MINIREVIEW

Facultative methanotrophy: false leads, true results, and suggestions for future research

Jeremy D. Semrau[1], Alan A. DiSpirito[2] & Stéphane Vuilleumier[3]

[1]Department of Civil and Environmental Engineering, The University of Michigan, Ann Arbor, MI, USA; [2]Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA, USA; and [3]Génétique Moléculaire, Génomique, Microbiologie, équipe Adaptations et Interactions Microbiennes dans l'Environnement, UMR 7156 CNRS, Université de Strasbourg, Strasbourg, France

Correspondence: Jeremy D. Semrau, Department of Civil and Environmental Engineering, The University of Michigan, 1351 Beal Avenue, Ann Arbor, MI 48109-2125, USA. Tel.: +1 734 764 6487; fax: +1 734 763 2275; e-mail: jsemrau@umich.edu

Received 18 March 2011; revised 8 May 2011; accepted 14 May 2011.
Final version published online 16 June 2011.

DOI:10.1111/j.1574-6968.2011.02315.x

Editor: Hermann Heipieper

Keywords
metabolism; gene regulation; pollutant degradation.

Abstract

Methanotrophs are a group of phylogenetically diverse microorganisms characterized by their ability to utilize methane as their sole source of carbon and energy. Early studies suggested that growth on methane could be stimulated with the addition of some small organic acids, but initial efforts to find facultative methanotrophs, i.e., methanotrophs able to utilize compounds with carbon–carbon bonds as sole growth substrates were inconclusive. Recently, however, facultative methanotrophs in the genera *Methylocella*, *Methylocapsa*, and *Methylocystis* have been reported that can grow on acetate, as well as on larger organic acids or ethanol for some species. All identified facultative methanotrophs group within the *Alphaproteobacteria* and utilize the serine cycle for carbon assimilation from formaldehyde. It is possible that facultative methanotrophs are able to convert acetate into intermediates of the serine cycle (e.g. malate and glyoxylate), because a variety of acetate assimilation pathways convert acetate into these compounds (e.g. the glyoxylate shunt of the tricarboxylic acid cycle, the ethylmalonyl-CoA pathway, the citramalate cycle, and the methylaspartate cycle). In this review, we summarize the history of facultative methanotrophy, describe scenarios for the basis of facultative methanotrophy, and pose several topics for future research in this area.

Introduction

Aerobic methanotrophs are widely distributed in the environment, found wherever methane : air interfaces develop, including in wetlands, bogs, agricultural, forest and urban soils, rice paddies, groundwater, landfill cover soils, among many other locations (Semrau *et al.*, 2010). These cells play a critical role in the global carbon cycle by utilizing methane as a source of carbon and energy – it is estimated that in soils, aerobic methanotrophs consume ~30 Tg methane $year^{-1}$ (Kolb, 2009).

It was initially widely believed that aerobic methanotrophs were obligate, i.e., that these microorganisms could only grow utilizing methane or methanol, and in some cases, other C_1 compounds such as formaldehyde, formate, and methylamine, but not compounds with carbon–carbon bonds (Bowman, 2006). The cause for obligate methanotrophy is still unresolved (Wood *et al.*, 2004), and, interestingly, many reports have recently been published of methanotrophs that also able to utilize multicarbon compounds as sole growth substrates (Semrau *et al.*, 2010). Hence, it appears that facultative methanotrophy may be more common than originally thought. In this review, the history and basis of facultative methanotrophy is summarized, as well as the implications and applications of such metabolism.

General review of phylogeny and physiology of methanotrophs

The defining characteristic of a methanotroph is its ability to utilize methane as its sole carbon and energy source, and there are at least two forms of the key enzyme involved in the initial oxidation of methane to methanol, the methane monooxygenase (MMO). Most but not all methanotrophs express a membrane-bound or particulate methane monooxygenase (pMMO), while some can either express in addition, or as the unique form, a cytoplasmic, or soluble methane monooxygenase (sMMO). Phylogenetically, aerobic

FEMS MICROBIOLOGY LETTERS

methanotrophs belong primarily to the *Alpha-* and *Gammaproteobacteria*, although recently aerobic methanotrophs have also been found that belong to the *Verrucomicrobia* phylum (Op den Camp *et al.*, 2009; Semrau *et al.*, 2010). The alphaproteobacterial methanotrophs can be further divided in the *Beijerinckiaceae* and *Methylocystaceae* families, while the gammaproteobacterial methanotrophs belong to the *Methylococcaceae* family.

There are many distinguishing characteristics between the major groups of aerobic methane-oxidizing bacteria, including predominant fatty acid composition, intracytoplasmic formation, and the mechanism by which carbon is assimilated into biomass (Op den Camp *et al.*, 2009; Semrau *et al.*, 2010). The latter issue may play a role in the ability of some methanotrophs to utilize multicarbon compounds, with alphaproteobacterial and verrucomicrobial methanotrophs utilizing the serine pathway for carbon assimilation, while *Gammaproteobacteria* methanotrophs utilize the ribulose monophosphate (RuMP) pathway (as discussed in more detail below).

Initial findings of facultative methanotrophy

As comprehensively reported in several recent reviews (Trotsenko & Murrell, 2008; Op den Camp *et al.*, 2009; Semrau *et al.*, 2010), methanotrophs were initially characterized over 100 years ago, and subsequent studies in the 1950s and 1960s indicated that these strains could only utilize methane or methanol for growth (Dworkin & Foster, 1956; Leadbetter & Foster, 1958; Brown *et al.*, 1964; Foster & Davis, 1966). In 1970, however, a first indication that methanotrophs could utilize multicarbon compounds to accentuate growth was reported (Whittenbury *et al.*, 1970). In this classic manuscript describing the isolation and characterization of methanotrophs from sites around the world, a wide variety of methanotrophs were reported to show enhanced growth on methane when malate, acetate, or succinate was also present in the culture medium. Such findings suggested that facultative methanotrophs may exist, i.e., strains that could utilize multicarbon compounds as well as methane as a sole growth substrate.

Shortly thereafter, the first facultative methanotrophic isolates from freshwater lake sediments and water were reported. These could utilize a wide range of multicarbon compounds as growth substrates, including many organic acids (malate, succinate, fumarate, and acetate) and sugars (glucose, galactose, sucrose, lactose, and ribose) (Patt *et al.*, 1974). One strain, later described as *Methylobacterium organophilum* (belonging to the *Alphaproteobacteria*), was further characterized, and had the complete tricarboxylic acid (TCA) cycle (Patt *et al.*, 1976). This strain, however, lost the ability to oxidize methane when grown repeatedly on glucose, and other workers subsequently did not succeed in growing the strain on methane (Green & Bousfield, 1983; Urakami *et al.*, 1993). Collectively, these findings suggested that these isolates were not facultative methanotrophs as originally surmised.

Other early studies reported the isolation of facultative methanotrophs from a rice paddy in South China, as well as from soils collected from an oil refinery in the Northeastern United States (Patel *et al.*, 1978; Zhao & Hanson, 1984a, b). These strains were found to have the complete TCA cycle and two of them, strains R6 and 761H, were able to grow solely on glucose, but not with other sugars such as fructose, galactose, or sucrose. In addition, a variant of strain 761H, strain 761M, could not grow on glucose as the sole carbon source, but glucose, as well as acetate and malate, were reported to enhance its growth on methane. Other sugars either inhibited or had no effect on growth of the strain on methane. Strain 761M, based on its 16S rRNA gene sequence, was later found to group with the *Gammaproteobacteria* (Bowman *et al.*, 1995). To the best of our knowledge, the phylogenetic grouping of strain R6 was never determined (although enzymatic analyses suggested its affiliation to *Alphaproteobacteria*). None of these strains appear to be still extant, making it impossible to repeat these experiments.

