Stappia indica sp. nov., isolated from deep seawater of the Indian Ocean

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A taxonomic study was carried out on strain B106^T, which was isolated from a polycyclic aromatic hydrocarbon-degrading consortium, enriched with deep seawater from the Indian Ocean. The isolate was Gram-negative, oxidase- and catalase-positive, rod-shaped and motile by means of one polar flagellum. Growth was observed at salinities of 0.5–11 ‰ and at temperatures of 4–42 °C, and the strain was capable of nitrate reduction, but was unable to degrade Tween 80 or gelatin. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain B106^T belonged to the genus *Stappia*, with a highest sequence similarity of 97.7 % to *Stappia stellulata* IAM 12621^T; similarity to other strains was below 95.2 %. DNA–DNA hybridization between strain B106^T and *S. stellulata* IAM 12621^T was 43 %. The major fatty acids were C_{16:0} (6.10 %), C_{18:1ω7c} (62.58 %), C_{18:0} (5.17 %), C_{18:1ω7c} 11-methyl (14.48 %) and C_{19:0ω8c} cyclo (4.70 %). The G + C content of the chromosomal DNA was 65.9 mol%. The combined genotypic and phenotypic data showed that strain B106^T represents a novel species of the genus *Stappia*, for which the name *Stappia indica* sp. nov. is proposed, with the type strain B106^T (=PR56-8^T=CCTCC AB 208228^T=LMG 24625^T=MCCC 1A01226^T).

In an attempt to investigate polycyclic aromatic hydrocarbon (PAH)-degrading bacteria in deep seawater of the Indian Ocean, many bacterial strains were isolated and characterized taxonomically. This study focused on one of these isolates, designated strain B106^T. Comparative 16S rRNA gene sequence analysis indicated that strain B106^T belonged to the genus *Stappia*, in the family *Rhodobacteraceae*. The genus *Stappia* was proposed by Uchino *et al.* (1998) and included *Stappia stellulata* (formerly *Agrobacterium stellulatum* ATCC 15215^T=IAM 12621^T) and *Stappia aggregata* (formerly *Agrobacterium stellulatum* ATCC 25650=IAM 12614). More recently, *Stappia alba* CECT 5095^T and *Stappia marina* mano 18^T were reported by Pujalte *et al.* (2005) and Kim *et al.* (2006), respectively. Subsequently, Biebl *et al.* (2007) created the genus *Labrenzia* to accommodate *Labrenzia alexandrii* and reclassified *S. aggregata*, *S. alba* and *S. marina* as *Labrenzia aggregata*, *Labrenzia alba* and *Labrenzia marina*, respectively. In addition, Weber & King (2007) proposed four species, '*Stappia carboxidovorans*' M4, '*Stappia conradae*'

MIO, '*Stappia kahanamokuae*' HI1 and '*Stappia meyeriae*' GA15, but the names have not been validly published. Accordingly, the aim of the present work was to determine the exact taxonomic position of strain B106^T by using a polyphasic approach.

Deep seawater was sampled with Niskin bottles attached to a CTD (conductivity, temperature and depth) circular rosette in December 2005 during cruise DY-105A of the research vessel *Da-Yang Yi-Hao*. The sampling site was at 31.0710° S 58.9945° E on the southwest Indian Ridge, and was designated as IR-CTD5-6; the depth of the water at the site was 4746 m, and a water sample from a depth of 4196 m was used for enrichment of PAH-degrading bacteria with 1 % (v/v) sterilized crude oil and a PAH mixture (final concentrations of 100 p.p.m. phenanthrene, 10 p.p.m. anthracene, 10 p.p.m. fluoranthene and 10 p.p.m. pyrene, dissolved in crude oil) as the carbon and energy source. A later enrichment with the PAH mixture as the sole carbon and energy source and bacterial isolation on 216L marine agar medium (comprising, per l⁻¹ 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) were performed using the method described by Lai *et al.* (2009). For morphological and biochemical characterization, strain B106^T was also cultivated on 216L medium.

Genomic DNA was prepared according to the method of Ausubel *et al.* (1995) and the 16S rRNA gene was amplified

Abbreviation: PAH, polycyclic aromatic hydrocarbon.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain B106^T is EU726271.

