

Ochrobactrum oryzae sp. nov., an endophytic bacterial species isolated from deep-water rice in India

Anil K. Tripathi,¹ Subhash C. Verma,¹ Soumitra Paul Chowdhury,¹ Michael Lebuhn,² Andreas Gattinger³ and Michael Schloter³

Correspondence
Michael Schloter
schloter@gsf.de

¹School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi – 221005, India

²Institute of Water Quality Control and Waste Management, Technical University of Munich, Am Coulombwall, 85748 Garching, Germany

³GSF–National Centre for Environment and Health, Institute of Soil Ecology, Ingolstaedter Landstrasse 1, 85764 Neuherberg, Germany

A non-pigmented, motile, Gram-negative bacterium designated MTCC 4195^T was isolated from surface-sterilized seeds and plant tissue from deep-water rice (*Oryza sativa*) cultivated in Suraha Tal Lake in northern India. This isolate was shown to reinfect and colonize deep-water rice endophytically. The highest level of 16S rRNA sequence similarity (96.8%) to strain MTCC 4195^T was shown by *Ochrobactrum gallinifaecis* DSM 15295^T. Strain MTCC 4195^T utilized γ -hydroxybutyric acid, adonitol, D-glucosaminic acid and arabinose as carbon sources, but failed to use gentiobiose or citrate. The cell-wall fatty acids of strain MTCC 4195^T were characterized by the presence of a relatively large proportion of C_{18:1 ω 7c} and a relative small proportion of C_{16:0} in comparison with *Ochrobactrum* species. DNA–DNA relatedness studies showed less than 52% binding with the DNAs of type strains of other species of the genus *Ochrobactrum*. On the basis of phenotypic and genotypic characteristics and the results of 16S rRNA gene sequence analysis, the novel species *Ochrobactrum oryzae* sp. nov. is proposed, with MTCC 4195^T (=DSM 17471^T) as the type strain.

A search for endophytic plant-growth-promoting bacteria in rice demonstrated the ability of some strains of *Herbaspirillum*, *Acetobacter*, *Azoarcus*, *Serratia* and *Pantoea* to colonize rice tissues endophytically (Cocking, 2003). Endophytes of rice include diverse types of nitrogen-fixing and non-nitrogen-fixing bacteria, which are found mainly in the roots, culms and seeds of various wild, traditional and cultivated varieties of rice (Barraquio *et al.*, 1997). The diversity of the endophytic bacteria associated with five varieties of deep-water rice has been characterized (Verma *et al.*, 2001). Analysis of the genotypic diversity of the endophytic bacterial communities present inside the seeds and other tissues of five varieties of deep-water rice revealed that seven different ARDRA (amplified ribosomal DNA restriction analysis) types were present in each rice variety. On the basis of the carbon-utilization pattern obtained, one of these seven ARDRA types showed greatest similarity to members of the genus *Ochrobactrum* (Kämpfer *et al.*, 2003).

Two other recent reports have described the isolation of *Ochrobactrum* strains from nodules of *Acacia mangium* (Ngom *et al.*, 2004) and *Lupinus albus* (Trujillo *et al.*, 2005). Comparison of the 16S rRNA gene sequences of *Ochrobactrum* isolates from *A. mangium* showed 98% sequence similarity with *Ochrobactrum intermedium* and *Ochrobactrum anthropi* (Ngom *et al.*, 2004), whereas the 16S rRNA gene sequence of the proposed type strain of '*Ochrobactrum lupini*' showed 100% similarity with that of *O. anthropi*, and further investigations are necessary to confirm the status of '*O. lupini*' as a distinct species (Trujillo *et al.*, 2005). Interestingly, analysis of the plasmid profile of '*O. lupini*' showed the presence of three plasmids carrying *nodD* and *nifH* genes.

