Methylobacterium oxalidis sp. nov., isolated from leaves of *Oxalis corniculata*

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A pink-pigmented, facultatively methylotrophic bacterium, strain 35a[T], was isolated from the leaves of *Oxalis corniculata*. Cells of strain 35a[T] were Gram-reaction-negative, motile, non-spore-forming rods. The highest 16S rRNA gene pairwise sequence similarities for strain 35a[T] were found with the strains of *Methylobacterium iners* 5317S-33[T] (96.7 %), '*Methylobacterium soli*' YIM 48816 (96.6 %) and *Methylobacterium jeotgali* S2R03-9[T] (96.3 %). 16S rRNA gene sequence similarities with the type strains of all other recognized species of the genus *Methylobacterium* were below 96 %. Major cellular fatty acids were $C_{18:1}\omega7c$, $C_{18:0}$ and $C_{16:0}$. The results of DNA–DNA hybridization experiments, analysis of *cpn60* gene sequences, fatty acid profiles, whole-cell MALDI-TOF/MS spectral pattern analysis, and physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain 35a[T] from its nearest phylogenetic neighbours. Strain 35a[T] is therefore considered to represent a novel species within the genus *Methylobacterium*, for which the name *Methylobacterium oxalidis* sp. nov. is proposed. The type strain is 35a[T] (=DSM 24028[T]=NBRC 107715[T]).

The genus *Methylobacterium* consists mostly of pink-pigmented, facultatively methylotrophic alphaproteobacteria, and at the time of writing, comprises of 35 species with validly published names (http://www.bacterio.cict.fr/m/methylobacterium.html). However, according to Kato *et al.* (2005), *Methylobacterium chloromethanicum* (McDonald *et al.*, 2001) and *Methylobacterium dichloromethanicum* (Doronina *et al.*, 2000) are later heterotypic synonyms of *Methylobacterium extorquens*, and *Methylobacterium rhodesianum* (Green *et al.*, 1988) is an earlier heterotypic synonym of *Methylobacterium lusitanum* (Doronina *et al.*, 2002) because they exhibited high DNA–DNA relatedness values (69–89 %). Also, the species names '*Methylobacterium dankookense*' (Lee *et al.*, 2009) and '*Methylobacterium goesingense*' (Idris *et al.*, 2006) were effectively but not validly published. Most recently, the species names *Methylobacterium gossipiicola* (Madhaiyan *et al.*, 2012), *Methylobacterium cerastii* (Wellner *et al.*, 2012), *Methylobacterium bullatum* (Hoppe *et al.*, 2011 and *Methylobacterium marchantiae* (isolated from a liverwort *Marchantia polymorpha* L.) (Schauer *et al.*, 2011) were validly published, while the name '*Methylobacterium soli*' (Cao *et al.*, 2011) has been only effectively, but not validly, published.

Methylobacteria can grow on single-carbon compounds such as methanol, formaldehyde and formate as sole carbon and energy sources, and also on a wide range of multi-carbon growth substances (Green, 1992). Members of the genus *Methylobacterium* are widespread, especially on plant surfaces, where they assimilate methanol emitted from plants as a product of pectin degradation (Corpe & Rheem, 1989). Analysis of the phyllospheric microbial community showed that members of the genus *Methylobacterium* are one of the predominant bacterial taxa on plant surfaces (Delmotte *et al.*, 2009); that site and plant species are important determinants of the composition of the bacterial community (Knief *et al.*, 2010a); and that isolates belonging to *M. extorquens* and *Methylobacterium tardum* are the most competitive colonizers on plants (Knief *et al.*, 2010b). Recently we isolated diverse strains of the genus *Methylobacterium* from plant leaf samples. One of the isolates, strain

$35a^T$ isolated from *Oxalis corniculata*, showed 96.7 % 16S rRNA gene sequence similarity to *M. iners* $5317S-33^T$ which is the closest related type strain. Here, strain $35a^T$ is described as a novel species of the genus *Methylobacterium*.

