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Streptomyces xinghaiensis sp. nov., a new actinomycete derived from marine sediment sample in Xinghai Bay, Dalian, China

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Running title: *Streptomyces xinghaiensis* sp.nov.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S187^T is EF577247.

A novel actinomycete strain S187^T was isolated from a marine sediment sample collected from Xinghai Bay, Dalian, China. Growth occurred on ISP medium 2 with 0-9% NaCl, pH 6.0-9.0 and 10-45 °C. The cell wall of strain S187 contained LL-Diaminopimelic acid (DAP) isomer. The predominant menaquinones were MK-9 (H_6) (40.8%), MK-9 (H_8) (38.2%), and MK-9 (H_2) (8.8%). The major fatty acids were iso-$C_{16:0}$ (29.6%), anteiso-$C_{15:0}$ (14.0%) and anteiso-$C_{17:0}$ (11.6%). Phospholipids of the cells contained phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol, phosphatidylinositol mannosides and one unknown phospholipid. The G+C content of the genomic DNA was 72.01 mol%. 16S rRNA gene sequence of the isolate had 98·1% sequence similarity with *Streptomyces flavofuscus* NRRL B-8036 (=DSM 41426^T) and 97·5% similarity with *Streptomyces albiaxialis* DSM 41799^T, respectively, showing that the new isolate should be assigned to the genus *Streptomyces*. Meanwhile, DNA–DNA hybridizations against the above-mentioned two *Streptomyces* species showed 31·4% and 46·9% relatedness, respectively. Moreover, the three strains differed in some physiological and biochemical properties. Thus, on the basis of phenotypic and genotypic analyses, it is proposed that strain S187^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces xinghaiensis* sp. nov. is proposed with the type strain S187^T (=NRRL B24674^T= CCTCC AA 208049^T= KCTC 19546^T).

Marine actinobacteria have been screened and identified intensively in recent years (Maldonado *et al*., 2005; Ward & Bora, 2006). It was demonstrated that a diverse assemblage of actinobacteria are present in marine environment, including marine sediment (Jensen *et al*., 2005; Bredholdt *et al*., 2007), especially deep sea sediment (Pathom-Aree *et al*., 2006), as well as marine sponges (Montalvo *et al*., 2005; Zhang *et al*., 2006). Dalian is a costal city located in the southernmost of Liaodong Peninsula of China, and has a very long coast line of 1906 km. However, the possible abundance of marine actinobacterial resources remains largely unexplored, except some reports on the sponge-derived marine actinobacteria (Zhang *et al*., 2006). During our screening of marine actinobacteria from the sediment samples of Dalian sea area, strain S187 was recovered from a sediment sample collected from Xinghai Bay. In this study, we performed polyphasic taxonomy on this strain, and proposed that strain S187^T is a new species of the genus *Streptomyces*, for which the name *Streptomyces xinghaiensis* sp. nov. is proposed.

Strain S187^T was isolated from a marine sediment sample from Xinghai Bay, Dalian, China, after 2 weeks incubation at 28°C on Bennett's agar (Margalith & Beretta, 1960). Morphological, physiological and biochemical characterization of strain S187^T were carried out following the standard protocol of the International *Streptomyces* Project (Shirling and Gottlieb, 1966; 1968 a, b, 1969). Colour determination was performed by comparing with the colour chips from ISCC-NBS COLOR CHARTS standard sample No. 2106 (Kelly, 1964). Spores and mycelia formation of strain S187^T grown on ISP medium 2 at 28°C for 4 weeks were observed under light microscopy (Olympus microscope BH-2) and scanning electron microscopy (JSM-5600LV; JEOL). Phenotypic properties of strain S187^T and its closely

related strains were examined using standard procedures (Shirling and Gottlieb, 1966; Williams *et al*., 1983). The tolerance to NaCl was determined on ISP medium 2 after incubation at 28 °C for 3-4 weeks, whereas cell growth at the temperature ranging from 10 °C to 45 °C was examined for temperature tolerance. Antimicrobial activities of strain S187^T and its closely related strains were tested as follows: Culture broth of the strains after cultivation in TSB medium at 28 °C for 6 days was centrifuged at 4 °C, 12000 rpm for 10 min, and 200 µl of the supernatant was applied to the agar blocks on the assay plates spread with test strains. Test strains were purchased from China General Microbiological Culture Collection Centre (CGMCC). The test strains are as following: *Escherichia coli* (CGMCC 1.797), *Staphylococcus aurea* (CGMCC 1.89), *Bacillus subtilis* (CGMCC 1.1849); *Pseudomonas aeruginosa* (CGMCC 1.2031), *Candida albicans* (CGMCC 2.538) and A*spergillus niger* (CGMCC 3.2915).

