Oceanibaculum pacificum sp. nov., isolated from hydrothermal field sediment of the south-west Pacific Ocean

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A taxonomic study was carried out on strain LMC2up-L3^T, which was isolated from a polycyclic aromatic hydrocarbon-degrading consortium enriched with a sediment sample collected from a hydrothermal field of the south-west Pacific Ocean. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain LMC2up-L3^T belonged to the genus *Oceanibaculum*, with the highest sequence similarity of 98.4 % to *Oceanibaculum indicum* P24^T; similarity to other strains was below 93.1 %. DNA–DNA hybridization between strain LMC2up-L3^T and *O. indicum* P24^T was 31 %. rep-PCR fingerprints also differentiated strain LMC2up-L3^T from *O. indicum* P24^T. The G + C content of the chromosomal DNA was 67.7 mol%. The principal fatty acids were C_{16:1} (17.8 %), C_{16:0} (21.2 %), C_{18:1}ω7c (23.6 %), C_{18:0} (4.1 %), C_{18:1} 2-OH (4.5 %) and C_{19:0} cyclo ω8c (17.4 %). The combined genotypic and phenotypic data show that strain LMC2up-L3^T represents a novel species of the genus *Oceanibaculum*, for which the name *Oceanibaculum pacificum* sp. nov. is proposed, with the type strain LMC2up-L3^T (=CCTCC AB 209059^T =LMG 24859^T =MCCC 1A02656^T). An emended description of the genus *Oceanibaculum* is also provided.

In an attempt to investigate polycyclic aromatic hydrocarbon (PAH)-degrading bacteria from sediments of a hydrothermal field in the Lau Basin of the south-west Pacific Ocean, many bacterial strains were isolated and identified by 16S rRNA gene sequence analysis. This study focuses on one of these isolates, designated strain LMC2up-L3^T. Comparative 16S rRNA gene sequence analysis indicated that strain LMC2up-L3^T formed a clade with *Oceanibaculum indicum* P24^T, but shared sequence similarity of only 98.4 %. Therefore, the aim of the present work was to determine the exact taxonomic position of strain LMC2up-L3^T by using a polyphasic characterization including the determination of phenotypic properties and a detailed phylogenetic analysis based on 16S rRNA gene sequences.

Abbreviation: PAH, polycyclic aromatic hydrocarbon.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LMC2up-L3^T is FJ463255.

Transmission electron micrographs of cells of strain LMC2up-L3^T, the cellular fatty acid profile of strain LMC2up-L3^T and a comparison of rep-PCR profiles are available as supplementary material with the online version of this paper.

Deep-sea sediment was sampled by a television multi-core sampler in May 2007 during cruise DY115-19 of the R/V *Da-Yang Yi-Hao*. The sediment sample was about 15 cm in depth and 10 cm in diameter and was numbered TVMC2. The sampling site was located at 22.5338° S 176.7999° W in the Lau Basin hydrothermal field, at the depth of 2725 m. The sample was sectioned aseptically into three subsamples and used for enrichment of PAH-degrading bacteria with 1 % (v/v) sterilized PAH mixture (final concentrations of 100 p.p.m. naphthalene, 100 p.p.m. phenanthrene and 20 p.p.m. pyrene) as the carbon and energy source. Enrichment was carried out on-board immediately after sampling. Within the laboratory, 2 months later, 1 ml enrichment culture was transferred into 100 ml fresh seawater medium containing (l⁻¹) 1.0 g NH₄NO₃, 0.5 g KH₂PO₄ and 2.8 mg FeSO₄·7H₂O, with the PAH mixture as the sole carbon and energy source. PAH stock solution in chloroform was added to flasks to the same concentrations given above and the flasks were then shaken at 160 r.p.m. for 2 days to evaporate the chloroform before inoculation. After incubation for 4 weeks at 28 °C and 160 r.p.m., 1 ml culture broth was transferred repeatedly to the same medium for

further enrichment. Three sequential transfers were performed at 4-week intervals. Bacteria were isolated by the plate-screening method on 216L medium [(1 seawater)⁻¹: 1.0 g sodium acetate, 10.0 g tryptone, 2.0 g yeast extract, 0.5 g sodium citrate, 0.2 g NH₄NO₃; pH 7.5]. This medium was also used for morphological and biochemical characterization.

