Microbacterium indicum sp. nov., isolated from a deep-sea sediment sample from the Chagos Trench, Indian Ocean

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Two bacterial strains, BBH6^T and BBH9, were isolated from a deep-sea sediment sample collected from the Chagos Trench, Indian Ocean, at a depth of 5904 m. The two strains were closely related in their 16S rRNA gene sequences (99.7 %), belonged to one genomic species and were virtually identical at the phenotypic level. *Microbacterium barkeri* DSM 20145^T was the nearest phylogenetic neighbour to the new isolates, with 16S rRNA gene sequence similarity levels of 97.2–97.4 %. The new isolates exhibited levels of DNA–DNA relatedness of 32–34 % to *M. barkeri* and differed from it in a number of phenotypic characteristics. Therefore, it is suggested that strains BBH6^T and BBH9 represent a novel species of the genus *Microbacterium*, for which the name *Microbacterium indicum* sp. nov. is proposed. The type strain is BBH6^T (=LMG 23459^T=IAM 15355^T).

A wide diversity of micro-organisms have been isolated from deep-sea sediments (Takami *et al.*, 1997, 1999; Luna *et al.*, 2004 and references therein). Raghukumar *et al.* (2004) reported on the microbial biodiversity in deep-sea sediment from the Chagos Trench, Indian Ocean, which forms part of the Chagos–Lacaddive ridge system, one of the deepest regions of the Indian Ocean. They isolated a fungus, *Aspergillus sydowii*, from a sub-seafloor sediment sample (3.4 m deep, 430 000 years old) and also reported the presence of bacteria. In the present paper, we describe isolates recovered from a deep-sea sediment sample collected from the Chagos Trench at a depth of 5904 m (from a 50–70 cm section of a deep sediment core of 4.6 m, approximately 50 000 years old). The age of the sediment was inferred from the sedimentation rates in the Central Indian Ocean, which is reported to be approximately 1.0 cm per 1000 years (Gupta, 1999, 2002).

Deep-sea sediment samples were collected as described by Raghukumar *et al.* (2004) and sterile containers with the samples were stored at 4 °C for 1 year prior to the present analysis. Approximately 100 mg of the sediment was suspended in 1 ml of 2 % NaCl, vortexed for 1 min and the suspension was allowed to settle for 2 min. Then, 100 µl of the top aqueous layer was spread onto a plate of yeast-extract/peptone (YP) agar (per litre: 5 g yeast extract, 10 g peptone, 30 g NaCl, 15 g agar). After 15 days incubation at 15 °C, bacterial growth ranged from 4.4×10^3 to 7.6×10^3 c.f.u. per gram of sediment. Seven morphotypes were observed among more than 400 colonies, and five representatives from each colony morphotype were subjected to total protein analysis by SDS-PAGE as described by Laemmli (1970). By using this approach, the morphotype representatives were divided into five groups. Strains from four of these groups were identified as representing members of the genera *Rothia*, *Micrococcus*, *Kocuria* and *Brachybacterium* on the basis of phenotypic characteristics and preliminary 16S rRNA gene sequence analysis (data not presented). Strains BBH6^T and BBH9, belonging to the fifth group, were characterized in detail to determine their taxonomic position. Reference strains *Microbacterium barkeri* JCM 1343^T and *Microbacterium gubbeenense* JCM 12075^T were used in experiments to determine the biochemical characteristics, fatty acid profile, polar lipid content and levels of DNA–DNA hybridization of the new strains.

Nutrient agar (HiMEDIA) was used for growth and maintenance of the strains and for determination of phenotypic, including chemotaxonomic, characteristics. To assess growth at different pH values, the pH of the sterile YP medium was adjusted from pH 4.0 to 10.0 at 0.5 pH units by using either 0.1 M HCl or NaOH. For tests of salt tolerance, 1, 3, 6, 8 and 10 % (w/v) NaCl was added

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains BBH6^T and BBH9 are AM158907 and AM286267, respectively.

Tables comparing the cellular fatty acid compositions and giving levels of DNA–DNA relatedness among strains BBH6^T and BBH9 and the type strains of *Microbacterium barkeri* and *Microbacterium gubbeenense* are available with the online version of this paper.

to YP medium lacking NaCl. Results were scored after 72 h incubation at 22 °C. A Leitz Diaplan phase-contrast microscope was used to ascertain the morphology and motility of the strains. Utilization of various carbon compounds as sole carbon source was tested at 22 °C for 15 days in mineral liquid medium as described by Reddy *et al.* (2000). Other physiological and biochemical characteristics were determined following the methods of Holding & Collee (1971) and Smibert & Krieg (1994). It was found that the two new isolates exhibited many similar phenotypic characteristics (see Table 1 and under the species description below) but were not identical. Strain

Table 1. Comparison of the phenotypic characteristics of strains BBH6^T and BBH9, *Microbacterium barkeri* JCM 1343^T and *Microbacterium gubbeenense* JCM 12075^T