Two methanotrophs isolated from freshwater lake sediments were also described as being facultative, i.e., able to utilize not only methane, but also casamino acids, nutrient agar, and a variety of organic acids and sugars for carbon and energy (Lynch *et al.*, 1980). However, one of these isolates, *Methylobacterium ethanolicum*, was later found by members of the same laboratory to actually consist of a stable syntrophic consortium of two methylotrophs, i.e., a *Methylocystis* strain capable of utilizing methane, and a *Xanthobacter* strain capable of utilizing a variety of multicarbon compounds for growth (Lidstrom-O'Connor *et al.*, 1983).

Collectively, the inability of putative facultative methanotrophs to grow on methane after growth on multicarbon substrates, the lack of extant strains, and evidence of stable mixed cultures initially originally described as pure methanotrophic strains all cast serious doubts on the possibility of facultative methanotrophy. As a result, research in this area was severely limited for the next 20 years.

Recent findings of facultative methanotrophy

Efforts to identify novel methanotrophs significantly regained momentum in the 1990s with the discovery of acidophilic methanotrophs from *Sphagnum* peat bogs (Dedysh *et al.*, 1998a, b). The first characterized acidophilic methanotroph was found to represent a new genus and

species within *Alphaproteobacteria*, *Methylocella palustris* (Dedysh *et al*., 2000), and subsequently two further strains of the same genus were isolated, *Methylocella silvestris* and *Methylocella tundrae* (Dunfield *et al*., 2003; Dedysh *et al*., 2004). All three strains were considered novel methanotrophs as their optimal pH for growth was < 6.0. Even more remarkably, all three isolates could only express the sMMO, and not the pMMO. This finding was quite unexpected as it showed that these were the first methanotrophs that did not express pMMO. Initial screens of each isolate showed that they could not grow on sugars or multicarbon substrates, but could grow on methane and methanol, as well as on methylamine to a variable degree, thus they were considered obligate methanotrophs.

These methanotrophs, however, were later shown to be facultative as they could utilize not only C_1 compounds for growth, but also acetate, pyruvate, succinate, malate, and ethanol (Dedysh *et al*., 2005 and Table 1). Cultures of these strains were unequivocally shown to be pure through a suite of rigorous assays, including: (1) phase-contrast analyses of thousands of cells grown with either acetate or methane; (2) sequence analyses of 50 16S rRNA gene clones from both acetate- and methane-grown cultures; and (3) whole-cell hybridizations of thousands of cells with probes specific for *Methylocella*. In no case was any evidence of contamination found. Furthermore, real-time PCR assays showed increases of the *mmoX* gene (encoding for the large hydroxylase subunit of the sMMO) that very closely corresponded with direct microscopic cell counts. Thus, for the first time, clear conclusive proof for the reality of facultative methanotrophy was provided. Remarkably, *M. silvestris* displayed higher yields, carbon conversion efficiency, and growth rates on acetate than on methane. Specifically, the growth rate of *M. silvestris* was 0.053 and 0.033 h^{-1} on acetate and on methane, respectively, suggesting that acetate may be the preferred growth substrate for this microorganism.

Shortly thereafter, another acidophilic methanotroph, *Methylocapsa aurea*, was also identified that could utilize acetate as the sole growth substrate (maximum $OD_{600\,nm} = 0.3$, $\mu = 0.006\,h^{-1}$). As shown in Table 1, neither larger organic acids (citrate, oxalate, malate) nor any tested sugar (glucose, fructose, maltose) could be used as a sole growth substrate (Dunfield *et al*., 2010). In contrast to *M. silvestris*, however, *M. aurea* only expresses pMMO. Strain purity was determined via: (1) phase-contrast and electron microscopy of acetate-grown cultures; (2) sequencing of more than 21 16S rRNA gene clones from both acetate- and methane-grown cultures; and (3) streaking onto medium with yeast extract and growing cultures with acetate in the absence of methane. In contrast to *M. silvestris*, however, *M. aurea* grew best on methane, with a maximum $OD_{600\,nm}$ of 1.2 and $\mu = 0.018\,h^{-1}$.

It is interesting to note that all these facultative methanotrophic species are not only acidophilic, but also members of the *Beijerinckiaceae* family known to include species with broad substrate ranges. It could thus be hypothesized that facultative methanotrophy will only thrive in a small subset of acidophilic methanotrophs of this family, in environments where organic acids such as acetate are found primarily in the protonated form due to the prevailing low pH, and are thereby more readily taken up (Axe & Bailey, 1995). Facultative methanotrophy, however, does not extend to all acidophilic methanotrophs of the *Beijerinckiaceae* family. For example, *Methylocapsa acidophila* cannot grow on multicarbon compounds such as malate, acetate, ethanol, succinate, or pyruvate (Dedysh *et al*., 2002, 2005; Dunfield *et al*., 2010).

As a result of these findings, more effort has been spent to find other facultative methanotrophs, and in the past year, other acidophilic methanotrophs of the genus *Methylocystis* (family *Methylocystaceae*) were found that could grow on either methane or acetate (Belova *et al*., 2011). Specifically, *Methylocystis* strain H2s, a mild acidophile (optimal growth pH of 6.0–6.5) with functional genes for both sMMO and pMMO (but shown to express pMMO only regardless of tested conditions), was found to not only utilize methane and methanol for growth, but acetate as well. As with studies on other acidophilic methanotrophs, culture purity was rigorously proven using a variety of microscopic and molecular analyses. Growth was greater on methane than on acetate (maximum $OD_{410\,nm}$ of 0.8–1.0 and 0.25–0.30, respectively), as was the growth rate ($\mu = 0.06$ and $0.006\,h^{-1}$, respectively). These data would suggest that methane is the preferred substrate of this strain. However, when both acetate and methane were used simultaneously, overall growth was enhanced, as first noted by Whittenbury *et al*. (1970) for other methanotrophs. Interestingly, strain H2s was not found to grow significantly on any other organic acid or sugar (Table 1).

With the finding of a facultative *Methylocystis* strain, Belova *et al*. (2011) screened validly described *Methylocystis* species for facultative methanotrophic growth, and found that another acidophilic species with an optimal pH range of 5.8–6.2, *Methylocystis heyeri* H2, also grew significantly on acetate. Most mesophilic *Methylocystis* species (i.e. growth pH of 6.8) did not grow on acetate, with the exception of *Methylocystis echinoides* IMET10491 which grew in the presence of acetate from an initial $OD_{410\,nm}$ of ~0.03 to a final $OD_{410\,nm}$ of 0.09 after 200 h of incubation.