Amino acids and peptides in cell-wall hydrolysates were analysed by the methods described by Hasegawa *et al*. (1983). Whole cell sugars were analyzed by the methods of Lechevalier and Lechevalier (1980). Menaquinones and phospholipids were extracted from freeze dried cells and analyzed according to Collins (1985) and Lechevalier *et al*. (1977). Fatty acid composition was determined according to the procedure described elsewhere (Sasser, 1990). Genomic DNA was extracted as described by Lee *et al*. (2003) and 16S rRNA gene was amplified according to Zhang *et al* (2006). The DNA G+C content of the strain S187^T was determined by using the HPLC method (Mesbah *et al*., 1989). Closely related reference strains were chosen from BLAST (Altschul *et al*., 1997) search results. Phylogenetic analysis was performed using the software package MEGA 2.1 version (Kumar *et al*., 2001) after multiple

alignment by CLUSTAL X (Thompson *et al*., 1997). A phylogenetic tree was reconstructed using neighbor-joining method (Saitou and Nei, 1987). The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

Morphological observation of a 28-day culture of strain S187^T grown on yeast extract/malt extract agar (ISP medium 2) revealed that strain S187^T had the typical characteristics of the genus *Streptomyces*. Aerial mycelium and substrate mycelium were well-developed without fragmentation. Long spore chains appeared as straight or flexuous, spores were smooth and non motile (Supplementary data Fig. S1). Strain S187^T grew well between pH 6.0-9.0, with an optimum pH of 7.0-8.0. The range of temperature is 10-45 °C, with the optimum growth temperature at 28°C. Strain S187^T grew in presences of 0-9% NaCl (w/v). Mycelia of strain S187^T were well developed on some media tested, including yeast extract/malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3), glycerol-asparagine agar (ISP medium 5), nutrient agar, and inorganic salt-starch agar (ISP medium 4), but the growth was poor on Czapek's agar and potato dextrose agar (Table 1). No diffusible pigments were observed on all test media.

The cell wall of strain S187^T contained LL-diaminopimelic acid and glycine, which indicates that this strain has a cell wall chemotype I (Lechevalier & Lechevalier, 1970). Whole-cell hydrolysates mainly contain ribose, glucose, galactose and small amount of mannose. The phospholipids of strain S187^T were revealed to be phosphatidylethanolamine,

phosphatidylinositol, phosphatidylglycerol, phosphatidylinositol mannosides and one unknown phospholipid. The predominant menaquinones were MK-9 (H_6)-40.8%, MK-9 (H_8)-38.2% and MK-9 (H_2)-8.8%. The major fatty acids found were iso-$C_{16:0}$ (29.2%), anteiso-$C_{15:0}$ (14.0%), antiso-$C_{17:0}$ (11.6 %), iso-$C_{14:0}$ (8.8%), iso-$C_{15:0}$ (5.5%) and iso-H $C_{16:1}$ (4.6%), anteiso-C $C_{17:1}$ (4.0%), $C_{16:0}$ (4.0%) and iso-$C_{17:0}$ (3.5%). All the morphological characteristics and chemotaxonomic data showed that strain S187^T should be assigned to the genus *Streptomyces*.

