Streptomyces vietnamensis sp. nov., a streptomycete with violet–blue diffusible pigment isolated from soil in Vietnam

Hong-hui Zhu,[1] Jun Guo,[1] Qing Yao,[2] Song-zhen Yang,[1] Ming-rong Deng,[1] Le Thi Bich Phuong,[3] Vo Thi Hanh[3] and Matthew J. Ryan[4]

Correspondence
Hong-hui Zhu
zhuhonghui66@yahoo.com.cn

[1]Guangdong Provincial Microbial Culture Collection and Application Key Laboratory, Guangdong Institute of Microbiology, Guangzhou, Guangdong 510070, China

[2]South China Agricultural University, Guangzhou, Guangdong 510642, China

[3]Institute of Tropical Biology, Vietnamese Academy of Science and Technology, Ho Chi Minh City, Vietnam

[4]CABI Bioscience UK Centre, Egham, Surrey, UK

An actinomycete, designated strain GIMV4.0001^T, was isolated from a forest soil sample in Vietnam. It produced white aerial mycelium and violet–blue diffusible pigment on Gause's synthetic agar. The substrate mycelium colour was not sensitive to pH. Micoscopic observations revealed that strain GIMV4.0001^T produced long, straight chains of cylindrical spores, and chemotaxonomic data confirmed that it belongs to the genus *Streptomyces*. Melanin was produced, but no antibacterial activity was evident against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albicans* or *Penicillium citrinum*. Analysis of the 16S rRNA gene sequence of strain GIMV4.0001^T revealed that the highest similarity (99.4 %) was to *Streptomyces bikiniensis* ATCC 11062^T. However, the DNA–DNA relatedness between strain GIMV4.0001^T and *S. bikiniensis* ATCC 11062^T was found to be 50.3 %. Strain GIMV4.0001^T could also be differentiated from *S. bikiniensis* ATCC 11062^T and other *Streptomyces* species showing high 16S rRNA gene sequence similarity (98–99 %) based on morphological, physiological and biochemical characteristics. On the basis of its physiological and molecular properties, it is evident that strain GIMV4.0001^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces vietnamensis* sp. nov. is proposed. The type strain is GIMV4.0001^T (=CCTCC M 205143^T=IAM 15340^T).

Streptomyces species are abundant in terrestrial environments and are easily isolated on simple laboratory media. They have broad metabolic capabilities and can produce pigments and antibiotics; they have use in various applications, especially in the food and pharmaceutical industries. Strain GIMV4.0001^T was isolated from the forest at Do Xongpha, Vietnam, in September 2004. [The soil samples were inoculated into Gause's synthetic agar medium (Atlas, 1993) and incubated for 5–7 days at 28 °C]. The strain produced large quantities of violet–blue diffusible pigment on Gause's synthetic agar medium.

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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Streptomyces vietnamensis* GIMV4.0001^T is DQ311081.

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 mycelia were conducted via light microscopy (Leica DM RAR) and scanning electron microscopy (Phillip FEI-XL30). Physiological tests were carried out at 28 °C (unless otherwise indicated). All carbon sources for carbon-utilization tests were filter-sterilized. Melibiose, glucose, sorbinose, sucrose, D-fructose, xylose, D-galactose, rhamnose, arabinose and D-mannitol were tested as sole carbon source at concentrations of 0.1 % (w/v). Colour determination was referenced against Kornerup & Wanscher (1978).

Analysis of the isomer of diaminopimelic acid (DAP) and the whole-cell sugar composition followed the procedure

Table 1. Cultural characteristics of strain GIMV4.0001^T on various media

ISP media (Shirling & Gottlieb, 1966) are described in the text. Diffusible pigment was observed on most of the media listed. Colour comparisons were made against Kornerup & Wanscher (1978).

