DOI 10.1099/ijs.0.002873-0

Novosphingobium indicum sp. nov., a polycyclic aromatic hydrocarbon-degrading bacterium isolated from a deep-sea environment

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A novel polycyclic aromatic hydrocarbon (PAH)-degrading bacterium, strain H25^T, which was isolated from deep-sea water of the Indian Ocean, was studied phenotypically, genotypically and phylogenetically. Strain H25^T can utilize several PAHs including phenanthrene and fluoranthene as sole carbon sources. The 16S rRNA gene sequence of strain H25^T showed the highest similarity with that of *Novosphingobium naphthalenivorans* TUT562^T (96.3 %), and showed lower similarities (92.1–96.0 %) with other members of the genus *Novosphingobium*. The major fatty acids of strain H25^T were $C_{14:0}$ 2-OH (3.2 %), $C_{16:0}$ (13.6 %), $C_{16:1}\omega7c$ (5.2 %), $C_{18:0}$ (13.4 %) and $C_{18:1}\omega7c$ (57.0 %), which accounted for 92.3 % of the total fatty acids. It had ubiquinone 10 as the major respiratory quinone and spermidine as the major polyamine. All these characteristics were consistent with those of recognized *Novosphingobium* species. Results of DNA–DNA hybridization experiments and BOX-PCR fingerprint comparisons also indicate that strain H25^T represents a novel *Novosphingobium* species, for which the name *Novosphingobium indicum* sp. nov. is proposed. The type strain is H25^T (=MCCC 1A01080^T =CGMCC 1.6784^T =LMG 24713^T).

Polycyclic aromatic hydrocarbons (PAHs) are hazardous environmental contaminants (Edwards, 1983; Harvey, 1991; Suess, 1976). Because of their hydrophobicity, they are difficult to remove. However, micro-organisms play a dominant role in the degradation and elimination of these contaminants from the environment (Alemayehu *et al.*, 2004). Many PAH-degrading bacteria have been isolated from marine environments, including species of *Novosphingobium* (Nohynek *et al.*, 1996; Balkwill *et al.*, 1997; Sohn *et al.*, 2004; Liu *et al.*, 2005; Cui & Shao, 2006; Wang *et al.*, 2008; Cui *et al.*, 2008).

In this study, we focused on an active PAH-degrading bacterium named H25^T, which was isolated from deep-sea water of the Indian Ocean (Yuan *et al.*, 2008). Strain H25^T can degrade several aromatic hydrocarbons including biphenyl, naphthalene, acenaphthene, 2-methylnaphthalene, dibenzofuran, dibenzothiophene, 2,6-dimethylnaphthalene, 4-methyldibenzothiophene, phenanthrene, anthracene, chrysene and fluoranthene (Yuan *et al.*, 2008). Sequence analysis of the 16S rRNA gene strongly suggested that strain H25^T represents a novel species of the genus *Novosphingobium*. In order to classify strain H25^T, further analyses were carried out, including DNA base composition and fatty acid composition, analyses of polyamines and quinones and DNA–DNA hybridization.

Deep-sea water was sampled from a depth of 4546 m below the surface, 200 m above the seabed, in December 2005 during cruise DY-105A of the R/V *Da-Yang Yi-Hao*, at site IR-CTD5 (16° 59.9412′ N 124° 58.2958′ E) on the Southwest Indian Ridge. The water sample was used for enrichment with crude oil as the carbon and energy source. Enrichment of an oil-degrading consortium was conducted on board immediately after sampling. In the laboratory, about 2 months later, 1 ml enriched culture was transferred into 100 ml fresh medium containing (l^{-1}) 1.0 g NH_4NO_3, 0.5 g KH_2PO_4, 2.8 mg $FeSO_4 . 7H_2O$ and 10 ml sterilized crude oil, prepared with aged deep-sea water, pH 7.5. After incubation for 3 weeks at 28 °C, 1 ml culture broth was transferred repeatedly to the same medium for further enrichment. Three sequential transfers

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Abbreviation: PAH, polycyclic aromatic hydrocarbon.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain H25^T is EF549586.

