Streptomyces fenghuangensis sp. nov., isolated from seawater

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An actinomycete, designated strain GIMN4.003^T, was isolated from seawater collected in Sanya, China. It produced white aerial mycelium and yellow substrate mycelium on Gause's synthetic agar medium no. 1. The substrate mycelium colour was not sensitive to pH. Scanning electron microscopy observations revealed that GIMN4.003^T produced straight to flexuous spore chains of rough to warty spores. LL-Diaminopimelic acid was present in the cell-wall hydrolysate. Based on chemotaxonomy and morphological features, strain GIMN4.003^T was identified as a member of the genus *Streptomyces*. Melanin was not produced. No antimicrobial activity was detected against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Penicillium citrinum* or *Candida albicans*. Analysis of the 16S rRNA gene sequence revealed that the highest sequence similarity was to *Streptomyces radiopugnans* R97^T (99.0%). However, DNA relatedness between GIMN4.003^T and *S. radiopugnans* DSM 41901^T was low (41.24 ± 1.47%). Furthermore, the morphological, physiological and biochemical characteristics of strain GIMN4.003^T were different from those of *S. radiopugnans* DSM 41901^T and the type strains of other closely related *Streptomyces* species. On the basis of its physiological and molecular properties, it is evident that strain GIMN4.003^T (=CCTCCM 208215^T =NRRL B-24801^T) represents the type strain of a novel species within the genus *Streptomyces*, for which the name *Streptomyces fenghuangensis* sp. nov. is proposed.

The marine biosphere is one of the richest habitats of micro-organisms. Marine microbes, particularly *Streptomyces* species, are considered important for their secondary metabolites and enzymes with novel properties (Sabu, 2003). Marine streptomycetes are widely recognized as a potential source of new drug candidates (Jensen *et al.*, 2005). Members of the genus *Streptomyces* are known to produce a wide variety of bioactive compounds, antibiotics and enzymes (Koshy *et al.*, 1997; Balagurunathan, 2004). The isolation and screening of novel streptomycetes as sources of novel natural products, bioactive compounds and therapeutic enzymes are therefore essential. Strain GIMN4.003^T was isolated from seawater collected in Sanya, China, in August 2007. Seawater samples were collected from five stations at Sanya and were stored in sterile 1 l

bottles. The samples were stored under environmental conditions and processed within 24 h in a laboratory; water samples (1 ml) were inoculated onto Gause's synthetic agar medium no. 1 (Atlas, 1993), prepared with artificial seawater, and incubated for 5–7 days at 28 °C.

International *Streptomyces* Project (ISP) media were prepared according to the methods of Shirling & Gottlieb (1966). Morphological and physiological characteristics were determined as recommended by Williams *et al.* (1989). Morphological observations of spores and mycelium were conducted by light microscopy (Leica DM RAR) and scanning electron microscopy (Hitachi S-3000N). Physiological tests were carried out at 28 °C (unless otherwise indicated). D-Glucose, sucrose, D-fructose, D-xylose, L-arabinose, inositol, mannitol, L-rhamnose and raffinose were tested as sole carbon sources at 0.1% (w/v); all carbon sources for carbon-utilization tests were filter-sterilized. Colour determination was referenced against the *Methuen Handbook of Colour* (Kornerup & Wanscher, 1978).

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Abbreviations: DAP, diaminopimelic acid; ISP, International *Streptomyces* Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GIMN4.003^T is GU356598.

Analysis of the diaminopimelic acid (DAP) isomer and the whole-cell sugar composition followed the procedure described by Hasegawa *et al.* (1983) with the exception that dried cells were used instead of colonies from agar plates. Fully hydrolysed cell-wall material was prepared from approximately 10 mg (dry weight) cells by the hot trichloroacetic acid trypsin rapid-screening method of Schleifer & Kandler (1972). Fatty acid methyl esters were prepared by the trimethyl sulphonium hydroxide method (Butte, 1983) and analysed by GC (6890; Hewlett Packard) using the Microbial Identification software package (Sasser, 1990). Menaquinones were extracted according to the method of Collins *et al.* (1977) and analysed by HPLC (Kroppenstedt, 1985). The genomic DNA base composition of strain GIMN4.003^T was determined in 0.1 × SSC by the method of Mandel & Marmur (1968). Genomic DNA was extracted (Cui *et al.*, 2001) and the 16S rRNA gene was amplified by PCR using universal bacterial 16S rRNA gene primers. The forward primer F27 (5'-AGAGTTTGAT-CCTGGCTCAG-3') and reverse primer 1522R (5'-AA-GGAGGTGATCCAGCCGCA-3') were adapted from primers pA and pH of Edwards *et al.* (1989). The 16S rRNA gene was sequenced with an automated capillary DNA sequencing system (ABI 3730) and ABI Big Dye Terminator cycle sequencing kit. DNA relatedness studies were done by the method described by De Ley *et al.* (1970).

