Microbacterium azadirachtae sp. nov., a plant-growth-promoting actinobacterium isolated from the rhizoplane of neem seedlings

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Microbacterium strain AI-S262^T was isolated from the rhizoplane of neem seedlings in the Botanical garden of Tamilnadu Agricultural University, Coimbatore, India, and subjected to phenotypic, chemotaxonomic and genetic characterization. Cells of this strain were Gram-stain-positive, motile, non-spore-forming, short rods and formed light-yellow-pigmented colonies on nutrient agar. Strain AI-S262^T contained MK-12 and MK-13 as the main respiratory quinones, anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} as the predominant fatty acids, peptidoglycan-type B2β with glycolyl residues, and had a DNA G + C content of 69.5 mol%. A phylogenetic analysis based on 16S rRNA gene sequences showed 98.0–98.6 % pair-wise similarity with respect to close relatives in the genus *Microbacterium*. DNA–DNA hybridization experiments revealed a low level of DNA–DNA relatedness (less than 39%) between strain AI-S262^T and its closest relatives. Data from DNA–DNA hybridization and phenotypic analyses supported the conclusion that strain AI-S262^T represents a novel species in the genus *Microbacterium*, for which the name *Microbacterium azadirachtae* sp. nov. is proposed. The type strain is AI-S262^T (=JCM 15681^T =LMG 24772^T =KCTC 19668^T).

Members of the genus *Microbacterium* can be isolated from a wide range of different environmental habitats from soil to insects to human clinical specimens to marine environments (Evtushenko & Takeuchi, 2006; Park *et al.*, 2008; Bakir *et al.*, 2008; Takeuchi & Hatano, 1998a, b; Lee *et al.*, 2006; Shivaji *et al.*, 2007; Collins & Bradbury, 1992) and plants, having been reported from the phyllosphere of sugar beet and spring wheat, and as endophytes in sweet corn and cotton (Thompson *et al.*, 1993; Legard *et al.*, 1994; McNroy & Kloepper, 1995). The genus *Microbacterium*, established by Orla-Jensen (1919) and emended

by Collins *et al.* (1983), comprises a diverse collection of Gram-positive, non-spore-forming, rod-shaped bacteria. More recently, the genus was further emended to include members of the genus *Aureobacterium* (Takeuchi & Hatano, 1998b) and, at the time of writing, it included 61 recognized species (<http://www.bacterio.cict.fr/m/microbacterium.html#aerolatum>) isolated from different environmental sources. This paper describes a novel, plant-growth-promoting species of the genus *Microbacterium*, *Microbacterium azadirachtae* sp. nov., isolated from the rhizoplane of neem seedlings.

Strain AI-S262^T was isolated from the rhizoplane of neem seedlings by the dilution-plating technique on R2A medium (Reasoner & Geldreich, 1985; Difco) and was maintained as 50 % glycerol stocks at –80 °C. Carbon source utilization pattern was studied by using Biolog GP2 Microplates following the manufacturer's instructions and acid production from various sugars was tested as described by Smith *et al.* (1952) and Madhaiyan *et al.*

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; IAA, indole-3-acetic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AI-S262^T is EU912487.

An extended phylogenetic tree, a scanning electron micrograph of cells of strain AI-S262^T, and cellular fatty acid compositions of strain AI-S262^T and type strains of related species are available with the online version of this paper.

