Paenibacillus arachidis sp. nov., isolated from groundnut seeds

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A Gram-stain-positive, endospore-forming, rod-shaped, facultatively anaerobic bacterium, designated as strain E3T, was isolated from groundnut seeds. Based on the 16S rRNA gene sequence analysis, strain E3T belongs to the genus Paenibacillus with Paenibacillus thailandensis S3-4A1 (96.0 %), Paenibacillus xanthiinlyticus 11N27T (95.7 %), Paenibacillus mendeli C2/2 (95.7 %) and other members of the genus Paenibacillus (<95.5 %) as its closest phylogenetic neighbours. The DNA G+C content of strain E3T was 53 mol%. Strain E3T was positive for gelatin hydrolysis, ammonification, catalase, chitinase production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, HCN production, siderophore production, biofilm formation, and urea and starch hydrolysis. Strain E3T had phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylcholine, an unidentified aminophospholipid, two unidentified aminolipids and two unidentified lipids as polar lipids. Strain E3T had diploptene, depolymerol and bacteriohapaneterol as major hopanoids. anteiso-C15:0 was the predominant cellular fatty acid with significant proportions of iso-C16:0, C16:0, C17:0, anteiso-C17:0, C18:1ω9c and iso-C14:0. Strain E3T had meso-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. On the basis of physiological, biochemical, chemotaxonomic and molecular analysis, strain E3T represents a novel species of the genus Paenibacillus, for which the name Paenibacillus arachidis sp. nov. is proposed. The type strain is E3T (=KCTC 33574T=LMG 28417T).

The genus Paenibacillus is a group of Gram-stain-positive, endospore-forming, rod-shaped, facultatively anaerobic bacteria, which branched off from the genus Bacillus (Ash et al., 1993; Shida et al., 1997; Achouak et al., 1999). Plant-associated species of the genus Paenibacillus have been isolated from roots (Nielsen & Sorensen, 1997; Berge et al., 2002), leaves (De Oliveira Costa et al., 2012) or seeds (Liu et al., 2010). During a survey on the bacteria associated with groundnut seeds, we isolated a strain (E3T) which belongs to the genus Paenibacillus based on 16S rRNA gene sequence analysis, and characterized it using a polyphasic taxonomic analysis as described by Heyndrickx et al. (1996). Species of the genus Paenibacillus are widely recognized as plant growth promoting rhizobacteria, which promote plant growth through an array of mechanisms and are thus exploited in agricultural practices (Oedjijono & Dragar, 1993; Lebuhn et al., 1997; Weid et al., 2002; Cheong et al., 2005; da Mota et al., 2008; Das et al., 2010a; Ghazalibigar et al., 2015).

Strain E3T was isolated from groundnut seed. Briefly, seed coat was removed and the seeds surface sterilized with 2.5 % (w/v) sodium hypochlorite (NaOCl) for 5 min, followed by washing with sterile double-distilled water. The washed seeds were triturated (using a mortar and pestle in a small volume of 1× PBS buffer, pH 7) and diluted to 15 ml with 1× PBS buffer and incubated at 37 °C for 15 min to maintain osmolarity. Two percent of the diluted triturate was added to Luria Bertani (LB) agar and incubated at 37 ±2 °C until colonies appeared. Only one type of colony appeared after 5 days of incubation. The culture was further purified by repeated streaking. The purified culture was...
maintained on LB agar plates and subjected to a polyphasic taxonomic analysis.