Good growth was observed on yeast extract/malt extract agar (ISP 2), Gause's synthetic agar medium no. 1, glucose-asparagine agar (Waksman, 1961) and Czapek's solution agar (Nakase, 1992). Moderate growth was observed on oatmeal agar (ISP 3), glycerol-asparagine agar (ISP 5) and starch agar (Waksman, 1961). Poor growth was observed on inorganic salts/starch agar (ISP 4). Diffusible pigments were produced only on ISP 5 medium and Gause's synthetic agar medium no. 1 (Table 1).

Morphological features were observed on Gause's synthetic agar medium no. 1 (Xiao *et al.*, 2009) and ISP 2. Cultures were incubated for 2 weeks at 28 °C. Strain GIMN4.003^T

had characteristics typical of the genus *Streptomyces*. Microscope studies revealed a straight to flexuous mycelium without verticils. The aerial mycelium produced straight to flexuous spore chains. Spores were rough to warty (Fig. 1). The colour of the spore mass was grey, and the strain produced white aerial mycelium and yellow to yellowish-white vegetative mycelium. Melanin was not produced on tyrosine agar (ISP 7).

Chemotaxonomic tests showed that the cell wall contained LL-DAP, indicating that it was of cell-wall type I (Lechevalier & Lechevalier, 1970). Whole-cell hydrolysates contained glucose. The predominant menaquinones were MK-10(H₂) (63.22 %), MK-9(H₈) (33.23 %), MK-10(H₄) (2.16 %) and MK-9(H₆) (1.01 %). Fatty acid analysis showed that strain GIMN4.003^T contained straight-chain and iso- and anteiso-branched fatty acids; the major cellular fatty acids (>4 %) were iso-C_{16:0} (39.30 %), anteiso-C_{15:0} (24.20 %), iso-C_{14:0} (12.10 %), iso-C_{16:1} H (6.21 %), anteiso-C_{17:0} (5.43 %) and iso-C_{15:0} (4.63 %). The G + C content of the genomic DNA was 72.94 mol%.

The morphological and physiological characteristics of strain GIMN4.003^T, for example its cell-wall type, whole-cell sugar pattern, fatty acid profile and menaquinones, were in line with its assignment to the genus *Streptomyces* (Williams *et al.*, 1989; Manfio *et al.*, 1995).

A partial 16S rRNA gene sequence (1427 nt) was determined for strain GIMN4.003^T. Sequence comparison with representatives of the family *Streptomycetaceae* confirmed that strain GIMN4.003^T is closely related to members of the genus *Streptomyces* (BLAST analysis; Altschul *et al.*, 1997). It was 97.0–99.67 % similar to the 16S rRNA gene sequences of *Streptomyces* species. High 16S rRNA gene sequence similarity was found with *Streptomyces radiopugnans* R97^T (GenBank accession no. DQ912930; 99.0 %). *Streptomyces macrosporus* DSM 41449^T (AB184616; 98.1 % similarity over 1399 bases) and *Streptomyces megasporus* NBRC 14749^T (AB184617; 97.0 % similarity over 1394 bases) showed lower

Table 1. Cultural characteristics of strain GIMN4.003^T on various media

ISP media (Shirling & Gottlieb, 1966) are described in the text. The colour of mycelium was compared according to Kornerup & Wanscher (1978). The strain gave the same result for growth and for sporulation on all tested media.

Medium	Growth and sporulation	Colour of:		Production of diffusible pigments
		Aerial mycelium	Substrate mycelium	
ISP 2	Good	White	Yellowish white	—
ISP 3	Moderate	White	Grey	—
ISP 4	Poor	None	Greyish white	—
ISP 5	Moderate	Greyish white	Yellowish white	Blue
Glucose-asparagine agar	Good	White	Yellowish white	—
Gause's synthetic agar medium no. 1	Good	White	Yellow	Pale yellow
Czapek's agar	Good	Yellowish white	White	—
Starch agar	Moderate	Orange–yellow	Yellow	—

Fig. 1. Scanning electron micrographs of strain GIMN4.003^T showing straight to flexuous (*rectiflexibiles*) spore chains of rough to warty spores after growth on yeast extract/malt extract agar (ISP 2) for 14 days at 28 °C. Bars, 5 µm (a) and 2 µm (b).

similarity; similarity to the 16S rRNA gene sequences of other *Streptomyces* species was less than 97 %.

