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

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Summary

Enrichment and elective culture for methylotrophs from sediment of the River Thames in central London yielded a diversity of pure cultures representing several genera of Gram-negative and Gram-positive bacteria, which were mainly of organisms not generally regarded as typically methylotrophic. Substrates leading to successful isolations included methanol, monomethylamine, dimethylamine, trimethylamine, methanesulfonate and dimethylsulfone. Several isolates were studied in detail and shown by their biochemical and morphological properties and 16S rRNA gene sequencing to be *Sphingomonas melonis* strain ET35, *Mycobacterium fluoranthenivorans* strain DSQ3, *Rhodococcus erythropolis* strain DSQ4, *Brevibacterium casei* strain MSQ5, *Klebsiella oxytoca* strains MMA/F and MMA/1, *Pseudomonas mendocina* strain TSQ4, and *Flavobacterium* sp. strains MSA/1 and MMA/2. The results show that facultative methylotrophy is present across a wide range of *Bacteria*, suggesting that turnover of diverse C_1-compounds is of much greater microbiological and environmental significance than is generally thought. The origins of the genes encoding the enzymes of methylotrophy in diverse heterotrophs need further study, and could further our understanding of the phylogeny and antiquity of methylotrophic systems.

Introduction

Bacteria with the ability to grow on one-carbon compounds as their sole source of energy and carbon have been known since the late 19th century (Loew, 1892). One of the earliest organisms isolated was the Gram-positive bacterium, *Bacillus methylicus* (later renamed *Bacterium methylicum*, but no longer available in culture): an aerobic, non-spore-forming, facultative methylotroph producing red pigmentation when grown on formate or methanol, and which also grew on formaldehyde (Loew, 1892; Migula, 1900; Bergey *et al.*, 1939). During the 20th century, numerous specialized methylotrophs were described, including a great diversity of methanotrophs, some of which were obligate methane-users, many also using methanol, and a few being capable of growth on multicarbon compounds (Whittenbury *et al.*, 1970; Dedysh *et al.*, 2005a). Methylotrophy came to be recognized as a property of specialized bacteria capable of growth on C_1-compounds (and some of which would only grow on C_1-compounds), with the names of numerous genera and species reflecting this concept (e.g. *Methylobacterium, Methylomonas, Methylosinus, Methyloversatilis, Bacillus methanolicus*; Whittenbury *et al.*, 1970; Arfman *et al.*,1992; Kalyuzhnaya *et al.*, 2006; Lidstrom, 2006). The occurrence of methylotrophy in prokaryotes was subsequently realized not to correlate with traditional bacterial classification (Brautaset *et al.*, 2004), as facultative methylotrophy was progressively shown to be a property of diverse typically heterotrophic genera including *Paracoccus, Hyphomicrobium, Micrococcus, Arthrobacter, Brevibacterium, Beijerinckia, Bacillus, Klebsiella, Afipia, Variovorax, Amycolatopsis, Mycobacterium,* and *Acinetobacter* (Nishio *et al.*, 1975; Bamforth and Quayle, 1978; Duménil *et al.*, 1979; Levering *et al.*, 1981; Nazina, 1981; Dijkhuizen *et al.*, 1988; Kato *et al.*, 1988; Nesvera *et al.*, 1991; Cercel, 1999; Mitsui *et al.*, 2000; Alves *et al.*, 2001; Borodina *et al.*, 2002; Anesti *et al.*, 2005; Moosvi *et al.*, 2005a, b; Dedysh *et al.*, 2005b; Kelly *et al.*, 2006; Lidstrom, 2006; Ghosh *et al.*, 2007). The ribulose monophosphate (RuMP) cycle and its key enzymes (3-

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

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

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

We have used classical enrichment culture and isolation on various elective C_1-substrates in a study of methylotrophs present in the River Thames, with the aim of obtaining a 'snapshot' overview of the diversity of such bacteria in the river, and to recover and identify novel organisms. Microbiological studies of the Thames date back to the 19th century, with numerous bacteria being reported by Frankland (1885, 1899) and Ward (1898, 1899). The river is routinely monitored for its bacterial load, especially with respect to faecal contamination, but prior to our studies, which have been conducted over a five-year period (2002-2006), there had been no report of any methylotrophs in the River Thames where it runs through central London.

