Pink-pigmented, facultatively methylotrophic bacteria, strains 87eT and 99bT, were isolated from the bryophytes Haplocladium microphyllum and Brachythecium plumosum, respectively. The cells of both strains were Gram-reaction-negative, motile, non-spore-forming rods. On the basis of 16S rRNA gene sequence similarity, strains 87eT and 99bT were found to be related to Methylobacterium organophilum ATCC 27886T (97.1 % and 97.7 %, respectively). Strains 87eT and 99bT showed highest 16S rRNA gene similarity to Methylobacterium gnaphalii 23eT (98.3 % and 99.0 %, respectively). The phylogenetic similarities to all other species of the genus Methylobacterium with validly published names were less than 97 %. Major cellular fatty acids of both strains were C_{18:1} \omega 7c and C_{18:0}. 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 mass spectrometry (MALDI-TOF/MS) analysis, and physiological and biochemical tests allowed genotypic and phenotypic differentiation of strains 87eT and 99bT from their phylogenetically closest relatives. We propose that strains 87eT and 99bT represent novel species within the genus Methylobacterium, for which the names Methylobacterium haplocladii sp. nov. (type strain 87eT = DSM 24195T = NBRC 107714T) and Methylobacterium brachythecii sp. nov. (type strain 99bT = DSM 24105T = NBRC 107710T) are proposed.

Species of the genus Methylobacterium can grow on single-carbon compounds such as methanol, formaldehyde and formate as 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 (Fall & Benson, 1996). Recently we isolated members of the genus Methylobacterium from plant leaf samples to evaluate their diversity. Their whole-cell mass spectrometry data (WC-MS) obtained by matrix-assisted, laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) were used to discriminate different species (Tani et al., 2012c). The WC-MS-based classification allowed rapid identification and classification without sequencing any marker genes, and consequently unique strains could be easily identified within hundreds of isolates. We have characterized two such unique isolates and proposed Methylobacterium oxalidis isolated from Oxalis corniculata (Tani et al., 2012a) and Methylobacterium gnaphalii isolated from Gnaphalium spicatum (Tani et al., 2012b). Here we describe a further two unique isolates, 87eT and 99bT.

The genus Methylobacterium consists mostly of pink-pigmented, facultative methylotrophs belonging to class Alphaproteobacteria, and at the time of writing, comprises 37 recognized species (http://www.bacterio.net/m/methylobacterium.html). Recently described species include Methylobacterium longum (Knief et al., 2012), Methylobacterium bulbatum (Hoppe et al., 2011), Methylobacterium gossipicola (Madhaiyan et al., 2012), Methylobacterium cerastii (Wellner et al., 2012), Methylobacterium marchantiae (Schauer et al., 2011) and Methylobacterium oxalidis (Tani et al., 2012a), while Methylobacterium sp. nov. (type strain 99bT = DSM 24105T = NBRC 107710T) proposed.

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

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 87eT and 99bT are AB698691 and AB703239, respectively. Those for the partial cpn60 gene sequences of strains 87eT and 99bT are AB713910 and AB713911, respectively.

Three supplementary figures and one supplementary table are available with the online version of this paper.

