Methylomonas lenta sp. nov., a methanotroph isolated from manure and a denitrification tank

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Two methanotrophic bacteria, strains R-45377T and R-45370, were respectively isolated from a slurry pit of a cow stable and from a denitrification tank of a wastewater treatment plant in Belgium. The strains showed 99.9 % 16S rRNA gene sequence similarity. Cells were Gram-negative, motile rods containing type I methanotroph intracytoplasmic membranes. Colonies and liquid cultures appeared white to pale pink. The pmoA gene encoding particulate methane monooxygenase (pMMO) and the nifH gene encoding nitrogenase were present. Soluble methane monooxygenase (sMMO) activity, the presence of the mmoX gene encoding sMMO and the presence of the ppxmA gene encoding a sequence-divergent pMMO were not detected. Methane and methanol were utilized as sole carbon sources. The strains grew optimally at 20 °C (range 15–28 °C) and at pH 6.8–7.3 (range pH 6.3–7.8). The strains grew in media supplemented with up to 1.2 % NaCl. The major cellular fatty acids were C16:1ω8c, C16:1ω5c, C16:1ω7c, C14:0, C15:0 and C16:0 and the DNA G+C content was 47 mol%. 16S rRNA gene and pmoA-based phylogenetic analyses showed that the isolates cluster among members of the genus Methylomonas within the class Gammaproteobacteria, with pairwise 16S rRNA gene sequence similarities of 97.5 and 97.2 % between R-45377T and the closest related type strains, Methylomonas scandinavica SR5T and Methylomonas paludis MG30T, respectively. Based on phenotypic characterization of strains R-45377T and R-45370, their low 16S rRNA gene sequence similarities and the formation of a separate phylogenetic lineage compared with existing species of the genus Methylomonas, we propose to classify these strains in a novel species, Methylomonas lenta sp. nov., with R-45377T (=LMG 26260T =JCM 19378T) as the type strain.

INTRODUCTION

Aerobic methanotrophic bacteria are grouped by their ability to oxidize the greenhouse gas methane and utilize it as a sole carbon and energy source. In the first step of methane oxidation, methane is converted to methanol by a particulate or soluble methane monooxygenase (pMMO or sMMO) (Semrau et al., 2010). The first isolation of a methanotroph in pure culture, ‘Bacillus methanicus’, later renamed Methylomonas methanica, was reported by Söhngen (1906), after which only three more methanotrophs were described until 1970 (Anthony, 1982). At that time, Whittenbury and colleagues isolated and characterized more than 100 methanotrophic cultures, establishing the basic phylogenetic framework of the intensively studied proteobacterial methanotrophs (Whittenbury et al., 1970), which are positioned within the classes Gammaproteobacteria and Alphaproteobacteria, although a few non-proteobacterial methane-oxidizing bacteria have also been described (Ettwig et al., 2010; Op den Camp et al., 2009). Five methanotrophic genera have been described within the class Alphaproteobacteria: Methylolocella, Methylolfacella and Methyllocapsa in the family Beijerinckiaceae and Methylosinus and Methylcocystis in the family Methylcystaceae (Dedys, 2009; Vorobev et al., 2011). At the time of writing, 12 genera of methanotrophic bacteria have been formally described within the family Methylococccaceae of the class Gammaproteobacteria: Methylomonas, Methylbacter, Methyllococcus, Methylococcus, Methylosarcina, Methylocaldum, Methylogea, Methylothermus, Methylobacter, Methylomonas, Methylophilum and Methylophaera (Geymonat et al., 2011).

Members of the genus Methylomonas are rod-shaped, motile by single polar flagella, neutrophilic and mesophilic and possess type I intracytoplasmic membranes. Colonies...
of strains of the genus *Methylomonas* are typically pink, orange or red due to the production of carotenoid pigments. Representatives of this genus have been isolated from a wide variety of environments, such as rice paddies, wetlands, wastewater and coal-mine drainage water (Bowman, 2006; Bowman et al., 1993; Danilova et al., 2013; Hanson & Hanson, 1996; Ogiso et al., 2012).