Characteristic	ISP2	ISP3	ISP4	ISP5	Czapek agar	Gause's synthetic agar
Growth	Good	Good	Moderate	Moderate	Poor	Good
Sporulation	Good	Good	Moderate	Moderate	Poor	Good
Colour of:						
Aerial mycelium	White	White	White	White	White	White
Substrate mycelium	Reddish brown	Reddish brown	Reddish brown	Reddish brown	Greyish orange	Reddish brown
Colour of diffusible pigment	Violet–blue	Violet–blue	Bluish violet	Bluish violet	–	Violet–blue

described by Hasegawa *et al.* (1983) with the exception that dried cells were used instead of colonies from agar plates. Fatty acid methyl esters were prepared by using the trimethyl sulfonium hydroxide method (Butte, 1983). The base composition of the genomic DNA of strain GIMV4.0001^T was determined in 0.1 × SSC following the method of Mandel & Marmur (1968). Genomic DNA was extracted (Cui *et al.*, 2001) and the 16S rRNA gene sequence was amplified by PCR by using universal bacterial 16S rRNA gene primers. The forward primer F27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1522R (5′-AAGGAGGTGATCCAGCCGCA-3′) were adapted from primers pA and pH of Edwards *et al.* (1989). The 16S rRNA gene was sequenced using an automated capillary DNA sequencing system (ABI 3730) and a Bigdye Terminator cycle sequencing kit. DNA relatedness studies were conducted by using the fluorometric microdilution plate method (Ezaki *et al.*, 1988; Sawabe *et al.*, 1998).

Strain GIMV4.0001^T grew well on yeast extract/malt extract agar (ISP2), oatmeal agar (ISP3) and Gause's synthetic agar. It exhibited moderate growth on inorganic salts/starch agar (ISP4) and glycerol–asparagine agar (ISP5), but poor growth on Czapek agar media (Atlas, 1993). Diffusible pigments of different colours were produced on the various test media (Table 1). The colour range was from bluish violet (18C8) to violet–blue (19C8).

Morphological features were observed on ISP2, ISP3, ISP4 and ISP5. Cultures were incubated for 2 weeks at 28 °C. Strain GIMV4.0001^T showed characteristics typical of the genus *Streptomyces*. Microscopical studies revealed a branched mycelium without verticils. The aerial mycelium produced flexuous (rectiflexibiles) spore chains. Spores were cylindrical and smooth (Fig. 1).

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 mainly mannose and small quantities of ribose and galactose. Fatty acid analysis showed that strain GIMV4.0001^T contained straight-chain, and iso- and anteiso-branched components and a high proportion of unsaturated components: iso-$C_{14:0}$ (6.995 %), anteiso-$C_{16:0}$ (20.1 %), $C_{18:0}$ (5.8 %), $C_{16:1}$ (17.8 %), $C_{18:1}$ (33.5 %) and $C_{18:2}$ (15.8 %). The G+C content of the genomic DNA was 73.9 mol%. Melanin was produced on tyrosine agar (ISP7).

A 1419-bp 16S rRNA gene sequence was determined for strain GIMV4.0001^T. A BLAST search (Altschul *et al.*, 1997) of the GenBank database using this sequence showed its similarity to that of many species of the genus *Streptomyces*. The 16S rRNA gene sequence of strain GIMV4.0001^T showed levels of similarity of 99.4 % (over 1410 bases) to that of *Streptomyces bikiniensis* ATCC 11062^T (GenBank accession no. X79851), 98.9 % (over 1404 bases) to that of *Streptomyces showdoensis* ATCC 15105^T (GenBank accession no. AY999741), 99.1 % (over 1406 bases) to that of *Streptomyces viridobrunneus* ATCC 43698^T (GenBank accession no. AJ781372) and <98 % to that of other *Streptomyces* species.

A phylogenetic tree based on 16S rRNA gene sequences of members of the genus *Streptomyces* was constructed

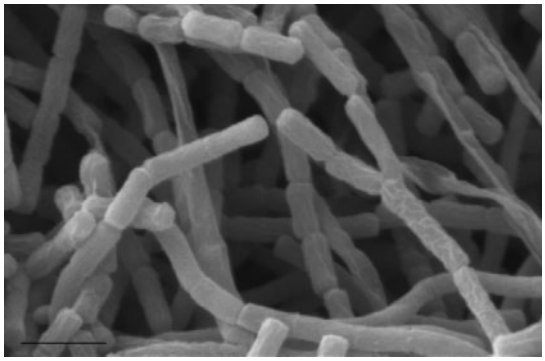

Fig. 1. Scanning electron micrograph of cells of strain GIMV4.0001^T grown on inorganic salts/starch agar (ISP4) at 28 °C for 14 days. Flexuous spore chains (rectiflexibiles) of cylindrical spores are evident. Bar, 2 μm.