Genomic DNA was prepared according to the method of Ausubel *et al.* (1995); the 16S rRNA gene was amplified by PCR using primers described previously (Liu & Shao, 2005). Sequences of related taxa were obtained from the GenBank database. Phylogenetic analysis was performed using MEGA version 4 (Tamura *et al.*, 2007) after multiple alignment of data by DNAMAN (version 6.0; Lynnon Biosoft). Distances (Kimura's two-parameter model) and clustering with the neighbour-joining method (Saitou & Nei, 1987) and minimum-evolution method (Rzhetsky & Nei, 1992, 1993) were determined by using bootstrap values based on 1000 replications. As the results were similar, results for the minimum-evolution method are not shown.

A nearly full-length 16S rRNA gene sequence (1451 nt) of strain LMC2up-L3^T was determined. The neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) showed that strain LMC2up-L3^T and *O. indicum* P24^T formed an independent monophyletic cluster with the highest sequence similarity of 98.4%; similarity to other strains was below 93.1%. The high 16S rRNA gene sequence similarity strongly indicated that strain LMC2up-L3^T belongs to the genus *Oceanibaculum*, proposed recently by Lai *et al.* (2009).

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain LMC2up-L3^T and representatives of some other related taxa, based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0.02 nucleotide substitution rate (K_{nuc}) units.

DNA–DNA hybridization experiments were performed with genomic DNA of strain LMC2up-L3^T and *O. indicum* P24^T as described previously (Liu & Shao, 2005). Genomic DNA from *Escherichia coli* DH5 α was used as an outgroup. Salmon-sperm DNA was used as a negative control. The results showed that strain LMC2up-L3^T and *O. indicum* P24^T possessed low DNA–DNA relatedness (31%), demonstrating their affiliation to different species in accordance with the cut-off value of 70% recognized by Wayne *et al.* (1987) for discrimination of bacterial species. Strain LMC2up-L3^T and *O. indicum* P24^T were further compared by rep-PCR. In this study, the primer BOX-A1R (5'-CTACGGCAAGGCGACGCTGACG-3') was used for rep-PCR fingerprint analysis (Versalovic *et al.* 1991). The PCR was carried out with the following cycle conditions: 5 min denaturation at 94 °C; 35 cycles of 15 s at 94 °C, 30 s at 53 °C and 8 min at 65 °C and a final extension at 65 °C for 8 min. PCR products were separated by 2% agarose gel electrophoresis. The rep-PCR results are shown in Supplementary Fig. S1 (available in IJSEM Online). Strain LMC2up-L3^T showed a different rep-PCR fingerprint pattern from *O. indicum* P24^T, supporting the DNA–DNA hybridization result.

General cell morphology was studied under an Olympus inverted microscope (IX-70) using a 1-day-old culture on 216L agar. For electron microscopy, exponential-phase cells were harvested and subsequently suspended and absorbed on a Formvar–carbon-coated grid and then stained with phosphotungstic acid (Supplementary Fig S2). The Gram reaction, catalase and oxidase activities, lipase (Tween 80) and amylase were assessed according to Dong & Cai (2001). The temperature for growth was determined over the range 4–55 °C on 216L agar. Tolerance of NaCl was tested by using Luria–Bertani medium (Sambrook *et al.*, 1989) supplemented with NaCl at 0, 0.5, 1–10 (in increments of 1%), 12, 15, 18 and 20% (w/v). The presence of bacteriochlorophyll *a* was assessed as described previously (Biebl *et al.*, 2005). Other biochemical tests were carried out by using API 20NE and API ZYM strips (bioMérieux) according to the manufacturer's instructions and by using the Biolog GN2 Microplate system according to Ivanova *et al.* (1998). The G + C content of the chromosomal DNA was determined according to the methods described by Mesbah & Whitman (1989) using a reversed-phase HPLC. These results are given in the species description and Table 1.

