Enterobacter arachidis sp. nov., a plant-growth-promoting diazotrophic bacterium isolated from rhizosphere soil of groundnut

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A methylotrophic nitrogen-fixing bacterial strain, Ah-143^T, isolated from the rhizosphere soil of field-grown groundnut was analysed by a polyphasic taxonomic approach. Comparative 16S rRNA gene sequence analysis combined with *rpoB* gene sequence analysis allocated strain Ah-143^T to the family *Enterobacteriaceae*, with *Enterobacter radicincitans* and *Enterobacter cowanii* as the closest relatives. The strain is Gram-stain-negative, non-spore-forming, aerobic and motile, having straight rod-shaped cells with a DNA G + C content of approximately 53.2 mol%. The strain utilizes methanol as a carbon source and the *mxoF* gene was closely related to the *mxoF* gene of members of the genus *Methylobacterium*. The fatty acid profile consisted of C_{16:0}, C_{17:0} cyclo, C_{18:1}ω7c, summed feature 2 (iso-C_{16:1} I and/or C_{14:0} 3-OH) and summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c) as the major components. DNA–DNA relatedness of strain Ah-143^T with its close relatives was less than 20 %. On the basis of the phylogenetic analyses, DNA–DNA hybridization data, and unique physiological and biochemical characteristics, it is proposed that the strain represents a novel species of the genus *Enterobacter* and should be named *Enterobacter arachidis* sp. nov. The type strain is Ah-143^T (=NCIMB 14469^T =KCTC 22375^T).

The genus *Enterobacter* is clearly separated from other groups, forming a distinct branch, and at the time of writing included 22 species with validly published names (<http://www.bacterio.cict.fr/e/enterobacter.html>), *Enterobacter cloacae* being the type species (Hormaeche & Edwards, 1960). Members of the genus *Enterobacter* are detected in

various environments, such as infant formula (Stephan *et al.*, 2007, 2008), clinical specimens (Brenner *et al.*, 1986; Hoffmann *et al.*, 2005) and in association with plants (Egamberdieva *et al.*, 2008; Kämpfer *et al.*, 2005). Several species of the genus *Enterobacter* are known to interact and exert beneficial effects on plant growth. The plant-growth-promoting effects of beneficial bacteria may occur through direct or indirect mechanisms that include production of phytohormones or enzymes that promote plant growth, increased nutrient uptake, and prevention of deleterious phytopathogens (Glick *et al.*, 1999; Whipps, 2001). The complex mixture of carbohydrates, amino acids, organic acids and other nutrients released from seeds and roots are thought to support the growth of beneficial bacteria such as *E. cloacae* in the spermosphere and rhizosphere. *E. cloacae* colonizes the spermospheres and rhizospheres of a number

Abbreviation: ACCD, 1-aminocyclopropane-1-carboxylate deaminase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoB*, *nifH* and *mxoF* gene sequences of strain Ah-143^T are EU672801, FJ768683, FJ460712 and EU912491, respectively.

Phase-contrast and scanning electron micrographs of cells of strain Ah-143^T, phylogenetic trees based on *rpoB* and *nifH* gene sequences, and cellular fatty acid compositions of strain Ah-143^T and related species of the genus *Enterobacter* are available with the online version of this paper.

of plant species and also suppresses *Pythium ultimum*-induced damping-off of seeds and seedlings of cucumber and other crops (Roberts *et al.*, 2009). Similarly, other species of the genus *Enterobacter* have been reported to exhibit plant-growth-promoting effects that include production of phytohormones, fixation of atmospheric nitrogen and biocontrol activity (Ruppel *et al.*, 1992; Ruppel & Merbach, 1997; Götz *et al.*, 2006). Further, application of species of the genus *Enterobacter* to plants has improved phosphate uptake and stimulated plant mycorrhization (Vassileva *et al.*, 1999). In this paper, we include the formal taxonomic description of strain Ah-143^T, a novel species of the genus *Enterobacter* isolated from rhizosphere soil of groundnut, which exhibits extensive plant-growth-promoting activities such as production of phytohormones and 1-aminocyclopropane-1-carboxylate deaminase (ACCD) and fixation of atmospheric nitrogen. The name *Enterobacter arachidis* sp. nov. is proposed.