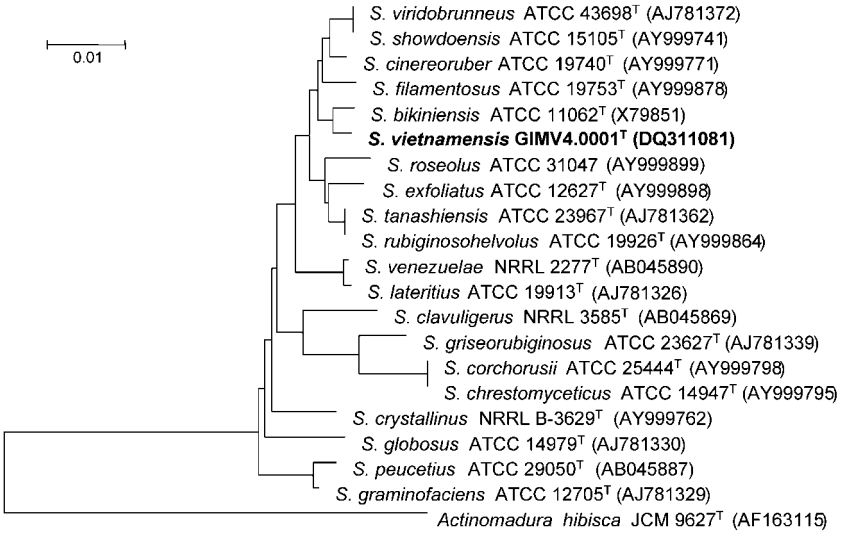

Fig. 2. Unrooted phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between strain GIMV4.0001^T and *Streptomyces* species belonging to the major, minor and single member clusters defined by Williams *et al.* (1983). The 16S rRNA gene sequence of *Actinomadura hibisca* JCM 9627^T was used as an outgroup. GenBank sequence accession numbers are given in parentheses. The tree was generated by using the neighbour-joining method. Bar, 0.01 substitutions per nucleotide position.

according to the neighbour-joining method of Saitou & Nei (1987) with CLUSTAL W (version 1.81) and MEGA (version 3.1; Kumar *et al.*, 2001) (Fig. 2). For the neighbour-joining analysis, a distance matrix was calculated according to Kimura's two-parameter correction model. This tree shows the close phylogenetic association of strain GIMV4.0001^T with certain other *Streptomyces* species.

The morphological and physiological characteristics of strain GIMV4.0001^T, for example its cell-wall type, whole-cell sugar pattern and fatty acid profile, were also consistent with those of members of the genus *Streptomyces*.

Strain GIMV4.0001^T produced reddish-brown substrate mycelium and grey spore mass, and developed cylindrical spores in flexuous chains. Melanin was produced. Bluish-violet or violet–blue diffusible pigments were produced. The pigment was pH-sensitive, and was stable at high temperature and under UV light. Comparison of the cultural characteristics of strain GIMV4.0001^T and its closest phylogenetic neighbours (Table 2), however, revealed significant differences from those *Streptomyces* species showing >99 % 16S rRNA gene sequence similarity. *S. bikiniensis* differed from strain GIMV4.0001^T in that it did not produce a diffusible pigment on ISP media; *S. showdoensis* did not produce diffusible pigment on ISP media and *S. viridobrunneus* produced a green pigment. Despite the high 16S rRNA gene sequence similarity between strain GIMV4.0001^T and *S. bikiniensis*, they could be differentiated based on morphological and cultural characteristics (Table 2), carbon-utilization patterns, fatty acid methyl esters and antibiotic resistance properties (Table 3), indicating that strain GIMV4.0001^T does not belong to *S. bikiniensis*. DNA–DNA hybridization studies confirmed that strain GIMV4.0001^T is unique. The level of DNA–DNA relatedness between strain GIMV4.0001^T and *S. bikiniensis* ATCC 11062^T was 50.3 %.

