**Methylobacterium gnaphalii** sp. nov., isolated from leaves of **Gnaphalium spicatum**

Akio Tani,¹ Nurettin Sahin² and Kazuhide Kimbara³

1Institute of Plant Science and Resources, Okayama University, Chuo 2-20-1, Kurashiki, 710-0046 Okayama, Japan
2Egitim Fakultesi, Mugla University, Kötekli 48000, Mugla, Turkey
3Faculty of Engineering, Shizuoka University, Shizuoka 422-8529, Japan

A pink-pigmented, facultatively methylotrophic bacterium, strain 23eᵀ, was isolated from the leaves of **Gnaphalium spicatum** (cudweed). The cells of strain 23eᵀ were Gram-reaction negative, motile and non-spore-forming rods. On the basis of 16S rRNA gene sequence similarities, strain 23eᵀ was related to **Methylobacterium organophilum** ATCC 27886ᵀ (97.1 %) and **Methylobacterium marchantiae** JT1ᵀ (97 %), and the phylogenetic similarities to all other **Methylobacterium** species with validly published names were less than 97 %. Major cellular fatty acids were C₁₈ : 1ω7c, C₁₆ : 0 and C₁₈ : 0. The results of DNA–DNA hybridization, phylogenetic analyses based on 16S rRNA and cpn60 gene sequences, fatty acid profiles, whole-cell matrix-assisted laser desorption/ionization time of flight/MS analysis, physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain 23eᵀ from the phylogenetically closest relatives. We propose that strain 23eᵀ represents a novel species within the genus **Methylobacterium**, for which the name **Methylobacterium gnaphalii** sp. nov. is proposed. The type strain is 23eᵀ (≡DSM 24027ᵀ=NBRC 107716ᵀ).

The genus **Methylobacterium** consists mostly of pink-pigmented, facultatively methylotrophic members of the class **Alphaproteobacteria**, and at the time of writing, comprises 35 recognized species (http://www.bacterio.cict.fr/m/methylobacterium.html). However, according to Kato et al. (2005), **M. chloromethanicum** (McDonald et al., 2001) and **M. dichloromethanicum** (Doronina et al., 2000) are later heterotypic synonyms of **M. extorquens**, and **M. rhodesianum** (Green et al., 1988) is an earlier heterotypic synonym of **M. lusitanum** (Doronina et al., 2002), since they exhibited high levels of DNA–DNA relatedness (69–89 %). Also, the species name ‘**M. dankoookense**’ (Lee et al., 2009) has been proposed but is currently not validly published. Recently, **M. goesingense** from **Thlaspi goesingense** (Idris et al., 2006), **M. marchantiae** from **Marchantia polymorpha** L. (Schauer et al., 2011), ‘**M. soli**’ from soil (Cao et al., 2011), **M. gossipicola** from the cotton phyllosphere (Madhaiyan et al., 2012), **M. bullatum** from the surface of a bryophyte gametophyte (Hoppe et al., 2011) and **M. cerastii** from the surface of a leaf (Wellner et al., 2012) were described as new species.

Members of the genus **Methylobacterium** can grow on single-carbon compounds such as methanol, formaldehyde and formate as the sole carbon and energy source, 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. Recent intensive studies on phyllospheric **Methylobacterium** species showed that members of this genus are the predominant bacterial species on plant surfaces (Delmotte et al., 2009). Recently, we isolated diverse strains of members of the genus **Methylobacterium** from plant leaf samples. We have characterized one of them and proposed the name **M. oxalidis** for the isolate from **Oxalis corniculata** (Tani et al., 2012). Another isolate, strain 23eᵀ, isolated from **Gnaphalium spicatum**, showed 97.1 % 16S rRNA gene sequence similarity with **M. organophilum**, the closest type strain. Here we describe isolate 23eᵀ as a novel species of the genus **Methylobacterium**.

Leaves of **G. spicatum** were collected at the Institute of Plant Science and Resources, Okayama University, in April 2008. Leaves were briefly washed with 50 ml sterile water.