The aim of the present study was to characterize the taxonomic position of strain MTCC 4195^T, isolated as an endophyte from samples taken from five varieties of deep-water rice, with respect to the type strains of species of *Ochrobactrum* with validly published names.

Seeds from deep-water rice (*Oryza sativa* varieties Jaisurya, Kariyawa, Supankhi, Tudihiwa and Siga) were surface-sterilized by treatment with 1% chloramine T for 15 min

Abbreviation: ARDRA, amplified ribosomal DNA restriction analysis.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MTCC 4195^T is AM041247.

(Barraquio *et al.*, 1997), thoroughly macerated, resuspended in 5 ml PBS (pH 7.0) and shaken for 15 min at 200 r.p.m. in an orbital shaker at room temperature. This suspension was used, after serial dilution, to inoculate nitrogen-free semi-solid medium containing malate as the carbon source (Albrecht & Okon, 1980) and incubated at 30 °C. A sub-surface pellicle appeared after 24 h. This was vortexed and transferred to fresh nitrogen-free semi-solid medium to enrich for microaerophilic, diazotrophic bacteria. After the fifth subculture, appropriate dilutions of the vortexed suspensions were spread on nutrient agar plates. Twenty randomly selected colonies isolated from the seeds of each of the five varieties were subjected to phylogenetic study. From a taxonomic point of view, 16 of the isolates were of great interest because of their ARDRA pattern (Heyndrickx *et al.*, 1996), which was very similar to those of members of the genus *Ochrobactrum*. REP-PCR (Versalovic *et al.*, 1994) and ERIC-PCR (Gillings & Holley, 1997) fingerprinting indicated that all 16 isolates were identical. The inability of the REP-PCR to reveal any genotypic differences among the 16 isolates could be because the endophytic environment resulted in genetic isolation and strong selection pressure preventing genotypic diversity within the species. A very low level of genotypic diversity was observed previously in *Ochrobactrum tritici* strains isolated from the rhizosphere of wheat (Lebuhn *et al.*, 2000). As genotypic diversity was not observed (Christensen *et al.*, 2001), we used only one isolate, MTCC 4195^T, for further identification. The characteristic ARDRA and genomic fingerprints of MTCC 4195^T were present only in the seeds and tissues of five varieties of deep-water rice and were absent from the water, sediments and soils of the rice-cultivation site.

The cells were stained according to the Gram procedure described by Doetsch (1981). Motility was investigated using phase-contrast microscopy after growth in nutrient broth at 30 °C for 48 h. The position of the flagellum and the cell size were determined using scanning electron microscopy. Cell growth on nutrient agar plates was tested at different temperatures for 48 h. Additionally, the pH range and pH optimum for growth of strain MTCC 4195^T were determined in liquid culture for 48 h at 30 °C, using buffers containing phosphate salts. The results are presented in the species description.

To determine the cellular fatty acid pattern, the procedure described by Gattinger *et al.* (2002) was used. Cells of reference type strains and strain MTCC 4195^T were grown in nutrient broth medium for 24 h at 30 °C. Clear differences were observed in the fatty acid methyl ester patterns of different *Ochrobactrum* species. Although fatty acid C_{18:1}ω7c was predominant in all extracts from the type strains of the five *Ochrobactrum* species and MTCC 4195^T, there were clear quantitative differences. Whereas the content of C_{18:1}ω7c in extracts of *O. anthropi* DSM 6882^T and *O. intermedium* LMG 3301^T constituted, respectively, only 45.1 and 41.4% of total fatty acid methyl ester content, the figure for strain MTCC 4195^T was 65.2%. The largest

content (70.9%) was found in *Ochrobactrum grignonense* DSM 13338^T. However, the most significant differences were found with respect to the saturated fatty acid fraction. The C_{16:0} content in strain MTCC 4195^T (5.1%) was significantly smaller than those of the type strains of *O. anthropi*, *O. tritici*, *Ochrobactrum gallinifaecis* and *O. intermedium*. A large content of the fatty acid C_{19:0} cyclo (16.5%) was characteristic of strain MTCC 4195^T and contrasted with the content in the type strain of *O. grignonense* (9.4%). The cellular fatty acid profiles of the strains investigated are given in Table 1.