Other *Streptomyces* species that showed >98 % 16S rRNA gene sequence similarity to strain GIMV4.0001^T revealed significant differences when grown on ISP media. For example, *Streptomyces tanashiensis*, *Streptomyces cinereoruber*, *Streptomyces filamentosus* and *Streptomyces venezuelae* differed from strain GIMV4.0001^T in that they did not produce pigments (Shirling & Gottlieb, 1968a, b, 1969); *Streptomyces exfoliatus* differed in producing pink aerial mycelium but no substrate mycelial pigment (Shirling &

Table 2. Cultural characteristics of strain GIMV4.0001^T and its phylogenetic neighbours

Data for reference species were taken from Shirling & Gottlieb (1968b, 1972), Preobrazhenskaya *et al.* (1983) and Williams *et al.* (1983).

Characteristic	Strain GIMV4.0001^T	*S. bikiniensis*	*S. showdoensis*	*S. viridobrunneus*
Colony colour on ISP2	Grey–white	Grey	Grey–white	Grey
Spore shape	Cylindrical	Oval	Ellipsoid or column	Oval or long round
Spore chain morphology	Straight or flexuous	Straight to flexuous	Long straight or flexuous	Straight or flexuous
Spore surface	Smooth	Smooth	Smooth	Smooth
Production of diffusible pigment	Violet–blue	–	–	Green

Table 3. Phenotypic properties of strain GIMV4.0001^T and *S. bikiniensis* ATCC 11062^T

Characteristic	Strain GIMV4.0001^T	*S. bikiniensis* ATCC 11062^T
Milk coagulation	+	+
Milk peptonization	+	+
Starch hydrolysis	+	Weak
H_2S production	+	+
Melanin	+	+
Growth on sole carbon source:		
Glucose	+	+
D-Fructose	+	+
Sucrose	+	−
D-Mannitol	−	+
Rhamnose	−	Weak
Xylose	+	Weak
Raffinose	Weak	−
Arabinose	+	−
Inositol	−	−
Whole-cell sugar composition	Mannose, ribose, galactose	Glucose
Fatty acids	iso-$C_{14:0}$ (6.995 %), anteiso-$C_{16:0}$ (20.1 %), $C_{18:0}$ (5.8 %), $C_{16:1}$ (17.8 %), $C_{18:1}$ (33.5 %), $C_{18:2}$ (15.8 %)	$C_{12:0}$ (6.209 %), $C_{14:0}$ (5.432 %), $C_{15:0}$ (4.649 %), $C_{16:0}$ (59.832 %), $C_{18:0}$ (23.877 %)
Antibiotic resistance	No antibiosis exhibited against *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 6538, *B. subtilis* ATCC 6633, *C. albicans* ATCC 10231, *Penicillium citrinum* AS3.2788	Antibiosis is exhibited against *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 6538, *B. subtilis* ATCC 6633, *C. albicans* ATCC 10231, *Penicillium citrinum* AS3.2788

Gottlieb, 1968a); *Streptomyces rubiginosohelvolus* produced either a light-yellow diffusible pigment and light-yellow aerial mycelium or no pigment at all (Shirling & Gottlieb, 1968b); *Streptomyces crystallinus* produced a light-brown or dark-brown diffusible pigment (Williams *et al.*, 1989).

These results support the classification of strain GIMV4.0001^T as representing a novel species of the genus *Streptomyces*, for which we propose the name *Streptomyces vietnamensis* sp. nov. Additional data from the phenotypic characterization of strain GIMV4.0001^T are presented below.

Description of *Streptomyces vietnamensis* sp. nov.

Streptomyces vietnamensis (vi.et.nam.en′sis. N.L. masc. adj. *vietnamensis* pertaining to Vietnam, the geographical location from where the type strain was isolated).

