Nocardioides furvisabuli sp. nov., isolated from black sand

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A Gram-positive, rod-shaped, yellow-pigmented actinomycete, designated strain SBS-26^T, was isolated from a sample of black sand from Samyang Beach on Jeju Island (Republic of Korea) and was subjected to polyphasic characterization to unravel its taxonomic status. Phylogenetic analyses based on 16S rRNA gene sequences revealed that the organism belongs to the genus *Nocardioides* but forms a distinct branch at the base of a *Nocardioides ganghwensis*–*Nocardioides oleivorans* cluster. The 16S rRNA gene sequence of strain SBS-26^T showed the highest levels of similarity to those of *N. ganghwensis* JC2055^T (97.7 %) and *N. oleivorans* DSM 16090^T (97.6 %). The levels of 16S rRNA gene sequence similarity between strain SBS-26^T and other members of the genera *Nocardioides* and *Marmoricola* were in the range 93.0–96.2 %. The following chemotaxonomic characteristics support the phylogenetic association of strain SBS-26^T with members of the genus *Nocardioides*: LL-diaminopimelic acid as the principal diamino acid of the peptidoglycan, MK-8(H₄) as the major menaquinone, iso-C_{16:0} as the predominant fatty acid and a DNA G + C content of 69.1 mol%. The polar lipids contained phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol and an unknown phospholipid. On the basis of the phenotypic, chemotaxonomic and phylogenetic data, strain SBS-26^T represents a novel species of the genus *Nocardioides*, for which the name *Nocardioides furvisabuli* sp. nov. is proposed. The type strain is strain SBS-26^T (=JCM 13813^T=NRRL B-24465^T).

The genera *Nocardioides* and *Marmoricola* are characterized chemotaxonomically within the family *Nocardioideaceae* by the combination of LL-diaminopimelic acid in the peptidoglycan and the presence of respiratory quinone MK-8(H₄) as the major menaquinone (O'Donnell *et al.*, 1982; Prauser, 1976; Urzì *et al.*, 2000). These genera are phylogenetically intermixed within the radiation of the family *Nocardioideaceae* but can be readily differentiated from each other on the basis of cell morphology and the cellular fatty acid profile (Urzì *et al.*, 2000).

Since the original description by Prauser (1976), the number of *Nocardioides* species described has increased rapidly because of improved classification resulting from the polyphasic approach. Currently, there are 17 species (isolated mainly from terrestrial and aquatic substrates) with validly published names. Of these, *Nocardioides ganghwensis* (Yi & Chun, 2004a) and *Nocardioides aestuarii* (Yi & Chun, 2004b) were isolated recently from tidal flat sediments.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SBS-26^T is DQ411542.

The cellular fatty acid compositions of strain SBS-26^T growing on different media are presented in a supplementary table available in IJSEM Online.

Strain SBS-26^T was isolated from samples of black sand collected around Samyang Beach on Jeju Island, Republic of Korea, and its taxonomic status was investigated by using a polyphasic approach. For bacterial isolation, a sand sample (1 g) was placed into a sterile tube containing 9 ml sterile seawater. After mixing for 30 min on a tube rotator, aliquots (100 µl) of the serial diluents of the samples were transferred directly onto ISP 4 medium (Shirling & Gottlieb, 1966) supplemented with 60 % (v/v) natural seawater. The agar plates were incubated at 30 °C for 14 days and the isolate was subcultured on ISP 2 medium (Shirling & Gottlieb, 1966) supplemented with 60 % (v/v) natural seawater (YE/SW agar). The pure culture was maintained at –20 and –80 °C in a 20 % (v/v) glycerol suspension supplemented with 60 % (v/v) natural seawater.

