cell morphology, with individual cells varying
155 between 2-6 µm in length and 0.5 µm diameter during the growth cycle, and typically 2 µm
156 length during rapid growth on DMA. Its 16S rRNA gene sequence (1418 bp; EU481631)
157 showed 99.6% sequence identity to *Rhodococcus erythropolis*. Its physiological properties
158 and the observed morphology were typical of *Rhodococcus* (Table 2; Fig. 2). The G+C
159 content of its DNA (67.2 mol%) was within the range seen for *Rhodococcus* (67-73 mol%
160 G+C). Methylophilic growth was obtained on DMA, but was very slow or absent on
161 methanol, formate, MMA, TMA, MSA, DMSO₂. It was moderately sensitive to sodium
162 chloride: growth yields on sucrose were depressed by 18, 42 and 69% by 1.25, 2.5 and 5%
163 (w/v) NaCl. Methylophilicity in *Rhodococcus* has not previously been studied.

164

165 *Brevibacterium casei* strain MSQ5

166

167 Strain MSQ5 was identified as *B. casei* from its physiological properties (Table 2) and a
168 partial 16S rRNA gene sequence (343 bp; EU815829), which showed 100% identity to the
169 corresponding nucleotides of the type strain (DSM 20657^T, AJ252418; NCDO 2048,

170 X76564) and strain FM1A (AY468375), and 99.7% identity (342/343 aligned nucleotides) to
171 *B. casei* strains 3Tg (AY468375) and 3S(a) (AY468374), all of which are known to be
172 methylotrophic (Anesti et al. 2004, 2005). It was a regular, aerobic, Gram positive rod (Fig
173 3), producing orange/pink colonies, which may be compared to *Brevibacterium linens*
174 (yellow to deep orange colonies) and *B. rufescens* (red-pink; Nazina, 1981). Possibly the red
175 growth on C₁-compounds produced by “*Bacillus methylicus*” (Loew, 1892) may indicate that
176 “*B. methylicus*” was in fact also a *Brevibacterium*. Methylotrophy had previously been
177 shown in several other *Brevibacterium* strains (Nesvera et al., 1991; Anesti et al., 2004,
178 2005).

179

180 [TABLE 2] [FIGURES 1, 2, 3]

181

182 *Sphingomonas melonis* strain ET35

183

184 A partial sequence (740 bp; EU416229) of the 16S rRNA gene of strain ET35 showed 99.9%
185 identity to that of *Sphingomonas melonis* (AB055863), 99.7% to *S. aquatilis* (AF131295),
186 and lower similarity (98-99%) to *S. pruni*, *S. mali* and *Caulobacter leidyi*. Interestingly, the
187 clones of methylotrophic putative sphingomonads recovered using ¹³C-stable isotope probing
188 were most closely related phylogenetically to *S. stygia* (Nercessian et al., 2005), but the ET35
189 sequence showed only 94% sequence identity to *S. stygia* (AB025013). The properties of
190 strain ET35 (Table 3), including colony colour, lack of motility, salt-tolerance, and other
191 physiological characteristics are consistent with those of *S. melonis* (Buonaurio et al., 2002;
192 Yabuuchi and Kosako, 2005; Yoon et al., 2006). Its methylotrophic growth was supported by
193 methanol or formate but not by other C₁-substrates, including dimethylsulfide and DMSO₂.
194 Growth on formate and methanol was stimulated by addition of bicarbonate (10 mM), which
195 raised the growth yields from 7 to 9 g dry wt (mol formate)⁻¹, and from 12 to 19 g dry wt

196 (mol methanol)⁻¹. Its growth rates (μ) on formate or methanol at 25°C were 0.05 h⁻¹ and 0.10
197 h⁻¹, respectively, stimulated to 0.12 h⁻¹ and 0.17 h⁻¹ by bicarbonate. It showed high growth
198 rates (0.30–0.46 h⁻¹) on sucrose, fructose, glucose, and C₄-acids, with typical growth yields
199 of 40 g dry wt (mol fructose)⁻¹, 70 g (mol sucrose)⁻¹, and 31 g (mol succinate)⁻¹. It grew with
200 2.5% (w/v) NaCl but growth was not sustained in the presence of 5% (w/v) NaCl.

