

Marinactinospora thermotolerans gen. nov., sp. nov., a marine actinomycete isolated from a sediment in the northern South China Sea

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A novel marine actinomycete, designated SCSIO 00652^T, was isolated from a marine sediment collected from the northern South China Sea at a depth of 3865 m. The strain formed branched substrate mycelia and no fragmentation was found. Abundant aerial mycelia differentiated into long spore chains and the spores had a wrinkled surface. Growth occurred on ISP medium 2 with 0–5% (w/v) NaCl and at 10–55 °C. The whole-cell hydrolysate contained *meso*-diaminopimelic acid and glucose as the whole-cell sugar. BLAST search results based on an almost-complete 16S rRNA gene sequence showed the novel strain had the highest similarity (96.5%) with *Nocardiopsis trehalosi* VKM Ac-942^T. The phylogenetic tree of the family *Nocardiopsaceae* indicated that strain SCSIO 00652^T formed a distinct lineage at the deepest branch with a high bootstrap value. Additionally, the profiles of menaquinones, phospholipids and fatty acids showed there were marked differences between strain SCSIO 00652^T and the recognized genera of the family *Nocardiopsaceae*. Based on the polyphasic data, a new genus, *Marinactinospora* gen. nov., is proposed within the family *Nocardiopsaceae* with the type species *Marinactinospora thermotolerans* sp. nov. The type strain of the type species is SCSIO 00652^T (=DSM 45154^T=CCTCC AA 208041^T).

The family *Nocardiopsaceae* was created, with *Nocardiopsis* as the type genus, by Rainey and co-workers in 1996 based on polyphasic data (Rainey *et al.*, 1996). At present it contains four genera, namely, *Nocardiopsis* (Meyer, 1976), *Thermobifida* (Zhang *et al.*, 1998), *Streptomonospora* (Cui *et al.*, 2001) and *Haloactinospora* (Tang *et al.*, 2008). Members of this family can form long or short spore chains or sporangia with abundant spores, which increase the survival rate of these organisms in many extreme environments. Members of the genus *Thermobifida* and some species of the genus *Nocardiopsis* are thermophilic actinomycetes, which can grow at high temperatures (40–65 °C) (Kroppenstedt & Evtushenko, 2006). Two genera, *Streptomonospora* and *Haloactinospora*, are strictly halo-

philic micro-organisms. Many *Nocardiopsis* strains have been isolated from hypersaline environments (Tang *et al.*, 2008), one *Nocardiopsis* strain has been isolated from an Antarctic glacier and a few species have been found in marine sediment (Dixit & Pant, 2000; Evtushenko *et al.*, 2000; Kroppenstedt & Evtushenko, 2006). Extreme environments are the general habitat of members of the family *Nocardiopsaceae*.

In this study, an abundant filamentous actinomycete strain was isolated on raffinose–histidine agar (Vickers *et al.*, 1984) from a deep-sea sediment sample collected from site E410 (17° 58.742' N 116° 00.228' E; black soft mud at 3865 m depth) in the northern South China Sea. Based on phylogenetic analysis, strain SCSIO 00652^T could be readily distinguished from the previously described genera of the family *Nocardiopsaceae* and should be recognized as representing a new genus and novel species.

The samples were collected aseptically with a grab-bucket collection sampler in September 2006 from the northern

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SCSIO 00652^T is EU698029.

A supplementary table showing patterns of selected 16S rRNA gene signature nucleotides in the genera of the family *Nocardiopsaceae* is available with the online version of this paper.

South China Sea. After collection, the surface layer of sediment, from 0–10 cm depth, was obtained as a subsample. Wet samples were first air-dried aseptically by being placed into a laminar flow hood and then a 2 g air-dried sample was suspended in 18 ml sterile seawater before 0.1 ml was spread on isolation media plates. After being incubated at 28 °C for 3 weeks, colonies were selected, purified and maintained on ISP 2 agar medium modified with seawater instead of distilled water. The purified strains were suspended in 20% (w/v) glycerol at –20 °C.

