**Jatrophihabitans endophyticus** gen. nov., sp. nov., an endophytic actinobacterium isolated from a surface-sterilized stem of *Jatropha curcas* L.

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A short rod-shaped Gram-stain-positive actinobacterium was isolated as an endophyte from the tissues of *Jatropha curcas* cv. KB27 and was investigated by means of a polyphasic taxonomic approach. An analysis of its 16S rRNA gene sequence indicated that strain S9-650T forms an individual line of descent and is related to certain members of the suborder **Frankineae**, order **Actinomycetales** (<95% sequence similarity). Distance-matrix and neighbour-joining analyses set the branching point of the novel isolate between two clades, one being represented by members of the genera **Frankia** (family **Frankiaceae**) and **Acidothermus** (family **Acidothermaeaceae**) and the other by members of the genera **Geodermatophilus**, **Blastococcus** and **Modestobacter** (family **Geodermatophilaceae**). The organism had meso-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. The acyl type was found to be N-glycolylated. The major menaquinone was MK-9(H4) and the fatty acid profile was characterized by the predominance of iso-C₁₆:0, C₁₈:1ω9c, anteiso-C₁₇:0 and C₁₇:1ω8c. The polar lipids comprised diphosphatidylglycerol, an unidentified glycolipid, phospholipids and aminolipids. The G+C content of the genomic DNA was 71.2 mol%. The distinct phylogenetic position and the phenotypic markers that clearly separate the novel organism from all other members of the suborder **Frankineae** indicate that strain S9-650T represents a novel species in a new genus, for which the name **Jatrophihabitans endophyticus** gen. nov., sp. nov. is proposed. The type strain of the type species is S9-650T (=DSM 45627T = KACC 16232T).

Members of the class **Actinobacteria** are very diverse in their physiological and metabolic properties and morphology, which ranges from coccoid to permanent and highly differentiated branched mycelium (Atlas, 1997; Schrempf, 2001). They are the primary antibiotic-producing organisms exploited by the pharmaceutical industry (Lechevalier & Lechevalier, 1967; Demain, 1998; Bérdy, 2005). They are known as pathogens, plant commensals, nitrogen-fixing symbionts (**Frankia**), non-**Frankia** actinobacteria nitrogen-fixers (Gtari et al., 2012), ferric uptake regulators (Santos et al., 2008) and decomposers of organic materials (Pankratov et al., 2006). **Frankineae** is a suborder below the order **Actinomycetales** (consisting of 13 suborders at the time of writing) within the class **Actinobacteria** which consists of morphologically and biochemically heterogeneous genera readily distinguished from one another by a combination of chemotaxonomic and morphological properties (Table 1).

Members of this suborder have been isolated from various specialized habitats, such as plants, hot springs, acidic rocks in a medieval alum slate mine, stone surfaces, activated sludge and geographically diverse soils. Most of the strains are characterized by low growth rates and fastidious growth requirements. The suborder **Frankineae** (Stackebrandt et al. 1997) currently accommodates 13 genera classified within six families (Garrity et al., 2007): **Frankiaceae** Becking 1970 emend. Hahn et al. 1989 emend. Normand et al. 1996 emend. Stackebrandt et al. 1997 (genus **Frankia** Brunchorst 1886); **Geodermatophilaceae** Normand et al. 1996 emend. Stackebrandt et al. 1997 (genera **Geodermatophilus** Luedemann 1968, **Blastococcus** Ahrens and Moll 1970 and **Modestobacter** Ahrens and Moll 1970).
Table 1. Characteristics that serve to differentiate strain S9-650T from members of genera classified in the suborder Frankineae