Results and Discussion

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

Samples were taken on six separate occasions and enrichments set up with methanol, monomethylamine (MMA), dimethylamine (DMA), trimethylamine (TMA), methanesulfonate (MSA), or dimethylsulfone ($DMSO_2$) as elective substrates. Numerous colony types were observed on subsequent plating on to agar media, with as many as five different morphologies and colours from one sampling. These ranged in colour from white to orange, but few appeared to give the characteristic pink pigmentation of the facultative methylotroph, *Methylobacterium* (Kelley *et al.*, 2004; Green, 2006; Lidstrom, 2006), suggesting that most methylotrophs recovered were examples of other genera. Of the new

isolates studied in detail, most were obtained from liquid shake-flask enrichment cultures, but strains DSQ3 and DSQ4 were obtained from direct plating of sediment suspensions from the Sugar Quay site on to DMA-agar medium. These two strains grew very poorly in liquid DMA shake-flask cultures and were routinely grown and maintained on DMA in static culture or on agar slants. These strains would thus have been unlikely to have been recovered by the shake-flask enrichment method. Only strains positively assigned to specific genera are described below, but additional strains of *Rhodococcus, Arthrobacter, Xanthobacter*, and *Paracoccus* were also tentatively identified among the numerous other methylotrophic isolates obtained, growing variously on methanol, formate, or $DMSO_2$.

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

[TABLE 1]

Mycobacterium fluoranthenivorans strain DSQ3

Strain DSQ3 showed short straight to curved rods during active growth (Fig. 1), but became more pleomorphic with changes from long thin rods to vibrioid and spiral morphology in older cultures. Its partial 16S rRNA gene sequence (944 bp; EU416230) showed 99.6% identity (937/941 nucleotides) to *Mycobacterium fluoranthenivorans* (AJ617741; Hormish *et al.*, 2004), which is a high G-C, acid-fast, but non-pigmented *Mycobacterium*. The orange pigmentation of strain DSQ3 is, however, common in *Mycobacterium* species (Holt *et al.*, 1994). The G+C content of its DNA (73.8 mol%) was slightly above the range reported for *Mycobacterium* (64-71 mol%; Wayne and Gross, 1968; Hartmans *et al.*, 2006). Unlike some *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 $DMSO_2$. 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). Methylotrophic growth was obtained on DMA, but was very slow or absent on
161 methanol, formate, MMA, TMA, MSA, $DMSO_2$. 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. Methylotrophy 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,

X76564) and strain FM1A (AY468375), and 99.7% identity (342/343 aligned nucleotides) to *B. casei* strains 3Tg (AY468375) and 3S(a) (AY468374), all of which are known to be methylotrophic (Anesti et al. 2004, 2005). It was a regular, aerobic, Gram positive rod (Fig 3), producing orange/pink colonies, which may be compared to *Brevibacterium linens* (yellow to deep orange colonies) and *B. rufescens* (red-pink; Nazina, 1981). Possibly the red growth on C_1-compounds produced by *"Bacillus methylicus"* (Loew, 1892) may indicate that *"B. methylicus"* was in fact also a *Brevibacterium*. Methylotrophy had previously been shown in several other *Brevibacterium* strains (Nesvera *et al.*, 1991; Anesti *et al.*, 2004, 2005).

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

Sphingomonas melonis strain ET35

A partial sequence (740 bp; EU416229) of the 16S rRNA gene of strain ET35 showed 99.9% identity to that of *Sphingomonas melonis* (AB055863), 99.7% to *S. aquatilis* (AF131295), and lower similarity (98-99%) to *S. pruni, S. mali* and *Caulobacter leidyi.* Interestingly, the clones of methylotrophic putative sphingomonads recovered using ^{13}C-stable isotope probing were most closely related phylogenetically to *S. stygia* (Nercessian *et al.*, 2005), but the ET35 sequence showed only 94% sequence identity to *S. stygia* (AB025013). The properties of strain ET35 (Table 3), including colony colour, lack of motility, salt-tolerance, and other physiological characteristics are consistent with those of *S. melonis* (Buonaurio *et al.*, 2002; Yabuuchi and Kosako, 2005; Yoon *et al.*, 2006). Its methylotrophic growth was supported by methanol or formate but not by other C_1-substrates, including dimethylsulfide and $DMSO_2$. Growth on formate and methanol was stimulated by addition of bicarbonate (10 mM), which 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 C_4-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

exhibited pale pink fluorescence under UVA illumination. *P. mendocina* was not previously reported as methylotrophic, but does use C_2-C_8 primary alcohols (Smith *et al.*, 2003). A facultatively methylotrophic strain of *P. alcaligenes* was described by Cercel (1999): the G+C content of the DNA of strain TSQ4 (64.2 mol%) was in the range for *Pseudomonas* (58-69 mol%), and similar to that for *P. alcaligenes* (64-68 mol%; Palleroni, 2005). Unlike strain TSQ4, the strain described by Cercel (1999) was not pigmented, lacked catalase, and did not produce acetoin, but did possess gelatinase, and lysine decarboxylase. In other physiological respects (Table 3) the strains were similar.

