Marispirillum indicum gen. nov., sp. nov., isolated from a deep-sea environment

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A taxonomic study was carried out on strain B142^T, which was isolated from a crude-oil-degrading microbial consortium via enrichment with deep water from the Indian Ocean. Cells of the isolate were Gram-negative, oxidase-negative, catalase-positive, helical in shape, motile by means of polar flagella (three per cell) and moderately halophilic. Growth was observed at salinities of 0.5–12‰ and at temperatures of 10–41 °C. The micro-organism was capable of denitrification, but was unable to degrade Tween 80 or gelatin. The predominant fatty acids were C_{16:1}ω7c and/or iso-C_{15:0} 2-OH (6.4%), C_{16:0} (15.7%), C_{18:1}ω7c (45%), C_{18:0} (6.8%) and C_{19:0}ω8c cyclo (6.7%). The G+C content of the chromosomal DNA was 67.3 mol%. Comparisons of 16S rRNA gene sequences showed that strain B142^T was most closely related to the type strains of two *Insolitospirillum peregrinum* subspecies (93.0–93.1% sequence similarity), two *Novispirillum itersonii* subspecies (92.8–92.9%) and *Caenispirillum bisanense* (91.7%); sequence similarities with respect to other taxa were below 90.5%. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain B142^T formed a distinct evolutionary lineage within the family *Rhodospirillaceae*. Strain B142^T was distinguishable from phylogenetically related genera with regard to several phenotypic properties. On the basis of phenotypic and phylogenetic data, therefore, strain B142^T represents a novel genus and species, for which the name *Marispirillum indicum* gen. nov., sp. nov. is proposed. The type strain is B142^T (=CCTCC AB 208225^T=LMG 24627^T=MCCC 1A01235^T).

In an attempt to investigate crude-oil-degrading bacteria in the deep water of the Indian Ocean, many bacterial strains were isolated and characterized taxonomically. Comparative 16S rRNA gene sequence analysis showed that one isolate, designated strain B142^T, formed a deep branch within the phylogeny of the family *Rhodospirillaceae*. Accordingly, it was subjected to further taxonomic study, using a polyphasic approach that included the determination of its phenotypic properties and a detailed phylogenetic analysis based on 16S rRNA gene sequences.

Seawater was sampled from a depth of 1714 m (1400 m above the sea floor) at the IR-CTD13 site (69.7944° E 24.2822° S) on the south-west Indian Ridge in December 2005 during the DY-105A cruise of the R/V *Da-Yang Yi-Hao*. The seawater sample was used for an enrichment with crude oil as the carbon and energy source. Enrichment for the oil-degrading bacterial consortium was conducted on board, immediately after sampling. In the laboratory

(2 months later), 1 ml enrichment culture was transferred into 100 ml fresh MM medium (see below), incubated at 28 °C with shaking at 160 r.p.m. The MM medium consisted of NH₄NO₃ at 1.0 g l⁻¹, KH₂PO₄ at 0.5 g l⁻¹, FeSO₄·7H₂O (filtered through a 0.22 µm film) at 2.8 mg l⁻¹ and 1% (v/v) crude oil, prepared with aged deep seawater. The medium was sterilized at 121 °C for 20 min and the crude oil and the KH₂PO₄ stock solution were sterilized separately under the same conditions. Sequential transfers were performed three times at intervals of 2 weeks. Bacteria were isolated using the plate screening method on 216L medium (containing, per litre seawater: CH₃COONa, 1.0 g; tryptone, 10.0 g; yeast extract, 2.0 g; sodium citrate, 0.5 g; NH₄NO₃, 0.2 g; pH 7.5). The 216L medium was used for all studies of strain B142^T, except where indicated otherwise.

The general cell morphology was studied using an inverted microscope (IX70; Olympus) with a 1-day-old culture of strain B142^T grown on 216L agar. For electron microscopy, exponential phase cells were harvested, suspended and absorbed onto a carbon-coated Formvar grid, then stained with phosphotungstic acid (Liu & Shao, 2005); the result is shown in Supplementary Fig. S1 (available in IJSEM Online). The Gram reaction, catalase and oxidase activities,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain B142^T is EU642410.

Transmission electron micrographs of cells of strain B142^T and a table showing the cellular fatty acid contents of strain B142^T and related taxa are available as supplementary material with the online version of this paper.

lipase activity (Tween 80) and amylase activity were determined using the methods described by Liu & Shao (2005). The optimal growth temperature was determined over the temperature range 4–55 °C in 216L. Tolerance of NaCl was tested by using Luria–Bertani medium (Sambrook *et al.*, 1989) supplemented with NaCl at the following concentrations: 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18 and 20 % (w/v). Antibiotic-susceptibility tests were performed using the disc diffusion method as described by Shieh *et al.* (2003). Other biochemical tests were carried out with API 20NE and API ZYM strips (bioMérieux) according to the manufacturer's instructions, and with the Biolog GN2 MicroPlate panel as described by Ivanova *et al.* (1998). The G+C content of the chromosomal DNA was determined according to the methods described by Mesbah & Whitman (1989) using reversed-phase HPLC. These results are given in the species description and in Table 1.