Antibiotic susceptibility tests were carried out by the disc-diffusion method as described by Shieh *et al.* (2003). Strain LMC2up-L3^T and *O. indicum* P24^T were tested in parallel. Both strains were sensitive to (per disc; Oxoid) chloromycetin (30 µg), ciprofloxacin (5 µg), kanamycin (30 µg), neomycin (10 µg), polymyxin B (30 U) and streptomycin (10 µg) and resistant to cefalexin (30 µg), cefazolin (30 µg), cefobid (30 µg), cephradine (30 µg), clindamycin (2 µg), erythromycin (15 µg), furazolidone (15 µg), lincomycin (2 µg), metronidazole (5 µg), minomycin (30 µg), norfloxacin (10 µg), ofloxacin (5 µg), oxacillin (1 µg),

Table 1. Characteristics that differentiate strain LMC2up-L3^T from *O. indicum* P24^T

Data for *O. indicum* P24^T were taken from Lai *et al.* (2009). +, Positive; w, weakly positive; –, negative.

Characteristic	LMC2up-L3 ^T	<i>O. indicum</i> P24 ^T
Catalase	+	–
Sensitivity to antimicrobial agents*		
Co-trimoxazole (25 µg)	–	+
Ampicillin (10 µg), carbenicillin (100 µg), gentamicin (10 µg), penicillin G (10 µg), rifampicin (5 µg), rocephin (30 µg), tetracycline (30 µg), vibramycin (30 µg)	+	–
API 20NE results		
Nitrate reduction	–	+
Arginine dihydrolase, urease	+	–
API ZYM results		
Esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, naphthol-AS-BI-phosphohydrolase	+	w
Trypsin, α-chymotrypsin	w	–
Biolog GN2 results		
<i>cis</i> -Aconitic acid, dextrin, D-fructose, glycogen, L-alaninamide, methyl pyruvate, α-cyclodextrin	+	–
L-Alanine, L-proline	+	w
L-Histidine, L-leucine, L-phenylalanine, Tween 80, uridine, urocanic acid	w	–
D-Arabitol, L-rhamnose	–	+
α-Ketoglutaric acid, D-saccharic acid, L-glutamic acid, glycyl L-glutamic acid, L-pyroglutamic acid	–	w
DNA G + C content (mol%)	67.7	64.8

*Amounts given are per disc.

piperacillin (100 µg) and vancomycin (30 µg). Differential susceptibility to other antibiotics is shown in Table 1.

Fatty acids from whole cells grown on 216L agar plate medium at 28 °C for 48 h were extracted, saponified and esterified, followed by GC analysis of the fatty acid methyl esters according to the instructions of the MIDI System (Sasser, 1990). The fatty acid profile of *O. indicum* P24^T was determined in parallel with that of strain LMC2up-L3^T (Supplementary Table S1). The principal fatty acids of strain LMC2up-L3^T were C_{16:1} (17.8%), C_{16:0} (21.2%), C_{18:1ω7c} (23.6%), C_{18:0} (4.1%), C_{18:1} 2-OH (4.5%) and C_{19:0} cyclo ω8c (17.4%). The two strains showed large differences (>5 percentage points) in the content of summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH), C_{18:1ω7c} and C_{19:0} cyclo ω8c.

The high 16S rRNA gene sequence similarity between strain LMC2up-L3^T and *O. indicum* P24^T indicated strongly that strain LMC2up-L3^T belongs to the genus *Oceanibaculum*. However, strain LMC2up-L3^T can be differentiated from *O. indicum* P24^T on the basis of phenotypic properties, DNA–DNA hybridization and rep-PCR fingerprint comparison. As shown in Table 1, strain LMC2up-L3^T was positive for catalase, urease and arginine dihydrolase, whereas *O. indicum* P24^T was negative. The two strains also give different utilization reactions for 22 carbon sources in the Biolog GN2 microplate. On the basis of the results described above, strain LMC2up-L3^T should be placed in a novel species of the genus *Oceanibaculum*, for

which the name *Oceanibaculum pacificum* sp. nov. is proposed.