**Correspondence**

Akio Tani

atani@rib.okayama-u.ac.jp

1Institute of Plant Science and Resources, Okayama University, Okayama, Japan

2Egitim Fakultesi, Mugla Sitki Kocman University, 48170 Kotekli, Mugla, Turkey

**Methylobacterium haplocladii** sp. nov. and **Methylobacterium brachythecii** sp. nov., isolated from bryophytes

**Akio Tani**¹ and **Nurettin Sahin**²

¹Institute of Plant Science and Resources, Okayama University, Okayama, Japan

²Egitim Fakultesi, Mugla Sitki Kocman University, 48170 Kotekli, Mugla, Turkey

"Pink-pigmented, facultatively methylotrophic bacteria, strains 87eT and 99bT, were isolated from the bryophytes Haplocladium microphyllum and Brachythecium plumosum, respectively. The cells of both strains were Gram-reaction-negative, motile, non-spore-forming rods. On the basis of 16S rRNA gene sequence similarity, strains 87eT and 99bT were found to be related to Methylobacterium organophilum ATCC 27886T (97.1% and 97.7%, respectively). Strains 87eT and 99bT showed highest 16S rRNA gene similarity to Methylobacterium gnaphalii 23eT (98.3% and 99.0%, respectively). The phylogenetic similarities to all other species of the genus Methylobacterium with validly published names were less than 97%. Major cellular fatty acids of both strains were C_{18:1} \omega 7c and C_{18:0}. 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 mass spectrometry (MALDI-TOF/MS) analysis, and physiological and biochemical tests allowed genotypic and phenotypic differentiation of strains 87eT and 99bT from their phylogenetically closest relatives. We propose that strains 87eT and 99bT represent novel species within the genus Methylobacterium, for which the names Methylobacterium haplocladii sp. nov. (type strain 87eT = DSM 24195T = NBRC 107714T) and Methylobacterium brachythecii sp. nov. (type strain 99bT = DSM 24105T = NBRC 107710T) are proposed.\"
and 99bT, as representative of the novel species *Methylobacterium haplocadii* sp. nov. and *Methylobacterium bra- chythecii* sp. nov., respectively.

Bryophyte samples, *Haplocladium microphyllum* and *Brachythecium plumosum* were collected at the Institute of Plant Science and Resources, Okayama University, in April 2008. Isolation procedures and media used were reported previously (Tani et al., 2012a). Physiological and biochemical tests were carried out at 28 °C. Conventional biochemical tests were done according to standard methods (Smibert & Krieg, 1994). Oxidation of various substrates was determined by using Biolog GN2 MicroPlates, as described previously (Tani et al., 2012a). Utilization of methylvamine (0.1%, w/v), salt tolerance and nitrate reduction with 0.2% KNO₃ (w/v) were examined as described previously (Tani et al., 2012a). The results of the nutritional tests are given in the species description.

The 16S rRNA gene of strains 87eT and 99bT was PCR-amplified, cloned in pCR-TOPO vector (Invitrogen), sequenced (Lane, 1991) and analysed using MEGA5 software (Tamura et al., 2011) after multiple alignment of data by the CLUSTALX2 program (Larkin et al., 2007). Genetic distances were obtained by the Kimura’s two-parameter distance model (Kimura, 1980). Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Nei & Kumar, 2000) and maximum-likelihood methods in PhyML (Guindon & Gascuel, 2003). 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 the neighbour-joining analysis indicated that the closest relatives of strain 87eT included *Methylobacterium organophilum* ATCC 27886T (97.1%) and *Methylobacterium marchantiae* JT1T (96.9%) and those of strain 99bT were *Methylobacterium organophilum* ATCC 27886T (97.7%) and *Methylobacterium marchantiae* JT1T (96.9%). Strains 87eT and 99bT showed highest 16S rRNA gene similarity to *Methylobacterium gnaphalii* 23eT (98.3% and 99.0%, respectively). The two strains showed 16S rRNA gene sequence similarity below 97.0% with other members of the genus *Methylobacterium*. The phylogenetic tree based on 16S rRNA gene sequence reconstructed by using the neighbour-joining method is shown in Fig. 1. Parsimony and maximum-likelihood trees also produced similar results (see Figs S1 and S2, available in IJSEM Online). Thus, the two strains are close relatives of *Methylobacterium gnaphalii* 23eT, and their sequences showed 98.3% identity to each other.

DNA–DNA hybridization between closely related strains was carried out at 50 °C for 3 h and measured fluorometrically as described by Ezaki et al. (1989). The DNA–DNA relatedness values obtained were 29.3% (87eT and *Methylobacterium organophilum* NBRC 15689T), 22.4% (99bT and *Methylobacterium organophilum* NBRC 15689T), 34.6% (87eT and *Methylobacterium gnaphalii* 23eT), 30.8% (99bT and *Methylobacterium gnaphalii* 23eT) and 37.3% (99bT and 87eT) (Table S1).