A transmission electron micrograph of cells of strain B106^T and figures showing polar lipids and rep-PCR comparisons of strain B106^T and *Stappia stellulata* IAM 12621^T, and a table detailing the cellular fatty acid contents of strain B106^T and *S. stellulata* IAM 12621^T are available as supplementary material with the online version of this paper.

by PCR using primers that have been described previously (Liu & Shao, 2005). Sequences of related taxa were obtained from the GenBank database. Phylogenetic analysis was performed by using MEGA version 4 (Tamura *et al.*, 2007) after multiple alignment of data by using DNAMAN (version 5.1; Lynnon Biosoft). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method of Saitou & Nei (1987) and minimum-evolution methods of Rzhetsky & Nei (1992, 1993) were determined by using bootstrap values based on 1000 replications. Clustering results using the minimum-evolution approach were similar to those obtained using neighbour-joining (data not shown).

An almost full-length 16S rRNA gene sequence (1448 nt) of strain B106^T was determined. As shown in Fig. 1, the phylogenetic tree based on 16S rRNA gene sequences showed that strain B106^T and *S. stellulata* IAM 12621^T formed an independent monophyletic cluster, with a high bootstrap value (100%). The two strains shared a 16S rRNA gene sequence similarity of 97.7%. The sequences of type strains of other species had <95.4% similarity to strain B106^T. This high similarity strongly confirmed that strain B106^T belonged to the genus *Stappia*. In addition, the phylogenetic comparisons performed in this study demonstrated that '*Stappia carboxidovorans*' M4, '*Stappia conradae*' MIO, '*Stappia kahanamokuae*' HI1 and '*Stappia meyeriae*' GA15 were more closely related to the genera *Labrenzia* and *Pannonibacter* (Fig. 1). Further studies are needed to determine their exact taxonomic positions.

DNA–DNA hybridization experiments were performed with genomic DNA from strain B106^T and *S. stellulata* IAM 12621^T using a method described by Liu & Shao (2005). Genomic DNA from *Escherichia coli* DH5 α was used as an

outgroup sample. Salmon sperm DNA was used as a negative control. The results showed that strain B106^T and *S. stellulata* IAM 12621^T had a low DNA–DNA relatedness (43%), demonstrating their affiliation to separate species in accordance with the cut-off value of 70% recognized by Wayne *et al.* (1987) for the discrimination of bacterial species. Strain B106^T and *S. stellulata* IAM 12621^T were further compared by using rep-PCR. In this study, the primer BOX-A1R (5'-CTACGGCAAGGCGACGCTGACG-3') was used for rep-PCR fingerprint analysis (Versalovic *et al.*, 1991). PCR was carried out with the following cycle conditions: denaturation for 5 min 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. The PCR products were separated by using agarose (2%) gel electrophoresis. The rep-PCR results are shown in Supplementary Fig. S1 (in IJSEM Online). Strain B106^T showed a unique pattern rep-PCR fingerprint in comparison with *S. stellulata* IAM 12621^T. These results confirmed the results of the DNA–DNA hybridization.

Gram reaction, catalase, oxidase and lipase (Tween 80) activities, hydrolysis of aesculin and starch, optimal growth temperature and pH, tolerance to NaCl, antibiotic susceptibility, general cell morphology and electron microscopy were studied as described previously (Lai *et al.*, 2009). Other biochemical tests were carried out using API 20NE and API ZYM strips (bioMérieux) and the Biolog GN2 MicroPlate test panel according to the manufacturers' instructions, except that the NaCl concentration was adjusted in all tests to 3.0%. *S. stellulata* IAM 12621^T was tested at the same time as strain B106^T for comparison. The results are given in the species description and Table 1.

