Paenibacillus methanolicus sp. nov., a xylanolytic, methanol-utilizing bacterium isolated from the phyllosphere of bamboo (Pseudosasa japonica)

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Strain BL24T, isolated from bamboo phyllosphere collected in Coimbatore, India, was studied for taxonomic classification. Cells of the strain were aerobic, Gram-stain-positive, motile, catalase- and oxidase-positive rods and grew on media containing methanol. In 16S rRNA gene sequence analysis, strain BL24T showed the highest sequence similarities with Paenibacillus phyllosphaerae KACC 11473T (97.8 %) and Paenibacillus sacheonensis SY01 (95.1 %). DNA–DNA hybridization with P. phyllosphaerae KACC 11473, phylogenetically the most closely related species, was 21.6 %; this value showed that strain BL24T belonged to a different species. The cell-wall peptidoglycan was found to possess meso-diaminopimelic acid and the G+C content of genomic DNA was 52.1 mol %. It contained menaquinone (MK)-7 as the predominant respiratory quinone and the major cellular fatty acids are C16:0, anteiso-C15:0, iso-C16:0, and anteiso-C17:0. Based on the molecular and chemotaxonomic markers and physiological properties, strain BL24T (=NRRL B-51698=CCM 7577T) is considered to represent a novel species of the genus Paenibacillus, for which the name Paenibacillus methanolicus is proposed.

The genus Paenibacillus is a monophyletic group (previously referred as RNA group 3) comprised of bacteria distinct from other endospore formers. The cells are rod-shaped, aerobic or facultative anaerobes, Gram-stain-positive and shows Gram-stain-variable or negative reaction and produce oval endospore (Ash et al., 1993). Currently, this genus is comprised of more than 182 species with validly published names occurring in a wide range of ecological niches, of which soil and plants seem to be important environments for this bacterial association. Interestingly, several plant-associated species of the genus Paenibacillus are able to hydrolyse xylan polymers. This property was reflected in most of the strains recovered from soil and plant environments. These include Paenibacillus barcinonensis, Paenibacillus cellulosolyticus, Paenibacillus sianensis, Paenibacillus septicrinonalis, Paenibacillus nanensis, Paenibacillus montaniiew, Paenibacillus panacisoli, Paenibacillus soli, Paenibacillus phyllosphaerae, Paenibacillus thailandensis and Paenibacillus woosongensis reported in the LPSN website (Parte, 2014). Facultative methylotrophy among bacteria is witnessed commonly in soil and plant-associated environments. However, up to date, only two facultatively methylotrophic species (Bacillus velezensis and

The GenBank/EMBL/DBJ accession number for the 16S rRNA sequence of strain BL24T is EU912465.

Four supplementary figures are available with the online Supplementary Material.
Bacillus methanolicus have been reported in Bacillaceae a family phylogenetically close to the genus Paenibacillus (Arfman et al., 1992; Madhaiyan et al., 2010).

In plant-associated environments, addition to members of the genera Pseudomonas and Bacillus, Paenibacillus are regarded as biocontrol agents (i.e. they suppress plant diseases), mainly due to presence of properties like production of phytohormones, hydrolytic enzymes and antibiotics. Their ubiquitous occurrence in different environments was recently demonstrated through metagenomic analysis of sequences recovered from different environmental sources (Rybakova et al., 2015; Kim et al., 2010).

In the present study, strain BL24<sup>T</sup> was isolated from leaf bunches by the leaf imprinting method using ammonium mineral salts (AMS) medium (Whittenbury et al., 1970) enriched with methanol (0.5 %); after sterilization, filter sterilized cycloheximide (10 μg ml<sup>-1</sup>) was added to the medium to prevent fungal contamination. For further long-term preservation, either AMS or R2A medium added with 0.5 % (v/v) methanol was used (Madhaiyan & Poonguzhali, 2014).

The morphological characteristics of the strain were determined as per the methods of Brown & Smith (2014). The potential of strain BL24<sup>T</sup> to degrade polymeric substrates (cellulose and xylan) was tested in solid medium amended with 5 g<sup>-1</sup> of either CM-cellulose or xylan (Amore et al., 2015). After spot inoculating of the test strain, the plates were incubated for up to 3 days, and the cellulolytic or xylanolytic activity was tested by adding 0.1 % congo red solution and incubating for 30 min; finally, the plates were washed with 5 M NaCl. Formation of clear halo against an opaque background indicated positive results (Amore et al., 2015). As xylanolytic potential has been widely observed in members of the genus Paenibacillus, activity of strain BL24<sup>T</sup> was further determined quantitatively as per the method of Bailey et al. (1992).