Strain SCSIO 00652^T grew well on ISP 2, ISP 3, ISP 4 and ISP 5 agars (Shirling & Gottlieb, 1966), Czapek solution agar (Waksman, 1961), nutrient agar (Difco) and potato agar (Waksman, 1961). During incubation at 28 °C for 7 days, white aerial mycelia developed well on agar plates of the above media (except ISP 2) and yellow–white substrate mycelia grew well on all of the media. After 14 days incubation, yellow–white aerial mycelia were observed on all media. By comparing the cultures with the most suitable colour chips from the ISCC-NBS colour charts (Kelly, 1964), it was evident that the novel strain did not produce diffusible pigments on any of the media. Micromorphology was observed by light microscopy (BH 2; Olympus) and electron microscopy (JEM-1010; JEOL) using cells incubated for 7, 14, 21 and 28 days. After 14 days, the white aerial mycelia were observed to be differentiated into long spore chains and the spores had a wrinkled surface (Fig. 1).

Physiological characteristics, including temperature and pH ranges for growth and tolerance to sodium chloride, were tested using ISP 2 as the basal medium. Carbon-source utilization for growth was carried out as described by Shirling & Gottlieb (1966). Tests for hydrolysis of cellulose, gelatin, starch and Tweens 20, 40, 60 and 80, nitrate reduction, utilization of urea, milk coagulation and peptonization and production of H₂S and melanin were performed as described by Gonzalez *et al.* (1978). Antibiotic susceptibility was examined as described by Groth *et al.* (2004) using antibiotic discs on modified ISP 2 agar. The detailed physiological properties of the strain are given in the species description.

Biomass for chemotaxonomic studies was obtained by cultivation using modified ISP 2 broth at 28 °C for 1 week and centrifugation at 150 r.p.m. Analysis of whole-cell sugars was performed according to the procedure described by Stanek & Roberts (1974). Amino acids and peptides in the cell-wall hydrolysate were analysed by the methods described by Hasegawa *et al.* (1983). Menaquinones were isolated using the methods of Minnikin *et al.* (1984) and separated by HPLC (Kroppenstedt *et al.*, 1981; Kroppenstedt, 1982). Phospholipids were extracted and examined by using published procedures (Collins & Jones, 1980; Minnikin *et al.*, 1979). Fatty acid analysis was performed by using standard methods (Sasser, 1990) and the results were compared with the database of fatty acids in the Microbial Identification System.

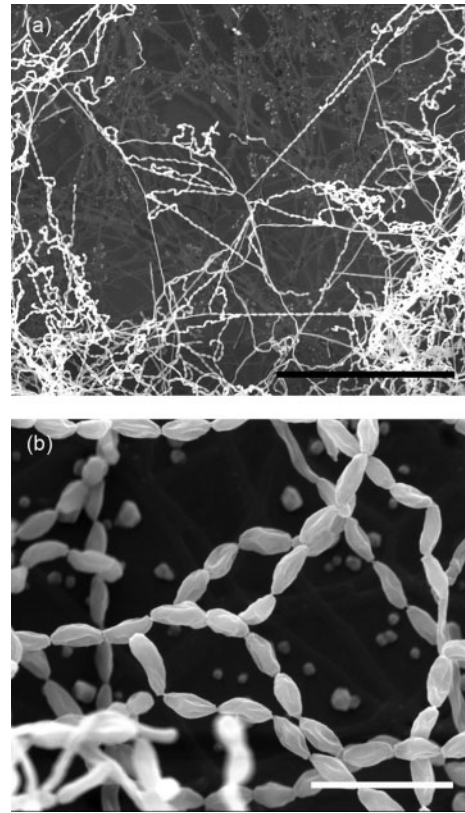


Fig. 1. Scanning electron micrographs (a, b) showing the long spore chains and spores of *Marinactinospora thermotolerans* sp. nov. SCSIO 00652^T after incubation on modified ISP 2 for 14 days at 28 °C.