Six species have been described within the genus *Methylomonas* (Danilova et al., 2013), and three of them no longer have extant type strains, namely *Methylomonas scandinavica*, *Methylomonas fodinarum* and *Methylomonas aurantiaca*. Two species of the genus *Methylomonas* were recently described, *Methylomonas koyamae* and *Methylomonas paludis* (Danilova et al., 2013; Ogiso et al., 2012). The type strain of *Methylomonas methanica*, the type species of the genus, represents the first isolated methanotroph (Anthony, 1982).

Here, we report the characterization of two new isolates of the genus *Methylomonas* from a manure pit and a denitrification tank of a wastewater treatment plant in Belgium that we propose to assign to a novel species, *Methylomonas lenta* sp. nov.

![Electron micrograph of actively growing cells of strain R-45377<sup>T</sup> showing rods with typical intracytoplasmic membrane arrangements (ICM) of gammaproteobacterial methanotrophs. Bar, 0.2 μm.](image)

**Fig. 1.**

![ML phylogenetic tree (1403 nt alignment; GTR + I substitution model; 1000-replicate bootstrapping) based on 16S rRNA gene sequences of strains R-45377<sup>T</sup> and R-45370, type strains of recognized species within the genus *Methylomonas* and type strains of the type species of the other genera within the family *Methylococcaceae*. The alphaproteobacterial methanotrophic type strains *Methylocystis parvus* NCIMB 11129<sup>T</sup> (GenBank accession no. Y18945), *Methylosinus trichosporium* OB3b<sup>T</sup> (Y18947), *Methyloferula stellata* AR4<sup>T</sup> (FR686343), *Methylocella palustris* K<sup>T</sup> (Y17144) and *Methylcapsa acidiphila* B2<sup>T</sup> (AJ278726) were used as an outgroup. Bar, 0.02 substitutions per nucleotide position.](image)

**Fig. 2.**
Heat resistance and desiccation resistance were assessed on turbid suspensions (OD$_{600}$ 1) of cultures grown to stationary phase. To measure heat resistance, suspensions were incubated for 15 min at 85 °C, cooled rapidly on ice and plated onto dNMS agar and incubated. For evaluation of desiccation resistance, drops of suspensions were air-dried on sterile glass slides and kept at 28 °C for 3 weeks. Desiccation resistance of the cultures was assessed by rehydration and incubation in dNMS (both on agar plates and in liquid cultures).

The ability to fix nitrogen was evaluated by incubation of the strains in duplicate in liquid nitrogen-free diluted mineral salts (dMS) medium with 20 % methane added to air at normal (21 %) and tenfold-reduced (2.1 %) levels of oxygen. Growth was determined by replacing nitrate in dNMS, with methane added to the headspace: methanol, formate, urea, methylamine, dimethylacetamide, ethanol, citrate, glycine, D-xylose, maltose and glucose. Utilization of the following nitrogen sources at 2 mM was evaluated by replacing nitrate in dNMS with methane added to the headspace: methanol, ammonium, dimethyl carbonate, formamide, acetate, pyruvate, succinate, malate, ethanol, citrate, glycine, D-xylose, maltose and glucose.

Utilization of the following carbon sources (0.1 %) was evaluated in liquid dNMS without methane added to the headspace: methanol, formate, urea, methylamine, dimethylamine, dimethyl carbonate, formamide, acetate, pyruvate, succinate, malate, ethanol, citrate, glycine, D-xylose, maltose and glucose. Utilization of the following nitrogen sources at 2 mM was evaluated by replacing nitrate in dNMS, with methane added to the headspace: nitrite, ammonium, [NH$_4$Cl] and (NH$_4$)$_2$SO$_4$, formamide, methylamine, glycine, hydroxylamine, urea, i-serine, i-proline, i-aspartate, i-glutamate, glutathione, dimethyl carbonate and dimethylacetamide.
R-45377T and R-45370 were aligned among a selection of methanotrophic type strains using the Classifier program of the BioNumerics 5.1 (Applied Maths). Preliminary phylogenetic identification was obtained by query in the Classifier program of the BioNumerics 5.1 (Applied Maths). Preliminary phylogenetic identification was obtained by query in the Classifier program of the BioNumerics 5.1 (Applied Maths).