Various carbon source utilization profile and characteristic biochemical features were identified using Biolog GP2 Microplates (Madhaiyan et al., 2007; Poonguzhali et al., 2006). Salt tolerance was tested in R2A medium containing 0, 1, 2, 3, 4 or 5 (w/v) NaCl after 72 h of incubation. The pH range (pH 2–10 at intervals of 1 pH unit) and temperature range (4, 10, 15, 25, 28, 30, 37 and 42 °C) for growth were assessed in R2A liquid medium for 72 h and recorded at OD<sub>600</sub>. For scanning electron microscope (SEM) observations, the samples were dehydrated in an increasing concentration of alcohol (50–100 %), critical-point dried and stabilized with fixative, and spray coated with gold/palladium adapting the method described by Bozzola & Russell (1998). Samples were then examined using a Hitachi S-2500C SEM.

Cells of strain BL24<sup>T</sup> were rod-shaped, aerobic, motile and formed spherical endospores centrally or subterminally. In AMS medium, cells formed creamy-white colonies, with convex appearance and an entire edge. Poor growth was observed on AMS medium in the absence of 0.5 % methanol. Cells of strain BL24<sup>T</sup> are shown in Fig. S1 (available in the online Supplementary Material). Further, carbon source utilization and biochemical features of strain BL24<sup>T</sup> were compared with those of other closely related species (Table 1). Utilization of one-carbon compounds (methanol, dimethylamine, methylamine, trimethylamine and diethanolamine) served as an important differentiating feature from its evolutionarily closest relatives.

Cellulose and xylanase activity demonstrated by strain BL24<sup>T</sup> showed a clear halo around the spot inoculation, which indicated a positive activity for xylanase (2.9 cm

### Table 1. Distinctive characteristics that differentiate novel strain BL24<sup>T</sup> from closely related species of the genus Paenibacillus

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Phyllosphere of bamboo</td>
<td>Phyllosphere of Phoenix dactylifera</td>
<td>Sediment</td>
</tr>
<tr>
<td>Gram stain</td>
<td>+</td>
<td>v</td>
<td>–</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>NaCl tolerance (%)</td>
<td>3</td>
<td>4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Hydrolisis of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>w</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>w</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>–</td>
<td>w</td>
<td>+</td>
</tr>
<tr>
<td>Aesculin</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Ribose</td>
<td>+</td>
<td>–</td>
<td>w</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>w</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methyl β-D-xylopyranoside</td>
<td>w</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>+</td>
<td>+*</td>
<td>–</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>+</td>
<td>+*</td>
<td>–</td>
</tr>
<tr>
<td>N-Acetylglucomamine</td>
<td>+</td>
<td>+*</td>
<td>+*</td>
</tr>
<tr>
<td>Amygdalin</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Arbutin</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Salicin</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Melezitose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Succinate</td>
<td>+</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>Genomic DNA G+C content (mol %)*</td>
<td>52.1</td>
<td>50.7</td>
<td>56.1</td>
</tr>
</tbody>
</table>

*Discrepancies found between this study and results reported by Rivas et al. (2005) and Moon et al. (2011).
diameter) and cellulase (2.1 cm diameter) (Fig. S2). The highest level (11.2±1.8 U ml⁻¹) of xylanase production in flask culture was achieved after 48 h of incubation at 37 °C.

For 16S rRNA gene sequencing, genomic DNA was extracted from strain BL24ᵀ (Wilson, 1997). Using the universal primers 27F and 1492R, a partial 16S rRNA gene sequence was amplified, and the gene fragment sequence was determined by using an ABI prism Big dyeterminator cycle sequencing ready reaction kit v.3.1 (Applied Biosystems). The sequences of type strains of the evolutionary nearest neighbours were subjected to pairwise alignment using the EzTaxon-e Database (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012). The phylogenetically closest sequences were aligned using the CLUSTAL W program of MEGA version 6.0 (Tamura et al., 2013). The evolutionary distance was obtained using the Kimura correction model by pairwise deletion (Kimura, 1980). Phylogeny was reconstructed, and the evolutionarily nearest neighbours were ascertained using different treeing methods including neighbour-joining, maximum-likelihood and maximum-parsimony (Saitou & Nei, 1987; Felsenstein, 1981; Fitch, 1971) present in MEGA version 6.0 with bootstrapping of 1000 replications (Felsenstein, 1985; Tamura et al., 2013).