Aerobic, Gram-positive, catalase-positive and forms a white aerial mycelium and a reddish-brown substrate mycelium. Verticils are not present. The mycelium does not fragment. Straight to flexuous chains of cylindrical spores are produced. Diffusible pigments are produced on ISP2, ISP3, ISP4 and ISP5 media and on Gause's synthetic agar, but not on Czapek solution agar. Melanin is produced on ISP7. Although growth on ISP4 is initially slow, very good growth with profuse sporulation is observed on this medium after 14 days. Very good growth occurs on ISP2, Gause's synthetic agar and ISP3. Moderate growth is observed on ISP5 but only poor growth on Czapek agar. The substrate mycelium is reddish brown on ISP2, ISP5, Gause's synthetic agar, ISP4 and ISP3, but greyish orange on Czapek medium. Cell wall contains LL-DAP (cell-wall type I). Whole-cell sugar pattern contains diagnostic sugars: mannose, small quantities of ribose and galactose. No antibiosis is exhibited against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231 or *Penicillium citrinum* AS3.2788. Utilizes melibiose, glucose, sorbinose, sucrose, D-fructose, xylose, D-galactose, rhamnose and arabinose. Positive for production of H_2S, but pectin is not hydrolysed. The DNA G+C content of the type strain is 73.9 mol%.

The type strain, GIMV4.0001^T (=CCTCC M 205143^T= IAM 15340^T), was isolated from a forest soil sample in Vietnam

Acknowledgements

This research was supported by the Guangdong Ministry of Science and Technology, PR China (project no. 2004B50201011). We thank Dr Tai-hui Li (Guangdong Institute of Microbiology) for collecting soil in Vietnam and Dr David P. Labeda (National Center for Agricultural Utilization Research) for his constructive suggestion and grammatical correction of the manuscript.

References

Altschul, S. F., Madden, T. L., Schaffer, 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.

Butte, W. (1983). Rapid method for the determination of fatty acid profiles from fats and oils using trimethylsulphonium hydroxide for transesterification. *J Chromatogr* **261**, 142–145.

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.

Edwards, U., Rogall, T., Blocker, H., Emde, M. & Bottger, E. C. (1989). Isolation and direct complete nucleotide determination of entire genes, characterization of gene coding for 16S ribosomal RNA. *Nucleic Acids Res* **17**, 7843–7853.

Ezaki, T., Hashimoto, Y., Takeuchi, N., Yamamoto, H., Liu, S.-L., Miura, H., Matsui, K. & Yabuuchi, E. (1988). Simple genetic method to identify viridans group streptococci by colorimetric dot hybridization and fluorometric hybridization in microdilution wells. *J Clin Microbiol* **26**, 1708–1713.

Hasegawa, T., Takizawa, M. & Tanida, S. (1983). A rapid analysis for chemical grouping of aerobic actinomycetes. *J Gen Appl Microbiol* **29**, 319–322.

Kornerup, A. & Wanscher, J. H. (1978). *Methuen Handbook of Colour*. London: Eyre Methuen.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: Molecular Evolutionary Genetics Analysis software. Tempe, AZ: Arizona State University. http://www.megasoftware.net

Lechevalier, M. P. & Lechevalier, H. A. (1970). A critical evaluation of the genera of aerobic actinomycetes. In *The Actinomycetales*, pp. 393–405. Edited by H. Prauser. Jena: Gustav Fischer.

Mandel, M. & Marmur, J. (1968). Use of ultraviolet absorbance-temperature profile for determining the guanine plus cytosine content of DNA. *Methods Enzymol* **12B**, 195–206.

Preobrazhenskaya, T. P., Sveshnikova, M. A., Terekhova, L. P. & Maximova, T. S. (1983). *A Guide for the Determination of Actinomycetes. Genera* Streptomyces, Streptoverticillium, *and* Chainia. Moscow: Nauka.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sawabe, T., Makino, H., Tatsumi, M., Nakano, K., Tajima, K., Iqbal, M. M., Yumoto, L., Ezura, Y. & Christen, R. (1998). *Pseudomoalteromonas bacteriolytica* sp. nov., a marine bacterium that is the causative agent of red spot disease of *Laminaria japonica*. *Int J Syst Bacteriol* **48**, 769–774.

Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.

Shirling, E. B. & Gottlieb, D. (1968a). Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. *Int J Syst Bacteriol* **18**, 69–189.

Shirling, E. B. & Gottlieb, D. (1968b). Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. *Int J Syst Bacteriol* **18**, 279–392.

Shirling, E. B. & Gottlieb, D. (1969). Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. *Int J Syst Bacteriol* **19**, 391–512.

Shirling, E. B. & Gottlieb, D. (1972). Cooperative description of type strains of *Streptomyces*. V. Additional descriptions. *Int J Syst Bacteriol* **22**, 265–394.

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.