Based on the 16S rRNA gene sequence alignment, a maximum likelihood tree was exported in Newick format and analyzed using theMEGA 5 software (Tamura et al., 2004). The best-scoring ML tree (285 nt alignment; GTR substitution model; 1000-replicate bootstrapping) based on nifH gene sequences of strains R-45377T and R-45370 with the available nifH sequences of the type strains within the genus Methylococcales and available nifH sequences within the family Methylococcales. The alphaproteobacterial methanotrophic type strains of Methylocystis parvus, Methylosinus trichosporium, Methyloferula stellata, Methylocella palustris and Methylocapsa acidiphila were used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

Fig. 4. ML phylogenetic tree (285 nt alignment; GTR + I substitution model; 1000-replicate bootstrapping) based on nifH gene sequences of strains R-45377T and R-45370 with the available nifH sequences of the type strains within the genus Methylococcales and available nifH sequences within the family Methylococcales. The alphaproteobacterial methanotrophic type strains of Methylocystis parvus, Methylosinus trichosporium, Methyloferula stellata, Methylocella palustris and Methylocapsa acidiphila were used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

L-leucine, L-cysteine, L-arginine and yeast extract [0.1% (w/v) yeast extract, instead of 2 mM]. Salt tolerance [tested at 0.23, 0.58, 1.0, 1.2, 5 and 10 % (w/v) NaCl spiked in dNMS], the pH optimum and range, tested at pH 4 and 5 (citric acid/Na2HPO4 buffer), pH 5.8, 6.3, 6.8, 7.3 and 7.8 (KH2PO4/Na2HPO4 buffer), and the temperature optimum and range (tested at 5, 15, 20, 25, 28, 33, 37, 45 and 52 °C) of the strains were assessed in liquid dNMS. The buffer strength was similar (2 mM) for all media, and the intended pH was confirmed after medium preparation. An additional 0.2 mM Na2HPO4 was added to media prepared with glycine buffer, to provide a source of phosphate.

sSMO activity was tested using a modified version of the naphthalene oxidation assay (Hoeft et al., 2012a). Cryopreservation of strains R-45377T and R-45370 was performed as described by Hoeft et al. (2012b).

DNA was extracted and 16S rRNA gene sequences were generated as described by Hoeft et al. (2012a). Sequences were assembled with BioNumerics 5.1 (Applied Maths). Preliminary phylogenetic identification was obtained by query in the Classifier program of the Ribosomal Database Project II (Cole et al., 2005). Sequences of strains R-45377T and R-45370 were aligned among a selection of methanotrophic type strains using the integrated aligner of ARB (Ludwig et al., 2004). Based on the 16S rRNA gene sequence alignment, a maximum-likelihood (ML) phylogenetic tree was reconstructed using RAxML version 7.3.5 based on the general time reversible substitution model with gamma-distributed rate variation (GTR + Γ). Bootstrap analysis based on 1000 replicates was performed. The best-scoring ML tree was exported in Newick format and analysed using the MEGA 5 software (Tamura et al., 2011).