<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>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Stem tissue</td>
<td>Alnus</td>
<td>Light red to red</td>
<td>Soil</td>
<td>Marble and calcareous stones</td>
<td>Soil</td>
<td>Soil</td>
<td>Soil</td>
<td>Acidic springs</td>
</tr>
<tr>
<td>Colony colour</td>
<td>White</td>
<td>NA</td>
<td>NA</td>
<td>Pink</td>
<td>Beige to pink</td>
<td>Light-yellow</td>
<td>Greyish-white</td>
<td>Creamy white</td>
<td>Yellow to orange</td>
</tr>
<tr>
<td>Cellular morphology</td>
<td>Short rods</td>
<td>Substrate hyphae; no aerial mycelium; multilocular sporangia</td>
<td>Thallus consisting of cuboid to oval cells; multilocular sporangia; rudimentary hyphae; no aerial mycelium</td>
<td>Rods and cocci</td>
<td>Cocci</td>
<td>Short aerial hyphae; no substrate mycelium</td>
<td>Slender rods, Substrate and aerial mycelia; sporangia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spore/bud formation</td>
<td>–</td>
<td>Sporangiospores</td>
<td>Zoospores</td>
<td>Buds</td>
<td>Buds</td>
<td>–</td>
<td>Coccoid to rod-shaped spores</td>
<td>–</td>
<td>Sporangiospores</td>
</tr>
<tr>
<td>Motility</td>
<td>–</td>
<td>–</td>
<td>+/-</td>
<td>+/-</td>
<td>meso-A2pm</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>A2pm, Ser, Ala</td>
</tr>
<tr>
<td>Cell-wall diamino acid(s)</td>
<td>meso-A2pm</td>
<td>meso-A2pm</td>
<td>–</td>
<td>–</td>
<td>meso-A2pm</td>
<td>–</td>
<td>–</td>
<td>meso-A2pm</td>
<td>meso-A2pm</td>
</tr>
<tr>
<td>Major menaquinone(s)</td>
<td>MK-9(H4), MK-8(H4), MK-9(H6)</td>
<td>MK-9(H4), MK-9(H6)</td>
<td>MK-9(H4)</td>
<td>MK-9(H4), MK-9(H6)</td>
<td>MK-9(H4), MK-8(H4), MK-9(H6)</td>
<td>MK-9(H4), MK-9(H6)</td>
<td>MK-9(H4), MK-8(H4)</td>
<td>MK-9(H4), MK-9(H6)</td>
<td>NA</td>
</tr>
<tr>
<td>Polar lipid(s)*</td>
<td>DPG, PL, AL, GL</td>
<td>PI, PIM, DPG</td>
<td>PE, PIM, PI, DPG</td>
<td>DPG, PG, PI, PE</td>
<td>DPG, PG, PI, DPG</td>
<td>DPG, PE, PE, dimethyl antimycocin</td>
<td>PI, PG, DPG, PE</td>
<td>PI, PG, DPG</td>
<td>PE</td>
</tr>
<tr>
<td>Predominant fatty acid(s)</td>
<td>iso-C16:0, iso-C15:0, anteiso-C17:0, anteiso-C19:0</td>
<td>C18:1ω9c, iso-C16:0, iso-C15:0, anteiso-C17:0, anteiso-C19:0</td>
<td>C18:1ω9c, iso-C16:0, iso-C15:0, anteiso-C17:0, anteiso-C19:0</td>
<td>C18:1ω9c, iso-C16:0, iso-C15:0, anteiso-C17:0, anteiso-C19:0</td>
<td>C18:1ω9c, iso-C16:0, iso-C15:0, anteiso-C17:0, anteiso-C19:0</td>
<td>C18:1ω9c, iso-C16:0, iso-C15:0, anteiso-C17:0, anteiso-C19:0</td>
<td>C18:1ω9c, iso-C16:0, iso-C15:0, anteiso-C17:0, anteiso-C19:0</td>
<td>C18:1ω9c, iso-C16:0, iso-C15:0, anteiso-C17:0, anteiso-C19:0</td>
<td>C18:1ω9c, iso-C16:0, iso-C15:0, anteiso-C17:0, anteiso-C19:0</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>71.2</td>
<td>66–71</td>
<td>73–75</td>
<td>74</td>
<td>68–70</td>
<td>73</td>
<td>71</td>
<td>61</td>
<td>70</td>
</tr>
</tbody>
</table>

*DPG, Diphostatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PE-dimethyl, phosphatidyl(dimethylethanolamine); PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; PL, unknown phospholipid(s); AL, unknown aminolipid(s); GL, unknown glycolipid(s).

Strain S9–650ᵀ was isolated from surface-sterilized stem tissue of Jatropha curcas L. cv. KB–27 collected from the Agrotechnology Experimental Station in Singapore in September 2009. The surface sterilized stem tissue were macerated, diluted serially with 10 mM MgSO₄ and plated onto ammonium mineral salts (AMS) medium (Whittenbury et al., 1970) supplemented with 0.5 % (v/v) methanol and incubated at 30 °C. Colonies that appeared on AMS plates after 96 h were subcultured to obtain pure cultures. The isolate S9–650ᵀ was routinely cultured on AMS or R2A (Difco Laboratories) plates at 30 °C under aerobic conditions and stored frozen at −80 °C in 15 % (v/v) DMSO. Strain S9–650ᵀ was deposited into the Korean Agricultural Culture Collection (KACC) as KACC 16232ᵀ and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) as DSM 45627ᵀ. All physiological and morphological characteristics were observed with cells cultured on R2A under optimal growth conditions, 28–30 °C and pH 6.0–7.0, unless otherwise noted.