201 Interestingly, although strain ET35 was isolated as a methylotroph, it also grew on
202 several substituted thiophenes, suggesting from its colour and physiology when first isolated
203 that it might have been a strain of *Xanthobacter* (Padden et al., 1997). It used a wider range
204 of substituted thiophenes than either *X. tagetidis* or *Rhodococcus* sp. strain TTD-1
205 (Kanagawa and Kelly, 1987; Padden *et al.*, 1997), with growth yields (g dry wt [mol
206 substrate]⁻¹) of: thiophene-2-carboxylate (20), thiophene-3-carboxylate (22), thiophene-2-
207 acetate (34), thiophene-3-acetate (20), 5-methyl-thiophene-2-carboxylate (24) and 3-methyl-
208 thiophene-2-carboxylate (14). These yields represented 8-20% conversion of substrate-
209 carbon to new biomass, which was similar to the carbon-conversion efficiency of 25% from
210 thiophene-2-carboxylate by *Rhodococcus* strain TTD-1 (Kanagawa and Kelly, 1987). The
211 difference between the yields of strain ET35 on thiophene-2-carboxylate and thiophene-2-
212 acetate was 14 g dry wt mol⁻¹, (indicating that about 25% of the carbon of the acetate moiety
213 of thiophene-2-carboxylate was converted to cell-carbon), and the growth yield on acetate
214 alone was about 14 g dry wt mol⁻¹, consistent with the contribution to growth of the acetate
215 moiety of thiophene-2-acetate.

216

217 *Pseudomonas mendocina* strain TSQ4

218

219 A partial sequence of the 16S rRNA gene of this strain (552 bp; EU416231) showed 99.5%
220 identity (549/552 aligned nucleotides) to the database sequence for *Pseudomonas mendocina*
221 (CP000680.1) and 98.6% (544/552 nt) to *P. pseudoalcaligenes* (DQ071558). Strain TSQ4

222 exhibited pale pink fluorescence under UVA illumination. *P. mendocina* was not previously
223 reported as methylotrophic, but does use C₂-C₈ primary alcohols (Smith *et al.*, 2003). A
224 facultatively methylotrophic strain of *P. alcaligenes* was described by Cercel (1999): the
225 G+C content of the DNA of strain TSQ4 (64.2 mol%) was in the range for *Pseudomonas* (58-
226 69 mol%), and similar to that for *P. alcaligenes* (64-68 mol%; Palleroni, 2005). Unlike strain
227 TSQ4, the strain described by Cercel (1999) was not pigmented, lacked catalase, and did not
228 produce acetoin, but did possess gelatinase, and lysine decarboxylase. In other physiological
229 respects (Table 3) the strains were similar.

230

231 *Klebsiella oxytoca* strains MMA/F and MMA/1

232

233 Strain MMA/F showed 99.6% 16S rRNA gene sequence (1126 bp; AY186181) identity to
234 *Klebsiella oxytoca* (AY150697) and 99.2% identity to *K. pneumoniae* (AY369139). The
235 partial 16S rRNA sequence of strain MMA/1 (606 bp; EF468682) showed 99.3% identity to
236 the *K. oxytoca* and 99.0% to the *K. pneumoniae* sequences. These reference sequences for *K.*
237 *oxytoca* and *K. pneumoniae* showed 99.6% identity to each other, so identification of strains
238 MMA/F and MMA/1 as *K. oxytoca*, rather than *K. pneumoniae*, were deduced by their being
239 positive for indole production, urease and lysine decarboxylase, which *K. pneumoniae* is not.
240 In all other properties they were similar to each other (Tables 1 and 3), and their
241 characteristics were consistent with those expected for *Klebsiella*: a capsulated, non-motile,
242 Gram-negative rod, able to denitrify with copious production of N₂ gas, positive for
243 gelatinase and catalase, and negative for oxidase, arginine dihydrolase and ornithine
244 decarboxylase. Like *Klebsiella*, growth was unaffected by 2.5% NaCl. Growth of each was
245 supported with similar growth rates by MMA ($\mu = 0.21\text{--}0.23\text{ h}^{-1}$; growth yield 11 g dry
246 wt/mol⁻¹), methanol ($\mu = 0.31\text{--}0.35\text{ h}^{-1}$), DMA and TMA, but not by formate, MSA or
247 DMSO₂.