Strain Ah-143^T was isolated from rhizosphere soil of groundnut (*Arachis hypogaea* L. 'ALR-2') collected from Tamilnadu Agricultural University experimental plot, Coimbatore, India, on selective ammonium mineral salts (AMS) medium (Whittenbury *et al.*, 1970) supplemented with filter-sterilized cycloheximide (10 µg ml⁻¹) and 0.5 % (v/v) methanol at 28 °C by the serial dilution technique. Cells were maintained on nutrient agar (NA, Difco) with 1 % (v/v) methanol, or on R2A medium with 0.5 % (v/v) methanol. Determination of morphological properties was performed using standard procedures (Gerhardt *et al.*, 1994). Gram staining was performed by using a Gram staining kit (Difco), and bacterial suspensions were examined by phase-contrast microscopy for cell morphology and motility. Motility test agar (nutrient broth with 0.5 % glucose and 0.5 % agar) and motility test medium (Edwards & Ewing, 1972) were also used. Preparation and examination of cells under a scanning electron microscope (SEM) were carried out according to Bozzola & Russell (1998) and the cells were visualized using a Hitachi S-2500C SEM with GEMINI column equipped with a field-emission electron source. Cells of strain Ah-143^T were Gram-stain-negative, straight rods and motile (Supplementary Fig. S1, available in IJSEM Online).

Nutritional features were determined using Biolog GN2 Microplates (Madhaiyan *et al.*, 2007a) and carbon-source utilization tests (excluding Biolog) were performed using standard protocols (Green & Bousfield, 1982). Antibiotic resistance and heavy metal tolerance of the strain was determined (Chanprame *et al.*, 1996; Tan *et al.*, 1999; Madhaiyan *et al.*, 2007b). The strain was catalase-positive, and negative or weakly positive for oxidase. The nutritional and physiological characteristics of strain Ah-143^T are given in the species description. Strain Ah-143^T was able to utilize methanol as a carbon source, which differentiated it from other close relatives. Furthermore, the strain utilized arabinol and L-fucose while other species showed weak or no growth (Table 1).

Strain Ah-143^T was screened for different plant-growth-promoting characteristics, such as the production of indole-3-acetic acid (IAA) and siderophores, ACCD activity and sulfur oxidation, through plate and quantitative assays using previously described procedures (Poonguzhali *et al.*, 2006; Madhaiyan *et al.*, 2006). Nitrogenase activity was estimated by using an acetylene reduction assay (Poonguzhali *et al.*, 2006). Strain Ah-143^T possessed ACCD activity when examined through plate assays, but was negative for sulfur oxidation and siderophore production. The ACCD activity of the cell-free extracts was 7.85 nmol α-ketobutyrate (mg protein)⁻¹ h⁻¹ and the IAA production and nitrogenase activity were 5.57 µg ml⁻¹ and 203.6 nmol C₂H₄ (mg protein)⁻¹ h⁻¹, respectively. Furthermore, in experiments using growth pouches (cyg seed germination pouch, Mega International), seed inoculation with strain Ah-143^T significantly increased the root length of Indian mustard, tomato and rice by 61.1, 21.6 and 10.4 %, respectively, when compared with uninoculated controls. Strain Ah-143^T produced methanol dehydrogenase at 34.16 nmol min⁻¹ (mg protein)⁻¹ when the enzyme was estimated quantitatively according to Dunfield *et al.* (2003).

Chromosomal DNA from strain Ah-143^T was extracted by using the QIAamp DNA mini kit (Qiagen) and amplified using the universal primers 27F and 1492R (Madhaiyan *et al.*, 2009). The partial gene sequence was determined by the fluorescent dye terminator method using the ABI prism Big dye terminator cycle sequencing ready reaction kit v.3.1 and products were run on an ABI3730XL capillary DNA sequencer (ABI Prism 310 Genetic Analyzer). The resultant 16S rRNA gene sequence was compared with sequences from representative organisms of the same and related genera from the GenBank database and was aligned by using CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic relationships were determined by the neighbour-joining method (Saitou & Nei, 1987) using the software package MEGA 3.1 (Kumar *et al.*, 2004). Bootstrap confidence values were obtained using 1000 resamplings. The 16S rRNA gene sequence analysis showed that strain Ah-143^T was phylogenetically affiliated to the genus *Enterobacter* within the *Gammaproteobacteria*, with the highest sequence similarity (97.2–98.7 %) with *Enterobacter radicincitans*, *Enterobacter cloacae* subsp. *cloacae*, *Enterobacter cloacae* subsp. *dissolvens* and *Enterobacter cowanii* (Fig. 1). However, based on 16S rRNA gene sequence analysis alone, the novel strains cannot be allocated unequivocally to a narrower taxonomic level within the family *Enterobacteriaceae* and hence *rpoB* gene sequence analysis was performed for species discrimination (Mollet *et al.*, 1997; Drancourt *et al.*, 2001; Li *et al.*, 2004; Kämpfer *et al.*, 2005). For analysis of *rpoB* sequence, total DNA was prepared according to Niemann *et al.* (1997) and the gene was amplified and sequenced following the protocol of Mollet *et al.* (1997). Sequence comparison and phylogenetic analysis were carried out as mentioned earlier. The highest *rpoB* sequence similarities were obtained with *E. radicincitans* (97.8 %), *E. cowanii* (95.8 %) and *Enterobacter*

