SHORT COMMUNICATION

Environmental distribution and abundance of the facultative methanotroph *Methylocella*

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***Methylocella* spp. are facultative methanotrophs, which are able to grow not only on methane but also on multicarbon substrates such as acetate, pyruvate or malate. *Methylocella* spp. were previously thought to be restricted to acidic soils such as peatlands, in which they may have a key role in methane oxidation. There is little information on the abundance and distribution of *Methylocella* spp. in the environment. New primers were designed, and a real-time quantitative PCR method was developed and validated targeting *Methylocella mmoX* (encoding the α -subunit of the soluble methane monooxygenase) that allowed the quantification of *Methylocella* spp. in environmental samples. We also developed and validated specific PCR assays, which target 16S rRNA genes of known *Methylocella* spp. These were used to investigate the distribution of *Methylocella* spp. in a variety of environmental samples. It was revealed that *Methylocella* species are widely distributed in nature and not restricted to acidic environments.**

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Introduction

Methylocella spp. are aerobic facultative methanotrophs (*Alphaproteobacteria*), with three validated species, *M. palustris*, *M. silvestris* and *M. tundrae*, isolated from acidic soils (Dunfield *et al.*, 2003; Dedysh *et al.*, 2000, 2004). *M. silvestris* BL2, which can grow on a variety of multicarbon substrates (Dedysh *et al.*, 2005; Theisen *et al.*, 2005), oxidizes methane using the soluble methane monooxygenase encoded by the operon *mmoXYBZDC*. *Methylocella* spp. lack the particulate methane monooxygenase encoded by the *pmoCAB* operon, which is present in all other methanotrophs (Dedysh *et al.*, 2000, 2004; Dunfield *et al.*, 2003). Although a few studies have detected *Methylocella*-related 16S rRNA genes in different environments (for example, Dunfield *et al.*, 2003; Dedysh *et al.*, 2000, 2004; Chen *et al.*, 2008a,b; Cockell *et al.*, 2009; Fabiani *et al.*, 2009;

Han *et al.*, 2009), there is little information on the distribution and abundance of *Methylocella*. Current molecular techniques for example, *pmoA* microarray (Bodrossy *et al.*, 2003) and real-time quantitative PCR (qPCR) (Kolb *et al.*, 2003; Tuomivirta *et al.*, 2009), that are commonly applied to study the diversity and abundance of methanotrophs, would not detect *Methylocella*, as these methods target *pmoA*. Therefore, we aimed in this study (1) to develop *Methylocella* 16S rRNA gene-targeting PCR-based assays to assess the environmental distribution of *Methylocella*, and (2) to design *Methylocella mmoX* primers and develop a *mmoX*-targeted qPCR assay to enumerate *Methylocella* in environmental samples.

Results and discussion

Methylocella-related 16S rRNA gene-targeting PCR assays were developed using the primers Type IIF (Chen *et al.*, 2007) and Mcell-1445 (Dedysh *et al.*, 2005). Primer Type IIF was originally designed to detect 16S rRNA genes of type II methanotrophs, including the genus *Methylocella*, whereas primer Mcell-1445 was originally designed as a *Methylocella* 16S rRNA gene fluorescent *in situ* hybridization probe. PCR conditions were optimized and validated with DNA from pure cultures of known methanotrophs (for methodology, see Supplementary Information). Specificity of these primers to

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Table 1 PCR-based survey for *Methylocella* in the environmental samples