The capacity to oxidize different carbon sources was analysed using the Microlog GN2 system (Biolog). The procedure, including growth, inoculation of plates and incubation, was performed according to the manufacturer's

Table 1. Fatty acid methyl ester compositions of members of the genus *Ochrobactrum*

Strains: 1, *O. anthropi* DSM 6882^T; 2, *O. intermedium* LMG 3301^T; 3, *O. tritici* DSM 13340^T; 4, *O. grignonense* DSM 13338^T; 5, *O. oryzae* sp. nov. MTCC 4195^T. Values are percentages of total fatty acid methyl esters.

Fatty acid methyl ester	1	2	3	4	5
C _{16:1} ω6	0.2	<0.1	0.3	<0.1	<0.1
C _{16:1} ω7c	1.7	1.1	1.6	1.6	0.94
C _{18:1} ω9	<0.1	0.1	0.5	0.1	<0.1
C _{18:1} ω7c	45.1	41.4	52.6	70.9	65.2
C _{18:1} ω7t	0.1	0.1	0.1	<0.1	0.1
C _{10:1} 9-OH	0.1	0.2	0.2	<0.1	0.1
C _{9:0} dihydroxy	<0.1	<0.1	0.1	<0.1	<0.1
C _{11:1} 11-OH	0.1	<0.1	0.1	<0.1	0.1
C _{12:0} 11-OH	0.1	<0.1	0.1	<0.1	<0.1
C _{12:1} 11-OH	0.1	0.2	0.3	<0.1	0.1
C _{13:1} 13-OH	0.2	0.1	0.2	<0.1	0.1
C _{16:0} methoxy	1.4	1.5	1.5	0.4	1.0
C _{15:1} 15-OH	0.3	0.2	0.2	<0.1	0.2
C _{16:1} 15-OH	0.9	0.8	0.1	<0.1	0.7
C _{18:0} cyclo 2-OH	0.3	1.7	1.0	1.3	1.8
C _{17:2} 16-OH	0.1	0.1	<0.1	<0.1	<0.1
C _{19:0} cyclo 2-OH	0.1	0.3	0.2	0.1	0.1
C _{14:0}	0.3	0.2	1.0	<0.1	0.1
C _{15:0} iso	<0.1	<0.1	0.1	<0.1	<0.1
C _{15:0} anteiso	0.1	<0.1	0.1	<0.1	<0.1
C _{15:0}	0.1	0.1	0.1	<0.1	<0.1
C _{16:0} iso	0.1	0.1	0.1	<0.1	<0.1
C _{16:0}	10.0	10.4	15.1	7.7	5.1
C _{17:0} anteiso	<0.1	0.1	0.1	<0.1	<0.1
C _{17:0} cyclo	0.4	0.4	0.1	<0.1	<0.1
C _{17:0}	0.6	0.3	0.4	0.3	0.3
C _{18:0}	8.4	7.8	8.1	7.7	5.6
C _{19:0} cyclo	26.4	30.7	14.1	9.4	16.5
C _{20:0}	0.1	0.1	0.1	<0.1	0.1

instructions. Strain MTCC 4195^T showed most similarity to *O. anthropi* DSM 6882^T (differences were apparent only in the utilization of gentiobiose and arabinose) and *O. tritici* DSM 13340^T (differences were found in the utilization of γ-hydroxybutyric acid and arabinose). Strain MTCC 4195^T could be distinguished from *O. intermedium* LMG 3301^T on the basis of utilization of gentiobiose, citrate and arabinose; *O. grignonense* DSM 13338^T differed from strain MTCC 4195^T in the utilization of adonitol, D-glucosaminic acid and citrate. *O. gallinifaecis* DSM 15295^T was the most distant relation of MTCC 4195^T and differed from the novel strain in terms of the utilization of γ-hydroxybutyric acid, adonitol, D-glucosaminic acid and gentiobiose. The results are summarized in Table 2.