A second recent study supports the finding of facultative mesophilic *Methylocystis* species, with the characterization of *Methylocystis* strain SB2, a novel methanotroph that can only express pMMO (Im *et al*., 2011). This isolate, collected from a spring bog with an optimal growth pH of 6.8, was able to utilize methane, ethanol, or acetate as growth substrates. Growth was highest on methane followed by ethanol and acetate (maximum $OD_{600\,nm}$ of 0.83, 0.45, and

Table 1. Substrate utilization and general characteristics of *Alphaproteobacteria* facultative methanotrophs

	Methylocella silvestris	*Methylocella palustris*	*Methylocella tundrae*	*Methylocapsa aurea*	*Methylocystis* strain H2s	*Methylocystis* strain SB2
Growth substrate						
Methane	+	+	+	+	+	+
Methanol	+	+	+	+	+	−
Formate	−	−	±	±	−	−
Formaldehyde	ND	ND	ND	−	−	ND
Methylamine	+	±	+	ND	ND	−
Urea	−	−	−	ND	ND	ND
Glucose	−	−	−	−	−	−
Fructose	−	−	−	−	−	−
Sucrose	−	−	−	−	ND	−
Lactose	−	−	−	−	−	ND
Galactose	−	−	−	−	ND	−
Xylose	−	−	−	ND	ND	−
Sorbose	−	−	−	ND	ND	ND
Maltose	−	−	−	−	−	−
Raffinose	−	−	−	ND	−	ND
Arabinose	−	−	−	−	−	−
Ribose	−	−	−	ND	ND	ND
Lactate	−	−	−	ND	ND	ND
Oxalate	−	−	−	−	−	−
Citrate	−	−	−	−	−	−
Acetate	+	+	+	+	+	+
Pyruvate	+	+	+	−	±	−
Succinate	+	+	+	−	−	−
Malate	+	+	+	−	−	−
Ethanol	+	+	+	ND	±	+
Mannitol	−	−	−	ND	−	ND
Sorbitol	−	−	−	ND	−	ND
Reported growth rates (μ, h^{-1})						
Methane	0.033	ND	ND	0.018	0.06	0.052
Acetate	0.053	ND	ND	0.006	0.006	Linear/ Exponential*
Ethanol	ND	ND	ND	ND	ND	0.022
General characteristics						
Family	*Beijerinckiaceae*	*Beijerinckiaceae*	*Beijerinckiaceae*	*Beijerinckiaceae*	*Methylocystaceae*	*Methylocystaceae*
Carbon fixation pathway	Serine cycle	Serine cycle	Serine cycle	Serine cycle	Serine cycle	Serine cycle
sMMO	+	+	+	−	+	−
pMMO	−	−	−	+	+	+
Optimal growth pH	5.5	5.5–6.0	5.0–5.5	6.0–6.2	6.0–6.5	6.8
Isolation location	Acidic forest cambisol	*Sphagnum* peat bog	*Sphagnum* tundra peatland	Acidic forest soil	*Sphagnum* peat bog	Spring bog

*Growth of *Methylocystis* strain SB2 on acetate could be modeled as either exponential or linear growth, thus no exponential growth rates have been reported.

+, Substantial growth; ±, trace growth; −, no growth; ND, not determined (data collected from: Dedysh *et al.*, 2000, 2004, 2005; Dunfield *et al.*, 2003, 2010; Chen *et al.*, 2010b; Belova *et al.*, 2011; Im *et al.*, 2011).

0.26, respectively). Interestingly, growth on methane and ethanol followed standard exponential kinetics ($\mu = 0.052$ and $0.022\,h^{-1}$, respectively), but growth on acetate could be modeled as either exponential or linear growth. Such a finding supports the hypothesis that acetate is transported into *Methylocystis* strain SB2 as the undissociated acid, and at this growth pH, the proton-motive force is dissipated for acetate uptake (Axe & Bailey, 1995). Finally, as with other investigations of facultative methanotrophy, culture purity was verified using a variety of microscopic and molecular techniques.

Gene expression in facultative methanotrophs

The recent findings of facultative methanotrophy raises some very interesting questions. Particularly, is the MMO

expressed when these strains are grown on multicarbon compounds in the absence of methane? Interestingly, acetate has been shown to repress MMO expression in some facultative methanotrophs, while others constitutively express MMO regardless of the growth substrate. Specifically, when using acetate as an alternative substrate, *M. silvestris* was clearly shown to repress expression of the sMMO (the only form of MMO it expresses) in either the absence or presence of methane (Theisen *et al.*, 2005). As shown by Dedysh *et al.* (2005), *M. silvestris* prefers acetate over methane as a growth substrate, possibly due to the requirement of reducing equivalents for the initial oxidation of methane to methanol, and because acetate concentrations can be quite high in *Sphagnum* peat bogs where this strain was isolated. As a result it appears that facultative methanotrophic *Methylocella* strains have an effective regulatory network to control MMO expression.

Conversely, the facultative *Methylocystis* strains H2s and SB2 were found to constitutively express pMMO regardless if these strains were grown on methane or acetate (Belova *et al.*, 2011; Yoon *et al.*, 2011). Expression of *pmoA*, a key functional gene of the pMMO however, was significantly greater when *Methylocystis* strain SB2 was grown on methane than on acetate (Yoon *et al.*, 2011). As described above, these strains show weaker growth on acetate. It may be that these strains use acetate as a secondary carbon or reducing source that enables the continued expression of MMO in the absence of methane such that these strains can readily utilize methane when it becomes more available (Dunfield, 2007; Belova *et al.*, 2011). These strains, however, were also isolated from bogs where acetate concentrations can be expected to be high (Duddleston *et al.*, 2002), thus, the ability to control MMO expression may have other origins. It is interesting to note that sMMO expression in *Methylococcus capsulatus* Bath is repressed at high copper concentrations, while pMMO is constitutively expressed but its expression increases with increasing copper concentration (Choi *et al.*, 2003). The finding that sMMO expression by *M. silvestris* is repressed in the presence of acetate while pMMO expression is constitutive and positively regulated by the carbon source in *Methylocystis* strain SB2 suggests that the regulatory pathway of sMMO/pMMO expression used by facultative and obligate methanotrophs have some similarities.

Speculations on the origin of facultative methanotrophy

It may be that *Methylocella* species were originally facultative methylotrophs, later generating the ability to utilize methane as a growth substrate through lateral gene transfer of the genes for the sMMO, and subsequently developed the ability to control MMO expression with respect to carbon source. This is intriguing as *Methylocella* species are the only known methanotrophs lacking pMMO. By extension, *M. aurea* may also have been methylotrophic, but through lateral gene transfer developed the ability to express pMMO. To the best of our knowledge, however, it should be stressed that it has not yet been reported whether *M. aurea* expresses pMMO when grown on acetate. Although the origin of facultative methanotrophy from methylotrophs in these strains is speculative, it is interesting to note that a facultative methylotroph, *Methylobacterium extorquens* AM1, when engineered to express the ammonia monooxygenase (AMO) of *Paracoccus denitrificans*, was able to grow on methane as the sole carbon source (Crossman *et al.*, 1997). The AMO is capable of oxidizing methane to methanol, thus these results suggest that AMO activity enabled *M. extorquens* AM1 to utilize methane as a sole carbon source. On the other hand, facultative *Methylocystis* species may have originally been obligate methanotrophs that constitutively expressed pMMO, but developed the ability to utilize acetate through selective pressure to either increase the expression of various enzymatic systems needed for effective acetate assimilation or through lateral gene transfer to complete corresponding pathways as required (see below for further discussion).

How do facultative methanotrophs assimilate multicarbon substrates?

Although empirical evidence definitively shows that facultative methanotrophy exists, the pathway(s) by which multicarbon compounds are assimilated by these strains is still unclear. Historically, an incomplete citric acid cycle in *Gammaproteobacteria* methanotrophs (2-ketoglutarate dehydrogenase activity is missing) and the absence of transporters for compounds with carbon–carbon bonds have been viewed as the primary reasons why this microbial group can only utilize C_1 compounds (Wood *et al.*, 2004). *Alphaproteobacteria* methanotrophs, of which all known facultative methanotrophs are members, however, have the complete TCA cycle, which removes one of the metabolic restrictions noted above (Trotsenko & Murrell, 2008). To date, facultative methanotrophs have been found to utilize C_2 to C_4 organic acids or ethanol as sole growth substrates. As these compounds are typically membrane permeable, the second metabolic restriction for methanotrophic growth is also removed. In the following discussion, we will consider several pathways by which facultative methanotrophic growth may occur on acetate as this compound can be used as a sole growth substrate by all currently known facultative methanotrophs.