The extraction of chromosomal DNA, the amplification and purification of the 16S rRNA gene by PCR and the direct sequencing of the purified PCR product were performed as described previously (Lee *et al.*, 2000; Lee, 2006). The almost-complete sequence (1392 nt) of the 16S rRNA gene of strain SBS-26^T determined in this study was compared, using the CLUSTAL X program (Thompson *et al.*, 1997), with those of representatives of the family *Nocardioideaceae*. The aligned sequences were manually adjusted according to the secondary structure of the *Escherichia coli* 16S rRNA gene

sequence (Brosius *et al.*, 1978). Phylogenetic analyses were carried out using the neighbour-joining (Saitou & Nei 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. The neighbour-joining tree was constructed with a distance matrix calculated using the method described by Jukes & Cantor (1969). *Streptomyces griseus* KCTC 9080^T (GenBank accession no. M76388) was used as an outgroup organism. The stability of the tree topology was evaluated by bootstrap analysis (Felsenstein, 1985). The neighbour-joining tree (Fig. 1) based on the comparison of 16S rRNA gene sequences revealed that the organism is related to, but distinct from, members of the genera *Nocardioideis* and *Marmoricola* of the family *Nocardioideaceae* and formed a distinct clade at the foot of the *N. ganghwensis*–*N. oleivorans* cluster, with a high level of bootstrap support (100 %). This branching pattern of the isolate was also found in the trees obtained using the maximum-parsimony and maximum-likelihood treeing algorithms. The 16S rRNA gene sequence of strain SBS-26^T showed the highest levels of similarity with those of *N. ganghwensis* JC2055^T (97.7 %) and *Nocardioideis oleivorans* DSM 16090^T (97.6 %). The levels of 16S rRNA gene sequence similarity between strain SBS-26^T and other members of the genera *Nocardioideis* and members of the genus *Marmoricola* were in the range 93.0–96.2 %.

Fig. 1. Neighbour-joining phylogenetic tree showing the relationships between strain SBS-26^T and members of the genera *Marmoricola* and *Nocardioideis*, based on 16S rRNA gene sequences. Asterisks indicate that the corresponding branch is also recovered in the maximum-likelihood and maximum-parsimony trees. Levels of bootstrap support, expressed as percentages of 1000 resampled datasets from a neighbour-joining analysis, are given at nodes if greater than 40 %. Bar, 1 nucleotide substitution per 100 nucleotides.

Growth on various culture media was tested by using ISP 2 medium (Shirling & Gottlieb, 1966), trypticase soy broth agar (TSBA; Difco) and nutrient agar (NA; Difco) and marine agar (MA; Difco). To test the requirement for seawater, culture media (with the exception of MA) were each supplemented with 60 % (v/v) natural seawater. The degree of growth was recorded after incubation for 7 days at 30 °C. The growth temperature and pH were tested at 4–45 °C and pH 4.1–12.1. NaCl tolerance during growth was determined in ISP 2 medium supplemented with 0–9, 10 and 15 % (w/v) NaCl. Cell morphology and motility were investigated under phase-contrast and transmission electron microscopy, using 3-day-old cultures. Colony pigmentation was observed visually and recorded using 5-day-old cultures growing on YE/SW agar at 30 °C. Carbohydrate utilization was tested using ISP 9 medium (Shirling & Gottlieb, 1966): each filter-sterilized carbon source was tested at a final concentration of 1 % (w/v) (for carbohydrates and alcohols) or 0.1 % (w/v) (for organic acids). The Gram reaction was determined by using a Gram-stain kit (bioMérieux) according to the instructions of the manufacturer. Oxidase activity was tested by assessing the oxidation of 1 % (w/v) *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Catalase activity was determined with a 3 % (v/v) H₂O₂ solution. The decomposition of hypoxanthine, DL-tyrosine and xanthine was determined as described previously (Gordon *et al.*, 1974). The degradation of elastin was investigated on ISP 2 medium supplemented with 0.3 % (w/v) elastin. The hydrolysis of aesculin, casein and starch was assessed using the methods of MacFaddin (1980). Other physiological and biochemical properties were tested with the API 20E, API 20NE and API ZYM systems (bioMérieux). For phenotypic comparisons, *N. ganghwensis* IMSNU 14028^T and *N. oleivorans* DSM 16090^T were used as reference strains and further investigated for physiological and biochemical properties not previously tested (Yi & Chun, 2004a; Schippers *et al.*, 2005).

Strain SBS-26^T showed good growth on ISP 2 medium, NA and MA. No growth occurred on TSBA irrespective of supplementation with natural seawater, whereas the reference strains, *N. ganghwensis* IMSNU 14028^T and *N. oleivorans* DSM 16090^T, showed good growth on TSBA. The cells of strain SBS-26^T were motile (flagellated) short rods (0.4–0.5 × 0.6–1.2 μm) but did not produce mycelium or spores (Fig. 2). On YE/SW agar, the colonies were circular, smooth, convex and yellow in colour. After incubation for 5 days, the colonies reached a diameter of 0.6–0.8 mm. The physiological and biochemical characteristics of strain SBS-26^T are given in the species description and are compared with those of *N. ganghwensis* and *N. oleivorans* in Table 1.

