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Design and Evaluation of Novel Primers for the Detection of Genes Encoding Diverse Enzymes of Methylotrophy and Autotrophy

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Abstract

The phylogenetic significance of the diversity of key enzymes of methylotrophic and autotrophic metabolism is discussed. Primers for these key enzymes were designed using gene sequences encoding methanol dehydrogenase (*mxaF*; using subsets from database sequences for 22 Bacteria), hydroxypyruvate reductase (*hpr*; 36 sequences), methylamine dehydrogenase (*mauA*; 12 sequences), methanesulfonate monooxygenase (*msmA*; four sequences), and the *ccbL* and *cbbM* genes of ribulose bisphosphate carboxylase (26 and 23 sequences). These were effective in amplifying the correct gene products for the target genes in reference organisms and in test organisms not previously shown to contain the genes, as well as in some methylotrophic *Proteobacteria* isolated from the human mouth. The availability of the new primers increases the probability of detecting diverse examples of the genes encoding these key enzymes both in natural populations and in isolated bacterial strains.

Key words: *Proteobacteria*, primers, methylotrophy, autotrophy, methanol dehydrogenase, hydroxypyruvate reductase, methylamine dehydrogenase, RuBisCO

List of abbreviations: C_1 – one-carbon, RuBisCO – ribulose bisphosphate carboxyase/oxygenase, *cbbL* – gene encoding the large subunit of type I RuBisCO, CbbL – amino acid sequence encoded by *cbbL*, *cbbM* – gene encoding the large subunit of type II RuBisCO, HPR – hydroxypyruvate reductase, *hpr* – gene encoding HPR, Hpr – amino acid sequence encoded by *hpr*, HPS – 3-hexulose 6-phosphate synthase, *Hps* – gene encoding HPS, Hps – amino acid sequence encoded by *hps*, MDH – methanol dehydrogenase, *mxaF* – gene encoding the large subunit of MDH, MxaF – amino acid sequence encoded by *mxaF*, MMA – methylamine, MADH – methylamine dehydrogenase, *mauA* – gene encoding the small subunit of MADH, MauA – amino acid sequence encoded by *mauA*, MSA – methanesulfonate, MSAMO – methanesulfonate monooxygenase, *msmA* – gene encoding the α-subunit of the hydroxylase of MSAMO, MsmA – amino acid sequence encoded by *msmA*, T_m – DNA melting point

Introduction

Methylotrophic bacteria use C_1-compounds (including methanol, methylamine and methanesulfonate) to provide energy and carbon for growth (Kelly and Murrell 1996; Lidstrom, 2006). Their metabolic processes have global significance in environmental bioremediation, climate homeostasis, and in the human body (Murrell and Kelly, 1996; Kelly and Wood, 2010; Wood and Kelly, 2010; Wood *et al*., 2010). They occur in diverse habitats including Antarctica, ubiquitously in soil, marine and freshwater environments, and in the human microbiome (Anesti *et al*., 2004, 2005; Moosvi *et al*., 2005a, b; Neufeld *et al*., 2007; Boden *et al*., 200 8; Hung *et al*., 2011). A powerful tool in molecular microbial ecology and biochemistry is the use of oligonucleotide primers to probe for genes encoding enzymes diagnostic of specific metabolic processes, and this approach has enabled detection of methylotrophic bacteria in diverse habitats. Primers for methanol dehydrogenase, methylamine dehydrogenase, and methanesulfonate monooxygenase, have all been described in recent years. The most widely used have been the primers to detect MDH developed by McDonald and Murrell (1997), and few variations on those original primers have been reported (Moosvi *et al*., 2005b; Neufeld *et al*., 2007). Only a few examples of primers for other genes of C_1-metabolism have been published, such as those for MADH (Neufeld *et al*., 2007), and two primer sets for MSAMO, producing either 783 or 233 bp products (Baxter *et al*., 2002; Moosvi *et al*., 2005b). One outcome of using molecular methods to detect and

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subsequently sequence the genes encoding MDH has been the demonstration of significant diversity among the MDH genes and enzymes of different methylotrophs (Fesefeldt and Gliesche 1997; Anesti *et al.*, 2004; Neufeld *et al.*, 2007; Vorob'ev *et al.*, 2009). While degeneracy of the nucleotide sequences of genes encoding MDH is expected because of the variety of codons encoding the same amino acid, significant diversity is also found in the encoded MDH and MADH proteins from different Bacteria (*e.g.*, Fesefeldt and Gliesche 1997; Chistoserdov 2001; Neufeld *et al.*, 2007; Vorob'ev *et al.*, 2009), in which the nucleotide and amino acid sequences can deviate by 20% or more. The active site of the MxaF subunit of MDH appears, however, to be highly conserved, and the primers targeting that region have been successful in detecting quite divergent genes encoding MxaF. In order both to increase the likelihood of detecting variant forms of MDH, and to increase the range and specificity of probes for MDH, MADH and MSAMO, we set out to design new primers for *mxaF*, *mauA* and *msmA*, as well as primers for genes encoding enzymes essential for assimilatory pathways for C_1-compounds (the serine and ribulose monophosphate cycles), namely *hpr* for hydroxypyruvate reductase, and *hps* for hexulose phosphate synthase. In addition, primers were designed for *cbbL* and *cbbM*, encoding the subunits of the key enzyme of the Calvin Cycle (RuBisCO), essential for autotrophic growth in some facultative methylotrophs. Our aim was to use the phylogeny of the genes encoding key enzymes of C_1-metabolism to guide the design of PCR primers for the detection of these genes, and to increase both the power and specificity of primers in the armoury of the molecular microbial ecologist and biochemist.

Experimental

Materials and Methods

Design of primers based on GenBank database sequences encoding enzymes of methylotrophy and autotrophy from Bacteria in various phylogenetic groups. Nucleotide sequences of *cbbL*, *cbbM*, *hpr*, *hps*, *mxaF*, *msmA*, and *mauA* genes of Bacteria from the phylogenetic groups detailed in the Results and Discussion section were obtained from the GenBank database, and aligned using ClustalW within the BioEdit suite (Hall 1999). Neighbor-joining phylogenetic trees were constructed from amino acid sequences to guide primer design, using MEGA4 (Tamura *et al.*, 2007). Nucleotide alignments were manually inspected for regions of homology and multiple degenerate primers were designed for clusters of related sequences for each gene, with G+C contents of between 45 and 55%. Self-complementarity and hairpin formation was avoided in primer design, and degeneracy introduced to a maximum of three bases per primer. Primers were synthesized by MWG Biotech (London, UK). For each gene, a panel of reference strains was assembled and the specificity of the primers checked using PCR.