A transmission electron micrograph of cells of strain H25^T and details of rep-PCR profiles, cellular fatty acid contents, API 20NE and API ZYM test results and susceptibility to antimicrobial agents of strain H25^T and related strains are available as supplementary material with the online version of this paper.

were performed at 2-week intervals. Bacteria including strain H25^T were isolated by the plate-screening method on 216L marine agar medium (per litre of sea water; 1.0 g sodium acetate, 10.0 g tryptone, 2.0 g yeast extract, 0.5 g sodium citrate, 0.2 g NH_4NO_3, pH 7.5). For morphological and biochemical characterization, strain H25^T was also cultivated on 216L medium.

Genomic DNA was prepared according to Ausubel *et al.* (1995) and the 16S rRNA gene was amplified by PCR using primers described previously (Liu & Shao, 2005). Sequences of related strains were obtained from the GenBank database. Phylogenetic analysis was performed by using DNAMAN (version 5.1; Lynnon Biosoft). Distances (distance options according to Kimura's two-parameter model) and clustering with the neighbour-joining (Saitou & Nei, 1987) and minimum-evolution (Rzhetsky & Nei, 1992, 1993) methods were determined by using bootstrap values based on 1000 replications. A tree constructed using the neighbour-joining method is shown in Fig. 1.

A nearly full-length 16S rRNA gene sequence (1453 nt) of strain H25^T was determined. Phylogenetic analysis of strain H25^T indicated that it belonged to the *Alphaproteobacteria*, forming a robust clade with the genus *Novosphingobium*. As shown in Fig. 1, the closest related strains included *Novosphingobium naphthalenivorans* TUT562^T (96.3 % 16S rRNA gene sequence similarity), *Novosphingobium pentaromativorans* US6-1^T (96.0 %), *Novosphingobium subarcticum* KF1^T (95.8 %) and *Novosphingobium resinovorum* NCIMB 8767^T (95.8 %); other strains shared sequence similarities below 94.7 %. The name *Novosphingobium resinovorum* was created by the reclassification of *Flavobacterium resinovorum* Delaporte and Daste 1956, with *Novosphingobium subarcticum* (Nohynek *et al.* 1996) Takeuchi *et al.* 2001 as a later heterotypic synonym (Lim *et al.*, 2007). Alignment of the 16S rRNA gene sequence of strain H25^T with those of members of the genus *Novosphingobium* confirmed the presence of the *Novosphingobium* signature nucleotides (52C, 134G, 359G, 593U, 987G, 990U, 1215A and 1218C; Takeuchi *et al.*, 2001) in strain H25^T. Although strain H25^T showed high 16S rRNA gene sequence similarity to *Altererythrobacter epoxidivorans* JCS350^T (95.8 %), they formed two different clades in the phylogenetic tree based on 16S rRNA gene sequences (Fig. 1). In general, a 16S rRNA gene sequence divergence greater than 2 % is accepted as a criterion for delineating different species (Stackebrandt & Goebel, 1994). The 16S rRNA gene sequence divergence between strain H25^T and the closest type strain *N. naphthalenivorans* TUT562^T was 3.8 %; the data therefore support the view that strain H25^T represents a novel species in the genus *Novosphingobium*.

DNA–DNA hybridization experiments were performed with genomic DNA from strain H25^T, *N. naphthalenivorans* DSM 18518^T and *N. pentaromativorans* US6-1^T using a previously described method (Liu & Shao, 2005). Genomic DNA from *Escherichia coli* DH5α was used as a reference sample, and salmon sperm DNA was used as a negative control. Strain H25^T showed low DNA–DNA relatedness of 31 and 38 % to *N. naphthalenivorans* DSM 18518^T and *N. pentaromativorans* US6-1^T. This demonstrates their affiliation to different species in accordance with the cut-off value of 70 % recognized by Wayne *et al.* (1987) for

