Streptomyces aomiensis sp. nov., isolated from a soil sample using the membrane-filter method

Aya Nagai,¹ Shams Tabrez Khan,¹ Tomohiko Tamura,² Motoki Takagi¹ and Kazuo Shin-ya³

Correspondence

Kazuo Shin-ya
k-shinya@aist.go.jp
Shams Tabrez Khan
shamsalig75@gmail.com

¹Biomedical Information Research Center (BIRC), Japan Biological Informatics Consortium (JBIC), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan

²NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE), 2-5-8 Kazusa-kamatari, Kisarazu, Chiba 292-0818, Japan

³Biomedical Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), 2-4-7 Aomi, Koto-ku, Tokyo, 135-0064, Japan

A Gram-positive actinobacterium, designated M24DS4^T, was isolated from a soil sample collected from Aomi, Tokyo, Japan, using the membrane-filter method. Strain M24DS4^T exhibited low 16S rRNA gene sequence similarity (96.1 %) with *Streptomyces scabrisporus* NBRC 100760^T. Cell hydrolysates contained the LL-isomer of diaminopimelic acid and the predominant quinones were MK-9(H₈) and MK-9(H₆). The genomic DNA G + C content was 75 mol%. Comparison of the characteristics of strain M24DS4^T and related members of the genus *Streptomyces* with validly published names showed that the strain represents a novel species of the genus, for which the name *Streptomyces aomiensis* sp. nov. is proposed. The type strain is M24DS4^T (=NBRC 106164^T =KACC 14925^T).

Actinomycetes are known to produce pharmaceutically important compounds and have been extensively studied (Bull *et al.*, 2000; McVeigh *et al.*, 1994; Newman & Hill, 2006). Soil has served as the primary isolation source of these actinomycetes. For the isolation of novel strains from a soil sample, the membrane-filter method described by Hirsch & Christensen (1983) is used. This method employs a membrane filter (0.22–0.45 µm pore size) to selectively eliminate non-actinomycete bacteria on the basis of their inability to form mycelia. Briefly, the agar plate for isolation is overlaid with a cellulose ester membrane filter and the filter surface is inoculated with a soil suspension. During incubation, the branched mycelia of actinomycetes penetrate the filter through the pores and grow on the underlying medium; bacteria that cannot penetrate the filter fail to grow. The membrane is removed after a few days of incubation and the plate is reincubated to allow the development of actinomycete colonies.

During our studies on the isolation of actinobacteria using the membrane-filter method from a soil sample, strain M24DS4^T was isolated from a soil sample collected from Aomi, Tokyo, Japan. A soil sample (1 g) was suspended in

PBS (10 ml) and the solution was serially diluted in sterile water. Aliquots (100 µl) of appropriate dilutions were plated on the surface of 0.3 µm-pore cellulose ester membrane filters (Advantec) on humic acid-vitamin agar (Hayakawa & Nonomura, 1987) without antibiotics. The membrane filters were removed after 4 days of incubation at 28 °C and the plates were reincubated at 28 °C for 2–3 weeks. Actinomycete colonies appearing on the plates were transferred to International *Streptomyces* Project (ISP) 2 agar (Shirling & Gottlieb, 1966) for further purification. For long-term preservation, the isolate was stored at –80 °C in 15 % glycerol (v/v).

For analysis of the 16S rRNA gene sequence, genomic DNA was prepared using the Prepman Ultra system (Applied Biosystems). The 16S rRNA gene was amplified using a universal primer set (9f and 1492r; Brosius *et al.*, 1978) and sequenced directly using a BigDye Terminator v3.1 Cycle Sequencing kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). In BLAST searches (Altschul *et al.*, 1990) comparing the almost-complete 16S rRNA gene sequence (1486 bp) of strain M24DS4^T against sequences available in the EzTaxon server (Chun *et al.*, 2007), strain M24DS4^T showed highest 16S rRNA gene sequence similarity (96.13 %, 59 bp difference) with *Streptomyces scabrisporus* NBRC 100760^T. 16S rRNA gene sequences related to strain M24DS4^T were downloaded from DDBJ and a multiple alignment (1430 bp) was created using CLUSTAL X (Thompson *et al.*, 1997). A phylogenetic tree was

Abbreviation: ISP, International *Streptomyces* Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain M24DS4^T is AB522686.