Klebsiella oxytoca strains MMA/F and MMA/1

Strain MMA/F showed 99.6% 16S rRNA gene sequence (1126 bp; AY186181) identity to *Klebsiella oxytoca* (AY150697) and 99.2% identity to *K. pneumoniae* (AY369139). The partial 16S rRNA sequence of strain MMA/1 (606 bp; EF468682) showed 99.3% identity to the *K. oxytoca* and 99.0% to the *K. pneumoniae* sequences. These reference sequences for *K. oxytoca* and *K. pneumoniae* showed 99.6% identity to each other, so identification of strains MMA/F and MMA/1 as *K. oxytoca*, rather than *K. pneumoniae*, were deduced by their being positive for indole production, urease and lysine decarboxylase, which *K. pneumoniae* is not. In all other properties they were similar to each other (Tables 1 and 3), and their characteristics were consistent with those expected for *Klebsiella*: a capsulated, non-motile, Gram-negative rod, able to denitrify with copious production of N_2 gas, positive for gelatinase and catalase, and negative for oxidase, arginine dihydrolase and ornithine decarboxylase. Like *Klebsiella*, growth was unaffected by 2.5% NaCl. Growth of each was supported with similar growth rates by MMA (μ = 0.21–0.23 h^{-1}; growth yield 11 g dry wt/mol^{-1}), methanol (μ = 0.31–0.35 h^{-1}), DMA and TMA, but not by formate, MSA or $DMSO_2$.

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

Flavobacterium sp. strains MSA/1 and MMA/2

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

3). Strain MSA/1 grew on MSA ($\mu = 0.20\ h^{-1}$; growth yield 11 g dry wt mol^{-1}), methanol ($\mu = 0.31\ h^{-1}$), and formate, but did not grow on MMA, DMA, TMA or $DMSO_2$. In contrast, strain MMA/2 could not grow on MSA, but did grow on MMA ($\mu = 0.20\ h^{-1}$), DMA and TMA, as well as methanol ($\mu = 0.28\ h^{-1}$). Because of these differences, some enzymes of methylotrophy were assayed in each strain: as expected, strain MMA/2 grown on MMA contained MMA dehydrogenase activity, which was absent from MSA-grown strain MSA/1. Both contained active methanol, formaldehyde and formate dehydrogenases, as expected for methylotrophic growth. Hydroxypyruvate reductase was present in cell-free extracts of both at activities of about 60 nmol NADH oxidized min^{-1} (mg protein) $^{-1}$. The only previous report of methylotrophy in a *Flavobacterium* strain was in an isolate from the Antarctic (Moosvi *et al.*, 2005b).

[TABLE 3]

Conclusions and prospects

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

This and other studies have now shown facultative methylotrophy in taxonomically unrelated members of the *Alpha-*, *Beta-* and *Gammaproteobacteria*, *Actinobacteria* and *Firmicutes* (Lidstrom, 2006). The view that "there has been an overemphasis on methylotrophy as a novel taxonomic trait" (Boucher *et al.*, 2003; Jakobsen *et al.*, 2006) is thus supported, and confirms that attempts to define "methylotrophs" as discrete taxonomic entities (Ghosh *et al.*, 2007) are invalid. Methylotrophy is an ancestral metabolic trait (Brocks *et al.*, 2003; Battistuzzi *et al.*, 2004), and it is possible that genes to confer methylotrophic capacity were acquired by taxonomically diverse organisms through lateral gene transfer over geological time. A mechanism for such transformation could be plasmid-borne gene transfer. A plasmid carrying genes for methanol dehydrogenase and some enzymes of C_1-assimilation occurs in numerous strains of *Bacillus methanolicus*, and has been studied in great detail (Brautaset *et al.*, 2004; Jakobsen *et al.*, 2006). To date, comparable "methylotrophy plasmids" have not been shown in Gram negative methylotrophs, although some contain cryptic plasmids (Warner and Higgins, 1977; Lidstrom and Wopat, 1984). There has, however, been a report of a bacterium containing such a plasmid, capable of replication in both Gram negative and Gram positive bacteria (Meganathan and Ranganathan, 2008). This plasmid enabled transfer of methylotrophy for use of methanol, dimethylsulfoxide, DMS and methylamines to a wide variety of non-methylotrophs. Such a plasmid in natural populations could explain the diaspora of methylotrophy across taxonomically-unrelated bacteria. Methylated compounds such as methylamines and DMS are ubiquitous in the environment, and their use as supplementary energy and carbon sources by bacteria normally regarded as "heterotrophic" has largely been overlooked to date. It is clearly worth further study. The phylogenetic origins of the enzymes of C_1-metabolism in diverse heterotrophs would also be a rewarding study.

