Methylocapsa aurea sp. nov., a facultative methanotroph possessing a particulate methane monooxygenase, and emended description of the genus *Methylocapsa*

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An aerobic, methanotrophic bacterium, designated KYG^T, was isolated from a forest soil in Germany. Cells of strain KYG^T were Gram-negative, non-motile, slightly curved rods that multiplied by binary fission and produced yellow colonies. The cells contained intracellular granules of poly- β -hydroxybutyrate at each cell pole, a particulate methane monooxygenase (pMMO) and stacks of intracytoplasmic membranes (ICMs) packed in parallel along one side of the cell envelope. Strain KYG^T grew at pH 5.2–7.2 and 2–33 °C and could fix atmospheric nitrogen under reduced oxygen tension. The major cellular fatty acid was C_{18:1 ω 7c} (81.5 %) and the DNA G+C content was 61.4 mol%. Strain KYG^T belonged to the family *Beijerinckiaceae* of the class *Alphaproteobacteria* and was most closely related to the obligate methanotroph *Methylocapsa acidiphila* B2^T (98.1 % 16S rRNA gene sequence similarity and 84.7 % *pmoA* sequence similarity). Unlike *Methylocapsa acidiphila* B2^T, which grows only on methane and methanol, strain KYG^T was able to grow facultatively on acetate. Facultative acetate utilization is a characteristic of the methanotrophs of the genus *Methylocella*, but the genus *Methylocella* does not produce pMMO or ICMs. Strain KYG^T differed from *Methylocapsa acidiphila* B2^T on the basis of substrate utilization pattern, pigmentation, pH range, cell ultrastructure and efficiency of dinitrogen fixation. Therefore, we propose a novel species, *Methylocapsa aurea* sp. nov., to accommodate this bacterium. The type strain is KYG^T (=DSM 22158^T =VKM B-2544^T).

All known aerobic methanotrophic bacteria belong to the phyla *Proteobacteria* or *Verrucomicrobia* (Op den Camp *et al.*, 2009). The proteobacterial methanotrophs are affiliated to two families of the *Alphaproteobacteria* (*Methylocystaceae* and *Beijerinckiaceae*) and one family of the *Gamma**proteobacteria* (*Methylococcaceae*). The family *Beijerinckiaceae* is particularly metabolically diverse and contains obligate and facultative methanotrophs and non-methanotrophic chemohetero-

trophs. The two methanotrophic genera in this family, *Methylocella* and *Methylocapsa*, are abundant in acidic soils and peats (Dedysh *et al.*, 2001, 2003) and are physiologically distinct from each other. *Methylocapsa acidiphila* is an obligate methanotroph capable of growth only on one-carbon substrates. It has a particulate methane monooxygenase (pMMO) and an extensive intracytoplasmic membrane (ICM) system (Dedysh *et al.*, 2002). In contrast, *Methylocella* species are the only known methanotrophs that lack pMMO and use only a soluble methane monooxygenase (sMMO) for methane oxidation. The genus *Methylocella* contains the first-described facultative methanotrophs and they are capable of growth on a few multicarbon substrates as well as methane (Dedysh *et al.*, 2005). Here, we describe the isolation of a new methanotroph in the family *Beijerinckiaceae*.

Abbreviations: ICM, intracytoplasmic membrane; pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *pmoA*, *mxoF* and *nifH* gene sequences of strain KYG^T are FN433469–FN433472, respectively.

A figure showing the effect of pH on growth of strain KYG^T is available as supplementary material with the online version of this paper.

Strain KYG^T was isolated from a soil sample collected in March 2003 from under a small ephemeral brook in a forest near Marburg, Germany. Plates of solid diluted nitrate mineral salts medium at pH 5.8 (DNMS; Dunfield *et al.*, 2003) were inoculated with soil and incubated at 25 °C in a closed glass desiccator containing a headspace of 20 % methane (v/v) and 5 % CO₂ in air for 2 months. During this time, yellowish colonies of irregular shape developed. Strain KYG^T was obtained from a single colony and purified by successive streaking. Once a pure culture had been obtained, strain KYG^T was maintained by transfer at 1-month intervals to 120-ml serum vials containing 20 ml liquid DNMS. Vials were sealed with rubber septa and 20 % methane and 5 % CO₂ were added aseptically using syringes equipped with disposable filters (0.22 µm). Vials were incubated on a rotary shaker (120 r.p.m.) at 24 °C.