Emended description of the genus *Oceanibaculum* Lai *et al.* 2009

Cells are rod-shaped, motile by a single polar flagellum, Gram-negative and oxidase-positive. Catalase activity and nitrate reduction are variable. The principal fatty acids are C_{16:1ω7c}, C_{16:0}, C_{18:1ω7c}, C_{18:0}, C_{18:1} 2-OH and C_{19:0} cyclo ω8c. Bacteriochlorophyll *a* is not detected. The DNA G + C content is 64.8–67.7 mol%. The type species is *Oceanibaculum indicum*.

Description of *Oceanibaculum pacificum* sp. nov.

Oceanibaculum pacificum (pa.ci'fi.cum. L. neut. adj. *pacificum* peaceful, pertaining to the Pacific Ocean, where the type strain was isolated).

Displays the following properties in addition to those given in the emended genus description. Cells are 1.7–2.1 µm long and 0.5–0.7 µm wide. Positive for catalase, urease and arginine dihydrolase; negative for lipase (Tween 80), amylase, gelatinase, indole production and nitrate reduction. On 216L agar plates, forms smooth, grey colonies with regular edges, 1–2 mm in diameter after 72 h incubation at 28 °C, non-pigmented and slightly raised in the centre. Moderately halophilic; grows in 0–9% NaCl (optimum 1.5–5.0%). Grows at 10–45 °C (optimum 28–

37 °C) but not at 4 or 50 °C after 7 days. Unable to ferment glucose. Sensitive to (per disc; Oxoid) ampicillin (10 µg), carbenicillin (100 µg), chloromycetin (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), kanamycin (30 µg), neomycin (10 µg), penicillin G (10 µg), polymyxin B (30 U), rifampicin (5 µg), rocephin (30 µg), streptomycin (10 µg), tetracycline (30 µg) and vibramycin (30 µg). Resistant to cefalexin (30 µg), cefazolin (30 µg), cefobid (30 µg), cephradine (30 µg), clindamycin (2 µg), co-trimoxazole (25 µg), erythromycin (15 µg), furazolidone (15 µg), lincomycin (2 µg), metronidazole (5 µg), minomycin (30 µg), norfloxacin (10 µg), ofloxacin (5 µg), oxacillin (1 µg), piperacillin (100 µg) and vancomycin (30 µg). In API ZYM tests, positive for acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase (C4), esterase lipase (C8), leucine arylamidase, lipase (C14), naphthol-AS-BI-phosphohydrolase and valine arylamidase, weakly positive for trypsin and α -chymotrypsin and negative for *N*-acetyl- β -glucosaminidase, α -fucosidase, α -galactosidase, α -glucosidase, α -mannosidase, β -galactosidase, β -glucosidase and β -glucuronidase. Of 95 substrates in the Biolog GN2 system, positive for utilization of *cis*-aconitic acid, dextrin, D-fructose, glycogen, L-alaninamide, L-alanine, L-proline, methyl pyruvate and α -cyclodextrin and weakly positive for bromosuccinic acid, L-histidine, L-leucine, L-phenylalanine, succinamic acid, Tweens 40 and 80, uridine and urocanic acid; negative for the other substrates. The DNA G+C content of the type strain is 67.7 mol%.

The type strain, LMC2up-L3^T (=CCTCC AB 209059^T =LMG 24859^T =MCCC 1A02656^T), was isolated from sediment retrieved from a hydrothermal vent field of the Lau Basin in the south-west Pacific Ocean.

Acknowledgements

This work was supported financially by the National Infrastructure of Natural Resources for Science and Technology Program of China (no. 2005DKA21209), the COMRA program (no. DYXM115-02-2-05), the National Basic Research Program of China (no. 2004CB719601) and the National Natural Science Foundation of China (nos 36100501 and 40376041).

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