**Abbreviations**: MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; POQ, pyrroloquinoline quinone.

The GenBank/EMBL/DBJ accession numbers for the nucleotide sequences of strain 23eᵀ 16S rRNA and partial cpn60 genes are AB627071 and AB627072, of **Methylobacterium marchantiae** JT1ᵀ for the partial cpn60 gene is AB627073, and that of **Methylobacterium organophilum** ATCC 27886ᵀ for the partial cpn60 gene is AB627074. Three supplementary figures are available with the online version of this paper.
and then washed vigorously with 10 ml sterile water. The wash solution was then spread on methanol medium (Tani et al., 2012). 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, and reactions were observed for 2, 3, 5, 7 and 10 days with a microplate reader (PowerScan HT; Dainippon Sumitomo Pharma). The results of the nutritional tests are shown in the species description. Methanol mineral agar medium was also used in tests for the utilization of methylamine (0.1 %, w/v) as the 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 % (w/v) KNO₃.

The 16S rRNA gene of strain 23eᵀ was amplified by PCR, cloned in the pCR-TOPO vector (Invitrogen) and sequenced (Lane, 1991). Sequencing was carried out with an automated DNA sequencer (model 3130; Applied Biosystems). Phylogenetic analysis was performed using MEGA4 software (Tamura et al., 2007), after multiple sequence alignment of the data by CLUSTAL X2 (Larkin et al., 2007). Genetic distances were obtained by the Kimura’s two-parameter distance model (Kimura, 1980). 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 nucleotide similarity calculations after a neighbour-joining analysis indicated that the closest relatives of strain 23eᵀ were M. organophilum ATCC 27886ᵀ (97.1 %) and M. marchantiae JT1ᵀ (97 %). Strain 23eᵀ showed 16S rRNA gene sequence similarities of below 97.0 % with other members of the genus Methylobacterium. The phylogenetic tree based on the 16S rRNA gene sequence, constructed by using the neighbour-joining method, is shown in Fig. 1. The tree inferred by using the maximum-parsimony method also produced similar results (see Fig. S1, available in IJSEM Online).

DNA–DNA hybridization was carried out at 50 °C for 3 h and measured fluorometrically as described by Ezaki et al. (1989). The DNA–DNA relatedness between strain 23eᵀ and M. organophilum ATCC 27886ᵀ and M. marchantiae JT1ᵀ was 26 % in both cases.

The cpn60 gene was selected for phylogenetic analysis as an alternative marker. The cpn60 gene sequences of strain 23eᵀ and its closest relatives were determined directly from PCR fragments using the method described by Hill et al. (2004). Experimental conditions for PCR amplification and sequencing were the same as described previously (Tani et al., 2012). Strain 23eᵀ showed 92.3 and 92.6 % cpn60 gene nucleotide sequence similarity with those of M. organophilum ATCC 27886ᵀ and M. marchantiae JT1ᵀ, respectively.

Whole-cell matrix-assisted laser desorption/ionization time of flight (MALDI-TOF)/MS analysis was also performed as described previously (Tani et al., 2012). The results of MALDI-TOF/MS analysis (Fig. S2) showed clearly that strain 23eᵀ has a different spectrum from the phylogenetically closest type strains, M. organophilum ATCC 27886ᵀ and M. marchantiae JT1ᵀ.

The selected physiological and biochemical differential characteristics of strain 23eᵀ are compared with those of related type strains in Table 1. Detailed phenotypic information is given in the species description.

The morphology of cells grown on R2A agar media for 5 days was observed with a confocal laser scanning microscope (FV-1000, Olympus) (Fig. S3). The cell size of strain 23eᵀ was 1.99 × 0.9 μm, while that of M. organophilum ATCC 27886ᵀ was 2.7 × 1.39 μm and that of M. marchantiae JT1ᵀ was 3.1 × 1.36 μm. The relatively smaller cell size is characteristic of strain 23eᵀ.

Fatty acid methyl ester (FAME) analysis of the whole cell was determined by the DSMZ Identification Service using GC (MIDI, Microbial ID). FAMEs were obtained from 40 mg cells, grown aerobically on R2A agar for 3 days at 28 °C and scraped from Petri dishes by saponification, methylation and extraction, using minor modifications of the methods described by Miller (1982) and Kuykendall et al. (1988), as noted previously (Tani et al., 2012). The major cellular fatty acids were C₁₈:₀ 10:7c (83.4 %), C₁₆:₀ 5.1 %) and C₁₈:₀ 4.1 %). C₁₈:₀ 3-OH (2.45 %) was the only hydroxylated fatty acid detected. In addition, a minor amount (0.42 %) of a C₁₂:₀ fatty acid and an unidentified fatty acid with an equivalent chain length of 11.799 (0.77 %), summed feature 2 (comprising C₁₄:₀ 3-OH and/or iso-C₁₆:₁ 2.55 %) and summed feature 3 (comprising C₁₆:₁ 10:7c or iso-C₁₅:₀ 2-OH, 1.22 %), were also detected. Thus, strain 23eᵀ could be distinguished from its phylogenetic relatives based on its fatty acid profile.

