gbm01413

genus **Methyloparacoccus**

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Keywords: aerobe, methanotroph, freshwater, mesophile, neutrophile

Abstract:

Cells are aerobic, Gram-negative, non-motile coccoids, 0.8-1.5 µm. Cells occur singly with a diplococcoid tendency. Reproduce by normal cell devision. Resting stages are not observed. Obligate utilizers of methane and methanol as sole carbon and energy source. Methane is oxidized by a particulate methane monooxygenase (pMMO), the soluble (sMMO) and alternative particulate (pXMO) form of this enzyme are absent. Cells possess the typical intracytoplasmic membrane system for gammaproteobacterial methanotrophs forming bundles of membrane vesicles. No growth occurs on compounds containing carbon-carbon bonds. Atmospheric nitrogen is not fixed. Cells are neutrophilic, growing between pH 5.8-9, with an optimal pH of 6.3-6.8 and mesophilic, non-thermotolerant, growing between 20-37°C, optimal growth temperature 25-33°C. The dominant cellular fatty acids are $\mathbf{C_{16:1w7c}}$ (52-54%) and $\mathbf{C_{16:0}}$ (24-25%). So far only isolated from pond water in Africa and Japan. Belongs to the Gammaproteobacteria as part of the Order Methylococcales, Family Methylococcaceae.

Defining publication: Hoefman, van der Ha, Iguchi, Yurimoto, Sakai, Boon, Vandamme, Heylen and De Vos, 2014, 2105^{VP}

Entymology: Me.thy.lo.pa.ra.coc'cus. N.L. n. methylum (from French méthyle), the methyl group; N.L. pref. methylo-, pertaining to the methyl radical; Gr. prep. para, beside, alongside of, near, like; N.L. masc. n. coccus (from Gr. n. kokkos), a grain or berry; N.L. masc. n. Methyloparacoccus referring to a methyl-using organism resembling but clearly different from other methyl-using cocci

Generic definition:

Strict aerobic, **Gram-negative, non-motile coccoids**, 0.8-1.5 µm. Cells occur singly with **a diplococcoid tendency**. Reproduce by normal cell devision. **Resting stages are not observed**. **Obligate utilizers of methane and methanol as sole carbon and energy source**. Methane is oxidized by a **particulate methane monooxygenase (pMMO)**, the soluble (sMMO) and alternative particulate (pXMO) form of this enzyme are absent. Cells possess the typical intracytoplasmic membrane system for gammaproteobacterial methanotrophs forming bundles of membrane vesicles. **No growth occurs on compounds containing carbon-carbon bonds.** The *nifH* gene is absent confirming the inability to fix atmospheric nitrogen. Cells are **neutrophilic**, growing between pH 5.8-9, with an optimal pH of 6.3-6.8 and **mesophilic, non-thermotolerant**, growing between 20-37°C, optimal growth temperature 25-33°C. The dominant cellular fatty acids are $\mathbf{C_{16:1w7c}}$ (52-54%) and $\mathbf{C_{16:0}}$ (24-25%). So far only isolated from pond water in Africa and Japan. Belongs to the Gammaproteobacteria as part of the Order Methylococcales, Family Methylococcaceae.

The mol% G+C of the DNA is: 65.6% (Tm).

Type species:

Type species: **Methyloparacoccus murrellii** Hoefman, van der Ha, Iguchi, Yurimoto, Sakai, Boon, Vandamme, Heylen and De Vos, 2014, 2105^{VP}

Number of species with validated names: 1

Family classification:

Methylococcaceae (fbm00225)

Further Descriptive Information

Cell morphology and ultrastructure.

Cells of these bacteria are gram-negative, nonmotile coccoids that occur singly with a diplococcoid tendency similar to what was observed by Foster and Davis (1966) in *Methylococcus capsulatus* cultures (Fig. 1). They reproduce by binary fission. *Methyloparacoccus* species contain standard type I intracytoplasmic membrane systems appearing as bundles of vesicular disks (Fig. 1), a typical feature of the gammaproteobacterial methanotrophs (Hanson and Hanson, 1996). Similarly as was observed for the type strain of *Methylogaea oryzae* (Geymonat et al., 2011), cells display low and high electron density inclusions (Fig. 1). These inclusions possibly represent poly-β-hydroxybutyrate and glycogen granules, respectively. Both are known to be produced by several methanotrophs (Helm et al., 2006; Heyer et al., 2005; Whittenbury et al., 1970). *Azotobacter*-type cysts are not observed and cells are neither heat resistant nor desiccation resistant.

