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

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Pink-pigmented, facultatively methylotrophic bacteria, strains 87e¹ and 99b¹, were isolated from the bryophytes *Haplocodium 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 87e¹ and 99b¹ were found to be related to *Methylobacterium organophilum* ATCC 27886T (97.1 % and 97.7 %, respectively). Strains 87e¹ and 99b¹ showed highest 16S rRNA gene similarity to *Methylobacterium gnaphalii* 23e² (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 C₁₈:₁₀7c and C₁₈:₀. The results of DNA–DNA hybridization, phylogenetic analyses based on 16S rRNA and *cpr*60 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 87e¹ and 99b¹ from their phylogenetically closest relatives. We propose that strains 87e¹ and 99b¹ represent novel species within the genus *Methylobacterium*, for which the names *Methylobacterium haplocadii* sp. nov. (type strain 87e¹=DSM 24195¹=NBRC 107714¹) and *Methylobacterium brachythecii* sp. nov. (type strain 99b¹=DSM 24105¹=NBRC 107710¹) are proposed.

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 bullatum* (Hoppe et al., 2011), *Methylobacterium gossipicola* (Madhaiyan et al., 2012), *Methylobacterium cerasti* (Wellner et al., 2012), *Methylobacterium marchantiae* (Schauer et al., 2011) and *Methylobacterium oxalidis* (Tani et al., 2012a), while *Methylobacterium soli* (Cao et al., 2011), ‘*Methylobacterium funariae’* (Schauer & Kutscher, 2011) and *Methylobacterium gnaphalii* (Tani et al., 2012b) have been published online.

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, 87e¹ and 99b¹.
and 99b\textsuperscript{T}, 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 methylamine (0.1%, w/v), salt tolerance and nitrate reduction with 0.2% KNO\textsubscript{3} (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 87e\textsuperscript{T} and 99b\textsuperscript{T} 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 87e\textsuperscript{T} included *Methylobacterium organophilum* ATCC 27886\textsuperscript{T} (97.1%) and *Methylobacterium marchantiae* JT1\textsuperscript{T} (96.9%) and those of strain 99b\textsuperscript{T} were *Methylobacterium organophilum* ATCC 27886\textsuperscript{T} (97.7%) and *Methylobacterium marchantiae* JT1\textsuperscript{T} (96.9%). Strains 87e\textsuperscript{T} and 99b\textsuperscript{T} showed highest 16S rRNA gene similarity to *Methylobacterium gnaphalii* 23e\textsuperscript{T} (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* 23e\textsuperscript{T}, 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% (87e\textsuperscript{T} and *Methylobacterium organophilum* NBRC 15689\textsuperscript{T}), 22.4% (99b\textsuperscript{T} and *Methylobacterium organophilum* NBRC 15689\textsuperscript{T}), 34.6% (87e\textsuperscript{T} and *Methylobacterium gnaphalii* 23e\textsuperscript{T}), 30.8% (99b\textsuperscript{T} and *Methylobacterium gnaphalii* 23e\textsuperscript{T}) and 37.3% (99b\textsuperscript{T} and 87e\textsuperscript{T}) (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 87e\textsuperscript{T} showed 91.9% (*Methylobacterium organophilum* NBRC 15689\textsuperscript{T}), 95.0% (*Methylobacterium marchantiae* JT1\textsuperscript{T}), 95.7% (strain 99b\textsuperscript{T}) and 94.6% (*Methylobacterium gnaphalii* 23e\textsuperscript{T}) cpn60 gene nucleotide sequence similarity. Strain 99b\textsuperscript{T} showed 92.1% (*Methylobacterium organophilum* NBRC 15689\textsuperscript{T}), 93.9% (*Methylobacterium marchantiae* JT1\textsuperscript{T}) and 96.8% (*Methylobacterium gnaphalii* 23e\textsuperscript{T}) cpn60 gene nucleotide sequence similarity.

The results of whole-cell MALDI-TOF/MS analysis showed that strains 87e\textsuperscript{T} and 99b\textsuperscript{T} have clearly different spectra from their closest relatives (Fig. S3). *Methylobacterium marchantiae* JT1\textsuperscript{T}, *Methylobacterium gnaphalii* 23e\textsuperscript{T} and strain 99b\textsuperscript{T} 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 87e\textsuperscript{T} and 99b\textsuperscript{T} 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 87e\textsuperscript{T} and 3 days for strain 99b\textsuperscript{T} as described previously (Tani et al., 2012a). Major cellular fatty acids of strain 87e\textsuperscript{T} were C\textsubscript{18}:1\textsuperscript{v7} (83.1%), C\textsubscript{18}:0 (7.15%) and C\textsubscript{16}:0 (3.0%). Summed feature 2 (comprising C\textsubscript{14}:0 3-OH and/or iso-C\textsubscript{16}:1\textsuperscript{w6}) and summed feature 3 (comprising C\textsubscript{16}:1\textsuperscript{w7}c and/or iso-C\textsubscript{15}:0 2-OH; 5.2%) were also detected. Major cellular fatty acids of strain 99b\textsuperscript{T} were C\textsubscript{18}:1\textsuperscript{v7}c (85.5%), C\textsubscript{18}:0 (5.92%) and C\textsubscript{16}:0 (2.7%). Summed feature 2 (comprising C\textsubscript{14}:0 3-OH and/or iso-C\textsubscript{16}:1\textsuperscript{v7}c (1.9%) and summed feature 3 (comprising C\textsubscript{16}:1\textsuperscript{w7}c and/or iso-C\textsubscript{15}:0 2-OH; 2.2%) were also detected. C\textsubscript{18}:0 3-OH (1.85%) was the only hydroxylated fatty acid detected. Thus, strain 87e\textsuperscript{T} can be distinguished from its closest phylogenetic relatives based on its lack of C\textsubscript{18}:0 3-OH 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
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 87eT, 99bT and related type strains of species of the genus Methylobacterium