248 Strains with the characteristics of *K. oxytoca* could be isolated repeatedly in
249 successive years, with one such strain differing from strain MMA/F only in being positive for
250 use of citrate. These results thus confirm the reports of methylotrophic strains of *Klebsiella*
251 by Nishio *et al.* (1975) and Cercel (1999). The presence of the gene for hexulose phosphate
252 synthase in *Klebsiella oxytoca* strain Msa1 (GenBank AF282849), suggests that these
253 organisms probably used the ribulose monophosphate pathway of formaldehyde assimilation.

254

255 *Flavobacterium* sp. strains MSA/1 and MMA/2

256

257 Analysis of the 16S rRNA gene sequences of strains MSA/1 and MMA/2 showed both to be
258 most closely related to *Flavobacterium* species. Strain MSA/1 (1291 bp; AY786182) showed
259 highest identity (up to 96.5%) to the GenBank sequences of *F. limicola*, *F. psychrolimnae*
260 and *F. frigoris*. A partial sequence from strain MMA/2 (379 bp; AY836678) showed 95%
261 identity to *F. geladicus* (AJ440996) and 93.4% to *F. degerlachei* (AY771756). The two
262 strains showed only 95% sequence identity to each other. The DNA G+C content of 26
263 *Flavobacterium* species ranges between 29-38 mol% (Bernardet and Bowman, 2006), so the
264 DNA G+C content of 34.4 and 30.2 mol% for strains MSA/1 and MMA/2 were consistent
265 with identification as *Flavobacterium* species. Some 26 valid species of *Flavobacterium* were
266 described by Bernardet and Bowman (2006), and up to 75 species have been reported
267 (Euzéby, 2008). Precise identification of species requires a polyphyletic approach and is not
268 simple (Bernardet *et al.*, 2002; Bernardet and Bowman, 2006), so we have necessarily only
269 characterized these strains to the genus level. Some older species, including some able to
270 metabolize xenobiotics, have been shown more likely to be strains of *Sphingomonas*
271 *paucimobilis* (Bernardet and Bowman, 2006), but the 16S rRNA gene sequences of strains
272 MSA/1 and MMA/2 showed only 73-75% identity to that of *S. paucimobilis* (D16144). The
273 two strains differed in their pigment colour, and their methylotrophic abilities (Tables 1 and

274 3). Strain MSA/1 grew on MSA ($\mu = 0.20 \text{ h}^{-1}$; growth yield $11 \text{ g dry wt mol}^{-1}$), methanol (μ
275 $= 0.31 \text{ h}^{-1}$), and formate, but did not grow on MMA, DMA, TMA or DMSO₂. In contrast,
276 strain MMA/2 could not grow on MSA, but did grow on MMA ($\mu = 0.20 \text{ h}^{-1}$), DMA and
277 TMA, as well as methanol ($\mu = 0.28 \text{ h}^{-1}$). Because of these differences, some enzymes of
278 methylotrophy were assayed in each strain: as expected, strain MMA/2 grown on MMA
279 contained MMA dehydrogenase activity, which was absent from MSA-grown strain MSA/1.
280 Both contained active methanol, formaldehyde and formate dehydrogenases, as expected for
281 methylotrophic growth. Hydroxypyruvate reductase was present in cell-free extracts of both
282 at activities of about $60 \text{ nmol NADH oxidized min}^{-1} (\text{mg protein})^{-1}$. The only previous report
283 of methylotrophy in a *Flavobacterium* strain was in an isolate from the Antarctic (Moosvi *et*
284 *al.*, 2005b).