Leaves of *Oxalis corniculata* were collected at the Institute of Plant Science and Resources, Okayama University, Japan in April 2008. A piece of leaf blade (2 cm) was washed briefly with 50 ml sterile water, washed vigorously with 10 ml sterile water and the wash solution was spread on methanol medium. Methanol medium contained (per litre): 0.3 g $(NH_4)_2HPO_4$, 0.1 g KCl, 0.05 g yeast extract, 0.1 mg $MgSO_4 . H_2O$, 10 ml vitamin solution, 10 ml metal solution, 5 ml methanol and 15 g agar. Vitamin solution contained (per litre): 0.4 g calcium pantothenate, 0.2 g inositol, 0.4 g niacin, 0.2 g *p*-aminobenzoic acid, 0.4 g pyridoxine HCl, 0.4 g thiamin HCl, 0.2 g biotin and 0.2 g vitamin B_{12}. The metal solution contained (per litre): 1.9 g $CoCl_2 . 6H_2O$, 1.0 g $MnCl_2 . 6H_2O$, 0.7 g $ZnCl_2$, 0.06 g H_3BO_3, 0.36 g $Na_2MoO_4 . H_2O$, 0.24 g $NiCl_2 . 6H_2O$, 0.02 g $CuCl_2 . 2H_2O$. After incubation at 28 °C for 3–5 days, a pink-pigmented colony was picked and purified by restreaking on agar plates of the same composition.

Physiological and biochemical tests were carried out at 28 °C. Conventional biochemical tests were performed according to standard methods (Smibert & Krieg, 1994). Oxidation of various substrates was determined by using Biolog GN2 MicroPlates according to the manufacturer's instructions, at 2, 3, 5, 7 and 10 days with a microplate reader (Powerscan HT, Dainippon Sumitomo Pharma). Data from the nutritional tests are shown in the species description. Modified methanol medium (solidified with agar and containing no yeast extract) was also used in tests for the utilization of methylamine (0.1 % w/v) as a carbon source. Salt tolerance was tested on R2A agar medium supplemented with 2 % (w/v) NaCl. Nitrate reduction was tested in R2A broth containing 0.2 % KNO_3.

Genomic DNA was extracted, purified (Marmur, 1961), and the 16S rRNA gene was amplified and sequenced (Lane, 1991). Sequencing was performed with an automated DNA sequencer (model 3130; Applied Biosystems) and ca. 1.5 kb sequence was determined. Phylogenetic analysis was performed using MEGA4 software (Tamura *et al.*, 2007) following multiple alignment of data by the CLUSTAL_X2 program (Larkin *et al.*, 2007) (Fig. S3). Genetic distances were obtained by the Kimura's two-parameter distance model (Kimura, 1980) and phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Nei & Kumar, 2000) methods. The robustness for individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985). Pairwise nucleotide sequence similarity values were calculated by using the algorithm of Myers & Miller (1988) using the EzTaxon server version 2.1 (http://www.eztaxon. org; Chun *et al.*, 2007). The alignment gap was not considered in the similarity calculation.

Pairwise 16S rRNA nucleotide similarity calculations indicated that the closest relatives of strain $35a^T$ were *M.*

iners $5317S-33^T$ (96.7 %), '*M. soli*' YIM 48816 (96.6 %) and *Methylobacterium jeotgali* $S2R03-9^T$ (96.3 %). The phylogenetic tree based on 16S rRNA gene sequences, constructed by using the neighbour-joining method, is shown in Fig. 1. The maximum-parsimony tree also produced similar results (Fig. S1, available in IJSEM online).

DNA–DNA hybridization was carried out at 50 °C for 3 h and analysed fluorometrically as described by Ezaki *et al.* (1989). The DNA–DNA relatedness between strain $35a^T$ and *M. iners* DSM 19015^T, that shared the highest 16S rRNA (96.7 %) pairwise gene sequence similarity was 26.7 % (mean of three replicate experiments).The DNA–DNA relatedness value between strain $35a^T$ and the recently described '*M. soli*' KCTC 22810, which showed 96.6 % 16S rRNA pairwise gene sequence similarity, was 20 %.

A 16S rRNA gene sequence similarity of less than 97 % has been suggested as the threshold for defining a genomic species (Wayne *et al.*, 1987), which corresponds to a DNA–DNA hybridization value of less than 70 % (Stackebrandt & Goebel, 1994). Recently, 98.7–99.0 % 16S rRNA gene sequence similarity was the threshold recommended for species delineation by Stackebrandt & Ebers (2006). Strain $35a^T$ showed 16S rRNA gene sequence similarity below 97.0 % with all recognized members of the genus *Methylobacterium*, and DNA–DNA hybridization experiments also confirmed that it represents a novel species.

