Nitratireductor pacificus sp. nov., isolated from a pyrene-degrading consortium

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Strain pht-3B^T was isolated from a pyrene-degrading consortium of an enriched sediment from the Pacific Ocean, collected during the screening of polycyclic aromatic hydrocarbon-degrading bacteria. Cells were Gram-negative, short rods that were motile by means of flagella. Growth was observed at 0–7 % NaCl and 10–41 °C. The isolate was able to reduce nitrate to nitrite, but not to nitrogen. 16S rRNA gene sequence comparisons showed that strain pht-3B^T was most closely related to *Nitratireductor aquibiodomus* NL21^T (97.3 % 16S rRNA gene sequence similarity), *N. indicus* C115^T (97.1 %), *N. basaltis* J3^T (96.8 %) and *N. kimnyeongensis* KY 101^T (96.7 %). DNA–DNA hybridization between strain pht-3B^T and these reference strains revealed 55, 54, 28 and 42 % DNA–DNA relatedness, respectively. The dominant fatty acids were $C_{19:0}\omega8c$ cyclo (22.6 %) and summed feature 8 (consisting of $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; 60.4 %). The G+C content of the chromosomal DNA was 63 mol%. These characteristics were in good agreement with those of members of the genus *Nitratireductor*. According to cell morphology, physiology, fatty acid composition, 16S rRNA gene sequence analysis and DNA–DNA relatedness, the isolate belonged to the genus *Nitratireductor* but could be readily distinguished from recognized species of the genus. Therefore a novel species is proposed to accommodate strain pht-3B^T, for which the name *Nitratireductor pacificus* sp. nov. is proposed. The type strain is pht-3B^T (=CCTCC AB 209302^T=LMG 25541^T=MCCC 1A01024^T).

The genus *Nitratireductor*, belonging to the family *Phyllobacteriaceae*, was proposed by Labbé *et al.* (2004) and it currently comprises four species: *Nitratireductor aquibiodomus*, isolated from a denitrification system (Labbé *et al.*, 2004), *Nitratireductor kimnyeongensis*, from seaweed (Kang *et al.*, 2009), *Nitratireductor basaltis*, from black sand (Kim *et al.*, 2009) and *Nitratireductor indicus*, from deep-sea water (Lai *et al.*, 2011b). In this study, we describe a novel strain, pht-3B^T, which was isolated from a pyrene-degrading consortium from an enriched deep-sea sediment sample collected from the western Pacific Ocean (Wang *et al.*, 2008) and shown to be phylogenetically related to members of the genus *Nitratireductor*. At the time of writing, four novel species from this pyrene-degrading consortium have been described: *Roseovarius pacificus* (Wang *et al.*, 2009), *Oceanicola pacificus* (Yuan *et al.*, 2009), *Bowmanella pacifica* (Lai *et al.*, 2009) and *Alcanivorax pacificus* (Lai *et al.*, 2011a).

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain pht-3B^T is DQ659453.

A supplementary figure and a supplementary table are available with the online version of this paper.

Genomic DNA was prepared according to the method of Ausubel *et al.* (1995) and the 16S rRNA gene was amplified by PCR using primers described previously (Liu & Shao, 2005). Amplification products were ligated into the pMD20-T vector and transformed into *Escherichia coli* DH5α. Sequencing was carried out on an automated DNA sequencer (model 3730; Applied Biosystems) using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems). 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 5.1 (Lynnon Biosoft). Distances were determined using options according to the Kimura two-parameter model and clustering was performed with the neighbour-joining method of Saitou & Nei (1987) and the minimum-evolution method of Rzhetsky & Nei (1992, 1993). Bootstrapping analysis based on 1000 resamplings was used to evaluate tree topology. A nearly full-length 16S rRNA gene sequence (1448 nt) of strain pht-3B^T was determined. The neighbour-joining phylogenetic analysis (Fig. 1) indicated that strain pht-3B^T belonged to the family *Phyllobacteriaceae* and formed a robust clade with the genus *Nitratireductor*; a similar result was obtained with the minimum-evolution analysis (data not shown). The closest relatives of strain