A supplementary figure and a supplementary table are available with the online version of this paper.

Fig. 1. A neighbour-joining tree based on almost-complete 16S rRNA gene sequences (1430 bp), showing the phylogenetic position of strain M24DS4^T in the genus *Streptomyces*. Bootstrap values (>500) based on 1000 resamplings are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony method. *Paraoerskovia marina* CTT-37^T was used as an outgroup. Bar, 0.01 K_{nuc} .

reconstructed using the neighbour-joining algorithm (Saitou & Nei, 1987) and the robustness of the tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) using 1000 resamplings of the sequences. Strain M24DS4^T clustered with *S. scabrisporus* NBRC 100760^T (Fig. 1) as a monophyletic clade supported by a high bootstrap value of 98%. This cluster was also obtained with maximum-parsimony analysis (Swofford, 2000).

Cultural and physiological characteristics of strain M24DS4^T and *S. scabrisporus* NBRC 100760^T were determined as described by Shirling & Gottlieb (1966). Growth at 4, 10, 15, 20, 28, 35 and 40 °C was assessed on ISP 2 agar for 6 days. Growth at pH 5 and 6 (adjusted with HCl) and pH 8, 9 and 10 (adjusted with NaOH) was examined on ISP 2 agar at 28 °C for 9 days. The growth characteristics on ISP 1 and ISP 3–9 were also determined (Shirling & Gottlieb, 1966). The API ZYM and API Coryne systems (bioMérieux) were used, according to the manufacturer's instructions, for biochemical characterization. Strain M24DS4^T could be differentiated phenotypically from *S. scabrisporus* NBRC 100760^T by a number of characteristics (Table 1), namely spore morphology, aerial mycelium on ISP 2, colony base colour, temperature range for growth, utilization of carbon sources, activities of alkaline phosphatase, α -chymotrypsin and urease, and hydrolysis of gelatin.

For strain M24DS4^T, the hyphal arrangement, spore chain morphology and spore surface ornamentation were observed by light microscopy (CX41LF; Olympus) and scanning electron microscopy (JSM-6060; JEOL) after growth on ISP 2 agar at 28 °C for 15–20 days. A micrograph showing the spore chain morphology of strain M24DS4^T is given in Supplementary Fig. S1 (available in IJSEM Online). Genomic DNA was extracted from cells grown to late exponential growth phase using the protocol

of Minamisawa (1990) and the G+C content of the genomic DNA was determined using the method described by Mesbah *et al.* (1989). Menaquinones, cellular fatty acids and the diaminopimelic acid isomer in whole-cell hydrolysates were determined as described by Tamura *et al.* (1994). The culture used to determine the fatty acid content was prepared on ISP 2 agar at 28 °C for 10 days. The fatty acid content of strain M24DS4^T is given in Supplementary Table S1. Other characteristics are given in the species description.

On the basis of significant differences shown in the 16S rRNA gene sequence analysis and the phenotypic differences between strain M24DS4^T and its closest phylogenetic neighbour (Stackebrandt & Ebers, 2006), we propose that strain M24DS4^T should be classified in a novel species of the genus *Streptomyces*, for which the name *Streptomyces aomiensis* sp. nov. is proposed.

Description of *Streptomyces aomiensis* sp. nov.

Streptomyces aomiensis (ao.mi.en'sis. N.L. masc. adj. *aomiensis* pertaining to Aomi, the place from where the strain was isolated).

Aerobic and Gram-positive actinomycete. Spore chains are *rectiflexibiles* and the spore surface is smooth. Spores and aerial mycelium are grey. The diaminopimelic acid isomer in the cell hydrolysate is the LL-type. Growth occurs at 15–37 °C (optimum 25–28 °C). Optimum pH is pH 6.0–8.0 (weak growth at pH 9.0 and 10.0). Growth is good on yeast extract-malt extract agar (ISP 2) and peptone-yeast extract iron agar (ISP 6), but is average-to-poor on tryptone yeast extract agar (ISP 1), oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5) and tyrosine agar (ISP 7). Melanin pigments are not produced on ISP 6 or ISP 7. With

Table 1. Characteristics that differentiate strain M24DS4^T from its closest recognized phylogenetic neighbour

All data were taken from this study, unless otherwise indicated. +, Positive; w, weakly positive; –, negative.

