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Characterization of *Methylobacterium* strains isolated from the phyllosphere and description of *Methylobacterium longum* sp. nov

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Abstract *Methylobacterium* strains are abundantly found in the phyllosphere of plants. Morphological, physiological and chemotaxonomical properties of 12 previously isolated strains were analyzed in order to obtain a more detailed overview of the characteristics of phyllosphere colonizing *Methylobacterium* strains. All strains showed the typical properties of the genus *Methylobacterium*, including pink pigmentation, facultative methylotrophy, a fatty acid profile dominated by C18:1 ω7c, and a high G+C content of 65 mol % or more. However, some strains showed only weak growth on methanol and pigmentation varied from pale pink to red. Strains grew best under mesophilic, neutrophilic conditions and low salt ($\leq$1%) concentrations, but variation was seen with respect to the temperature and pH range under which growth occurred. Likewise, differences were seen with respect to carbon source utilization. Some strains were versatile and utilized diverse organic acids, amino acids and sugars, while others could only metabolize a restricted number of organic acids. The strains that were most distinct from existing type strains based on 16S rRNA gene sequence analysis were selected for DNA–DNA hybridization experiments to analyze whether they are sufficiently different at the genomic level from existing type strains to justify their classification as new species. This resulted in the delineation of strain 440 and its description as *Methylobacterium longum* sp. nov. strain 440 (=DSM 23933^T = CECT 7806^T). A main characteristic of this species is the formation of relatively long rods compared to other *Methylobacterium* species.

Keywords Phyllosphere · *Methylobacterium longum* sp. nov

Introduction

The alphaproteobacterial genus *Methylobacterium* comprises aerobic, Gram-negative and pink pigmented bacteria with a facultative methylotrophic lifestyle (Green 2006). Members of this genus can grow on diverse one-carbon compounds such as methanol, formaldehyde or formate and some of them on methylated amines or halogenated one-carbon compounds (Kayser et al. 2002; Schäfer et al. 2007), but none of them on methane (Dedysh et al. 2004). *Methylobacterium* strains occur widespread in nature;

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e. g., they have been isolated from freshwater, soil, dust, sediments, air, or hospital environments (Green 2006). Remarkable is their consistent occurrence in association with plants (Corpe and Rheem 1989; Knief et al. 2010b). *Methylobacterium* strains colonize the aerial and below ground parts of plants as epi- and endophytes (e. g., Andreote et al. 2009; Elbeltagy et al. 2000; Mano et al. 2007; Pirttilä et al. 2000; Schauer and Kutschera 2008). Upon plant colonization, they express genes involved in methylotrophy (Delmotte et al. 2009; Gourion et al. 2006) and benefit from methanol utilization (Abanda-Nkpwatt et al. 2006; Schmidt et al. 2010; Sy et al. 2005). Potential advantages of this association for the plant host, in particular with regard to growth stimulation, have been discussed in different reports (e. g., Fedorov et al. 2011; Holland et al. 2002; Idris et al. 2004; Koenig et al. 2002; Kutschera 2007; Lidstrom and Chistoserdova 2002; Madhaiyan et al. 2007; Omer et al. 2004). Diverse members of the genus *Methylobacterium* are found in association with plants and the *Methylobacterium* community composition has been shown to vary on different plant species and in dependence on the location of the plant (Knief et al. 2010a; Knief et al. 2010b). Among the retrieved *Methylobacterium* isolates from the plant phyllosphere are phylogenetic lineages that are not well described (Knief et al. 2010a). In order to obtain a more detailed picture about the physiology and morphology of the diverse plant associated *Methylobacterium* lineages, we characterized different strains that were isolated from the phyllosphere of plants.

Methods

Enrichment and isolation of *Methylobacterium* strains

Methylobacterium strains were isolated from the phyllosphere of *Arabidopsis thaliana*, *Cardamine hirsuta* or *Medicago truncatula* (Table 1) in previous studies (Knief et al. 2008; Knief et al. 2010a). These plants grew at different natural sampling sites in Spain or France, except the greenhouse grown *A. thaliana* plant from which strain F15 was isolated (Table 1). All strains were enriched and isolated on a mineral salt medium supplemented with 120 mM methanol at 28°C (Knief et al. 2010b). Due to the application of different enrichment strategies in that previous study, strains 108, 109, 113, 189, 440, 679 and 812 were initially cultivated on a fivefold diluted mineral salt medium, but were shown to grow equally well or better at the higher substrate concentration. Thus, all strains were cultivated on normal strength media in this study. All strains characterized in this study were deposited in the German and Spanish culture collection; their deposition numbers are included in Table 1.

Reference strains *Methylobacterium aquaticum* DSM 16371^T, *Methylobacterium variabile* DSM 16961^T, *Methylobacterium brachiatum* DSM 19569^T, *Methylobacterium mesophilicum* DSM 1708^T, *Methylobacterium tardum* DSM 19566^T, *Methylobacterium radiotolerans* DSM 1819^T, *Methylobacterium oryzae* DSM 18207^T, *Methylobacterium fujisawaense* DSM 5686^T, *Methylobacterium phyllosphaerae* DSM 19779^T and *Methylobacterium adhaesivum* DSM 17169^T were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). They were cultured under the conditions described above.

Morphological characterization

Morphology of the isolates is described for cultures grown on mineral salt medium agar plates with methanol as sole carbon source after 4 days of incubation at 28°C. For microscopical observations and cell size measurements an Axiophot microscope and the Axiovision 4.7 software (Zeiss) were used. Motility of the strains was tested in swarming assays using tenfold diluted R2A medium (Difco) soft agar plates (0.15% agar), which were inoculated with cell material in the center of the plate. Swarming of the cells was evaluated after 5 days of incubation.