Genomic DNA was extracted and purified according to the method of Marmur (1961) and the G+C of the DNA of strain E3 was 53.0 mol% as determined by HPLC (Mesbah et al., 1989). The almost complete length of the 16S rRNA gene sequence of strain E3 was obtained by PCR and subsequent sequencing with primers, F-27 (5′-GTGTTGATCCTGGGCTCAG-3′), and R-1494 (5′-CTACGGGYTACCTTGTTACGAC-3′) as described by Weisburg et al. (1991). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e server (Kim et al., 1999). The relatedness of strain E3 with 16S rRNA gene sequence similarity was calculated using a bootstrap procedure. The combined phylogenetic tree (NJ, ME, ML; Fig. 1) indicated that strain E3 had highest 16S rRNA gene sequence similarity with Paenibacillus thailandensis S3-4A (96.0 %), Paenibacillus xanthinolyticus 11N27 (95.7 %), Paenibacillus mendelli C2T (95.7 %) and other members of the genus Paenibacillus (<95.5 %). The CLUSTAL_W algorithm of MEGA (Tamura et al., 2011) was used for sequence alignments. The phylogenetic analysis of the individual sequences was performed using MEGAS. Distances were calculated by using the Kimura correction in a pairwise deletion manner (Kimura, 1980). Neighbour-joining (NJ), minimum-evolution (ME) and maximum-likelihood (ML) methods in the MEGA-6 software were used to reconstruct phylogenetic trees. Percentage support values were obtained using a bootstrap procedure. The combined phylogenetic tree (NJ, ME, ML; Fig. 1) indicated the relatedness of strain E3 with members of the genus Paenibacillus; however, strain E3 represented a distinct lineage within the paenibacilli. Although P. thailandensis S3-4A showed the highest sequence similarity with strain E3 according to the Ez-Taxon-e result, this species fell altogether into a different clade away from strain E3 (Fig. 1).

Plant growth promoting activities (in vitro) were determined employing standard protocols, viz. phosphate solubilization (Nautiyal, 1999), zinc solubilization (Saravanan et al. (1999), siderophore production (Swyn & Neilands, 1987), HCN production (Lorck, 1948; Joseph et al., 2007), indole-acetic acid production (Brick et al., 1991), 1-aminocyclopropane-1-carboxylate (ACC) deaminase production (Li et al., 2011), chitinase production (Das et al., 2010b) and biofilm formation (O’Toole, 2011). Phenotypic characterization was performed according to standard methods. Morphological characteristics were observed using an Olympus model BH-2 phase-contrast light microscope. Cells of strain E3 were rod shaped, 0.5–0.7 µm in width and 1.4–1.7 µm in length and Gram-stain-positive. Cells of strain E3 appeared as single individuals and were motile. Growth of strain E3 was tested from pH 5 to 10 with an interval of 0.5 with CH₃ COONa/CH₃COOH buffer for pH 4–5, K₂HPO₄/KH₂PO₄ buffer for pH 5.5–8 and NaHCO₃/NaOH buffer for pH 8.5–10. Growth of strain E3 occurred from pH 6.5 to 8.0 (optimum pH 7.0–7.5). Although strain E3 had no obligate requirement for NaCl for growth, it could tolerate up to 2 % when tested from 0 to 5 % (w/v) with an interval of 1 %. Optimum growth occurred at 30 °C (range 30–40 °C) when tested at 4, 10, 15, 25, 30, 35, 40, 45, 50 and 55 °C. Doubling time of strain E3 was 1 h. Various biochemical tests such as hydrolysis of starch, casein, Tween 80, gelatin and urea, oxidase, catalase, nitrate and nitrite reduction, methyl red, Voges–Proskauer, production of H₂S, citrate utilization and acid and gas production from carbohydrates were carried out in the prescribed media as outlined by Cappuccino & Sherman (1998). Strain E3 showed positive reactions for gelatin hydrolysis, ammonification, catalase, urease production and starch hydrolysis. The reaction was negative for methyl red, Voges–Proskauer and citrate utilization. Strain E3 produced chitinase, ACC deaminase, HCN, siderophore and formed biofilm. Growth of strain E3 was independent of yeast extract or added vitamins. Strain E3 could utilize D-fructose, D-ribose, sucrose, starch, trehalose dehydrate, N-acetylglucosamine, D-glucose, sodium pyruvate and D-sorbitol, whereas growth did not occur with L-rhamnose, inositol, D-mannitol, maltose or maleic acid (Table 1). The API ZYM (bioMérieux) kit data analysis indicated that strain E3 was positive for esterase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase and N-acetyl-β-glucosaminidase but negative for alkaline phosphatase, esterase lipase (C8), lipase (C4), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase.