A phylogenetic tree of 16S rRNA gene sequences of members of the genus *Streptomyces* was constructed using the neighbour-joining method (Saitou & Nei, 1987) with CLUSTAL W version 1.81 (Thompson *et al.*, 1994) and MEGA version 3.1 (Kumar *et al.*, 2008) (Fig. 2). For the neighbour-joining analysis, a distance matrix was calculated according to Kimura's two-parameter correction model. The minimum-evolution and maximum-parsimony methods were also used for tree reconstruction. Fig. 2 shows the close phylogenetic relationship of strain GIMN4.003^T with members of the genus *Streptomyces*.

Phenotypic properties of strain GIMN4.003^T were compared with those of its closest phylogenetic neighbours (Table 2) by using procedures described above. The results showed that GIMN4.003^T has some characteristics that differed from those of its closest relatives; *S. radiopugnans* DSM 41901^T, *S. macrosporus* NBRC 14748^T and *S. megasporus* NBRC 14749^T differed from strain GIMN4.003^T in that they produced spiral spore chains and that diffusible pigments were not formed on any of the tested media (Table 2).

It is evident from the phylogenetic analysis (Fig. 2) that strain GIMN4.003^T forms a distinct phyletic line together with *S. radiopugnans* R97^T, a relationship that is supported by both of the other tree-making algorithms employed and by a 91 % bootstrap value in the neighbour-joining analysis. Despite the higher 16S rRNA gene sequence similarity between GIMN4.003^T and *S. radiopugnans* R97^T, DNA relatedness between strain GIMN4.003^T and *S. radiopugnans* DSM 41901^T was found to be 41.24 ± 1.47 %, a value far below the 70 % cut-off point recommended for the delineation of genomic species (Wayne *et al.*, 1987). DNA–DNA hybridization studies therefore confirmed that strain GIMN4.003^T represents a novel species within the genus *Streptomyces*. The result is in agreement with the results of the 16S rRNA gene sequence analyses.

Furthermore, *S. radiopugnans* DSM 41901^T also differs from the new isolate in that it lacked characteristic sugars, and formed white to pale-grey aerial spore mass on Gause's synthetic agar medium no. 1 and yeast extract-malt extract (ISP 2) and pale-yellow aerial spore mass on glycerol-asparagine agar (ISP 5). Substrate mycelium was grey to greyish-white on Gause's synthetic agar medium no. 1 and ISP 2, ISP 3, ISP 4, ISP 5 (Table 2).

The genotypic and phenotypic data presented clearly demonstrate that strain GIMN4.003^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces fenghuangensis* sp. nov. is proposed.

Description of *Streptomyces fenghuangensis* sp. nov.

Streptomyces fenghuangensis (feng.hu.ang.en'sis. N.L. masc. adj. *fenghuangensis* pertaining to Fenghuang, where the seawater was collected from which the type strain was isolated).

Aerobic, Gram-stain-positive, catalase-positive actinomycete that forms an extensively branched substrate mycelium which carries aerial hyphae that differentiate into straight to flexuous chains of spores with rough to warty surfaces. Milk coagulation, milk peptonization and starch hydrolysis are negative. Good growth on yeast extract/malt extract agar (ISP 2), Gause's synthetic agar medium no. 1, glucose-asparagine agar and Czapek's solution agar and moderate growth on oatmeal agar (ISP 3), glycerol-asparagine agar (ISP 5) and starch agar. Poor growth on inorganic salts/starch agar (ISP 4). White aerial mycelium is formed on ISP 2, ISP 3, glucose-asparagine agar and Gause's synthetic agar medium no. 1. Greyish-white, yellowish-white and orange-white aerial mycelium is formed on ISP 5, Czapek's agar and starch agar, respectively. Substrate mycelium is white on Czapek's agar, yellow on Gause's synthetic agar medium no. 1 and starch agar, yellowish white on ISP 2, ISP 5 and glucose-asparagine agar, grey on ISP 3 and greyish white on ISP 4. Blue diffusible pigment is formed on ISP 5. Melanin pigment is not produced on ISP 7. D-Glucose, D-fructose, inositol, mannitol and L-arabinose are utilized as sole carbon sources. H₂S is not produced. The cell wall contains LL-DAP (cell-wall type I) and glucose is detected as a diagnostic sugar in the whole-cell sugar