(2007b). Gram staining was performed by using the Gram staining kit (Difco), and bacterial suspensions were examined by phase-contrast microscopy for cell morphology and motility. The pH range for growth was determined on R2A agar. The growth temperature range (4, 10, 20, 25, 30, 37 and 45 °C) and NaCl tolerance [0, 1, 2, 3, 4, 5 and 10 % (w/v)] were tested on R2A medium. All tests were incubated at 30 °C, unless otherwise indicated. Growth on nutrient agar, trypticase soy agar (Difco) and MacConkey agar was also evaluated at 30 °C. Catalase activity was determined by assessing bubble production in 3 % (v/v) H₂O₂, and oxidase activity was determined with 1 % (w/v) tetramethyl-*p*-phenylenediamine, by using Difco BBL catalase and oxidase reagent droppers according to the manufacturer's instructions. Tests for the hydrolysis of starch, casein, lipids, CM-cellulose, gelatin, aesculin and pectin were performed and evaluated after 5 days (Atlas, 1993; Kouker & Jaeger, 1987; Ten *et al.*, 2004). Colony and cell morphology were determined by using phase-contrast microscopy. Sample preparation and other procedures for scanning electron microscope (SEM) observations were performed as described by Madhaiyan *et al.* (2007b) and the samples were visualized using a Hitachi S-2500C SEM with GEMINI column equipped with a field emission source. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v) and purified by using TLC on Kieselgel 60 F₂₅₄ plates (20 × 20 cm, 0.5 mm thick; Merck) with petroleum ether/diethyl ether (9:1, v/v) as the solvent. The quinones were identified by using reversed phase HPLC analysis, as described by Shin *et al.* (1996). Purified cell-wall preparations were obtained as described by Schleifer & Kandler (1972). Amino acids and peptides in cell-wall hydrolysates were analysed by two-dimensional TLC on cellulose plates by using solvent systems described by Schleifer & Kandler (1972). The cellular fatty acids were extracted from cultures grown in R2A medium at 28 °C for 4 to 5 days, derivatized to methyl esters, and analysed by a Gas Chromatograph (Hewlett Packard 6890) using the Microbial Identification System (MIDI; Microbial ID) software package, according to standard protocols (Sasser, 1990). Determination of minimal inhibitory concentrations of heavy metals and antibiotic resistance patterns was carried out as previously described (Madhaiyan *et al.*, 2007a; Chanprame *et al.*, 1996). The quantitative assay for estimation of indole-3-acetic acid (IAA) and plate assays for detecting the presence of siderophore production, phosphate solubilization, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and sodium thiosulfate oxidation were carried out as previously described (Poonguzhali *et al.*, 2006). A gnotobiotic assay using growth pouches was performed to measure the root elongation induced by ACC deaminase. Tomato and canola seeds were used for this root elongation assay, since they are representative of ethylene sensitive plants (Glick *et al.*, 1994; Li *et al.*, 2000; Penrose & Glick, 2003; Ghosh *et al.*, 2003; Madhaiyan *et al.*, 2006, 2007b).

Cells of strain AI-S262^T were aerobic, motile, non-endospore forming, Gram-stain-positive short rods (0.3–0.4 × 0.6–0.7 µm), occurring singly or in pairs on solid R2A medium and forming light-yellow, smooth and circular colonies with entire margins. Physiological, biochemical and chemotaxonomic characteristics of strain AI-S262^T are given in the species description; those that serve to distinguish this strain from its closest phylogenetic neighbours are listed in Table 1. The major fatty acids of strain AI-S262^T were anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{16:0} and iso-C_{15:0} (Supplementary Table S1, available in IJSEM Online). Strain AI-S262^T exhibited ACC deaminase activity, phosphate solubilization and sulfur oxidation when examined through plate assays but was negative for siderophore production. Strain AI-S262^T produced IAA and ACC deaminase activity at a concentration of 12.3 µg ml⁻¹ and a rate of 20.2 nmol α-ketobutyrate min⁻¹ (mg protein)⁻¹, respectively. From the gnotobiotic growth pouches assay, the root lengths of the AI-S262^T-treated tomato (16.2 % increase over control) and canola (45.7 % increase over control) seeds were comparatively greater when compared with the uninoculated control (data not shown).

Chromosomal DNA was extracted according to the kit instructions (QIAamp DNA mini kit, Qiagen). The 16S rRNA gene was amplified using the universal primers 27F and 1492R as described by Madhaiyan *et al.* (2009) and the partial gene sequence was determined by the fluorescent dye terminator method using the ABI prism Big dye terminator cycle sequencing ready reaction kit (version 3.1); products were run on an ABI3730XL capillary DNA sequencer (ABI Prism 310 Genetic Analyzer). The resultant 16S rRNA gene sequence was compared with representative organisms from the same and related genera from GenBank and was aligned by using CLUSTAL W (Thompson *et al.*, 1994) multiple alignments. Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods, using the MEGA 4.1 program (Tamura *et al.*, 2007), and bootstrap values were based on 1000 replications (Felsenstein, 1985). DNA–DNA hybridizations were carried out on nitrocellulose membrane filters according to the method of Seldin & Dubnau (1985). The DIG-High prime system and DIG luminescent detection kit (Roche Diagnostics) were used for labelling the probe DNA and visualization. Hybridization temperatures were 60 and 65 °C and the DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad). The G + C content of the genomic DNA was determined by HPLC analysis using a reversed-phase column (Supelcosil LC-18 S, Supelco) of individual nucleosides as described by Mesbah *et al.* (1989).