Biomass for the chemotaxonomic characterization was obtained from 3-day-old cultures growing on YE/SW broth at 30 °C. The isomer type of the cell-wall diaminopimelic acid was analysed as described previously (Staneck & Roberts, 1974). Isoprenoid quinones were extracted as described by Minnikin *et al.* (1984) and analysed using the

Fig. 2. Transmission electron micrograph of cells of strain SBS-26^T grown on YE/SW agar for 3 days at 30 °C. Bar, 500 nm.

method described by Kroppenstedt (1985). Polar lipids were separated using 6.6 × 6.6 cm TLC silica gel 60 F₂₅₄ plates (Merck) according to the method described by Minnikin *et al.* (1977). Individual phospholipids were identified by using several spray reagents (Embley & Wait, 1994) and through co-migration with authentic standards (Sigma). To determine the cellular fatty acid composition, cells were cultivated on YE/SW agar and MA at 30 °C for 5 days. Fatty acid methyl esters were prepared and analysed according to the instructions of the Microbial Identification System (MIDI). The G + C content of the DNA was determined by using HPLC with a Supelcosil LC-18-S (150 × 4.6 mm) column, as described by Mesbah *et al.* (1989). Strain SBS-26^T contained LL-type diaminopimelic acid as the diagnostic cell-wall diamino acid. MK-8(H₄) was the predominant menaquinone. The DNA G + C content, determined by HPLC, was 69.1 mol%. Most of the chemotaxonomic features of strain SBS-26^T were consistent with those of the genus *Nocardioides* (Collins *et al.*, 1994; Lawson *et al.*, 2000; O'Donnell *et al.*, 1982; Schippers *et al.*, 2005; Yi & Chun, 2004a, b; Yoon *et al.*, 1997, 1999, 2004, 2005, 2006). Strain SBS-26^T had a cellular fatty acid profile characterized by the presence of large amounts of straight-chain, branched and unsaturated fatty acids, and a major amount of iso-C_{16:0} irrespective of the culture medium used (see Supplementary Table S1 available in IJSEM Online). In general, cellular fatty acid compositions can vary according to the culture medium and growth conditions used (Kroppenstedt, 1985); under the conditions used in our study, the other major fatty acid was C_{18:1ω9c} (YE/SW agar) but, on MA, C_{18:0} and C_{16:0} were also detected as principal components. The polar lipids comprised phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol and an unknown phospholipid. The presence of phosphatidylcholine in the phospholipid profile can serve as a key marker for differentiating the novel

isolate from members of the genus *Nocardioides* (O'Donnell *et al.*, 1982).

The phylogeny based on 16S rRNA gene sequences suggested that the isolate belonged to the genus *Nocardioides*; however, the strain consistently formed a distinct clade at the base of the *N. ganghwensis*–*N. oleivorans* cluster (Fig. 1), indicating that the organism could be a novel member of the genus. This relationship is clear in that a number of phenotypic characteristics sharply distinguish between the isolate and its phylogenetic relatives (Table 1), as do chemotaxonomic markers such as the phospholipid and fatty acid compositions. On the basis of the combination of the physiological and chemotaxonomic features and its phylogenetic distinctness, strain SBS-26^T represents a novel species of the genus *Nocardioides*, for which the name *Nocardioides furvisabuli* sp. nov. is proposed.

Description of *Nocardioides furvisabuli* sp. nov.

Nocardioides furvisabuli (fur.vi.sa'bu.li. L. neut. adj. *furvum* black-coloured; L. neut. n. *sabulum* gravel, sand; N.L. gen. n. *furvisabuli* of black-coloured sand, the source of isolation of the type strain).