Table 1. Major characteristics that allow differentiation among members of the genus *Enterobacter*

Taxa: 1, *Enterobacter arachidis* sp. nov.; 2, *E. cloacae* subsp. *cloacae*; 3, *E. cloacae* subsp. *dissolvens*; 4, *E. radicincitans*; 5, *E. cowanii*; 6, *E. cancerogenus*; 7, *E. asburiae*; 8, *E. turicensis*; 9, *E. helveticus*; 10, *E. gergoviae*; 11, *E. kobei*; 12, *E. hormaechei*; 13, *E. pyrinus*; 14, *E. ludwigii*; 15, *E. pulveris*; 16, *E. aerogenes*; 17, *E. nimipressuralis*; 18, *E. amnigenus* biovar 1; 19, *E. amnigenus* biovar 2. Data in columns 1–10 from this study. Data in columns 10–17 from Izard *et al.* (1981), Hoffmann *et al.* (2005), Kämpfer *et al.* (2005) and Stephan *et al.* (2007, 2008). The percentage of strains giving a positive result is scored as: –, 0–10%; +/–, 10–20%; v, 20–80%; +/–, 80–90%; +, 90–100%. ND, No data available; SR, straight rods; CR, coccoid rods; R, rods.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Pigmentation	–	–	–	+	–	–	–	+	+	–	–	–	+	–	+	–	–	–	–
Cell shape	SR	R	R	R	R	SR	R	CR	CR	R	R	R	SR	R	CR	R	R	SR	SR
Motility	+	+	+	+	+	+	+/–	+	+	+	+/–	+/–	+	+	+	+	–	+	+
Aesculin hydrolysis	–	–	–/+	+	+	+	+	+	+	+	–	–	+	–/+	+	+	+	+	+
Arginine dihydrolase	+	+	+	v	+	+	v	–	–	–	+	v	–	+	–	–	v	–	v
Ornithine decarboxylase	+	+	+	–	–	+	+	–	–	+	+	+	+	+	–	+	–	v	+
Lysine decarboxylase	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	+	ND	–	–
Methyl red test	–	–	–	–	–	–	+	+	+	–	ND	v	v	ND	+	–	–	–	v
Voges–Proskauer test	+	+	+	+	+	+	–	–	–	+	+	+	v	+	–	+	+	+	+
Carbon source utilization																			
Sucrose	+	+	+	+	+	–	+	–	–	+	+	+	+	+	+	+	–	+	–
Citrate	+	+	+	+	+	+	+	–	–	+	+	+	–	+	–	+	ND	+	+
Sorbitol	+	+	+	+	+	–	+/–	–	–	–	+	–	–	–	–	+	+	–	+
Succinate	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	ND
Dulcitol	+	–	–	+	+	–	–	+	+	–	v	+	–	–	v	–	–	–	–
D-Melibiose	–	+	+	–	+	–	–	+	+	+	+	–	+	+	+	+	+	+	+
D-Arabinol	+	–/+	–	–	–	–	–	–	–	+	–	–/+	+	–	+	+	–	–	–
L-Fucose	+	–	v	–	–	+	–	–	–	v	–	+	–	v	–	–	–	–	ND
L-Rhamnose	+	+	+	+	+	+	+/–	+	+	+	+	+	+	+	+	+	+	+	+
Putrescine	–	+	+	–	–	+	–/+	–	+	v	v	–	+	–	v	v	–	–	–
Mucate	–	v	+	–	+	+	v	+	+	–	v	v	–	+	+	+	+	v	+
1-O-Methyl	+	+	+	+	+	–	+/–	+	+	+	+	–	–	+	+	+/–	–	+	ND
α-galactopyranoside	–	–	–	–	–	+	–	–	–	–	–	v	–	+/–	–	–	–	–	ND
3-O-Methyl	–	–	–	–	–	+	–	–	–	–	–	v	–	+/–	–	–	–	–	ND
D-glucopyranoside	–	–	–	–	–	+	–	–	–	–	–	v	–	+/–	–	–	–	–	ND