285

286 [TABLE 3]

287

288 **Conclusions and prospects**

289

290 Two novel findings from this study of the River Thames are (1) that among several hundred
291 colonies of methylotrophs observed qualitatively on elective-agar plates and on plating from
292 enrichment cultures, pink-pigmented facultative methylotrophs (*Methylobacterium* species;
293 Kelley *et al.*, 2004; Green, 2006) were uncommon; and (2) the range of randomly-selected
294 pure cultures contained examples of heterotrophic genera already known to harbour
295 methylotrophic strains, but also revealed methylotrophy in other genera. The latter included
296 *Sphingomonas*, no pure cultures of which had previously been reported to exhibit
297 methylotrophy, and genera from three phyla of the *Bacteria* with a limited previous history of
298 methylotrophy (*Klebsiella*, *Flavobacterium* and *Mycobacterium*).

299 This and other studies have now shown facultative methylotrophy in taxonomically
300 unrelated members of the *Alpha-*, *Beta-* and *Gammaproteobacteria*, *Actinobacteria* and
301 *Firmicutes* (Lidstrom, 2006). The view that “there has been an overemphasis on
302 methylotrophy as a novel taxonomic trait” (Boucher *et al.*, 2003; Jakobsen *et al.*, 2006) is
303 thus supported, and confirms that attempts to define “methylotrophs” as discrete taxonomic
304 entities (Ghosh *et al.*, 2007) are invalid. Methylotrophy is an ancestral metabolic trait
305 (Brocks *et al.*, 2003; Battistuzzi *et al.*, 2004), and it is possible that genes to confer
306 methylotrophic capacity were acquired by taxonomically diverse organisms through lateral
307 gene transfer over geological time. A mechanism for such transformation could be plasmid-
308 borne gene transfer. A plasmid carrying genes for methanol dehydrogenase and some
309 enzymes of C₁-assimilation occurs in numerous strains of *Bacillus methanolicus*, and has
310 been studied in great detail (Brautaset *et al.*, 2004; Jakobsen *et al.*, 2006). To date,
311 comparable “methylotrophy plasmids” have not been shown in Gram negative
312 methylotrophs, although some contain cryptic plasmids (Warner and Higgins, 1977; Lidstrom
313 and Wopat, 1984). There has, however, been a report of a bacterium containing such a
314 plasmid, capable of replication in both Gram negative and Gram positive bacteria
315 (Meganathan and Ranganathan, 2008). This plasmid enabled transfer of methylotrophy for
316 use of methanol, dimethylsulfoxide, DMS and methylamines to a wide variety of non-
317 methylotrophs. Such a plasmid in natural populations could explain the diaspora of
318 methylotrophy across taxonomically-unrelated bacteria. Methylated compounds such as
319 methylamines and DMS are ubiquitous in the environment, and their use as supplementary
320 energy and carbon sources by bacteria normally regarded as “heterotrophic” has largely been
321 overlooked to date. It is clearly worth further study. The phylogenetic origins of the enzymes
322 of C₁-metabolism in diverse heterotrophs would also be a rewarding study.

323

324 **Experimental procedures**

325

326 *The River Thames*

327 The River Thames, in the south-east of England, is 346 km long, of which 237 km is non-
328 tidal. The tidal section of the river (109 km) has a tidally-variable salinity ranging from about
329 0.3 g l^{-1} (5 mM NaCl) at Barnes in S.W. London, through 0.6 g l^{-1} (10 mM) at London
330 Bridge (central London), to 20 g l^{-1} (340 mM) near the estuary at Chapman Buoy. Tidally
331 and seasonally, the depth of the river in the section sampled varies between about 2 to 9 m.
332 The river is now one of the cleanest metropolitan rivers in the world, after having had a
333 history of extreme pollution, especially during the nineteenth century: concern about
334 pollution can even be traced back to the 14th century, but today seals and more than 100 fish
335 species have returned to the central London reaches of the river. While there is still pollution,
336 particularly when storm water overwhelms the sewage system, the natural oxygenation of the
337 river is enhanced by two purpose built vessels the '*Thames Bubbler*' (commissioned in 1989)
338 and the '*Thames Vitality*' (1997), which can pump 30 tonnes of oxygen per day directly into
339 the River as necessary. Oxygenation on an emergency basis is also achieved by injection of
340 hydrogen peroxide.