Gram-positive, aerobic, oxidase-negative, catalase-positive. Cells are motile, short rods (0.4–0.5 × 0.6–1.2 μm). Colonies are circular, smooth, convex, yellow in colour and 0.6–0.8 mm in diameter after 5 days incubation on YE/SW agar at 30 °C. The temperature range for growth is 4–37 °C, with optimum growth at 30 °C. The pH range for growth is pH 5.1–10.1, with optimum growth at pH 7.1. Growth occurs in the presence of 0–6 % NaCl. Good growth occurs on ISP 2, NA (with/without the addition of natural seawater) and marine agar. Growth does not occur on TSBA medium. Nitrate is reduced to nitrite. H₂S and indole production are not detected. Urease activity is not detected. Voges–Proskauer reaction is weakly positive. Casein and starch are hydrolysed but gelatin is not hydrolysed. Elastin is degraded. Decomposition of hypoxanthine, DL-tyrosine or xanthine does not occur. Acid is not produced from glucose. Positive for esterase lipase (C8). Weakly positive for valine arylamidase and α-galactosidase. Negative for lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, esterase (C4), cystine arylamidase and acid phosphatase. L-Arabinose, inulin, methyl α-D-mannoside, D-raffinose, D-xylitol, adonitol, D-dulcitol, glycerol and D-mannitol are utilized as sole carbon and energy sources. D-Arabinose, dextran, D-melezitose, methyl α-D-glucoside, sucrose, L-rhamnose, salicin, L-sorbose, 2,3-butanediol, *meso*-erythritol and D-xylitol are not utilized. Assimilation of citrate, formate, malate, succinate and tartrate is observed. Weakly positive for the assimilation of acetate and the utilization of *myo*-inositol, 1,2-propanediol and D-sorbitol. The predominant fatty acids are iso-C_{16:0} and C_{18:1ω9c} (on YE/SW agar) or iso-C_{16:0}, C_{18:0} and C_{16:0} (on MA). Polar lipids contain phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol and an unknown phospholipid. The DNA

Table 1. Characteristics that differentiate strain SBS-26^T from phylogenetic relatives from the genus *Nocardioides*

Strains: 1, SBS-26^T; 2, *N. ganghwensis* IMSNU 14028^T; 3, *N. oleivorans* DSM 16090^T. Data were taken from Yi & Chun (2004a), Schippers *et al.* (2005) and this study. Symbols: +, positive; –, negative; w, weakly positive; v, variable. All strains are positive for the Gram reaction, catalase and β -galactosidase and negative for arginine dihydrolase, glucose fermentation, indole production from tryptophan and urease (API 20NE kit). Casein and DNA are degraded by all strains but cellulose or hypoxanthine are not. Alkaline phosphatase and leucine arylamidase are positive for all of the strains, but lipase (C14), trypsin, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, β -glucuronidase, β -glucosidase, *N*-acetyl- β -glucosamidase, α -mannosidase or α -fucosidase are negative (API ZYM kit). All strains utilize D-cellobiose, D-fructose, D-galactose, D-glucose, D-lactose, maltose, D-mannitol, D-mannose and D-trehalose but not benzoate or D-ribose.

Characteristic	1	2	3
Motility	+	–	–
NaCl tolerance for growth (%)	0–6	0–8	0–2
Nitrate reduction	+	+	–
Hydrolysis of:			
Aesculin	–	w	+
Gelatin	–	+	–
Starch	+	+	–
Tween 80	–	+	–
Degradation of:			
Tyrosine	–	+	–
Xanthine	–	w	–
API ZYM tests			
Esterase (C4)	–	v	w
Esterase lipase (C8)	+	+	w
Valine arylamidase	w	+	+
Cystine arylamidase	–	w	w
Acid phosphatase	–	w	–
α -Galactosidase	w	+	–
α -Glucosidase	–	+	+
Utilization of carbon sources			
Acetate	w	+	+
Adonitol	+	–	–
L-Arabinose	+	+	–
Citrate	+	+	–
Formate	+	–	–
Glycerol	+	+	–
Inositol	w	–	–
Inulin	+	–	–
Malate	+	–	+
Melibiose	–	+	+
D-Raffinose	+	+	–
L-Rhamnose	–	+	+
D-Salicin	–	+	–
D-Sorbitol	w	–	–
Sucrose	–	+	+
D-Xylose	+	+	–
Acid production from D-glucose	–	–	+

G + C content is 69.1 mol%. Other characteristics are given in Table 1.

The type strain, SBS-26^T (=JCM 13813^T=NRRL B-24465^T), was isolated from black sand from Samyang Beach on Jeju Island, Republic of Korea.

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