Phylogenetic reconstruction using the neighbour-joining algorithm showed that the 16S rRNA gene sequence of strain BL24ᵀ forms a separate and distinct branch, clustering within the P. phyllosphaerae clade (Fig. 1). A similar tree topology was observed in the trees generated through the maximum-parsimony and maximum-likelihood algorithms. Collectively all these phylogenetic data confirmed that strain BL24ᵀ belongs to a novel member of the genus Paenibacillus (see Figs S3 and S4).

Cellular fatty acid methyl ester analysis was conducted for strain BL24ᵀ and the phylogenetically related strains (P. phyllosphaerae DSM 17399ᵀ and P. saceonensis DSM 23054ᵀ). All these strains were grown aerobically in nutrient agar (NA, Difco Laboratories, Detroit, MI, USA) at 28 °C for 48 h. Fatty acids were extracted, methylated, separated using a gas chromatograph (model 6890, Hewlett Packard), and the peaks were identified on comparison with the standard peaks of fatty acids and their percentages were arrived at using the Microbial Identification System (Microbial ID; 6.0 version) (Sasser, 1990).

The bacterial menaquinones were separated and analysed on a reverse-phase column as per the method of Kroppenstedt (1982). The genomic DNA was isolated and analysed using HPLC (Supelcosil LC-18-S, Supelco), and the DNA G+C content was obtained by measuring the distinct ratios of deoxyguanosine and thymidine (Mesbah et al., 1989). The cell wall (peptidoglycan) components were analysed by disrupting the cells and preparing the cell wall components as per the method of Schleifer (1985), and the hydrolysates were analysed by TLC using cellulose plates.

The taxonomic relationship of strain BL24ᵀ with the evolutionarily closest strains was determined by DNA–DNA hybridization on nitrocellulose membranes (Seldin & Dubnau, 1985). Probes used for hybridization were non-radioactively labelled using DIG-High Prime systems (Roche diagnostics). The hybridization temperature was set at 60 and 65 °C, and the chemiluminescent reaction was detected using a DIG hybridization kit (Roche diagnostics).}

![Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparison showing the position of strain BL24ᵀ and close relatives in the genus Paenibacillus. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points. Bar, 0.01 substitutions per nucleotide position.](https://example.com/fig1.png)

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**Paenibacillus methanolicus** BL24ᵀ (EU912465)

**Paenibacillus phyllosphaerae** PALXL04ᵀ (AY598818)

**Paenibacillus saceonensis** SY01 (GU124597)

**Paenibacillus taihimensis** THMBG22ᵀ (UO398861)

**Paenibacillus marinum** THE-22 (FR865169)

**Paenibacillus pinihumi** S23ᵀ (GG423057)

**Paenibacillus wooponensis** WPCB018 (EU939687)

**Paenibacillus humicus** PC-147ᵀ (AM411528)

**Paenibacillus oenotherae** DT7-4ᵀ (KF900218)

**Paenibacillus ranthilyticus** 11N27ᵀ (JX089326)

**Paenibacillus kobensis** DSM 10249ᵀ (AB073363)

**Paenibacillus endophyticus** PECAE04ᵀ (KC447384)

**Paenibacillus glycanilyticus** DS-1ᵀ (AB042938)

**Paenibacillus catalpae** D75ᵀ (HQ657320)

**Paenibacillus popilliae** ATCC 14706ᵀ (AF071859)

**Bacillus subtilis** NCDO 1769ᵀ (X60646)
Table 2. Cellular fatty acid composition of strain BL24\(^T\) and phylogenetically related species of the genus Paenibacillus