341

342 *The River Thames sampling sites*

343 Sediment and water samples were taken on numerous occasions from five locations on the
344 River Thames during September to November, 2002-2005. Surface sediment samples with
345 river water were taken at low tide from (1) "Tower Beach", on the North Bank of the river,
346 opposite the museum warship "HMS Belfast", East of the Sugar Quay Wharf walkway; and
347 the following South Bank sites: (2) a tidal sand bank West of Waterloo Bridge, adjacent to
348 the Royal Festival Pier; (3) the tidal beach adjacent to Tower Bridge; (4) adjacent to
349 "Gabriel's Wharf"; and (5) adjacent to the pier in front of the Tate Modern Art Gallery. The
350 median map reference location of these sites was around $51^{\circ} 30' 20'' \text{ N}$ and $0^{\circ} 05' 00'' \text{ W}$.

351 Water temperatures during this period were 4-9°C, at about pH 7.9, with salinity in the stretch
352 of the river sampled was typically around 0.6-1.0 g l⁻¹ (10-17 mM NaCl).

353 Enrichment cultures were set up with one of six elective methylotrophic substrates:
354 methanol, MMA, DMA, TMA, DMSO₂, or MSA.

355

356 *Culture media, elective culture, and assessment of growth substrates*

357 The mineral salts medium contained (grams per litre in distilled water): KH₂PO₄ (1.5),
358 Na₂HPO₄·2H₂O (7.9), NH₄Cl (0.8), MgSO₄·7H₂O (0.1), trace metal solution (10 ml), initial
359 pH 7.3, was prepared as described by Kelly and Wood (1998), sterilized at 121°C for 15 min.

360 For some enrichment cultures on MMA, DMA or TMA, the NH₄Cl was omitted, to force
361 selection of organisms that used the methylamines as sources of nitrogen as well as carbon
362 and energy. One-carbon growth substrates were supplied as (mM): MMA (20), DMA (10),
363 TMA (10), methanol (20), DMSO₂ (10), sodium MSA (15), or sodium formate (25). To test
364 for growth on multicarbon substrates, trisodium citrate was used at 5 mM and other organic
365 acids at 10 mM, monosaccharides and amino acids at 10 mM, and disaccharides at 5 mM.

366 Agar media were prepared by addition of Oxoid agar No. 1 (15 g l⁻¹). For culture with
367 methanol on solid medium, agar mineral medium was inoculated and a sterile filter paper
368 with 50 µl methanol placed in the inverted Petri dish lids, before incubating in a gas-tight
369 box. For liquid culture in shake-flasks with methanol, the flasks were sealed with “Subaseal”
370 vaccine stoppers.

371 Liquid medium enrichment cultures (50 ml in 250 ml Erlenmeyer flasks) on elective
372 media were inoculated with about 10 g of sediment samples and shaken in an orbital shaker
373 at 30°C. These cultures were subcultured (10 % v/v, without transfer of sediment) into fresh
374 medium after 4-6 days. After 2-4 transfers, aliquots were spread on to agar media for
375 isolation of pure cultures by subculture of single colonies.

376 Elective culture was also achieved by direct inoculation on to agar media: sediment (10 g)
377 was shaken with sterile deionized water, the sediment allowed to settle, and aliquots of the
378 suspension spread on media with MMA, DMA or TMA. Plates were incubated aerobically
379 for up to eight days and the range of colony types assessed. Colonies were repeatedly
380 subcultured on to new plates to obtain pure cultures. These cultures were also plated on to
381 substrate-free agar to ensure that growth was methylotrophic and not simply due to use of the
382 agar or its impurities.

