Allokutzneria oryzae sp. nov., isolated from rhizospheric soil of *Oryza sativa* L.

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The taxonomic status of a rhizospheric soil actinomycete, designated R8-39¹, was established using a polyphasic approach. The organism had phenotypic and morphological characteristics consistent with its classification in the genus *Allokutzneria*. Phylogenetic analysis based on an almost complete 16S rRNA gene sequence showed that the strain formed a monophyletic clade with the type strains of members of the genus *Allokutzneria*. Strain R8-39¹ displayed the highest levels of 16S rRNA gene sequence similarity to *Allokutzneria albata* DSM 44149¹ (98.8 %) and *Allokutzneria multivorans* YIM 120521¹ (98.3 %). However, the DNA–DNA hybridization values between strain R8-39¹ and *A. albata* and *A. multivorans* were clearly below the 70 % threshold. The organism was found to have chemical characteristics consistent with its classification in the genus *Allokutzneria*. Whole-cell hydrolysates contained meso-diaminopimelic acid, arabinose, galactose, glucose, mannose, rhamnose and ribose. The main menaquinone was MK-9(H4). No mycolic acid was detected. The G+C content of the genomic DNA was 71.8 mol%. In addition, strain R8-39¹ had a phenotypic profile that readily distinguished it from recognized representatives of the genus *Allokutzneria*. It is evident from the combined genotypic and phenotypic properties that strain R8-39¹ represents a novel species of the genus *Allokutzneria*. The proposed name for this species is *Allokutzneria oryzae* sp. nov.; the type strain is R8-39¹ (=BCC 60399¹=NBRC 109649¹).

The genus *Allokutzneria* belongs to the family *Pseudonocardiaeae*, with *Allokutzneria albata* as the type species. In 1993, Tomita *et al.* proposed a novel species, *Kibdelosporangium albatum*, which produced the highly active antiviral antibiotics, cycloviracins (Tomita *et al.*, 1993). Later, Labeda & Kroppenstedt (2008) found that this species was misplaced within the genus *Kibdelosporangium* based on 16S rRNA gene sequence and chemotaxonomic analyses. The strain was then transferred to the newly proposed genus *Allokutzneria*, referring to the fact that it was phylogenetically close to *Kutzneria* but chemotaxonomically distinct. Members of this genus are Gram-positive bacteria that form well-branched substrate and aerial mycelia. The aerial mycelia contain long chains of spores and sporangium-like globular bodies containing hyphae. The whole-cell sugars consist of arabinose, galactose, glucose, mannose, rhamnose and ribose. The main menaquinone was MK-9(H₄). No mycolic acid was detected. The G+C content of the genomic DNA was 71.8 mol%. In addition, strain R8-39¹ had a phenotypic profile that readily distinguished it from recognized representatives of the genus *Allokutzneria*. It is evident from the combined genotypic and phenotypic properties that strain R8-39¹ represents a novel species of the genus *Allokutzneria*. The proposed name for this species is *Allokutzneria oryzae* sp. nov.; the type strain is R8-39¹ (=BCC 60399¹=NBRC 109649¹).
2008) and *Allokutzneria multivorans*, isolated from soil at the banks of the Nujiang River, south-west China (Cao et al., 2013).

During a programme of isolation of actinomycetes from the rhizospheric soil of rice plants (*Oryza sativa* L.), strain R8-39\(^T\) was isolated using the dilution plating method on acidified starch casein agar plates (pH 5.5; Küster & Williams, 1964) supplemented with ketoconazole, nalidixic acid and nystatin at final concentrations of 100, 25 and 50 \(\mu\)g ml\(^{-1}\), respectively. The plates were incubated at 28 °C for 4 weeks. The strain was isolated and purified on glucose yeast extract agar [GYE agar; containing 1.0 % (w/v) glucose, 1.0 % (w/v) yeast extract and 1.5 % (w/v) agar], pH 5.5. Pure culture was maintained as a 20 % (v/v) glycerol suspension at \(-20\) °C or as lyophilized cells for long-term storage. The type strains of species of the genus *Allokutzneria*, *A. albata* NBRC 101910\(^T\) and *A. multivorans* DSM 45532\(^T\), were used in this study for comparison of physiological, morphological and chemotaxonomic properties and DNA–DNA hybridization.