Colonial and cultural characteristics.

Liquid cultures of *Methyloparacoccus* species display white turbidity. A surface pellicle is not formed. All *Methyloparacoccus* strains available form colonies on solid

media made with high purity grade agar. One-week old colonies are round, convex, white to creamy colored and smooth with entire edges. Older colonies remain white but might display a distinct green shine. Cells exhibit catalase and cytochrome c oxidase activities.

Nutrition and growth conditions.

Methyloparacoccus species are obligate methane oxidizing bacteria (MOB), with their sole carbon and energy source restricted to methane and methanol. Medium amended with 0.1% methanol does not support growth, however strains grow on methanol fumes as sole carbon source when incubated on diluted Nitrate Mineral Salts medium (dNMS) plates with a few drops of methanol placed on the inside lid of the petri dish. Methane is oxidized to methanol by a pMMO. The genes encoding a sMMO or a pXMO are absent.

Nitrogen sources include ammonium, nitrate, nitrite, urea, proline, aspartate, arginine and yeast extract. Strains exhibit a high tolerance to the inorganic nitrogen species up to 100mM, 40mM and 5mM for ammonium, nitrate and nitrite respectively (Hoefman et al., 2014). The *nifH* gene could not be detected and isolates could not grow in nitrogen-free medium at high (21%) or low (2.1%) oxygen tension. Strains produce nitrous oxide from ammonium and nitrate (only under low oxygen tension). Nitrite is only formed as an intermediate during the production of nitrous oxide from nitrate (Hoefman et al., 2014).

Growth factors are not required for the cultivation of *Methyloparacoccus* strains, however growth of some strains can be improved by the addition of cobalamin during cultivation (Iguchi et al., 2011).

These methanotrophs are mesophilic, non thermotholerant growing at temperatures between 20°C and 37°C (optimum: 25-33°C), neutrophilic with pH between 5.8 to 9

(optimum 6.3-6.8) and salt resistant up to additions of 100 mM NaCl. Under optimal conditions in dNMS, the doubling time is in the range of 15-28h.

Chemotaxonomic characteristics.

Phospholipid fatty acid analysis identified $C_{16:1}$w7c and $C_{16:0}$ as the two major fatty acids. This finding is consistent with the other members of the *Methylococcus-Methylocaldum-Methylogaea-Methylomagnum* clade. The major fatty acid of *Methyloparacoccus* and *Methylomagnum* is $C_{16:1}$w7c, while for the other members of the clade $C_{16:0}$ is more dominant than $C_{16:1}$w7c (Table 1).

Ecology.

Ecological insights concerning the genus *Methyloparacoccus* are limited. Currently, only one described species is reported in this genus that was isolated from pond water. Based on molecular studies, using the *pmoA* gene sequences as a molecular marker, members of the genus *Methyloparacoccus* are restricted to aquatic ecosystems, occurring preferentially in the sediment (Knief, 2015).

Thus, based on molecular insights and the neutrophilic, mesophilic and moderate halotolerant nature of the currently available strains, it is highly probable that the habitat for the genus *Methyloparacoccus* is confined to freshwater ecosystems.

Enrichment and Isolation Procedures

Methyloparacoccus strains can be enriched and isolated from aerobic water samples in aquatic environments. Ideally a small amount of sample is collected and added to NMS liquid mineral medium in serum vials (Iguchi et al., 2011). Alternatively, a diluted Ammonium Mineral Salts (dAMS) medium, which is a modification of dNMS, (Hoefman et al., 2012a) amended with 2 mM NH_4Cl as sole nitrogen source, 4 mM

phosphate buffer and 10 µM $CuSO_4$ can be used. Additionally cobalamin might be added to stimulate the selective enrichment of *Methyloparacoccus* strains, however this seems to be strain specific (Iguchi et al., 2011). After inoculation, methane should be aseptically added to the headspace to achieve a headspace gas-mixing ration of about 25%. Next, the serum vials should be incubated up to five weeks at 20°C-28°C while shaking. Alternatively a serial dilution series of the environmental samples can made where the highest dilution found positive for the oxidation of methane can then be used to further enrich and isolate the methane oxidizers. Successful enrichments of *Methyloparacoccus* strains develop a white turbidity in combination with a methane and oxygen consumption and carbon dioxide production.