Microbial uptake of acetate is known to occur both through a specific permease as well as by passive diffusion through the cell membrane (Gimenez *et al.*, 2003). Growth characteristics of facultative methanotrophs

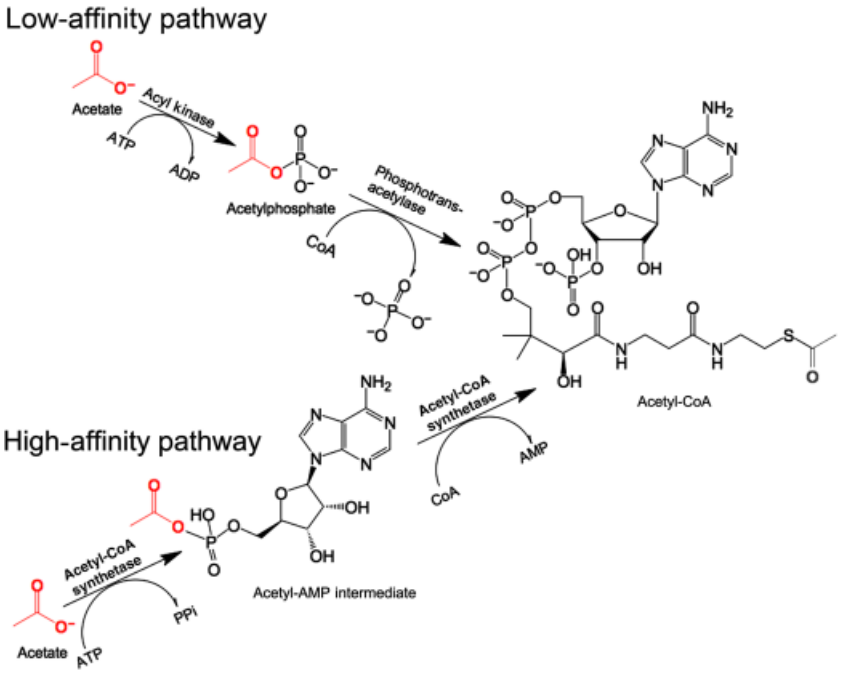

Fig. 1. Mechanisms for the production of acetyl-CoA from acetate (modified from Starai & Escalante-Semerena, 2004).

and observations that most facultative methanotrophs are isolated from acidic environments with high acetate concentrations suggest acetate enters via passive diffusion. Following uptake, acetate must first be activated to acetyl-CoA before assimilation into biomass (Starai & Escalante-Semerena, 2004). In environments with high concentrations of acetate (i.e. $>$ 30 mM) or in cells with active transport systems, acetate can be activated via a kinase and a phosphotransacetylase to acetyl-CoA (Fig. 1). In the absence of these enzymes or under lower acetate concentrations, acetate can be activated via the acetyl-CoA synthetase (either AMP or ADP forming) (Starai & Escalante-Semerena, 2004). Once activated, acetyl-CoA can then be assimilated via a variety of pathways including, but not limited to the glyoxylate shunt (Fig. 2), the ethylmalonyl-CoA pathway (Fig. 3), the methylaspartate cycle (Fig. 4), or the citramalate cycle (Fig. 5) (Howell *et al.*, 1999; Dunfield *et al.*, 2003; Theisen *et al.*, 2005; Erb *et al.*, 2007, 2009; Berg & Ivanovsky, 2009; Peyraud *et al.*, 2009; Alber, 2011; Khomyakova *et al.* 2011). It is interesting that some intermediates of these assimilatory pathways, for example malate and glyoxylate, are also intermediates in the serine cycle and as such may afford easy coupling with utilization of the serine cycle.

Identification of acetate utilization pathways in methanotrophs, however, has been challenging. For example, early enzymatic work on *M. silvestris* found no evidence for the key enzymatic activities in the glyoxylate cycle, i.e., isocitrate lyase and malate synthase (Dunfield *et al.*, 2003; Theisen *et al.*, 2005). Genomic analyses, however, show that genes encoding for these enzymes are present (Chen *et al.*, 2010a). Subsequent deletion of the gene encoding for isocitrate lyase severely limited growth of *M. silvestris* on acetate, and abolished it on methane (Crombie & Murrell, 2011). As discussed by the authors, such data suggest that the glyoxylate shunt may be vital to *M. silvestris* for regeneration of glyoxylate in the serine cycle used for carbon assimilation from C_1 compounds as well as from C_2 compounds. These findings also suggest that this microorganism may have multiple mechanisms to utilize multicarbon compounds, as growth still occurred on acetate when the gene encoding for isocitrate lyase was deleted. However, homologs of known key genes of ethylmalonyl-CoA, citramalate, and methylaspartate pathways for carbon assimilation from acetate are not readily apparent in the genome sequence of *M. silvestris*. In contrast, phylogenetically closely related methylotrophs such as the alphaproteobacterium *M. extorquens* AM1 were often shown to utilize the coupled serine and ethylmalonyl-CoA pathways for growth (Peyraud *et al.*, 2009; Ŝmejkalová *et al.*, 2010). Preliminary analysis of publicly available genome sequences of obligate methanotrophs [i.e. *Alphaproteobacteria Methylosinus trichosporium* OB3b (Stein *et al.*, 2010), *Methylocystis* sp. strain ATCC 49242 (Stein *et al.*, 2011), *Gammaproteobacteria M. capsulatus* Bath (Ward *et al.*, 2004), *Methylobacillus flagellatus* KT (Chistoserdova *et al.*, 2007), *Methylobacter tundripaludum* SV96, *Methylomicrobium album* BG8, *Methylomonas methanica* MC09, as well as *Candidatus* Methylomirabilis oxyfera (Ettwig *et al.*, 2010) and *Methylacidiphilum infernorum* V4 (Hou *et al.*,