The cpn60 gene was selected for phylogenetic analysis as an alternative marker. Experimental conditions are the same as previously described (Tani et al., 2012a). Strain 87eT showed 91.9% (*Methylobacterium organophilum* NBRC 15689T), 95.0% (*Methylobacterium marchantiae* JT1T), 95.7% (strain 99bT) and 94.6% (*Methylobacterium gnaphalii* 23eT) cpn60 gene nucleotide sequence similarity. Strain 99bT showed 92.1% (*Methylobacterium organophilum* NBRC 15689T), 93.9% (*Methylobacterium marchantiae* JT1T) and 96.8% (*Methylobacterium gnaphalii* 23eT) cpn60 gene nucleotide sequence similarity.

The results of whole-cell MALDI-TOF/MS analysis showed that strains 87eT and 99bT have clearly different spectra from their closest relatives (Fig. S3). *Methylobacterium marchantiae* JT1T, *Methylobacterium gnaphalii* 23eT and strain 99bT share an identical most prominent peak at m/z 8355.2, but most of the other peaks were not found in common.

The selected physiological and biochemical differential characteristics of strains 87eT and 99bT are compared with those of related type strains in Table 1. Detailed phenotypic descriptions are given in the species descriptions.

Fatty acid methyl ester (FAME) analysis of whole cells was determined by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) identification service using GC (MIDI; Microbial ID). FAMEs were obtained from 40 mg cells grown aerobically on R2A agar at 28 °C after 9 days for strain 87eT and 3 days for strain 99bT as described previously (Tani et al., 2012a). Major cellular fatty acids of strain 87eT were C₁₈:₁ω7c (83.1%), C₁₈:₀ (7.15%) and C₁₆:₀ (3.0%). Summed feature 2 (comprising C₁₄:₀ 3-0H and/or iso-C₁₆:₁:1 1.6%) and summed feature 3 (comprising C₁₆:₁ω7c and/or iso-C₁₅:₀ 2-0H; 5.2%) were also detected. Major cellular fatty acids of strain 99bT were C₁₈:₁ω7c (85.5%), C₁₈:₀ (5.92%) and C₁₆:₀ (2.7%). Summed feature 2 (comprising C₁₄:₀ 3-0H and/or iso-C₁₆:₁:1 1.9%) and summed feature 3 (comprising C₁₆:₁ω7c and/or iso-C₁₅:₀ 2-0H; 2.2%) were also detected. C₁₈:₀ 3-0H (1.85%) was the only hydroxylated fatty acid detected. Thus, strain 87eT can be distinguished from its closest phylogenetic relatives based on its lack of C₁₈:₀ 3-0H fatty acid (Table 1).