The complete 16S rRNA gene sequence of strain MTCC 4195^T showed the highest level of similarity (96.8 %) with *O. gallinifaecis* DSM 15295^T. Comparison and alignment of the complete 16S rRNA gene sequence of strain MTCC 4195^T was done according to Lebuhn *et al.* (2000). A phylogenetic tree was constructed by using neighbour-joining analysis (Fig. 1). The tree was rooted using *Mycoplana dimorpha* IAM 13154^T as the outgroup. Identical tree topologies were obtained using other algorithms, such as maximum parsimony and maximum likelihood (puzzle) (not shown). The results clearly indicate that strain MTCC 4195^T is distantly related to *O. anthropi*, *O. tritici*, *O. intermedium* and *O. grignonense*. On the basis of the 16S rRNA gene sequence, the closest relative of strain MTCC 4195^T is *O. gallinifaecis*.

As the 16S rRNA gene sequence similarity between the novel isolate and *Ochrobactrum* species was less than 97 %, strain MTCC 4195^T may represent a novel species of the genus *Ochrobactrum*. DNA–DNA hybridization studies of the strain with standard reference strains of the five *Ochrobactrum* species were performed according to Ziemke *et al.* (1998). The results showed that strain MTCC 4195^T did not have sufficient DNA relatedness to be assigned to any known species of the genus *Ochrobactrum*. The highest level of DNA relatedness (52.7 %) was found with respect to *O.*

Table 2. Capacity to oxidize carbon sources by members of the genus *Ochrobactrum*, as determined using the Biolog system

Strains: 1, *O. anthropi* DSM 6882^T; 2, *O. intermedium* LMG 3301^T; 3, *O. tritici* DSM 13340^T; 4, *O. grignonense* DSM 13338^T; 5, *O. gallinifaecis* DSM 15295^T; 6, *O. oryzae* MTCC 4195^T. +, Positive; −, negative; (+), weakly positive.

Carbon source	1	2	3	4	5	6
γ-Hydroxybutyric acid	+	+	−	+	−	+
Adonitol	+	+	+	−	−	+
D-Glucosaminic acid	+	+	+	−	−	+
Gentiobiose	+	+	−	−	(+)	−
Citrate	−	+	−	+	−	−
Arabinose	−	−	−	+	+	+

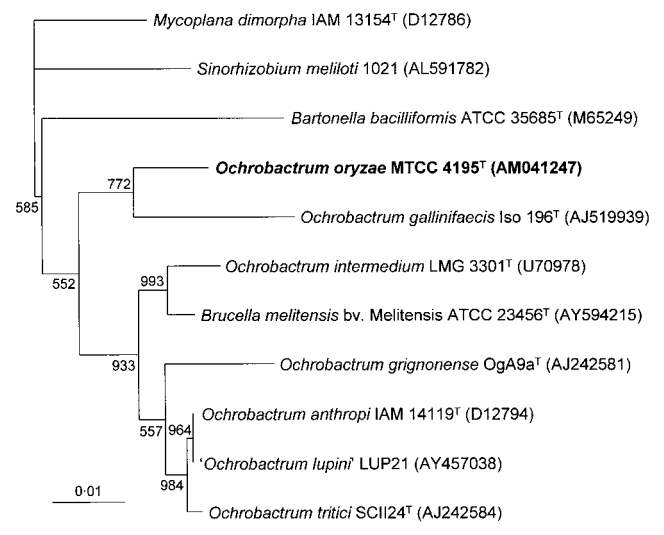


Fig. 1. CLUSTREE neighbour-joining tree based on 16S rRNA gene sequences (1374 nt) of *Ochrobactrum oryzae* sp. nov. MTCC 4195^T and related species of the genus *Ochrobactrum*. The Kimura two-parameter model was used as a substitution model, and multiple substitutions were not considered. Bootstrap probabilities were obtained from 1000 subsets. Gaps were treated as a fifth nucleotide state. Bar, 0.01 substitutions per site.

