Enterobacter oryzae sp. nov., a nitrogen-fixing bacterium isolated from the wild rice species *Oryza latifolia*

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Twelve facultatively anaerobic, endophytic diazotrophs were isolated from surface-sterilized roots of the wild rice species *Oryza latifolia* and characterized by phenotypic and molecular methods. Six isolates were grouped together as group A by phenotypic characters, and this grouping was confirmed by SDS-PAGE whole-cell protein patterns and insertion sequence-based PCR (IS-PCR) methods. Phylogenetic analysis of the 16S rRNA gene sequence indicated that group A, represented by strain Ola 51^T, is closely related to *Enterobacter radicincitans* D5/23^T (98.9 % similarity, except that *E. radicincitans* D5/23^T has a 70 bp insertion) and *Enterobacter cloacae* (98.0 % similarity to the type strain). *rpoB* gene sequence analysis also showed strain Ola 51^T has the highest sequence similarity to *E. radicincitans* DSM 16656^T (98.3 %), but supported the distinct position. Biological and biochemical tests, protein patterns, genomic DNA fingerprinting, antibiotic resistance and comparison of cellular fatty acids showed differences among group A, *E. radicincitans* DSM 16656^T and *E. cloacae* ATCC 13047^T. DNA–DNA hybridization distinguished strain Ola 51^T from closely phylogenetically related *Enterobacter* species. Based on these data, the novel species *Enterobacter oryzae* sp. nov. is proposed, with strain Ola 51^T (=LMG 24251^T =CGMCC 1.7012^T) as the type strain.

The wild rice species *Oryza latifolia* belongs to the *Oryza officinalis* complex, which is the largest in the genus *Oryza*. *O. latifolia*, *O. alta* and *O. grandiglumis* are three closely related tetraploid species. *O. latifolia* is widely distributed in Central and South America and on the Caribbean islands (Vaughan, 1989). Endophytic diazotrophic bacteria, known to supply biologically fixed nitrogen directly to these non-legume plants, may have great potential to improve sustainable rice production (Chaintreuil *et al.*, 2000).

Diazotrophic bacteria belonging to the species *Enterobacter cloacae* and *Klebsiella oxytoca* and the genera *Alcaligenes*

and *Azospirillum* have previously been isolated from the rhizosphere of wetland rice (Baldani & Doebereiner, 1980; Fujie *et al.*, 1987; Tou & Zhou, 1989). A number of diazotrophic bacteria, including strains of *Herbaspirillum rubrisubalbicans* (from *Oryza barthii*), *Herbaspirillum seropedicae* (*O. officinalis*, *O. rufipogon*), *Ideonella dechloratans* (*O. sativa*), *Enterobacter cancerogenus* (*O. rufipogon*), *Azospirillum lipoferum* (*O. grandiglumis*, *O. sativa*) and *Azospirillum brasilense* (*O. rufipogon*), were isolated from stems of wild rice species by Elbeltagy *et al.* (2001). In addition to these diazotrophs isolated from rice, a remarkable diversity of uncultured diazotrophs has also been detected in association with rice roots (Engelhard *et al.*, 2000; Hurek *et al.*, 2002; Tan *et al.*, 2001; Ueda *et al.*, 1995). These diazotrophic isolates from wild rice may serve as valuable bioresource micro-organisms that could potentially supply nitrogen to rice and promote rice growth. Little work has been reported on nitrogen-fixing isolates from the wild rice species *O. latifolia*.

Samples of *O. latifolia* were collected from the Wild Rice Core Collection Nursery at South China Agricultural University. Procedures for sterilization of rice roots and

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene sequences of strains Ola 50, Ola 51^T and Ola 01 are respectively EF488758–EF488760 and the accession number for the partial *rpoB* gene sequence of strain Ola 51^T is EU249553.