Analysis of growth characteristics

Growth at different temperatures, pH and NaCl concentrations was tested on mineral salt medium agar plates with methanol as carbon source. Cells from plate-grown cultures were suspended in medium to an OD_{600} of 1 and a tenfold serial dilution was done in a microtiter plate. 5 µl of each dilution was spotted onto agar plates. Each test was performed twice based on independently grown precultures. Growth under the different conditions was evaluated after 1 week and up to 4 weeks in case of slow growth. Growth was tested

Table 1 Origin of the *Methylobacterium* strains

Strain	Sampling site	Location	Ecosystem	Type of sample	Sampling date	Culture collection identifier
31	Menasalbas, Spain	39°39.536′N 4°20.654′W	Mediterranean xeric grassland and scrubland	Leaf of *Arabidopsis thaliana*	April 2005	DSM 23928 CECT 7804
32	Menasalbas, Spain	39°39.536′N 4°20.654′W	Mediterranean xeric grassland and scrubland	Leaf of *Arabidopsis thaliana*	April 2005	DSM 23929 CECT 7805
85	Ciruelos de Coca, Spain	41°12.882′N 4°32.664′W	Sandy site at the edge of a pine forest	Leaf of *Arabidopsis thaliana*	April 2005	DSM 23930 CECT 7807
108	Marjaliza, Spain	39°34.951′N 3°55.526′W	Trackside in a Mediterranean forest of evergreen *Quercus* species	Leaf of *Arabidopsis thaliana*	April 2005	DSM 23934 CECT 7808
109	Marjaliza, Spain	39°34.951′N 3°55.526′W	Trackside in a Mediterranean forest of evergreen *Quercus* species	Leaf of *Arabidopsis thaliana*	April 2005	DSM 23977 CECT 7812
113	Marjaliza, Spain	39°34.951′N 3°55.526′W	Trackside in a Mediterranean forest of evergreen *Quercus* species	Leaf of *Cardamine hirsuta*	April 2005	DSM 23935 CECT 7814
189	La Chopera, Spain	40°30.878′N 3°53.810′W	Trackside adjacent to a xeric grassland and nearby a residential area	Fruit surface of *Arabidopsis thaliana*	April 2005	DSM 23931 CECT 7810
337	Polan, Spain	39°49.515′N 4°15.000′W	Mediterranean scrubland of evergreen *Quercus*	Leaf of *Arabidopsis thaliana*	April 2005	DSM 23932 CECT 7809
440	Ciruelos de Coca, Spain	41°12.882′N 4°32.664′W	Sandy site at the edge of a pine forest	Leaf of *Arabidopsis thaliana*	April 2005	DSM 23933 CECT 7806
679	Gruissan, France	43°07.297′N 3°04.572′E	Boundary ridge of a vineyard	Leaf of *Medicago truncatula*	May 2005	DSM 23936 CECT 7803
812	Marjaliza, Spain	39°34.951′N 3°55.526′W	Trackside in a Mediterranean forest of evergreen *Quercus* species	Leaf of *Cardamine hirsuta*	April 2005	DSM 23937 CECT 7811
F15[a]	Castanet-Tolosan, France	43°31.650′N 1°30.158′E	Greenhouse	Leaf of *Arabidopsis thaliana*	April 2004	DSM 23938 CECT 7813

[a] This strain was isolated in the study published by Knief et al. in 2008, while the other strains were isolated in the study of Knief et al. (2010a)

at 4, 13, 20, 28 and 37°C. NaCl tolerance was tested by adding 0.5, 1, 1.5, 2, 2.5, 3 and 4% NaCl to the medium. To analyze acid and alkali tolerance, the pH of the medium was adjusted to values between 4.0 and 9.0 by changing the amount of mono- and dibasic phosphates in the buffer of the medium and by addition of H_3PO_4 for more acid media. To reach higher alkalinity (7.5–9.5) a 10 mM TRIS/HCl buffer was used and phosphate supplied in form of dibasic hydrogen phosphate. The actual pH of each medium was determined after autoclaving and was as follows: 4.8, 5.5, 5.7, 6.1, 7.1, 7.6, 7.9 and 8.3 for the phosphate based media and 7.6, 8.2, 8.4, 8.6 and 8.7 for the TRIS based media. Nitrogen source utilization was tested by replacing the ammonium sulfate in the medium by potassium nitrate, urea or potassium glutamate (equal amount of N supplied under all conditions) and using the trace element solution described in Peyraud et al. (2009). For this test, cells were resuspended in 120 mM phosphate buffer. Carbon source utilization

was tested as described by Green and Bousfield (1982) using cultures grown on R2A medium plates supplemented with 60 mM methanol. Moreover, the api 20NE identification system (bioMérieux) and Biolog plates were used as described by the manufacturer but with increased incubation times. Api strips were analyzed after 2 or 3 days of incubation, Biolog plates after 1 and 2 weeks. All tests were performed twice using independently grown precultures.

Fatty acid analysis

A whole cell fatty acid analysis was done with 4-day old cultures grown on mineral medium agar plates supplemented with methanol. Fatty acids were extracted and methylated according to the method of Sasser (1990) and analyzed on a GC-FID as described by Dedysh et al. (2007). Identification of fatty acid methyl esters was based on their retention times relative to known standards.

DNA extraction

For DNA based analyses DNA was extracted using the Master Pure™ DNA Purification Kit (Epicentre Biotechnologies) according to the kit instructions, but with an additional mechanical lysis step for 3 min at 30 Hz in a tissue lyser (Qiagen) after Proteinase K digestion. For cell disruption 0.1 mm zirconia silica beads (BioSpec Products) were added to the extraction suspension.

Determination of the G+C content

The G+C content of the DNA was determined based on fluorimetric measurements during thermal denaturation according to the methods of Xu et al. (2000) and Gonzalez and Saiz-Jimenez (2002). The thermal denaturation curve and thus the melting temperature (T_m) of the DNA was determined in a Rotor-Gene 3000 Real-Time PCR Cycler (Corbett). 500 ng of DNA were mixed with 10 µl of 2× SensiMix *Puls* SYBR green (Quantace), 2 µl of 1× SSC and filled up with sterile water to a volume of 20 µl. The temperature profile consisted of an initial incubation step at 60°C for 1 min and a sequential increase by 0.5°C per step up to 99°C with a hold of 5 s at each step. The G+C content was calculated from a calibration curve that was set up with experimentally determined T_m values of the following genome sequenced strains whose known G+C contents cover a range from 59 to 71 mol %: *Pseudomonas syringae* pv. *syringae* B728a (59.23 mol % G+C), *Bradyrhizobium japonicum* USDA 110 (64.06 mol %), *Methylobacterium extorquens* CM4 (68.12 mol %) and *Methylobacterium radiotolerans* 0–1 (71.50 mol %). The analysis was done in three independent runs and triplicates for each run.