gallinifaecis DSM 15295^T. The levels of DNA relatedness with *O. anthropi* DSM 6882^T (43.5 %), *O. intermedium* LMG 3301^T (22.7 %), *O. tritici* DSM 13340^T (23.5 %) and *O. grignonense* DSM 13338^T (21.8 %) were significantly lower. Standard deviations for all hybridization experiments did not exceed 15 %.

As *Ochrobactrum* belongs to the *Rhizobiales*, and as rhizobia are known to possess the ability to fix nitrogen, it was considered worthwhile to investigate whether *nifH* sequences were present in the genome of strain MTCC 4195^T, particularly as there are entries in the NCBI database that suggest that some *Ochrobactrum* strains might be able to fix nitrogen. Amplification of *nifH* sequences was performed by using the primers described by Ueda *et al.* (1995). An amplicon of 390 bp was generated from all of the known nitrogen-fixing bacteria but not from strain MTCC 4195^T. The ability of strain MTCC 4195^T to colonize deep-water rice endophytically was demonstrated by genetically tagging the strain with a constitutively expressed GFP (green fluorescent protein) reporter, re-infecting gnotobiotically grown rice seedlings with *gfp*-tagged MTCC 4195^T and localizing expression within the plant tissues by means of confocal laser scanning microscopy (Verma *et al.*, 2004).

Description of *Ochrobactrum oryzae* sp. nov.

Ochrobactrum oryzae (L. fem. gen. n. *oryzae* of rice, pertaining to the habitat from which the first strains were isolated).

Cells are Gram-negative, aerobic (oxidase- and catalase-positive), non-spore-forming rods ($0.8 \times 1.4 \mu\text{m}$), producing non-pigmented, translucent to milky white, circular, convex, smooth colonies on nutrient agar; typical growth occurs between 10 and 37°C (optimum, 30°C) and between pH 4 and 9 (optimum, pH 6–7). Cells are motile by means of a polar flagellum. Utilization of various carbon sources was determined using the Biolog test system. Positive test results were obtained for the following: α -cyclodextrin, glycogen, Tweens 40 and 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, i-erythritol, D-fructose, L-fucose, D-galactose, α -D-glucose, myo-inositol, maltose, D-mannitol, D-mannose, psicose, L-rhamnose, D-sorbitol, sucrose, turanose, methyl pyruvate, succinic acid monomethyl ester, acetic acid, cis-aconitic acid, D-galactonic acid lactone, D-gluconic acid, D-glucosaminic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, γ -hydroxybutyric acid, α -ketoglutaric acid, DL-lactic acid, propionic acid, succinic acid, bromosuccinic acid, 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, hydroxy-L-proline, L-leucine, L-ornithine, D-serine, L-threonine, γ -aminobutyric acid, inosine and uridine. Negative Biolog reactions were obtained for dextrin, gentiobiose, D-melibiose, methyl β -D-glucosaminide, D-raffinose, xylitol, citric acid, D-galacturonic acid, p-hydroxyphenylacetic acid, itaconic acid, D-saccharic acid, L-phenylalanine, L-pyroglutamic acid, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol and glucose 1-phosphate. Predominant fatty acids are $C_{18:1\omega7c}$ (65.2%) and $C_{19:0}$ cyclo (16.5%). In addition, significant amounts of $C_{16:0}$ (5.1%) and $C_{18:0}$ cyclo 2-OH (1.8%) are detected.

The type strain, MTCC 4195^T (=DSM 17471^T), was isolated from surface-sterilized seeds and plant tissue from deep-water rice (*Oryza sativa*) cultivated in Suraha Tal Lake in northern India.