A comparison of the biological and biochemical characteristics of strain Ola 51^T with those of related strains, detailed DNA–DNA hybridization results, a transmission electron micrograph of cells of strain Ola 51^T, comparisons of IS-PCR and whole-cell protein SDS-PAGE profiles of selected novel strains and related strains and an *rpoB* gene sequence-based neighbour-joining tree are available as supplementary material with the online version of this paper.

isolation and purification of nitrogen-fixing bacteria on VM medium (Reinhold-Hurek *et al.*, 1993) were described previously (Peng *et al.*, 2006). The nitrogen-fixing ability of all isolates was checked by using the acetylene-reduction assay as described by Eckert *et al.* (2001) and confirmed by PCR amplification of the *nifH* gene (Peng *et al.* 2006), which codes for the nitrogenase reductase enzyme involved in nitrogen fixation. The primers and the protocol for *nifH* gene amplification were described by Peng *et al.* (2006). A total of 12 putatively endophytic nitrogen-fixing bacteria (isolates Ola 01, Ola 05, Ola 08, Ola 10, Ola 12, Ola 15, Ola 28, Ola 32, Ola 37, Ola 46, Ola50 and Ola 51) were isolated from root samples of *O. latifolia*. These isolates were obtained with VM medium supplied with DL-malic acid as the sole carbon source. All of them were Gram-negative, facultatively anaerobic, motile, straight or curved rods. Acetylene reduction was detected in all the isolates within 20 h of acetylene injection. All strains were able to reduce acetylene to ethylene in the range of 60–115 nmol ethylene h^{-1} per 10^8 cells at 28 °C without addition of yeast extract. Following PCR amplification of the *nifH* gene, the expected fragment of about 360 bp was obtained from all the isolates and the positive-control strain *A. brasilense* Sp7^T, which further confirmed the nitrogen-fixing capacity of these isolates.

The phenotypic features described previously (Chen *et al.*, 1988; Gao *et al.*, 1994) were used to group the isolates. Phenotypic analyses covered utilization of sugars, amino acids, alcohols and organic acids as sole carbon sources, resistance to various antibiotics (Tan *et al.*, 1999), ranges of NaCl concentration and pH for growth and biochemical characteristics tested by using the API 20E kit (bioMérieux). The results were converted into a binary matrix. Phenotypic similarity (simple matching coefficient; S_{SM}) between each pair of strains was estimated and used in clustering analysis to produce a dendrogram using the unweighted pair group method with arithmetic means (UPGMA) as described by Sneath & Sokal (1973). The 12 diazotrophic isolates were separated into three groups at a similarity of 76 % (Fig. 1). Group A included six strains, Ola 01, Ola 10, Ola 12, Ola 28, Ola 50 and Ola 51^T, group B comprised strains Ola 05 and Ola 37 and strains Ola 08, Ola 15 and Ola 46 belonged to group C. One diazotrophic isolate, Ola 32, could not be included in any cluster. 16S rRNA gene sequences of about 900 bp were obtained from representative strains from each group (Ola 51^T, Ola 05, Ola 46 and Ola 32). Similarities over this partial sequence between the strain pairs Ola 05 (group B) and *Pantoea agglomerans* DSM 3493^T (GenBank accession no. AJ233423), Ola 46 (group C) and *Pseudomonas putida* ATCC 17485 (AF094739) and Ola 32 and *Azospirillum brasilense* ATCC 29145^T (AY324110) were more than 99 %. Strain Ola 51^T of group A shared 96.8 % sequence similarity (over 900 bp) with *Enterobacter cloacae* ATCC 13047^T (GenBank accession no. AJ251469) and *Enterobacter radicincitans* D5/23^T (AY563134), although the sequence of *E. radicincitans* D5/23^T has a 70 bp

Fig. 1. Dendrogram showing phenotypic similarities among the diazotrophic isolates from *O. latifolia*. The UPGMA method was used for cluster analysis (Sneath & Sokal, 1973).

insertion. The biological and biochemical characters and antibiotic resistance of *E. radicincitans* DSM 16656^T and *E. cloacae* ATCC 13047^T were also tested and compared with those of the representative strain Ola 51^T of group A (Supplementary Table S1, available in IJSEM Online).

Negatively stained cells (Cole & Popkin, 1981) from cultures growing in liquid medium were used for the examination of flagella under a transmission electron microscope. Cell morphology was observed by light microscopy and transmission electron microscopy (Hitachi 600). Cells of strain Ola 51^T are straight or slightly curved rods, 0.8–1.1 × 1.4–2.0 µm, with subpolar flagella (Supplementary Fig. S1 in IJSEM Online).

