Methylocella Species Are Facultatively Methanotrophic

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Received 31 January 2005/Accepted 29 March 2005

All aerobic methanotrophic bacteria described to date are unable to grow on substrates containing carbon-carbon bonds. Here we demonstrate that members of the recently discovered genus Methylocella are an exception to this. These bacteria are able to use as their sole energy source the one-carbon compounds methane and methanol, as well as the multicarbon compounds acetate, pyruvate, succinate, malate, and ethanol. To conclusively verify facultative growth, acetate and methane were used as model substrates in growth experiments with the type strain Methylocella silvestris BL2. Quantitative real-time PCR targeting the mmoX gene, which encodes a subunit of soluble methane monooxygenase, showed that copies of this gene increased in parallel with cell counts during growth on either acetate or methane as the sole substrate. This verified that cells possessing the genetic basis of methane oxidation grew on acetate as well as methane. Cloning of 16S rRNA genes and fluorescence in situ hybridization with strain-specific and genus-specific oligonucleotide probes detected no contaminants in cultures. The growth rate and carbon conversion efficiency were higher on acetate than on methane, and when both substrates were provided in excess, acetate was preferably used and methane oxidation was shut down. Our data demonstrate that not all methanotrophic bacteria are limited to growing on one-carbon compounds. This could have major implications for understanding the factors controlling methane fluxes in the environment.

Aerobic methanotrophic bacteria occupy a key position in the global methane cycle. In aerobic interfaces of flooded soils and wetlands their activity limits potential methane efflux to the atmosphere, and in well-aerated upland soils they consume atmospheric methane directly (8, 25). The 11 described genera of methanotrophic bacteria include species with diverse environmental tolerances and biochemical properties, but one trait considered common to all is the inability to grow on substrates containing carbon-carbon bonds (5, 18, 20). Growth is limited to methane, methanol, and in some cases formate, formaldehyde, and methylamines (5). Experiments with 14C-labeled substrates have shown that small amounts of some organic acids can also be assimilated during exponential growth on one-carbon substrates. However, this accounts for at most 5 to 10% of the total C assimilation, no energy is gained, and no growth occurs on these compounds alone (18).

Recently, the new methanotrophic species Methylocella palustris (13, 14), Methylocella silvestris (17), and Methylocella tundrae (9) were isolated from acidic peat, forest, and tundra soils, respectively. Together with Methylocapsa acidiphila (12), these species form a distinct taxonomic cluster of acidophilic, aerobic methanotrophs, having only a less-extensive series of membrane-bound vesicles adjacent to the inner cell membrane (9, 13, 17).

Like other methanotrophs, Methylocella spp. do not grow on a range of sugars or other complex multicarbon substrates (9, 13, 17). However, in further tests we have observed growth on acetate, pyruvate, succinate, malate, and ethanol. Since this ability, if properly verified, would be unique among methanotrophs, experiments were undertaken to examine more closely the metabolism of acetate and methane and to confirm beyond doubt the purity of the cultures. Similar results were obtained with all three Methylocella species but are shown in detail for Methylocella silvestris strain BL2 only, since this strain grows most robustly. Acetate was chosen as the model multicarbon substrate because this is a major product of fermentation in flooded soils.

MATERIALS AND METHODS

Growth experiments. The use of the carbon substrates acetate, pyruvate, succinate, malate, and ethanol by Methylocella silvestris BL2T (DSM 15510T = NCIMB 13906T), Methylocella palustris K7T (ATCC 700799T), Methylocella tundrae T4T (DSM 15673T = NCIMB 13949T), and Methylocapsa acidiphila B2 (DSM 13967T = NCIMB13765T) were tested in the same way that other...
substrate utilization patterns were tested for taxonomic descriptions of the organisms (9, 12, 13, 17).