To date, MOB are still mostly isolated via the classical plate method. For this end, growth from the enrichments is serially diluted onto solid media prepared with high purity agar or gellum gum and incubated under a 1:1 methane/air atmosphere at 20-28°C. Single colonies form these spread plates should be picked and transferred to liquid media to evaluate the potential of the colony to utilize methane. Moreover, as MOB cross-feed non-methanotrophs with methane-derived carbon (Krause et al., 2017), methanotrophic enrichments are highly contaminated with non-methanotrophic bacteria and thus one or several liquid enrichment steps, followed by a serial dilution onto plates (Dedysh et al., 2004; Dunfield et al., 2003; Heyer et al., 2005; Tsubota et al., 2005; Wartiainen et al., 2006) are required to obtain a pure culture. This makes the classical plate procedure very laborious and time consuming without guarantee for successfully obtaining a pure MOB culture. Alternatively, a miniaturized extinction culturing (Hoefman et al., 2012a) procedure in 96-well plates can be applied. This miniaturized approach allows straightforward isolation preventing the need to transfer cultures over plates and in the case of fast-growing

methanotrophs yielding an immediate monocultures making laborious purification redundant (Hoefman et al. 2012a). MOB purity can be evaluated by (i) colony morphology, (ii) phase-contrast microscopy, and (iii) absence of growth on ten-fold diluted trypticase soy agar and dNMS plates supplemented with 0.1% glucose, 0.1% fructose and 0.1% yeast extract under air.

Maintenance Procedures

Strains can be routinely cultured at 20°C-28°C in (d)NMS or (d)AMS medium (liquid or solidified with high purity agar) with a modified copper concentration (10 µM Cu^{2+}) under a CH_4:air atmosphere in gastight flasks (20% methane in air) or jars (CH_4:air, 1:1). Liquid cultures are best incubated while shaking (100rpm). Periodic subcultivation should be performed every 1.5-2 months. For short term storage (up to several months) cultures can be kept at low metabolic rates (4°C, under a methane/air atmosphere). However, continuous subcultivation and storage at low temperature of the cultures requires periodic maintenance, time and physical space. Therefore, mid to long term storage (>1 months) is recommended to prevent the risk of contamination and loss of authenticity of the culture over time. All methanotrophic strains can be long term cryopreserved without a significant loss in viability and culturability using 1% trehalose in ten-fold diluted trypticase soy broth as preservation medium and 5% DMSO as cryoprotectant (Hoefman et al., 2012b). Additional cryopreservation and lyophilization conditions for long term storage of methanotrophic bacteria are described by Hoefman et al. (2012b).

Differentiation of the genus *Methyloparacoccus* from other genera

Phenotypic characteristics distinguishing *Methyloparacoccus* from other members of the *Methylococcus-Methylocaldum-Methylogaea-Methylomagnum* clade are shown in table2.

The cells of *Methyloparacoccus murrellii* are clearly different in appearance when compared to *Methylogae, Methylocaldum* and *Methylomagnum* cells, since rod-shapes, pleomorphism and motility are absent for all *Methyloparacoccus* strains. The non-motile cocci most closely resemble the appearance of cells of *Methylococcus* strains. Although *Methylococcus* and *Methylocaldum* strains are thermotolerant, *Methyloparacoccus* strains, *Methylogaea oryzae* E10^T and *Methylomagnum ishizawai* RS11D-Pr^T are not. Further distinctions between *Methyloparacoccus* strains and *Methylococcus* strains include the sensitivity to 1% NaCl, sensitivity to 0.1% methanol and the absence of the *nifH* and *mmoX* genes, respectively, for *Methyloparacoccus*. Further, *Methyloparacoccus* strains seem to be capable of nearly complete methane oxidation when 1%, 0.1% or 0.01% methane are amended to the headspace. In contrast, Knief and Dunfield (2005) found that the strains *Methylococcus capsulatus* Bath and *Methylocaldum* sp. E10a, lost their methane oxidation activity when incubated with 0.1% CH_4 in the headspace.