Previous studies indicated that the *cpn60* gene, also known as *groEL* or *hsp60* (encoding the universally conserved 60 kDa chaperonin) is a more phylogenetically informative target than 16S rRNA (Sahin *et al.*, 2010). Therefore *cpn60* was selected for phylogenetic analysis as an alternative marker. *cpn60* sequences of strain $35a^T$ and its closest relatives were determined directly from PCR fragments using the method described by Hill *et al.* (2004). Primers H1594 and H1595 amplifying nucleotide positions 274–828 of *Escherichia coli cpn60* were used to amplify the universal target region of the gene in strain $35a^T$ and the type or reference strains of *M. iners*, *M. jeotgali* and '*M. soli*'. The PCR conditions for amplification were 1 min at 96 °C, 30 cycles of 30 s at 96 °C, 30 s at 58 °C and 30 s at 72 °C, followed by 5 min at 72 °C. Strain $35a^T$ showed 86.1 %, 87 % and 86.1 % *cpn60* nucleotide sequence similarity with *M. iners* DSM 19015^T, *M. jeotgali* LMG 23639^T and '*M. soli*' KCTC 22810, respectively (data not shown).

For whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis (Freiwald & Sauer, 2009), biomass from methanol agar cultures (grown for 3–5 days) was spotted on a stainless steel target plate. After air-drying, 1.5 µl matrix solution [saturated solution of sinapinic acid in 50 % (v/v) acetonitrile and 2.5 % (v/v) trifluoroacetic acid] was applied to each spot. Mass spectra were acquired using a MALDI-TOF Ultraflex mass spectrometer (Bruker Daltonics) in linear positive mode. The measured mass range of spectra was 2–20 kDa. Data from MALDI-TOF/MS analysis (Fig. S2) showed that strain $35a^T$ had a distinctly
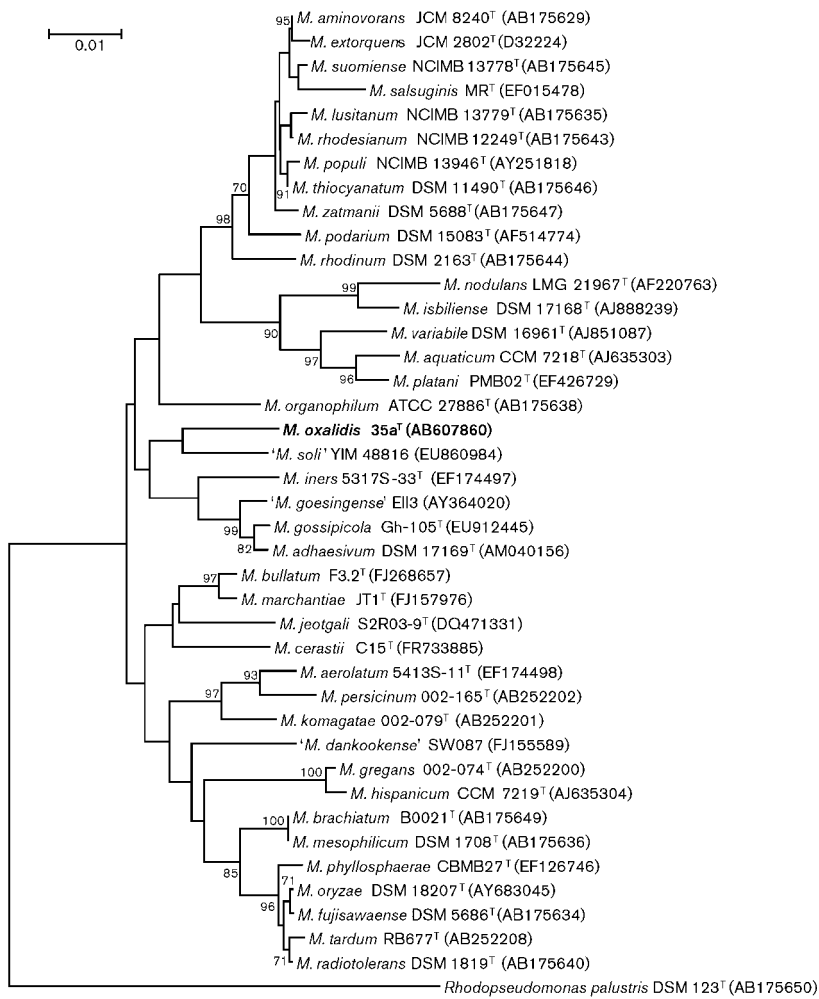
Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences constructed after multiple alignment of data (1291 nt) and clustering with neighbour-joining method. Bootstrap values $\geqslant 70\%$ based on 1000 replications are listed as percentages at the branching points. Bar, number of substitutions per nucleotide position. The sequence of *Rhodopseudomonas palustris* DSM 123[T] was used as an outgroup.

different spectrum from those of *M. jeotgali* LMG 23639[T], *M. iners* DSM 19015[T], and '*M. soli*' KCTC 22810.