Experimental procedures

The River Thames

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

The River Thames sampling sites

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

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

Enrichment cultures were set up with one of six elective methylotrophic substrates: methanol, MMA, DMA, TMA, $DMSO_2$, or MSA.

Culture media, elective culture, and assessment of growth substrates

The mineral salts medium contained (grams per litre in distilled water): KH_2PO_4 (1.5), $Na_2HPO_4.2H_2O$ (7.9), NH_4Cl (0.8), $MgSO_4.7H_2O$ (0.1), trace metal solution (10 ml), initial pH 7.3, was prepared as described by Kelly and Wood (1998), sterilized at 121˚C for 15 min. For some enrichment cultures on MMA, DMA or TMA, the NH_4Cl was omitted, to force selection of organisms that used the methylamines as sources of nitrogen as well as carbon and energy. One-carbon growth substrates were supplied as (mM): MMA (20), DMA (10), TMA (10), methanol (20), $DMSO_2$ (10), sodium MSA (15), or sodium formate (25). To test for growth on multicarbon substrates, trisodium citrate was used at 5 mM and other organic acids at 10 mM, monosaccharides and amino acids at 10 mM, and disaccharides at 5 mM. Agar media were prepared by addition of Oxoid agar No. 1 (15 g l^{-1}). For culture with methanol on solid medium, agar mineral medium was inoculated and a sterile filter paper with 50 µl methanol placed in the inverted Petri dish lids, before incubating in a gas-tight box. For liquid culture in shake-flasks with methanol, the flasks were sealed with “Subaseal” vaccine stoppers.

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

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

Growth substrates used by the pure culture isolates were assessed at 25˚C by inoculation into 5 ml medium in 25 ml sterile plastic Universal tubes and incubated without shaking for up to 14 days. Growth was assessed visually and as OD_{440nm} after vortex-mixing of the cultures to obtain homogeneous suspensions. Growth rates and growth yields of some organisms were determined in shake-flask cultures by following increase in OD_{440nm} at different temperatures with various substrates. Growth with KNO_3 (25 mM) as respiratory oxidant was tested in completely filled tubes with sucrose or methanol as substrate. Nitrogen production (as gas bubbles) was checked visually; and determinations made of nitrate disappearance and nitrite formation (Cawse 1987; Kelly and Wood 1998). Aerobic growth with alternative nitrogen sources was assessed on sucrose through several subcultures in the absence of NH_4Cl with KNO_3, MMA, DMA, TMA, cyanate, thiocyanate, or EDTA, each at 2.5 mM. Tolerance of NaCl was tested in cultures on sucrose with salt concentrations between 107-860 mM. Growth over a range of temperatures was assessed at 4, 15, 20, 25, 30, 37 and 45˚C. All growth determinations were carried out at least in duplicate, with repeat experiments for some tests.

Characterization of pure culture isolates of methylotrophic bacteria

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

and Fulton, 1933; Barrow and Feltham, 1995). Flagella staining used the method of Kodaka *et al.* (1982). Biochemical characterization was done using API®20E test strips (BioMérieux SA, Marcy-l'Etoile, France) according to the manufacturer's instructions. Assay of enzymes involved in C_1-substrate metabolism all used previously described methods (Anesti *et al.*, 2005; Moosvi *et al.*, 2005a). Scanning electron microscopy of gold sputter-coated preparations was carried out by the Electron Microscopy Unit of King's College London. For determination of the G+C content of chromosomal DNA, about 0.5 g wet-weight of bacteria were used for DNA isolation (Beji *et al.*, 1987), which was then resuspended in 100 µl sterile deionized water for assay. The purified DNA samples showed high purity $A_{260nm}/_{A280nm}$ ratios of 1.8-2.0. The G+C content was determined by the acetic acid method of Fredericq *et al.* (1961), assayed in triplicate to give virtually identical replicate values.

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

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Legends to Figures

Fig. 1. Scanning electron micrograph of *Mycobacterium fluoranthenivorans* strain DSQ3. Bar is 5 µm.

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

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