Isolation of DNA and PCR protocol. DNA was extracted from strains by means of the GenElute bacterial genomic DNA kit (Sigma-Aldrich Company Ltd., Poole, Dorset, UK) using the manufacturer's protocol for Gram-positive Bacteria. For PCR, DNA template (1 μL) and 5 pmol of each primer were added to 23 μL Reddy Mix PCR Master Mix (ABGene, Epsom, Surrey, UK). The PCR cycle conditions were: denaturation at 94°C for 15 min, then 30 cycles at 94°C for 45s, 54°C for 45 s, and 72°C for 90 s, followed by one cycle of elongation for 15 min at 72°C. Amplicons were bi-directionally sequenced by means of the BigDye Terminator 3.1 cycle sequencing kit (Applied Biosystems) with the primers used for the original PCRs using a 3730xl DNA Analyzer (Applied Biosystems) according to the manufacturer's instructions, and sequences assembled in BioEdit. The predicted annealing temperatures for the primer pairs used was estimated from their mean T_m values, and ranged over 50–60°C. Use of the primers with DNA from the reference bacterial strains (see below) was tested within the range 42–61°C for different primer pairs. The optimum for the reference strains was taken as 54°C. Further detail is given in the Results and Discussion.

Reference bacterial strains cultured to test the specificity of the primers generated in this study. The following strains were cultured on agar or liquid media, incubated aerobically at 26° or 30°C, using the conditions and media specified for the organisms on the website of the Deutsche Sammlung von Mikroorganismen und Zellkuturen (http://www.dsmz.de), or as described below: *Methylobacterium extorquens* AM1 (DSM 1338), *Methylococcus capsulatus* Bath (ATCC 33009; Warwick collection, grown at 45°C, on ATCC medium 1309: http://www.lgcstandards-atcc.org/Attachments/2554.pdf), and *Xanthomonas campestris pv. campestris* (DSM 3586) for *mxaF*; *Polaromonas naphthalenivorans* CJ2 (DSM 15660) and *Ralstonia eutropha* H16 (DSM 428) for *hpr*; *Methylobacterium extorquens* AM1 and *Methylobacillus flagellatus* KT (DSM 6875) for *mauA*; *Afipia felis* 25E1 (Ann Wood collection; cultured as described by Moosvi *et al.*, 2005a) for *msmA*; *Burkholderia xenovorans* LB400 (DSM 17367), *Ralstonia eutropha* H16 and *Methylococcus capsulatus* Bath for *cbbL*; and *Rhodobacter sphaeroides* 2.4.1 (DSM 158), *Polaromonas naphthalenivorans* CJ2 and *Thiomicrospira kueneni* (DSM 12350) for *cbbM*.

Bacterial strains probed using the new primers. Six methylotrophic bacterial strains from the War-

wick Collection, known to express activities of MDH, HPR or RuBisCO, were tested to determine if the target genes could be detected in their DNA using the new *mxaF*, *hpr*, *cbbL* and *cbbM* primers. These were *Aminobacter aminovorans* MA (ATCC 23819), *Xanthobacter tagetidis* TagT2C (DSM 11105), *Methylocella silvestris* BL2 (DSM 15510), *Methylocystis sporium* strain 5 (ATCC 35069), *Methylocystis parvus* OBBP (ATCC 35066), and *Methylosinus trichosporium* OB3b (ATCC 35070).

In addition, eleven strains of methylotrophic bacteria previously isolated from the mouth (Hung *et al*., 2011) were tested for the presence of the target genes by PCR using the new *msmA, mxaF*, *hpr*, *cbbL* and *cbbM* primers. Six of these were *Actinobacteria* (*Brevibacterium*, *Gordonia*, *Leifsonia, Microbacterium*, *Micrococcus*, *Rhodococcus*,) and five were *Alpha-, Beta-* and *Gammaproteobacteria* (*Achromobacter*, *Klebsiella*, *Methylobacterium*, *Pseudomonas*, *Ralstonia*). The culture and properties of all these were described previously (Hung *et al*., 2011).

Results and Discussion

Phylogenetic significance of the diversity of genes encoding enzymes of autotrophy and methylotrophy. Nucleotide sequence diversity was expected among genes encoding the same protein in different organisms because the same amino acid can be encoded by more than one triplet of nucleotides (*e.g.* six different synonymous codons exist for each of arginine, leucine and serine). For this reason, comparison of the encoded amino acid sequences was a more useful means of making comparisons of functional genes among different Bacteria. In the case of the genes considered by us, however, considerable diversity also existed among the encoded proteins, as was initially observed for MDH and MADH from different *Proteobacteria* (McDonald and Murrell 1997; Chistoserdov 2001; Neufeld *et al*., 2007). We found that this diversity applied to the amino acid sequences for all the strains we used for the design of primers for the genes encoding CbbL, CbbM, Hpr, MauA, and MxaF (Fig. 1–5). This diversity was