The whole-cell hydrolysate of the novel isolate contained *meso*-diaminopimelic acid as the diagnostic diamino acid and glucose. The menaquinones consisted of MK-11(H₈) (43.8%), MK-11(H₁₀) (18.7%), MK-10(H₈) (16.5%), MK-12(H₈) (8.8%), MK-10(H₁₀) (7.9%) and MK-11(H₆) (4.4%). Phospholipids comprised diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol mannosides, phosphatidylinositol and unknown phosphoglycolipids. The G+C content of the genomic DNA was 72 mol%, as determined by using the HPLC method (Mesbah *et al.*, 1989). The fatty acid profile mainly contained 10-methyl-C_{18:0} (24.5%), *i*-C_{16:0} (24.5%), *i*-C_{16:1}G (10.9%) and *ai*-C_{17:0} (9.5%).

Genomic DNA extraction and PCR amplification of the 16S rRNA gene were conducted as described by Li *et al.* (2007). Multiple alignments with sequences of closely related taxa were carried out using CLUSTAL_X (Thompson *et al.*, 1997). A phylogenetic tree was reconstructed using the neighbour-joining method of Saitou & Nei (1987) from *K*_{nuc} values (Kimura, 1980, 1983) and MEGA version 4.0 (Tamura *et al.*, 2007). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

BLAST results for the 16S rRNA gene sequence of strain SCSIO 00652^T showed that its closest relatives were members of the genus *Nocardiopsis* in the family *Nocardiopsaceae*, with the highest gene sequence similarity of 96.5% with *Nocardiopsis trehalosi* VKM Ac-942^T. The phylogenetic tree (not shown) of the family *Nocardiopsaceae* based on 16S rRNA gene sequences showed that the novel isolate formed a distinct but unstable lineage among members of the family *Nocardiopsaceae*, with a bootstrap value of <30%. However, in the reconstructed phylogenetic tree (Fig. 2) based on the 16S rRNA gene sequences of type strains of species of the family *Nocardiopsaceae*, strain SCSIO 00652^T formed a distinct and stable lineage at the deepest branch with a bootstrap value of 100%. The chemotaxonomic characteristics of the novel strain could be readily distinguished from those of the recognized genera in the family *Nocardiopsaceae* (Table 1). Additionally, analysis of the nucleotide signatures also showed differences in some signature sites between SCSIO 00652^T and the other genera in the family *Nocardiopsaceae* (see Supplementary Table S1, available in IJSEM Online). Based on these polyphasic data, strain SCSIO 00652^T represents a novel genus for which the name *Marinactinospora* gen. nov. is proposed. The type species of the genus is *Marinactinospora thermotolerans* sp. nov.

Description of *Marinactinospora* gen. nov.

Marinactinospora (Ma.rin.ac.ti.no'spo.ra. L. adj. *marinus* of the sea; Gr. n. *actis actinos* a ray; Gr. n. *spora* a seed; N.L. fem. n. *Marinactinospora* marine and spored ray, referring to marine spore-forming actinomycete).

Gram-positive-staining, aerobic, moderately thermotolerant, filamentous actinomycetes. Forms are branched but non-fragmented substrate mycelia and yellow–white aerial mycelia that differentiate into long spore chains composed of spores with a wrinkled surface. No diffusible pigments are produced. Whole-cell hydrolysates contain *meso*-diaminopimelic acid with no diagnostic sugar. The predominant menaquinones are MK-10(H₈), MK-11(H₈) and MK-11(H₁₀). The phospholipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol mannosides, phosphatidylinositol and unknown phosphoglycerolipids. The major fatty acids are 10-methyl-C_{18:0}, i-C_{16:0}, i-C_{16:1} G and ai-C_{17:0}. The G + C content of the genomic DNA is 72 mol%. The type species is *Marinactinospora thermotolerans*.