Taxonomic comments

16S rRNA gene based phylogenetic analysis indicates that *Methyloparacoccus murrellii* is a member of the deep lineage of the *Methylococcus-Methylocaldum-Methylogaea-Methylomagnum* clade included among gammaproteobacterial methanotrophs within the family *Methylococcaceae* (Bowman et al., 1993). The closest phylogenetic neighbors of *Methyloparacoccus* are methanotrophs belonging to the genus *Methylomagnum* (93.8-94.4% sequence similarity). Despite their close

phylogenetic relationship they are phenotypically clearly distinct. The 16S rRNA gene sequence results are in line with the phylogenetic analysis of the *pmoA* gene, confirming that members of the genus *Methyloparacoccus* form a line of descent different from members of the *Methylococcus-Methylocaldum-Methylogaea-Methylomagnum* clade (Fig. 3).

List of species of the genus *Methyloparacoccus*

1. Methyloparacoccus murrellii

Hoefman, van der Ha, Iguchi, Yurimoto, Sakai, Boon, Vandamme, Heylen and De Vos, 2014, 2105^{VP}

mur.rel'li.i. *murrellii* of Murrell, named in honor of the British microbiologist Colin Murrell, for his numerous contributions to expanding the knowledge on methanotroph physiology, biochemistry, diversity and molecular ecology.

Displays all properties described for the genus *Methyloparacoccus*. In addition, cocci have a diameter of 0.8 to 1.5 µm. Cells still display methane oxidation activity with 0.01 % CH_4 amended to the headspace. Cells grow optimally between 25°C to 33°C at a pH between 6.3 and 6.8 and can utilize ammonium, nitrate, nitrite, urea, proline, aspartate, arginine and yeast extract as sole nitrogen source. Cells grow with 0.58% NaCl amended to the medium, but are sensitive to 1% NaCl additions.

Two currently available strains, R-49797^T (= LMG 27482^T = JCM 19379) and OS501 (=LMG 27483), of this species are isolated from a facultative waste stabilization, high inorganic nitrogen containing pond in South Africa and the Inukai pond in Suita City, Osaka, Japan respectively.

DNA G+C content (mol %): 65.6% (Tm).

Type strain: R-49797, LMG 27482, JCM 19379.

The EMBL/GenBank accession (16S rRNA gene): HF558990

TABLES

Table 1: Composition of cellular fatty acids distinguishing *Methyloparacoccus* strains from other genera within the *Methylococcus-Methylocaldum-Methylogaea-Methylomagnum* clade. Values are percentages of the total fatty acids.

Fatty acid	***Methyloparacoccus murrellii***	***Methylococcus* spp.***	***Methylogaea* oryzae†**	***Methylocaldum* spp.‡**	***Methylomagnum ishizawai*¥**
$C_{12:0}$	-	NR	2.11	0-0.10	NR
$C_{14:0}$	3.77-4.71	0.8-6.2	5.84	1.8-3.26	15.8
$C_{15:1}$w8c	0.29-0.34	NR	NR	NR	0.22
$C_{15:0}$	3.19-3.34	0-12.7	1.03	2.49-3.51	1.6
$C_{16:1}$w9c	5.14-6.45	-	7.36	NR	NR
$C_{16:1}$w7c§	52.4-54.2	10.6-45.9	10.33	43-46.8	47.3
$C_{16:1}$w5c	4.17-5.70	0-9.0	NR	0-0.24	-

$C_{16:1}$	-	-	-	11.9-46.77	-
$C_{16:0}$	23.7-24.8	33.5-56	62.05	43.22-64.99	19.6
$C_{16:0}$ 3-OH	2.61-2.72	NR	2.93	0-0.37	1.8
iso-$C_{16:0}$ 3-OH	-	NR	3.69	NR	NR
$C_{17:0}$ cyclo	-	0-15.1	-	1.3-8.99	NR