Preliminary sequence comparisons with 16S rRNA gene sequences held in GenBank indicated that the isolate was closely related to the genus *Microbacterium*. In the phylogenetic tree (Fig. 1; an extended version of this tree is available as Supplementary Fig. S1), strain AI-S262^T clustered within the genus *Microbacterium* of the class

Table 1. Phenotypic characteristics that differentiate strain AI-S262^T from related species of the genus *Microbacterium*

Strains: 1, AI-S262^T; 2, *Microbacterium resistens* DMMZ 1710^T; 3, *M. testaceum* DSM 20166^T; 4, *M. aerolatum* V-73^T; 5, *M. esteraromaticum* DSM 8609^T; 6, *M. arabinogalactanolyticum* DSM 8611^T; 7, *M. foliorum* DSM 12966^T; 8, *M. phyllosphaerae* DSM 13468^T; 9, *M. paraoxydans* CF36^T; 10, *M. natoriense* TNJL143-2^T. The cell wall contains ornithine as diamino acid and the peptidoglycan type is B2 β for all strains except *M. resistens* DMMZ 1710^T (B2 α). +, Positive; –, negative; w, weakly positive; y, yellow; yo, yellow–orange; yw, yellow–white; ly, light-yellow. All data were obtained in this study.

Characteristic	1	2	3	4	5	6	7	8	9	10
Colony colour	LY	Y	YO	Y	YW/Y	YW/w	Y	Y	LY	Y
Growth in 6.5 % NaCl	–	–	+	+	w	w	–	–	+	+
Growth at 37 °C	+	+	–	+	+	–	w	w	+	+
Motility	+	–	+	–	+	–	+	+	–	–
H ₂ S production	–	–	+	–	+	–	–	–	–	–
Nitrate reduction	–	–	+	+	–	+	–	–	–	–
Catalase	+	+	+	+	–	+	+	+	+	+
Oxidase	–	–	–	–	–	–	–	–	–	–
Hydrolysis of:										
Casein	+	+	+	–	–	–	–	+	+	–
Gelatin	–	+	+	–	–	+	+	+	+	+
Starch	–	–	–	+	+	+	w	w	–	+
Aesculin	+	+	–	–	–	–	+	+	+	+
Assimilation of:										
L-Alanine	+	w	+	–	+	+	+	+	w	+
L-Arabinose	+	–	+	+	+	w	+	+	+	+
Citrate	+	+	+	–	–	–	w	–	+	w
D-Malate	+	–	+	–	+	+	+	+	–	–
Propionate	–	+	w	+	+	+	+	+	+	w
Acid production from:										
Glucose	+	+	–	+	–	+	+	+	+	+
Rhamnose	+	+	–	+	–	+	–	+	–	–
Menaquinones (MK)	12,13	12,13	10,11	12,13	12,13	12,13	10,11,12	10,11,12	11,12	9,10,11,12
G + C content (mol%)	69.5	71.6	67.7	69.7	68.8	69.3	67	64	69.9	69.1

Actinobacteria. Strain AI-S262^T showed the highest 16S rRNA gene sequence similarity to the type strains of *Microbacterium aerolatum* (98.6 % pairwise similarity),

M. resistens (98.5 %), *M. testaceum* (98.3 %), *M. foliorum* (98.3 %), *M. phyllosphaerae* (98.3 %), *M. natoriense* (98.3 %), *M. paraoxydans* (98.2 %), *M. esteraromaticum*

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparison showing the position of strain AI-S262^T and other related species of the genus *Microbacterium*. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets. Bootstrap values below 50 % are not shown. Bar, 0.005 substitutions per site.

(98.2%) and *M. arabinogalactanolyticum* (98.0%). Strain AI-S262^T showed a low level of DNA–DNA relatedness, that ranged between 11 and 39%, with its closest relatives *M. aerolatum* V-73^T (39 ± 2.9%), *M. resistens* DMMZ 1710^T (34 ± 2.1%), *M. foliorum* DSM 12966^T (32 ± 2.3%), *M. paraoxydans* CF36^T (31 ± 2.0%), *M. esteraromaticum* DSM 8609^T (28 ± 1.4%), *M. testaceum* DSM 20166^T (24 ± 1.6%), *M. phyllosphaerae* DSM 13468^T (17 ± 1.0%), *M. arabinogalactanolyticum* DSM 8611^T (16 ± 1.2%) and *M. natoriense* TNJL143-2^T (11 ± 0.8%). The DNA–DNA hybridization data revealed that strain AI-S262^T did not belong to any of the previously described species of the genus *Microbacterium*, when the recommendation of a threshold value of 70% DNA–DNA similarity for species definition is considered (Wayne *et al.*, 1987). The DNA G + C content of strain AI-S262^T was 69.5 mol%, which falls within the range described for the genus *Microbacterium* (Orla-Jensen, 1919; Skerman *et al.*, 1980). On the basis of the results described, strain AI-S262^T represents a novel species of the genus *Microbacterium*, for which the name *Microbacterium azadirachtae* sp. nov. is proposed.