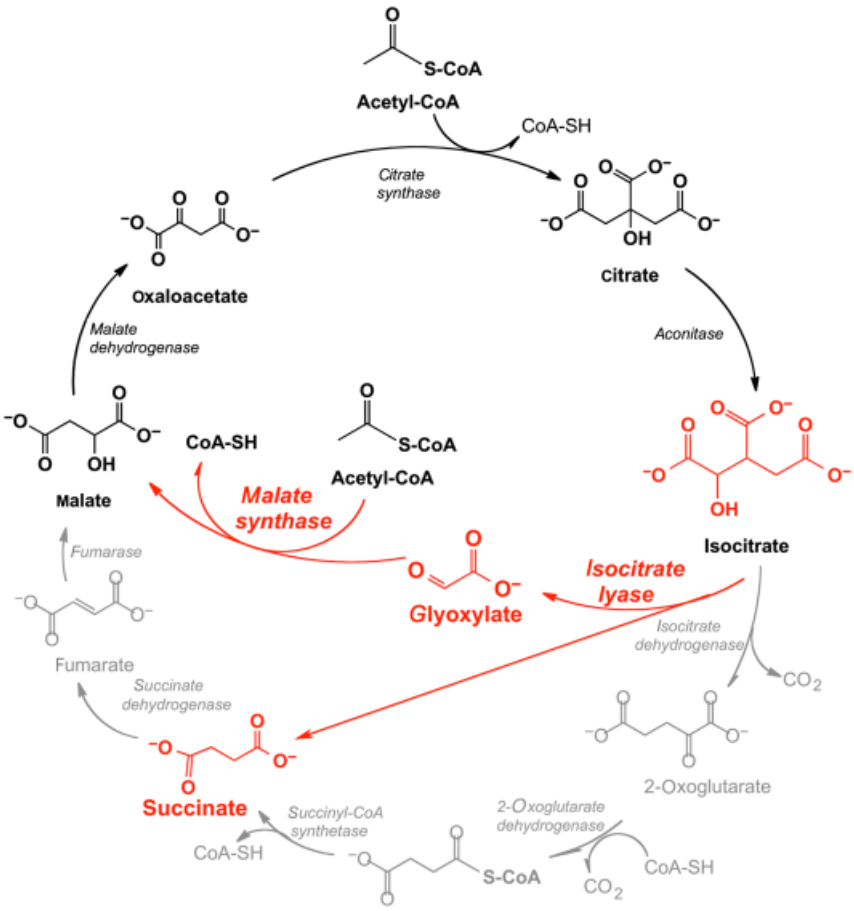

Fig. 2. Acetyl-CoA assimilation by the glyoxylate cycle (modified from Theisen *et al.*, 2005). Key enzymatic intermediates and enzymes in this pathway are noted in red.

2008)], indicates that the key genes of the ethylmalonyl-CoA pathway (Fig. 3) are only present in the two alphaproteobacterial methanotrophs that were sequenced so far, and are found in synteny in the *Methylocystis* strain. Further, no evidence was observed for the presence of the set of key genes defining citramalate (Fig. 4) or methylaspartate pathways (Fig. 5) for multicarbon assimilation in any methanotroph for which a genome sequence is available. At present, however, such observations should be treated with caution. First, sequence information is still lacking for some reactions (e.g. mesaconate hydratase and mesaconate-CoA ligase of the citramalate pathway, Berg & Ivanovsky, 2009). Second, several publically available methanotroph genomes are not yet completely assembled, and absence of evidence does not provide evidence of absence. Third, the required pathway reactions could be performed by proteins whose sequence bears little or no resemblance to experimentally characterized enzymes. Clearly, more research is needed to elucidate how facultative methanotrophs assimilate carbon from multicarbon compounds into biomass, and the increasing availability of genome sequences represents as much a great asset as a sobering reminder of our ignorance.

Facultative methanotrophy: implications and applications

It has been confirmed that facultative methanotrophy does indeed exist, but corresponding isolates can only utilize a small number of organic acids and ethanol to support growth, i.e., sugars cannot be used, possibly due to lack of sugar transporters and/or lack of key steps of the glycolytic pathway. Also, to date, no methanotrophs of the gammaproteobacterial phylum have conclusively been shown to be facultative. These methanotrophs present several key differences to *Alphaproteobacteria* methanotrophs including, as noted above, the lack of a complete TCA cycle, as well as their utilization of the RuMP pathway for growth. One *Gammaproteobacteria* methanotroph, *M. capsulatus* Bath, has been found to have genes for the E1 and E2 subunits of the 2-ketoglutarate dehydrogenase (Ward *et al.*, 2004). At this time, it is unclear under what conditions, if any, these genes are transcribed, and active enzyme synthesized. The absence of 2-ketoglutarate dehydrogenase activity may limit growth of *Gammaproteobacteria* methanotrophs with alternative multicarbon compounds, as well as the fact that

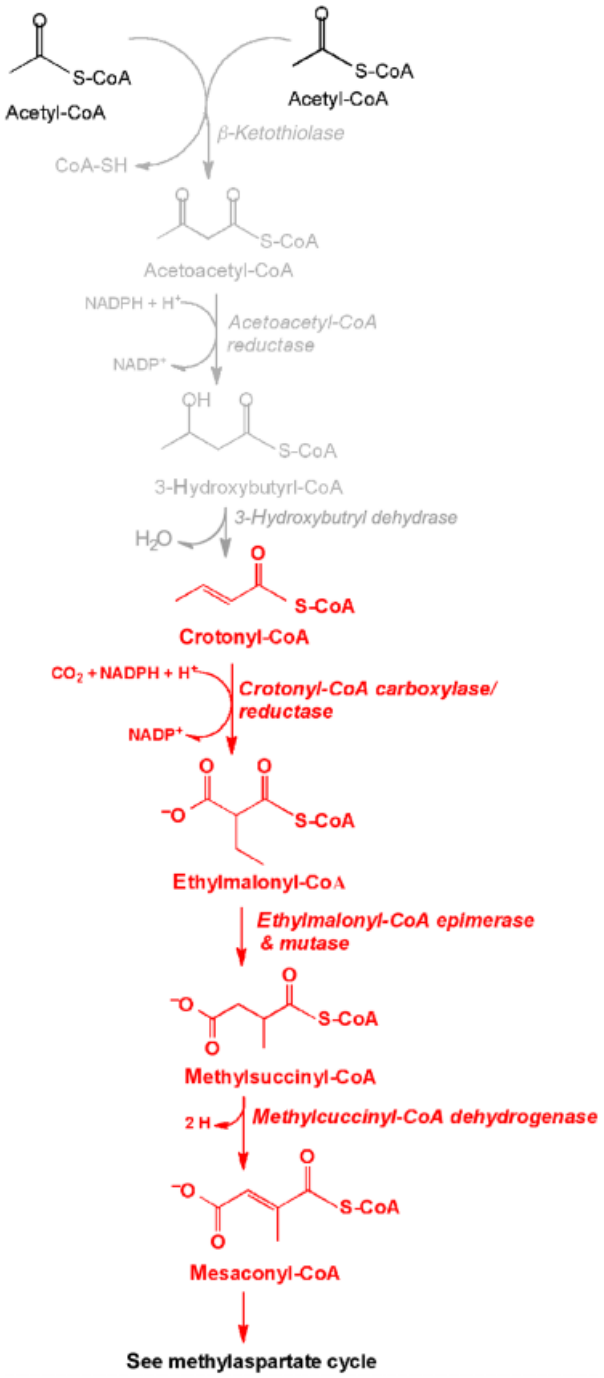

Fig. 3. Acetyl-CoA assimilation by the ethylmalonyl-CoA pathway (modified from Peyraud *et al.*, 2009). Key enzymatic intermediates and enzymes in this pathway are noted in red.

isocitrate lyase and malate synthase are apparently missing in these microorganisms (Trotsenko & Murrell, 2008).

Further, the acetate assimilation pathways described above do not lead to the production of intermediates of the RuMP pathway. Accordingly, and unlike *Alphaproteobacteria* methanotrophs that utilize the serine cycle, *Gammaproteobacteria* methanotrophs appear to be unable to use these pathways for carbon assimilation from multicarbon compounds. This may help explain why all known facultative methanotrophs utilize the serine cycle and not the RuMP pathway for carbon assimilation. We suggest that more effort be invested to isolate *Gammaproteobacteria* methanotrophs from environments with high acetate concentrations, for example, peat bogs and acidic forest soils, to determine if such conditions promote facultative growth in a broader phylogenetic range of methanotrophs. Molecular evidence indicates that such methanotrophs exist in these environments, particular peat bogs, but that they do not represent a significant fraction of the overall methanotrophic population (Dedysh, 2009).

