Microbacterium profundi sp. nov., isolated from deep-sea sediment of polymetallic nodule environments

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A Gram-positive, aerobic, neutrophilic and rod-shaped bacterium, strain Shh49^T, was isolated from a deep-sea sediment sample collected from the East Pacific polymetallic nodule region. The strain was able to grow within a temperature range of 4–35 °C and tolerated up to 7.5 % (w/v) NaCl. Strain Shh49^T was characterized chemotaxonomically by having MK-12 and MK-13 as predominant isoprenoid quinones, anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$ as major fatty acids and ornithine as cell-wall diamino acid. The genomic DNA G+C content was 66.8 mol%. On the basis of 16S rRNA gene sequence similarities, the closest phylogenetic neighbours were the type strains of *Microbacterium phyllosphaerae* (98.3 %) and *Microbacterium keratanolyticum* (98.0 %), but strain Shh49^T could be clearly distinguished from its phylogenetic relatives with reference to a broad range of physiological and biochemical markers. DNA–DNA relatedness of strain Shh49^T with *M. phyllosphaerae* DSM 13468^T and *M. keratanolyticum* DSM 8606^T was 56 and 31 %, respectively. On the basis of phenotypic and genotypic data presented in this study, strain Shh49^T represents a novel species of the genus *Microbacterium*, for which the name *Microbacterium profundi* sp. nov. is proposed. The type strain is Shh49^T (=CGMCC 1.6777^T =JCM 14840^T).

Ocean polymetallic nodules form a vast metallic resource on the sea floor. The interesting environment of the deep-sea polymetallic nodule regions has only rarely been explored for the presence of novel bacterial species (Liu & Shao, 2005). Here we present a polyphasic study describing a novel *Microbacterium* strain isolated from a deep-sea sediment sample collected from a polymetallic nodule area. At the time of writing, the genus *Microbacterium*, belonging to the family *Microbacteriaceae* within the class *Actinobacteria*, contains 49 species of Gram-positive, non-spore-forming, rod-shaped bacteria (Euzéby, 1997; Takeuchi & Hatano, 1998a). Members of the genus *Microbacterium* are widespread and can be isolated from various habitats, including air, soil, water, milk products and plant, insect and human specimens (Evtushenko & Takeuchi, 2006).

Deep-sea sediment samples were collected by a multicorer from the East Pacific polymetallic nodule region (station ES0301; 8° 22′ 38″ N 145° 23′ 56″ W) at a depth of 5280 m (temperature 2 °C; salinity 34 ‰) during cruise DY105-12&14 of the vessel *Da Yang Yi Hao* in 2003. Aboard ship, sediment samples were subsampled aseptically and stored at −20 °C until use.

Approximately 200 mg of the subsample was suspended in 3 ml sterile seawater and vortexed for 15 min. The dispersed sediment suspension was then added to modified ZoBell medium (ZoBell, 1941) and plated by using a tenfold dilution series. The modified ZoBell agar contained (l^{-1} distilled water): 19.45 g NaCl, 8.8 g $MgCl_2$, 3.24 g Na_2SO_4, 1.8 g $CaCl_2$, 0.55 g KCl, 0.16 g $NaHCO_3$, 0.1 g ferric citrate pentahydrate, 80 mg KBr, 34 mg $CsCl_2$, 22 mg H_3BO_3, 4.0 mg Na_2SiO_3, 2.4 mg NaF, 1.6 mg

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Shh49^T is EF623999.

An extended 16S rRNA gene sequence-based tree, a phenotypic comparison with related type strains and a comparison of fatty acid profiles are available as supplementary material with the online version of this paper.

NH_4NO_3, 8.0 mg Na_3PO_4, 0.5 g peptone, 0.1 g yeast extract, 20 g agar (pH 5.5, adjusted with HCl). After 2 weeks of aerobic incubation at 25 °C, a yellow colony, named Shh49^T, was picked. The strain was purified by repeated restreaking; purity was confirmed by the uniformity of colony morphology. Unless otherwise stated, strain Shh49^T was maintained on trypticase soy yeast extract (TSBYE) medium (DSMZ medium 92) at 28 °C.

The optimal conditions for growth were determined in TSBYE medium with different NaCl concentrations (0, 0.5, 1, 3, 5, 7.5, 10, 15, 20 and 30 %, w/v). The pH range for growth was determined by adding MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5) or CAPSO (pH 9.0–10.0) to TSBYE medium at a concentration of 25 mM. The temperature range for growth was determined by incubating from 4 to 48 °C. Strain Shh49^T could grow at 32 °C after 2 days and at 35 °C after 10 days but not at 37 °C after 10 days. Cell morphology and motility were examined by optical microscopy (Olympus BX40) and electron microscopy (Cambridge S260 and JEM-1230) (Fig. 1).