Further experiments on methane and acetate metabolism by *Methylocella silvestris* BL2 used the basal salts medium DNMS (dilute nitrate mineral salts, pH 5.8) (17). For growth curve experiments, cultures were grown in 400-ml amounts of medium in 1-liter Erlenmeyer flasks capped gas tight with silicone stoppers. Methane (5 to 15%, vol/vol) was added to the headspace by using a syringe and a sterile filter (0.22 μm). Alternatively, sodium acetate was added to DNMS at 0.04%, wt/vol (6.8 mM), before sterilization, and no methane was added to the headspace. Cultures were grown to an optical density at 600 nm (OD600) of >0.1 (about 10^5 cells ml^-1) from an inoculation OD600 of <0.001 (<10^2 cells ml^-1). Inoculant cells were obtained from plates grown under CH_4 (17) and were washed three times in sterile water before inoculation. Cultures were grown at 25°C with shaking on a rotary shaker at 120 rpm. Uninoculated controls of each medium were included as blanks for leakage and sterility control, and inoculated DNMS medium without any added carbon source was included to verify that no cryptic growth occurred. Samples were taken for determination of methane and acetate concentrations, direct microscopic cell counts, OD600, and occasionally DNA extraction and quantitative real-time PCR. Methane was measured using an SRI 8610C gas chromatograph (SRI Instruments, Torrance, CA) equipped with a flame ionization detector (detector, 140°C; 6 ft by 1/8 in. diameter 80/100 mesh Porapak Q column; oven, 100°C). The optical density at 600 nm was measured on an Eppendorf (Hamburg, Germany) BioPhotometer. Direct cell counts were made with a Haber cytomter. Acetate was measured on a Sykam (Göching, Germany) high-performance liquid chromatography system with a retraction index detector (19).

The molar growth yield Y_{\text{CH}_4} (g dry cell material mol^-1 substrate) and the efficiency of carbon to cell material (g cell mass g^-1 substrate C) were determined in triplicate 400-ml amounts of medium in 1-liter flasks containing exactly 0.0654 g of CH_4 or 0.115 g of acetate. *Methylocella silvestris* was inoculated at an initial density of about 6 × 10^6 cells ml^-1 and incubated until the substrate was exhausted. Cells were harvested by centrifugation (20 min, 16,900 × g) and dried for 24 h at 110°C. Cell material was assumed to be 47% carbon (C4H8O2N).

Serial (10-fold) dilutions for most-probable-number (MPN) counts were made in quadruplicate 20-ml test tubes containing 5 ml of medium (giving a 95% confidence interval of about 1 order of magnitude). The medium either contained 6.8 mM acetate for the estimation of the acetate-utilizing population or was incubated in closed glass desicators under a headspace containing 10% (vol/vol) CH_4 for estimation of the methane-utilizing population. MPNs were calculated using an Excel-based program (6).

**Whole-cell hybridization.** Whole-cell hybridization with 16S RNA-targeted fluorescently labeled oligonucleotide probes was performed with methane-, acetate-, and succinate-grown cultures of *Methylocella silvestris* BL2. Cell fixation, hybridization, probe design, and optimization of hybridization conditions were all performed as described previously (10).

For each cell preparation, two probes labeled with different fluorescent dyes were used. The first, Mcells-1024 (5'-TCCCGGCAGCCTACTGCA-3'), was developed to specifically target *Methylocella silvestris* and was labeled with indocarbocyanine dye (Cy3). The second probe was labeled with 5(-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) and was either (i) a newly designed probe that targeted both *M. silvestris* and *M. palustris*, Mcell-1445 (5'-GCTCTCTCTCTTTTGCGTTT-3'), or (ii) the universal bacterial probe EUB338 (1). Oligonucleotide probes were purchased from MWG Biotech (Ebersberg, Germany). The oligonucleotide probes Mcells-1024 and Mcell-1445 were developed using the probe design tool of the ARB program package (version 2.5b available at http://www.arb-home.de), and the target specificity of these probes was verified using the probe match tool of the Ribosomal Database Project (28, 31). Cell hybridization and the hybridization of fluorescently labeled oligonucleotide probes were performed as described previously (10). For optimizing specific hybridization conditions, *Beijerinckia indica* subsp. indica and *Azorhizobium caulinodans* were used as the nontarget control organisms, as these displayed the smallest number of mismatches within the target regions of the probes Mcells-1024 and Mcell-1445, respectively. The optimal hybridization temperature that provided high target specificity was 50°C. Cell preparations were examined with a Zeiss 1200M microscope (Zeiss, Jena, Germany) equipped with the HQ light filters AHF/AF 41001 (AHF Analysentechnik, Tübingen, Germany) for FLUOS-labeled probes and AHF/F 41007 for Cy3-labeled probes. The same procedure was used to examine the purity of methane-, acetate-, and succinate-grown cultures of *Methylocella palustris* K, using the M. palustris-specific probe Mcell-1026 (10) instead of Mcells-1024.