Three strains of *Methylocapsa acidiphila* were used as reference strains in this study: B2^T, V1 and N2. Strains V1 and N2 were newly isolated from an acidic peat soil (pH 3.8) sampled at a depth of 10 cm of the oligomesotrophic fen Torfjanoye, Archangelsk region, European North Russia (65° 01' N 35° 44' E) in June 2006. These strains were isolated using liquid mineral medium at pH 5.0 (M2; Dedysh *et al.*, 1998) and were identified as *Methylocapsa acidiphila* on the basis of 99.9–100 % 16S rRNA gene sequence similarity with *Methylocapsa acidiphila* B2^T.

Morphological observations and cell size measurements were made with an Axioplan 2 microscope and Axiovision 4.2 software (Carl Zeiss). Ultrathin sections were prepared as described previously (Dedysh *et al.*, 2000) and examined on a JEM-100C transmission electron microscope (JEOL). The absence of heterotrophic satellites in strain KYG^T was checked by phase-contrast and electron microscopy and growth on solid DNMS amended with 0.05 % (w/v) glucose, fructose or yeast extract. Physiological tests were carried out on cultures grown in liquid DNMS with methane as the sole substrate. Growth of strain KYG^T under a variety of conditions, including 2–37 °C, pH 3.9–8.0 and 0–3.0 % (w/v) NaCl, was monitored by nephelometry at 410 nm using a Specol spectrophotometer (Carl Zeiss) for 3 weeks. Utilization of the following carbon sources (0.05 %, w/v) was examined: methanol, ethanol, formate, formaldehyde, glucose, fructose, arabinose, lactose, sucrose, maltose, galactose, acetate, citrate, oxalate, malate, pyruvate and succinate. The capacity to utilize 0.01–5 % (v/v) methanol was determined in liquid DNMS supplemented with methanol. Nitrogen sources were tested in liquid DNMS by replacing NaNO₃ individually with 0.05 % (w/v) (NH₄)₂SO₄, NaNO₂, urea, hydroxylamine, peptone, L-serine, L-proline, L-alanine, L-asparagine and yeast extract. For all substrate utilization tests, growth was examined after 1 month and confirmed by comparison to a negative control. Biomass for cellular fatty acid analysis and DNA extraction was obtained from batch cultures grown at 24 °C for 10 days. The fatty acid profiles were analysed by

the Identification Service of the DSMZ, Braunschweig, Germany, as described by Kämpfer & Kroppenstedt (1996). The DNA base composition of strains was determined by thermal denaturation using a Unicam SP1800 spectrophotometer with a heating rate of 0.5 °C min⁻¹. The DNA G+C content was calculated according to Owen *et al.* (1969). The 16S rRNA gene sequence and partial sequences of *pmoA* and *mxoF* of strain KYG^T were determined as described by Heyer *et al.* (2002). The partial sequence of *nifH* was determined as described by Dedysh *et al.* (2004a). Phylogenetic analysis was carried out using the ARB program package (Ludwig *et al.*, 2004).