Characteristic	<i>S. aomiensis</i> sp. nov. M24DS4 ^T	<i>S. scabrisporus</i> NBRC 100760 ^T
Spore morphology (SEM)	<i>Rectiflexibiles</i> , smooth	Spiral, rugose*
Characteristics on ISP 2		
Aerial mycelium	Grey	–
Colony base colour	Raised, cream–yellow	Wrinkled, light ivory
Temperature range for growth (°C)	15–37	10–37
Utilization of:		
L-Arabinose	w	–
Inositol	–	+
API ZYM		
Alkaline phosphatase	–	+
α-Chymotrypsin	–	+
Valine arylamidase	w	+
Cystine arylamidase	w	+
API Coryne		
Pyrrolidonyl arylamidase	w	–
Urease	–	+
Gelatin hydrolysis	–	+

*Data taken from Ping *et al.* (2004).

API ZYM and API Coryne, positive for acid phosphatase, catalase, β-glucosidase, leucine arylamidase, *N*-acetyl-β-glucosaminidase, naphthol-AS-BI-phosphohydrolase, pyrazinamidase and nitrate reduction and weakly positive for cystine arylamidase, pyrrolidonyl arylamidase and valine arylamidase, but negative for alkaline phosphatase, esterase, esterase lipase, α-chymotrypsin, α-fucosidase, α- and β-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase, lipase, trypsin, urease and gelatin hydrolysis. As sole carbon sources, L-rhamnose, D-glucose, lactose and D-xylose are utilized and L-arabinose, D-fructose, sorbitol and sucrose are weakly utilized, but *myo*-inositol, D-mannitol and raffinose are not utilized. The predominant fatty acids are iso-C_{16:0}, anteiso-C_{15:0}, iso-C_{15:0} and C_{16:0}. The major respiratory quinones are MK-9(H₈) and MK-9(H₆); a small amount of MK-9(H₄) is also present. The DNA G+C content of the type strain is 75 mol%.

The type strain, M24DS4^T (=NBRC 106164^T =KACC 14925^T), was isolated from a soil sample using the membrane-filter method.

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References

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Brosius, J., Palmer, M. L., Kennedy, P. J. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci U S A* **75**, 4801–4805.
- Bull, A. T., Ward, A. C. & Goodfellow, M. (2000). Search and discovery strategies for biotechnology: the paradigm shift. *Microbiol Mol Biol Rev* **64**, 573–606.
- Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y.-W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259–2261.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Hayakawa, M. & Nonomura, H. (1987). Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. *J Ferment Technol* **65**, 501–509.
- Hirsch, C. F. & Christensen, D. L. (1983). Novel method for selective isolation of actinomycetes. *Appl Environ Microbiol* **46**, 925–929.
- McVeigh, H. P., Divers, M., Warwick, S., Munro, J. & Embley, T. M. (1994). Exploration of actinomycete diversity using ribosomal RNA sequences. *Biotechnologia* **7–8**, 253–260.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Minamisawa, K. (1990). Division of rhizobitoxine-producing and hydrogen-uptake positive strains of *Bradyrhizobium japonicum* by *nifDKE* sequence divergence. *Plant Cell Physiol* **31**, 81–89.
- Newman, D. J. & Hill, R. T. (2006). New drugs from marine microbes: the tide is turning. *J Ind Microbiol Biotechnol* **33**, 539–544.
- Ping, X., Takahashi, Y., Seino, A., Iwai, Y. & Ōmura, S. (2004). *Streptomyces scabrisporus* sp. nov. *Int J Syst Evol Microbiol* **54**, 577–581.

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

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

Stackebrandt, E. & Ebers, J. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* **33**, 152–155.

Swofford, D. L. (2000). PAUP*: phylogenetic analysis using parsimony (and other methods), version 4. Sunderland, MA: Sinaur Associates.

Tamura, T., Nakagaito, Y., Nishii, T., Hasegawa, T., Stackebrandt, E. & Yokota, A. (1994). A new genus of the order *Actinomycetales*, *Couchioplanes* gen. nov., with descriptions of *Couchioplanes caeruleus* (Horan and Brodsky 1986) comb. nov. and *Couchioplanes caeruleus* subsp. *azureus* subsp. nov. *Int J Syst Bacteriol* **44**, 193–203.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.