Facultative methanotrophy may not only be of fundamental interest but also yield some interesting applications, in particular in the field of bioremediation approaches, for example, enhanced degradation of halogenated hydrocarbons (Im & Semrau, 2011; Yoon *et al.*, 2011). Methanotrophs had previously been widely examined for pollutant degradation through the activity of the MMO (Semrau *et al.*, 2010), and the finding of at least two facultative methanotrophs that constitutively express pMMO effectively allows for the uncoupling of pollutant degradation from carbon assimilation. This strategy could enhance overall methanotroph-mediated pollutant degradation, as competition for binding to MMO between the pollutant(s) and the growth substrate is avoided if alternative substrates such as ethanol or acetate are used to support growth. Issues such as substrate and product toxicity of chlorinated hydrocarbons may still limit overall methanotrophic growth, however, regardless of the growth substrate (Im & Semrau, 2011). It is recommended that future work takes care to determine the abundance and distribution of facultative methanotrophs *in situ*, as well as the ability of such strains to compete for alternative growth substrates in environments where heterotrophs also exist.

How can facultative methanotrophy be verified in future isolates?

As noted above, initial reports of facultative methanotrophy were later disproven. Given that facultative methanotrophy does indeed exist, this implies that more as yet undiscovered facultative methanotrophs also exist. The conclusion of facultative methanotrophy, however, should be drawn only after rigorously characterizing putative isolates. The reader is directed to Dedysh & Dunfield (2011) for a thorough description of suggested assays that we only briefly describe here. Putative methanotrophic isolates should first be cultivated on relatively simple growth media with methane as the sole carbon and energy source, followed by determination of

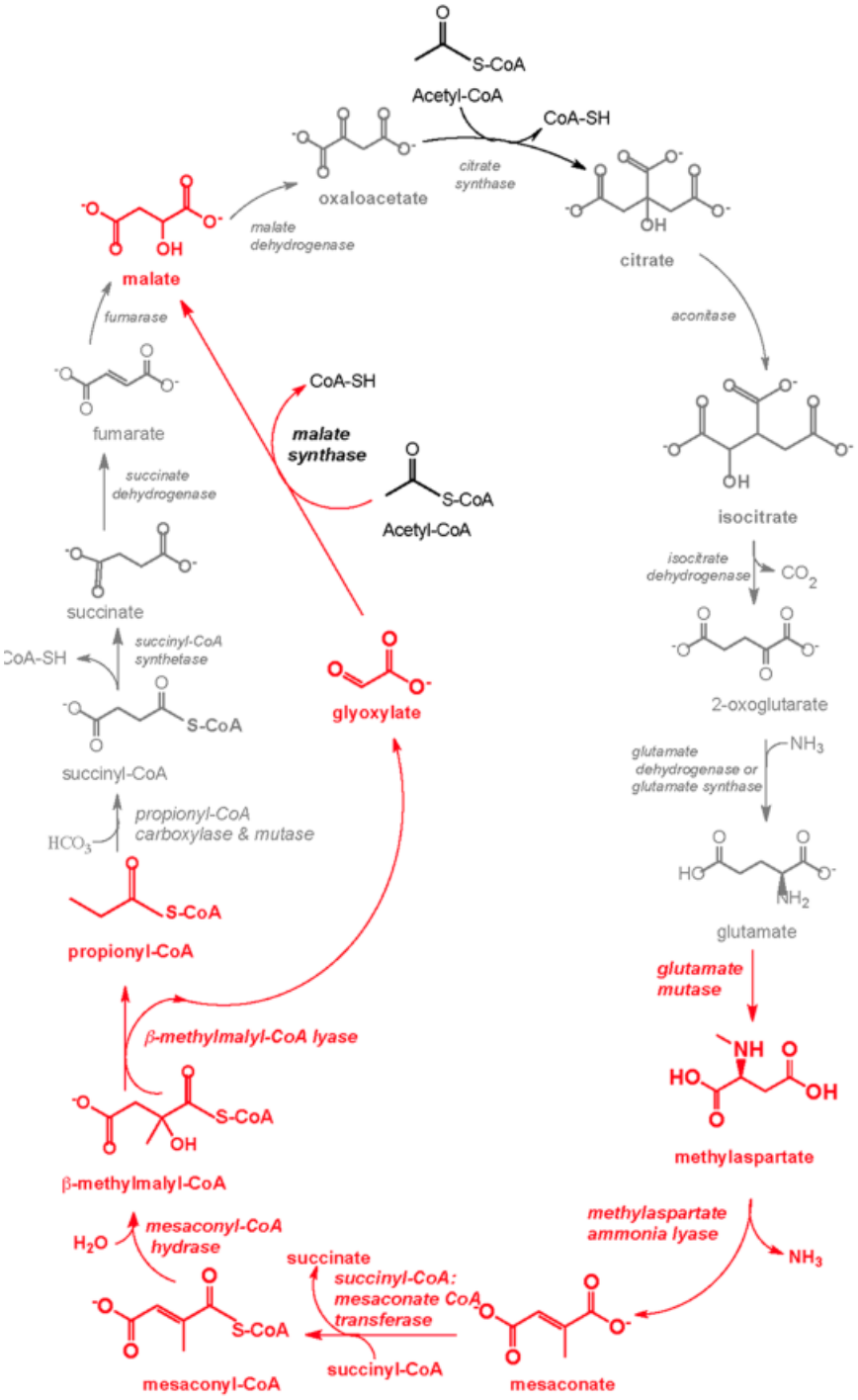

Fig. 4. Acetyl-CoA assimilation by the methylaspartate cycle (modified from Khomyakova *et al.*, 2011). Key enzymatic intermediates and enzymes in this pathway are noted in red.

the presence of genes for sMMO and/or pMMO using specific PCR primer sets. Following such initial characterization, the ability of methanotrophic isolates to grow on various multicarbon compounds should next be determined. If facultative methanotrophy is suspected, one should then verify culture purity by performing most if not all of the following assays: (1) plating onto complex organic media; (2) phase-contrast and electron microscopy; (3) whole-cell hybridization with genus/species-specific probes; (4) 16S rRNA gene library sequence analysis of scores of clones; (5) dilution–extinction experiments using both methane and multicarbon compounds as the sole carbon source; and (6) quantification of MMO gene(s) when grown on multicarbon compounds.

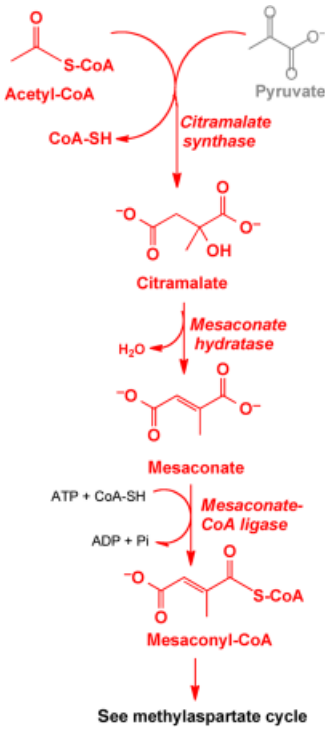

Fig. 5. Acetyl-CoA assimilation by the the citramalate cycle (modified from Howell *et al.*, 1999). Key enzymatic intermediates and enzymes in this pathway are noted in red.