On solid DNMS, strain KYG^T formed small, yellow colonies. Colony diameter after 2 weeks was 1–2 mm and after 6 weeks was 3–5 mm. Colonies were pleomorphic in appearance, ranging from raised, slimy and transparent with a light yellow tint to flat and cream-yellow. Liquid cultures displayed white turbidity. Cells of strain KYG^T were Gram-negative, non-motile, encapsulated, slightly curved rods, 0.7–1.2 µm wide and 1.8–3.1 µm long after incubation for 10 days (Fig. 1a). They reproduced by binary fission and occurred singly or in irregular aggregates. The formation of rosettes was not observed. Cells from older cultures (3–4 weeks) were shorter (1.1–1.7 µm) and had a cyst-like shape. Similarly to the genus *Methylocella*, cells of strain KYG^T possessed a distinctive bipolar appearance due to highly refractile intracellular granules of poly-β-hydroxybutyrate at each cell pole (Fig. 1a, b). However, in contrast to the genus *Methylocella*, thin-sectioned cells of strain KYG^T displayed well-developed ICMs packed in parallel to one side of the cell (Fig. 1b). This type of arrangement has been revealed previously in the cells of only one other methanotroph, *Methylocapsa acidiphila* B2^T, and has been termed a type III arrangement (Dedysh *et al.*, 2002).

Strain KYG^T grew on methane and methanol as sole carbon and energy sources. Weak growth was also observed on formate. Methanol supported growth only at concentrations below 0.1 % (v/v); the most active growth occurred at 0.05 % (v/v). The preference for a low methanol concentration is characteristic of *Methylocapsa acidiphila* B2^T. In addition to these one-carbon compounds, strain KYG^T was also able to grow on acetate (Fig. 2). Cultures could be maintained continuously on acetate as the sole carbon and energy source without loss of viability. Acetate is also utilized by the facultative methanotrophs of the genus *Methylocella*, which possess an sMMO but no pMMO (Dedysh *et al.*, 2005). However, in contrast to members of the genus *Methylocella*, which display a clear preference for acetate, strain KYG^T grew better on methane (maximum OD₆₀₀ 1.2, µ=0.018 h⁻¹) than on acetate (maximum OD₆₀₀ 0.3, µ=0.006 h⁻¹). No growth was observed on the other multicarbon compounds tested.

The purity of acetate-grown cultures of strain KYG^T was tested by means of phase-contrast and electron microscopy and by plating onto solid DNMS amended with 0.05 % (w/v) glucose, fructose or yeast extract and incubation

Fig. 1. (a) Phase-contrast micrograph of cells of strain KYG^T, grown in liquid DNMS with methane for 10 days. (b) Electron micrograph of an ultrathin section of a methane-grown cell of strain KYG^T. ICM, Intracytoplasmic membrane; PHB, poly-β-hydroxybutyrate. Bars, 2 μm (a) and 1 μm (b).

under a methane-free atmosphere. No growth occurred on these substrates, demonstrating that cultures were not contaminated with organotrophs. Morphology of acetate-grown cells was essentially the same as for methane-grown cells, except that acetate-grown cells were more refractile and the cyst-like shape was attained much earlier, after 1.5–2 weeks of growth. ICMs were maintained in acetate-grown cells, although they were present in a reduced form (not shown). Finally, DNA was extracted from acetate-grown and methane-grown cultures and the 16S rRNA gene sequence was amplified by PCR and cloned into *Escherichia coli* TOP10 using a TA cloning kit (Invitrogen). At least 500 bp from the 5' end was sequenced from >21 clones of each culture. All cloned sequences were the same as that of strain KYG^T, indicating that the growth observed in the acetate culture did not represent a contaminating bacterium.

Fig. 2. Growth of strain KYG^T with methane (●) or acetate (■) as the sole carbon and energy source, shown as means of triplicate experiments. ▲, Control with no carbon source. Error bars (where visible) represent the SEM.

The ability to grow on acetate has not been shown for *Methylocapsa acidiphila* B2^T (Dedysh *et al.*, 2002, 2005). In this study, we were able to examine two newly isolated members of this species, strains V1 and N2. Both of these, as well as *Methylocapsa acidiphila* B2^T, were tested for the ability to grow on acetate, ethanol, malate, succinate and pyruvate. For all three strains of *Methylocapsa acidiphila*, no growth was detected on any of these substrates after 1 month, whereas good growth was observed in the positive controls containing methane. Therefore, we were able to confirm the previous findings that *Methylocapsa acidiphila* is unable to grow using multicarbon compounds.