**Molecular analyses.** DNA to be used in cloning was extracted using a mechanical disruption procedure (17). A portion of the 16S rRNA gene was amplified by PCR using universal bacterial primers targeting *Escherichia coli* positions 907 to 926 and 1513 to 1494 (31). The products were cloned into *E. coli* by using the TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany). A direct PCR amplification was made from positive clones by using vector primers as per the manufacturer's instructions. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced (at least 550 base pairs) on an ABI 377 DNA sequencer by using BigDye terminator chemistry (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany).

To achieve maximum extraction of DNA for quantitative PCR, 2-ml amounts of culture were lysed by combining mechanical cell disruption (17) with incubation with proteinase K and three cycles of rapid freezing in liquid N_2 and thawing (15). A phenol chloroform extraction (15) was then performed in 2-ml Phase Lock Gel tubes (Eppendorf). For each extract, real-time quantitative PCR based on SybrGreen detection was performed in triplicate on an i-Cycler (Bio-Rad, Munich, Germany), using detection principles and PCR mixtures described previously (22). A 16S rRNA gene fragment was quantified using universal primers targeting all members of the *Bacteria* (28, 31). An *mnoX* gene (encoding a subunit of soluble methane monooxygenase) fragment was quantified using the primer mnoXB-1401b (3) in combination with a forward primer (mnoX-ms-945, 5'-TGGGCGCAATCTGGATGAT-3') in a three-step PCR consisting of 3 min of initial denaturation at 94°C followed by 45 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and 1 min of elongation at 72°C. The identity of the products was confirmed by agarose gel electrophoresis or sequencing. Calibration standards were prepared from dilution series of PCR products of the 16S rRNA and *mnoX* genes of *Methylocella silvestris* (22). The counts based on the universal 16S rRNA real-time PCR assay always closely paralleled direct microscopic cell counts but were an average factor of 6.7 lower (coefficient of variation, 87%; total range in individual measurements, 1 to 21), indicating that not all cells were completely lysed. This ratio of direct cell counts to 16S rRNA gene counts was therefore used as a correction factor for extraction efficiency when quantifying the *mnoX* gene (see Fig. 1).

**RESULTS**

All three described species of *Methylocella* were able to grow on acetate, pyruvate, succinate, malate, and ethanol as the sole energy substrate, as well as on the one-carbon substrates methane and methanol (Table 1). The phylogenetically closely related methanotroph *Methylcyclops acidiphila* was not able to grow on any of the multicarbon substrates tested. *Methylocella silvestris* cultures grew on either acetate or methane as the sole carbon and energy source, consuming acetate to below the detection limit (<10 μM) and methane to about 500 parts per million of volume (ppmv) (Fig. 1). Control cultures without added substrate (acetate or methane) did not grow. Growth was estimated by direct cell counts and also by quantitative real-time PCR of the methanotroph-specific *mnoX* gene, which encodes the alpha subunit of the hydroxylase of sMMO. After correction for DNA extraction efficiency by using a real-time PCR assay of the 16S rRNA gene (see Materials and Methods), the *mnoX* gene target counts corresponded almost exactly with direct microscopic cell counts (i.e., equal numbers of 16S rRNA and *mnoX* genes were obtained over the entire growth curves) (Fig. 1). This is a useful purity control, suggesting that all lysed cells were methanotrophs.

More to the point, the parallel increase of *mnoX* copies and total cells clearly demonstrates that cells possessing genes that encode methane monooxygenase were proliferating on acetate as well as on methane. Cultures could be maintained continuously on acetate as the sole carbon and energy source without loss of viability.

Standard tests of culture purity were presented in earlier work (9, 13, 17), and *Methylocella* cultures have been accepted as pure by three major culture collections (Deutsche Sammlung von Mikroorganismen und Zellkulturen, American Type Cul-
TABLE 1. Substrate utilization by the methanotrophs Methylocella silvestris BL2T (= DSM 15510T = NCIMB 13906T) Methylocella palustris K (= ATCC 700799T) Methylocella tundreae T4T (= DSM 15673T = NCIMB 13940T), and Methylocapsa acidiphila B2 (= DSM 13967T = NCIMB13765T)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Methylocella silvestris BL2</th>
<th>Methylocella palustris K</th>
<th>Methylocella tundreae T4</th>
<th>Methylocapsa acidiphila B2</th>
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<td>Methane</td>
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* Data are summarized from references 9, 12, 13, and 17 and the present work.