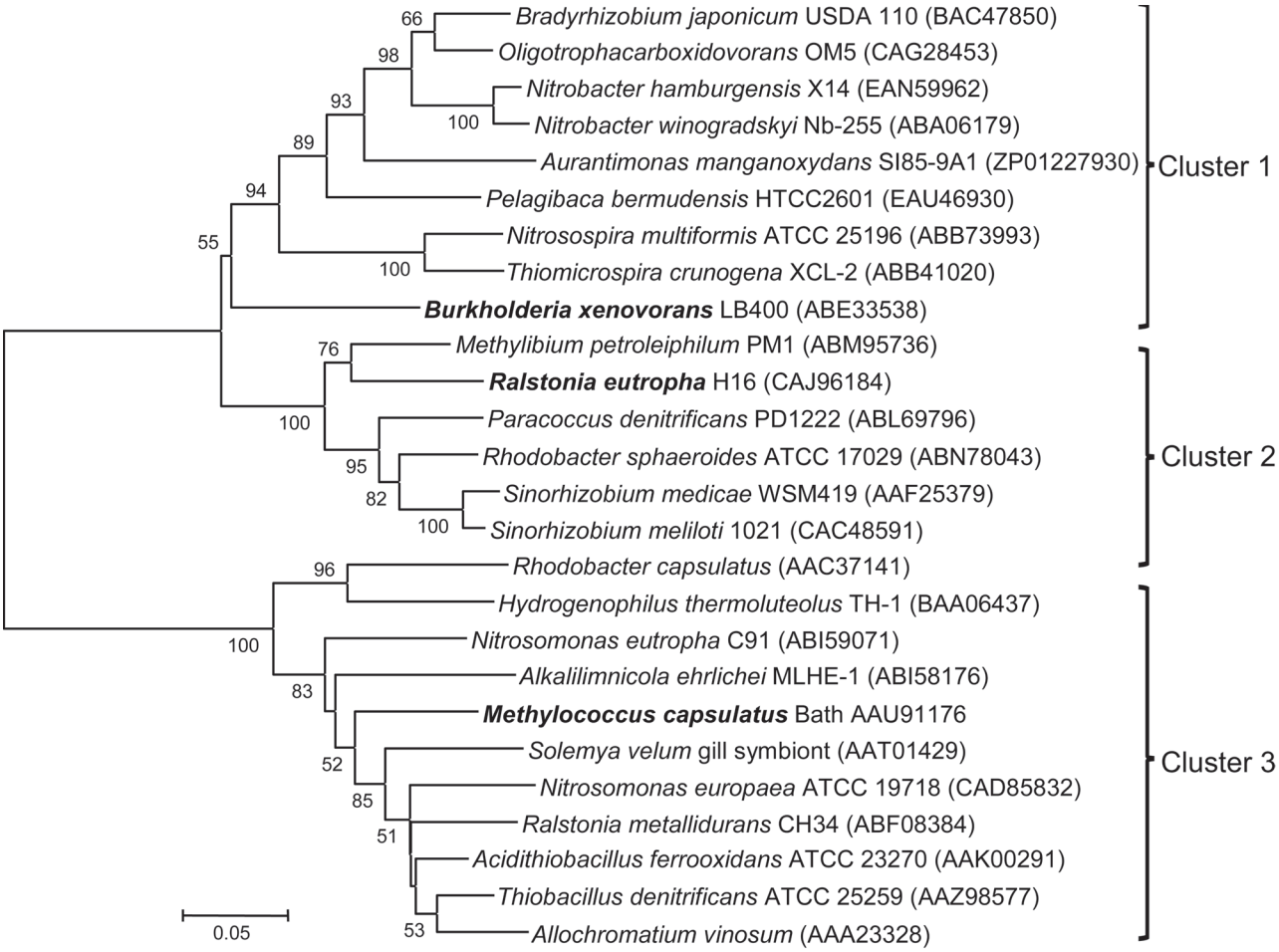

Fig. 1. Phylogenetic tree based on CbbL amino acid sequence comparisons constructed using the neighbor-joining method with the Poisson correction from an alignment of 470 amino acids. Numbers represent bootstrap values for each branch based on data for 1000 trees. Accession numbers for amino acid sequences are given for each strain. Scale bar shows number of amino acid substitutions per site. Organisms shown in bold type were reference strains used to test the primers.

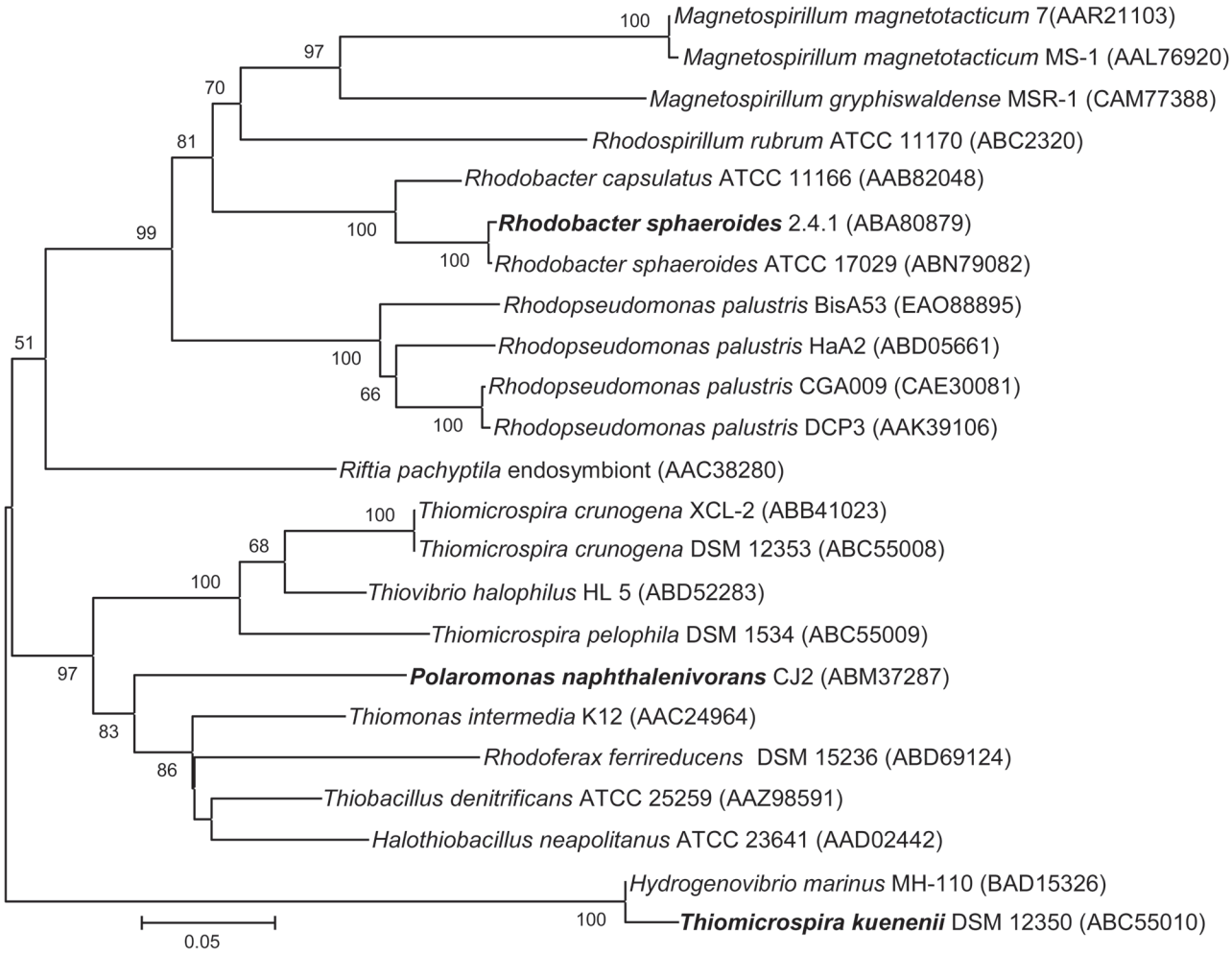

Fig. 2. Phylogenetic tree based on CbbM amino acid sequence comparisons constructed from an alignment of 457 amino acids. [All other details as in legend to Fig. 1]

well-illustrated by the CbbL tree in which three distinct clusters could be identified (Fig. 1). Similarly, clustering into two or more groups was seen for the CbbM, Hpr, MauA, and MxaF trees (Fig. 2–5), with further probable subdivision into several clades, between which considerable sequence diversity was seen. The distribution of sequences from different Bacteria was frequently not congruent with their 16S rRNA gene sequence phylogeny, with distinct clusters or groups containing mixtures of *Alpha- Beta-* and *Gammaproteobacteria*. This was particularly marked in the case of cluster 3 of the CbbL tree, which contained examples of *Alpha-, Beta-* and *Gammaproteobacteria* (Fig. 1: *e.g. Rhodobacter, Thiobacillus, Methylococcus*); and for CbbM and HPr (in which considerable 'sub-clustering' was seen) with different proteobacterial genera exhibiting highly similar proteins (Fig. 2: *e.g. Rhodoferax, Thiobacillus,* and *Halothiobacillus*; Fig. 3: *e.g. Acidiphilium, Methylibium*). McDonald and Murrell (1997) originally observed consistency between proteobacterial groups and the *mxaF*-encoded amino acid sequences, which was largely supported by our analysis although with some occurrence of both *Alpha-* and *Beta- and Gammaproteobacteria* within the same cluster (Fig. 5: *e.g. Granulibacter*, *Methylophaga* and *Paracoccus*). Similarly, there was clustering of *Beta-* and *Gammaproteobacteria* in our MauA tree (*Methylophaga, Methylophilus, Methylobacillus*), with *Alphaproteobacteria* forming a separate cluster (Fig. 4: *Paracoccus, Methylobacterium* and *Hyphomicrobium*). Clustering of similar amino acid sequences for genera from different phylogenetic classes suggested that horizontal gene transfer (HGT) had taken place in the acquisition of genes for at least some of these functional enzymes (Kalyuzhnaya *et al.*, 2008), while clusters of members the same proteobacterial group with similar proteins was consistent with retention of an ancestral form. Using MxaF as an example (Fig. 5), the proteins from *Methylophaga* and *Methylococcus* show only 43% sequence identity to that from *Xanthobacter* (all being *Gammaproteobacteria*), but show 70–73% identity to that from *Paracoccus* (*Alphaproteobacteria*), highly indicative of HGT having occurred.