Growth of strain KYG^T occurred over a relatively narrow pH range, pH 5.2–7.2 (optimum pH 6.0–6.2), and no growth occurred below pH 5.0 (Supplementary Fig. S1, available in IJSEM Online). The temperature range for growth was 2–33 °C, with an optimum at 25–30 °C. Strain KYG^T was extremely sensitive to NaCl: 0.1% (w/v) NaCl inhibited growth by 90% and 0.2–0.3% (w/v) NaCl inhibited growth completely. Sole nitrogen sources (0.05%, w/v) included ammonium salts, nitrates, urea, L-proline, L-alanine, L-asparagine, peptone and yeast extract. Strain KYG^T was also able to fix dinitrogen and a partial *nifH* sequence was obtained by PCR. However, in contrast to *Methylocapsa acidiphila* B2^T, strain KYG^T was not capable of active growth in nitrogen-free medium under fully aerobic conditions (Dedysh *et al.*, 2004a).

The cellular fatty acid profile of strain KYG^T was very similar to those of *Methylocapsa acidiphila* B2^T and members of the genus *Methylocella* (Table 1). The major cellular fatty acid in all of these methanotrophs was 11-*cis*-octadecanoic acid (C_{18:1}ω7*c*), comprising 59–82% of the total fatty acids. Despite small differences in some minor components (e.g. C_{17:1}ω7*c*, C_{18:0} and C_{19:0} cyclo ω8*c*), strain KYG^T and *Methylocapsa acidiphila* B2^T were nearly indistinguishable from each other on the basis of their cellular fatty acid profiles.

Table 1. Cellular fatty acid compositions of strain KYG^T and related taxa

Values are percentages of total fatty acids and were taken from this study, Dedysh *et al.* (2000, 2002, 2004b) and Dunfield *et al.* (2003). —, Not detected/not reported.

Fatty acid	Strain KYG ^T	<i>Methylocapsa acidiphila</i> B2 ^T	<i>Methylocella</i>
C _{13:1}	0.8	—	—
C _{14:0}	—	—	0–4.1
iso-C _{15:0}	0.3	0.1	0.2–1.2
C _{15:0}	—	—	0–0.1
C _{16:1} ω7c	6.3	4.7	6.8–11.3
C _{16:1} ω7t	—	—	0–5.8
C _{16:1} ω5c	—	0.1	0–0.1
C _{16:0}	5.9	7.3	3.0–7.7
iso-C _{17:1} ω9c	0.2	—	0–0.3
iso-C _{17:0}	0.9	0.6	0–2.5
C _{17:1} ω8c	—	—	0–0.3
C _{17:1} ω7c	—	1.0	—
C _{17:0} cyclo	—	—	0–6.5
C _{17:0}	—	0.1	0–0.1
iso-C _{18:0}	—	—	0–0.5
C _{18:1} ω9c	0.4	—	—
C _{18:1} ω7c	81.5	78.3	59.2–82.2
C _{18:0}	0.8	7.6	0.4–1.2
C _{19:0} cyclo	2.5	—	0–13.6
ω8c	—	—	—
C _{19:0}	—	—	0–0.6

Comparative analysis of 16S rRNA gene sequences showed that strain KYG^T belonged to the family *Beijerinckiaceae* of the class *Alphaproteobacteria* and was most closely related to the obligate methanotroph *Methylocapsa acidiphila* B2^T (98.1 % 16S rRNA gene sequence similarity). Other closely related organisms were strains of species of the genera *Beijerinckia* (97.5–97.7 %) and *Methylocella* (97.5–97.8 %) and *Methylovirgula ligni* (96.8 %). In a neighbour-joining analysis, strain KYG^T clustered most closely with *Methylocapsa acidiphila* B2^T, although the node was not supported by a high bootstrap value (40 %; Fig. 3). Tree topologies were constructed using the maximum-likelihood, maximum-parsimony and maximum-likelihood quartet puzzling methods with different distance corrections but failed to produce a consistent affiliation of strain KYG^T to any particular genus in the family *Beijerinckiaceae*, and bootstrap and support values were typically <50 %. In some constructions, strain KYG^T affiliated more closely to the genera *Methylocella*, *Methylovirgula* or *Beijerinckia* than to *Methylocapsa acidiphila* B2^T. On the basis of the physiological data, we assign strain KYG^T to the genus *Methylocapsa*; however, it may be evolutionarily intermediate to the genus *Methylocapsa* and other genera.