Respiratory lipoquinones were extracted from 100 mg of freeze-dried cell material based on the two-stage method described by Tindall (1990a, b) and analysis was carried out by the Identification Service of the DSMZ as described previously (Tani et al., 2012a). The major ubiquinone system of strains belonging to the genus *Methylobacterium* reported to date is ubiquinone Q-10. The quinone group of
Methylobacterium oryzae DSM 18207T (AY683045)
Methylobacterium fujisawaense DSM 5686T (AB175634)
Methylobacterium phyllosphaerae CBMB27T (EF126746)
Methylobacterium radiotolerans DSM 1819T (AB175640)
Methylobacterium tardum RB677T (AB252208)
Methylobacterium longum 440T (FN868949)
Methylobacterium brachiatum B0021T (AB175649)
Methylobacterium mesophilicum DSM 1708T (EF174498)
Methylobacterium persicum 002-165T (AB252202)
Methylobacterium dankookense SW08-77T (FJ155589)
Methylobacterium hispanicum CCM 7219T (AJ635304)
Methylobacterium cerastii C15T (FR733885)
Methylobacterium jeotgalii S2R03-9T (DQ471331)
Methylobacterium marchantiae JT1T (FJ157976)
Methylobacterium funariae F3.2 FJ157975
Methylobacterium bullatum F3.2T (FJ268657)
Methylobacterium organophilum ATCC 27866T (AB175638)
Methylobacterium hapiocladii 87eT (AB698691)
Methylobacterium brachythecii 99bT (AB703239)
Methylobacterium gnaphalii 23eT (AB607860)
Methylobacterium soli YIM48816T (EU860984)
Methylobacterium adhaesivum DSM 17169T (AM040156)
Methylobacterium rhdium DSM 2163T (AB175644)
Methylobacterium salsuginis MRT (EF015478)
Methylobacterium podarium DSM 15083T (AF514774)
Methylobacterium aminovorans JCM 8240T (AB175629)
Methylobacterium extorquens JCM 2802T (D32224)
Methylobacterium suomiense NCTC 13778T (AB175645)
Methylobacterium adhaesivum DSM 17169T (AM040156)
Methylobacterium thiocyanatum DSM 11490T (AB175646)
Methylobacterium organophilum ATCC 27866T (AB175638)
Methylobacterium plankti PMBO2T (EF426729)
Microvirga flocculans TFB (AB098515)

**Fig. 1.** Phylogenetic analysis based on 16S rRNA gene sequences reconstructed after multiple alignment of data (1291 nt) and clustering with neighbour-joining method. Bootstrap values greater than 70 % based on 1000 replications are listed as percentages at the branching points. Bar, number of substitutions per nucleotide position. The sequence of *Microvirga flocculans* TFB (AB098515) was used as an outgroup. Solid circles indicate that corresponding nodes were seen in both the maximum-parsimony and maximum-likelihood alternative treeing methods.
Table 1. Differential characteristics of strains 87e<sup>T</sup>, 99b<sup>T</sup> and related type strains of species of the genus Methylobacterium

<table>
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<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<td>BG</td>
<td>FS</td>
<td>Leaf</td>
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<td>LR</td>
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<td>Light pink</td>
<td>Pink</td>
<td>Pink</td>
<td>Red</td>
<td>Red</td>
<td>Non-pigmented</td>
<td>Pinkish</td>
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<td>Pink</td>
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<td>Growth on peptone rich media</td>
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<td>+</td>
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<td>Growth at 35 °C</td>
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<td>+</td>
<td>+*</td>
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<td>+*</td>
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<td>Nitrate reduction</td>
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<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>(+)</td>
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<td>+*</td>
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<td>NA</td>
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<td>Growth with 2% NaCl</td>
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<td>Utilization of:</td>
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<tr>
<td>D-Glucose</td>
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<tr>
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<td>(+*)</td>
<td>-</td>
<td>NA</td>
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<td>-</td>
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<td>L-Arabinose</td>
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<td>+</td>
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<td>Citrate</td>
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<td>(+)</td>
<td>V</td>
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<td>Hydroxy fatty acids (% of total)</td>
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<tr>
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<tr>
<td>DNA G + C content (mol%)</td>
<td>67.3</td>
<td>66.5</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>
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</table>

strain 87e<sup>T</sup> was Q-10 (100%) and that of strain 99b<sup>T</sup> was also Q-10 (100%).

DNA base composition analysis was done based on thermal denaturation temperature (Sahin et al., 2008). A side-rophore assay was performed according to the published method of Schwyn & Neilands (1987). Pyrroloquinoline quinone (PQQ) content (Tani et al., 2012a) and indole acetate content were measured as reported by Glickmann & Dessaux (1995). Carotenoid extraction and pigment spectral analysis were determined according to Sahin (2011). The results are given in the species description.

On the basis of results described above, strains 87e<sup>T</sup> and 99b<sup>T</sup> represent novel species within the genus Methylobacterium, for which the names Methylobacterium haplocladii sp. nov., and Methylobacterium brachythecii sp. nov., respectively, are proposed.