The almost-complete 16S rRNA gene sequence (1490 nt) of strain S187^T was aligned manually with corresponding 16S rRNA sequences of representative *Streptomyces* type strains retrieved from the GenBank/EMBL/DDBJ databases using BLAST search at http://blast.ncbi.nlm.nih.gov/Blast.cgi (Altschul *et al*., 1997). Phylogenetic analyses based on 16S rRNA gene sequence showed that the novel isolate fell into one distinct subclade with type strains of *Streptomyces flavofuscus* NRRL B-8036^T (=DSM 41426^T) and *Streptomyces albiaxialis* DSM 41799^T with which it shares the 16S rRNA gene sequence similarity of 98.1% and 97.5%, respectively. Another close strain is *Streptomyces ferralitis* SFOp68^T, the 16S rRNA gene sequence of which showed 95.6% similarity (1425 out of 1490 nucleotides) with that of S187^T. The phylogenetic trees based on the 16S rRNA gene sequences of strain S187^T and most closely related type strains of the genus *Streptomyces* are shown by neighbour-joining method (Fig. 1) and maximum parsimony method (Supplementary figure Fig. S2), respectively. DNA-DNA relatedness tests were performed between strain S187^T and its closest match, *S. flavofuscus* DSM 41426^T (=NRRL B-8036^T) and *S. albiaxialis* DSM 41799^T using the optical renaturation method (De Ley *et al*., 1970; Huss *et al*., 1983; Jahnke

1992). The determined DNA-DNA relatedness values between S187^T and strains *S. flavofuscus* NRRL B-8036 (DSM 41426^T) and *S. albiaxialis* DSM 41799^T were 31.4% and 46.9% respectively, which was significantly lower than 70%, the threshold value for the delineation of genomic species (Wayne *et al*., 1987). Antimicrobial activities of the three strains were investigated, and it was showed that strain S187^T displayed excellent activities against the four bacterial strains tested, while *S. flavofuscus* DSM 41426^T showed no activity against *P. aeruginosa* (Supplementary data Table S1). The phenotypic comparison between strain S187^T and its closely related species of the genus *Streptomyces* were performed to differentiate them (Table 2). On the basis of morphological, chemotaxonomic and phylogenetic evidence and its physiological and its biochemical distinctiveness, it is confirmed that strain S187^T should be a new member of the genus *Streptomyces*, for which the name *Streptomyces xinghaiensis* sp.nov. is proposed.

Description of *Streptomyces xinghaiensis* sp. nov.

Streptomyces xinghaiensis (xing.hai.en'sis. N.L. masc. adj. xinghaiensis pertaining to Xinghai Bay of Dalian, China, the site from which the type strain was isolated).

Gram-positive, aerobic and non-motile. Grows well on glycerol aspaginate agar, Oatmeal agar, yeast extract/malt extract agar and inorganic salt-starch agar media, but poor on nutrient agar, potato dextrose agar and Czapek's agar media (Table 1). Colonies are whitish to yellow, ruffle. Aerial mycelium and substrate mycelium are well-developed without fragmentation. Long spore chains appear as straight or flexuous, and spores are smooth with non-motile. Casein, pectin and cellulose are degraded, but starch is not. Positive for catalase production and gelatin liquefaction. No soluble pigments are produced on all test media. Temperature range

for growth is 10 to 45°C with an optimum at 28°C.Growth occurs at pH 6.0-9.0 and in the presence of 0-9% NaCl (w/v). Good growth is found on almost all nitrogen sources tested, and can use D-glucose, sucrose, mannitol, fructose, mannose, galactose, ribose and rhamnose as carbon source, while cannot use trehalose, L-arabinose, inositol, raffinose, callobiose and sorbitol. More detailed phenotypic properties are mentioned in Table 2. Cell wall contains LL-diaminopimelic acid. Whole-cell hydrolysates mainly contain ribose, glucose, galactose and small amount of mannose. The chemotaxonomic properties are typical of the genus *Streptomyces*. The DNA G+C content of the type strain is 72.01 mol %.

The type strain, S187^{T}(=NRRL B24674^{T}= CCTCC AA 208049^{T}= KCTC 19546^{T}), was isolated from the marine sediment sample collected from Xinghai Bay, Dalian, Liaoning Province, China.