Single-carbon-source assimilation tests were performed using a minimal medium (Takeuchi & Hatano, 1998b). Acid production was tested using modified MOF medium supplemented with 0.5 % sugars or alcohols (Leifson, 1963; Xu *et al.*, 2008). Biochemical tests were performed using the methods described by Mata *et al.* (2002). API 20NE and API 50CH tests (bioMérieux) were also used to determine physiological and biochemical characteristics. Detailed results are given in the species description.

Fatty acid methyl esters were obtained from cells grown in TSBYE medium for 24 h at 28 °C and analysed by using GC/MS (Kuykendall *et al.*, 1988). Isoprenoid quinones were analysed as described previously (Komagata & Suzuki, 1987) using reversed-phase HPLC, with menaquinones from *Microbacterium keratanolyticum* DSM 8606^T (MK-12 and 13) and *Microbacterium phyllosphaerae* DSM 13468^T (MK-10, 11 and 12) as reference standards. Cell-wall peptidoglycan was prepared and hydrolysed by the methods of Kawamoto *et al.* (1981) and the amino acid composition was analysed with an automatic amino acid analyser (Hitachi L-8900).

The 16S rRNA gene was amplified as described previously (Xu *et al.*, 2007). Sequence data were aligned with CLUSTAL W 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods with the MEGA 3 program package (Kumar *et al.*, 2004). Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980) for the neighbour-joining method. The DNA G+C content was determined by thermal denaturation (T_m) (Marmur & Doty, 1962) using *Escherichia coli* K-12 DNA as calibration standard. DNA–DNA hybridizations were performed by the thermal denaturation and renaturation method of De Ley *et al.* (1970) as modified by Huß *et al.* (1983), using a Beckman DU 800 spectrophotometer.

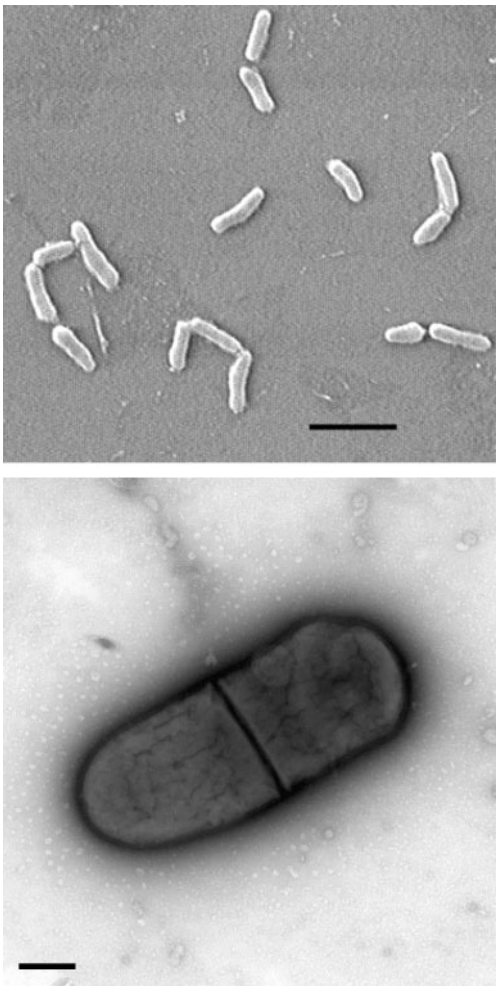

Fig. 1. Scanning electron photomicrograph (top) and transmission electron photomicrograph (bottom) of cells of *Microbacterium profundi* sp. nov. Shh49^T grown on TSBYE agar. Bars, 2 μm (top) and 0.2 μm (bottom).

The almost-complete 16S rRNA gene sequence (1488 nt) of strain Shh49^T was compared with closely related sequences of reference organisms from the EzTaxon service (Chun *et al.*, 2007). Strain Shh49^T showed the highest sequence similarity to the type strain of *M. phyllosphaerae* (98.3 %). Phylogenetic analysis based on 16S rRNA gene sequence comparison showed that strain Shh49^T formed a coherent cluster with the type strain of *M. keratanolyticum* (98.0 % sequence similarity) (Fig. 2 and Supplementary Fig. S1, available in IJSEM Online). Comparison of phenotypic properties (Table 1 and Supplementary Tables S1 and S2) indicated differences between strain Shh49^T and the other *Microbacterium* species, such as colony pigmentation, salt or temperature range for growth, H_2S formation, hydrolysis of substrates, utilization of hydrocarbons, production of acids from sugars, cell-wall diamino acid composition, menaquinone composition, fatty acids and G+C contents. The DNA relatedness values of strain Shh49^T to *M.*