383 Growth substrates used by the pure culture isolates were assessed at 25°C by inoculation
384 into 5 ml medium in 25 ml sterile plastic Universal tubes and incubated without shaking for
385 up to 14 days. Growth was assessed visually and as OD_{440nm} after vortex-mixing of the
386 cultures to obtain homogeneous suspensions. Growth rates and growth yields of some
387 organisms were determined in shake-flask cultures by following increase in OD_{440nm} at
388 different temperatures with various substrates. Growth with KNO₃ (25 mM) as respiratory
389 oxidant was tested in completely filled tubes with sucrose or methanol as substrate. Nitrogen
390 production (as gas bubbles) was checked visually; and determinations made of nitrate
391 disappearance and nitrite formation (Cawse 1987; Kelly and Wood 1998). Aerobic growth
392 with alternative nitrogen sources was assessed on sucrose through several subcultures in the
393 absence of NH₄Cl with KNO₃, MMA, DMA, TMA, cyanate, thiocyanate, or EDTA, each at
394 2.5 mM. Tolerance of NaCl was tested in cultures on sucrose with salt concentrations
395 between 107-860 mM. Growth over a range of temperatures was assessed at 4, 15, 20, 25, 30,
396 37 and 45°C. All growth determinations were carried out at least in duplicate, with repeat
397 experiments for some tests.

398

399 *Characterization of pure culture isolates of methylotrophic bacteria*

400 Gram staining, acid-fast staining, spore and capsule stains, motility in hanging drops, and
401 catalase, oxidase and phosphatase activities were all assessed by standard methods (Schaeffer

402 and Fulton, 1933; Barrow and Feltham, 1995). Flagella staining used the method of Kodaka
403 *et al.* (1982). Biochemical characterization was done using API®20E test strips (BioMérieux
404 SA, Marcy-l’Etoile, France) according to the manufacturer’s instructions. Assay of enzymes
405 involved in C₁-substrate metabolism all used previously described methods (Anesti *et al.*,
406 2005; Moosvi *et al.*, 2005a). Scanning electron microscopy of gold sputter-coated
407 preparations was carried out by the Electron Microscopy Unit of King’s College London. For
408 determination of the G+C content of chromosomal DNA, about 0.5 g wet-weight of bacteria
409 were used for DNA isolation (Beji *et al.*, 1987), which was then resuspended in 100 µl sterile
410 deionized water for assay. The purified DNA samples showed high purity A_{260nm}/A_{280nm} ratios
411 of 1.8-2.0. The G+C content was determined by the acetic acid method of Fredericq *et al.*
412 (1961), assayed in triplicate to give virtually identical replicate values.

413

414 *Determination of the sequences of the 16S ribosomal RNA genes of some isolates.*

415 Genomic DNA was isolated and 16S ribosomal gene sequences determined as described by
416 Schäfer (2007). Phylogenetic relationships of the sequences were determined using the
417 BLASTN and BLAST2 on-line programs of the NCBI (www.ncbi.nlm.nih.gov/blast), and
418 from neighbor-joining distance trees produced using BLAST pairwise alignments. Accession
419 numbers for the 16S ribosomal RNA gene sequences have been deposited with GenBank as:
420 strain ET35 (EU416229), strain DSQ3 (EU416230), strain MSQ5 (EU815829), strain TSQ4
421 (EU416231), strain MMA/F (AY186181), strain MMA/1 (EF468682), strain MSA/1
422 (AY786182), and strain MMA/2 (AJ836678).

423

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425

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429

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647

648

Legends to Figures

649

650 **Fig. 1.** Scanning electron micrograph of *Mycobacterium fluoranthenvorans* strain DSQ3.

651 Bar is 5 μm .

652

653 **Fig. 2.** Scanning electron micrograph of *Rhodococcus erythropolis* strain DSQ4. Bar is 5 μm .

654

655 **Fig. 3.** Scanning electron micrograph of *Brevibacterium* sp. strain MSQ5. Bar is 5 μm .

656