Description of *Marinactinospora thermotolerans* sp. nov.

Marinactinospora thermotolerans (ther.mo.tol'er.ans. Gr. n. *therme* heat; L. pres. part. *tolerans* tolerating; N.L. part. adj. *thermotolerans* able to tolerate a high temperature).

Morphological, chemotaxonomic and general characteristics are as given above for the genus. Aerial mycelia are yellow–white on most media after 14 days incubation and no diffusible pigment is produced. Grows at pH 6.0–9.0 and 10–55 °C with 0–5% (w/v) NaCl, with optimum growth at pH 7.0–8.0 and 28 °C with 0–1% (w/v) NaCl. Utilizes cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannitol, D-mannose, L-rhamnose, D-ribose, D-sorbitol, sucrose and D-xylose as sole carbon sources, but

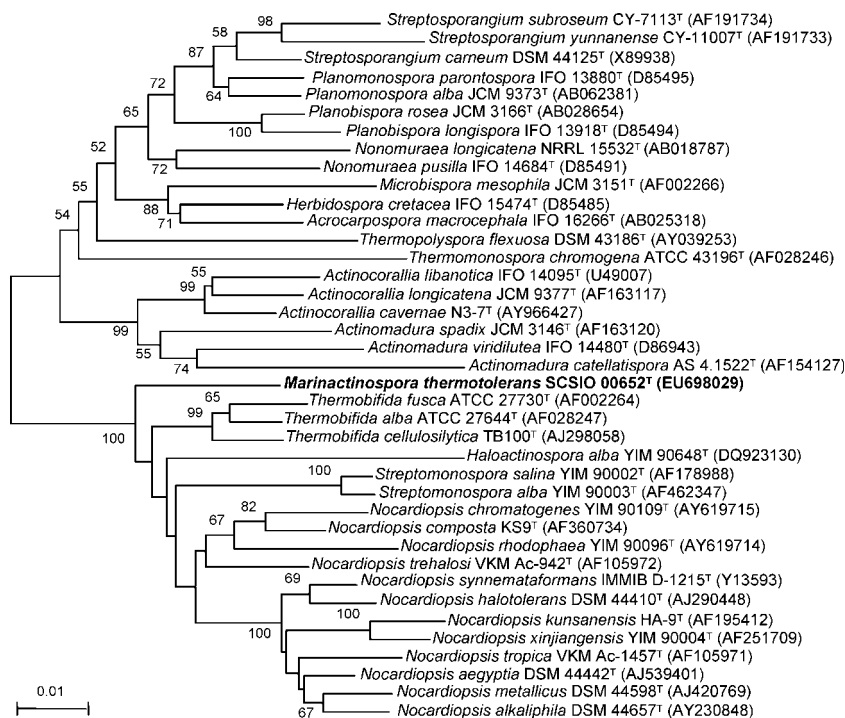


Fig. 2. Phylogenetic tree, based on 16S rRNA gene sequences of 1440 nt, of strain SCSIO 00652^T and related strains in the family *Nocardiopsaceae*, reconstructed by using the neighbour-joining method. Numbers at nodes are bootstrap values based on 1000 resamplings (only values >50% are indicated). Bar, 1% sequence divergence.