Country and location	Latitude and longitude	Vegetation	Site description (pH) (soil depth)	Methylocella (16S rRNA genes; amplicon size ~ 1 kbp)		Methylocella (mmoX genes; amplicon size ~ 450 bp)	
				Direct PCR approach	Nested PCR approach	Confirmed by clone library*	Confirmed by clone library*
Aberdeen, UK	57° 11' N, 2° 12' W	<i>Solanum tuberosum</i> L.	Arable land (4.5) (0–10 cm)	–	+	NT	NT
			Arable land (7.5) (0–10 cm)	–	+	NT	NT
Ufton landfill, UK	52° 25' N, 1° 44' W	Grassland	Landfill cover soil (7.4) (0–20 cm)	–	–	NA	NA
Austria	Not recorded	Grassland	Landfill cover soil (7.4) (0–20 cm)	–	+	NT	NA
Svalbard, Arctic	Not recorded	Not available	Arctic soil (6.2) (0–10 cm)	+	+	+	NT
Sourhope, UK	55° 28' N, 2° 14' W	<i>Agrostis capillaris</i> L., <i>Festuca rubra</i> L., <i>Nardus stricta</i> L.	Upland grassland (7.7) (0–10 cm)	–	–	NA	NA
Moor House, UK	54° 42' N, 2° 18' W	<i>Sphagnum</i> spp., <i>Eriophorum</i> spp., <i>Calluna vulgaris</i> L.	Peatland (4.3) (0–10 cm)	+	+	+	+
Xistral, Spain	42° 48' N, 8° 6' W	<i>Eriophorum angustifolium</i> L., <i>Carex durieui</i> L., <i>Carex echinata</i> L., <i>Molinia caerulea</i> L., <i>Erica mackaiana</i> L., <i>Calluna vulgaris</i> L.	Peatland (4.3) (0–10 cm)	–	+	NT	NA
Finland	62° 11' N, 25° 40' E	<i>Betula pubescens</i> Ehrh., <i>Picea abies</i> L., <i>Sphagnum angustifolium</i> L., <i>Sphagnum girgensohnii</i> L., <i>Polytrichum commune</i> Hedw., <i>Carex</i> spp., <i>Calamagrostis purpurea</i> Trin., <i>Equisetum sylvaticum</i> L., <i>Vaccinium myrtillus</i> , <i>Vaccinium vitis-idaea</i> L.	Boreal forest soil (5.9) (0–10 cm)	–	+	NT	NA
Wales, UK	52° 26' N, 3° 46' W	<i>Sphagnum</i> spp., <i>Eriophorum</i> spp., <i>Calluna vulgaris</i> L.	Peatland (4.6) (0–10 cm)	–	+	NT	NA
Peru Wayqecha	13° 11' S, 71° 24' W	<i>Clusiaceae</i> spp., <i>Cunoniaceae</i> spp.	Montane cloud forest soil (5.5) (0–10 cm)	–	–	NA	NA
Pillahuata	13° 7' S, 71° 25' W	<i>Cunoniaceae</i> spp.	Montane cloud forest soil (6.2) (0–10 cm)	–	–	NA	NA
San Pedro	13° 02' S, 71° 32' W	<i>Clethraceae</i> spp.	Cloud forest soil (4.8) (0–10 cm)	+	+	+	+

Table 1 (Continued)

Country and location	Latitude and longitude	Vegetation	Site description (pH) (soil depth)	Methylocella (16S rRNA genes; amplicon size ~1 kbp)		Methylocella (mmoX genes; amplicon size ~450 bp)	
				Direct PCR approach	Nested PCR approach	Confirmed by clone library*	PCR Confirmed by clone library*
Tono	12° 53' S, 71° 33' W	<i>Elaeocarpaceae</i> spp., <i>Fabaceae</i> spp.	Highland rainforest soil (4.9) (0–10 cm)	+	+	NT	+
Tambopata	12° 49' S, 69° 16' W	<i>Moraceae</i> spp., <i>Fabaceae</i> spp.	Lowland rainforest soil (6.4) (0–10 cm)	–	+	NT	NT
Hornavan, Sweden	66° 13' N, 17° 78' E	<i>Vaccinium myrtillus</i> L., <i>Vaccinium vitis-idaea</i> L., <i>Empetrum hermaphroditum</i> L., <i>Pinus sylvestris</i> L., <i>Betula pubescens</i> Ehrh., <i>Picea abies</i> L.	Boreal forest soil (4.5) (0–20 cm)	+	+	+	+
Uddjaure, Sweden	65° 60' N, 17° 48' E	<i>Vaccinium myrtillus</i> L., <i>Vaccinium vitis-idaea</i> L., <i>Empetrum hermaphroditum</i> L., <i>Pinus sylvestris</i> L., <i>Betula pubescens</i> Ehrh., <i>Picea abies</i> L.	Boreal forest soil (4.5) (0–20 cm)	+	+	NT	+
Move Cave, Romania	43° 82' N, 28° 55' E	NA	Fresh water (7.5)	–	–	NA	–
Lonar Lake, India	19° 97' N, 76° 51' E	NA	Sediment (10.0) (0–10 cm)	–	+	+	+
Colne Estuary, Colchester, Essex, UK	51° 82' N, 0° 98' E	NA	Marine sediment (7.2) (0–5 cm)	–	+	+	NT
Colne Estuary, Brightlingsea, Essex, UK	51° 80' N, 1° 02' E	NA	Marine sediment (7.1) (0–5 cm)	–	–	NA	–
Atlantic Ocean	42° 04' N, 22° 11' W	NA	Sea water (7.4)	–	–	NA	–
	2° 47' S, 26° 51' W	NA	Sea water (7.4)	–	–	NA	–
	26° 33' N, 24° 59' W	NA	Sea water (7.4)	–	–	NA	–