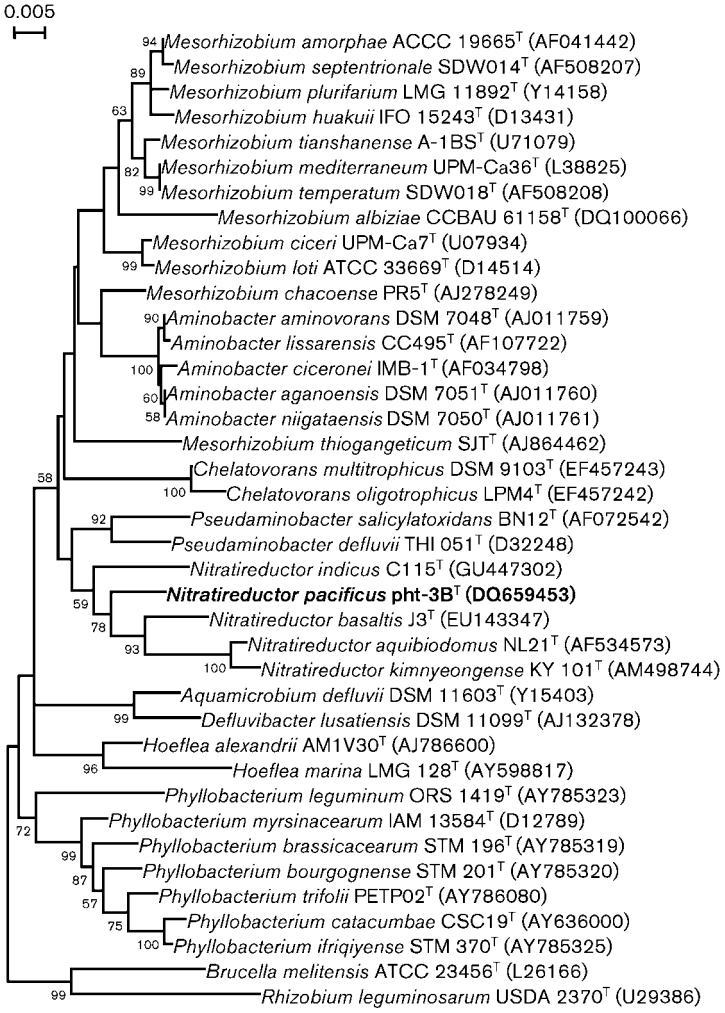

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic positions of strain pht-3B^T and representatives of other related taxa. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. Bar, 0.005 substitution rate (K_{nuc}).

pht-3B^T were *N. aquibiodomus* NL21^T (97.3 % 16S rRNA gene sequence similarity), *N. indicus* C115^T (97.1 %), *N. basaltis* J3^T (96.8 %) and *N. kimnyeongensis* KY 101^T (96.7 %).

DNA–DNA hybridization experiments were performed with genomic DNA from strain pht-3B^T, *N. aquibiodomus* NL21^T, *N. indicus* C115^T, *N. basaltis* J3^T and *N. kimnyeongensis* KY 101^T using a previously described method with five replicates (Coram & Rawlings, 2002; Tønjum *et al.*, 1998). Genomic DNA from *Escherichia coli* DH5α was used as a reference sample and salmon sperm DNA was used as a negative control. The DIG High Prime DNA Labelling and Detection Starter Kit II (Roche) was used, the hybridization temperature was 42 °C and the formamide concentration was 50 %. Strain pht-3B^T exhibited low DNA–DNA relatedness with *N. aquibiodomus* NL21^T, *N. indicus* C115^T, *N. basaltis* J3^T and *N. kimnyeongensis* KY 101^T (55±3, 54±2, 28±4 and 42±5 %, respectively), which, in accordance with the 70 % cut-off value recognized by Wayne *et al.* (1987) for discrimination of bacterial species, demonstrated the isolate's affiliation to a separate species.