DNA–DNA hybridization experiments

DNA–DNA hybridization experiments were performed using the method of Mehlen et al. (2004). The hybridization was carried out with 42% of formamide in the hybridization buffer at a temperature of 55°C. Washing solutions A, B, D, F and H were used as described by Mehlen et al. (2004). Solutions with lower stringency were used instead of solutions C, E and G: 1 (5% formamide, 2.0× SSC), 2 (25% formamide, 1.5× SSC) and 3 (40% formamide, 1.2× SSC). This allowed a better analysis of thermal denaturation curves, especially for more distantly related strains. Standardized curves were analyzed by performing a sigmoidal curve fit using SigmaPlot 11.0 based on the following formula: $y = y_0 + a/(1 + \exp(-(x - x_{1/2})/b))$, where y_0 corresponds to the background absorption, a to the maximum absorption, b to the slope of the curve and $x_{1/2}$ to the melting temperature of the DNA hybrids (Rutledge 2004). All experiments were performed in 3–5 replicates per strain combination using unlabeled reference DNA from independently prepared PCR-products. Moreover, differences in the melting temperature between homologous and heterologous DNA–DNA hybrids (ΔT_m) were determined twice independently for each strain combination using as reference for the melting temperature of a homologous DNA–DNA hybrid either the melting temperature of one or the other strain. The mean values of these independent analyses are given.

Results and discussion

16S rRNA gene sequence phylogeny of the strains selected for this study

Diverse *Methylobacterium* strains were isolated from different plant species in our previous studies (Knief

et al. 2008; Knief et al. 2010a). They were identified based on 16S rRNA gene sequence analysis. Among the different 16S rRNA genotypes that were obtained we selected the 12 most distinct and representative strains for a detailed characterization. In particular isolates related to strains 31, 32, 108, 109 and 337 were frequently obtained from diverse plant species at different locations in our previous study (Knief et al. 2010a), suggesting that these are common plant colonizers. The phylogenetic relationship of the selected strains to all validly described type strains and currently proposed type strains representing new species within the genus *Methylobacterium* was analyzed (Fig. 1). Strains with closely related validly described type strains within the genus *Methylobacterium* were strain 440, which is related to *M. tardum* Rb677^T (99.4% sequence identity), strain 189 to *M. aquaticum* GR16^T (98.8%), strain 31 to *M. mesophilicum*A47^T (99.2%) and strain 32 to *Methylobacterium marchantiae* JT1^T (99.8%). The other strains were most closely related to strains that were recently proposed as novel species but that are still awaiting validation at the time of writing this manuscript: strain F15 was most closel y related to '*Methylobacterium dankookense*' SW08-7 (100%), strain 812 to '*Methylobacterium soli*' YIM 48816 (99.8%), strain 85 to '*Methylobacterium bullatum*' F3.2 (100%), strains 109 and 679 to '*Methylobacterium gossipiicola*' Gh-105 (99.1–99.2%) and strains 108, 113 and 337 to '*Methylobacterium goesingense*' iEII3 (99.4–99.6%). Besides the relationship to these species, the 16S rRNA gene sequences of strains 108, 109, 113, 337 and 679 were most similar to the sequence of *M. adhaesivum* AR27^T (>98.6%).

Morphological characterization

All strains showed a characteristic pink pigmentation. Pigmentation was rather weak (pale pink) for strain 440 and most intense (red) for strains 109 and 189. Strains 109, 113 and to lesser extend 679 had a matt appearance and formed rather crumbly irregular colonies. Cells of all strains were Gram negative and rod-shaped (Figure S1). These rods were for most strains between 2.3 and 3.1 μm long and between 1.4 and 1.6 μm wide (Table 2). The mean cell size of strain 85 was the smallest (2.3 × 1.3 μm). Strains 189 and 440 had the longest cells; the mean length was 4.2 and 4.3 μm, respectively, and the longest cell of strain 440 observed was 12.2 μm. Upon aging, the cells of several strains (31, 109, 113, 440, 679, 812, F15) became longer, clubbed, curved, branched or even pleomorphic (Figure S1), an observation that has been reported for *Methylobacterium* strains before (Green 2005). Inclusion bodies, most likely consisting of poly-β-hydroxybutyrate (PHB), were clearly visible in strains 108, 109, 189, 440 and 679. In strain 189 they were localized at both poles of the cells. For some of the strains (31, 32, 85, 108, 337 and 679) PHB production and accumulation was additionally analyzed by gas chromatography according to the method of Braunegg et al. (1978) using methanol grown liquid cultures. The detection of PHB in these strains supports the assumption that the visible inclusion bodies are indeed consisting of PHB. All strains were motile, but to different extent. Strain 108 showed highest motility while stains 109, 113, 679 and 189 were least motile under the applied experimental conditions.

Growth characteristics

All strains were able to grow on mineral salt medium agar plates supplemented with 120 mM methanol as sole carbon and energy source, though the growth of strains 113 and 189 was rather weak. While strain 189 showed slightly better growth when the culture medium was supplemented with 30 mM succinate instead of methanol, strain 113 showed almost no growth under this condition (Table 2). Particularly suitable for the cultivation of all different strains was R2A agar (Difco) with or without the addition of 60 mM methanol, since all strains grew well on this medium. Other tested culture media were less suitable. One third of the strains showed weak or no growth on nutrient broth and most strains were unable to growth on tryptic soy broth (Table 2). All strains except strain 109 could be cultivated in liquid mineral salt medium supplemented with methanol. Cells of strain 109 could not be resuspended homogenously in liquid and remained aggregated in the medium. Moreover, they attached to the glass wall at the interphase between medium and air during incubation, even though cultures were shaken (180 rpm). Weak growth was observed for isolates 189, 337 and 440 in liquid mineral salt medium supplemented with methanol. Strains 108, 113 and 679 remained aggregated when resuspended into liquid medium. In case of strain 113