Abbreviations: NA, not applicable; NT, not tested.

+, presence; –, absence.

The salinity of Colne Estuary sediment collected from Colchester (Essex, UK), Brightlingsea (Essex, UK) and Atlantic Ocean sea water was approx 1 g NaCl L⁻¹, 20 g NaCl L⁻¹ and 35 g NaCl L⁻¹ respectively.*Number of clones that were positive for *Methylocella* out of the total number of clones analyzed.

detect *Methylocella* 16S rRNA genes in DNA extracted from environmental samples was verified by constructing clone libraries using seven samples that were found to be positive for the 16S rRNA gene PCR products of *Methylocella* either by a direct and/or by a nested PCR approach (see Table 1 for samples analyzed by clone libraries). These seven samples were chosen to cover a wide range of pH values ranging from moderately acidic to very alkaline. Over 82% of the 16S rRNA gene sequences from these clone libraries were closely related (>97% identity) to *Methylocella*, and the remainder were related to 16S rRNA genes from *Alphaproteobacteria* and *Gammaproteobacteria* (*Hyphomicrobium*, *Methylobacter*) or *Bacteroidetes* (*Flexibacter*) (data not shown). The distribution of these non-*Methylocella* sequences was random across these samples. DNA extracted from various environmental samples was PCR-screened for *Methylocella*-related 16S rRNA genes (Table 1). Among 23 samples originating from diverse locations, *Methylocella*-related 16S rRNA gene PCR products were detected in 15 samples. Of these, six were positive for *Methylocella*-related 16S rRNA genes using a direct PCR approach. All these six samples were acidic, suggesting a higher abundance of *Methylocella* in these environments compared with other samples that were more neutral or alkaline in nature and which required a nested PCR approach for detection of *Methylocella*. In fact *Methylocella*-related 16S rRNA genes were detected not only from acidic and neutral environments, but also from alkaline environments such as Lonar Lake sediment, India (pH 10.0) (Table 1). These findings suggest that *Methylocella* is not limited to acidic pH in nature, despite being originally isolated from an acidic soil. Recently Fabiani *et al.* (2009) and Han *et al.* (2009) detected *Methylocella*-related 16S rRNA gene sequences in DNA extracted from an alkaline waste-treatment plant soil (pH 9), Italy, and an alkaline Chinese coal mine soil (pH 9), respectively. On the basis of our survey and the findings of these authors, it is evident

that *Methylocella* are more widely distributed in nature than previously thought. It is interesting to note that no sequences were detected from high-salinity environments. To the best of our knowledge, there are no previously published reports of marine *Methylocella* sequences, suggesting that salinity may restrict the diversity of this genus. We found *Methylocella* in soils associated with a wide diversity of vegetation (Table 1), therefore these data do not permit any conclusions to be drawn regarding particular *Methylocella*-plant associations.