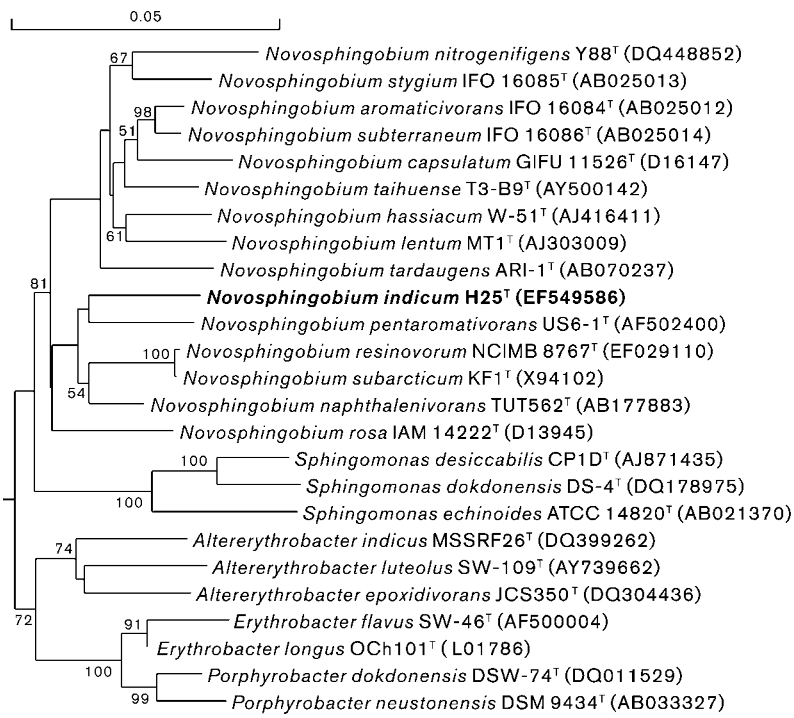

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain H25^T and representatives of some other related taxa, based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0.05 nucleotide substitution rate (K_{nuc}) units.

discrimination of bacterial species. Strain H25ᵀ, *N. naphthalenivorans* DSM 18518ᵀ, *N. pentaromativorans* US6-1ᵀ and *N. subarcticum* KF1ᵀ 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 followed by 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. The PCR products were separated by agarose (2 %) gel electrophoresis. The rep-PCR results are shown in Supplementary Fig. S1, available in IJSEM Online. Strain H25ᵀ yielded a unique BOX-PCR fingerprint compared with related strains. These results confirm the result of DNA–DNA hybridization.

General cell morphology was observed under an Olympus inverted microscope using a 1-day-old culture grown on 216L agar. For electron microscopy, exponential-phase cells were harvested, resuspended and absorbed on a Formvar–carbon-coated grid and then stained with phosphotungstic acid. The Gram reaction, catalase and oxidase activities, lipase (Tween 80 hydrolysis), gelatinase and hydrolysis of aesculin were examined according to standard methods (Dong & Cai, 2001). The optimal growth temperature was determined over the temperature range 4–45 °C on 216L agar. The major respiratory quinone was determined by HPLC analysis according to Collins (1985). Polyamines were extracted and analysed according to Busse & Auling (1988) and Busse *et al.* (1997). Other biochemical tests were carried out using API 20NE and API ZYM strips (bioMérieux) and the Biolog GN2 MicroPlate panel according to the manufacturers' instructions, with the NaCl concentration adjusted to 3.0 %. These results are given in the species description, Table 1 and Supplementary Table S1.

Fatty acids of cells grown aerobically in 216L broth at 28 °C for 48 h were extracted, freeze-dried, saponified and esterified according to the methods described by Mrozik *et al.* (2004). Analysis of fatty acid methyl esters was performed on a GC-MS (Shimadzu model QP2010) equipped with an RTX-5MS column. As shown in Supplementary Table S2, the major fatty acids of strain H25ᵀ were $C_{14:0}$ 2-OH (3.2 %), $C_{16:0}$ (13.6 %), $C_{16:1}\omega7c$ (5.2 %), $C_{18:0}$ (13.4 %) and $C_{18:1}\omega7c$ (57.0 %), which accounted for 92.3 % of the total fatty acids; this profile is consistent with those of recognized *Novosphingobium*

Table 1. Physiological characteristics of strain H25ᵀ and type strains of related *Novosphingobium* species

