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 51T, is closely related to Enterobacter radicincitans D5/23T (98.9 % similarity, except that E. radicincitans D5/23T has a 70 bp insertion) and Enterobacter cloacae (98.0 % similarity to the type strain). rpoB gene sequence analysis also showed strain Ola 51T has the highest sequence similarity to E. radicincitans DSM 16656T (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 16656T and E. cloacae ATCC 13047T. DNA–DNA hybridization distinguished strain Ola 51T from closely phylogenetically related Enterobacter species. Based on these data, the novel species Enterobacter oryzae sp. nov. is proposed, with strain Ola 51T (=LMG 24251T =CGMCC 1.7012T) 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 lipoforum (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
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, Ola 50 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 Sp7T, 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; SMM) 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 51T, 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 51T, Ola 05, Ola 46 and Ola 32). Similarities over this partial sequence between the strain pairs Ola 05 (group B) and Pantoea agglomerans DSM 3493T (GenBank accession no. AJ233423), Ola 46 (group C) and Pseudomonas putida ATCC 17485 (AF094739) and Ola 32 and Azospirillum brasilense ATCC 29145T (AY324110) were more than 99%. Strain Ola 51T of group A shared 96.8% sequence similarity (over 900 bp) with Enterobacter cloacae ATCC 13047T (GenBank accession no. AJ251469) and Enterobacter radiiicintans D5/23T (AY563134), although the sequence of E. radiiicintans D5/23T has a 70 bp insertion. The biological and biochemical characters and antibiotic resistance of E. radiiicintans DSM 16656T and E. cloacae ATCC 13047T were also tested and compared with those of the representative strain Ola 51T 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 51T 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 genotype 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 13048T, E. cloacae ATCC 13047T and E. radiiicintans DSM 16656T (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 13047T and E. aerogenes ATCC 13048T (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 51T, representing the...
diazotrophs of group A, and the related strains *E. radicincitans* DSM 16656<sup>T</sup> and *E. cloacae* ATCC 13407<sup>T</sup> had 11 components in common, at retention times of 6.2, 8.8, 12.0, 12.5, 14.3, 14.5, 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<sup>T</sup>, *E. radicincitans* DSM 16656<sup>T</sup> and *E. cloacae* ATCC 13407<sup>T</sup> (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<sup>T</sup> and *E. cloacae* ATCC 13407<sup>T</sup>. 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 (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 TREESCON software package (Van de Peer & De Wachter, 1994). Three strains from group A, Ola 01, Ola 50 and Ola 51<sup>T</sup>, were sequenced, and they shared more than 98.8 % 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<sup>T</sup> (98.9 % similarity over 1481 bp) than to other species. Bootstrap confidence also indicated that strain Ola 51<sup>T</sup> and *E. radicincitans* D5/23<sup>T</sup> 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 x 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.

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**Fig. 2.** Phylogenetic tree constructed by neighbour-joining analysis of 16S rRNA gene sequences showing that strain Ola 51<sup>T</sup> is most closely related to *E. radicincitans* D5/23<sup>T</sup>. 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\textsuperscript{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 \textit{E. radicicutans} DSM 16656\textsuperscript{T}, \textit{E. cloacae} ATCC 13047\textsuperscript{T} and three strains of group A was 33–43 %. Relatedness between \textit{Enterobacter asburiae} JCM 6051\textsuperscript{T} and three strains of group A was 28–35 %.

