Streptomyces nanhaiensis sp. nov., a marine streptomycete isolated from a deep-sea sediment

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A novel aerobic streptomycete, strain SCSIO 01248^T, was isolated from a sample of deep-sea sediment collected from the northern South China Sea, at a depth of 1632 m. This isolate formed yellow–white substrate mycelium and grey–white aerial hyphae. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain SCSIO 01248^T was most closely related to *Streptomyces radiopugnans* R97^T (98.8 % sequence similarity), *S. macrosporus* NBRC 14748^T (97.5 %) and *S. megasporus* NBRC 14749^T (97.3 %). The novel strain could, however, be readily differentiated from *S. radiopugnans* DSM 41901^T on the basis of some physiological and cellular chemical characteristics; the level of DNA–DNA relatedness between these two strains was only 40 %. Based on phylogenetic and phenotypic evidence, strain SCSIO 01248^T represents a novel species, for which the name *Streptomyces nanhaiensis* sp. nov. is proposed. The type strain is SCSIO 01248^T (=DSM 41926^T =KCTC 19401^T =CCTCC AA 208007^T).

Streptomyces is the largest genus of the phylum *Actinobacteria* and is the type genus of the family *Streptomycetaceae* (Kämpfer, 2006). The members of the genus *Streptomyces* are Gram-positive bacteria with high G+C contents and are characterized by a complex secondary metabolism. They produce over two-thirds of the clinically useful antibiotics of natural origin (Kieser *et al.*, 2000). Streptomycetes are found predominantly in soil and in decaying vegetation, and most produce spores. A gram of soil, an important habitat for this group of bacteria, typically contains 10⁴–10⁷ c.f.u. of streptomycetes, representing 1–20 %, or an even larger proportion, of the total counts of viable bacteria (Schrenpf, 2006). In contrast, the total numbers of culturable actinobacteria in fresh samples of marine sediments are very low (usually about 10²–10³ c.f.u. g^{−1}), especially in samples collected more than 1000 m below the sea surface. In the South China Sea, for example, *Streptomyces* species are scarce in sediment samples collected at depths beyond 1000 m, even though *Streptomyces* and *Micromonospora* species predominate in sediments collected at depths of 30–300 m (unpublished results).

Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; PL, phospholipid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SCSIO 01248^T is GQ871748.

During our investigation of marine actinobacterial resources in the South China Sea, a novel isolate, strain SCSIO 01248^T, was obtained from a sediment sample collected at a depth of 1632 m, a pH of about 7.8 and a temperature of 5 °C. Here we report the classification and identification of this new isolate based on a polyphasic taxonomic approach. Strain SCSIO 01248^T was isolated from a sediment sample collected below the northern South China Sea, at 118° 57.006' N 22° 0.574' E, using methods described by Tian *et al.* (2009). A grey–white colony was picked after incubation at 28 °C for 3 weeks on humic acid–vitamin agar (Hayakawa & Nomura, 1987) that had been made with 70 % (v/v) natural seawater in distilled water (instead of pure distilled water). The purified strain was maintained both on ISP medium 2 and as 20 % (w/v) glycerol suspensions at −80 °C. Biomass for chemotaxonomic and molecular systematic studies was obtained by cultivation in ISP 2 broth or, for the fatty acid analysis, trypticase soy broth (Difco) at 28 °C for 1 week, with continuous shaking at 150 r.p.m.

For morphological and cultural characterization, strain SCSIO 01248^T was incubated at 28 °C for 28 days on ISP media 2, 3, 4 and 5 and Czapek's solution agar (Waksman, 1961), nutrient agar (Difco) and potato agar (Waksman, 1961). Cell morphology was examined using a light microscope (BH2; Olympus), a transmission electron microscope

(JEM-1010; JEOL) and a scanning electron microscope (JSM 5600LV; JEOL) and cells that had been incubated for 14 or 28 days on ISP medium 2 modified by replacement of the pure distilled water with 50 % (v/v) seawater in distilled water. Diffusible pigments were investigated by comparing cultures against the most suitable colour chips from the ISCC-NBS Color Charts (Kelly, 1964).

The Gram-reaction test was performed as described by Smibert & Krieg (1994). Tests for hydrolysis of starch, gelatin, cellulose and Tweens 20, 40, 60 and 80, milk coagulation and peptonization and utilization of urea were carried out as described by Tindall *et al.* (2007). The methods used for determination of H₂S and melanin production, nitrate reduction, production of soluble pigments and use of various substrates (each at a final concentration of 1 %, w/v) as sole carbon sources were those described by Williams *et al.* (1989). Catalase activity was evaluated by the production of oxygen bubbles in 3 % (v/v) H₂O₂ and oxidase activity by the use of oxidase reagent (bioMérieux). Ranges of temperature and pH for growth and tolerance of NaCl were evaluated as described by Xu *et al.* (2005) using ISP medium 2 as the basal medium, made

with 50 % (v/v) natural seawater in distilled water (instead of pure distilled water). The medium pH was adjusted using acetate/citrate buffer (pH 4–6), KH₂PO₄/NaOH (pH 6–8), NaHCO₃/Na₂CO₃ (pH 9–10) or Na₂HPO₄/NaOH (pH 11). Antibiotic susceptibility was examined as described by Groth *et al.* (2004), using antibiotic discs on ISP medium 2. The morphological, cultural, physiological and biochemical characteristics of strain SCSIO 01248^T are given in Table 1, Fig. 1 and the species description.