To enumerate *Methylocella* populations in environmental samples, a *Methylocella mmoX* gene-targeting SYBR-Green based qPCR assay was developed targeting *mmoX* specific for *Methylocella* spp. Functional gene primers *mmoXLF* (5'-GAA GATTGGGGCGGCATCTG-3') and *mmoXLR* (5'-CCC AATCATCGCTGAAGGAGT-3') were designed based on *mmoX* sequences available in GenBank using the ARB program (Ludwig *et al.*, 2004). Although activity cannot be inferred from its presence, the use of *mmoX* rather than the 16S rRNA gene, by restricting the investigation to the functional group being studied, enables a much higher sensitivity of detection in complex environments (McDonald *et al.*, 2008). PCR conditions were optimized and validated with DNA from pure cultures of known methanotrophs (for methodology, see Supplementary Information). Clone library analysis of *mmoX* PCR products retrieved from five environmental samples (Table 1) indicated that over 84% of the *mmoX* sequences were closely related (>97% identity) to *Methylocella* spp. and the remainder were related to *mmoX* genes of other methanotrophs, for example *Methylobacter*, *Methylocystis* or *Methylococcus* (data not shown). Detection of a certain level of non-target sequences by primers designed to target a particular bacterial genus has earlier been observed (Fierer *et al.*, 2005). The qPCR assay was validated by spiking Ufton landfill cover soil (United Kingdom) with known amounts of *M. silvestris* BL2 cells (ranging from 10^3 to 10^5

Figure 1 (a) The number of *mmoX* copies detected by qPCR in DNA extracted from soil and sediment. (b) Detection of *mmoX* copies from the spiked soil. The dotted line shows the theoretical 100% detection efficiency. Error bars indicate the s.d. of triplicate measurements.

cells g⁻¹ soil), and by detecting the *mmoX* copies from the spiked soil (Figure 1b). Ufton landfill cover soil was chosen for the spiking study because we did not detect any *Methylocella*-related 16S rRNA genes from this soil, which supports the earlier observation of Héry *et al.* (2008), who also did not detect *Methylocella* spp. from this location. The detection limit of the qPCR assay was 10³ cells g⁻¹ of soil. The amplification efficiency of the reactions was between 80% and 104% (slope -3.92 to -3.27). Extraction and detection efficiency from the spiked soil was 88–111% (Figure 1b). The presence of *Methylocella* in amplified qPCR products was confirmed by clone library analysis (data not shown). The abundance of *Methylocella* detected in selected environmental samples varied from 0.9 (± 0.2) × 10⁶ (Colne Estuary sediment, Essex, UK) to 3.3 (± 0.6) × 10⁶ cells g⁻¹ (Hornavan, Sweden) (Figure 1a). *Methylocella* in Lonar Lake sediment (India) and Arctic soil (Svalbard, Arctic) were below the limits of detection. However, it was surprising to see that the abundance of *Methylocella* in the Svalbard Arctic soil was below the limits of detection, as we detected *Methylocella* 16S rRNA gene PCR products with these samples using the direct PCR approach. Failure to detect *Methylocella*-related *mmoX* genes in Arctic soil (Svalbard, Arctic) might be accounted for by the fact that there is one copy of *mmoX* in the genome of *M. silvestris* compared with two copies of the 16S rRNA gene. There are no previous qPCR data on the abundance of *Methylocella* in the environment; however, using fluorescent *in situ* hybridization, Dedysh *et al.* (2001) showed that the numbers of *Methylocella* spp. in *Sphagnum* peat was ~ 10⁶ cells g⁻¹ of wet soil. The abundance of *Methylocella* that we detected in peat soil (Moor House peat) was 2.3 (± 0.6) × 10⁶ copies g⁻¹ of wet soil (Figure 1a), which is in good agreement with the data of Dedysh *et al.* (2001). In contrast, the total number of *pmoA* copies in DNA extracted from these selected environmental samples varied from 1.8 (± 0.1) × 10⁷ (Colne Estuary sediment, Essex, UK) to 3.8 (± 0.5) × 10⁹ *pmoA* copies g⁻¹ (Hornavan, Sweden) (Supplementary Table 1). The qPCR protocol developed for *Methylocella* spp., in parallel with investigations into the activity of these organisms, can now be applied to study the effects of environmental parameters, for example availability of alternative carbon sources, salinity, vegetation type or pH, on the distribution and abundance of *Methylocella* in many environments.

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