pmoA was detected in strain KYG^T by PCR using primers A189f and A682r as described by Heyer *et al.* (2002), which indicated the presence of pMMO. Amplification from

strain KYG^T was weak compared with DNA extracts from other methanotrophs, which suggested that this primer set may not be well targeted to *pmoA* in strain KYG^T. To determine the presence of *pmoX*, which encodes a subunit of sMMO, four PCR primer pairs, 166f and 1401r (*pmoX*A and *pmoX*D), 166f and 1353r [*pmoX*A and *pmoX*mc1 (5'-VCGYTCGCCCCARTCRCTC-3')], 166f and 1272r [*pmoX*A and *pmoX*mc2 (5'-VGTCGGGCAGAA-SGGCAC-3')] and 786f and 1353r [*pmoX*mc3 (5'-CC-GGCSGCSCAGAAATAT-3') and *pmoX*mc1], were used with a PCR protocol consisting of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min and extension at 72 °C for 1 min for 35 cycles, with a final extension step of 7 min. Primers 166f and 1401r target a wide range of *pmoX* sequences in methanotrophs (Auman *et al.*, 2000; Heyer *et al.*, 2002). Primers 1353r, 1272r and 786f were designed to target *pmoX* sequences specific to methanotrophs in the family *Beijerinckiaceae* on the basis of all known published sequences plus several unpublished sequences from *Methylocella*-like isolates in our collection. DNA from *Methylocella silvestris* BL2^T was used as a positive control. No *pmoX* sequences were detected in strain KYG^T. The colorimetric naphthalene oxidation test (Graham *et al.*, 1992) for sMMO activity was performed with cells of strain KYG^T grown on copper-free medium and also gave a negative result. The results strongly suggested that sMMO was not present in strain KYG^T.

A phylogenetic tree based on partial *pmoA* and *amoA* sequences (495 nt positions) was constructed using TreePuzzle, a maximum-likelihood quartet-puzzling method, with the Schöniger–von Haeseler distance correction method (Schmidt *et al.*, 2002). Support values for nodes were calculated on the basis of 5000 puzzling steps. The tree showed that strain KYG^T belonged to the lineage of pMMO-possessing methanotrophs defined by *Methylocapsa acidiphila* B2^T (84.7 % *pmoA* sequence similarity, 91.4 % deduced amino acid sequence identity) (Fig. 4). The *pmoA* sequences from the two *Methylocapsa* species were closely related to *pmoA* sequences detected by cultivation-independent methods from several soils and peats, including the upland soil cluster alpha that is widely distributed in acidic upland soils and is assumed to represent methanotrophs adapted to consuming the trace level of methane in the atmosphere (Holmes *et al.*, 1999; Knief *et al.*, 2003). So far, these methanotrophs have eluded all attempts at cultivation.

The partial *nifH* sequence from strain KYG^T displayed the highest similarity with those of *Methylocapsa acidiphila* B2^T and *Beijerinckia indica* subsp. *indica* ATCC 9039^T (92.0 % *nifH* sequence similarity, 99.0 % deduced amino acid sequence identity). However, unexpectedly, the partial *mxoF* sequence of strain KYG^T was most closely related to *mxoF* fragments from members of the genera *Methylobacterium* (79.8–83.2 % *mxoF* sequence similarity, 83.4–88.3 % deduced amino acid sequence identity) and *Methylocella* (79.0–80.1 and 84.2–85.3 %, respectively) and was less related to *Methylocapsa acidiphila* B2^T (76.1 and 78.4 %, respectively).