Fatty acids from whole cells grown on 216L plate medium at 28 °C for 48 h were extracted, saponified and esterified; gas chromatography analysis of the fatty acid methyl esters was then performed according to the instructions of the MIDI system (Sasser, 1997). The major fatty acids of strain B142^T were C_{16:1}ω7c and/or iso-C_{15:0} 2-OH (6.4 %), C_{16:0} (15.7 %), C_{18:1}ω7c (45.0 %), C_{18:0} (6.8 %) and C_{19:0}ω8c cyclo (6.7 %).

Genomic DNA was prepared according to the method of Ausubel *et al.* (1995) and the 16S rRNA gene was amplified using PCR with primers that have been described previously (Liu & Shao, 2005). Sequences of related taxa were obtained from the GenBank database. A phylogenetic analysis was performed using MEGA, version 4 (Tamura *et al.*, 2007), after multiple alignment of the data by DNAMAN (version 5.1; Lynnon Biosoft). Distance options according to the Kimura two-parameter model (Kimura, 1980) and clustering with the neighbour-joining method of Saitou & Nei (1987) and the minimum-evolution method of Rzhetsky & Nei (1992, 1993) were determined by using bootstrap percentages based on 1000 replications. The minimum-evolution data are not shown here, as the clustering obtained was similar to that found with the neighbour-joining method.

An almost-complete 16S rRNA gene sequence (1450 nt) was determined for strain B142^T. A phylogenetic analysis of strain B142^T indicated that it belonged to the family *Rhodospirillaceae* (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strain B142^T was positioned in the phylogenetic clade comprising type strains of subspecies of *Insolitispirillum peregrinum* and *Novispirillum itersonii* and *Caenispirillum bisanense*, with which the strain exhibited the highest 16S rRNA gene sequence similarities (91.7–93.1 %). The sequence similarity values between strain B142^T and the other species used in the phylogenetic analysis were in the range 85.6–90.4 % (Fig. 1). In addition, when subjected to BLAST analysis (<http://blast.ncbi.nlm.nih.gov/blast>), strain B142^T showed the highest 16S rRNA gene sequence similarity (97.1 %) with an uncultured

Table 1. Characteristics that serve to differentiate strain B142^T from related genera

Strains: 1, B142^T (*Marispirillum indicum* gen. nov., sp. nov.); 2, *N. itersonii* subsp. *itersonii* LMG 4337^T; 3, *N. itersonii* subsp. *nipponicum* LMG 7370^T; 4, *I. peregrinum* subsp. *peregrinum* LMG 4340^T; 5, *I. peregrinum* subsp. *integrum* LMG 5407^T; 6, *C. bisanense* K92^T. Data for 2–6 were from Yoon *et al.* (2007a, b). +, Positive; –, negative; w, weak.

Characteristic	1	2	3	4	5	6
Oxidase	–	+	+	+	+	+
No. of flagella	1–3	1	1	1	1	1
Maximum growth temperature (°C)	41	43	41	39	39	47
Nitrate reduction	+	+	+	–	–	+
Anaerobic growth with KNO ₃	+	+	+	–	–	+
Hydrolysis of:						
Starch	–	–	–	–	–	+
Tween 80	–	–	–	–	–	+
Enzyme activity (API ZYM)						
Acid phosphatase	–	w	–	–	w	–
Alkaline phosphatase	+	+	+	+	+	–
Esterase (C4)	–	+	+	–	–	+
Esterase lipase (C8)	–	+	+	–	–	+
Leucine aminopeptidase	+	w	–	–	–	–
Naphthol-AS-BI-phosphoamidase	w	+	+	+	+	w
β-Glucosidase	–	–	–	+	+	–
Susceptibility to:						
Ampicillin	–	+	+	+	+	+
Carbenicillin	+	–	–	+	+	+
Lincomycin	–	–	+	–	–	–
Penicillin G	–	+	+	+	+	+
Polymyxin B	–	w	–	+	+	+
Tetracycline	–	+	+	+	+	+
Utilization of:						
Glycerol	–	–	+	–	–	+
Citrate	–	–	–	–	–	+
D-Fructose	–	+	+	+	+	–
Glycogen	+	–	–	–	–	–
DNA G+C content (mol%)	67.3	63.2	64.7	62.4	62.3	70

proteobacterial clone isolated from subsurface water from the Kalahari Shield in South Africa (GenBank accession no. DQ234645.1; Gihring *et al.*, 2006).