Cultural characteristics of strain R8-39\(^T\) were examined on ISP media 2, 3, 4, 5, 6 and 7 (Shirling & Gottlieb, 1966) and GYE agar. The colour of mycelium and soluble pigment was determined by comparing its colour with the colour chips from the Colour Harmony Manual (Jacobson et al., 1958). Morphological characteristics were observed by using a light microscope fitted with a long-working-distance objective and a scanning electron microscope (model JSM-5600, JEOL), after cultivation on ISP medium 5 (Shirling & Gottlieb, 1966) at 27 °C for 10 days. The temperature range for growth was determined on ISP medium 2 using a temperature gradient incubator (Tokyo Kagaku Sangyo). The ability to grow over a range of pH was examined on ISP medium 2 adjusted to an appropriate pH with the following buffer system: 0.1 M citric acid/0.1 M sodium citrate (pH 4–5); 0.1 M KH\(_2\)PO\(_4\)/0.1 M NaOH (pH 6–8); 0.1 M NaHCO\(_3\)/0.1 M Na\(_2\)CO\(_3\) (pH 9–10). Acid production from carbon sources was determined using media and methods described by Gordon et al. (1974). Enzyme activity profiles were determined using the API ZYM system (bioMerieux) according to the manufacturer’s instructions. Urease activity was determined by a colour change on urea agar (Gordon et al., 1974). Hydrolysis of casein and nitrate reduction were examined using the methods of Gordon & Mihm (1957). The degradation of (w/v) cellulose (1.0 %), guanine (0.4 %), hypoxanthine (0.4 %), starch (1.0 %), Tween 80 (1.0 %), L-tyrosine (0.4 %), xanthine (0.4 %) and xylan (0.4 %) was assessed using standard procedures (Gordon & Mihm, 1957; Gordon et al., 1974). Degradation of adenine, aesculin, allantoin, arbutin and gelatin was determined as described by Gordon et al. (1974) and Williams et al. (1983). Catalase and oxidase tests were determined using 3 % (v/v) hydrogen peroxide and 1 % (w/v) tetrathymethyl-p-phenylenediamine dihydrochloride solution, respectively. Melanin production was determined on ISP media 6 and 7 by the method of the International *Streptomyces* Project (Shirling & Gottlieb, 1966). The production of hydrogen sulphide was detected on trypticase soy broth (Merck) by using lead acetate strips. NaCl tolerance was examined on ISP medium 2 containing NaCl at final concentrations of 0–10 % (w/v) (at intervals of 1.0 %).

Biomass for chemotaxonomic analyses was obtained by growing the strain in DSMZ medium no. 554 broth [containing 1.0 % (w/v) glucose, 2.0 % (w/v) soluble starch, 0.5 % (w/v) yeast extract, 0.5 % (w/v) N-Z-amine and 0.1 % (w/v) CaCO\(_3\)], incubated with shaking at 27 °C for 5 days. The cells were washed three times with distilled water before freeze-drying. Standard procedures were used to determine the isomers of diaminopimelic acid (Hasegawa et al., 1983). The acyl type of the cell wall was analysed according to the method of Uchida & Aida (1984). Whole-cell sugars were analysed according to the method of Becker et al. (1965). Polar lipids were examined by TLC using the method of Minnikin et al. (1977). Menaquinoes were extracted and purified by the method of Collins et al. (1977), and isoprene units were subsequently analysed by LC/MS (JMS-T100LP, JEOL) with a PEGASIL ODS column (2\(\times\)50 mm) using methanol/2-propanol (7:3, v/v). Mycolic acids were detected by TLC according to the method of Tomiyasu (1982). Fatty acid methyl esters were determined by GLC according to the instructions of the Sherlock Microbial Identification System (Microbial ID; MIDI version 6.1) (Sasser, 1990) and identified with the RTSBA6 database. The G+C content (mol%) of the DNA was determined by HPLC according to the method of Tamaoka & Komagata (1984). DNA–DNA hybridization was performed by the photobiotin-labelling method of Ezaki et al. (1989).

Total DNA of strain R8-39\(^T\) was extracted and purified following the method of Kieser et al. (2000). The 16S rRNA gene was amplified as described by Mingma et al. (2014). The PCR products were purified and sequenced (Macrogen, Korea) using universal primers (Lane, 1991). An almost complete 16S rRNA gene sequence (1465 bp) was aligned with the corresponding sequences of representative genera in the family *Pseudonocardia*, retrieved from the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). Evolutionary trees were inferred using maximum-parsimony (Fitch, 1971), maximum-likelihood (Felsenstein, 1993) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms drawn from the MEGA 5 package (Tamura et al., 2011).