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences, constructed using the neighbour-joining method with Jukes–Cantor correction, showing the position of strain KYG^T in relation to representatives of the families *Beijerinckiaceae* and *Methylocystaceae*. Bootstrap values (>50%) based on 1000 resamplings are shown at branch nodes. Sequences from the type-I methanotrophs *Methylobacterium album* ACM 3314^T (GenBank accession no. X72777), *Methylobacter luteus* NCIMB 11914^T (AF304195), *Methylomonas methanica* S1^T (AF304196) and *Methylococcus capsulatus* Texas^T (AJ563935) were used as an outgroup (not shown). Bar, 0.05 substitutions per nucleotide position.

In summary, strain KYG^T was shown to be most similar to *Methylocapsa acidiphila* B2^T on the basis of pMMO-based methanotrophy, possession of a well-developed ICM system, utilization of methanol at low concentrations and preference for growth on methane. These phenotypic similarities were supported by phylogenetic analyses of the 16S rRNA gene, *pmoA* and *nifH* sequences. However, there were differences in substrate utilization patterns, cell morphology and pigmentation, pH range for growth and efficiency of dinitrogen fixation. The key difference between strain KYG^T and *Methylocapsa acidiphila* B2^T was the facultative methanotrophy in strain KYG^T versus the obligate methanotrophy in *Methylocapsa acidiphila*

B2^T. Using two novel isolates of *Methylocapsa acidiphila*, strains V1 and N2, we were able to confirm that obligate methanotrophy is a feature consistent for the species *Methylocapsa acidiphila*. Therefore, we propose that strain KYG^T be classified in a novel species of the genus *Methylocapsa*, with the name *Methylocapsa aurea* sp. nov.

Emended description of the genus *Methylocapsa* Dedysh et al. 2002

Gram-negative, curved coccoids or thick curved rods that occur singly or in conglomerates, but do not form rosettes. Reproduce by normal cell division. Non-motile.

Fig. 4. Phylogenetic tree based on partial (495 nt) *pmoA* and *amoA* sequences, constructed using a quartet maximum-likelihood method and Schöniger–von Haeseler distance correction, showing the relationships between strain KYG^T, other methane- or ammonia-oxidizing bacteria and sequences obtained from cultivation-independent studies of various environments. Support values (>50%) based on 5000 puzzling steps are shown at branch nodes. Where the support value is <50% a multifurcation is drawn. Bar, 0.1 changes per nucleotide position.

Encapsulated. Produce intracellular poly- β -hydroxybutyrate granules. Cells contain a well-developed ICM system of type III arrangement, which appears as stacks of membrane vesicles packed in parallel on only one side of the cell membrane. Strictly aerobic. Possess pMMO and do not express sMMO. Moderately acidophilic and mesophilic. Prefer dilute media with low salt content. Some members of the genus are obligate utilizers of one-carbon compounds, while others are also capable of growth on acetate, but sugars are not utilized. One-carbon compounds are utilized via the serine pathway. Capable of atmospheric nitrogen fixation. The major cellular fatty acid is C_{18:1} ω 7c. The G + C content of the DNA is 61.4–63.1 mol%. Member of the family *Beijerinckiaceae* in the class *Alphaproteobacteria*. Known habitats are acidic and nearly neutral wetlands, soils and sediments. The type species is *Methylocapsa acidiphila*.

Description of *Methylocapsa aurea* sp. nov.

Methylocapsa aurea (au're.a. L. fem. adj. *aurea* golden, referring to the colony colour).

Displays the properties described for the genus, including those in the emended description above, with the following additional traits. Cells are 0.7–1.2 μ m wide and 1.8–3.1 μ m long. Colonies are yellow. Carbon sources include methane, methanol, formate and acetate. Nitrogen sources are ammonium salts, nitrates, urea, L-proline, L-alanine, L-asparagine, peptone and yeast extract. Dinitrogen is fixed via oxygen-sensitive dinitrogenase. Optimal growth occurs at 25–30 °C and pH 6.0–6.2. Growth is inhibited with 0.3% (w/v) NaCl. The DNA G + C content of the type strain is 61.4 mol%.

The type strain is KYG^T (=DSM 22158^T =VKM B-2544^T), isolated from a forest soil near Marburg, Germany.

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