Table 1. Differentiating chemotaxonomic characteristics of strain SCSIO 00652^T and recognized genera of the family Nocardioseae

Taxa: 1, strain SCSIO 00652^T; 2, *Thermobifida*; 3, *Nocardioseae*; 4, *Haloactinospora*; 5, *Streptomonospora*. Data are from this study, Cai *et al.* (2008), Cui *et al.* (2001), Kroppenstedt & Evtushenko (2006), Tang *et al.* (2008), Yang *et al.* (2008) and Zhang *et al.* (1998). Cell walls of all taxa contain meso-diaminopimelic acid. Gal, galactose; Rib, ribose; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PIM, phosphatidylinositol mannoside; PME, phosphatidylmethylethanolamine; PL, unknown phospholipids.

| Characteristic | 1 | 2 | 3 | 4 | 5 |
|--------------------------|---|---|---|---|--|
| Diagnostic sugars | None | None | None | Gal, Rib | Gal |
| Predominant menaquinones | MK-11(H ₈ , H ₁₀), MK-10(H ₈) | MK-10(H ₆), MK-10(H ₈) | MK-10(H ₂ , H ₄ , H ₆), or MK-9 (H ₄ , H ₆) | MK-10(H ₈), MK-11(H ₄ , H ₆ , H ₈) | MK-10(H ₆), MK-10(H ₈) |
| Diagnostic phospholipids | DPG, PC, PG, PIM, PI, PL | DPG, PME, PC, PI, PG, PL | PC, PME | DPG, PG, PC, PIM | PE, PG, PI |
| Major fatty acids (>10%) | i-C _{16:0} , i-C _{16:1G} , 10-methyl-C _{18:0} | i-C _{16:0} , ai-C _{17:0} | i-C _{16:0} , ai-C _{17:0} , 10-methyl-C _{18:0} | i-C _{16:0} , ai-C _{17:0} | i-C _{15:0} , i-C _{16:0} , i-C _{17:0} |
| DNA G+C content (mol%) | 72 | 66–72 | 64–76 | 68 | 72–75 |

acetate, D-arabinose, citrate, dulcitol, inositol, D-lactose, raffinose, trehalose and xylitol are not utilized. Susceptible to (µg per disc): amikacin (30), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), gentamicin (10), neomycin (10), netilmicin (30), norfloxacin (10), novobiocin (30), rifampicin (5), tetracycline (30), tobramycin (10), trimethoprim (1.25) and vancomycin (30). Resistant to (µg per disc): amoxicillin (10), ampicillin (10), lincomycin (2), penicillin G (10) and streptomycin (10). Positive for hydrolysis of starch, cellulose and Tweens 20, 40, 60 and 80, nitrate reduction, melanin production, utilization of urea, catalase and oxidase. Negative for gelatin liquefaction, milk coagulation, milk peptonization and H₂S production. The fatty acid profile contains 10-methyl-C_{18:0}, i-C_{16:0}, i-C_{16:1G}, ai-C_{17:0}, C_{16:0}, C_{18:1 cis 9}, C_{18:0}, ai-C_{17:1A}, C_{16:1 cis 9}, 10-methyl-C_{16:0}, 10-methyl-C_{17:0}, C_{18:2 cis 9,12}/C_{18:0} a, i-C_{18:0}, ai-C_{15:0}, i-C_{17:0} and i-C_{14:0}.

The type strain, SCSIO 00652^T (=DSM 45154^T=CCTCC AA 208041^T), was isolated from deep-sea sediment at about 3865 m depth. The DNA G+C content of the type strain is 72 mol%.

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References

Cai, M., Zhi, X. Y., Tang, S. K., Zhang, Y. Q., Xu, L. H. & Li, W. J. (2008). *Streptomonospora halophila* sp. nov., a halophilic actinomycete isolated from a hypersaline soil. *Int J Syst Evol Microbiol* **58**, 1556–1560.

Collins, M. D. & Jones, D. (1980). Lipids in the classification and identification of coryneform bacteria containing peptidoglycans based on 2, 4-diaminobutyric acid. *J Appl Bacteriol* **48**, 459–470.

Cui, X. L., Mao, P. H., Zeng, M., Li, W. J., Zhang, L. P., Xu, L. H. & Jiang, C. L. (2001). *Streptomonospora salina* gen. nov., sp. nov., a new member of the family Nocardioseae. *Int J Syst Evol Microbiol* **51**, 357–363.