IS-PCR (insertion sequence-based PCR) fingerprinting was performed to evaluate the genotypic diversity of the isolates. The conditions of PCR amplification and electrophoresis were described previously (Peng *et al.*, 2006). The amplified fragments were between 100 and 600 bp long. The six diazotrophic isolates of group A showed very similar patterns (total six fragments, five common fragments), but these patterns differed from those of *Enterobacter aerogenes* ATCC 13048^T, *E. cloacae* ATCC 13047^T and *E. radicincitans* DSM 16656^T (Supplementary Fig. S2).

Methods of cell preparation and protein extraction and analysis of whole-cell protein SDS-PAGE patterns were described previously (Tan *et al.*, 2001). The six diazotrophic isolates had identical protein patterns in the range 14–116 kDa. The main intense protein bands differed between the six novel isolates and the reference strains *E. cloacae* ATCC 13047^T and *E. aerogenes* ATCC 13048^T (Supplementary Fig. S3).

Harvesting of cells and extraction of fatty acids were performed as described by Sasser (1990). Fatty acid analysis methods were described by Peng *et al.* (2006). The cellular fatty acid profiles of strain Ola 51^T, representing the

diazotrophs of group A, and the related strains *E. radicincitans* DSM 16656^T and *E. cloacae* ATCC 13407^T had 11 components in common, at retention times of 6.2, 8.8, 12.0, 12.5, 14.5, 16.5, 17.5, 18.4, 19.5, 22.6 and 25.6 min. These common components respectively made up 81.2, 97.3 and 98.6 % of the components detected in Ola 51^T, *E. radicincitans* DSM 16656^T and *E. cloacae* ATCC 13407^T (Supplementary Table S2). Strain Ola 51 had five components, at retention times of 9.5, 10.5, 13.4, 15.8 and 21.2 min, which were absent or trace peaks (less than 2 %) in the profiles of *E. radicincitans* DSM 16656^T and *E. cloacae* ATCC 13407^T. The relative abundance of each compound was also different among the three strains.

The partial 16S rRNA gene sequences of representative isolates, together with some closely related sequences from GenBank of strains of species with validly published names, were aligned by using the CLUSTAL W program package (Thompson *et al.*, 1994) and the alignment was adjusted manually using GeneDoc (Nicholas & Nicholas, 1997). Ambiguous bases were excluded from the calculation of

similarity. The tree topology was inferred by the neighbour-joining method (Saitou & Nei, 1987) and the phylogenetic tree was visualized and bootstrapped by using the TREECON software package (Van de Peer & De Wachter, 1994). Three strains from group A, Ola 01, Ola 50 and Ola 51^T, were sequenced, and they shared more than 99.8 % 16S rRNA gene sequence similarity. In the reconstructed phylogenetic tree (Fig. 2), these three strains formed a monophyletic group within the genus *Enterobacter* and were related more closely to *E. radicincitans* D5/23^T (98.9 % similarity over 1481 bp) than to other species. Bootstrap confidence also indicated that strain Ola 51^T and *E. radicincitans* D5/23^T formed a phylogenetic group (with a bootstrap value of 100 %).

DNA–DNA relatedness was determined by the initial renaturation rate method (De Ley *et al.*, 1970) in 2 × SSC with three repetitions and mean values were calculated. DNA was isolated and purified as described by Marmur (1961) and DNA base composition was determined spectrophotometrically. DNA from *Escherichia coli* K-12 was used

Fig. 2. Phylogenetic tree constructed by neighbour-joining analysis of 16S rRNA gene sequences showing that strain Ola 51^T is most closely related to *E. radicincitans* D5/23^T. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; only values greater than 55 % are shown. Bar, 1 % nucleotide substitutions.

as the standard for estimation of G + C content. The G + C content of the genomic DNA of strain Ola 51^T was 55.0 ± 0.4 mol%. The results of DNA–DNA hybridization are presented in Supplementary Table S3. DNA–DNA relatedness among the strains of group A varied from 88 to 100 %, indicating that they were members of the same genomic species. DNA–DNA relatedness among *E. radicincitans* DSM 16656^T, *E. cloacae* ATCC 13047^T and three strains of group A was 33–43 %. Relatedness between *Enterobacter asburiae* JCM 6051^T and three strains of group A was 28–35 %.