Description of *Microbacterium azadirachtae* sp. nov.

Microbacterium azadirachtae (a.za.di.rach'ta.e. N.L. n. *azadirachta* a botanical genus name; N.L. gen. n. *azadirachtae* of *Azadirachta*, isolated from *Azadirachta indica*).

Cells are Gram-positive, strictly aerobic, motile and short rods, approximately 0.3–0.4 µm wide and 0.6–0.7 µm long, occurring singly or in pairs (see Supplementary Fig S2). Spore formation is not observed. Colonies are 1–2 mm in diameter, smooth, circular and light-yellow after 48 h at 28 °C on R2A agar. NaCl concentration for growth is 0–5.5% (w/v), with an optimum at 0–1%. pH and temperature ranges for growth are pH 5.0–10.0 and 10–37 °C (optimum at pH 7.0–8.0 and 28 °C). Catalase-positive, oxidase-negative. Nitrate is not reduced to nitrite. Aesculin, casein and tributyrin are hydrolysed. Starch, gelatin and urea are not hydrolysed. No growth on MacConkey agar. Tolerates up to 1 mM NiCl₂ and <0.5 mM CdCl₂ in plate assays. The type strain is highly resistant to bacitracin, cephalosporin, nalidixic acid and polymyxin B (all at 500 µg ml⁻¹), but sensitive to ampicillin (25 µg ml⁻¹), carbenicillin (300 µg ml⁻¹), cefotaxime (25 µg ml⁻¹), chloramphenicol (100 µg ml⁻¹), doxycycline (50 µg ml⁻¹), erythromycin (25 µg ml⁻¹), gentamicin (50 µg ml⁻¹), kanamycin (25 µg ml⁻¹), novobiocin (25 µg ml⁻¹), penicillin G (25 µg ml⁻¹), rifampicin (100 µg ml⁻¹), spectinomycin (300 µg ml⁻¹), tetracycline (500 µg ml⁻¹), trimethoprim (100 µg ml⁻¹) and vancomycin (25 µg ml⁻¹). The following carbon substrates are assimilated for growth (Biolog GP2 Microplates): β-cyclodextrin, dextrin, Tween 40, Tween 80, N-acetyl-D-galactosamine, N-acetyl-β-D-mannosamine, amygdalin, L-arabinose, D-arabitol, arbutin, cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, D-gluconic

acid, α-D-glucose, myo-inositol, maltose, maltotriose, D-mannitol, D-mannose, melezitose, methyl β-D-glucoside, palatinose, D-psicose, L-rhamnose, salicin, D-sorbitol, sucrose, D-tagatose, trehalose, turanose, acetic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, ρ-hydroxy phenylacetic acid, α-ketobutyric acid, D-lactic acid methyl ester, L-lactic acid, D-malic acid, L-malic acid, pyruvic acid methyl ester, pyruvic acid, succinamic acid, succinic acid, N-acetyl-L-glutamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-serine, putrescine, glycerol, adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine and adenosine-5'-monophosphate. Negative for α-cyclodextrin, glycogen, inulin, mannan, D-galacturonic acid, lactose, lactulose, melibiose, methyl α-D-galactoside, methyl β-D-galactoside, 3-methyl-D-glucose, methyl α-D-glucoside, methyl α-D-mannoside, raffinose, D-ribose, sedoheptulosan, stachyose, xylitol, D-xylose, α-hydroxybutyric acid, α-ketovaleric acid, lactamide, succinic acid monomethyl ester, propionic acid, L-pyrogutamic acid, 2,3-butanediol, thymidine-5'-monophosphate, uridine-5'-monophosphate, D-fructose 6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate and DL-α-glycerol phosphate. Positive for plant-growth-promoting traits including IAA production, P-solubilization, ACC deaminase activity and sulfur oxidation in plate assays, but negative for siderophore production. The predominant menaquinones are MK-12 and MK-13. The cell wall contains ornithine as diamino acid, the peptidoglycan type is B2β with glycolyl residues. The major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{16:0} and iso-C_{15:0}. The G + C content of the genomic DNA of the type strain is 69.5 mol%.

The type strain, AI-S262^T (=JCM 15681^T =LMG 24772^T =KCTC 19668^T), was isolated from the rhizoplane of neem seedlings at Tamilnadu Agricultural University (TNAU), Coimbatore, Tamilnadu, India.

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