As \textit{rpoB} sequence analysis has been reported previously by several authors to be useful for species discrimination within the family \textit{Enterobacteriaceae} (Drancourt et al., 2001; Kämpfer et al., 2005; Li et al., 2004; Mollet et al., 1997), the forward primer CM7 (5'-AACCGATTCCGGTTGGCCTTG-3') and reverse primer CM31b (5'-CCTGGACACGCTCGGGA-3') (Mollet et al., 1997) were used to amplify the \textit{rpoB} gene from strain Ola 51\textsuperscript{T}. PCR fragments were sequenced directly following the protocol of Mollet \textit{et al.} (1997) with the forward sequencing primer CM81 (5'-CAGTCCGCGTTGGCCTG-3') and reverse primer CM32b (5'-CGGAACGCGCTAGTTGAC-3'). The \textit{rpoB} gene sequence obtained from strain Ola 51\textsuperscript{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\textsuperscript{T} on the basis of \textit{rpoB} gene sequence analysis is similar to that derived from 16S rRNA gene sequence analysis. The highest \textit{rpoB} gene sequence similarity (following comparison of 948 bp of the \textit{rpoB} gene sequence) of strain Ola 51\textsuperscript{T} was found with \textit{E. radicicutans} D5/23\textsuperscript{T} (98.2 % similarity). The similarities between strain Ola 51\textsuperscript{T} and \textit{E. cloacae} subsp. \textit{cloacae} ATCC 13407\textsuperscript{T} and \textit{E. cloacae} subsp. \textit{dissolvens} ATCC 23373\textsuperscript{T} were 92.5 and 92.2 %. \textit{rpoB} gene sequence analysis, in addition to DNA–DNA hybridization experiments and phenotypic characterization, indicates that strain Ola 51\textsuperscript{T} represents a novel species of the genus \textit{Enterobacter}.

### Table 1. Phenotypic characteristics that differentiate the group A strains (\textit{Enterobacter oryzae} sp. nov.) from related species of the genus \textit{Enterobacter}

| Taxa: 1, \textit{Enterobacter oryzae} sp. nov.; 2, \textit{E. radicicutans} (data from Kämpfer et al., 2005); 3, \textit{E. turicensis} (Stephan et al., 2007); 4, \textit{E. helveticus} (Stephan et al., 2007); 5, \textit{E. gergoviae} (Farmer et al., 1985; Stephan et al., 2007); 6, \textit{E. asburiae} (Brenner et al., 1986; Hoffmann et al., 2005a); 7, \textit{E. kobei} (Hoffmann et al., 2005a; Kosako et al., 1996); 8, \textit{E. hornaeciei} (Kämpfer et al., 2005; O’Hara et al., 1989); 9, \textit{E. cancerogenus} (Hoffmann et al., 2005a; Stephan et al., 2007); 10, \textit{E. pyrinus} (Hoffmann et al., 2005a; Stephan et al., 2007); 11, \textit{E. ludwigii} (Hoffmann et al., 2005b; Kämpfer et al., 2005); 12, \textit{E. cloacae} subsp. \textit{cloacae} (Hoffmann et al., 2005a; Kämpfer et al., 2005); 13, \textit{E. cloacae} subsp. \textit{dissolvens} (Brenner et al., 1986; Hoffmann et al., 2005a); 14, \textit{E. cloacae} ATCC 13047\textsuperscript{T} (data from this study). The percentage of strains giving a positive result is scored as: −, 0–10 %; /−, 10–20 %; +, 20–80 %; ++, 80–90 %; +++, 90–100 %. ND, No data available.

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* 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. muaythiensis, C. dublinensis subsp. dublinensis, C. dublinensis subsp. lausannensis 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 51T of group A is closely related to E. radicicinans D5/23T, with 100% bootstrap support. The low to moderate DNA–DNA relatedness with the type strains of E. radicicinans, 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’zæ, 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 aerobic 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 below 3%. Resistant to erythromycin, neomycin and ampicillin (300 μg ml⁻¹), chloramphenicol (50 μg ml⁻¹), streptomycin, tetracycline and gentamycin (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. radicicinans and E. cloacae.

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

**Acknowledgements**

This work was supported by National Natural Science Foundation of China (NSFC; 30470002, 30770001), the Scientific Research Foundation for the Returned Overseas Chinese Scholars of the State Education Ministry (SRF for ROCS, SEM) and the Program for New Century Excellent Talents in University (NCET).

**References**