Scanning electron microscope observations were performed on fixed material that was prepared for routine examination as described by Bozzola & Russell (1998). Samples were critical-point-dried, mounted on stubs, sputter-coated with gold/palladium and observed under a JEOL-JSM-6360 electron microscope. For transmission electron microscope (TEM) observations, the samples were fixed in 2.5 % glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.2) overnight at 4 °C, washed four times in 0.1 M phosphate buffer and post-fixed in 1 % osmium tetroxide in 0.1 M phosphate buffer for 1 h at 4 °C. The samples were then gradually dehydrated with ethanol, embedded in Spurr’s resin and sectioned on a Leica Ultracut UCT ultramicrotome equipped with diamond knives. The sections were stained with uranyl acetate and lead citrate and observed under a JEOL JEM-12300 electron microscope. Milk coagulation and peptonization were determined by using 20 % (w/v) skimmed milk as the medium and incubation for 3 weeks at 28 °C. Indole and H₂S production were performed in SIM agar (BD) at 30 °C. Voges–Proskauer and methyl red tests were determined as described by Krieg & Padgett (2011).

Optimum conditions for growth were determined by culturing at different temperatures, pH values and NaCl concentrations for up to 14 days. Growth was monitored on R2A broth at 4, 10, 20, 28, 30, 37 and 45 °C. The pH range (pH 4.0–10.0 at intervals of 1.0 pH units) for growth was determined in R2A broth that was buffered with citrate/phosphate buffer or Tris/hydrochloride buffer (Breznak & Costilow, 1994). Tolerance to NaCl (0–5 % w/v) and temperature (4–45 °C) was determined in R2A broth spectrophotometrically from OD₆₀₀ using an Ultraspec 2100 pro UV/Vis Scanning Spectrophotometer (Amersham Biosciences). A Gram stain kit (Difco Laboratories) was used for Gram staining, and oxidase reagent (bioMérieux) was used for the measurement of oxidase activity. Catalase activity was determined by observing the production of oxygen (bubbles) after the application of a 3 % (v/v) hydrogen peroxide solution. Motility was tested by culturing the organism in R2A media that contained 0.4 % soft agar (w/v). Starch hydrolysis was determined with Lugol’s iodine solution after cultivation on R2A plates containing 0.2 % (w/v) starch. For the hydrolysis of carboxymethyl-cellulose and xylan, the isolate was cultured on R2A plates supplemented with 0.5 % (w/v) carboxymethyl-cellulose or 0.5 % (w/v) xylan, respectively. After culturing, the plates were stained with 0.2 % aqueous Congo red dye solution and washed with 1 M NaCl solution in order to observe the zone of clearing. The hydrolysis of casein and L-tyrosine was measured after culturing on R2A plates supplemented with 3 % (w/v) casein and 0.5 % (w/v) L-tyrosine, respectively. A zone of clearing around the bacterial colonies indicated positive activity. Enzyme activities, utilization of carbon sources and acid production from substrates were tested with commercial API ZYM, API 20NE and API 32GN kits (bioMérieux) according to the manufacturer’s protocols. Susceptibility to antibiotics was determined by spread plate techniques. A bacterial suspension (OD₆₀₀ ~1.0) was spread on an R2A plate supplemented with different concentrations (0, 25, 50, 100 and 250 μg ml⁻¹) of the antibiotics kanamycin, ampicillin, carbenicillin, tetracycline, hygromycin, chloramphenicol, rifampicin, spectinomycin and nalidixic acid. Antibiotic sensitivity was observed after 3 days at 30 °C.

Strain S9–650ᵀ was Gram-stain-positive, strictly aerobic, catalase-positive and oxidase-negative and non-motile. Cells of the strain S9–650ᵀ are short rods that are 1.0–1.4 μm long and 0.4–0.63 μm wide and do not have flagella according to TEM examination (Fig. 1a–d). Colonies were white-coloured with a round, convex shape after 3 days of growth at 30 °C on R2A agar. Growth occurred on R2A, nutrient agar (NA; Difco), King’s B medium and M869 (Mergeay et al., 1985), but not on trypticase soy agar (TSA; Difco), LB and 2 × YT. The diameters of colonies on R2A agar plates are 0.2–0.4 mm after 3 days at 30 °C. Strain S9–650ᵀ grew at 20–30 °C,
optimally at 30 °C, but not at over 37 °C. The initial media pH range for the growth of strain S9-650T was pH 5.0–10.0; the optimal pH was 7.0, but strain S9-650T did not grow at under pH 5.0 or over pH 10.0. The strain grew in R2A media that contained 0–2 % (w/v) NaCl, but not in media containing >3 % (w/v) NaCl. Other phenotypic characteristics are given in detail in the species description.