Strain B142^T is oxidase-negative and the cells have bipolar flagella: thus, it is distinguishable from *I. peregrinum*, *N. itersonii* and *C. bisanense*, which are oxidase-positive and consist of cells with single polar flagella. The fatty acid profiles of strain B142^T were similar to those of *I. peregrinum*, *N. itersonii* and *C. bisanense* (see Supplementary Table S1, available in IJSEM Online). However, strain B142^T showed minor differences in the proportions of several fatty acids and only strain B142^T

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strain B142^T and representatives of some related taxa in the family *Rhodospirillaceae*. Bootstrap percentages (based on 1000 replications) are shown at branch points. Bar, 0.01 nucleotide substitution rate (K_{nuc}) units.

contained C_{17:0} cyclo (4.7%) (see Supplementary Table S1 in IJSEM Online). Strain B142^T was also distinguishable from *I. peregrinum*, *N. itersonii* and *C. bisanense* in terms of several phenotypic properties, as shown in Table 1. The low levels of 16S rRNA gene sequence similarity between strain B142^T and all other members of the family *Rhodospirillaceae*, together with the differential phenotypic properties, suggest that strain B142^T represents a novel genus and species within the family *Rhodospirillaceae*, for which the name *Marispirillum indicum* gen. nov., sp. nov. is proposed.

Description of *Marispirillum* gen. nov.

Marispirillum (Ma.ri.spi.ril'lum. L. neut. n. *mare* the sea; N.L. dim. neut. n. *spirillum* a small spiral; N.L. neut. n. *Marispirillum* a small spiral of the sea).

Cells are Gram-negative, oxidase-negative, catalase-positive and helical in shape. Major fatty acids are C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, C_{16:0}, C_{18:1}ω7c, C_{18:0} and C_{19:0}ω8c cyclo. The type species is *Marispirillum indicum*.

Description of *Marispirillum indicum* sp. nov.

Marispirillum indicum (in.di'cum. L. neut. adj. *indicum* Indian, referring to the Indian Ocean, where the strain was isolated).

Exhibits the following properties in addition to those given in the genus description. Cells are 1.9–2.7 μm long and 0.6–0.7 μm wide and motile, by means of 1–3 flagella (one flagellum at each pole). Positive for denitrification, lipase (Tween 80), amylase, urease, gelatinase, arginine dihydro-lase and indole production. On 216L agar plates, colonies are smooth, grey and yellowish with regular edges, 1–2 mm in diameter and slightly raised in the centre after 72 h

incubation at 28 °C. Moderately halophilic, growing with NaCl at 0.5–12% (optimally at 2–8%). Grows at 10–41 °C (optimally at 25–37 °C), but not at 4 or 45 °C, within 7 days. Unable to ferment glucose. Sensitive to the following antibiotics (Oxoid) (μg per disc): carbenicillin (100), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), gentamicin (10), kanamycin (30), minocycline (30), neomycin (10), norfloxacin (10), ofloxacin (5), rifampicin (5), ceftriaxone (30), streptomycin (10) and doxycycline (30). Resistant to the following antibiotics (μg per disc, unless indicated otherwise): ampicillin (10), cefalexin (30), cefazolin (30), cefoperazone (30), cefradin (30), clindamycin (2), co-trimoxazole (25), furazolidone (15), lincomycin (2), metronidazole (5), oxacillin (1), penicillin G (10), piperacillin (100), polymyxin B (30 U), tetracycline (30) and vancomycin (30). In the API ZYM test, positive for alkaline phosphatase and leucine aminopeptidase, weakly positive for naphthol-AS-BI-phosphatase and negative for acid phosphatase, cystine aminopeptidase, esterase (C4), esterase lipase (C8), lipase (C14), *N*-acetyl-β-glucosaminidase, trypsin, valine aminopeptidase, α-chymotrypsin, α-fucosidase, α-galactosidase, α-glucosidase, α-mannosidase, β-galactosidase, β-glucosidase and β-glucuronidase. In the API 20NE test, positive for utilization of potassium gluconate and malic acid, but not D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, capric acid, adipic acid, trisodium citrate or phenylacetic acid. In the Biolog GN2 system (96 substrates), positive for L-alaninamide, L-alanine, bromosuccinic acid, DL-lactic acid, DL-α-glycerol phosphate, D-galactonic acid lactone, D-gluconic acid, D-psicose, glycogen, L-glutamic acid, L-phenylalanine, L-proline, L-threonine, pyruvic acid methyl ester, succinic acid monomethyl ester, *p*-hydroxyphenylacetic acid, propionic acid, succin-

amic acid, succinic acid, Tween 40, α -hydroxybutyric acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, β -hydroxybutyric acid and γ -hydroxybutyric acid. The DNA G+C content of the type strain is 67.3 mol%.

The type strain, B142^T (=CCTCC AB 208225^T=LMG 24627^T=MCCC 1A01235^T), was isolated from deep-water samples from the Indian Ocean.

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