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Table 1. Culture characteristics of strain S187^T and its most closely related neighbours

Culture medium	*S. xinghaiensis* S187^T			*S. flavofuscus* DSM 41426^T			*S. albiaxialis* DSM 41799^T		
	Growth	Arial mycelium	Substrate mycelium	Growth	Arial mycelium	Substrate mycelium	Growth	Arial mycelium	Substrate mycelium
Yeast extract/malt extract	Good	White	Dark orange yellow	Good	White	Dark orange yellow	Good	Strong olive green	Moderate reddish brown
Oatmeal agar	Good	White	Yellow white	Good	White	Strong olive green	Good	White	Deep orange yellow
Potato Dextrose agar	Poor	-	Pale orange yellow	Good	White	Yellow white	Good	Vivid bluish green	Moderate reddish brown
Nutrient agar	Moderate	White	Light orange yellow	Good	White	Moderate orange yellow	Poor	-	Light orange yellow
Czapek's agar	Poor	-	Light yellow brown	moderate	White	Strong olive green	Good	Strong olive green	Light yellow brown
Glucose/Asparagine agar	Good	Vivid bluish green	Yellow white	Good	White	Pale orange yellow	Good	Vivid yellowish pink	Moderate reddish brown
Inorganic salts starch agar	Good	White	Pale orange yellow	Good	White	Yellow white	Good	Deep olive green	Pale orange yellow

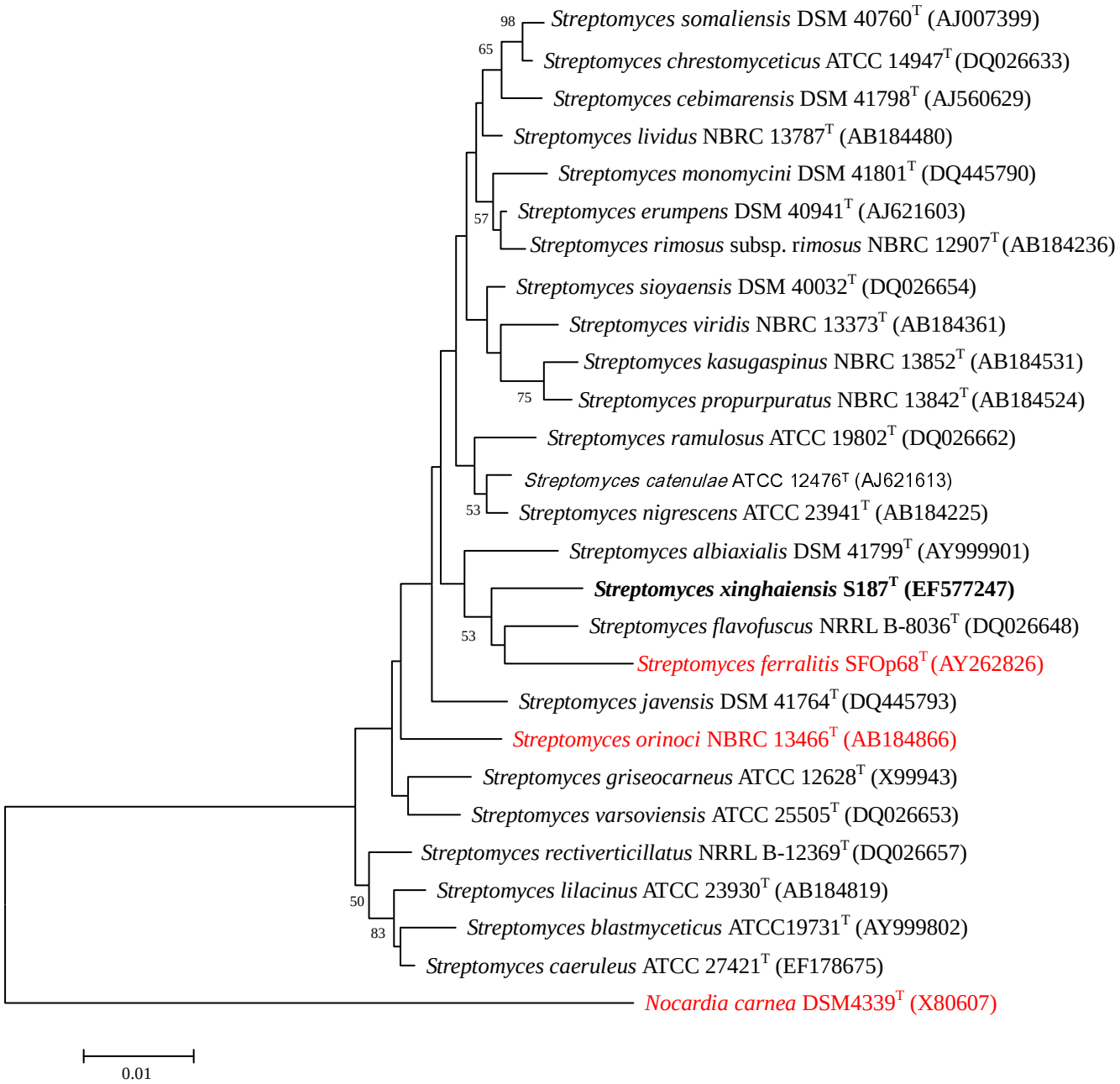
Note: '-', no growth. Diffusible pigment was not observed on any of the media listed. Colors were taken from ISCC-NBS COLOR CHARTS standard sample No. 2106 (Kelly, 1964). Plates were incubated for 4 weeks at 28°C.