Designing the primers. For primer design, groups of sequences from within the amino acid trees (Fig. 1–5)

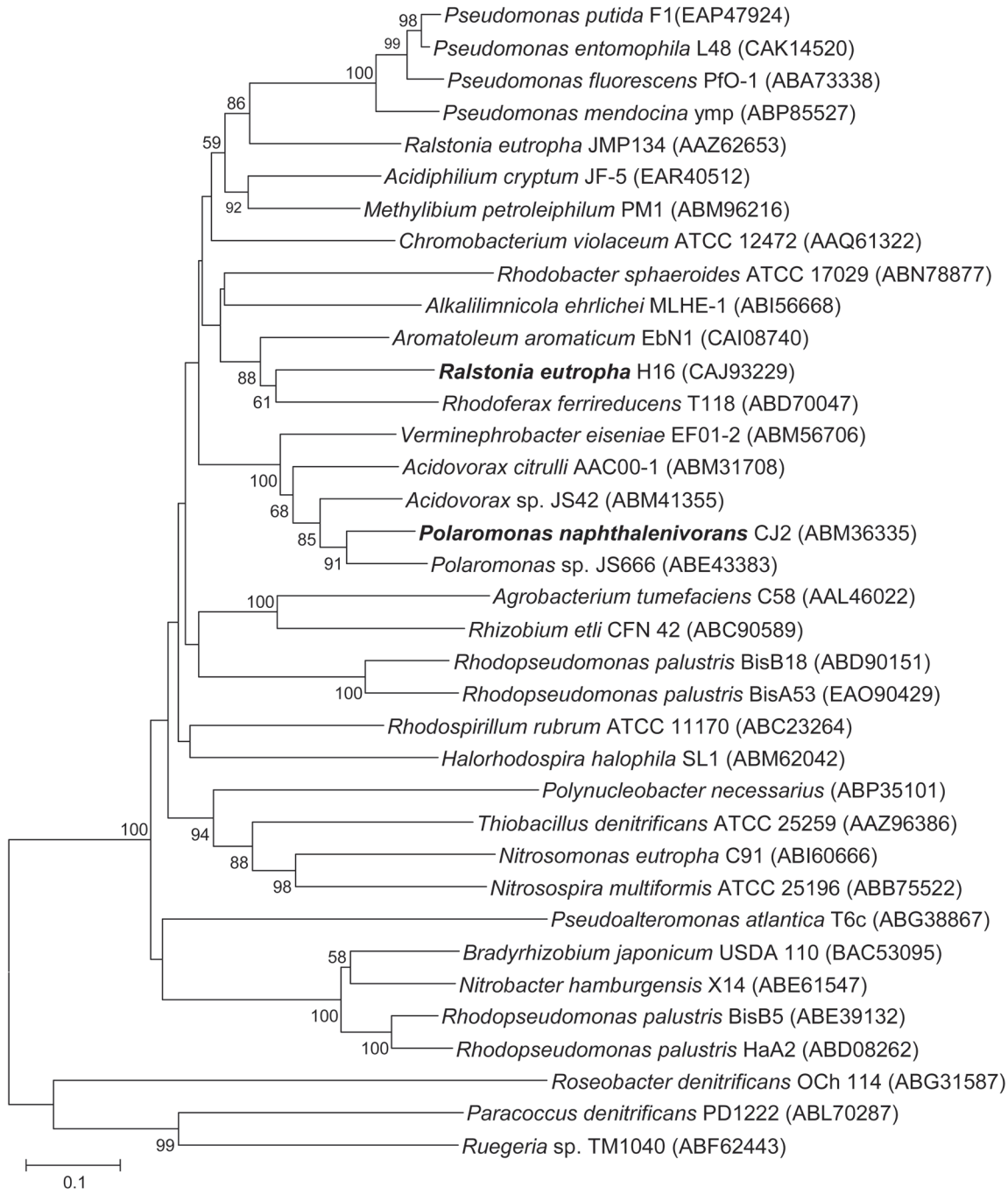

Fig. 3. Phylogenetic tree based on Hpr amino acid sequence comparisons constructed from an alignment of 315 amino acids. [All other details as in legend to Fig. 1]

were selected to enable targeting of the presumed diverse genes encoding the enzyme proteins. For *cbbL*, for example, nucleotide sequences were retrieved for the three clusters of the 26 strains of *Proteobacteria* (Fig. 1). Sub-alignments were created for each of the three clusters, and there was sufficient sequence homology between strains within the clusters to allow design of cluster-specific degenerate PCR primers. Primers were designed in a similar way for the genes encoding the other clusters for 23 *cbbM*, 36 *hpr*, and 12 *mauA* nucleotide sequences. All sequences found for these genes were from strains of *Proteobacteria*, except for *mauA* for which one sequence was from the verrucomicrobium, *Methylacidiphilum infernorum*.

Amino acid sequences encoded by the 22 *mxaF* genes subdivided into three, possibly four, clusters or clades (Fig. 5) and primers were designed for the *Granulibacter-Bradyrhizobium* group (*mxaF* I), and for the *Xanthobacter-Xanthomonas* clade (*mxaF* III). For the *Hyphomicrobium-Methylobacillus* clade(s), we found that the primers designed by McDonald and Murrell (1997) and Neufeld *et al.*, (2007) gave good