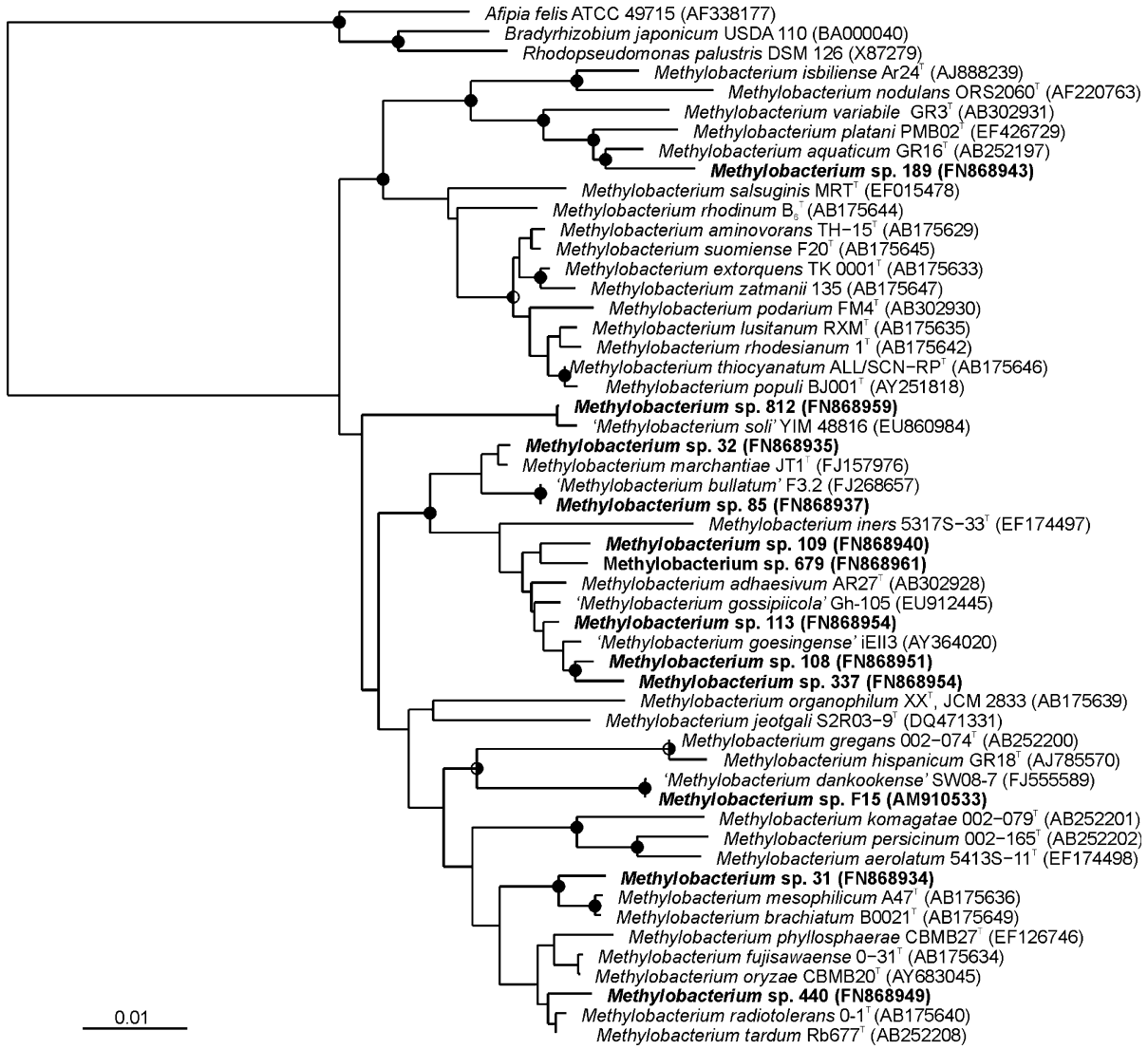

Fig. 1 Phylogenetic tree based on 16S rRNA gene sequence data. The tree shows the placement of the twelve strains that were characterized relative to all species and proposed novel species within the genus. The tree was calculated based on 1447 aligned nucleotide positions using Jukes–Cantor correction. It was compared to a maximum-likelihood tree (RAXML) and a maximum-parsimony tree. Concordant results between the trees are indicated by *circles*: *left half filled* = branching was seen in the maximum-parsimony tree; *right half filled* = branching was seen in the maximum-likelihood tree; *full circle filled* = branching was seen in both alternative treeing methods). The *bar* indicates 1% sequence dissimilarity. All trees were calculated using the default settings in the ARB software package if not otherwise stated

these aggregates were very small. Strains 812 and F15 could be homogenously resuspended but formed aggregates upon incubation during growth. Cells of strains F15, 31 and 32 grew in the liquid medium but also attached to the glass wall.

All strains grew well at temperatures of 20 and 28°C. Strain F15 is distinct from the other strains by the ability to grow at 37°C (Table 2). At temperatures below 20°C growth was delayed Thus, growth at 4°C was evaluated after 3 and 4 weeks of incubation. All strains except strain 812 were able to grow at 4°C. Little variation was seen in terms of salt tolerance, as no strain was able to grow with more than 1% NaCl (Table 2). Strains 31, 679 and 113 did not even

Table 2 Morphology, growth conditions and chemical composition of the *Methylobacterium* strains