Conclusions

The discovery of facultative methanotrophs marks another major milestone in the field of methanotrophy. The conclusive identification and characterization of facultative methanotrophs provides us with opportunities to answer some important questions. In particular why are some methanotrophs obligate for C_1 compounds and others facultative, i.e., what is the metabolic basis for facultative methanotrophy? More thorough analyses of methanotrophic genomes may provide some resolution regarding this issue, particularly if closely related strains that exhibit differential ability to grow on multicarbon compounds would be sequenced (e.g. the obligate methanotroph *Methylocystis parvus* OBBP and the facultative methanotroph *Methylocystis* strain H2s). Comparison of the genomes of obligate and facultative methanotrophs with those of facultative methylotrophs could also prove useful in this endeavor. In addition, proteomic and/or metabolomic strategies could be applied to help deduce metabolic pathway(s) used for uptake of multicarbon compounds.

Other important questions that remain to be answered include:

(1) What environmental conditions promote obligate vs. facultative methanotrophy?

(2) What competitive advantage(s) does either obligate or facultative methanotrophy provide?

(3) Do facultative methanotrophs effectively compete with other microorganisms for multicarbon substrates *in situ*?

(4) How does facultative methanotrophy affect the ability of methanotrophs to consume methane, particularly at atmospheric concentrations?

(5) Are there facultative methanotrophs among *Gammaproteobacteria*?

(6) Can facultative methanotrophy be used to enhance methanotrophic-mediated bioremediation of halogenated hydrocarbons?

Finally, it is interesting to note that not only *Methylocystis* strains are found in many different environments, but also *Methylocella* strains. Members of both genera are widely distributed throughout the globe, found not only in peat bogs, but also in acidic forest and arctic soils as well as environments with pH values > 7.0 (Bowman, 2006; Dedysh, 2009; Rahman *et al.*, 2011). Such findings indicate that facultative methanotrophy may be widespread.

References

Alber BE (2011) Biotechnological potential of the ethylmalonyl-CoA pathway. *Appl Microbiol Biot* **89**: 17–25.

Axe DE & Bailey JE (1995) Transport of lactate and acetate through the energized cytoplasmic membrane of *Escherichia coli*. *Biotechnol Bioeng* **47**: 8–19.

Belova SE, Baani M, Suzina NE, Bodelier PLE, Liesack W & Dedysh SN (2011) Acetate utilization as a survival strategy of peat-inhabiting *Methylocystis* spp. *Environ Microbiol Rep* **3**: 36–46.

Berg IA & Ivanovsky RN (2009) Enzymes of the citramalate cycle in *Rhodospirillum rubrum*. *Microbiology* **78**: 16–24.

Bowman J (2006) The methanotrophs – the families *Methylococcaceae* and *Methylocystaceae*. *Prokaryotes*, *Vol. 5*, pp. 266–289. Springer, New York.

Bowman JP, Sly LI & Stackebrandt E (1995) The phylogenetic position of the family *Methylococcaceae*. *Int J Syst Bacteriol* **45**: 182–185.

Brown LR, Strawinski RJ & McCleskey CS (1964) The isolation and characterization of *Methanomonas methanooxidans* Brown and Strawiniski. *Can J Microbiol* **10**: 791–799.

Chen Y, Crombie A, Rahman MT, Dedysh SN, Liesack W, Stott MB, Alam M, Theisen AR, Murrell JC & Dunfield PF (2010a) Complete genome sequence of the aerobic facultative methanotroph *Methylocella silvestris* BL2. *J Bacteriol* **192**: 3840–3841.

Chen Y, Scanlan S, Song L, Crombie A, Rahman MT, Schäfer H & Murrell JC (2010b) γ-Glutamethylamide is an essential intermediate in the metabolism of methylamine by *Methylocella silvestris*. *Appl Environ Microb* **76**: 4530–4537.

Chistoserdova L, Lapidus A, Han C *et al.* (2007) Genome of *Methylobacillus flagellatus*, molecular basis for obligate

methylotrophy, and polyphyletic origin of methylotrophy. *J Bacteriol* **189**: 4020–4027.

Choi D-W, Kunz RC, Boyd ES, Semrau JD, Antholine WE, Han J-I, Zahn JA, Boyd JM, de la Mora AM & DiSpirito AA (2003) The membrane-associated methane monooxygenase (pMMO) and pMMO-NADH: quinine oxidoreductase complex from *Methylococcus capsulatus* Bath. *J Bacteriol* **185**: 5755–5764.

Crombie A & Murrell JC (2011) Development of a system for genetic manipulation of the facultative methanotroph *Methylocella silvestris* BL2. *Method Enzymol* **495B**: 119–133.

Crossman LC, Moir JWB, Enticknap JJ, Richardson DJ & Spiro S (1997) Heterologous expression of heterotrophic nitrification genes. *Microbiology* **143**: 3775–3783.

Dedysh SN (2009) Exploring methanotroph diversity in acidic northern wetlands: molecular and cultivation-based studies. *Microbiology* **78**: 655–669.

Dedysh SN & Dunfield PF (2011) Facultative and obligate methanotrophs: how to identify and differentiate them. *Method Enzymol* **495B**: 31–44.

Dedysh SN, Panikov NS, Liesack W, Großkopf R, Zhou J & Tiedje JM (1998a) Isolation of acidophilic methane-oxidizing bacteria from northern peat wetlands. *Science* **282**: 281–284.

Dedysh SN, Panikov NS & Tiedje JM (1998b) Acidophilic methanotrophic communities from *Sphagnum* peat bogs. *Appl Environ Microb* **64**: 922–929.

Dedysh SN, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD, Liesack W & Tiedje JM (2002) *Methylocapsa acidiphila* gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from *Sphagnum* bog. *Intl J Syst Evol Micr* **52**: 2561–2661.

Dedysh SN, Liesack W, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD, Bares AM, Panikov NS & Tiedje JM (2000) *Methylocella palustris* gen. nov., a new methane-oxidizing acidophilic bacterium from peat bogs, representing a novel subtype of serine pathway methanotrophs. *Int J Syst Evol Micr* **50**: 955–969.

Dedysh SN, Berestovskaya YY, Vasylieva LV, Belova SE, Khmelenina VN, Suzina NE, Trotsenko YA, Liesack W & Zavarzin GA (2004) *Methylocella tundrae* sp. nov., a novel methanotrophic bacterium from acidic tundra peatlands. *Int J Syst Evol Micr* **54**: 151–156.

Dedysh SN, Knief C & Dunfield PF (2005) *Methylocella* species are facultatively methanotrophic. *J Bacteriol* **187**: 4665–4670.

Duddleston KN, Kinney MA, Kiene RP & Hines ME (2002) Anaerobic microbial biogeochemistry in a northern bog: acetate as a dominant metabolic end product. *Global Biogeochem Cy* **16**: 1063.

Dunfield P (2007) The soil methane sink. *Greenhouse Gas Sinks* (Reay DS, Hewitt CN, Smith KA & Grace J, eds), pp. 152–170. CAB International, Wallingford.

Dunfield PF, Khmelenina VN, Suzina NE, Trotsenko Y & Dedsyh SN (2003) *Methylocella silvestris* sp. nov., a novel methanotroph isolated from an acidic forest cambisol. *Int J Syst Evol Micr* **53**: 1231–1239.

Dunfield PF, Belova SE, Vorob'ev AV, Cornish SL & Dedysh SN (2010) *Methylocapsa aurea* sp. nov., a facultative methanotroph possessing a particulate methane monooxygenase and emended description of the genus *Methylocapsa*. *Int J Syst Evol Micr* **60**: 2659–2664.

Dworkin M & Foster JW (1956) Studies on *Pseudomonas methanica* (Söhngen) nov. comb. *J Bacteriol* **72**: 646–659.