Respiratory lipoquinones were extracted from 100 mg freeze-dried cell material based on the two-stage method described by Tindall (1990a, b) and carried out by the Identification Service and Dr Brian Tindall, DSMZ, Braunschweig, Germany. Respiratory lipoquinones were separated into their different classes (menaquinones and ubiquinones) by TLC on silica gel (MACHEREY-NAGEL), using hexane: tert-butylmethyl ether (9:1, v/v) as the solvent. UV-absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analysed by HPLC. This step was carried out on an LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (MACHEREY-NAGEL, 2 × 125 mm, 3 μm, RP18)
using methanol as the eluant. Respiratory lipoquinones were detected at 269 nm.

The major ubiquinone system of strains of members of the genus *Methylobacterium* reported to date is ubiquinone Q-10. Similarly, strain 23eT had major ubiquinone system Q-10 (95 %) and minor Q-9 (5 %). The occurrence of ubiquinones Q-8, Q-9 and Q-11 as minor components in *M. extorquens*, *M. fujisawaense* and *M. radiotolerans* has been reported by Urakami et al. (1993). Ubiquinone system Q-9 has also been reported as a minor (2–7 %) component in type strains of *M. mesophilicum*, *M. komagatae*, *M. brachiatum*, *M. tardum* and *M. hispanicum* (Kato et al., 2008) and ‘*M. soli*’ (Cao et al., 2011). Thus strain 23eT had an additional chemotaxonomic difference from its phylogenetic relatives based on the presence of ubiquinone system Q-9.

DNA base composition analysis based on the thermal denaturation temperature, siderophore production and carotenoid extraction and pigment spectral analysis were determined as described by Sahin et al. (2008), Schwyn & Neilands (1987) and Sahin (2011), respectively. Members of the genus *Methylobacterium* 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

---

**Fig. 1.** Phylogenetic analysis based on 16S rRNA gene sequences constructed after multiple alignment of data (1291 nt) and clustering with the neighbour-joining method. Bootstrap values greater than 70 % based on 1000 replications are listed as percentages at the branching points. The sequence of *Microvirga flocculans* TFB1 (AB098515) was used as an out-group. The solid circles indicate corresponding nodes with maximum-parsimony trees. Bar, number of substitutions per nucleotide position.
### Table 1. Differential characteristics of strain 23e<sup>T</sup> and related species of the genus *Methylobacterium*