turicensis (86.9%) (Supplementary Fig. S2). The similarity values with the nearest neighbours were rather low, ranging from 91.4 to 97.8%, when compared with the intraspecies similarity found within the family *Enterobacteriaceae* (Mollet *et al.*, 1997), thus confirming a possibility that the strain might represent a novel species within the family *Enterobacteriaceae*.

The methanol dehydrogenase (*mxhF*) gene required for methanol utilization and the *nifH* gene responsible for nitrogenase activity in strain Ah-143^T were amplified from DNA extracts using the specific primer pairs *mxhF* f1003 and *mxhF* r1561 (McDonald & Murrell, 1997), and 19F and 407R (Ueda *et al.*, 1995), respectively. The products were sequenced directly and analysed as already mentioned. Analysis of the *mxhF* gene sequence (556 bp) from Ah-143^T revealed 99.4 and 94.1% gene sequence similarity with sequences from the type strains of *Methylobacterium zatmanii* and *Methylobacterium lusitanum*, respectively. The *nifH* gene (390 bp) from Ah-143^T was closely related to those of the strains of *Serratia marcescens* (99.2% similarity) and *Klebsiella pneumoniae* (93.3%), and other

members of the family *Enterobacteriaceae* (Supplementary Fig. S3). Furthermore, PCR amplification was performed to determine the presence of the genes *mmoX* (methane monooxygenase) and *acdS* (ACCD) in strain Ah-143^T using specific primer pairs *mmoX*B-1401b and *mmoX*-ms-945f (Auman *et al.*, 2000; Dedysh *et al.*, 2005), and F1936 and F1939, respectively (Blaha *et al.*, 2006). The results showed the presence of an expected 1230 bp fragment for *mmoX* and 558 bp fragment for ACCD (data not shown).

Fatty acid methyl ester analysis was carried out using a culture grown on tryptic soy agar [TSA, tryptic soy broth (Difco) with 1.5% agar] at 28 °C for 48 h and the Microbial Identification System (MIDI; Microbial ID), according to standard protocols (Sasser, 1990). Only those species located in the monophyletic group of close relatives with the new species and supported by a pairwise sequence similarity ranging from 97.0 to 98.7% were included in addition to the type species of the genus. The fatty acid profile of strain Ah-143^T contained C_{16:0}, C_{18:1} ω7c, summed feature 2 (iso-C_{16:1} I and/or C_{14:0} 3-OH), summed feature 3 (C_{16:1} ω7c and/or iso-C_{15:0} 2-OH) and

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparison showing the position of strains belonging to the genus *Enterobacter* and other related genera. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; only values greater than 50 % are shown. Bar, 0.005 substitutions per nucleotide position.

C_{17:0} cyclo as major constituents (Supplementary Table S1). DNA–DNA hybridization was carried out to determine the relatedness of strain Ah-143^T with its closest relatives following the filter hybridization method (Seldin & Dubnau, 1985). Hybridization temperature was 65 °C and DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad). Probe labelling was conducted by using the non-radioactive DIG-High Prime system (Roche Diagnostics), and hybridized DNA was visualized using the DIG luminescent detection kit according to the manufacturer's instructions (Roche Diagnostics). Strain Ah-143^T showed a low level of DNA–DNA relatedness, less than 20 %, with its close relatives *E. radicincitans* (16 %), *E. cloacae* subsp. *cloacae* (12 %), *E. cloacae* subsp. *dissolvens* (14 %) and *E. cowanii* (19 %), indicating that the new isolate is genotypically different from these type strains. The G+C content of genomic DNA was determined by HPLC analysis using a reverse-phase column (Supelcosil LC-18 S, Supelco) of individual nucleosides as described previously (Mesbah *et al.*, 1989). The DNA G+C content of strain Ah-143^T was 53.2 mol%, which falls within the range described for the genus *Enterobacter* (Richard, 1984; Inoue *et al.*, 2000).

The 16S rRNA and *rpoB* gene sequence similarity data and DNA–DNA hybridization values differentiated strain Ah-143^T from other members of the genus *Enterobacter*.