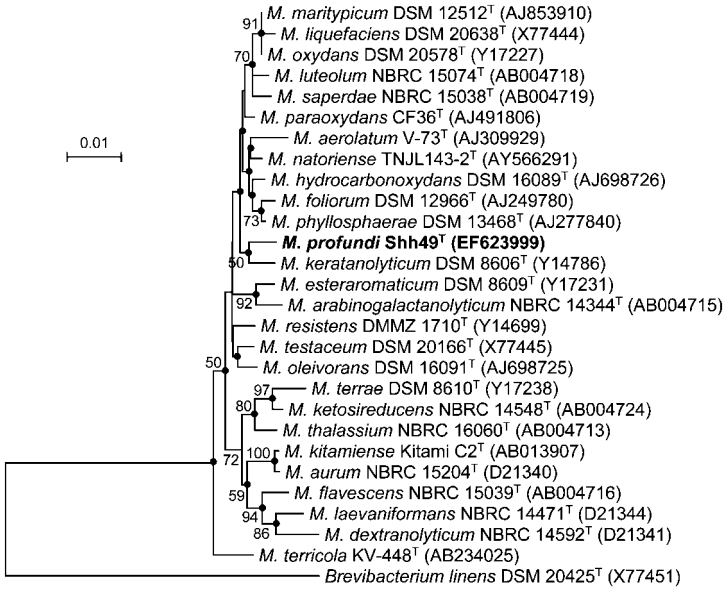

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships of the novel isolate and related taxa. Bootstrap percentages are based on 1000 replicates; only values >50 % are shown. Bar, 0.01 substitutions per nucleotide position. Dots indicate branches of the tree that were also found using the maximum-parsimony method.

keratanolyticum DSM 8606^T and *M. phyllosphaerae* DSM 13468^T were 31 and 56 %, respectively.

Therefore, based on the 16S rRNA gene sequence analysis as well as DNA–DNA hybridization data and differential phenotypic properties, we propose that strain Shh49^T represents a novel species within the genus *Microbacterium*, for which we propose the name *Microbacterium profundi* sp. nov.

Description of *Microbacterium profundi* sp. nov.

Microbacterium profundi (pro.fun′di. L. gen. n. *profundi* of the depths of the sea, of the deep-sea).

Aerobic and Gram-positive. Spore formation is not observed. Young cultures show short rod-like cells (1.0–2.0 × 0.4–0.6 μm), occurring singly or in pairs. Colonies are 1–2 mm in diameter, smooth, circular, elevated and yellow in colour after 48 h at 28 °C. NaCl concentration for growth is 0–7.5 % (w/v), with an optimum at 0–1 %. pH and temperature ranges for growth are pH 6.0–9.5 and 4–35 °C (optimum at pH 7.0–8.0 and 28 °C). Catalase-positive, oxidase-negative. Nitrate is not reduced to nitrite. Aesculin, DNA and gelatin are hydrolysed. Casein, starch, Tweens 20 and 80, tyrosine and urea are not hydrolysed. No growth on MacConkey agar or cetrimide agar. Indole and H_2S are not formed. *o*-Nitrophenyl β-D-galactopyranosidase and selenite reduction are positive. Negative for arginine dihydrolase, β-galactosidase, lecithinase and gluconate oxidation. Chemo-organotrophic. The following substrates are utilized for growth: acetate, L-arabinose, L-arginine, L-aspartate, cellobiose, D-fructose, fumarate, D-galactose, D-glucose, glycerol, isoleucine, lactate, lactose, lysine, malate, maltose, D-mannitol, D-mannose, propionate, pyruvate, raffinose, rhamnose, ribose, salicin, L-serine, succinate, sucrose, trehalose, L-valine and xylose. The following compounds are not utilized as sole carbon and energy sources: *N*-acetylglucosamine, adipic acid, L-alanine, capric acid, citrate, L-cysteine, ethanol, formate, glycine, L-histidine, *myo*-inositol, malonate, L-methionine, potassium gluconate, phenylacetic acid, sorbitol, sorbose and trisodium citrate. According to results from the API 50 CH test, acid is produced from D- and L-arabinose, arbutin, cellobiose, erythritol, aesculin ferric citrate, D-galactose, D-glucose, D-fructose, L-fucose, glycerol, D-lactose, maltose, D-mannitol, D-mannose, methyl β-D-xylopyranoside, L-rhamnose, D-ribose, salicin and D-xylose. Negative for the production of acid from sucrose and trehalose, but positive when tested according to the method of Leifson (1963). Susceptible to ampicillin (10 μg), bacitracin (0.04 IU), carbenicillin (100 μg), cefalexin (30 μg), cefoxitin (30 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), minocycline (30 μg), neomycin (30 μg), novobiocin (30 μg), penicillin (10 μg), polymyxin B (300 IU), rifampicin (5 μg), streptomycin (10 μg) and tetracycline (30 μg), but not to kanamycin (30 μg), nitrofurantoin (300 μg), nystatin (100 μg) or tobramycin (10 μg). The cell-wall diamino acid is ornithine; alanine, glycine and two unidentified amino acids are also found. The predominant menaquinones are MK-12 and MK-13. Major fatty acids are anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$. The DNA G+C content of the type strain is 66.8 mol% (T_m).