Erb TJ, Berg IA, Brecht V, Müller M, Fuchs G & Alber BE (2007) Synthesis of C_5-dicarboxylic acids from C_2-units involving crotonyl-CoA carboxylase/reductase: the ethylmalonyl-CoA pathway. *P Natl Acad Sci USA* **104**: 10631–10636.

Erb TJ, Fuchs G & Alber BE (2009) [2S]-Methylsuccinyl-CoA dehydrogenase closes the ethylmalonyl-CoA pathway for acetyl-CoA assimilation. *Mol Microbiol* **73**: 992–1008.

Ettwig K, Butler MK, Le Paslier D *et al.* (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464**: 543–548.

Foster JW & Davis RH (1966) A methane-dependent coccus, with notes on classification and nomenclature of obligate, methane-utilizing bacteria. *J Bacteriol* **91**: 1924–1931.

Gimenez R, Nuñez MF, Badia J, Aguilar J & Baldoma L (2003) The gene yjcG, cotranscribed with the gene *acs*, encodes an acetate permease in *Escherichia coli*. *J Bacteriol* **185**: 6448–6455.

Green PN & Bousfield IJ (1983) Emendation of *Methylobacterium* Patt, Cole, and Hanson 1976; *Methylobacterium rhodinum* (Heumann 1962) comb. nov. corrig.; *Methylobacterium radiotolerans* (Ito and Iizuka 1971) comb. nov. corrig.; and *Methylobacterium mesophilicum* (Austin and Goodfellow 1979) comb. nov. (1983). *Int J Syst Bacteriol* **33**: 875–877.

Hou S, Makarova KS, Saw JH *et al.* (2008) Complete genome sequence of the extremely acidophilic methanotroph isolate V4, *Methylacidiphilum infernorum*, a representative of the bacterial phylum Verrucomicrobia. *Biol Direct* **3**: 26.

Howell DM, Xu H & White RH (1999) (*R*)-Citramalate synthase in methanogenic archaea. *J Bacteriol* **181**: 331–333.

Im J & Semrau JD (2011) Pollutant degradation by *Methylocystis* strain SB2 grown on ethanol: bioremediation via facultative methanotrophy. *FEMS Microbiol Lett* **318**: 137–142.

Im J, Lee S-W, Yoon S, DiSpirito AA & Semrau JD (2011) Characterization of a novel facultative *Methylocystis* species capable of growth on methane, ethanol, and acetate. *Environ Microbiol Rep* **3**: 174–181.

Khomyakova N, Bükmez Ö, Thomas LK, Erb TJ & Berg IA (2011) A methylaspartate cycle in haloarchaea. *Science* **331**: 334–337.

Kolb S (2009) The quest for atmospheric methane oxidizers in forest soils. *Environ Microbiol Rep* **1**: 336–346.

Leadbetter ER & Foster JW (1958) Studies on some methane utilizing bacteria. *Arch Mikrobiol* **30**: 91–118.

Lidstrom-O'Connor ME, Fulton GL & Wopat AE (1983) '*Methylobacterium ethanolicum*': a syntrophic association of two methylotrophic bacteria. *J Gen Microbiol* **129**: 3139–3148.

Lynch MJ, Wopat AE & O'Connor ML (1980) Characterization of two new facultative methanotrophs. *Appl Environ Microb* **40**: 400–407.

Op den Camp HJM, Islam T, Stott MB, Harhangi HR, Hynes A, Schouten S, Jetten MSM, Birkeland N-K, Pol A & Dunfield PF (2009) Environmental, genomic and taxonomic perspectives on methanotrophic *Verrucomicrobia*. *Environ Microbiol Rep* **1**: 293–306.

Patel RN, Hou CT & Felix A (1978) Microbial oxidation of methane and methanol: isolation of methane-utilizing bacteria and characterization of a facultative methane-utilizing isolate. *J Bacteriol* **136**: 352–358.

Patt TE, Cole GC, Bland J & Hanson RS (1974) Isolation and characterization of bacteria that grow on methane and organic compounds as sole sources of carbon and energy. *J Bacteriol* **120**: 955–964.

Patt TE, Cole GC & Hanson RS (1976) *Methylobacterium*, a new genus of facultatively methylotrophic bacteria. *Int J Syst Bacteriol* **26**: 226–229.

Peyraud R, Kiefer P, Christen P, Massou S, Portals J-C & Vorholt JA (2009) Demonstration of the ethylmalonyl-CoA pathway by using ^{13}C metabolomics. *P Natl Acad Sci USA* **106**: 4846–4851.

Rahman MT, Crombie A, Chen Y, Stralis-Pavese N, Bodrossy L, Meir P, McNamara NP & Murrell JC (2011) Environmental distribution and abundance of the facultative methanotroph *Methylocella*. *ISME J* **5**: 1061–1066.

Semrau JD, DiSpirito AA & Yoon S (2010) Methanotrophs and copper. *FEMS Microbiol Rev* **34**: 496–531.

Ŝmejkalová H, Erb TJ & Fuchs G (2010) Methanol assimilation in *Methylobacterium extorquens* AM1: demonstration of all enzymes and their regulation. *PLoS One* **5**: e13001.

Starai VJ & Escalante-Semerena JC (2004) Acetyl-coenzyme A synthetase (AMP forming). *Cell Mol Life Sci* **61**: 2020–2030.

Stein LY, Yoon S, Semrau JD *et al.* (2010) Genome sequence of the obligate methanotroph *Methylosinus trichosporium* OB3b. *J Bacteriol* **192**: 6497–6498.

Stein LY, Bringel F, Dispirito AA *et al.* (2011) Genome sequence of the methanotrophic alphaproteobacterium *Methylocystis* sp. strain Rockwell (ATCC 49242). *J Bacteriol* **193**: 2668–2669.

Theisen AR, Ali MH, Radajewski S, Dumont MG, Dunfield PF, McDonald IR, Dedysh SN, Miguez CB & Murrell JC (2005) Regulation of methane oxidation in the facultative methanotroph *Methylocella silvestris* BL2. *Mol Microbiol* **58**: 682–692.

Trotsenko YA & Murrell JC (2008) Metabolic aspects of aerobic obligate methanotrophy. *Adv Appl Microbiol* **63**: 183–229.

Urakami T, Araki H, Suzuki K-I & Komagata K (1993) Further studies of the genus *Methylobacterium* and description of *Methylobacterium aminovorans* sp. nov. *Int J Syst Bacteriol* **43**: 504–513.

Ward N, Larsen Ø, Sakwa J *et al.* (2004) Genomic insights into methanotrophy: the complete genome sequence of *Methylococcus capsulatus* (Bath). *PLoS Biol* **2**: e303.

Whittenbury R, Phillips KC & Wilkinson JF (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. *J Gen Microbiol* **61**: 205–218.

Wood AP, Aurikko JP & Kelly DP (2004) A challenge for 21st century molecular biology and biochemistry: what are the causes of obligate autotrophy and methanotrophy? *FEMS Microbiol Rev* **28**: 335–352.

Yoon S, Im J, Bandow N, DiSpirito AA & Semrau JD (2011) Constitutive expression of pMMO by *Methylocystis* strain SB2 when grown on multi-carbon substrates: implications for biodegradation of chlorinated ethenes. *Environ Microbiol Rep* **3**: 182–188.

Zhao S-J & Hanson RS (1984a) Variants of the obligate methanotroph isolate 761M capable of growth on glucose in the absence of methane. *Appl Environ Microb* **48**: 807–812.

Zhao S-J & Hanson RS (1984b) Isolate 761M: a new type I methanotroph that possesses a complete tricarboxylic acid cycle. *Appl Environ Microb* **48**: 1237–1242.