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HM</th>
<th>BP</th>
<th>GS</th>
<th>Lake</th>
<th>Thallus</th>
<th>BG</th>
<th>FS</th>
<th>Leaf</th>
<th>PC</th>
<th>LR</th>
<th>LT</th>
<th>PO</th>
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<td>Hm</td>
<td>Bp</td>
<td>Gs</td>
<td>Lake</td>
<td>Thallus</td>
<td>BG</td>
<td>FS</td>
<td>Leaf</td>
<td>PC</td>
<td>LR</td>
<td>LT</td>
<td>PO</td>
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<tr>
<td>Colony pigmentation</td>
<td>Light pink</td>
<td>Light pink</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>
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<td>Growth on peptone rich media</td>
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<td>–</td>
<td>+</td>
<td>+*</td>
<td>+*</td>
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<td>–</td>
<td>+</td>
<td>+</td>
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<td>Growth at 35 °C</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+*</td>
<td>–</td>
<td>–</td>
<td>+*</td>
<td>–</td>
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<tr>
<td>Nitrate reduction</td>
<td>( + )</td>
<td>( + )</td>
<td>–</td>
<td>( + )</td>
<td>–</td>
<td>–</td>
<td>+*</td>
<td>NA</td>
<td>–</td>
<td>–</td>
<td>NA</td>
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<td>Growth with 2 % NaCl</td>
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<td>D-Glucose</td>
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<td>–</td>
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<td>( + )</td>
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<td>Methylamine</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+*</td>
<td>NA</td>
<td>+*</td>
<td>–</td>
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<td>( + )</td>
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<td>L-Arabinose</td>
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<td>NA</td>
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<td>V</td>
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<td>D-Xylose</td>
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<td>NA</td>
<td>+</td>
<td>NA</td>
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<td>Citrate</td>
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<td>Hydroxy fatty acids (% of total)</td>
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<td>iso-C17:0 3-OH</td>
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<tr>
<td>C18:0 3-OH</td>
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<td>1.85</td>
<td>2.5</td>
<td>2.5</td>
<td>0.9</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.9</td>
<td>3.46</td>
<td>1.8</td>
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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>
</tr>
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</table>

Strains 1, Methylobacterium haplocadii sp. nov. 87eT; 2, Methylobacterium brachythecii sp. nov. 99bT; 3, Methylobacterium gnaphali sp. 23eT (data from Tani et al., 2012b); 4, Methylobacterium organophilum JCM 2833T (Kato et al. 2005); 5, Methylobacterium marchantiae T1T (Schauer et al., 2011); 6, Methylobacterium bullatum F3.2T (Hoppe et al., 2011); 7, Methylobacterium jeotgali S2R03-9T (Aslam et al. 2007); 8, Methylobacterium caesii C15T (Wellner et al., 2012); 9, Methylobacterium gossipicola Gh-105T (Madhaiyan et al., 2012); 10, Methylobacterium phyllophaeae CMBB27T (Madhaiyan et al., 2009); 11, Methylobacterium platani PMB02T (Kang et al., 2007); and 12, Methylobacterium oxalis 35aT (Tani et al., 2012a). +, Positive; –, negative; (+), weakly positive; NA, data not available; V, variable reaction; *, data from this study. Hm, Haplocladium microphyllum; Bp, Brachythecium plumosum; Gs, leaves of Onaphilium spicatum L.; Lake, lake sediment; Thallus, thallus of a liverwort; BG, surface of a bryophyte gametophyte; FS, fermented seafood; Leaf, leaf surface; PC, cotton phyllosphere; LR, leaf surface of rice; LT, leaf from a tree; PO, phyllosphere of Oxalis corniculata.

Methylobacterium haplocadii sp. nov.

Methylobacterium haplocadii (hap.lo.ca’di.i. N.L. gen. n. haplocadii of the epilithic moss Haplocladium microphyllum, referring to the plant from which the type strain was isolated).

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 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 87eT and 99bT represent novel species within the genus Methylobacterium, for which the names Methylobacterium haplocadii sp. nov., and Methylobacterium brachythecii sp. nov., respectively, are proposed.
The type strain is 876T (=DSM 24195T=NBRC 107714T), isolated from _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 Methylobacterium brachytheicum sp. nov.**

_Methylobacterium brachytheicum_ (bra.chy.the’ci.i. N.L. gen. n. brachytheicum of Rusty feather-moss _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 RA2 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, x-D-glucose, turanose, methyl pyruvate, acetic acid, formic acid, β-hydroxybutyric acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, propionic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinic acid, L-asparagine, L-aspartic acid, L-glutamic acid, glycerol and DL-α-glycerophosphate. Methylamine is utilized as a sole carbon source. DNase is negative and urease is positive. Absorbance of the tree

North Carolina state is 99bT (=DSM 24105T=NBRC 107710T), isolated from _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**