Furthermore, the strain showed methylotrophic properties with an ability to utilize methanol and the *mxhF* gene was closely related to those of methylotrophic bacteria. Based on the recommendation of Wayne *et al.* (1987) and on the data presented here, it is concluded that strain Ah-143^T represents a novel species of the genus *Enterobacter* for which we propose the name *Enterobacter arachidis* sp. nov.

Description of *Enterobacter arachidis* sp. nov.

Enterobacter arachidis (a.ra.chi'dis. N.L. gen. n. arachidis, of Arachis, isolated from groundnut).

Cells are Gram-stain-negative, straight rods, motile, 0.5–0.7 µm wide, 2.0–3.7 µm long and occur singly or in pairs. Grows on AMS, TSA, R2A, NA+1 % methanol and MacConkey agar. Catalase-positive and weakly oxidase-positive. Grows in the presence of 0–5 % (w/v) NaCl, but not in the presence of 6 % (w/v) NaCl or higher concentrations. Growth occurs between 20 and 30 °C with an optimum at 28 °C. The pH range for growth is pH 4–10, with optimal growth at pH 7. Starch hydrolysis is positive, but aesculin and gelatin hydrolysis are absent. Nitrate reduction is positive and indole production is negative. Positive for protease, lipase, pectinase, cellulase, ornithine decarboxylase and arginine dihydrolase enzymes and negative for urease. Positive for Voges–Proskauer and negative for methyl red tests. Able to tolerate and grow in the presence of

NiCl₂ or CdCl₂ up to 1 mM or 4 mM, respectively, when supplemented in the growth plates. Positive for utilization of dextrin, glycogen, Tween 40, Tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α -D-glucose, *myo*-inositol, lactose, lactulose, maltose, D-mannitol, D-mannose, methyl β -D-glucoside, 1-O-Methyl α -galactopyranoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, dulcitol, sucrose, trehalose, turanose, xylitol, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, α -ketoglutaric acid, DL-lactic acid, malonic acid, D-saccharic acid, succinic acid, bromosuccinic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, L-ornithine, L-proline, L-pyrogutamic acid, D-serine, L-serine, L-threonine, urocanic acid, inosine, uridine, thymidine, glycerol, DL- α -glycerol phosphate, α -D-glucose 1-phosphate and D-glucose 6-phosphate. The following compounds are not utilized as sole carbon sources: α -cyclodextrin, D-melibiose, putrescine, mucate, 3-O-methyl D-glucopyranoside, D-galactonic acid lactone, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketobutyric acid, α -ketovaleric acid, propionic acid, quinic acid, sebacic acid, succinamic acid, L-hydroxyproline, L-leucine, L-phenylalanine, DL-carnitine, γ -aminobutyric acid, phenylethylamine, putrescine, 2-aminoethanol and 2,3-butanediol. The type strain is highly resistant (up to 500 μ g ml⁻¹) to bacitracin, penicillin G, novobiocin and cephalosporin, but sensitive (lowest concentration tolerated) to spectinomycin (25 μ g ml⁻¹), carbenicillin (500 μ g ml⁻¹), kanamycin (25 μ g ml⁻¹), nalidixic acid (25 μ g ml⁻¹), chloramphenicol (25 μ g ml⁻¹), erythromycin (50 μ g ml⁻¹), polymyxin B (25 μ g ml⁻¹), rifampicin (25 μ g ml⁻¹), vancomycin (500 μ g ml⁻¹), trimethoprim (25 μ g ml⁻¹), cefotaxime (125 μ g ml⁻¹), doxycycline (125 μ g ml⁻¹), ampicillin (500 μ g ml⁻¹), gentamicin (250 μ g ml⁻¹) and tetracycline (25 μ g ml⁻¹). The *mxrA* and *nifH* genes for methanol dehydrogenase and nitrogenase activity, respectively, are present. The major fatty acids are C_{16:0}, C_{18:1} ω 7c, C_{17:0} cyclo and summed features 2 (iso-C_{16:1} I and/or C_{14:0} 3-OH) and 3 (C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH).

The type strain, Ah-143^T (=NCIMB 14469^T =KCTC 22375^T), was isolated from rhizosphere soil of groundnut (*Arachis hypogaea* L. 'ALR-2'), the sample being collected at the pod initiation stage from an experimental field, Tamilnadu Agricultural University (TNAU), Coimbatore, Tamilnadu, India. The DNA G+C content of the type strain is 53.2 mol%.

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