FIG. 1. Growth curve of Methylocella silvestris on methane (upper panel) or acetate (lower panel) as the sole energy and carbon source. Closed circles represent the decline of substrate (% [vol/vol] methane or mM acetate) over time. Uninoculated controls (not shown) did not show any decline in substrate concentrations. Direct microscopic cell counts (open circles) were closely paralleled by mmoX gene targets estimated using a quantitative real-time PCR assay (triangles). The dotted line represents cell counts in inoculated medium without an added carbon source. Data are means for duplicate (methane treatment) or triplicate (all other treatments) cultures ± 1 SEM. Where error bars are not seen they are contained within the symbol.
1 SEM) were all higher on acetate ($Y_{\text{mm}} = 20.5 \pm 1.24$, efficiency $= 40.1 \pm 2.43$, and $\mu = 1.26 \pm 0.035$) than on methane ($Y_{\text{mm}} = 3.59 \pm 0.104$, efficiency $= 13.2 \pm 0.698$, and $\mu = 0.78 \pm 0.053$). One would therefore predict that *Methylocella* species might possess regulatory mechanisms to preferentially metabolize acetate over methane when both are present, and experimental evidence indicates that this is indeed so. A culture growing exponentially on methane nearly ceased methane oxidation when acetate was added and resumed only after the acetate had been consumed (Fig. 3). The quickness of this response suggests that the enzyme activity is directly regulated, rather than its expression. After the acetate was fully consumed, methane oxidation resumed at a higher maximum rate than in the control treatment, and methane was reduced to a lower threshold. Therefore, although the initial effect of acetate on methane oxidation was inhibitory, acetate addition also appeared to have a longer-term stimulatory effect on methane oxidation in the culture. It is likely that the inhibitory effect of acetate on methane consumption was a direct regulatory response of the bacteria to a preferred substrate, while the long-term stimulation resulted from population growth.

### DISCUSSION

The physiological and evolutionary reasons behind obligate methanotrophy are still the subject of considerable speculation. A physiological explanation for the inability of methanotrophic bacteria to grow on sugars and organic acids is that...
several key enzymes and membrane transporters needed for uptake and metabolism of these substrates are absent or non-functional (26, 27, 33). The genomes of the methanotroph *Methylococcus capsulatus* and of the nitrifier *Nitrosomonas europaea* (which presents a similar case because it grows only on ammonia, a structural analogue of methane) are both available (7, 30). Analyses of these genomes indicate that heterotrophy may be restricted because the organisms possess limited membrane transporters for organic molecules. In many type I methanotrophs of the *Gammaproteobacteria*, another key factor in obligate methylotrophy is the absence of functional α-ketoglutarate dehydrogenase (33). However, all type II methanotrophs of the *Alphaproteobacteria*, including the genus *Methyllocella*, possess a complete tricarboxylic acid cycle, including α-ketoglutarate dehydrogenase (5, 13, 17). The fundamental question is perhaps not why *Methyllocella* grows on organic acids such as acetate but why the other type II methanotrophs *Methylocystis*, *Methylosinus*, and *Methylocapsa* do not. Interestingly, *Methylococcus capsulatus* also possesses in principle all genes necessary for sugar metabolism, although the organism cannot be cultivated on sugars (30).

Enzyme and gene profiles have therefore provided only a partial explanation for obligate methanotrophy. The ultimate evolutionary reasons for why methanotrophs have concentrated on one-carbon metabolism also remain speculative. Presumably in many environments a selective advantage is gained by specialization on methane metabolism. However, it is now clear that not all methanotrophs are so restricted and that members of the genus *Methyllocella* have more metabolic flexibility. In lacking an extensive internal membrane system containing pMMO, *Methyllocella* is fundamentally different morphologically from all other methanotrophs, and these structural differences may be related to the observed metabolic differences.