The major respiratory quinone of strain B106^T was determined to be ubiquinone 10 (Q10) by using HPLC analysis according to Collins (1985). This trait is in accordance with the properties of *S. stellulata* IAM 12621^T. Fatty acids in whole cells grown on 216L 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, 1997). The fatty acid profile of *S. stellulata* IAM 12621^T was determined in parallel with strain B106^T in this study. The results of both strains are shown in Supplementary Table S1 in IJSEM Online. The major fatty acids in both strains were C_{18:1} ω 7c and C_{18:1} ω 7c 11-methyl, which accounted for >77% of the total fatty acids. The fatty acid profile of *S. stellulata* IAM 12621^T was similar to that determined by Kim *et al.* (2006). The two strains differed greatly (>7%) in the content of C_{18:1} ω 7c 11-methyl. As the fatty acid profiles of strain B106^T and *S. stellulata* IAM 12621^T were determined under the same conditions, this difference in proportion could be used to distinguish strain B106^T from *S. stellulata* IAM 12621^T.

Polar lipids of strain B106^T and *S. stellulata* IAM 12621^T were extracted and separated according to the methods

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain B106^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, 0.005 nucleotide substitution rate (K_{nuc}) units.

Table 1. Physiological characteristics that differentiate strain B106^T and *Stappia stellulata* IAM 12621^T

Strains: 1, B106^T (*Stappia indica* sp. nov.); 2, *S. stellulata* IAM 12621^T. w, Weak; +, positive; –, negative.

Characteristic	1	2*
API 20NE		
D-Glucose fermentation	–	w
Utilization of D-glucose, D-mannitol, adipic acid, trisodium citrate	+	–
API ZYM		
Esterase lipase (C8)	w	+
Lipase (C14), cystine aminopeptidase	w	–
Valine aminopeptidase	+	w
Reaction to antimicrobial agents		
Ampicillin (10 µg), cefalexin (30 µg), cefazolin (30 µg), cefobid (30 µg), cephradine (30 µg), furazolidone (15 µg), minomycin (30 µg), penicillin G (10 µg), piperacillin (100 µg), tetracycline (30 µg), vibramycin (30 µg)	–	+
Biolog GN2		
D-Fructose, L-alaninamide, L-histidine	w	+
Acetic acid, dextrin, propionic acid, Tween 40, Tween 80, α-ketoglutaric acid, α-ketovaleric acid	–	+
Bromosuccinic acid, L-glutamic acid, L-leucine, L-threonine, succinamic acid, succinic acid, urocanic acid, γ-aminobutyric acid	+	w
D-Mannose, pyruvic acid methyl ester, γ-hydroxybutyric acid	–	w
cis-Aconitic acid, citric acid, D-alanine, D-arabitol, D-galacturonic acid, D-gluconic acid, D-mannitol, D-sorbitol, itaconic acid, L-alanine, L-asparagine, L-phenylalanine, myo-inositol, succinic acid monomethyl ester, quinic acid	+	–
D-Serine, L-ornithine, L-serine, malonic acid, thymidine	w	–
DNA G + C content (mol%)	65.9	59

*The characteristics of strain IAM 12621^T were analysed at the same time as those of strain B106^T. Other characteristics tested by using API 20NE, API ZYM and Biolog and susceptibility to antibiotics not listed were the same as those of strain B106^T.

described by Tindall (1990). The results are shown in Supplementary Fig. S2 in IJSEM Online. The major polar lipids of strain B106^T and *S. stellulata* IAM 12621^T were an unidentified aminolipid, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine and phosphatidylethanolamine. Some unidentified lipids were detected in both strains. These results were similar to those of Biebl *et al.* (2007).

Antibiotic susceptibility tests were performed by using disc diffusion methods as described by Shieh *et al.* (2003). Strain B106^T and *S. stellulata* IAM 12621^T were tested at the same time in this study. Both strains were sensitive to carbenicillin (100 µg per disc; Oxoid), chloramphenicol (30 µg), ciprofloxacin (5 µg), co-trimoxazole (25 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), neomycin (10 µg), norfloxacin (10 µg), ofloxacin (5 µg) and rifampicin (5 µg); resistant to clindamycin (2 µg), lincomycin (2 µg), metronidazole (5 µg), oxacillin (1 µg), polymyxin B (30 IU), rocephin (30 µg), streptomycin (10 µg) or vancomycin (30 µg). The susceptibility patterns to antibiotics of the two strains studied are shown in 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 B106^T was 65.9 mol%, which is higher than that of *S. stellulata* IAM 12621^T (59 mol%).