Strains: 1, strain H25ᵀ; 2, *N. naphthalenivorans* DSM 18518ᵀ; 3, *N. pentaromativorans* US6-1ᵀ; 4, *N. aromaticivorans* SMCC F199ᵀ; 5, *N. capsulatum* DSM 30196ᵀ; 6, *N. rosa* DSM 7285ᵀ; 7, *N. stygium* SMCC B0712ᵀ; 8, *N. subarcticum* KF1ᵀ; 9, *N. subterraneum* SMCC B0478ᵀ; 10, *N. hassiacum* DSM 14552ᵀ; 11, *N. tardaugens* JCM 11434ᵀ; 12, *N. lentum* DSM 13663ᵀ; 13, *N. taihuense* T3-B9ᵀ; 14, *N. nitrogenifigens* Y88ᵀ. Data for reference strains were taken from Sohn *et al.* (2004), Tiirola *et al.* (2005), Liu *et al.* (2005), Suzuki & Hiraishi (2007), Addison *et al.* (2007) and Lim *et al.* (2007). +, Positive; –, negative; W, weakly positive; ND, no data available. All strains were positive for catalase and negative for arginine dihydrolase, indole production, denitrification and assimilation of citrate, D-mannitol and L-phenylalanine.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Oxidase	–	+	–	–	–	–	–	–	–	+	–	–	–	–
Urease	–	–	–	–	–	–	–	–	–	–	–	–	–	+
β-Galactosidase	–	–	–	+	+	+	+	+	+	–	–	–	ND	–
Gelatin hydrolysis	+	–	–	–	+	–	–	–	+	–	–	–	–	–
Aesculin hydrolysis	–	+	+	+	+	W	+	+	+	–	ND	–	+	ND
Nitrate reduction	+	+	+	+	+	+	–	+	+	–	–	+	+	+
Assimilation of:														
N-Acetyl-D-glucosamine	–	–	–	+	+	W	–	+	–	–	–	–	–	+
L-Arabinose	–	+	–	–	W	+	–	+	W	–	–	–	+	–
L-Alanine	+	ND	–	–	–	–	–	–	–	–	–	–	–	–
Cellobiose	–	ND	–	+	+	+	–	–	+	+	–	–	+	+
D-Galactose	–	ND	–	–	+	–	–	+	–	–	–	–	–	+
D-Glucose	+	+	–	+	+	+	–	+	+	+	–	–	+	+
D-Fructose	–	ND	+	–	–	+	–	–	–	+	–	–	+	+
D-Mannose	+	–	–	+	W	+	–	–	+	–	–	–	+	+
Maltose	+	+	–	+	+	+	–	+	+	–	–	–	+	+
Melibiose	–	ND	–	–	–	+	–	–	+	–	–	–	–	–
L-Rhamnose	–	ND	+	+	+	+	–	+	+	–	–	–	+	–
Sucrose	–	ND	+	+	+	+	–	–	+	+	–	–	+	+
L-Proline	+	ND	+	+	–	–	–	+	+	+	–	–	+	–
Trehalose	–	ND	+	–	+	–	–	–	–	–	–	–	–	+
G+C content (mol%)	62	64.6	61.1	64	63	65	65	66	60	ND	61	66	63.3	ND

species. Strain H25^T, *N. naphthalenivorans* DSM 18518^T and *N. pentaromativorans* US6-1^T contained similar proportions of $C_{18:1}\omega7c$ (57.0–64.0 %) and $C_{16:1}\omega7c$ (5.2–8.8 %), but they differed significantly in the content of $C_{14:0}$ 2-OH (3.2–19.7 %) and $C_{16:0}$ (1–13.6%).

The G+C content of the chromosomal DNA was determined according to the method described by Mesbah & Whitman (1989) using reversed-phase HPLC. The G+C content of strain H25^T was 62 mol%, which is within the range described for the genus *Novosphingobium* (60–66 mol%).

Antibiotic susceptibility tests were performed by the disc-diffusion method as described by Shieh *et al.* (2003). Strain H25^T, *N. naphthalenivorans* DSM 18518^T, *N. pentaromativorans* US6-1^T and *N. subarcticum* KF1^T were tested at the same time in this study. All of them were sensitive to (per disc; Oxoid) ampicillin (10 μg), carbenicillin (100 μg), cefobid (30 μg), chloromycetin (30 μg), co-trimoxazole (25 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), minomycin (30 μg), neomycin (10 μg), penicillin G (10 μg), piperacillin (100 μg), rifampicin (5 μg) and rocephin (30 μg), but resistant to lincomycin (2 μg), metronidazole (5 μg), oxacillin (1 μg) and streptomycin (10 μg). Differential susceptibility of the four strains to 12 other antibiotics is detailed in Supplementary Table S3.