Selected physiological and biochemical differential characteristics of strain 35a[T] are compared with those of related type strains in Table 1. Detailed phenotypic descriptions are given in the species description.

Fatty acid methyl ester (FAME) analysis of the whole-cell fatty acids was determined by the Identification Service of the DSMZ using GC (MIDI). A 40 mg sample of cells grown aerobically on R2A agar for 3 days at 28 °C was scraped from Petri dishes and fatty acid methyl esters were obtained by saponification, methylation and extraction using minor modifications of the methods of Miller (1982) and Kuykendall *et al.* (1988). The fatty acid methyl ester mixtures were separated using the Sherlock Microbial Identification System (MIS) (MIDI; Microbial ID) which consisted of a GC (6890N; Agilent) fitted with a 5% phenyl-methyl silicone capillary column (0.2 mm × 25 m), a flame-ionization detector, automatic sampler (7683A; Agilent), and a computer with the MIDI database (Hewlett Packard). Peaks were automatically integrated and fatty acid names and percentages calculated by the MIS Standard

Software (Microbial ID). The GC parameters were as follows: carrier gas, ultrahigh-purity nitrogen; column head pressure 60 kPa; injection volume 2 µl; column split ratio, 100 : 1; septum purge 5 ml min^{-1}; column temperature, 170–270 °C at 5 °C min^{-1}; injection port temperature, 240 °C; and detector temperature, 300 °C.

The major cellular fatty acids were $C_{18:1}\omega 7c$ (90.8%), $C_{18:0}$ (3.3%) and $C_{16:0}$ (1.95%). $C_{18:0}$ 3-OH (1.75%) was the only hydroxylated fatty acid detected. In addition, an unidentified fatty acid with an equivalent chain-length of 14.959 (0.35%), Summed feature 2 (comprising $C_{14:0}$ 3-OH or iso-$C_{16:1}$; 1.37%) and Summed feature 3 (comprising $C_{16:1}\omega 7c$ or iso-$C_{15:0}$ 2-OH; 0.46%) were also detected. Thus, strain 35a[T] could be distinguished from its phylogenetic relatives based on fatty acid profiles, in particular the absence of $C_{16:0}$ 2-OH and the presence of $C_{18:0}$ 3-OH.

Analysis of respiratory quinones was carried out by the Identification Service and Dr Brian Tindall, DSMZ, Braunschweig, Germany. DNA base composition analysis based on thermal denaturation temperature, carotenoid extraction and pigment spectral analysis were determined

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Table 1. Differential characteristics of strain 35a^T and related members of the genus *Methylobacterium*

Strains: 1, *M. oxalidis* sp. nov. 35a^T; 2, '*M. soli*' YIM 48816, (data from Cao *et al.*, 2011); 3, *M. iners* 5317S-33^T (Weon *et al.*, 2008); 4, *M. jeotgali* S2R03-9^T (Aslam *et al.*, 2007); 5, *M. organophilum* JCM 2833^T (Kato *et al.*, 2005); 6, *M. marchantiae* JT1^T (Schauer *et al.*, 2011); 7, *M. cerastii* C15^T (Wellner *et al.*, 2012); 8, *M. bullatum* F3.2^T (Hoppe *et al.*, 2011); 9, *M. gossipiicola* Gh-105^T (Madhaiyan *et al.*, 2012); 10, *M. phyllosphaerae* CBMB27^T (Madhaiyan *et al.*, 2009); 11, *M. platani* PMB02^T (Kang *et al.*, 2007). +, Positive; −, negative; (+), weakly positive; NA, data not available; V, variable reaction.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Isolation source	Phyllosphere of *Oxalis corniculata* L.	Forest soil	Urban air	Fermented seafood	Lake sediment	Thallus of a liverwort	Leaf surface	Surface of a bryophyte	Cotton phyllosphere	Leaf surface of rice	Leaf from a tree
Colony pigmentation	Pink	Pink	Pink	Non-pigmented	Pink	Red	Pinkish	Red	Light pink	Pink	Pink
Growth on/at:											
Peptone-rich media	+	+	−*	+	+	+	+	+	+	+	+
35 °C	+	+	−*	+/−*	+*	−	−	−	−	−	−
2 % NaCl	−	−	−	(+)	−	−	−	−	−	−	−
Nitrate reduction	−	−/+*	−*	+*	−	(+)	NA	−	−	−	NA
Utilization of:											
D-Glucose	−	+	−	−	+	−	−	−	+	(+)	−
Methylamine	−	(+)*	−*	(+)*	+*	−	−	NA	−	(+)	NA
D-Fructose	+	+	−	−	+	+	−	+	(+)	+	+
L-Arabinose	−	−	−	−	−	−	V	NA	+	+	+
D-Xylose	−	−	NA	+	+	−	−	−	NA	+	NA
Citrate	−	NA	−	−	−	(+)	−	V	+	+	−
Hydroxy fatty acids (% of total):											
C$_{16:0}$ 2-OH	−	−	1.2	−	−	−	−	−	−	−	−
iso-C$_{17:0}$ 3-OH	−	−	−	−	−	−	−	−	−	11.5	−
C$_{18:0}$ 3-OH	1.8	−	−	−	2.5	−	−	0.9	−	0.9	3.46
DNA G+C content (mol%)	70.2	66.2	68.0	64.9	69.6	68.0	NA	67.1	64.2	66.8	68.5