Strain R8-39\(^T\) was a Gram-stain-positive, aerobic, filamentous actinobacterium. The strain exhibited good growth on ISP medium 2, ISP medium 3 and GYE agar, with brownish-grey aerial mycelium and deep-brown substrate mycelium after 14 days at 27 °C. Moderate growth occurred on ISP medium 4, ISP medium 5 and ISP medium 7. No soluble pigment was detected in any of the media tested except GYE agar, on which the strain produced a pale yellowish-brown, soluble pigment. The aerial mycelium formed long straight chains of spores. The spores were non-motile rods with smooth surfaces (Fig. 1a). Sporangium-like bodies were formed on aerial mycelium (Fig. 1b).

The phylogenetic analysis of an almost complete 16S rRNA gene sequence (1465 bp) of strain R8-39\(^T\) indicated that
the isolate formed a distinct cluster with *A. albata* and *A. multivorans*, with a 92 % bootstrap value (Fig. 2). It was evident that this strain was affiliated within the genus *Allokutzneria*. The highest 16S rRNA gene sequence similarities were found between strain R8-39<sup>T</sup> and its nearest neighbours, *A. albata* DSM 44149<sup>T</sup> (98.8 %, which corresponds to 17 nt differences over 1464 positions) and *A. multivorans* YIM 120521<sup>T</sup> (98.3 %, which corresponds to 24 nt differences over 1461 positions). DNA–DNA relatedness studies provide a reliable way of distinguishing between representatives of species that share high 16S rRNA gene sequence similarity (Stackebrandt & Ebers, 2006). In this study, DNA–DNA hybridization experiments were repeated twice, on different days. Strain R8-39<sup>T</sup> showed a mean DNA–DNA relatedness value of 41.5 % with *A. albata* NBRC 101910<sup>T</sup> and 35 % with *A. multivorans* DSM 45532<sup>T</sup>; values well below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the delineation of genomic species.

The results of chemical analyses indicated that strain R8-39<sup>T</sup> has chemotaxonomic markers typical of the genus *Allokutzneria* (Labeled & Kroppenstedt, 2008). The strain contained *meso*-diaminopimelic acid in its peptidoglycan. The menaquinones found were MK-9(H<sub>4</sub>) (88.0 %), MK-8(H<sub>4</sub>) (8.5 %), MK-9(H<sub>2</sub>) (1.2 %), MK-9(H<sub>6</sub>) (1.2 %) and MK-9(H<sub>8</sub>) (1.1 %). Arabinose, glucose, galactose, mannose, rhamnose and ribose were detected in the whole-cell hydrolysates. Polar lipid analysis showed that this organism contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidyldimethylmethylethanolamine, hydroxyphosphatidyldimethylmethylethanolamine and phosphatidylinositol. The cellular fatty acid profile was represented by the predominance of iso-C<sub>16:0</sub> (48.4 %). Fatty acids found in smaller proportions included iso-C<sub>15:0</sub> (9.2 %), iso-C<sub>17:0</sub> (4.7 %), C<sub>17:0</sub> 3-OH (4.6 %), anteiso-C<sub>17:0</sub> (4.5 %), iso-C<sub>16:1</sub> H (4.0 %), C<sub>16:1</sub>ω7c, C<sub>16:1</sub>ω6c (3.5 %), C<sub>17:1</sub>ω8c (1.8 %), iso-C<sub>14:0</sub> (1.7 %), anteiso-C<sub>17:1</sub> A (1.2 %), anteiso-C<sub>15:1</sub> (1.22 %), C<sub>15:1</sub>ω6c (0.8 %), iso-C<sub>16:0</sub> 3-OH (0.7 %), C<sub>18:1</sub>ω9c (0.7 %), C<sub>17:0</sub> cyclo (0.6 %) and iso-C<sub>14:0</sub> 3-OH (0.5 %). Mycolic acids were not detected. The G+C content of the DNA was 71.8 mol%.

The assignment of strain R8-39<sup>T</sup> to the genus *Allokutzneria* was supported by the biochemical, chemotaxonomic, morphological and physiological properties. Strain R8-39<sup>T</sup> was positive for catalase and oxidase. Melanin pigment was not produced. Observation of cultures over 14 days revealed growth in a temperature range of 14 to 41 °C, with the optimum temperature for growth at 26–36 °C. The strain was able to grow at pH 5.0–10.0. NaCl was tolerated up to 5 %. Strain R8-39<sup>T</sup> was readily differentiated from its closest phylogenetic neighbours, *A. albata* and *A. multivorans*, on the basis of physiological properties (Table 1), including the colour of aerial spore mass and substrate mycelium on ISP medium 2. Strain R8-39<sup>T</sup> produced a brownish-grey aerial spore mass, whereas those of *A. albata* and *A. multivorans* were white to light yellow. Strain R8-39<sup>T</sup> produced acid from melibiose and raffinose, whereas *A. albata* and *A. multivorans* did not. Strain R8-39<sup>T</sup> and *A. multivorans* did not produce acid from (±)-l-arabinose or β-lactose, which supported acid production in *A. albata*. Enzyme activity tests of strain R8-39<sup>T</sup> were positive for β-glucosidase, α-mannosidase and trypsin, whereas *A. albata* gave negative results. Moreover, strain R8-39<sup>T</sup> could tolerate up to 5 % NaCl compared to 4 % in both *A. albata* and *A. multivorans*.