Table 2. Physiological and biochemical features that differentiate strain S187^T from closely related *Streptomyces* species

1, Strain S187^T; 2, *S. flavofuscus* DSM 41426^T; 3, *S. Albiaxialis* DSM 41799^T.
+; Positive, –; Negative. Only the items that showed differences in the strains are presented.

Characteristics	1	2	3
Degradation			
Casein	+	+	–
Cellulose	+	–	+
Tolerance to NaCl (%, w/v)	0-9	0-10	0-6
Optimum NaCl (%, v/v)	0-5	0-5	0-3
Growth at 5°C	–	–	+
Growth at pH	7-9	6-9	7-9
Carbon utilization			
L-arabinose	–	–	+
ribose	+	+	–
i-inositol	–	–	+
L-rhamnose	+	+	–
callobiose	–	+	+
sorbitol	–	–	+
Nitrogen utilization			
lysine	–	+	–
phenylalanine	+	–	+
serine	+	+	–
xanthine	+	+	–

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence analysis and constructed using the neighbor-joining method showing the position of strain S187^T among its phylogenetic neighbors. Numbers at the branch nodes are bootstrap values, expressed as a percentage of 1000 replicates (only values above 50% are shown). Bar 0.01 substitutions per nucleotide position.



Supplementary data

Table S1. Antimicrobial activities of S187^T and closely related strains *S. flavofuscus* DSM 41426^T and *S. albiaxialis* DSM 41799^T. The value indicated the inhibition zone diameter (mm) against the test strain.

Test strains	S187^T	*S. flavofuscus* DSM 41426^T	*S. albiaxialis* DSM 41799^T
E. coli	31	19	-
S. aureus	30	19	-
B. subtilis	38	21	-
P. aeruginosa	22	-	-
C. albicans	-	-	-
A. niger	-	-	-

Fig. S1. Scanning electron micrograph indicates the spore chain morphology of strain S 187^{T} grown on ISP medium 2 for 4 weeks at 28 °C. Bar 5 µm.

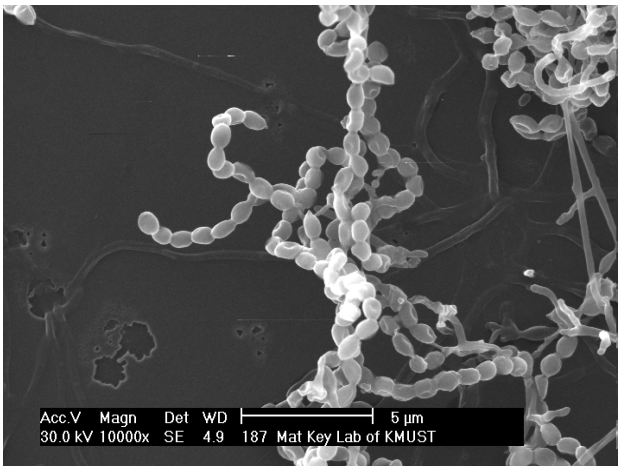


Fig. S2. Phylogenetic tree based on 16S rRNA gene sequence analysis and constructed using the maximum parsimony method showing the position of strain S187^T among its phylogenetic neighbors. Numbers at the branch nodes are bootstrap values, expressed as a percentage of 1000 replicates (only values above 50% are shown). Bar 0.02 substitutions per nucleotide position.