Fatty acids of strain E3 and P. thailandensis KCTC 13043 were analysed from cells grown in LB broth at 37 °C. Cells were harvested by centrifugation (10 000 g for 15 min at 4 °C) on reaching a cell density of 70 % of the maximum optical density (100 % = 0.8 OD₅₄₀ late exponential phase), and the lyophilized pellet was used for analysis. Cellular fatty acids were methylated, separated and identified according to the instructions for the Microbial Identification System (6.0 version; method, RTSBa6; MIDI) (Sasser, 1990; revised78; www.midi-inc.com) which was outsourced through Royal Research Labs, Secunderabad, India. Strain E3 had anteiso-C₁₅:0 and iso-C₁₆:0 as major (>10 %) fatty acids. Minor (>1 and <10 %) amounts of iso-C₁₄:0, iso-C₁₇:0, iso-C₁₉:0, anteiso-C₁₅:0 and anteiso-C₁₇:0 and C₁₆:0/16:0 were also present in strain E3 (Table 1, available in the online Supplementary Material). Major fatty acids were in concurrence with the type species of the genus, Paenibacillus polymyxa LMG 13294. However, strain E3 differed from P. thailandensis KCTC 13043 by the presence of iso-C₁₄:₀, iso-C₁₆:₀, C₁₇:₀ and by the absence of anteiso-C₁₃:₀, cyclo-Δ²₀₈c, iso-C₁₂:₀ and C₁₇:₀ (Table S1).

Cell-wall amino acids were extracted, derivatised and identified using HPLC (McKerrow et al., 2000). Strain E3 has genus-specific meso-diaminopimelamic acid as the diagnostic diamino acid in the cell-wall peptidoglycan (Fig S1). Polylipids were extracted from 1 g freeze-dried cells with methanol/chloroform/saline (2 : 1 : 0.8, by vol.) as described by Kates (1986). Lipids were separated using silica gel TLC (Kieselgel 60 F254; Merck) by two-dimensional chromatography and visualized using standard methods (Kates, 1972;
Novel Paenibacillus with PGPR activities

Paenibacillus arachidis (a.ra’chi.dis. N.L. gen. n. arachidis of Arachis, isolated from Arachis hypogaea).

Colonies on LB media are white. Cells are rod shape, 0.5–0.7 µm wide and 1.4–1.7 µm long, motile and Gram-stain-positive. Mesophilic and facultatively anaerobic. Positive for gelatin hydrolysis, ammonification, catalase, chitinase, ACC deaminase, HCN production, siderophore production, biofilm formation, and urea and starch hydrolysis. Negative reaction for methyl red, indole production, Voges–Proskauer and citrate utilization. Preferred substrates for growth are D-fructose, D-ribose, sucrose, starch, trehalose dehydrate, N-acetylglucosamine, D-glucose, sodium pyruvate and D-sorbitol. Growth does not occur with L-rhamnose, inositol, D-mannitol, maltose or maleic acid. Positive for esterase, acid phosphatase, naphthol-AS-Bl-phosphohydrolase, β-galactosidase, α-glucosidase.

Paenibacillus arachidis sp. nov.

On the basis of physiological, biochemical, chemotaxonomic and molecular properties, strain E3\(^T\) can be differentiated from other members of the genus Paenibacillus and represents a novel species of the genus Paenibacillus, for which the name Paenibacillus arachidis sp. nov. is proposed. As shown in Tables 1 and S1, there are sufficient characteristics which distinguish strain E3\(^T\) from the related species of the genus Paenibacillus to describe a novel species.

Description of Paenibacillus arachidis sp. nov.

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Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain E3\(^T\) and others within the genus Paenibacillus. The tree was reconstructed by the NJ method using the MEGA6 software and rooted by using Bacillus subtilis NCDO 1769\(^T\) (X60646) as the out-group. Numbers at nodes represent bootstrap values (based on 1000 resamplings). Bootstrap percentages refer to NJ/ME/ML analysis. The GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 1 nucleotide substitution per 100 nucleotides.

modified after Tindall, 1990 a, b; Oren et al., 1996). Whole-cell polar lipid analysis revealed the presence of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylcholine as the major polar lipids, with minor amounts of two aminolipids, aminophospholipid and two unidentified lipids (Fig. S2).