Description of Methylobacterium haplocladii sp. nov.

Methylobacterium haplocladii (hap.lo.кла’дий). N.L. gen. n. haplocladii of the epilithic moss Haplocladium microphyllum, referring to the plant from which the type strain was isolated.

Cells are Gram-negative, motile, rods (2.5 x 1.1 μm) and strictly aerobic. Colonies are light pink, convex and translucent with regular edges, very slow-growing and 0.1 mm in diameter after 9 days on R2A plates at 28 °C. Growth occurs at 28 °C and not at 32 °C. Nitrate reduction is negative and motility is negative. Oxidase-negative and catalase-positive; other characteristics are given in Table 1. The following substrates produce positive results in Biolog GN2 plates: α-D-Glucose, L-rhamnose, turanose, methyl pyruvate, formic acid, β-hydroxybutyric acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, propionic acid, bromosuccinic acid and glycerol. Methylamine is not utilized as sole carbon sources. DNase and urease are negative. Absorbance spectra of the pigment extracts in acetone/methanol (3:1, v/v) have absorbance maxima at 384, 470, 496 and 528 nm. Type strain has the ability to produce indole acetate (0.7 μg ml<sup>-1</sup>). PQQ production is not observed due to poor growth on methanol. Siderophore production is negative. Ubiquinone Q-10 is the only detected isoprenoid quinone. Major cellular fatty acids are C<sub>18:1</sub>ω7c, C<sub>18:0</sub> and C<sub>16:0</sub>-
The type strain is $87^\top$ (= DSM 24195$^\top$=NBRC 107714$^\top$), isolated from \textit{Haplocladium microphyllum} collected on the premises of the Institute of Plant Science and Resources, Okayama University, Japan. The $G+C$ content of DNA of the type strain is 67.3 mol% ($T_m$ method).

**Description of \textit{Methylobacterium brachythecii} sp. nov.**

\textit{Methylobacterium brachythecii} (bra.chy.the’ci.i. N.L. gen. n. \textit{brachythecium} of Rusty feather-moss \textit{Brachythecium plumosum}, referring to the plant from which the type strain was isolated).

Cells are Gram-reaction-negative, motile, rods (2.5 × 1.2 μm) and strictly aerobic. Colonies are light pink, convex and translucent with regular edges, and 0.2 mm in diameter after 5 days on R2A plates at 28 °C. Growth occurs at 28 °C and not at 32 °C. Nitrate reduction is weakly positive and motility is positive. Oxidase-positive and catalase-positive; other characteristics are given in Table 1. The following substrates produce positive results in Biolog GN2 plates: dextrin, glycogen, L-arabinose, \( \alpha \)-D-glucose, turanose, methyl pyruvate, acetic acid, formic acid, \( \beta \)-hydroxybutyric acid, \( \alpha \)-ketoglutaric acid, DL-lactic acid, malonic acid, propionic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinic acid, L-asperagine, L-asparatic acid, L-glutamic acid, glycerol and DL-\( \beta \)-glycerol-phosphate. Methylamine is utilized as a sole carbon source. DNase is negative and urease is positive. Absorbance spectra of the pigment extracts in acetone/methanol (3:1, v/v) have absorbance maxima at 472, 496 and 528 nm. Type strain has the ability to produce PQP (13.4 μg ml$^{-1}$) and indole acetate (1.3 μg ml$^{-1}$). Siderophore production is negative. Ubiquinone Q-10 is the only detected isoprenoid quinone. Major cellular fatty acids are C$_{18:1}$ω7c, C$_{18:0}$ and C$_{16:0}$ C$_{18:3}$ 3-0H hydroxylated fatty acid was also detected.

The type strain is 99b$^\top$ (= DSM 24105$^\top$=NBRC 107710$^\top$), isolated from \textit{Brachythecium plumosum} collected on the premises of the Institute of Plant Science and Resources, Okayama University, Japan. The $G+C$ content of DNA of the type strain is 66.5 mol% ($T_m$ method).

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**References**