As *rpoB* sequence analysis has been reported previously by several authors to be useful for species discrimination within the family Enterobacteriaceae (Drancourt *et al.*, 2001; Kämpfer *et al.*, 2005; Li *et al.*, 2004; Mollet *et al.*, 1997), the forward primer CM7 (5'-AACCAGTTCCGCG-TTGGCCTGG-3') and reverse primer CM31b (5'-CCTG-AACAACACGCTCGGA-3') (Mollet *et al.*, 1997) were used to amplify the *rpoB* gene from strain Ola 51^T. PCR fragments were sequenced directly following the protocol

of Mollet *et al.* (1997) with the forward sequencing primer CM81 (5'-CAGTTCCGCGTTGGCCTG-3') and reverse primer CM32b (5'-CGGAACGGCCTGACGTTGCAT-3'). The *rpoB* gene sequence obtained from strain Ola 51^T was aligned with related sequences from GenBank, and phylogenetic analysis was performed as described above for 16S rRNA gene sequence analysis. A phylogenetic tree constructed by neighbour-joining analysis is shown in Supplementary Fig. S4. The phylogenetic position of strain Ola 51^T on the basis of *rpoB* gene sequence analysis is similar to that derived from 16S rRNA gene sequence analysis. The highest *rpoB* gene sequence similarity (following comparison of 948 bp of the *rpoB* gene sequence) of strain Ola 51^T was found with *E. radicincitans* D5/23^T (98.2 % similarity). The similarities between strain Ola 51^T and *E. cloacae* subsp. *cloacae* ATCC 13407^T and *E. cloacae* subsp. *dissolvens* ATCC 23373^T were 92.5 and 92.2 %. *rpoB* gene sequence analysis, in addition to DNA–DNA hybridization experiments and phenotypic characterization, indicates that strain Ola 51^T represents a novel species of the genus *Enterobacter*.

Table 1. Phenotypic characteristics that differentiate the group A strains (*Enterobacter oryzae* sp. nov.) from related species of the genus *Enterobacter*

Taxa: 1, *Enterobacter oryzae* sp. nov.; 2, *E. radicincitans* (data from Kämpfer *et al.*, 2005); 3, *E. turicensis* (Stephan *et al.*, 2007); 4, *E. helveticus* (Stephan *et al.*, 2007); 5, *E. gergoviae* (Farmer *et al.*, 1985; Stephan *et al.*, 2007); 6, *E. asburiae* (Brenner *et al.*, 1986; Hoffmann *et al.*, 2005a); 7, *E. kobei* (Hoffmann *et al.*, 2005a; Kosako *et al.*, 1996); 8, *E. hormaechei* (Kämpfer *et al.*, 2005; O'Hara *et al.*, 1989); 9, *E. cancerogenus* (Hoffmann *et al.*, 2005a; Stephan *et al.*, 2007); 10, *E. pyrinus* (Hoffmann *et al.*, 2005a; Stephan *et al.*, 2007); 11, *E. ludwigii* (Hoffmann *et al.*, 2005b; Kämpfer *et al.*, 2005); 12, *E. cloacae* subsp. *cloacae* (Hoffmann *et al.*, 2005a; Kämpfer *et al.*, 2005); 13, *E. cloacae* subsp. *dissolvens* (Brenner *et al.*, 1986; Hoffmann *et al.*, 2005a); 14, *E. cloacae* ATCC 13047^T (data from this study). 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.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Voges–Proskauer reaction*	+	+	–	–	+	–	+	+	+	v	+	+	+	+
Motility	+	+	+	+	+	+/–	+/–	+/–	+	+	+	+	+	+
Aesculin hydrolysis	–	+	+	+	+	+	–	–	+	+	–	–	+	–
Arginine dihydrolase*	+	+	–	–	–	+	+	v	+	–	+	+	+	+
Ornithine decarboxylase*	+	–	–	–	+	+	+	+	+	+	+	+	+	+
Lysine decarboxylase*	+	–	–	–	+	–	–	–	–	+	–	–	–	–
Acid from sorbitol	+	+	–	–	–	+/–	+	–	–	–	+	+	+	+
Methyl red test	+	ND	+	+	–	+	ND	v	–	v	ND	–	–	–
Carbon source utilization:														
Sucrose	+	+	–	–	+	+	+	+	–	+	+	+	+	+
Citrate	+	+	–	–	+	+	+	+	+	–	+	+	+	+
Dulcitol	+	+	+	+	–	–	v	+	–	–	–	–	–	–
Putrescine	–	–	–	+	v	–/+	v	–	+	+	–	+	+	+
α-Melibiose	+	–	+	+	+	+/–	+	–	–	+	+	+	+	+
L-Fucose	+	–	–	–	v	–	–	+	+	–	v	–	v	+
D-Arabinol	–	–	–	–	+	–	–	–/+	–	+	–	–	–	–
L-Rhamnose	–	+	+	+	+	+/–	+	+	+	+	+	+	+	+
Mucate	+	–	+	+	–	v	v	v	+	–	+	v	+	+
3-Hydroxybutyrate	+/–	–	ND	ND	v	+	+/–	–	–	–	v	+	+	+
Methyl α-D-glucoside	+	–	ND	ND	–	+	v	+	–	–	+	+	+	+
1-O-Methyl α-galactopyranoside	ND	ND	+	+	+	+/–	+	–	–	–	+	+	+	ND
3-O-Methyl D-glucopyranoside	ND	ND	–	–	–	–	–	v	+	–	+/–	–	–	ND