Strain B106^T was a Gram-negative, non-pigmented, rod-shaped bacterium that was motile by means of a polar flagellum (see Supplementary Fig S3, in IJSEM Online).

The differences in physiological, biochemical and chemotaxonomic characteristics between strain B106^T and *S. stellulata* IAM 12621^T are given in Table 1. The high 16S rRNA gene similarity between strain B106^T and *S. stellulata* IAM 12621^T indicated strongly that strain B106^T belonged to the genus *Stappia*. However, strain B106^T could be differentiated from *S. stellulata* IAM 12621^T based on physiological and chemotaxonomic characteristics, DNA–DNA hybridization and rep-PCR fingerprint comparison. On the basis of the data described above, strain B106^T represents a novel species of the genus *Stappia*, for which the name *Stappia indica* sp. nov. is proposed.

Description of *Stappia indica* sp. nov.

Stappia indica (in.di'ca. L. fem. adj. *indica* Indian, referring to the Indian Ocean, where the strain was isolated).

Cells are rod-shaped, about 1.0 µm wide and 1.0 µm long, and motile by means of a single polar flagellum. Positive for oxidase and catalase, but negative for the Gram reaction, indole production, starch hydrolysis, gelatinase, β-glucosidase, β-galactosidase, urease and arginine dihydrolase. On 216L agar plates, forms smooth grey-white colonies with regular edges, 2–3 mm in diameter after 72 h incubation at 28 °C, non-pigmented and slightly raised in the centre. Moderately halophilic, grows in 0.5–11 % NaCl (optimum, 1–5 %) and at 4–42 °C (optimum, 25–37 °C), but not at 45 °C. Reduces nitrate, but does not ferment glucose. Major fatty acids are C_{16:0}, C_{18:1ω7c}, C_{18:0},

C_{18:1ω7c} 11-methyl and C_{19:0ω8c} cyclo. Sensitive to (per disc; Oxoid): carbenicillin (100 µg), chloromycetin (30 µg), ciprofloxacin (5 µg), co-trimoxazole (25 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), neomycin (10 µg), norfloxacin (10 µg), ofloxacin (5 µg) and rifampicin (5 µg); resistant to ampicillin (10 µg), cefalexin (30 µg), cefazolin (30 µg), cefobid (30 µg), cephradine (30 µg), clindamycin (2 µg), furazolidone (15 µg), lincomycin (2 µg), metronidazole (5 µg), minomycin (30 µg), oxacillin (1 µg), penicillin G (10 µg), piperacillin (100 µg), polymyxin B (30 IU), rocephin (30 µg), streptomycin (10 µg), tetracycline (30 µg), vancomycin (30 µg) and vibramycin (30 µg). In API ZYM tests, positive for acid phosphatase, alkaline phosphatase, esterase (C4), leucine aminopeptidase, trypsin and valine aminopeptidases; weakly positive for cystine aminopeptidase, esterase lipase (C8), lipase (C14) and naphthol-AS-BI-phosphoamidase; and negative for *N*-acetyl-β-glucosaminidase, α-chymotrypsin, 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 bromosuccinic acid, *cis*-aconitic acid, citric acid, DL-lactic acid, D-alanine, D-arabitol, D-galacturonic acid, D-gluconic acid, D-mannitol, D-sorbitol, glycerol, glycyl L-glutamic acid, itaconic acid, L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, L-leucine, L-phenylalanine, L-proline, L-threonine, *myo*-inositol, succinic acid monomethyl ester, quinic acid, succinamic acid, succinic acid, urocanic acid, α-D-glucose, α-hydroxybutyric acid, α-ketobutyric acid, β-hydroxybutyric acid and γ-aminobutyric acid; weakly positive for D-fructose, D-serine, glycogen, glycyl L-aspartic acid, hydroxy-L-proline, L-alaninamide, L-histidine, L-ornithine, L-serine, malonic acid and thymidine. The G + C content of the DNA of the type strain is 65.9 mol%.

The type strain, B106^T (=PR56-8^T=CCTCC AB 208228^T=LMG 24625^T=MCCC 1A01226^T), was isolated from deep seawater of the Indian Ocean.

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