Characteristic	189	812	F15	31	440	85	32	679	109	113	108	337
Morphology												
Colony pigmentation	Pink to red	Pink	Pink	Pink to red	Pale pink	Pink	Pink	Pink	Red	Pink	Pink	Pink
Cell width (μm)												
Minimum	1.3	1.1	1.2	1.1	1.2	1.0	1.2	1.1	1.2	1.1	1.2	1.2
Maximum	3.1	2.0	1.7	1.6	2.1	1.9	2.8	2.4	1.9	2.4	1.8	1.7
Mean ± SD ($n = 30$)	1.5 ± 0.1	1.4 ± 0.2	1.5 ± 0.1	1.3 ± 0.1	1.6 ± 0.2	1.3 ± 0.2	1.9 ± 0.5	1.6 ± 0.3	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
Cell length (μm)												
Minimum	2.4	2.0	2.0	2.3	2.4	1.8	2.3	2.1	1.8	2.0	1.8	2.0
Maximum	8.8	5.0	3.6	4.8	12.2	2.9	4.7	5.6	6.3	9.0	4.7	3.1
Mean ± SD ($n = 30$)	4.2 ± 1.1	2.3 ± 0.2	2.7 ± 0.4	3.1 ± 0.5	4.3 ± 1.1	2.3 ± 0.3	3.0 ± 0.6	2.9 ± 0.5	2.2 ± 0.2	2.8 ± 0.4	2.5 ± 0.3	2.4 ± 0.2
Motility	w	+	+	+	+	+	+	w	w	w	+	+
Growth characteristics												
Mineral salt medium with methanol	w	+	+	+	w	+	+	+	+	w	+	+
Mineral salt medium with succinate	+	w	+	+	w	+	+	–	w	–	+	+
Nutrient broth	+	w	+	+	+	+	+	w	+	–	w	+
Tryptic soy broth	+	–	+	w	–	–	–	–	–	–	–	–
Temperature range (°C)	4–28	13–28	4–37	4–28	4–28	4–28	4–28	4–28	4–28	4–28	4–28	4–28
pH range	4.8–8.7	5.5–7.6	5.5–8.7	7.0–8.2	5.5–7.6	5.7–8.3	5.7–8.7	7.0–7.6	6.1–7.6	7.0–7.6	5.7–8.7	5.7–7.6
NaCl tolerance (%), growth until	1.0	0.5	1.0	0	0.5	0.5	0.5	0	0.5	0	0.5	0.5
Chemical composition												
Fatty acid composition												
12:0	tr	0	0	0	0	0	0	0	tr	tr	0	0
14:0	tr	0	0	0	0	0	0	tr	tr	tr	0	0
16:0	3.4	1.4	3.2	1.6	4.4	1.3	1.2	3.2	3.8	6.9	1.9	2.1
16:1ω7c	1.0	2.9	3.7	4.1	4.9	7.2	8.6	12.4	13.9	11.3	9.3	7.8
17:0	0	0	0	0	0	0	0	0	0	tr	0	tr
18:0	1.5	3.6	4.0	2.1	2.8	4.3	1.4	2.8	3.3	5.5	1.8	2.4
18:1 ω 9c	0	0	0	0	0	0	0	0	0	tr	0	0
18:1 ω 9t	0	0	0	0	0	0	0	tr	0	tr	0	0
18:1 ω 7c	91.5	90.6	87.2	91.3	87.8	83.3	86.1	78.9	77.7	73.0	86.0	84.8

Table 2 continued

Characteristic	189	812	F15	31	440	85	32	679	109	113	108	337
18:0 3-OH	1.2	1.2	2.0	tr	tr	2.3	1.9	1.9	tr	1.2	tr	1.5
ECL 23.450 (3-OH fatty acid)	1.0	tr	0	tr	0	1.6	tr	tr	0	tr	0	1.3
DNA G+C content (mol%) ± SEM (n = 3)	68.6 ± 0.6	70.8 ± 1.8	71.9 ± 0.4	69.4 ± 0.2	68.6 ± 0.3	67.0 ± 0.4	66.0 ± 0.7	68.3 ± 0.5	68.0 ± 0.4	69.5 ± 0.5	68.4 ± 0.8	69.5 ± 0.8

w weak, *tr* trace, *ECL* equivalent chain length

All strains grew on R2A and R2A supplemented with 60 mM methanol

tolerate 0.5% NaCl in the medium. All strains were neutrophilic, but the tolerance towards alkali or acidic conditions was highly variable (Table 2). Strain 189 was most tolerant against acid and grew at pH 4.8. Strains 31, 679 and 113 did not tolerate acidic conditions; they did not grow on media with a pH $\leq$ 6.1. Tolerance towards alkali conditions was highest for strains 32, 108, 189 and F15; these strains grew even under the strongest condition that was tested (pH 8.7). Half of the strains did not grow on media with a pH above 7.6. Strain 189 showed the broadest range of pH-adaptation.

Fatty acid composition and G+C content

The fatty acid patterns of the strains were in agreement with previously reported patterns for the genus (Table 2). C18:1ω7c was the major fatty acid (73–91%) in all strains; further fatty acids that were present in all isolates were C16:0, C16:1ω7c, C18:0 and small amounts of C18:0 3-OH.

The G+C content of all strains was between 66 and 72 mol % and thus in the range of other *Methylobacterium* strains (Table 2) (Gallego et al. 2006; Green 2005).

Carbon and nitrogen source utilization

Physiological characteristics of the strains were studied based on different approaches. Utilization of a broad range of carbon compounds was analyzed using Biolog GN2 microplates. Carbon compounds that resulted in highest respiratory activities of one or more strains included asparagine, aspartic acid, alaninamide, D-fructose, D-galactonic acid lactone, D-gluconic acid, glutamic acid, hydroxybutyric acid, a-ketoglutaric acid, pyruvate, saccharic acid and succinamic acid. A two-dimensional cluster analysis was performed based on the results of the Biolog analysis to identify strains with similar carbon source utilization patterns and to identify carbon compounds that are used by certain groups of strains. Data of some closely related *Methylobacterium* type strains were included in this analysis. It revealed that the strains can be classified into four major groups (Fig. 2). The first group (cluster 1) is formed by strains 32 and 85. Especially strain 32 showed a limited capability of utilizing different carbon sources. Respiratory activity was only seen in the presence of some organic acids

and glutamic acid; compounds that are part of the central metabolism of *Methylobacterium*. In agreement with the similar carbon source utilization patterns is the close relatedness of these two strains seen in the 16S rRNA gene based tree (Fig. 1). Carbon utilization cluster 2 is formed by all those strains that were closely related to *M. adhaesivum* AR27^T according to 16S rRNA gene sequence analysis (strains 113, 109, 679, 337, 108), plus strains 812 and F15. The latter formed a separate branch within this second group of isolates, which is in agreement with the distinct branching seen in the 16S rRNA gene sequence based phylogenetic tree (Fig. 1). Besides using C_1–C_5 organic acids and few amino acids most of the strains in this group used D-fructose, L-asparagine and glycerol. A broader substrate range characterizes the strains grouped into cluster 3. Strains 31 and 440 fell into this cluster. D-gluconic acid, D-galactonic acid lactone, succinic acid monomethyl ester and L-arabinose were used in addition by all strains in this cluster, and further compounds including L-pyroglutamic acid, D-saccharic acid, D-galactose, α-keto butyric acid and α-hydroxybutyric acid by the majority of strains in cluster 3. Together with *M. aquaticum* GR16^T strain 189 fell into the fourth cluster, which contained the most versatile strains. In addition to the compounds that were also used by the other strains, these strains showed respiratory activity in the presence of several additional amino acids, organic acids and glucose. Taken together, the major clustering deduced from carbon source utilization reflects evolution as seen in the 16S rRNA gene sequence based tree; a finding that supports the statement of P.N. Green who pointed out carbon source utilization as important differential feature for species within the genus *Methylobacterium* (Green 2006). Moreover, it suggests that plant associated *Methylobacterium* strains did not develop a characteristic carbon source utilization pattern in comparison to strains that were isolated from other ecosystems like *M. aquaticum* GR16^T, *M. brachiatum* B0021^T or *M. adhaesivum* AR27^T.