Fig. 2. Unrooted neighbour-joining tree constructed from 16S rRNA gene sequences, showing phylogenetic relationships between strain GIMN4.003^T and related *Streptomyces* species belonging to the major, minor and single-member clusters defined by Williams *et al.* (1983). *Actinomadura glomerata* IMSNU 22179^T was used as the outgroup. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are given at nodes. The minimum evolution and maximum-parsimony methods were also used for tree reconstruction. Branches marked with an asterisk were conserved in all methods used. Bar, 0.01% sequence divergence.

Table 2. Phenotypic properties of strain GIMN4.003^T and its phylogenetic neighbours

Data were obtained in this study under the same conditions. +, Positive; w, weakly positive; –, negative; ai, anteiso-branched; i, iso-branched.

Characteristic	Strain GIMN4.003 ^T	<i>S. radiopugnans</i> DSM 41901 ^T	<i>S. macrosporus</i> NBRC 14748 ^T	<i>S. megasporus</i> NBRC 14749 ^T
Colour of spore mass/ substrate mycelium on:				
ISP 2	Grey/yellowish white	White/greyish white	Light grey/greyish grey	None/yellowish grey
ISP 3	Pinkish white/grey	None/greyish white	Olive/grey	None/greyish white
ISP 4	Pale yellow/greyish white	None/grey	None/greyish white	None/white
ISP 5	Greyish white/yellowish white	Pale yellow/greyish white	White/white	Pale white/pale yellow
Spore-surface ornamentation	Rough to warty	Rough to warty	Warty	Warty
Spore-chain morphology	Straight to flexuous	Spiral	Spiral	Spiral
Colour of diffusible pigment on ISP 5	Blue	None	None	None
Growth on sole carbon sources				
L-Rhamnose	–	+	+	w
D-Glucose	+	–	+	+
Sucrose	–	–	+	–
D-Fructose	+	–	+	–
Raffinose	–	–	–	+
Mannitol	+	+	–	–
D-Xylose	–	+	+	–
L-Arabinose	+	+	+	w
Inositol	+	–	+	+
Major cellular fatty acids (>4%)	i-C _{16:0} (39.3%), ai-C _{15:0} (24.2%), i-C _{14:0} (12.1%), i-C _{16:1} H (6.2%), ai-C _{17:0} (5.4%), i-C _{15:0} (4.6%)	i-C _{16:0} (35.0%), ai-C _{15:0} (15.6%), ai-C _{17:0} (13.5%), i-C _{15:0} (9.9%), i-C _{16:1} H (8.3%), ai-C _{17:1} ω9c (4.5%)	i-C _{16:0} (38.8%), ai-C _{15:0} (16.0%), i-C _{16:1} H (11.6%), i-C _{14:0} (8.9%), ai-C _{17:0} (6.6%), i-C _{15:0} (6.1%)	i-C _{16:0} (42.5%), ai-C _{15:0} (12.5%), i-C _{16:1} H (10.0%), i-C _{15:0} (10.0%), ai-C _{17:0} (8.0%), i-C _{14:0} (5.4%)

hydrolysate. The predominant menaquinones are MK-10(H₂), MK-9(H₈), MK-10(H₄) and MK-9(H₆). The type strain contains straight-chain and iso- and anteiso-branched fatty acids and a proportion of unsaturated fatty acids. The major cellular fatty acids (>4%) are iso-C_{16:0}, anteiso-C_{15:0}, iso-C_{14:0}, iso-C_{16:1} H, anteiso-C_{17:0} and iso-C_{15:0}. No antimicrobial activity is detected against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231 or *Penicillium citrinum* AS 3.2788. The DNA G + C content of the type strain is 72.94 mol%.

The type strain, GIMN4.003^T (=CCTCCM 208215^T =NRRL B-24801^T), was isolated from seawater.