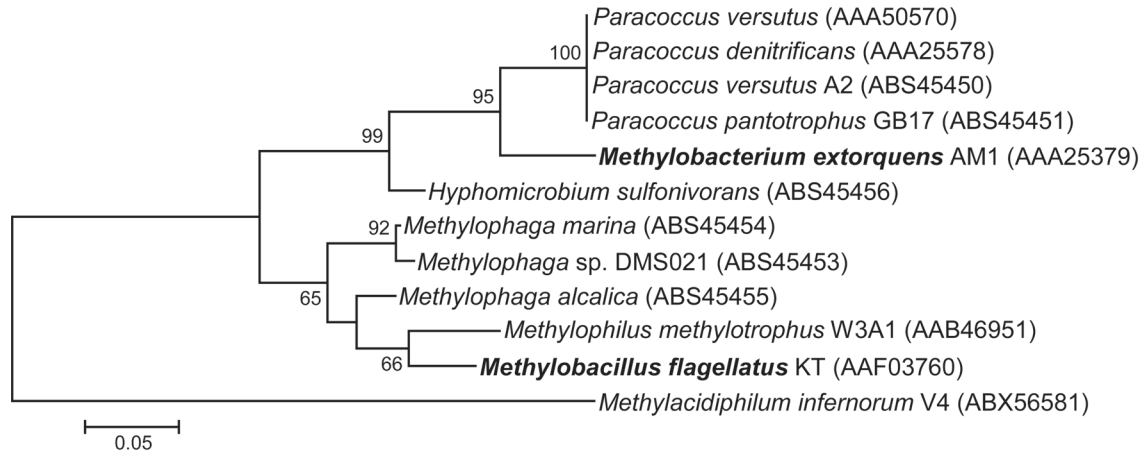

Fig. 4. Phylogenetic tree based on MauA amino acid sequence comparisons constructed from an alignment of 91 amino acids. [All other details as in legend to Fig. 1]

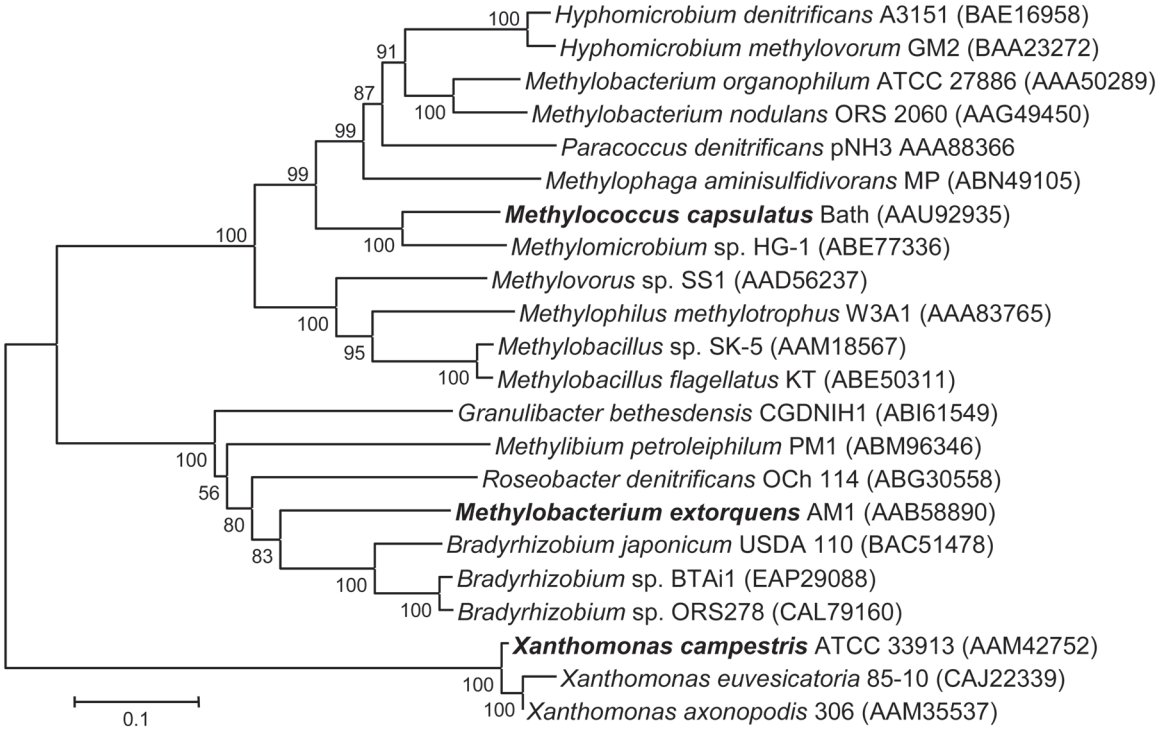

Fig. 5. Phylogenetic tree based on MxaF amino acid sequence comparisons constructed from an alignment of 155 amino acids. [All other details as in legend to Fig. 1]

matches to these sequences (differing in the main by 0–2 mismatches, and by 3–4 only in three of 36 primer matches for the 12 sequences). This contrasted with up to 12 mismatches for the other two groups. Consequently, it was unnecessary to design new primers for *Hyphomicrobium-Methylobacillus* group, and the available primers were used as *mxaF* II.

For *msmA*, four alphaproteobacterial GenBank sequences were used for primer design (*Methylobacterium nodulans*, ZP_02124686; *Afipia felis*, ABO41866; *Methylosulfonomonas methylovora*, AAD26619; and *Marinosulfonomonas methylotropha*, AAK84301). These were quite divergent, showing only 83–87% encoded amino acid sequence similarity to each other, but enabled nine primers to be designed.

Attempts to design primers for the hexulose phosphate synthase gene, *hps*, were unsuccessful. Database sequences were compared for 12 species of eight genera, all members of the *Gammaproteobacteria* (*Escherichia, Haemophilus, Klebsiella, Mannheimia, Methylococcus, Methylomonas, Shigella* and *Vibrio*), whose amino acid sequences fell into four distinct clades on a tree, but did not allow for the design of useful primers: this may have reflected the diversity of function known for Hps, which

is an assimilatory enzyme of RuMP-pathway methylotrophs, but functions for formaldehyde detoxification in non-methylotrophs (Kato *et al*., 2006), and possibly for pentose synthesis in other Bacteria and Archaea (*e.g.* Yurimoto *et al*., 2009).

Testing the new primers with DNA from the reference strains. A total of 100 primers were designed for the functional genes of RuBisCO (*cbbL* I, II, III and *cbbM* I, II), MDH (*mxaF* I, II), HPR (*hpr* I, II), MADH (*mauA* I, II), and MSAMO (*msmA*), all of which were tested in various combinations against the relevant reference organisms (see Materials and Methods and Fig. 1–5). Most generated the predicted size product, and amplicons were sequenced to confirm that the correct gene had been amplified. A reduced set of 44 primers yielding the correct amplicons was selected for recommended use (Table I). Pairs of these were tested in various combinations at different annealing temperatures (42–61°C): for example, ten *cbbL* I pairs tested with *Burkholderia xenovorans* DNA gave the correct-sized products at two or more of five temperatures between 45° and 57°C, nine of them at 54°C. Similarly, four *cbbL* II and nine *cbbL* III primer pairs gave products over the same range with *Ralstonia eutropha* and *Methylococcus capsulatus* at 54°C. For primer pairs designed for the other genes, the optimum was also 54°C, with few reactions at lower or higher temperatures. This established 54°C as the most likely temperature for annealing with the primer pairs.

Sequencing of the amplicons of the predicted size generated by the new primers with the reference Bacteria showed 99–100% sequence identity to the expected products (Table II).