Growth on further carbon compounds was analyzed based on the method described by Green and Bousfield (1982). In particular carbon compounds that are known to be metabolized by at least some *Methylobacterium* species and that are not included in the Biolog assay were tested. As expected, all strains were able to grow on methanol, ethanol, acetate, pyruvate and fumarate. None of the strains used methane, glycolate, tartrate, L-valine and trehalose. Differences were seen in the presence of methylamine, formate, formamide, ethylamine, betaine, glyoxalate and glycine under the tested conditions (Table 3).

The hydrolysis of further compounds was analyzed using the api 20 NE identification system. Urea was hydrolyzed by all strains (Table 3); it is a common feature of the members of this genus (Green 2006). Several strains showed β-glucosidase activity and several others protease activity. β-galactosidase activity was absent in all strains. Growth on selected carbon sources was also included in the api identification system and revealed that all compounds except *N*-acetyl-glucosamine, D-maltose and capric acid were used as carbon and energy source by at least some of the strains (Table 3). Again, strain 189 was most versatile. All strains were catalase and oxidase positive. A weak response in the oxidase test was observed for strains 189, 440, 85 and 32. Weak oxidase activity is known from other members of the genus *Mehylobacterium* (Green 2006). All strains utilized ammonium, nitrate, urea and glutamate as source of nitrogen, while none was able to grow on nitrogen-free medium.

Delineation of new species and DNA–DNA hybridization experiments

Based on 16S rRNA gene sequence analysis some of the characterized strains may represent novel species within the genus *Methylobacterium*, even though none of the strains is sufficiently different (i. e. has less than 97% sequence identity) from the type strains of validly described or proposed *Methylobacterium* species to be considered as member of a new species according to the definition of Stackebrandt and Goebel (1994). However, several species with more than 97% sequence identity to each other exist within the genus *Methylobacterium*. This applies for instance to all members within the monophyletic cluster consisting of *Methylobacterium podarium*, *Methylobacterium salsuginis*, *Methylobacterium rhodinum*, *Methylobacterium aminovorans*, *Methylobacterium suomiense*, *Methylobacterium lusitanum*, *Methylobacterium rhodesianum*, *Methylobacterium extorquens*, *Methylobacterium zatmanii*, *Methylobacterium thiocyanatum* and *Methylobacterium populi* (Fig. 1). To demonstrate the distinctiveness between strains analyzed in this

study and existing species on a genomic level, DNA–DNA hybridization experiments were performed using the method of Mehlen et al. (2004). Thereby, differences in the melting behavior of homologous versus heterologous DNA–DNA hybrids are determined based on DNA denaturation curves. According to the definition by Wayne et al. (1987), strains of different species show a difference of more than 5°C in the melting temperature between homologous and heterologous DNA hybrids (ΔT_m).

Hybridization experiments were performed with those strains that were most closely related to currently validly described type strains within the genus *Methylobacterium* and that display less than 99.5% sequence identity on 16S rRNA gene level to these strains. For strain 440 DNA–DNA hybridization experiments were performed against seven different type strains (Table 4). They resulted in thermal differences above 7°C for all these type strains, suggesting that strain 440 is sufficiently different at genomic level to represent a new species. The major phenotypic distinctive feature between strain 440 and the existing species is the cell length, in particular the observed maximum cell length (Table 2). Further differential features with respect to morphology, growth and chemotaxonomy were compiled in Table S1. Differences in carbon source utilization between strain 440 and the most closely related type strains can be seen in Fig. 2. Based on all these findings we propose that strain 440 represents a new species within the genus *Methylobacterium*. Strain 440 is the only representative available in our collection of strains representing this proposed new species.

In case of strain 189 the most closely related strain and thus the first comparison was made against *M. aquaticum* GR16^T. The hybridization experiments resulted in a thermal difference of 2.7°C between these strains (Table 3). Thus, strain 189 cannot be differentiated from *M. aquaticum* at genomic level. In agreement with this finding is the high morphological similarity of strain 189 to *M. aquaticum* GR16^T (Gallego et al. 2005). However, the two strains display numerous differences in terms of carbon source utilization (Fig. 2). Thus, to further verify this classification, two additional strains with identical 16S rRNA gene sequence to strain 189 but from different sampling sites were analyzed in hybridization experiments (isolates 686 and 692, Knief et al. 2010a). The ΔT_m-values between *M. aquaticum* GR16^T and these strains was also below 5°C (data not shown), confirming that strains of this 16S rRNA gene sequence type should not be considered as representatives of a new species.

Fig. 2 Carbon source utilization of the twelve characterized strains and their most closely related reference type strains. *Gray* shaded areas represent carbon sources that were metabolized while *black squares* indicate respiratory inactivity of the cells in the presence of the respective carbon source. The matrix was calculated in Systat Version 12. The clustering was done based on Jaccard distances and an UPGMA clustering algorithm. Data for *M. mesophilicum*, *M. fujisawaense*, *M. oryzae*, *M. phyllosphaerae*, and *M. radiotolerans* were taken from Madhaiyan et al. (2007, 2009), data for *M. aquaticum* and *M. adhaesivum* from Gallego et al. (2005, 2006). The latter were complemented and confirmed by data generated in this study. Carbon compounds that were not used by any of the tested strains are excluded in the figure. These are *N*-acetyl-D-galactosamine, 2,3-butandiol, D,L-carnitine, D-cellobiose, α-cyclodextrin, dextrin (was excluded due to precipitation), i-erythriol, α-D-glucose-1-phosphate, D-glucose-6-phosphate, glucuronamide, glycogen, L-histidine, hydroxyl-L-proline, *p*-hydroxy phenylacetic acid, inosine, m-inositol, α-D-lactose, lactulose, L-leucine, maltose, D-mannitol, D-mannose, D-melibiose, β-methyl-D-glucoside, L-ornithine, phenylethylamine, D-psicose, putrescine, D-raffinose, D-serine, D-sorbitol, sucrose, thymidine, D-trehalose, turanose, urocanic acid, uridine and xylitol

The DNA–DNA relatedness of strain 31 was firstly analyzed to its closest relatives, *M. mesophilicum* A47^T and *M. brachiatum* B0021^T (Table 3). While strain 31 could be differentiated from *M. mesophilicum* A47^T ($\Delta T_m = 7.2$°C), it could not be differentiated from *M. brachiatum* B0021^T ($\Delta T_m = 4.0$°C). Thus, also strain 31 is not considered as representative of a new species despite several differences in carbon source utilization to *M. brachiatum* B0021^T (Fig. 2).