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
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<tbody>
<tr>
<td>C14:0</td>
<td>1.9</td>
<td>1.7</td>
<td>–</td>
</tr>
<tr>
<td>C15:0</td>
<td>–</td>
<td>–</td>
<td>3.8</td>
</tr>
<tr>
<td>C16:0</td>
<td>10.0</td>
<td>11.4</td>
<td>8.9</td>
</tr>
<tr>
<td>iso-C14:0</td>
<td>2.3</td>
<td>2.3</td>
<td>3.9</td>
</tr>
<tr>
<td>anteiso-C15:0</td>
<td>63.2</td>
<td>53.7</td>
<td>49.8</td>
</tr>
<tr>
<td>iso-C15:0</td>
<td>4.27</td>
<td>8.9</td>
<td>13.2</td>
</tr>
<tr>
<td>iso-C16:0</td>
<td>10.3</td>
<td>12.1</td>
<td>11.3</td>
</tr>
<tr>
<td>anteiso-C17:0</td>
<td>6.2</td>
<td>6.8</td>
<td>5.8</td>
</tr>
<tr>
<td>C16:1ω7c alcohol</td>
<td>1.7</td>
<td>1.3</td>
<td>–</td>
</tr>
<tr>
<td>C16:1ω11c</td>
<td>–</td>
<td>1.2</td>
<td>–</td>
</tr>
<tr>
<td>C14:1ω5c</td>
<td>–</td>
<td>–</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Luminous Detection kit (Roche Applied Science, 68298 Mannheim, Germany).

The predominant cellular fatty acids are C16:0, anteiso-C15:0 and iso-C16:0 (summarized in Table 2). The G+C content of the DNA of strain BL24\(^T\) was 52.1 mol\%, which is well within the values (36–59 mol\%) observed in strains belonging to the genus *Paenibacillus* (De Vos *et al.*, 2009). Strain BL24\(^T\) contained meso-diaminopimelic acid, the diagnostic diamino acid of the cell-wall peptidoglycan of strains belonging to the genus *Paenibacillus* (De Vos *et al.*, 2009). Species-level identity was further delineated using DNA–DNA hybridization with the evolutionarily related clade member *P. phyllosphaerae* KACC 11473\(^T\). The value (21.6±2.7 %) recorded was well within the species-level recommendation (70 % or greater DNA–DNA relatedness being assigned to the same species) proposed for classification of novel strains (Wayne *et al.*, 1987).

Characteristic features identified in this polyphasic study, including genetic (16S rRNA gene, DNA G+C content and DNA relatedness), phenotypic, chemotaxonomic (peptidoglycan, fatty acid profiles and menaquinones) and other biochemical characteristic features (utilization of C\(_1\) compounds including methanol, dimethylamine, methylamine, trimethylamine and diethanolamine, NaCl tolerance, utilization of ribose and succinate), allowed the clear differentiation of strain BL24\(^T\) from its phylogenetic neighbours. Therefore, based upon these features, strain BL24\(^T\) is proposed a represent a novel species, for which the name *Paenibacillus methanolicus* sp. nov., is proposed.

**Description of Paenibacillus methanolicus sp. nov**

*Paenibacillus methanolicus* (me.tha.nol. -olis methanol; N.L. n. *methanol* -olis methanol; N.L. masc. adj. *methanolicus* pertaining to methanol).

Cells are Gram-staining-positive, motile, produce extracellular polysaccharide and are rod-shaped (0.7–0.9 µm wide and 2.2–3.2 µm long). Grows on NA, R2A, AMS/MMS, synthetic minimal medium containing 20 mM succinate (Harder *et al.*, 1973) and NA+methanol, and weakly on tryptic soy agar. After 48 h of incubation at 30 °C on R2A agar plates, colonies are 0.8–1.8 mm in diameter, smooth, round, raised and translucent/white. The strain grew within the temperature range 5–42 °C (optimum temperature 30 °C), at pH 4.8–8.5 (optimum pH 7.0) and in the presence of 3.0 % (w/v) NaCl. Xylan, starch, CM-cellulose and casein are hydrolysed but gelatin and ascinulin are not hydrolysed. Pectinase, cellulase, oxidase and catalase are positive. Gluc- erol, l-arabinose, α-cyclodextrin, D-glucose, D-fructose, D-galactose, maltose, maltotriose, D-mannose, D-mannit, L-rhamnose, melibiose, D-sorbitol, D-ribose, tagatose, γ-hydroxybutyric acid, α-ketobutyric acid, succinic acid monomethyl-ester, succinic acid, methylamine, diethy- amine, trimethylamine, methanol, l-asparagine, adenosine and inosine are utilized as sole sources of carbon. The main cellular fatty acids are anteiso-C15:0, iso-C16:0 and C16:0. The predominant menaquinone is MK-7.

The type strain, BL24\(^T\) (=NRRL B-51698\(^T\)=CCM 7577\(^T\)), was isolated from phyllosphere of Arrow bamboo (*P. japonica*) collected from Tamilnadu Agricultural University campus, Coimbatore, India (11° N 77° E). The DNA G+C content of the type strain is 52.1 mol\%.

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**References**


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