Testing the methylotrophic strains from the human mouth with the primers. The primers were tested against DNA from the eleven methylotrophs isolated from the mouth and described in detail by Hung *et al*. (2011). Six of these were *Actinobacteria*, none of which gave the predicted PCR products with any of the primers. As the design of the functional primers was based on proteobacterial sequences, it seems that they were not suitable for detecting the corresponding genes in the *Actinobacteria*. Alternatively, those strains may use different metabolic pathways: information on methylotrophy in Gram-positive organisms is relatively limited (Arfman *et al*., 1992), as methylotrophic metabolism research has mainly focused on pathways in Gram-negative organisms. For example, methanol oxidation by a thermophilic *Bacillus* uses an NAD-dependent MDH unrelated to the proteobacterial PQQ enzyme (de Vries, 1992); and a *Mycobacterium* strain used a novel methanol-oxidizing enzyme for its methylotrophic growth (Park *et al*., 2011). Also, methylamine dehydrogenase is not a unique diagnostic enzyme for methylamine-degraders, as

Table I
PCR primers for the amplification of *cbbL*, *cbbM*, *hpr*, *mxaF*, *msmA*, and *mauA* genes (5'-3'; F forward primer; R reverse primer)

Target gene	Primer	Sequence
cbbL	cbbLI-310F	TGGACCRYVGTVTGGACCGA
	cbbLI-763F	TTYATGCRYTGGCGCGAYCG
	cbbLI-1331R	SGTGCCRCCRCCRAAYTGCA
	cbbLII-446F	TCGCCTTCSAGYTTGCCVAC
	cbbLII-1254R	GTGTGGACCGAYCKSCTGAC
	cbbLIII-354F	GGYAACGTVTTCGGMTTYAA
	cbbLIII-615F	CAGCCNTTCATGCGYTGGMG
	cbbLIII-909R	ACVCGGAAGTGRATRCCGTG
cbbM	cbbMI-899F	TSATGAAGATCGCSTAYCCS
	cbbMI-1062F	GCAACAACCAGGGCATGGGC
	cbbMI-1472R	TCGSCRAAGGTYTCVAGGAT
	cbbMI-1706R	TCCATCTTGCCRWARCCCAT
	cbbMIII-517F	TCATYAAGAACGAYGARCCV
	cbbMIII-1169R	TCCATYTTRCCGTARCCCAT
	cbbMIII-1298R	AGVGCGTTCATRCCRCCRGA
hpr	hprI-463F	TGATCTCSGGCGGCGGCTCG
	hprI-606F	ATCAAGGGCGGGCGGCTVGC
	hprI-1069R	GTSACCGTSGTYTCGCCGCC
	hprI-1071R	ANGGTSACCGTSGTYTCGCC
	hprI-1389R	CKGAARTCRTTVACRTTGGT
	hprII-236F	CCRTCRATGCCGTCGGTRTC
	hprII-239F	TCGAYGCCGTCGGTGTCGSC
	hprII-369F	TCACBGTGGTYTCGCCRCCV
	hprII-776R	TCSGAYGTSCCSGGCGAYGA
	hprII-851R	CAYCTGTCSGCSATCAAGGG
	hprII-986R	CTGMTSTCBGGCGGCGGNTC
	hprII-1239R	VTYGGCGCBGGCAAGGCCKC
mxaF	mxaFI-356F	GTACGAGCCGAAGCAGGATC
	mxaFI-778F	GCGATCAGTGGAAGAYCGGY
	mxaFI-888F	CAGCGTCCGGGCGACAACAA
	mxaFI-1323R	TGGTTGGTSGGSACGTAGAA
	mxaFI-1447R	CCAGGCGATGAAGTTGCCCA
	mxaFIII-269F	AGATCAATCGCGACAACGTC
	mxaFIII-588F	CGGCAAGGAAGTGTGGAAGC
	mxaFIII-1024R	GTCATCGACCACTTGTTGTC
	mxaFIII-1507R	GGCTTCATCATCACGTTGGC
msmA	msmA-686R	CCGGTTATGGTAGTGCATGA
	msmA-854R	CCACTGGTTCGGCGGCAGAT
	msmA-421F	AAGGAAGGCTATCAGGACCG
	msmA-202F	GCATCGCCAACGAGCCGATC
mauA	mauAI-252F	GCACTGTTCCATCGACGGCA
	mauAI-490R	GCGCCGAAGCACCAGATGAT
	mauAII-232F	AAGTCTTGCGATTACTGGCG
	mauAII-526R	GACCGTGCAATGGTAGGTCA

Table II
Testing of new primers against reference strains known to contain the target genes. The top matches are shown from BLASTX homology interrogation of the protein database with the amino acid sequences encoded by the amplicons obtained from PCR with the primers

Gene	DNA template organism	Primer pair	BLASTX top match	Identity (% and amino acids aligned) to database sequence
mxaF I	*Methylobacterium extorquens* AM1	356F+1447R	PQQ-dependent dehydrogenase *M. extorquens* PA1 (ZP_02054561)	99 (248/250)
		888F+1323R	PQQ-dependent dehydrogenase *M. extorquens* PA1 (ZP_02054561)	100 (147/147)
mxaF III	*Xanthomonas campestris* pv *campestris*	588F+1024R	MDH heavy chain, *X. campestris* pv *campestris* ATCC 33913 (NP_638828)	100 (151/151)
		269F+1507R	MDH heavy chain, *X. campestris* pv *campestris* ATCC 33913 (NP_638828)	99 (283/285)
hpr I	*Polaromonas naphthalenivorans* CJ2	606F+1071R	Hydroxypyruvate reductase *P. naphthalenivorans* CJ2 (YP_981256)	100 (155/155)
		606F+1389R	Hydroxypyruvate reductase *P. naphthalenivorans* CJ2 (YP_981256)	99 (255/256)
hpr II	*Ralstonia eutropha* H16	236F+1239R	Hydroxypyruvate reductase *R. eutropha* H16 (YP_728027)	100 (301/301)
		369F+776R	Hydroxypyruvate reductase *R. eutropha* H16 (YP_728027)	99 (132/133)
cbbL I	*Burkholderia xenovorans* LB400	763F+1331R	RuBisCO B. xenovorans LB400 (YP_552888)	99 (194/195)
		310F+1331R	RuBisCO *B. xenovorans* LB400 (YP_552888)	100 (304/304)
cbbL II	*Ralstonia eutropha* H16	446F-1254R	RuBisCO *R. eutropha* H16 (YP_840914)	100 (238/238)
cbbL III	*Methylococcus capsulatus* Bath	615F+909R	RuBisCO *M. capsulatus* Bath (YP_115143)	100 (104/104)
		354F+909R	RuBisCO *M. capsulatus* Bath (YP_115143)	100 (189/189)
cbbM I	*Rhodobacter sphaeroides* 2.4.1	899F+1706R	RuBisCO large chain form II *R. sphaeroides* 2.4.1 (YP_354780)	99 (233/234)
		1061F+1472R	RuBisCO large chain form II *R. sphaeroides* 2.4.1 (YP_354780)	99 (141/142)
cbbM II	*Polaromonas naphthalenivorans* CJ2	427F+1054R	RuBisCO *P. naphthalenivorans* CJ2 (YP_982208	100 (183/183)
		811F+1054R	RuBisCO *P. naphthalenivorans* CJ2 (YP_982208	100 (87/87)
cbbM III	*Thiomicrospira kuenenii*	517F+1169R	RuBisCO large subunit form II *T. kuenenii* (ABC55010)	100 (212/212)
		517F+1298R	RuBisCO large subunit form II *T. kuenenii* (ABC55010)	100 (166/166)
mauA I	*Methylobacterium extorquens* AM1	252F-490R	Methylamine dehydrogenase light chain, *M. chloromethanicum* CM4 (ZP_02059240)	100 (62/62)
mauA II	*Methylobacillus flagellatus* KT	232F+526R	MauA (methylamine dehydrogenase small subunit) *M. flagellatus* KT (AAF03760)	100 (91/91)
msmA	*Afipia felis*	202F+854R	Methanesulfonate monooxygenase (hydroxylase alpha subunit) *Afipia felis* 25E1 (ABO41866)	100 (146/146)
		421F+686R	Methanesulfonate monooxygenase (hydroxylase alpha subunit) *Afipia felis* 25E1 (ABO41866)	100 (83/83)