Since strains 108, 109, 113, 337 and 679 are closely related to strains representing proposed but not yet validated novel species within the genus ('*M. gossipiicola*' Gh-105, '*M. bullatum*' F3.2 and '*M. goesingense*' iEII3) (Hoppe et al. 2011; Idris et al. 2006; Madhaiyan et al. 2011), they were not yet further analyzed. Whether some of these strains represent novel species has to be proven very carefully. Besides performing DNA–DNA hybridization experiments between all these strains including in addition *M. adhaesivum* AR27^T and *Methylobacterium iners* 5317S-33^T, an extensive comparative analysis of morphological, chemotaxonomical and physiological traits will be necessary for species delineation. As far as strains are available, this analysis should moreover

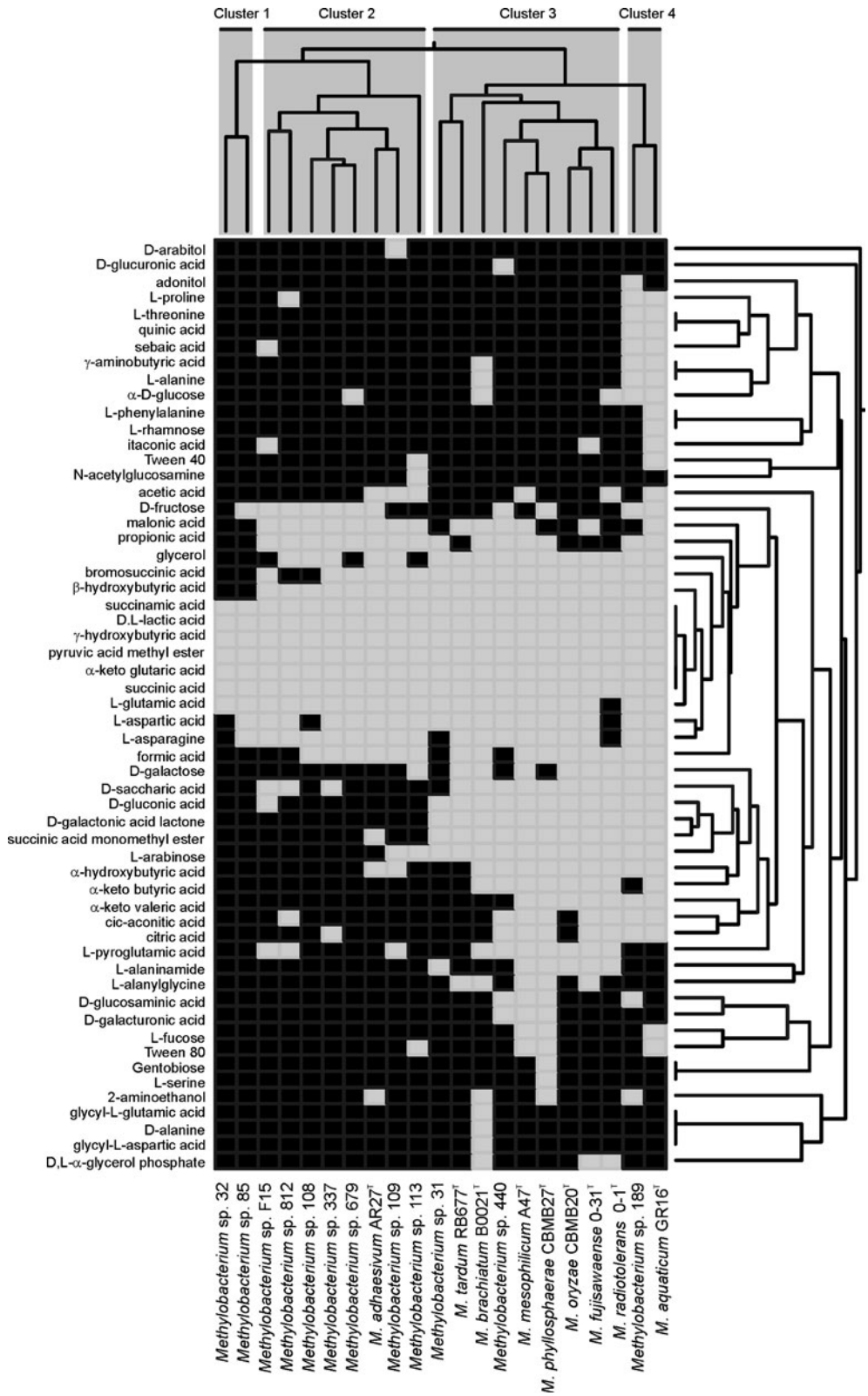
Cluster 1
Cluster 2
Cluster 3
Cluster 4
D-arabitol
D-glucuronic acid
adonitol
L-proline
L-threonine
quinic acid
sebaic acid
γ-aminobutyric acid
L-alanine
α-D-glucose
L-phenylalanine
L-rhamnose
itaconic acid
Tween 40
N-acetylglucosamine
acetic acid
D-fructose
malonic acid
propionic acid
glycerol
bromosuccinic acid
β-hydroxybutyric acid
succinamic acid
D,L-lactic acid
γ-hydroxybutyric acid
pyruvic acid methyl ester
α-keto glutaric acid
succinic acid
L-glutamic acid
L-aspartic acid
L-asparagine
formic acid
D-galactose
D-saccharic acid
D-gluconic acid
D-galactonic acid lactone
succinic acid monomethyl ester
L-arabinose
α-hydroxybutyric acid
α-keto butyric acid
α-keto valeric acid
cis-aconitic acid
citric acid
L-pyroglutamic acid
L-alaninamide
L-alanylglycine
D-glucosaminic acid
D-galacturonic acid
L-fucose
Tween 80
Gentobiose
L-serine
2-aminoethanol
glycyl-L-glutamic acid
D-alanine
glycyl-L-aspartic acid
D,L-α-glycerol phosphate
Methylobacterium sp. 32
Methylobacterium sp. 85
Methylobacterium sp. F15
Methylobacterium sp. 812
Methylobacterium sp. 108
Methylobacterium sp. 337
Methylobacterium sp. 679
M. adhaesivum AR27T
Methylobacterium sp. 109
Methylobacterium sp. 113
Methylobacterium sp. 31
M. tardum RB677T
M. brachiatum B0021T
Methylobacterium sp. 440
M. mesophilicum A47T
M. phyllosphaerae CBMB27T
M. oryzae CBMB20T
M. fujisawaense 0-31T
M. radiotolerans 0-1T
Methylobacterium sp. 189
M. aquaticum GR16T