References

- Albrecht, S. L. & Okon, Y. (1980). Culture of *Azospirillum*. *Methods Enzymol* **69**, 740–749.
- Barraquio, W. L., Revilla, L. & Ladha, J. K. (1997). Isolation of endophytic diazotrophic bacteria from wetland rice. *Plant Soil* **194**, 15–24.
- Christensen, H., Bisgaard, M., Frederiksen, W., Mutters, R., Kuhnert, P. & Olsen, J. E. (2001). Is characterization of a single isolate sufficient for valid publication of a new genus or species? Proposal to modify recommendation 30b of the Bacteriological Code (1990 Revision). *Int J Syst Evol Microbiol* **51**, 2221–2225.
- Cocking, E. C. (2003). Endophytic colonization of plant roots by nitrogen-fixing bacteria. *Plant Soil* **252**, 169–175.
- Doetsch, R. N. (1981). Determinative methods of light microscopy. In *Manual of Methods for General Bacteriology*, pp. 21–33. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.
- Gattinger, A., Schloter, M. & Munch, J. C. (2002). Phospholipid etherlipid and phospholipid fatty acid fingerprints in selected euryarchaeotal monocultures for taxonomic profiling. *FEMS Microbiol Lett* **213**, 133–139.
- Gillings, M. & Holley, M. (1997). Repetitive element PCR fingerprinting (rep-PCR) using enterobacterial repetitive intergenic consensus (ERIC) primers is not necessarily directed at ERIC elements. *Let Appl Microbiol* **25**, 17–21.
- Heyndrickx, M., Vauterin, L., Vandamme, P., Kersters, K. & De Vos, P. (1996). Applicability of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. *J Microbiol Methods* **26**, 247–259.
- Kämpfer, P., Buczolits, S., Albrecht, A., Busse, H. J. & Stackebrandt, E. (2003). Towards a standardized format for the description of a novel species (of an established genus): *Ochrobactrum gallinifaecis* sp. nov. *Int J Syst Evol Microbiol* **53**, 893–896.
- Lebuhn, M., Achouak, W., Schloter, M., Berge, O., Meier, H., Barakat, M., Hartmann, A. & Heulin, T. (2000). Taxonomic characterization of *Ochrobactrum* sp. isolates from soil samples and wheat roots, and description of *Ochrobactrum tritici* sp. nov. and *Ochrobactrum grignonense* sp. nov. *Int J Syst Evol Microbiol* **50**, 2207–2223.
- Ngom, A., Nakagawa, Y., Sawada, H. & 8 other authors (2004). A novel symbiotic nitrogen-fixing member of the *Ochrobactrum* clade isolated from root nodules of *Acacia mangium*. *J Gen Appl Microbiol* **50**, 17–27.
- Trujillo, M. E., Willems, A., Abril, A., Planchuelo, A. M., Rivas, R., Ludena, D., Mateos, P. F., Martinez-Molina, E. & Velazquez, E. (2005). Nodulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov. *Appl Environ Microbiol* **71**, 1318–1327.
- Ueda, T., Suga, Y., Yahiro, N. & Matsuguchi, T. (1995). Remarkable N₂-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. *J Bacteriol* **177**, 1414–1417.
- Verma, S. C., Ladha, J. K. & Tripathi, A. K. (2001). Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. *J Biotechnol* **90**, 127–141.
- Verma, S. C., Singh, A., Chowdhury, S. P. & Tripathi, A. K. (2004). Endophytic colonization ability of two deep-water rice endophytes, *Pantoea* sp. and *Ochrobactrum* sp. using green fluorescent protein reporter. *Biotechnol Lett* **26**, 425–429.
- Versalovic, J., Schneider, M., de Bruijn, F. J. & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). *Methods Mol Cell Biol* **5**, 25–40.
- Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998). Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.