Strains: 1, 23<sup>e</sup><sup>T</sup>; 2, *M. organophilum* JCM 2833<sup>T</sup> (data from Kato <em>et al.</em>, 2005); 3, *M. marchantiae* JT1<sup>T</sup> (Schauer <em>et al.</em>, 2011); 4, *M. bullatum* F3.2<sup>T</sup> (Hoppe <em>et al.</em>, 2011); 5, *M. jeotgali* S2R03-9<sup>T</sup> (Aslam <em>et al.</em>, 2007); 6, *M. cerastii* C15<sup>T</sup> (Wellner <em>et al.</em>, 2012); 7, *M. gossipiicola* Gh-105<sup>T</sup> (Madhaiyan <em>et al.</em>, 2012); 8, *M. phyllosphaereae* CBMB27<sup>T</sup> (Madhaiyan <em>et al.</em>, 2009); 9, *M. platani* PMB02<sup>T</sup> (Kang <em>et al.</em>, 2007); 10, *M. oxalidis* 35a<sup>T</sup> (Tani <em>et al.</em>, 2012). All strains grew on peptone-rich media and were negative for C<sub>16:0</sub> 2-OH. +, Positive; −, negative; (+), weakly positive; NA, data not available; V, variable reaction.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation source</td>
<td>Leaves of <em>G. spicatum</em> L.</td>
<td>Lake sediment</td>
<td>Thallus of a liverwort</td>
<td>Surface of a bryophyte gametophyte</td>
<td>Fermented seafood</td>
<td>Leaf surface</td>
<td>Cotton phyllosphere</td>
<td>Leaf surface of rice</td>
<td>Leaf from a tree</td>
<td>Phyllosphere of <em>Oxalis corniculata</em></td>
</tr>
<tr>
<td>Colony pigmentation</td>
<td>Pink</td>
<td>Pink</td>
<td>Red</td>
<td>Red</td>
<td>Non-pigmented</td>
<td>Pinkish</td>
<td>Light pink</td>
<td>Pink</td>
<td>Pink</td>
<td>Pink</td>
</tr>
<tr>
<td>Growth on/ at:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth at 35 °C</td>
<td>+</td>
<td>+*</td>
<td>−</td>
<td>(−)</td>
<td>−</td>
<td>+*</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>(−)</td>
<td>(−)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>(−)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2 % NaCl</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Glucose</td>
<td>−</td>
<td>(−)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>(+)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Methylamine</td>
<td>−</td>
<td>+*</td>
<td>−</td>
<td>NA</td>
<td>+*</td>
<td>−</td>
<td>−</td>
<td>(−)</td>
<td>NA</td>
<td>−</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>(−)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>NA</td>
<td>−</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>NA</td>
<td>+</td>
<td>NA</td>
<td>−</td>
</tr>
<tr>
<td>Citrate</td>
<td>−</td>
<td>−</td>
<td>(−)</td>
<td>V</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Hydroxy fatty acids (% of total)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iso C&lt;sub&gt;17:0&lt;/sub&gt; 3-OH</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>C&lt;sub&gt;18:0&lt;/sub&gt; 3-OH</td>
<td>2.5</td>
<td>2.5</td>
<td>0.9</td>
<td>2.5</td>
<td>−</td>
<td>−</td>
<td>11.5</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Quinone type</td>
<td>Q-10, Q-9</td>
<td>Q-10</td>
<td>NA</td>
<td>NA</td>
<td>Q-10</td>
<td>NA</td>
<td>Q-10</td>
<td>NA</td>
<td>Q-10</td>
<td>Q-10</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>67.2</td>
<td>69.6</td>
<td>68</td>
<td>67.1</td>
<td>64.9</td>
<td>NA</td>
<td>64.2</td>
<td>66.8</td>
<td>68.5</td>
<td>70.2</td>
</tr>
</tbody>
</table>

*Data from this study.
a favourable effect on plant growth (Duine & Frank, 1990; Choi et al., 2008). Urakami et al. (1992) reported the amount of extracellular PQQ content in strains of members of the genus Methylobacterium as between 0.34 and 0.75 µg ml⁻¹, by using methanol as the carbon and energy sources. PQQ production (Tani et al., 2012) and auxin (indole acetate) production were assayed as described by Glickmann & Dessaux (1995). The results are given in the species description.

On the basis of results described above, strain 23eᵀ represents a novel species within the genus Methylobacterium, for which the name Methylobacterium gnaphalii sp. nov. is proposed.

**Description of Methylobacterium gnaphalii sp. nov.**

*Methylobacterium gnaphalii* (gna.pha’li.i. N.L. gen. n. gnaphalii of a cudweed *G. spicatum*, referring to the leaves from which the type strain was isolated).

Cells are Gram-reaction negative, motile rods (1.99 × 0.9 µm) and strictly aerobic. Colonies are pink, convex and translucent with regular edges, slow-growing and 0.4 mm in diameter after 5 days on R2A plates at 28 °C. Growth occurs at 28–37 °C. Nitrate reduction is weakly positive. Oxidase negative, catalase positive and other characteristics are given in Table 1. The following substrates produce DL-lactic acid, malonic acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid and L-glutamic acid. Methylamine and dimethylamine are not utilized as sole carbon sources. DNase test is negative and urease is positive. Absorbance spectra of the pigment extracts in an acetone–methanol mixture (3:1, v/v) have absorbance maxima at 496 and 526 nm. The type strain also has the ability to produce PQQ (24.6 µg ml⁻¹) and indole acetic acid (2.6 µg ml⁻¹). Siderophore production is negative. Ubiquinone Q-10 (95%) is the predominant isoprenoid quinone, the other is Q-9 (5%).

The type strain is 23eᵀ (=DSM 24027ᵀ=NBRC 107716ᵀ). The G+C content of DNA is 67.2 mol% (Tm method).

**Acknowledgements**

This work was supported by a Research for Promoting Technological Seeds grant from the Japan Science and Technology Agency and grants from SEKISUI CHEMICAL and the Institute of Fermentation, Osaka (IFO). We wish to thank Ms Y. Fujitani for excellent technical assistance and T. Enomoto for plant identification.

**References**