Table 3 Physiological characteristics of the *Methylobacterium* strains based on the analysis according to the method of Green and Bousfield (1982) and the api 20 NE identification system

Characteristic	189	812	F15	31	440	85	32	679	109[a]	113	108	337
Carbon source utilization (0.1%)[b]												
Methylamine	−	+	−	−	w	+	+	w		w	−	−
Formate	−	+	−	+	w	+	−	−		−	+	−
Formamide	−	−	−	+	−	+	+	w		−	+	+
Acetate	+	+	+	+	+	+	+	+		+	+	+
Ethylamine	−	+	+	−	−	+	−	−		+	−	−
Betaine	+	w	w	w	−	w	−	w		−	−	−
Glycolate	−	−	−	−	−	−	−	−		−	−	−
Glyoxalate	w	+	+	−	+	+	−	+		+	−	−
Glycine	−	w	−	−	−	−	−	w		−	−	−
Api 20 NE identification system[c]												
Hydrolysis of												
L-Arginine (by arginindihydrolase)	−	−	−	+	−	−	−	−	−	−	−	−
Esculin (by β-glucosidase)	+	+	+	+	+	−	−	−	−	−	+	−
Gelatin (by protease)	−	−	−	−	−	−	−	w	w	w	w	w
Growth on												
D-glucose	+	−	−	−	−	+	+	−	+	−	+	+
L-arabinose	+	−	−	+	w	+	−	−	+	+	−	−
D-mannose	+	−	−	−	−	−	w	−	w	−	−	−
D-mannitol	+	+	−	+	−	−	−	−	−	−	−	−
Potassium gluconate	+	−	+	+	+	+	+	−	−	−	−	+
Adipic acid	+	−	+	+	−	−	+	−	−	−	−	−
Malic acid	+	+	−	+	−	+	+	−	+	−	+	+
Trisodium citrate	+	−	−	−	−	−	−	−	w	−	w	−
Phenylacetic acid	+	−	−	w	−	−	−	−	−	−	−	−

w weak

[a] Strain 109 did not grow well enough in liquid medium to be analyzed by the method of Green and Bousfield (1992)

[b] All strains grew on methanol, ethanol, pyruvate and fumarate, but none of the strains grew on methane, tartrate, L-valine and trehalose

[c] All strains were urease positive, but none of the strains was β-galactosidase positive, able to grow on *N*-acetyl-glucosamine, D-maltose and capric acid, and able to form indol from tryptophane

include additional closely related isolates so that each species is represented by multiple isolates in order to identify species-specific characteristics, e.g. in terms of carbon source utilization, morphological variation and growth conditions such as temperature, pH, or salt-tolerance. Finally, strains 32, 85, 812 and F15 were not further analyzed as they will most likely be representatives of recently proposed novel species: strain 32 is probably a member of the species *M. marchantiae*, strain 85 of '*M. bullatum*', strain 812 of '*M. soli*' and strain F15 of '*M. dankookense*'.

Description of *Methylobacterium longum* sp. nov

Methylobacterium longum (lon'gum. L. neut. adj. *longum*, long; because of the formation of long rods).

Cells are Gram-negative, motile, long rods (1.2–2.1 × 2.4–12.2 μm) that appear singly. They form shiny light pink colonies. Upon aging cells become slightly form variable. They accumulate light refracting inclusions (PHB) during growth in the presence of methanol. Growth occurs between 4 and 28°C, but not at 37°C. The species is neutrophilic (pH

Table 4 Thermal differences between the denaturation curves of homologous and heterologous DNA–DNA hybrids

Strain versus type strain	16S rRNA gene sequence identity (%)	ΔT_m (°C)
Methylobacterium sp. 440 vs.		
M. tardum RB677^T	99.4	8.9
M. radiotolerans 0-1^T	99.3	10.2
M. phyllosphaerae CBMB27^T	98.9	13.9
M. oryzae CBMB20^T	98.9	9.1
M. fujisawaense 0-31^T	98.8	7.8
M. brachiatum B0021^T	98.2	11.6
M. mesophilicum A47^T	98.2	7.1
Methylobacterium sp. 189 vs.		
M. aquaticum GR16^T	98.8	2.7
M. variabile GR3^T	97.1	6.6
Methylobacterium sp. 31 vs.		
M. mesophilicum A47^T	99.2	7.2
M. brachiatum B0021^T	99.1	4.0

5.5–7.6) and tolerates salt concentrations up to 0.5%. Growth in liquid mineral salt medium is rather weak, but cells grow well on nutrient broth and R2A medium. The type strain utilizes acetate, ethanol, fumarate, glyoxalate, methanol and pyruvate as carbon sources. Weak growth occurs in the presence of 0.1% formate and methylamine. The Biolog assay revealed a broad substrate utilization pattern including the metabolism of various C_6 compounds: growth was observed on cis-aconitic acid, L-arabinose, L-asparagine, L-aspartic acid, bromosuccinic acid, citric acid, D-fructose, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, L-glutamic acid, glycerol, α-, β- and γ-hydroxybutyric acid, α-keto butyric acid, α-keto glutaric acid, D,L-lactic acid, malonic acid, propionic acid, L-pyroglutamic acid, pyruvic acid methyl ester, D-saccharic acid, succinamic acid, succinic acid and succinic acid monomethyl ester. Carbon compounds that are not metabolized by this strain under the given conditions are shown in Fig. 2 and listed in Table 3. The strain can use ammonium, nitrate, urea and glutamate as source of nitrogen. The major fatty acids are C18:1 ω7c (88%), C16:1 ω 7c (5%), C16:0 (4%) and C18:0 (3%).

The type strain is 440^T (=DSM 23933^T = CECT 7806^T) and has a G+C content of 68.6%. It was isolated from a leaf of *Arabidopsis thaliana* plant grown in a pine forest in central Spain.

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