Functional genes were amplified and sequenced when applicable. Amplification of the pmoA gene encoding the 27 kDa peptide of pMMO was carried out with the primer set A189f/mb661r according to Costello & Lidstrom (1999). Amplification of the mmoX gene encoding the z-subunit of the hydroxylase component of sMMO was tested with primer sets 534f/1393r (Horz et al., 2001), mmoXA/ mmoXB (Auman et al., 2000) and mmoX206f/mmoX886r (Hutchens et al., 2004). Amplification of the ppxA (encoding a sequence-divergent particulate monooxygenase (pXMO) and nifH (encoding the highly conserved Fe protein of nitrogenase) genes was performed using the primer sets pmoA189f/ppxA634r (Tavormina et al., 2008), F1/nifH439R (De Meyer et al., 2011) and f1003/r1561 (McDonald et al., 1995), respectively. For each of the above primer sets, the PCR mixture and temperature programme recommended in the original literature were applied and success of each assay was verified using a set of methanotrophic type strains as positive and negative controls.

When functional gene amplification was positive, sequences were generated as explained for 16S rRNA gene sequence analysis. Protein translation analysis using Transeq (http://www.ebi.ac.uk/tools/emboss/transeq) and pBLAST (Altschul et al., 1990) confirmed that the sequences encoded part of the evaluated enzymes. Sequences were aligned using the MEGA 5 software based on translated protein sequences and DNA-based ML phylogenetic trees were subsequently reconstructed using RAxML as explained above.

DNA was extracted and purified as described by Logan et al. (2000) to determine the G + C content by HPLC (Mesbah et al., 1989). Phospholipid fatty acid (PFLA) analysis was carried out as described by Vancanneyt et al. (1996). Fatty acids were identified using the Microbial Identification System (MIDI) software (Sherlock Microbial Identification Systems, version 3.0; library TSBA 50, version 5.0). For identification of the methanotroph-specific fatty acid C16:0, not present in the database of the MIDI system, in-house-generated data from the type strains Methyloferula stellata, Methylocapsa acidiphila and Methylococcus capsulatus were used as references.
**RESULTS AND DISCUSSION**

Strains R-45377\textsuperscript{T} and R-45370 were respectively isolated from a slurry pit of a cow stable and from a denitrification tank of a WWTP. These inocula, both sampled from the oxic surface layer above an anoxic zone assumed to support methanogenic activity, were selected because active methanotrophs were expected in these environments, due to the assumed high levels of methane, nitrogen and oxygen.

Colonies of strains R-45377\textsuperscript{T} and R-45370 started to develop after 1 week of incubation under optimal conditions. After 1 to 2 weeks of incubation, colonies of strain R-45377\textsuperscript{T} were small (<1 mm), round and white with a light pink sheen, whereas colonies of strain R-45370 were similar in appearance, although they were clearly pink. The colonies took longer to develop (4–5 days difference) and appeared less slimy than those of typical cultures of Methylomonas methanica NCIMB 11130\textsuperscript{T} and Methylomonas koyamae NCIMB 14606\textsuperscript{T}, cultivated in parallel under identical conditions. Cultures were more easily maintained by plating than via liquid culturing, as cultivation success in liquid dNMS was often variable between replicates grown under identical conditions, resulting in increased difficulty in interpreting the results of different growth experiments performed in liquid dNMS. Cells of strains R-45377\textsuperscript{T} and R-45370 were Gram-stain-negative rods (1.3–2.0 \(\mu\)m long and 0.6–0.9 \(\mu\)m wide), motile by a single polar flagellum, exhibiting intracytoplasmic membrane systems appearing as stacks of vesicular discs (Fig. 1). Cells were sensitive to heat and desiccation, and Azotobacter-type cysts were not observed. Cells tested positive for cytochrome \(c\) oxidase, but negative for catalase. Both strains could be preserved successfully for longer periods at \(-80^\circ\text{C}\) and in liquid nitrogen using 5% DMSO as a cryoprotectant.