Cells of strain H25^T were Gram-negative, yellow-pigmented rods without polar flagella (Supplementary Fig. S2). Strain H25^T had $C_{14:0}$ 2-OH as the major 2-hydroxy fatty acid and displayed nitrate reductase activity, ubiquinone 10 as the major respiratory quinone and spermidine as the major polyamine. These characteristics supported the phylogenetic evidence that strain H25^T belongs to the genus *Novosphingobium*. Differences in physiological, biochemical and chemotaxonomic characteristics between strain H25^T and the type strains of related species are given in Table 1 and Supplementary Tables S1–S3. Strain H25^T could be distinguished from the two closest type strains, *N. naphthalenivorans* DSM 18518^T and *N. pentaromativorans* US6-1^T, in oxidase activity, colony colour, aesculin hydrolysis and some results from API and antibiotic susceptibility tests. On the basis of morphological, physiological and chemotaxonomic characteristics, together with data from 16S rRNA gene sequence analysis and DNA–DNA hybridization, strain H25^T should be placed in a novel species, for which the name *Novosphingobium indicum* sp. nov. is proposed.

Description of *Novosphingobium indicum* sp. nov.

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

Cells are non-motile rods without polar flagella, 1.1–1.4 μm long and 0.6–0.7 μm wide. Positive for catalase, gelatinase and utilization of D-glucose, D-mannose and maltose using API 20NE, but negative for Gram-staining, oxidase, lipase (Tween 80 hydrolysis), urease, aesculin hydrolysis, β-glucosidase, β-galactosidase, arginine dihydrolase, indole production and utilization of adipic acid, N-acetylglucosamine, capric acid, D-mannitol, L-arabinose, malic acid, phenylacetic acid, potassium gluconate and trisodium citrate. In API ZYM tests, positive for acid and alkaline phosphatases, esterase (C4), esterase lipase (C8), leucine aminopeptidase, naphthol-AS-BI-phosphoamidase, valine aminopeptidase and α-chymotrypsin, weakly positive for cystine aminopeptidase, lipase (C14) and α-glucosidase and negative for *N*-acetyl-β-glucosaminidase, trypsin, α-fucosidase, α-galactosidase, α-mannosidase, β-galactosidase, β-glucosidase and β-glucuronidase. Growth occurs at 10–41 °C (optimum 25–30 °C), but not at 4 or 45 °C. Acid is not produced from D-glucose. Able to reduce nitrate to nitrite, but incapable of denitrification. On 216L agar plates, produces smooth, round, yellow colonies with regular edges, slightly raised in the centre, 1–2 mm in diameter after 72 h incubation at 28 °C. Principal fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$, $C_{18:0}$, $C_{16:1}\omega7c$ and $C_{14:0}$ 2-OH. Of 95 substrates in the Biolog GN2 system, positive for utilization of α-cyclodextrin, dextrin, glycogen, α-D-glucose, α-ketovaleric acid, D-mannose, succinamic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-glutamic acid, glycyl L-glutamic acid, L-leucine, L-proline, Tweens 40 and 80, maltose, methyl pyruvate and β-hydroxybutyric acid. Sensitive to (per disc) ampicillin (10 μg), carbenicillin (100 μg), cefazolin (30 μg), cefobid (30 μg), cephradin (30 μg), chloromycetin (30 μg), ciprofloxacin (5 μg), co-trimoxazole (25 μg), erythromycin (15 μg), rifampicin (5 μg), gentamicin (10 μg), kanamycin (30 μg), minomycin (30 μg), neomycin (10 μg), ofloxacin (5 μg), penicillin G (10 μg), piperacillin (100 μg), rocephin (30 μg), tetracycline (30 μg) and vancomycin (30 μg); resistant to cefalexin (30 μg), clindamycin (2 μg), furazolidone (15 μg), lincomycin (2 μg), metronidazole (5 μg), norfloxacin (10 μg), oxacillin (1 μg), polymyxin B (30 U), streptomycin (10 μg) and vibramycin (30 μg). The G+C content of the DNA of the type strain is 62 mol%. The major respiratory quinone is ubiquinone 10 and the major polyamine is spermidine. Table 1 shows characteristics used to distinguish the type strain from type strains of related species.

The type strain, H25^T (=MCCC 1A01080^T =CGMCC 1.6784^T =LMG 24713^T), was isolated from deep-sea water in December 2005.

Acknowledgements

This work was supported financially by the National Natural Science Foundation of China (40376041), the National Infrastructure of Natural Resources for Science and Technology Program of China (no. 2005DKA21209), the COMRA program (no. DYXM115-02-2-05) and the National Basic Research Program of China (nos 2004CB719601, 2006CB708200). J. Y. is a PhD student of Xiamen University co-supervised by T. Z. and Z. S.; all work was carried out at the Third Institute of Oceanography, State Oceanic Administration.

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