*Determined by using the API 20E kit.

The taxonomic relationships among genera and species within the family *Enterobacteriaceae* are still poorly understood and are being continually updated. For example, *Enterobacter intermedius* was transferred to the genus *Kluyvera* as *Kluyvera intermedia* and *Kluyvera cochleae* was reclassified as a later synonym of *Kluyvera intermedia* (Pavan *et al.*, 2005), and the genomospecies of *Enterobacter sakazakii* were described as *Cronobacter sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis* subsp. *dublinensis*, *C. dublinensis* subsp. *laurannensis* and *C. dublinensis* subsp. *lactaridi* (Iversen *et al.*, 2008). In the present study, analysis of partial sequences of the *rpoB* and 16S rRNA genes showed that strain Ola 51^T of group A is closely related to *E. radicincitans* D5/23^T, with 100% bootstrap support. The low to moderate DNA–DNA relatedness with the type strains of *E. radicincitans*, *E. cloacae* and *E. aerogenes* and the differences in IS-PCR fingerprinting, SDS-PAGE of proteins, fatty acids and biological tests indicated that the strains of group A were distinct from currently defined *Enterobacter* species (Table 1). Based on these results and the current definition of bacterial species, we propose the classification of the novel diazotrophic bacteria of group A isolated from the wild rice species *O. latifolia* as members of *Enterobacter oryzae* sp. nov.

Description of *Enterobacter oryzae* sp. nov.

Enterobacter oryzae (o.ry'zae. L. gen. n. *oryzae* of rice, from which the first strains were isolated).

Cells are straight or slightly curved rods, 0.8–1.1 × 1.4–2.0 µm (Supplementary Fig. S1). Gram-negative and motile. Facultatively anaerobic and chemo-organotrophic. Colonies on VM medium are circular, convex and translucent, with a diameter of 3 mm within 3 days at 28 °C. Growth occurs at 10–40 °C (optimum 28–37 °C) and at pH 3.5–10. NaCl inhibits growth at concentrations above 5%, but acetylene reduction activity is displayed at concentrations below 3%. Resistant to erythromycin, neomycin and ampicillin (300 µg ml⁻¹), chloramphenicol (50 µg ml⁻¹), streptomycin, tetracycline and gentamicin (5 µg ml⁻¹), but not resistant to kanamycin (5 µg ml⁻¹). Shows a positive reaction for lysine decarboxylase; negative for urease and reduction of nitrate to dinitrogen. Adonitol, L-aspartic acid, dulcitol, sodium oxalate, vanillic acid, sodium benzoate, sodium hippurate, D-fructose, malate, mannitol, maltose and sorbitol can be used as sole carbon sources. The G + C content of genomic DNA of the type strain is 55.0 ± 0.4 mol%. According to 16S rRNA gene sequence analysis, the closest phylogenetic relatives are *E. radicincitans* and *E. cloacae*.

Strain Ola 51^T (=LMG 24251^T =CGMCC 1.7012^T) is the type strain. The group A strains represented by strain Ola 51^T were isolated as endophytic nitrogen-fixing bacteria from the wild rice species *Oryza latifolia*.

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