Only methane and methanol were used as sole carbon and energy sources. Cells were sensitive to 0.1 and 0.5% methanol amendments, but grew with 0.04% methanol added to the medium. Both strains grew with nitrate, nitrite, ammonium [both \(\text{NH}_4\text{Cl}\) and \((\text{NH}_4)\text{2SO}_4\)], urea, serine, leucine and yeast extract as sole nitrogen sources. Although the \(nifH\) gene could be amplified, the strains did not grow after 3 weeks of incubation in nitrogen-free medium under tested conditions, at neither high nor low oxygen tension. This was determined by a lack of visual turbidity, confirmed by OD\textsubscript{600}, analysis, the absence of a decline in methane and \(O_2\) concentrations and the absence of an increase in \(CO_2\) concentrations measured by GC. In parallel, growth and methane oxidation were observed for the strains with 10 mM \(\text{KNO}_3\) and 10 mM \(\text{NH}_4\text{Cl}\) added to the medium. Using the same setup, the positive control strain, Methylomonas methanica NCIMB 11130\textsuperscript{T}, was positive for \(N_2\) fixation at low oxygen tension. Strains R-45377\textsuperscript{T} and R-45370 grew optimally at 20 °C (range 15–28 °C). The optimal pH for growth was pH 6.8–7.3 (range pH 6.3–7.8). The strains tolerated additions of up to 1.2% NaCl, but were sensitive to the addition of 5% NaCl. The \(pmoA\) gene was present, while both strains were negative for sMMO activity and the \(mmoX\) gene could not be amplified. Although the \(pxmA\) gene dataset is not extensive, several gammaproteobacterial methanotrophs, including some strains of the genus Methylomonas, possess the \(pxmA\) gene, encoding a sequence-divergent pMMO with unknown function (Kip et al., 2011; Tavormina et al., 2008, 2011). Based on our in-house results, the \(pxmA\) gene was detected in several strains of Methylomonas methanica and Methylomonas koyamae, including the type strains Methylomonas methanica NCIMB 11130\textsuperscript{T} and Methylomonas koyamae NCIMB 14606\textsuperscript{T}. The presence of this gene in strains of Methylomonas koyamae has not yet been reported. In contrast, the presence of the \(pxmA\) gene could not be demonstrated for strains R-45377\textsuperscript{T} and R-45370.

16S rRNA gene sequences (1486 bp) of strains R-45377\textsuperscript{T} and R-45370 used in a phylogenetic analysis showed that the two strains formed a separate cluster within the genus Methylomonas (Fig. 2) adjacent to Methylomonas scandinavica SR5\textsuperscript{T}. Pairwise 16S rRNA sequence comparison

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
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<th>4</th>
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<td>22.90</td>
<td>18.9–24.6</td>
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<td>NR</td>
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\*Fatty acid C<sub>16</sub>:0<sub>O</sub>C was identified using in-house-generated data for Methylomonas methanica NCIMB 11130\textsuperscript{T} and Methylomonas koyamae NCIMB 14606\textsuperscript{T} as references.

\*This peak in the chromatogram represents C<sub>16</sub>:0<sub>7</sub>C and/or iso-C<sub>15</sub>:0<sub>2</sub>-OH according to the MIDI system; however, in this study, the peak is assigned to C<sub>16</sub>:0<sub>7</sub>C since this fatty acid is common among the studied methanotrophs.
revealed sequence similarities for R-45377T of 97.5 % with Methylomonas scandinavica SR5T, 97.2 % with Methylomonas paludis MG30T, 96 % with Methylomonas methanica S1T, 95.9 % with Methylomonas koyamae Fw12E-Y1T, 95 % with Methylomonas aurantiaca JB103T, 94.9 % with Methylomonas fodinarum JB13T and 99.9 % with strain R-45370. The results of 16S rRNA gene sequence analysis are supported by phylogenetic analysis of the pmoA gene (Fig. 3) and the nifH gene (Fig. 4), which confirms a distinct line of descent for strains R-45377T and R-45370 different from the other members of Methylomonas, although several species of the genus Methylomonas could not be included in the analysis since their pmoA and nifH genes were never reported, and since the type strains of Methylomonas scandinavica, Methylomonas aurantiaca and Methylomonas fodinarum are no longer extant [personal communication from the Australian (ACM) and Russian (VKM) collections of micro-organisms].