Strain R8-39<sup>T</sup> differed from other closely related species of the genus *Allokutzneria* not only by physiological characteristics, but also by chemotaxonomic characteristics. Glucose and rhamnose were found in whole-cell hydrolysates of strain R8-39<sup>T</sup> and *A. multivorans* but not in *A. albata*. In addition, MK-8(H<sub>4</sub>) was present only in R8-39<sup>T</sup>, while MK-9 was present only in *A. multivorans*.

On the basis of phenotypic, chemotaxonomic and phylogenetic data, strain R8-39<sup>T</sup> can be distinguished from *A. albata* and *A. multivorans*. We conclude that strain R8-39<sup>T</sup> represents a novel species of the genus *Allokutzneria* for which the name *Allokutzneria oryzae* sp. nov. is proposed.
**Description of *Allokutzneria oryzae* sp. nov.**

*Allokutzneria oryzae* (o.ry’zae. L. gen. n. oryzae of rice).

Aerobic, Gram-stain-positive, non-acid-fast actinomycete. Cells are filamentous; well-branched substrate and aerial mycelia. The aerial mycelium is brownish grey, and the substrate mycelium is deep brown on ISP medium 2, 3, 4 and GYE agar. No soluble pigment is produced on tested media except GYE agar, in which pale yellowish-brown soluble pigment is produced. Melanin is not detected. The aerial mycelium produces long chains of spores and sporangium-like globular bodies, 8–20 μm in diameter, containing hyphae. The spores are cylindrical with smooth surfaces. Catalase, nitrate reduction, oxidase and urease are positive. Hydrogen sulphide is not produced. Acid is produced from adonitol, (+)-cellobiose, (-)-d-fructose, fucose, (+)-d-galactose, glucose, myo-inositol, levulose, maltose, (-)-d-mannitol, melibiase, raffinose, (-)-l-rhamnose, (-)-d-ribose, sucrose and (+)-d-xylene. No acid production from (+)-l-arabinose, β-lactose, (-)-d-sorbitol, sorbose or xylitol. Aesculin, arbutin, casein (skimmed milk), hypoxanthine, L-tyrosine, starch and Tween 80 are degraded. Adenine, allantoin, cellulose and xylan are not degraded. Growth occurs at pH 5.0–10.0 with an optimum at pH 7.0–8.0. The temperature range for growth in ISP medium 2 is 14–41 °C with an optimum at 26–36 °C. Good growth occurs in ISP medium 2 with 0–2 % NaCl; no growth with 6 % NaCl. Acid phosphatase, alkaline phosphatase, chymotrypsin, cystine arylamidase, esterase (C4), β-galactosidase, z-glucosidase, β-glucosidase, leucine arylamidase, esterase lipase (C8),...
N-acetyl-β-glucosaminidase, naphthol-AS-BI-phosphohydrolase, α-mannosidase, trypsin and valine arylamidase are detected with the API ZYM enzyme assay. α-Fucosidase, β-galactosidase, β-glucuronidase and lipase (C14) are negative. The diagnostic diamino acid of the peptidoglycan is meso-diaminopimelic acid. Whole-cell sugars are arabinose, glucose, galactose, mannose, rhamnose and ribose. The glycan moiety of the murein is acetylated. Phospholipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylmethylethanolamine, hydroxyphosphatidylmethylethanolamine and phosphatidylinositol. The major menaquinone is MK-9(H4). Mycolic acids are not detected. The fatty acids profile is represented by the predominance of iso-C16:0.

The type strain, R8-39\textsuperscript{T} (=BCC 60399\textsuperscript{T}=NBRC 109649\textsuperscript{T}), was isolated from rhizospheric soil of a rice plant (*Oryza sativa* L.), collected from Pathumthani province, Thailand. The G+C content of the type strain DNA is 71.8 mol%.

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### References