*Data from this study.

according to Sahin *et al.* (2008). A siderophore assay was performed according to the published method of Schwyn & Neilands (1987). Methylobacteria oxidize methanol to formaldehyde through methanol dehydrogenase (MDH). MDH is a pyrroloquinoline quinone (PQQ)-linked enzyme. It plays an essential role in the first step of methanol oxidation by converting methanol to formaldehyde. In addition, PQQ has a favourable effect on plant growth (Duine & Frank, 1990; Choi *et al.*, 2008). Also, pink-pigmented methylobacteria have been found to be good hopanoid producers (Knani *et al.*, 1994). PQQ content in a 13 day culture in methanol medium was measured by HPLC [column, µBondasphere, 5µm, C18, 300Å (Waters); flow rate, 0.3 ml min^{-1}; temperature, 40 °C; isocratic elution with 10 % (v/v) methanol and 90 % (v/v) 6:4 mixture of 100 mM ammonium acetate and 100 mM acetic acid; detection, fluorescence (excitation 360 nm and emission 480 nm)]. Auxin (indole acetate) was measured as reported by Glickmann & Dessaux, 1995).

On the basis of the results described above, strain 35a^T represents a novel species within the genus *Methylobacterium*, for which the name *Methylobacterium oxalidis* sp. nov., is proposed.

Description of *Methylobacterium oxalidis* sp. nov.

Methylobacterium oxalidis [o.xa′li.dis. L. n. *oxalis* -*idis*, a sorrel, and also a scientific generic name (Oxalis); L. gen. n. *oxalidis* of a sorrel *Oxalis corniculata*, referring to the leaves from which the type strain was isolated].

Cells are Gram-reaction-negative, motile rods (1.3 × 1.8 µm), and are strictly aerobic. Colonies are pink, convex and translucent with regular edges, slow-growing and 0.4 mm in diameter after 5 days on R2A agar plates at 28 °C. Growth occurs at 28–35 °C, but not at 37 °C. Nitrate is not reduced to nitrite. Oxidase-negative and catalase-positive; additional characteristics are given in Table 1. The following substrates produce positive results in Biolog GN2 plates: D-fructose, methyl pyruvate, acetic acid, formic acid, β-hydroxybutyric acid, DL-lactic acid, malonic acid, propionic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinamic acid and L-glutamic acid. Methylamine and dimethylamine are not utilized as sole carbon sources. DNase and urease tests are negative. Absorbance spectra of the pigment extracts in acetone-methanol mixture (3:1, v/v) have absorbance maxima at 528, 498 and 394 nm. Also has the ability to produce the siderophore, PQQ (2.3 µg ml^{-1}) and indole-3-acetic acid (1.2 µg ml^{-1}). Ubiquinone Q-10 (100 %) is the predominant isoprenoid quinone. Major cellular fatty acids are C$_{18:1}$ω7c, C$_{18:0}$ and C$_{16:0}$.

The type strain, 35a^T (=DSM 24028^T=NBRC 107715^T), was isolated from the leaves of *Oxalis corniculata*. The DNA G+C content of the type strain is 70.2 mol% (T_m method).

Acknowledgements

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