Both strains displayed PLFA profiles typical of the genus Methylomonas, with C16:0,10:8c, C16:1,10:5c, C16:1,10:7c, C14:0 and C16:0 as major cellular fatty acids (Table 1). However, the strains possessed unusually large amounts of the fatty acid C15:0 (5.3–5.8 %) compared with other strains of the genus Methylomonas (0–1.2 %). Moreover, the strains possessed large amounts of C16:1,10:5c (11.7–18.3 %), similar to the type strain of Methylomonas koyamae, compared with other Methylomonas strains (1.8–6.3 %). Since the production of cellular fatty acids might be dependent on the applied growth conditions, the type strains of Methylomonas koyamae and Methylomonas methanica as well as several recently isolated strains belonging to both species were included for PLFA analysis in parallel with R-45377T and R-45370. PLFA analysis appeared to be highly robust for strains of the genus Methylomonas, since the PLFA profiles generated in house for strains of Methylomonas koyamae and Methylomonas methanica were highly similar to data reported in the literature for strains of these species, supporting the above-mentioned differences in PLFA profiles between R-45377T and R-45370 and other members of the genus Methylomonas.

The major morphological and physiological characteristics that distinguish strains R-45377T and R-45370 from other species within the genus Methylomonas are presented in Table 2. Both strains differ from other species of the genus...
Methylomonas by their sensitivity to 0.1 % methanol. Their DNA G+C content of 47 mol% is the lowest reported so far for members of the genus Methylomonas. Similar to the type strains of Methylomonas paludis and Methylomonas scandinavica, R-45377T and R-45370 have a lower temperature optimum than most strains of the genus Methylomonas. Strains R-45377T and R-45370 differ from Methylomonas paludis MG30T by (i) their motility by single polar flagella, (ii) their inability to form chains, (iii) their tolerance of 1 % NaCl and (iv) their higher sensitivity to acidic conditions. The strains are distinct from Methylomonas scandinavica SR5T by their inability to grow at 5 °C. Based on the above-mentioned genotypic and phenotypic differences between strains R-45377T and R-45370 and other members of the genus Methylomonas, we propose the addition of a novel species of the genus Methylomonas, Methylomonas lenta sp. nov., with R-45377T as the type strain.

Description of Methylomonas lenta sp. nov.

Methylomonas lenta (len’ta. L. fem. adj. lenta slow, referring to the slow growth of the organism).

Cells are aerobic, Gram-stain-negative rods (1.3–2.0 μm long and 0.6–0.9 μm wide), motile by a single polar flagellum, and possess intracytoplasmic membrane systems typical of gammaproteobacterial methanotrophs. Colonies appear white to pink. Resting stages are not observed. Cultures grow optimally at 20 °C (range 15–28 °C) and at an optimal pH of 6.8–7.3 (range pH 6.3–7.8). Cells utilize methane and methanol as sole carbon and energy sources but are sensitive to methanol concentrations ≥0.5 %. Grows with nitrate, nitrite, ammonium [both NH4Cl and (NH4)2SO4], urea, serine, leucine and yeast extract as sole nitrogen sources and tolerates NaCl amendments up to 1.2 %. Cultures are positive for cytochrome c oxidase but negative for catalase activity. The pmoA and nifH genes are present. The pxmA gene is not detected. The mmoX gene and sMMO activity are not detected. The DNA G+C content is 47 mol% and the major fatty acids are C16:1ω8c, C16:1ω5c, C16:1ω7c, C14:0, C15:0 and C16:0.

The type strain, R-45377T (=LMG 26260T =JCM 19378T), was isolated from a covered but aerobic slurry pit of a water lake sediment. An additional member of the species, strain R-45370 (=LMG 26613), showing 99.9 % 16S rRNA gene sequence similarity to the type strain, was isolated from the top layer of a denitrification tank of a wastewater treatment plant (Ossemeersen, Gent, Belgium).

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