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References

- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Atlas, R. M. (1993). *Handbook of Microbiological Media*. Edited by L. C. Parks. Boca Raton, FL: CRC Press.
- Balagurunathan, R. (2004). Marine actinomycetes: a promising future of pharmaceutical industry. *Adv Biotechnol* **20**, 16–20.
- Butte, W. (1983). Rapid method for the determination of fatty acid profiles from fats and oils using trimethylsulphonium hydroxide for transesterification. *J Chromatogr A* **261**, 142–145.
- Collins, M. D., Pirouz, T., Goodfellow, M. & Minnikin, D. E. (1977). Distribution of menaquinones in actinomycetes and corynebacteria. *J Gen Microbiol* **100**, 221–230.
- Cui, X. L., Mao, P. H., Zeng, M., Li, W. J., Zhang, L. P., Xu, L. H. & Jiang, C. L. (2001). *Streptimonospora salina* gen. nov., sp. nov., a new member of the family Nocardiopsaceae. *Int J Syst Evol Microbiol* **51**, 357–363.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Edwards, U., Rogall, T., Blöcker, H., Emde, M. & Böttger, E. C. (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* **17**, 7843–7853.
- Hasegawa, T., Takizawa, M. & Tanida, S. (1983). A rapid analysis for chemical grouping of aerobic actinomycetes. *J Gen Appl Microbiol* **29**, 319–322.
- Jensen, P. R., Mincer, T. J., Williams, P. G. & Fenical, W. (2005). Marine actinomycete diversity and natural product discovery. *Antonie van Leeuwenhoek* **87**, 43–48.
- Kornerup, A. & Wanscher, J. H. (1978). *Methuen Handbook of Colour*, 3rd edn. London: Eyre Methuen.
- Koshy, A., Dhevendaran, K., Georgekutty, M. I. & Matarajan, P. (1997). L-Asparaginase activity in *Streptomyces plicatus* isolated from the alimentary canal of the fish, *Gerres filamentosus* (Cuvier). *J Mar Biotechnol* **5**, 181–185.
- Kroppenstedt, R. M. (1985). Fatty acid and menaquinone analysis of actinomycetes and related organisms. In *Chemical Methods in Bacterial Systematics* (Society for Applied Bacteriology Technical Series vol. 20), pp. 173–199. Edited by M. Goodfellow & D. E. Minnikin. New York: Academic Press.
- Kumar, S., Nei, M., Dudley, J. & Tamura, K. (2008). MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* **9**, 299–306.
- Lechevalier, H. A. & Lechevalier, M. P. (1970). A critical evaluation of the genera of aerobic actinomycetes. In *The Actinomycetales*, pp. 393–405. Edited by H. Prauser. Jena: VEB Gustav Fischer.
- Mandel, M. & Marmur, J. (1968). Use of ultraviolet absorbance temperature profile for determining the guanine plus cytosine content of DNA. *Methods Enzymol* **12**, 195–206.
- Manfio, G. P., Zakrzewska-Czerwinska, J., Atalan, E. & Goodfellow, M. (1995). Towards minimal standards for the description of *Streptomyces* species. *Biotechnologia* **7–8**, 242–263.
- Nakase, T. (editor) (1992). *JCM Catalogue of Strains*, 5th edn. Wako: Japan Collection of Microorganisms and Life Science Research Information Section, Institute of Physical and Chemical Research.
- Sabu, A. (2003). Sources, properties and applications of microbial therapeutic enzymes. *Indian J Biotechnol* **2**, 334–341.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sasser, M. (1990). *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc.
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Waksman, S. A. (1961). *Classification, Identification and Descriptions of Genera and Species* (The Actinomycetes, vol. 2). Baltimore: Williams & Wilkins.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Williams, S. T., Goodfellow, M., Alderson, G., Wellington, E. M. H., Sneath, P. H. A. & Sackin, M. J. (1983). Numerical classification of *Streptomyces* and related genera. *J Gen Microbiol* **129**, 1743–1813.
- Williams, S. T., Goodfellow, M. & Alderson, G. (1989). Genus *Streptomyces* Waksman and Henrici 1943, 339^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 4, pp. 2452–2492. Edited by S. T. Williams, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Xiao, J., Wang, Y., Luo, Y. X., Xie, S. J., Ruan, J. S. & Xu, J. (2009). *Streptomyces avicenniae* sp. nov., a novel actinomycete isolated from the rhizosphere of the mangrove plant *Avicennia mariana*. *Int J Syst Evol Microbiol* **59**, 2624–2628.