The four strains are positive for nitrate reduction, β-galactosidase and aesculin hydrolysis, but negative for catalase, oxidase, urease, lysine decarboxylase, ornithine decarboxylase, phenylalanine deamination, Voges–Proskauer reaction, indole production and methyl red test. All produced acid from D-glucose, fructose, L-arabinose, D-arabinose, D-xylose, D-cellobiose, maltose, trehalose, melezitose and sucrose. All four are able to utilize D-glucose, D-fructose, D-galactose, L-arabinose, D-cellobiose, lactose, maltose, sucrose, acetate, L-serine, L-arginine and L-lysine, but not erythritol, adonitol, α-keto-glutarate, lactate, L-proline, L-cystine, L-isoleucine, L-aspartic acid or L-tyrosine as the sole carbon source. Menaquinones found are MK11 and MK12. All are sensitive to amikacin (30 μg), cefuroxime (30 μg), rifampicin (25 μg), bacitracin (10 μg), roxithromycin (30 μg), tobramycin (15 μg), gentamicin (30 μg), erythromycin (15 μg), ciprofloxacin (30 μg), chloramphenicol (30 μg) and kanamycin (30 μg), but resistant to nalidixic acid (30 μg). PG, Phosphatidylglycerol; DPG, diphosphatidylglycerol, PI, phosphatidylinositol. +, Positive; −, negative; S, sensitive; R, resistant.

Phenotypic characteristic	Strains BBH6^T and BBH9	*M. barkeri* JCM 1343^T	*M. gubbeenense* JCM 12075^T
Colony size	1.5–2 mm	2–2.5 mm	0.5–1 mm
Colony colour	Pale yellow	Yellow	Pale yellow
Growth temperature range (°C)	8–30	15–30	8–30
Optimum temperature (°C)	22	28	28
Growth pH range	5–9	5–11	5–10
NaCl tolerance (6 %)	+	−	+
NaCl tolerance (8 %)	−	−	+
Lipase	−	+	−
Amylase	−	+	−
Acid production from:			
D-Galactose	−	+	+
L-Rhamnose	−	+	+
Mannose	−	+	+
Sorbitol	−	+	+
Carbon source utilization:			
L-Rhamnose	−	+	+
D-Xylose	+	+	−
L-Xylose	+	−	−
D-Raffinose	+	−	−
Mannose	−	+	+
D-Ribose	+	−	−
Melezitose	+	+	−
Inositol	+	−	−
Mannitol	+	−	+
D-Glucuronate	−	−	+
Malonate	+	+	−
Citrate	−	+	+
L-Asparagine	+	+	−
L-Alanine	−	+	−
Sensitivity to antibiotic (μg per disc):			
Penicillin (10 μg)	S	R	S
Ampicillin (25 μg)	R	R	S
Polar lipids	PG, PI	PG, PI	PG, DPG, PI
Diamino acid in the cell wall	D-Orn	D-Orn*	L-Lys†
Peptidoglycan type	B2β	B2*	B1†
DNA G+C content (mol%)	65.5	68.3	71.6

All the data were acquired in the present study, except *Takeuchi & Hatano (1998) and †Brennan *et al.* (2001).

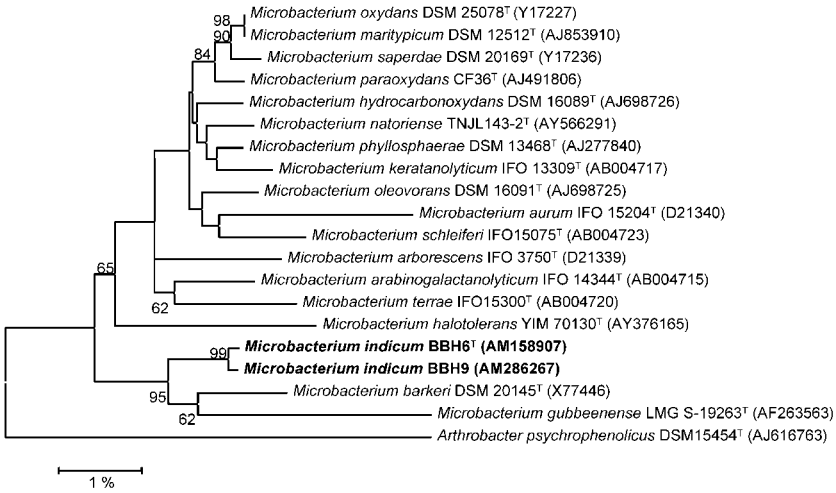

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship between strains BBH6^T and BBH9 and other closely related species of the genus *Microbacterium*. Bootstrap values (>50 %), expressed as percentages of 1000 replications, are given at the branching points. Accession numbers are given in parentheses. Bar, one substitution per 100 nt.

BBH6^T differed from BBH9 based on its ability to produce acid from D-glucosamine and lactose and by its inability to assimilate L-aspartate or L-tyrosine as a sole carbon and energy source.