Table III
PCR amplification of *mxaF*, *hpr*, *cbbL* and *cbbM* genes in three mouth isolates and six known methylotrophic or autotrophic strains

Primers	Strains								
	Pseudomonas NaF-B-1	*R. pickettii* MMA-BI-3	*Methylobacterium* MMA-CI-1	*Aminobecter aminovorans* MA	*Xanthobacter tagetidis* TagT2C	*Methylocella silvestris* BL2	*Methylocystis sporium* 5	*Methylocystis parvus* OBBP	*Methylosinus trichosporium* OB3b
mxaF 356F + 1447R	–	–	+	–	–	+	–	–	+
mxaF 778F + 1323R	+	+	nd[a]	–	–	+	+	–	+
mxaF 888F + 1323R	–	–	nd	+	–	+	–	–	–
mxaF 1003F + 1555R[b]	–	–	+	–	–	+	+	+	+
hpr 463F + 1069R	–	+	+	+	–	+	–	–	–
hpr 239F + 851R	+	–	–	–	–	+	–	–	–
hpr 239F + 986R	+	–	–	–	–	–	–	–	–
cbbL 763F + 1331R	–	–	–	nd	–	+	nd	nd	nd
cbbL 446F + 1254R	–	–	–	nd	+	+	nd	nd	nd

[a] nd not determined
[b] Neufeld *et al.* (2007)

methylamine oxidase operates in Gram-positive organisms (Chistoserdova *et al.*, 2009).

The five *Proteobacteria* were tested with 9, 3 and 9 primer pairs for *cbbL* I, *cbbL* II, and *cbbL* III, 7 and 5 pairs for *cbbM* I and *cbbM* II, and 6 pairs for *cbbM* III. All PCRs were run at an annealing temperature of 54°C, as well as 45–57°C for *cbbL*, and 50 and 57°C for *cbbM*. No PCR products were obtained from any of the five strains: the absence of the RuBisCO genes under all the PCR conditions was consistent with none of the strains having been shown to be autotrophic.

Several pairs of primers were tested for *hpr* I (4 pairs), *hpr* II (7), *mxaF* I (8), *mxaF* II (2), *mauA* (1) and *msmA* (2), with an annealing temperature of 54°C. Predicted products were obtained with *mxaF* and *hpr* primers for three strains (NaF-B-1, MMA-BI-3 and MMA-C-1; Table III). The PCR products from *Pseudomonas* sp. NaF-B-1 and *Ralstonia pickettii* MMA-BI-3 (*mxaF* II 778F+1323R), *Methylobacterium* sp. MMA-CI-1 (*mxaF* I 356F+1447R and *mxaF* II 1003F+1555R), *Ralstonia picketti* and the *Methylobacterium* strain (*hpr* I 463F+1069R), and the *Pseudomonas* strain (with *hpr* II 239F+986R) were sequenced, and in all cases identified as the expected sequences (Table IV). These results were consistent with the reported enzyme activities (Hung *et al.*, 2011).

No PCR products were obtained from any of the five proteobacterial strains for the *mauA* or *msmA* genes. All the strains were known to grow on MSA and MMA (Hung *et al.*, 2011), so the apparent absence of *msmA* and *mauA* genes could mean that satisfactory PCR conditions had not been achieved, or might indicate that novel enzymes were involved. It is known, for example, that methylamine metabolism in *Rhodopseudomonas capsulata*, *Methylovorus mays* and *Methylocella silvestris* involves glutamine synthetase (GS) to produce γ-glutamylmethylamide (Yoch *et al.*, 1983; Yamamoto *et al.*, 2008; Chen *et al.*, 2010a), while N-methylglutamate synthase and N-methylglutamate dehydrogenase have been proposed as alternatives in *Methyloversatilis universalis* FAM5 (Chistoserdova *et al.*, 2009). A recent study of a lake habitat also failed to detect the *mauA* gene in DNA from methylamine-enriched populations (Antony *et al.*, 2010), consistent with the existence of diverse dissimilatory systems.

In the case of the *Achromobacter* strain (NaF-BI-3), the database genome sequence was available for *Achromobacter xylosoxidans* strain A8, which contains putative genes for *mauABDE* (NC_014640). BLASTP of the encoded MauA amino acid sequence showed only 43–48% identity to the MauA sequences of *Methylacidiphilium infernorum, Methylobacillus flagellatus, Methylobacterium extorquens* and *Paracoccus denitrificans* shown in Fig. 4. It was surprising that the *Achromobacter* genome MauA sequence showed essentially the same low similarity to all four of these sequences, suggesting it was actually close to none of them. In contrast, the reference sequences tested showed 86–91% amino acid sequence identity to those of neighboring species (*e.g. P. denitrificans*/*H. sulfonivorans*; *M. flagellatus*/*M. alcalica*; and *M. extorquens*/*H. sulfonivorans*), whereas identity between more remote pairs, such as *M. infernorum*/*M. alcalica* and *M. flagellatus*/*P. denitrificans*, showed lower sequence similarities of 40% and 66%. Failure to detect the *mauA* gene in *Achromobacter* strain NaF-BI-3 thus suggested that the gene differs sufficiently from the known proteobacterial genes not to be detected by the primers under the conditions used.

Table IV
TBLASTX homology search of the protein database for PCR products obtained from three mouth isolates and six known methylotrophic strains using functional primer sets designed in this study.