Genomic DNA extractions were carried out according to standard protocol (Wilson, 1997). The 16S rRNA gene was amplified using universal primers 27F and 1492R were used (DeLong, 1992). Cycling conditions were as followed: 95°C for 10min, 30 cycles of 95°C for 1.5 min, 55°C for 1.5 min and 72°C for 1.5 min and final extension for 10 min at 72°C. Sequencing was carried out with a Big Dye Terminator cycle sequencing kit (Perkin Elmer) using primers 27F, 1492R, 785F, 518R and 1100R with an Applied Biosystems 3730 XL DNA sequencer (AB Applied Biosystems). Sequence similarity was analysed against the EzTaxon-e Database (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012) and aligned using the CLUSTAL W tool in MEGA version 4 (Kumar et al., 2004). Phylogenetic analyses were carried out using three treeing algorithms, the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods using MEGA version 5.05 (Tamura et al., 2011) and bootstrap values based on 1000 replications (Felsenstein, 1985). To determine the closest phylogenetic neighbours of strain S9-650T, a continuous stretch (1484 bp) of its 16S rRNA gene sequence was analysed using the EzTaxon-e Database (Kim et al., 2012). Similarity values with type strains of related species indicated that the novel strain is a member of the suborder Frankineae (Garrity et al., 2007; Stackebrandt et al., 1997) with Frankia alni (95.4 % similarity to the type strain), Sporichthya brevicatena (94.1 %), Sporichthya polymorpha (94.0 %), Geodermatophilus ruber (93.6 %), Acidothermus cellulosilyticus (93.6 %), Blastococcus jejuensis (93.3 %), Blastococcus saxobsidens (93.3 %), Modestobacter versicolor (93.2 %) and Saxebacter lacteus (93.1 %). Neighbour-joining, maximum-likelihood and maximum-parsimony methods clearly indicated an individual line of descent for strain S9-650T (Figs 2, S1 and S2, available in IJSEM Online); in view of its phylogenetically deep branching point within the suborder Frankineae, the strain could be considered to represent a novel species in a new genus.

Menaquinones and polar lipids were extracted and analysed by the protocol of Minnikin et al. (1984). For analysis of the whole-cell fatty acid composition, the strain was grown on R2A agar plates for 48 h at 30 °C. The physiological age of the biomass harvested for fatty acid analysis was standardized as far as possible by observing growth development during incubation of the plates and choosing the moment of harvest according to growth state. The cellular fatty acids were extracted, methylated and separated by GC (model 6890; Hewlett Packard) according to the protocol of the Sherlock Microbial Identification System (MIDI; Sasser, 1990). The fatty acid methyl esters were identified and quantified by using the TSBA 6 database (version 6.10) of the Sherlock Microbial Identification System. The fatty acid profile contained iso-C16:0 (68.7 %), C18:1ω9c (5.6 %), anteiso-C17:0 (4.7 %), C17:1ω8c (4.6 %), C16:1ω7c/C16:1ω6c (2.9 %), C17:1ω6c (2.6 %), 10-methyl C17:0 (2.1 %), C18:0 (1.4 %), C16:0 (1.4 %), iso-C18:0
(1.2\%) and \(<1\%\) of each of 11 other fatty acids. The polar lipid profiles contained diphosphatidylglycerol (DPG), unidentified phospholipids (PL1–PL2), aminolipids (AL1–AL2) and glycolipid (GL) (Fig. S3). The quinones of strain S9-650 contained MK-9(H4) as the predominant menaquinone, and also had trace amounts of MK8(H4) and MK-9(H6). The whole-cell hydrolysate of the peptidoglycan preparation contained the amino acid meso-diaminopimelic acid (meso-A2pm). These data indicate that the peptidoglycan type of strain S9-650\(^{T}\) is A1\(_{c}\) (meso-A2pm directly cross-linked) (DSMZ, 2001). The acyl type was N-glycolylated. DNA G+C content was determined by HPLC analysis of deoxyribonucleosides as described by Mesbah et al. (1989), using a reverse-phased column (Supelcosil LC-18 S; Supelco). The DNA G+C content of strain S9-650\(^{T}\) is 71.2 mol%.