General cell morphology was studied under an inverted microscope (IX70; Olympus) using 1-day-old cultures grown on marine agar 2216 (BD) medium. For electron microscopy, exponential-phase cells were harvested, resuspended and absorbed on a Formvar–carbon-coated grid and then stained with phosphotungstic acid. Cells exhibited more than one flagellum (Supplementary Fig. S1, available in IJSEM Online). Gram-reaction, catalase, oxidase and lipase (Tween 80) activities and hydrolysis of aesculin and starch were tested according to Dong & Cai (2001). Growth at 4, 10, 20, 25, 37, 42 and 55 °C and at pH 3–12 was determined in 216L medium (containing l^{-1} seawater: 1.0 g CH_3COONa, 10.0 g tryptone, 2.0 g yeast extract, 0.5 g sodium citrate, 0.2 g NH_4NO_3; pH 7.5 unless otherwise stated). Growth with 0, 0.5, 1, 3, 5, 7, 8, 9, 10, 12 and 15 % (w/v) NaCl was tested using Luria–Bertani medium (Sambrook *et al.*, 1989). Antibiotic susceptibility tests were performed using disc diffusion methods according to Shieh *et al.* (2003). Other biochemical tests were carried out using the API 20 NE and API ZYM systems (bioMérieux) and GN2 MicroPlates (Biolog), according to the manufacturers' instructions, except that the NaCl concentration in all tests was adjusted to 3.0 %

Table 1. Characteristics that differentiate strain pht-3B^T from type strains of species of the genus *Nitratireductor*

Strains: 1, pht-3B^T; 2, *N. aquibiodomus* NL21^T (data from Labbé *et al.*, 2004); 3, *N. indicus* C115^T (Lai *et al.*, 2011b); 4, *N. basaltis* J3^T (Kim *et al.*, 2009); 5, *N. kimnyeongensis* KY 101^T (Kang *et al.*, 2009). All strains are positive for nitrate reduction, acid phosphatase, alkaline phosphatase, esterase (C4), leucine aminopeptidase, trypsin and valine aminopeptidase, utilization of citric acid, DL-lactic acid, cellobiose, L-glutamic acid, L-proline, L-pyroglutamic acid, L-serine, sucrose and β-hydroxybutyric acid and resistant to kanamycin, lincomycin and metronidazole. All strains are negative for indole production, D-glucose fermentation, gelatin hydrolysis, α-fucosidase, α-mannosidase and β-glucuronidase, utilization of 2,3-butanediol, 2-aminoethanol, adonitol, DL-carnitine, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, melibiose, D-psicose, raffinose, D-saccharic acid, D-serine, formic acid, gentiobiose, α-D-glucose 1-phosphate, glucose 6-phosphate, glucuronamide, i-erythritol, lactulose, L-rhamnose, malonic acid, phenylethylamine, *p*-hydroxyphenylacetic acid, putrescine, quinic acid, sebacic acid, xylitol, α-cyclodextrin, lactose, α-ketovaleric acid and methyl β-D-glucoside and resistant to cefalexin, cefobid, chloromycetin, erythromycin, rifampicin and rocephin. +, Positive/sensitive; W, weakly positive; −, negative/resistant; ND, not determined.

Characteristic	1	2	3	4	5
Cell shape	Rods	Rods	Rods	Cocci, rods	Rods
Cell dimensions (μm)					
Width	0.8–0.9	1	1.3	0.6–0.7	0.4–0.5
Length	1.4–1.5	3	3	0.6–2.0	1.2–2.7
Motility	+	+	+	−	+
NaCl for growth (%, w/v)					
Range	0–7	0–5	0–7	0–8	0–7
Optimum	3	1	3	ND	ND
Temperature for growth (°C)					
Range	10–41	ND	10–37	15–45	10–45
Optimum	25–30	30–35	25–30	ND	30
API 20 NE*					
Arginine dihydrolase	−	W	+	−	W
Urease	−	W	+	−	W
Aesculin hydrolysis	+	+	+	W	+
β-Galactosidase	W	W	W	+	−
Capric acid	+	−	−	−	W
Maltose, L-arabinose	−	W	−	−	−
D-Mannitol	−	W	−	+	W
Potassium gluconate	−	W	−	+	−
Adipic acid	W	−	+	+	−
D-Glucose, *N*-acetylglucosamine	+	+	+	+	W
D-Mannose	W	+	+	−	+
Malic acid, phenylacetic acid	−	−	+	−	−
Trisodium citrate	+	+	+	−	−
API ZYM*					
Cystine aminopeptidase, naphthol-AS-BI-phosphoamidase, α-chymotrypsin, α-glucosidase	+	+	+	W	+
Esterase lipase (C8)	+	+	−	+	+
Lipase (C14)	+	W	+	+	W
N-Acetyl-β-glucosaminidase	+	+	−	−	−
α-Galactosidase, β-galactosidase	W	−	−	+	−
β-Glucosidase	+	−	−	−	−
Susceptibility to antibiotics*					
Ampicillin, carbenicillin, piperacillin	+	−	+	+	−
Cefazolin, penicillin G	+	−	+	+	+
Cephradin, streptomycin, tetracycline, vibramycin	−	+	−	+	+
Ciprofloxacin, minomycin	−	−	−	+	+
Clindamycin, co-trimoxazole, gentamicin, norfloxacin, ofloxacin, oxacillin, polymyxin B, vancomycin	−	−	−	+	−
GN2 MicroPlates*					
Acetic acid	+	W	W	+	−
Bromosuccinic acid, turanose	−	−	W	−	−
cis-Aconitic acid, succinic acid	−	−	+	W	−
DL-α-Glycerol phosphate, propionic acid	−	−	+	−	−