-, not detected; NR, not reported

Data was extracted from * Bowman et al. (1993), † Geymonat et al. (2011), ‡ Knief et al. (2003), Eshinimaev et al. (2004), Takeuchi et al. (2014) and ¥ Khalifa et al. (2015)

§ This peak in the chromatogram represents $C_{16:1}$w7c and/or iso-$C_{15:0}$ 2-OH according to the MIDI system, however in this study the peak is assigned to $C_{16:1}$w7c since this fatty acid is common among the studied methanotrophs

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Table 2: Phenotypic characteristics distinguishing *Methyloparacoccus* strains from other genera within the *Methylococcus-Methylocaldum-Methylogaea-Methylomagnum* clade.

Characteristic	***Methyloparacoccus murrellii***	***Methylococcus* spp.***	***Methylogaea oryzae*†**	***Methylocaldum* spp.‡**	***Methylomagnum ishizawai*¥**
Cell shape	Cocci	Cocci-rods	Curved rods	Rods-pleomorphic	Rods
Cell size (µm)	0.8-1.5	0.8-1.5 x 1.0-1.5	0.5-0.7 x 2.0-2.2	0.4-1.2 x 1.0-2.0	2.0-4.0 x 1.5-2.0
Pigmentation	White	White to brown	White	Cream to Brown	White
Motility	-	Variable	-	+/-	+
Chain formation	-	-	NR	Variable	-
Cyst formation	-	+	-	+	+
Temperature range (°C)	20-37	28-55	20-37	20-62	20-37

Temperature optimum (°C)	25-33	37-50	30-35	42-55	31-33
pH range	5.8-9	5.5-9.0	5-8	6-8.5	5.5-9.0
pH optimum	6.3-6.8	NR	6.5-6.8	7.1-7.2	6.8-7.4
Tolerance to 1% NaCl	-	+	-	+/-	-
nifH presence	-	+	+	+[§]	-
N_2 Fixation	-	+	-	NR	-
sMMO	-	+	-	-	+
Methanol 0.1%	-	+	+	-	-
G+C content (mol%)	65.6	59-66	63.1	57-59.7	64.1

+, positive result; -, negative result; +/-, strain dependent result; NR, not reported

Data was extracted from [*] Bowman et al. (1993), [†] Geymonat et al. (2011), [‡] Bodrossy et al. (1997), Eshinimaev et al.

(2004), Takeuchi et al. (2014) and ¥ Khalifa et al. (2015)

§ Data on *nifH* presence of type strains of *Methylocaldum* has not been reported, however *nifH* sequences of strains reported in (Eshinimaev et al., 2004) are available

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FIGURE CAPTIONS

Figure 1. Electron micrographs of ultrathin sections of methane growing cells of strain R-49797^T showing a cell shape resembling *Methylococcus* cultures. ICM, intracytoplasmic membranes; PHB, poly-β-hydroxybutyrate. Bar, 0.2 μm.

Figure 2. 16SrRNA gene-based neighbor joining tree (1348 nt alignment), showing the position of *Methyloparacoccus murrellii* relative to other gammaproteobacterial methanotrophs. An alphaproteobacterial methanotroph, *Methylocystis parvus* OBBP^T (GenBank accession number Y18945), was used as an outgroup. Bootstrap values (1000 data resamplings) lower than 50 % are not shown. Scale bar indicates the evolutionary distance.

Figure 3. *pmoA* gene-based neighbor joining tree (146 nt alignment), showing the position of *Methyloparacoccus murrellii* relative to other gammaproteobacterial methanotrophs. An alphaproteobacterial methanotroph, *Methylocystis parvus* OBBP^T (GenBank accession number U31651), was used as an outgroup. Bootstrap values (1000 data resamplings) lower than 50 % are not shown. Scale bar indicates the evolutionary distance.

ACKNOWLEDGEMENTS

B.V. is supported by the European Research Council Grant 640422 (assigned to Dr. Boran Kartal)

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