The type strain, Shh49^T (=CGMCC 1.6777^T =JCM 14840^T), was isolated from a deep-sea sediment sample collected from a polymetallic nodule region in the East Pacific Ocean.

Table 1. Phenotypic characteristics that differentiate strain Shh49^T from related *Microbacterium* species

Species/strain: 1, Shh49^T; 2, *M. keratanolyticum*; 3, *M. phyllosphaerae*; 4, *M. foliorum*; 5, *M. oleivorans*; 6, *M. hydrocarbonoxydans*; 7, *M. paraoxydans*; 8, *M. oxydans*; 9, *M. natoriense*; 10, *M. esteraromaticum*; 11, *M. halotolerans*; 12, *M. indicum*. Data are based on our comparative studies with *M. keratanolyticum* DSM 8606^T and *M. phyllosphaerae* DSM 13468^T as well as on literature data derived from Yokota *et al.* (1993), Takeuchi & Hatano (1998a, b), Schumann *et al.* (1999), Behrendt *et al.* (2001), Laffineur *et al.* (2003), Schippers *et al.* (2005), Li *et al.* (2005), Liu *et al.* (2005), Evtushenko & Takeuchi (2006) and Shivaji *et al.* (2007). +, Positive; (+), weakly positive; −, negative; d, reaction differs among strains; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Colony colour*	Y	Y	Y	Y	O	Y	LY	Y	Y	YW/Y	W	PY
Growth in 6.5 % NaCl	+	−	ND	ND	−	−	ND	ND	ND	−	+	ND
Growth at 37 °C	−	−	d	d	+	+	+	+	+	d	+	−
H_2S production	−	+	−	−	−	−	ND	+	ND	+	−	ND
Hydrolysis of:												
Casein	−	+	+	−	ND	ND	+	ND	−	ND	−	ND
Gelatin	+	+	+	+	ND	ND	+	+	+	−	−	ND
Starch	−	−	(+)	(+)	ND	ND	ND	−	+	+	−	−
Assimilation of:												
L-Alanine	−	+	+	ND	−	+	ND	ND	ND	ND	ND	−
Arabinose	+	−	+	+	+	+	+	−	+	+	+	+
Citrate	−	−	−	d	−	+	ND	+	ND	−	ND	−
Malate	+	−	+	+	+	+	ND	+	ND	+	+	ND
Propionate	+	+	+	ND	−	+	ND	ND	ND	+	ND	ND
Rhamnose	+	−	+	(+)	+	+	+	ND	−	ND	−	−
Acid production from:												
Glucose	+	−	+	+	−	−	+	+	+	−	+	+
Rhamnose	+	−	+	−	−	+	ND	d	−	ND	ND	−
Major cellular fatty acids†	ai15, i15, i16, ai17	ai15, i16, i15, ai17,	ai15, i16 i15, ai17	ai15, ai17, i16	ND	ND	ai15, ai17, i16, i15, i17	ai15, ai17, i16	ai15, i15, ai17	ai15, ai17, i16	ai15, ai17, i16	i16, ai15, 16, ai17
Menaquinones (MK)	12, 13	12, 13	10, 11, 12	10, 11, 12	11, 12	11, 12	ND	11, 12	9, 10, 11, 12	12, 13	10, 11	11, 12
Cell-wall diamino acid	Orn	Orn	Orn	Orn	Lys	Orn	Orn	Orn	Orn	Orn	Orn	Orn
G+C content (mol%)	66.8	66.5	64	67	ND	ND	69.9	67	69.1	68.8	63.8	65.5

*LY, Lemon yellow; O, orange; PY, pale yellow; W, white; Y, yellow; YW, yellow–white.
†ai, Anteiso-branched acid; i, iso-branched acid.

Acknowledgements

This work was supported by grants from the Major State Basic Research Development Program of China (973 Program) (2004CB719604-3), the China Ocean Mineral Resources R & D Association (COMRA) Special Foundation (DYXM-115-01-3-01, DYXM-115-01-3-02) and the Scientific Research Fund of the Second Institute of Oceanography, SOA (JT0709).

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