Table 1. cont.

Characteristic	1	2	3	4	5
D-Alanine, L-alanine	+	+	+	+	−
D-Arabitol, D-mannose	−	−	−	−	+
Dextrin, itaconic acid, thymidine	−	−	−	w	−
D-Fructose, D-mannitol	−	−	−	+	+
D-Galactose	−	+	−	w	+
D-Glucosaminic acid	−	+	−	−	−
D-Sorbitol, glycogen, inosine, L-arabinose, Tween 40, uridine, γ-hydroxybutyric acid	−	−	−	+	−
Trehalose	w	+	+	−	+
Glycerol	+	+	w	+	+
Glycyl L-aspartic acid, L-alanyl glycine	+	+	−	w	w
Glycyl L-glutamic acid	+	w	+	+	w
Hydroxyl-L-proline	w	+	w	+	+
L-Alaninamide	+	+	−	+	−
L-Asparagine	+	+	+	w	+
L-Aspartic acid	−	−	w	+	+
L-Fucose	−	+	−	w	−
L-Histidine, L-ornithine	−	+	w	−	+
L-Leucine	+	−	w	+	−
L-Phenylalanine	+	+	−	−	−
L-Threonine	−	+	w	+	+
Maltose	w	−	−	+	+
Pyruvic acid methyl ester	w	+	+	w	+
myo-Inositol	−	+	+	−	w
Succinic acid monomethyl ester, *N*-acetyl-D-glucosamine	+	+	+	−	+
N-Acetyl-D-galactosamine	−	+	−	−	w
Succinamic acid	−	−	+	+	w
Tween 80	w	−	−	+	−
Urocanic acid	+	−	+	−	+
α-D-Glucose	−	+	+	+	+
α-Hydroxybutyric acid	−	+	+	w	w
α-Ketobutyric acid	w	−	w	−	−
α-Ketoglutaric acid	+	w	+	w	+
γ-Aminobutyric acid	w	w	+	+	w
DNA G+C content (mol%)	63	57	59	56.7	60.4

*Data for all strains were obtained in this study under the same conditions.

(Lai *et al.*, 2011b). Data for the four reference strains were obtained in this study for catalase, oxidase, API 20 NE, API ZYM, GN2 MicroPlates and antibiotic resistance under the same conditions as the novel strain. The results are given in the species description and Table 1.

The G+C content of the chromosomal DNA was determined according to the method described by Mesbah & Whitman (1989) using reversed-phase HPLC. The DNA G+C content of strain pht-3B^T was 63 mol%, which was a little higher than the values obtained for the four reference strains (56.7–60.4 mol%).