Menaquinones and polar lipids were determined in freeze-dried cells. Menaquinones were purified by preparative TLC (Collins, 1994) and were identified via MS (Komagata & Suzuki, 1987). Polar lipids were analysed according to the procedure of Minnikin *et al.* (1975) by using pure lipids as standards. Whole-cell sugars of strain BBH6^T were determined by TLC (Staneck & Roberts, 1974) in samples prepared following the recommendations of Brennan *et al.* (2001). DNA was isolated according to the procedure of Marmur (1961) and the DNA G+C content was determined from the melting point (T_m) curves (Sly *et al.*, 1986) obtained by using a Lambda 2 UV-vis spectrophotometer (Perkin Elmer) equipped with the Templab 2.0 software package (Perkin Elmer). Results of the above experiments are reported in the species description below and/or in Table 1. Cell walls were prepared from 500 mg (dry weight) cell mass and purified according to Komagata & Suzuki (1987). Amino acids and peptides in the cell wall were analysed by two-dimensional TLC on cellulose plates by using the solvent systems described by Schleifer & Kandler (1972). The presence of L-Hse, D-Glu and D-Orn in the hydrolysed cell wall was detected by HPLC (Kageyama *et al.*, 2006). The peptidoglycan of strains BBH6^T and BBH9 was of B2β type [(L-Hse)–D-Glu–Gly–D-Orn] (Schleifer & Kandler, 1972).

Fatty acids were analysed as described by Reddy *et al.* (2002) in cells grown in trypticase soy agar (M322; HiMEDIA) at 28 °C and harvested in the exponential phase (24 h). Fatty acids of strain BBH6^T (anteiso-$C_{13:0}$, 0.2 %; iso-$C_{15:0}$, 6.6 %; $C_{15:0}$, 1.5 %; anteiso-$C_{15:0}$, 25.3 %; iso-$C_{16:0}$, 36.7 %; $C_{16:0}$, 15.2 %; anteiso-$C_{17:0}$, 14.3 %) were typical of the genus *Microbacterium*, but differed from those of *M. barkeri* and *M. gubbeenense* in the proportions of the predominant components (see Supplementary Table S1 available in IJSEM Online).

Genomic DNA was isolated from freshly grown culture following the methods of Sambrook *et al.* (1989). The 16S rRNA genes of the isolates were amplified, purified and sequenced according to Reddy *et al.* (2000). Alignment was performed with related sequences retrieved from the GenBank database by using CLUSTAL_X (Thompson *et al.*, 1997) and corrected manually. A neighbour-joining phylogenetic tree (Saitou & Nei, 1987) was constructed by using MEGA 3.1 (Kumar *et al.*, 2004) based on evolutionary distances determined with Kimura's two-parameter model (Kimura, 1980). Bootstrap analysis (Felsenstein, 1985) was performed for 1000 replications.

16S rRNA gene sequence similarity between strains BBH6^T and BBH9 was 99.7 %; their nearest phylogenetic neighbour was *M. barkeri* DSM 20145^T, with levels of similarity of 97.2 and 97.4 %, respectively. The two new strains formed a robust clade with *M. barkeri* and *M. gubbeenense* with bootstrap support of 95 % (Fig. 1). DNA–DNA dot-blot hybridization experiments were performed with a DIG DNA Labelling and Detection kit (Roche Diagnostics) as described by Bhadra *et al.* (2005). Strains BBH6^T and BBH9 showed 92 % DNA–DNA relatedness to each other. However, the two new strains exhibited levels of DNA–DNA relatedness of 32–34 % to *M. barkeri* and *M. gubbeenense* (see Supplementary Table S2 available in IJSEM Online), a level well below the 70 % value accepted as the cut-off point for species delineation (Wayne *et al.*, 1987). Therefore, based on the differences observed in the phenotypic and chemotaxonomic characteristics of strains BBH6^T and BBH9 with *M. barkeri* and *M. gubbeenense* (Table 1), it is suggested that they be classified as two strains of a novel species of the genus *Microbacterium*, for which the name *Microbacterium indicum* sp. nov. is proposed.

Description of *Microbacterium indicum* sp. nov.

Microbacterium indicum (in.di′cum. L. neut. adj. *indicum* pertaining to India).

On YP agar after 2 days incubation at 22 °C, forms circular, pale yellow colonies with an entire margin. Cells are Gram-positive rods. Growth occurs between 8 and 30 °C, but not at 5 or 37 °C. Growth occurs between pH 5 and 9, but not at pH 4 or 11. Tolerates up to 6 % (w/v) NaCl at pH 6.8. Whole-organism sugars are rhamnose, galactose and glucose. Major menaquinones are MK11 and MK12. Polar lipids are phosphatidylglycerol and phosphatidylinositol. Cells contain type B2β peptidoglycan and the diamino acid present in the cell wall is D-Orn. Other phenotypic characteristics are as given in Table 1.

The type strain, BBH6^T (=LMG 23459^T=IAM 15355^T), was isolated from a 50−70 cm section of a deep-sea sediment core of 4.6 m length obtained from the Chagos Trench, Indian Ocean, at a water depth of 5904 m.

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