Phylogenetic analysis performed on the basis of the 16S rRNA gene sequence revealed strain S9-650\(^{T}\) to be a member of the family Frankiaceae, but it did not form a reliable clade with any of the other members of this family. The strain S9-650\(^{T}\) can be differentiated from closely related species such as Geodermatophilus obscurus DSM 4316\(^{T}\) (CP001867), Geodermatophilus ruber CPCC 201356\(^{T}\) (EU438905), Blastococcus jejensis KST3-10\(^{T}\) (DQ200998), Blastococcus saxobsidens BC448 (AJ316571), Modestobacter marinus 42H12-1\(^{T}\) (EU181225), and Modestobacter versicolor CP153-2\(^{T}\) (AJ871304).

**Fig. 2.** Phylogenetic dendrogram, based on 16S rRNA gene sequences and constructed from evolutionary distances (Kimura, 1980), showing the position of *Jatrophihabitans endophyticus* strain S9-650\(^{T}\) within the radiation of members of the suborder Frankineae, order Actinomycetales (Stackebrandt et al., 1997). Filled circles at nodes indicate generic branches that were also recovered by using maximum-parsimony algorithms. Numbers at branching points refer to bootstrap percentages (based on 1000 resamplings); only values above 50% are shown. GenBank accession numbers for the sequences are in parentheses. Bar, 0.01 substitutions per nucleotide position.
related genera within the family Frankiaceae by its possession of unique fatty acids and also on the basis of other chemotaxonomic characteristics such as fatty acids, polar lipids, menaquinones and peptidoglycan structure (Table 1). On the basis of the phylogenetic analysis results and chemotaxonomic characteristics, strain S9-650T represents a novel species in a new genus, for which the name Jatrophihabitans endophyticus gen. nov., sp. nov. is proposed.

**Description of Jatrophihabitans gen. nov.**

*Jatrophihabitans* (Ja.tro.phi.ha’bi.tans. N.L. n. *Jatropha* scientific name of a botanical genus; L. part. adj. *habitan* inhabiting; N.L. part. adj. used as a masc. n. *Jatrophihabitans* inhabitant of *Jatropha*).

Gram-stain-positive, aerobic, non-motile, non-spore-forming rod-shaped, catalase-positive and oxidase-negative. The predominant menaquinone is MK-9(H4). The polar lipids comprise diphosphatidylglycerol and unidentified glycolipid, phospholipids (PL1–PL2) and aminolipids (AL1–AL2). The peptidoglycan contains *meso*-A2pm and is of the A1γ type. The acyl type is *N*-glycolylated. The cellular fatty acid profile is characterized by the predominance of iso-C16:0, C18:1ω9c, anteiso-C17:0 and C17:1ω8c. Phylogenetically, the genus is affiliated to the suborder Frankineae. The type species is *Jatrophihabitans endophyticus* sp. nov.

**Description of Jatrophihabitans endophyticus sp. nov.**

*Jatrophihabitans endophyticus* (en.do.phy’ti.cus. Gr. pref. *endo* within; Gr. n. *phyton* plant; L. masc. suff. -icus adjectival suffix used with the sense of belonging to; N.L. masc. adj. *endophyticus* within plant, endophytic, pertaining to the original isolation from plant tissues).

Gram-stain-positive, aerobic, non-motile, short rods (1.0–1.4 μm long, 0.4–0.63 μm wide), strictly aerobic, catalase-positive and oxidase-negative. Colonies are white-coloured with a round, convex shape after 3 days of growth at 30°C on R2A agar. Growth occurs on M869 agar, KB and nutrient agar at 30°C or higher. Good growth occurs between 20 and 30°C. The pH range for growth is pH 5–10, with optimal growth at pH 7. Starch, casein and peptonization. In API-32GN assays, positive for aesculin hydrolysis and negative for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease, gelatin hydrolysis, β-galactosidase, D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. In API ZYM assays, positive for alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase and β-glucosidase. Negative for lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase α-mannosidase and α-fucosidase. The type strain is highly resistant (up to 250 μg ml⁻¹) to kanamycin, ampicillin and carbenicillin and but sensitive (lowest concentration) to tetracycline (25 μg ml⁻¹), hygromycin (50 μg ml⁻¹), chloramphenicol (50 μg ml⁻¹), rifampicin (25 μg ml⁻¹), spectinomycin (50 μg ml⁻¹) and nalidixic acid (100 μg ml⁻¹). The DNA G+C content is 71.2 mol%.

The type strain, S9-650T (=DSM 45627T=KACC 16232T), was isolated from surface sterilized stem tissue of *Jatropha curcas* L. KB-27 collected from Jatropha farm, Agrotechnology Experimental Station at Lim Chu Kang, Singapore. The G+C content of the genomic DNA of the type strain is 71.2 mol%.

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**References**


Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using