For whole-cell fatty acid analysis, cells were prepared by culture on marine agar 2216 at 28 °C for 48 h. Fatty acids were extracted, saponified and esterified and fatty acid methyl esters were analysed by GC according to the instructions of the MIDI system (Sasser, 1997). The fatty acids of the four reference strains were analysed in parallel with those of strain pht-3B^T. The results of the fatty acid analysis are shown in Supplementary Table S1. The major fatty acids of strain pht-3B^T were $C_{19:0}\omega8c$ cyclo (22.6 %) and summed feature 8 (consisting of $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; 60.4 %), which accounted for 83.0 % of the total fatty acids. Strain pht-3B^T was similar to *N. aquibiodomus* NL21^T and *N. kimnyeongensis* KY 101^T, but different from *N. indicus* C115^T and *N. basaltis* J3^T in the proportions of summed feature 8 and $C_{19:0}\omega8c$ cyclo (>17 % difference).

On the basis of morphological, physiological and chemotaxonomic characteristics, together with data from 16S rRNA gene sequence analysis and DNA–DNA hybridization studies, it is concluded that strain pht-3B^T represents a novel species within the genus *Nitratireductor*, for which the name *Nitratireductor pacificus* sp. nov. is proposed.

Description of *Nitratireductor pacificus* sp. nov.

Nitratireductor pacificus (pa.ci′fic.us. L. masc. adj. *pacificus* pacific, pertaining to the Pacific Ocean).

Cells are short rods, 1.4–1.5 μm long and 0.8–0.9 μm wide. Motile by means of flagella. Positive for catalase, oxidase, β-galactosidase (weak), β-glucosidase (aesculin hydrolysis) and nitrate reduction, but negative for Gram reaction, indole production, D-glucose fermentation, urease, lipase (Tween 80), arginine dihydrolase, amylase and gelatinase. On marine agar, produces smooth grey non-pigmented colonies with regular edges and slightly raised in the centre, 2–3 mm in diameter after 3 days at 28 °C. Grows with 0–7 % NaCl (optimum 3 % NaCl), at 10–41 °C (optimum 25–30 °C), but not at 45 °C. The major fatty acids are $C_{19:0}\omega8c$ cyclo and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$). Sensitive to (μg per disc, unless otherwise indicated) ampicillin (10), carbenicillin (100), cefalexin (30), cefazolin (30), cefobid (30), chloromycetin (30), erythromycin (15), penicillin G (10), piperacillin (100), rifampicin (5) and rocephin (30). Resistant to cephadrin (30), ciprofloxacin (5), clindamycin (2), co-trimoxazole (25), gentamicin (10), kanamycin (30), lincomycin (2), metronidazole (5), minomycin (30), norfloxacin (10), ofloxacin (5), oxacillin (1), polymyxin B (30 IU), streptomycin (10), tetracycline (30), vancomycin (30) and vibramycin (30). With API ZYM, positive for acid phosphatase, alkaline phosphatase, cystine aminopeptidase, esterase (C4), esterase lipase (C8), leucine aminopeptidase, lipase (C14), *N*-acetyl-β-glucosaminidase, naphthol-AS-BI-phosphoamidase, trypsin, valine aminopeptidase, α-chymotrypsin, α-galactosidase (weakly), α-glucosidase, β-galactosidase (weakly) and β-glucosidase; negative for α-fucosidase, α-mannosidase and β-glucuronidase. With API 20 NE, utilizes adipic acid (weakly), capric acid, D-glucose, D-mannose (weakly), *N*-acetylglucosamine and trisodium citrate, but not maltose, D-mannitol, L-arabinose, malic acid, phenylacetic acid or potassium gluconate. With GN2 MicroPlates, positive for acetic acid, citric acid, DL-lactic acid, D-alanine, L-alanine, cellobiose, glycerol, glycyl L-aspartic acid, L-alanyl glycine, glycyl L-glutamic acid, L-alaninamide, L-asparagine, L-glutamic acid, L-leucine, L-phenylalanine, L-proline, L-pyroglutamic acid, L-serine, succinic acid monomethyl ester, *N*-acetyl-D-glucosamine, sucrose, urocanic acid, α-ketoglutaric acid and β-hydroxybutyric acid; weakly positive for trehalose, hydroxy-L-proline, maltose, pyruvic acid methyl ester, Tween 80, α-ketobutyric acid and γ-aminobutyric acid; negative for all other substrates.

The type strain, pht-3B^T (=CCTCC AB 209302^T=LMG 25541^T=MCCC 1A01024^T), was isolated from sediment of the Pacific Ocean. The DNA G+C content of the type strain is 63 mol%.

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