Diaminopimelic acid and whole-cell sugars were analysed according to the procedures developed by Hasegawa *et al.* (1983). Menaquinones were isolated using the methods of Minnikin *et al.* (1984) and separated by HPLC (Kroppenstedt, 1982). Polar lipids were extracted and examined by using published procedures (Minnikin *et al.*, 1979; Collins & Jones, 1980). Fatty acids were analysed by using standard methods (Sasser, 1990) and version 6.0 of the Sherlock Microbial Identification System (MIDI). The genomic DNA G+C content was determined by HPLC (Mesbah *et al.*, 1989).

The cell-wall peptidoglycan of strain SCSIO 01248^T contains LL-diaminopimelic acid, glycine, asparagine, glutamic acid

Table 1. Comparison of characteristics of strain SCSIO 01248^T and *S. radiopugnans* DSM 41901^T

All data were obtained in this study with the exception of the major fatty acid profile of *S. radiopugnans* R97^T (=DSM 41901^T), which was taken from Mao *et al.* (2007).

Characteristic	SCSIO 01248 ^T	<i>S. radiopugnans</i> DSM 41901 ^T
Spore chain morphology	Straight to spiral	Spiral
Spore surface ornamentation	Smooth to rough	Rough to warty
Utilization of:		
Arabinose	—	+
Cellobiose	+	—
D-Mannitol	—	+
L-Rhamnose	—	+
D-Ribose	—	+
Trehalose	+	—
Xylitol	—	+
Citrate	—	+
Hydrolysis of Tween 20	+	—
Temperature range for growth (°C)	10–45	20–50
NaCl concentration for growth (%)	0–7.5	0–7
Growth at pH 5 and 11	—	+
Major menaquinones (% of total)*		
MK-9(H ₆)	36	65
MK-9(H ₈)	55	31
Major fatty acids (% of total)†	i-C _{16:0} (29.9), ai-C _{15:0} (14.9), i-C _{16:1} H (14.3), i-C _{14:0} (10.4), ai-C _{17:0} (7.8), i-C _{15:0} (10.9), C _{16:1} cis9 (7.6), 9-methyl C _{16:0} (4.2)	i-C _{16:0} (34.5), ai-C _{15:0} (15.4), i-C _{16:1} H (14.2), i-C _{14:0} (5.6), ai-C _{17:0} (9.1), ai-C _{17:1} cis8 (5.2)
Phospholipids*‡	DPG, PE, PG, PI, PIM, PLs	DPG, PE, PG, PI, PLs
DNA G+C content (mol%)*	71.9	73.3

*Data for the two strains collected under the same culture conditions.

†ai, Anteiso-branched; i, iso-branched.

‡DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; PLs, unknown phospholipids.

Fig. 1. Scanning electron micrographs of the spore chains (a) and aerial mycelium (b) of strain SCSIO 01248^T, as seen after incubation for 14 days on ISP medium 2 at 28 °C. Bars, 2 µm (a) and 10 µm (b).

and alanine but no detectable sugar. The polar lipids were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides and three unknown phospholipids. The menaquinones were MK-9(H₈) (55.5 %), MK-9(H₆) (36.8 %), MK-9(H₁₀) (3.8 %), MK-9(H₄) (1.6 %), MK-10(H₈) (1.5 %) and MK-10(H₆) (0.8 %). The fatty acids consisted mainly of iso-C_{16:0} (29.9 %), anteiso-C_{15:0} (14.9 %), iso-C_{16:1} H (14.3 %), iso-C_{14:0} (10.4 %), iso-C_{15:0} (10.9 %), anteiso-C_{17:0} (7.8 %), C_{16:1}cis9 (7.6 %) and 9-methyl C_{16:0} (4.2 %). The genomic DNA G + C content was 71.9 mol%.

Extraction of genomic DNA and PCR-mediated amplification and sequencing of the 16S rRNA gene of strain SCSIO 01248^T were carried out according to Li *et al.* (2007). Multiple alignments with sequences of the recognized *Streptomyces* species that appeared most closely related to the novel strain and evaluation of the levels of sequence similarity were carried out using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic analyses were performed using neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A phylogenetic tree was constructed using the neighbour-joining method of Saitou & Nei (1987), *K*_{nuc} values (Kimura, 1983) and the MEGA5 software package (Tamura *et al.*, 2011). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985), with 1000 replicates.

DNA–DNA hybridization experiments were performed between strain SCSIO 01248^T and the type strain of the most closely related established species using the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992) and a UV1601 spectrophotometer (Shimadzu).