Hopanoids were extracted as previously described (Tushar et al., 2014) from 0.2 g lyophilized cells. Cells were sonicated and extracted twice with 10 ml methanol/dichloromethane (DCM)/water (10:5:4, by vol.). The pooled supernatants were finally extracted with DCM. The organic phase was collected and evaporated to dryness, and the lipids extracted were dissolved in 1 ml DCM. Total lipid extract was acetylated using acetic anhydride/pyridine (1:1, v/v) incubated for 30 min at 80 °C. Acetylated extract was analysed by GC-MS (model 7890; Agilent). Separation was achieved using a DB-1HT column (30 m × 0.25 mm i.d.; 0.1 µm film thickness) with helium as carrier gas. The ramping programme was started with 100 °C hold for 2 min, then ramped to 200 °C at 10 °C min\(^{-1}\), from 200 to 360 °C at 6 °C min\(^{-1}\) (held for 10 min). The system was operated with the following parameters: electron voltage 70 eV in electron impact at positive mode, source temperature 200 °C, back inlet temperature 350 °C, and acquisition delay was for 120 s. The mass spectrometer was operated in full scan mode (m/z 30–1000). Strain E3\(^T\) has diploptene, diplopterol and bacteriohopaneterol as major hopanoids.

On the basis of physiological, biochemical, chemotaxonomic and molecular properties, strain E3\(^T\) can be differentiated from other members of the genus Paenibacillus and represents a novel species of the genus Paenibacillus, for which the name Paenibacillus arachidis sp. nov. is proposed. As shown in Tables 1 and S1, there are sufficient characteristics which distinguish strain E3\(^T\) from the related species of the genus Paenibacillus to describe a novel species.

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Table 1. Differentiating characteristics between strain E3\(^T\) and closely related type strains of the genus Paenibacillus

<table>
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<th>2</th>
<th>3</th>
<th>4</th>
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<th>6</th>
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<th>8</th>
<th>9</th>
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<td>Cell size (W×L μm)</td>
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<td>NA</td>
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<td>1.4–1.7</td>
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<td>NaCl optimum (range) (%)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
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<td>0</td>
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<td>NA</td>
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<td>(0–2)</td>
<td>(0–5)</td>
<td>(NA)</td>
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<td>(0–0.5)</td>
<td>(0–2)</td>
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<td>(NA)</td>
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<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
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<td>7</td>
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<td>(6.5–8.0)</td>
<td>(7.5–)</td>
<td>(7–8.5)</td>
<td>(6.0–)</td>
<td>(6–7)</td>
<td>(6–7)</td>
<td>(6.5–9)</td>
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<td>Temperature optimum (range) (°C)</td>
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<td>35</td>
<td>30</td>
<td>25</td>
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<td>–</td>
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<td>+</td>
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<tr>
<td></td>
<td>N-Acetyl glucosamine</td>
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<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>53</td>
<td>50</td>
<td>50.8</td>
<td>50</td>
<td>50.3</td>
<td>50.1</td>
<td>51</td>
<td>51.1</td>
<td>52.9</td>
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and N-acetyl-β-glucosaminidase. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylycholine, two unidentified aminolipids, an aminophospholipid and two unidentified lipids are the polar lipids. Diplopterol, diploptene and bacteriohopaneterol are the major hopanoids. anteiso-C\(_{15:0}\) is the predominant cellular fatty acid with significant proportions of iso-C\(_{16:0}\), iso-C\(_{14:0}\), C\(_{14:0}\) iso-C\(_{15:0}\), C\(_{16:0}\), iso-C\(_{17:0}\), anteiso-C\(_{17:1}\) and C\(_{18:1ω9c}\). meso-diaminopimelic acid is the diagnostic diamino acid in the cell-wall peptidoglycan.

The type strain, E3\(^T\) (=KCTC 33574\(^T\)=LMG 28417\(^T\)), was isolated from groundnut seeds. The DNA G+C content of the type strain is 53 mol%.

Acknowledgements

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