Gene	Template	Primer pair	TBLASTX	% identity (amino acid match)
mxaF	*Pseudomonas* sp. NaF-B-1	778F+1323R	PQQ-dependent dehydrogenase *Pseudomonas mendocina* ymp (CP000680)	99 136/138
	Ralstonia pickettii MMA-BI-3	778F+1323R	PQQ-dependent dehydrogenase *Ralstonia pickettii* 12J (YP_001900058)	100 128/128
	Methylobacterium sp. MMA-CI-1	356F+1447R	Methanol dehydrogenase subunit 1 *Methylobacterium extorquens* DM4 (YP_003070571)	99 146/147
	Aminobacter aminovorans MA	888F + 1323R	PQQ-dependent dehydrogenase *Xanthobacter autotrophicus* Py2 (CP000781)	80 47/59
	Methylobacterium sp. MMA-CI-1	1003F+1555R[a]	Methanol dehydrogenase subunit 1 *Methylobacterium extorquens* DM4 (YP_003070571)	99 173/174
	Methylocella silvestris BL2	356F + 1447R	PQQ-dependent dehydrogenase *Methylocella silvestris* BL2 (CP001280)	100 43/43
		1003F+1555R[a]	PQQ-dependent dehydrogenase *Methylocella silvestris* BL2 (CP001280)	100 139/139
	Methylocystis sporium strain 5	778F+1323R	PQQ-dependent dehydrogenase *Methylobacterium nodulans* ORS 2060 (CP001349)	100 148/148
		1003F+1555R	Methanol dehydrogenase alpha subunit *Methylosinus sporium* (AJ459083)	85 90/106
	Methylocystis parvus OBBP	1003F+1555R[a]	Methanol dehydrogenase alpha subunit *Methylocystis* sp. KS12 (AJ459094)	99 144/145
	Methylosinus trichosporium OB3b	356F + 1447R 778F + 1323R	PQQ-dependent dehydrogenase *Methylobacterium* sp. 4-46 (CP000943)	81 142/176
	Methylosinus trichosporium OB3b	1003F+1555R[a]	Methanol dehydrogenase alpha subunit *Methylosinus trichosporium* (AJ459058)	99 159/160
hpr	*Ralstonia pickettii* MMA-BI-3	463F+1069R	Hydroxypyruvate reductase *Ralstonia eutropha* H16 (AM260479)	100 163/163
	Methylobacterium sp. MMA-CI-1	463F+1069R	Hydroxypyruvate reductase *Methylobacterium populi* BJ001 (ACB82755)	98 165/168
	Pseudomonas sp. NaF-B-1	236F+986R	Hydroxypyruvate reductase *Pseudomonas mendocina* ymp (YP_001188259)	100 168/168
	Aminobacter aminovorans MA	463F + 1069R	mlr5144 (hypothetical protein BAB51643) *Mesorhizobium loti* MAFF303099 (BA000012)	82 136/165
	Methylocella silvestris BL2	463F + 1069R and 239F + 851	Hydroxypyruvate reductase *Methylocella silvestris* BL2 (ACK50662)	100 122/122
cbbL	*Xanthobacter tagetidis* TagT2C	446F + 1254R	Ribulose bisphosphate carboxylase *Xanthobacter flavus* (X17252)	98 184/188
cbbL	*Methylocella silvestris* BL2	763F + 1331R and 446F + 1254R	Ribulose bisphosphate carboxylase *Methylocella silvestris* BL2 (CP001280)	100 236/236

[a] Neufeld *et al.* (2007)

A complete genome sequence was also available for a *Klebsiella pneumoniae* strain (NC_012731), enabling comparison with the mouth strain of *Klebsiella* (M-AI-2), in which *mxaF*, *mauA* and *hpr* were not detected. The *Klebsiella* genome contained no genes annotated as either MDH or MADH, suggesting the absence of these genes also from strain M-AI-2. The genome contained genes encoding nine alcohol dehydrogenases, but none showed any identity to the *Methylobacterium mxaF* gene. A gene encoding a putative HPR was

present (KP1_2293), and the encoded protein for this gene (YP_002919048; 419 amino acids) was compared by BLASTP with the amino acid sequences for four of the organisms shown in Fig. 3 (*Polaromonas, Pseudoalteromonas, Ralstonia* H16, and *Roseobacter*). It showed no significant similarity to the *Polaromonas* sequence, and negligible similarity to the other three, with only 6–10 aligned amino acids. This supported the view that the *Klebsiella* strain did not contain a typical *hpr* gene, detectable with the primers used.

Testing the primers with strains of known methylotrophic and autotrophic Bacteria. DNA from six methylotrophic or autotrophic strains was probed with the *mxaF*, *hpr* and *cbbL* primers (Table III), and one or more of the target genes were detected in all of the strains. Sequencing these amplicons revealed identity to the expected gene products (Table IV). The positive 'hits' for *cbbL*, *hpr* and *mxaF* to *Methylocella silvestris* BL2 were obtained before publication of the genome sequence of this organism (Chen *et al*., 2010b), but were then shown to match the relevant loci in the genome, including confirming the presence of RuBisCO genes in this facultatively methanotrophic organism. Finding *cbbL* in *Xanthobacter tagetidis* was expected as it, like other *Xanthobacter* species, grew autotrophically with methanol as the energy source (Meijer *et al*., 1990; Padden *et al*., 1997), and was consistent with the apparent absence of *hpr*. That strain grew weakly on methanol, so the failure to detect *mxaF* might indicate absence of the expected PQQ MDH. It was interesting that the product for *Aminobacter aminovorans*, using *hpr* I primers 463F+1069R showed 82% sequence identity to a hypothetical protein (mlr 5144; BAB51643) in the complete genome of a *Mesorhizobium loti* strain. The genome does not contain a gene annotated as *hpr* for this strain but this result suggests that the gene is present in the *Mesorhizobium loti* strain, and subjecting the BAB51643 amino acid sequence to BLASTP against seven authentic Hpr sequences gave partial sequence matches of 29–42%.

Conclusions. Our study provides evidence of significant diversity among the nucleotide and encoded amino acid sequences for essential genes of methylotrophy and autotrophy. While some sequences correlate with the proteobacterial phylogeny, some show clear evidence of horizontal gene transfer, thereby crossing the phylogenetic boundaries. Genes identified in the genomes of some genera (*e.g. hpr* in *Klebsiella*, *mauA* in *Achromobacter*) appeared to encode as yet uncharacterized novel proteins.

Our study has provided a set of novel primers for genes important in methylotrophy and autotrophy, which detected the target genes in reference strains and in phylogenetically diverse groups of methylotrophic strains for the first time, confirming their potential usefulness in future studies with *Proteobacteria* in pure culture or in consortia in natural environments. Rapid and inexpensive next generation genome sequencing may reduce the need for the separate detection of specific genes in pure cultures of Bacteria, although detection with appropriate primers will still be the minimum-cost method of choice when large numbers of isolates are to be tested. The use of specific primers will continue to be needed for the detection of functional genes in heterogeneous environmental samples. In this respect, the primers for *mau*A have already been applied successfully to demonstrate methylamine-users in environmental samples of the microbial mat from Movile Cave, Romania (D. Wischer, R. Boden, J.C. Murrell, personal communication). For maximum likelihood of detecting possibly variant forms of genes, such as *hpr*, *mau*A, and *mxa*F, it is advisable to use more than one pair of primers when probing target DNA preparations from pure cultures or environmental samples. It is clear that work is also needed to develop a range of primers for the detection of the enzymes that enable methylotrophy in *Actinobacteria*.

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