The results of BLAST analysis of 16S rRNA gene sequences indicated that strain SCSIO 01248^T was most closely related to *S. radiopugnans* R97^T (98.8 % sequence similarity; Mao *et al.*, 2007) followed by *S. macrosporus* NBRC 14748^T (97.5 %) and *S. megasporus* NBRC 14749^T (97.3 %). Several characteristics of strain SCSIO 01248^T, the production of long, spirally coiled chains of spores, the presence of LL-diaminopimelic acid in the cell-wall peptidoglycan, MK-9(H₈) and MK-9(H₆) as the predominant menaquinones and iso-C_{14:0}, iso-C_{16:0}, anteiso-C_{15:0} and anteiso-C_{17:0} as major fatty acids, were consistent with those of established *Streptomyces* species.

In a phylogenetic tree based on the 16S rRNA gene sequences of strain 01248^T and its closest known relatives, the novel strain formed a distinct subclade and clustered with *S. radiopugnans* R97^T, close to *S. macrosporus* NBRC 14748^T, *S. megasporus* NBRC 14749^T, *S. glaucosporus* NBRC 15416^T and *S. thermolineatus* NBRC 14750^T (Fig. 2). The topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms were similar to those of the tree constructed using neighbour-joining analysis (Fig. 2).

Several years ago, Stackebrandt & Ebers (2006) recommended an increase of about 2 % (from 97 % to 98.7–99 %) in the threshold for 16S rRNA gene sequence similarity used to determine the uniqueness of a novel isolate, provided that this level of difference in the sequences was supported by clear phenotypic differences. In this study, therefore, DNA–DNA relatedness experiments were only carried out between strain SCSIO 01248^T and the type strain of established species to which it appeared most closely related: *S. radiopugnans* DSM 41901^T. The mean level of DNA–DNA relatedness recorded between these two strains (over three replicate experiments) was 40 % and therefore well below the 70 % cut-off point recommended for the delineation of genomic species (Stackebrandt & Goebel, 1994). In addition, strain SCSIO 01248^T can be readily differentiated from *S. radiopugnans* DSM 41901^T on the basis of pH and temperature ranges for growth, sole carbon sources for growth, cellular phospholipids and predominant menaquinones and fatty acids (Table 1). Based on phylogenetic and phenotypic evidence, strain SCSIO 01248^T represents a novel species within the genus *Streptomyces*, for which the name *Streptomyces nanhaiensis* sp. nov. is proposed.

Description of *Streptomyces nanhaiensis* sp. nov.

Streptomyces nanhaiensis [nan.hai.en'sis. N.L. masc. adj. *nanhaiensis* of or pertaining to Nanhai (South China Sea), where the type strain was isolated].

Fig. 2. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences, showing the relationships between strain SCSIO 01248^T and its closely related neighbours within the genus *Streptomyces*. Filled circles indicate nodes that were also obtained in both maximum-parsimony and maximum-likelihood trees. Bootstrap values (expressed as percentages of 1000 replications) >50% are given at branching points. Bar, 0.005 substitutions per nucleotide position.

Gram-reaction-positive, aerobic actinomycete that forms yellow–white substrate mycelium and grey–white aerial hyphae. The hyphae differentiate into straight to spiral chains of spores, each chain comprising about 10 spores. The spores are 1–2 µm in diameter, with a smooth to rough surface. Grows well on ISP 2, ISP 3, ISP 4 and ISP 5 media, Czapek's solution agar and nutrient agar at 28 °C, but aerial mycelium is not easily observed on potato agar. Soluble pigments are not produced. Acetate, cellobiose, fructose, fucose, D-galactose, D-glucose, maltose, sucrose and D-xylose can be used as sole carbon sources for growth, but not arabinose, citrate, dulcitol, inositol, lactose, D-mannitol, D-mannose, L-rhamnose, D-ribose, D-sorbitol, xylitol or raffinose. Positive for hydrolysis of starch and Tweens 20, 40 and 60, nitrate reduction and catalase and weakly positive for hydrolysis of cellulose but negative for hydrolysis of gelatin and Tween 80, milk coagulation and peptonization, H₂S and melanin production, utilization of urea and oxidase activity. Temperature, pH and NaCl concentration ranges for growth are 10–45 °C, pH 6–10 and 0–7.5% (w/v); the corresponding optima are 28–37 °C, pH 7–8 and 3% (w/v) NaCl. Susceptible to (µg per disc) penicillin G (10), erythromycin (15), gentamicin (10), streptomycin (10), tetracycline (30), vancomycin (30), novobiocin (30), lincomycin (2), rifampicin (5), netilmicin (30), amoxicillin (10), ciprofloxacin (5), amikacin (30), tobramycin (10), neomycin (10) and chloramphenicol (30) but resistant to sulfamethoxazole (23.75), ampicillin (10) and norfloxacin (10). The cell wall contains LL-diaminopimelic acid, glycine, asparagine, glutamate and alanine. The polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides and three unknown phospholipids. The predominant menaquinones are MK-9(H₈) and MK-9(H₆). The major fatty acids are iso-C_{16:0}, anteiso-C_{15:0} and iso-C_{16:1} H.

The type strain, SCSIO 01248^T (=DSM 41926^T =KCTC 19401^T =CCTCC AA 208007^T), was isolated from a

sediment sample collected at a depth of 1632 m from the northern South China Sea. Its genomic DNA G + C content is 71.9 mol%.

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