There have been previous claims that certain species of *Methylobacterium* are also facultatively methanotrophic, but these are not widely accepted (11, 20). *Methylobacterium organophilum* is a facultative methylothroph that grows on methanol and a broad range of multicarbon substrates. However, early reports that it could also grow on methane have proven difficult to reproduce (11). A recent taxonomic description of *Methylobacterium populi* also describes this species as methanotrophic (29), but this claim is not supported by sufficient data (11). In contrast to *Methylobacterium*, *Methyllocella* has been demonstrated to possess both the genetic and enzymatic machineries of methane oxidation via sMMO (9, 13, 17), and methane uptake in pure culture can be demonstrated (Fig. 1). *Methyllocella* is therefore the first unequivocal example of a facultative methanotroph. Type strains of *Methyllocella* have been deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen, American Type Culture Collection, and National Collections of Industrial, Food and Marine Bacteria culture collections, and we encourage others to reproduce our growth experiments.

*Methyllocella* species are widely distributed in acidic soil environments, including peat bogs, forest soils, and arctic tundra (9, 13, 14, 17). In *Sphagnum* peat, *Methyllocella* is one of the numerically dominant methanotroph populations (10). Factors that affect *Methyllocella* are therefore also likely to affect methane fluxes in the environment. Acetate may be one such factor. Unlike methanol and other C1 substances (formate, formamide, and methylamines) used by a few methanotrophs (5), acetate is a major intermediate of carbon turnover in soil environments. It can exceed methane as an end product of anaerobic metabolism in wetlands, reaching concentrations of up to 1 mM (16). Like methane, acetate diffuses upwards from anaerobic zones to an aerobic surface sink, where *Methyllocella* is active (16). Although methane has a high energy of combustion, reducing power is required by MMO in an initial hydroxylation step, and growth on methane is strongly limited by reductant supply (2). Probably as a result of this, *Methyllocella silvestris* grows faster and more efficiently on acetate and shuts off methane oxidation when excess acetate is present. The immediate effect of acetate on methane oxidation is therefore negative. However, the long-term effect on methane oxidation potential in the field could be positive if acetate enhances growth and survival of the *Methyllocella* population. Providing an alternate energy source such as methanol to methanotrophs has been shown to increase methane oxidation activity (4, 21), and recent evidence indicates that acetate also stimulates methane oxidation in tundra soil (32). Based on previous knowledge of methanotroph physiology, the latter could only be explained as a secondary effect via enhanced methanogenesis, but our results demonstrate that direct stimulation of the methanotroph population by acetate is also possible.

Acetate can also be produced to millimolar levels in forest litter (23). It is therefore tempting to postulate that the methanotrophs responsible for the net uptake of the trace level of atmospheric methane (1.7 ppmv) by forest soils (8) may also benefit from growth on multicarbon substrates. The addition of acetate did lower the methane oxidation threshold for *Methyllocella* in our experiments. However, this threshold remained very high (>100 ppmv), even with repeated acetate addition (data not shown). The soluble MMO has a lower affinity for methane than the particulate form of the enzyme (20) and is unlikely to be involved in atmospheric methane uptake. Whether there are pMMO-containing facultative methanotrophs remains to be seen.

The findings presented here could have major implications for the way in which we view the methane cycle in the environment. It is now evident that there exist two physiologically distinct populations of methanotrophs: a specialist population that grows only on methane (and other C1 compounds) and a generalist *Methyllocella* population with the ability to metabolize several multicarbon compounds besides methane. The latter may hold a competitive advantage in natural environments where methane production is temporarily heterogeneous due to fluctuations in temperature, water content, and water table level (25). Studies have until now only addressed the specialist group and have worked from the assumption that methanotroph populations are energetically limited only by the supply of methane. We have demonstrated that this basic assumption is in error. Population sizes, survival, and methane oxidation activity of methanotrophs can be controlled by the availability of some multicarbon substrates as well.

**ACKNOWLEDGMENTS**

This work was supported in part by the Deutsche Forschungsgemeinschaft (grant DÜ 377/1-1) and by the “Molecular and Cell Biology” Program of the Russian Academy of Sciences.
We thank Johannes Scholten, Reiner Hedderich, Ralf Conrad, and Steffen Kolb for helpful discussions and Nina Ringleff for technical assistance.

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