Dixit, V. S. & Pant, A. (2000). Hydrocarbon degradation and protease production by *Nocardioseae* sp. NCIM 5124. *Lett Appl Microbiol* **30**, 67–69.

Evtushenko, L. I., Taran, V. V., Akimov, V. N., Kroppenstedt, R. M., Tiedje, J. M. & Stackebrandt, E. (2000). *Nocardioseae tropica* sp. nov., nov. rev., *Nocardioseae trehalosi* sp. nov., nom. rev. and *Nocardioseae dassonvillei* subsp. *albirubida* subsp. nov., comb. nov. *Int J Syst Evol Microbiol* **50**, 73–81.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Gonzalez, C., Gutierrez, C. & Ramirez, C. (1978). *Halobacterium vallismortis* sp. nov., an amyolytic and carbohydrate-metabolizing, extremely halophilic bacterium. *Can J Microbiol* **24**, 710–715.

Groth, I., Rodriguez, C., Schütze, B., Schmitz, P., Leistner, E. & Goodfellow, M. (2004). Five novel *Kitasatospora* species from soil: *Kitasatospora arboriphila* sp. nov., *K. gansuensis* sp. nov., *K. nipponensis* sp. nov., *K. paranensis* sp. nov. and *K. terrestris* sp. nov. *Int J Syst Evol Microbiol* **54**, 2121–2129.

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

Kelly, K. L. (1964). *Inter-Society Color Council – National Bureau of Standards Color Name Charts Illustrated with Centroid Colors*. Washington, DC: US Government Printing Office.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequence. *J Mol Evol* **16**, 111–120.

Kimura, M. (1983). *The Neutral Theory of Molecular Evolution*. Cambridge: Cambridge University Press.

Kroppenstedt, R. M. (1982). Separation of bacterial menaquinones by HPLC using reverse phase (RP18) and a silver loaded ion exchanger as stationary phases. *J Liq Chromatogr* **5**, 2359–2367.

- Kroppenstedt, R. M. & Evtushenko, L. I. (2006).** The family *Nocardiopsaceae*. In *The Prokaryotes: A Handbook on the Biology of Bacteria*, 3rd edn, vol. 3, pp. 754–795. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. New York: Springer-Verlag.
- Kroppenstedt, R. M., Korn-Wendisch, F., Fowler, V. J. & Stackebrandt, E. (1981).** Biochemical and molecular genetic evidence for transfer of *Actinoplanes armeniacus* into the family *Streptomycetaceae*. *Zentralbl Bakt Hyg Abt Orig C* 2, 254–262.
- Li, W. J., Xu, P., Schumann, P., Zhang, Y. Q., Pukall, R., Xu, L. H., Stackebrandt, E. & Jiang, C. L. (2007).** *Georgenia ruanii* sp. nov., a novel actinobacterium isolated from forest soil in Yunnan (China) and emended description of the genus *Georgenia*. *Int J Syst Evol Microbiol* 57, 1424–1428.
- 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.
- Meyer, J. (1976).** *Nocardiopsis*, a new genus of the order *Actinomycetales*. *Int J Syst Bacteriol* 26, 487–493.
- Minnikin, D. E., Collins, M. D. & Goodfellow, M. (1979).** Fatty acid and polar lipid composition in the classification of *Cellulomonas*, *Oerskovia* and related taxa. *J Appl Bacteriol* 47, 87–95.
- Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A. & Parlett, J. H. (1984).** An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* 2, 233–241.
- Rainey, F. A., Ward-Rainey, N., Kroppenstedt, R. M. & Stackebrandt, E. (1996).** The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam. nov. *Int J Syst Bacteriol* 46, 1088–1092.
- 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. *USFCC Newsl* 20, 16.
- Shirling, E. B. & Gottlieb, D. (1966).** Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* 16, 313–340.
- Staneck, J. L. & Roberts, G. D. (1974).** Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* 28, 226–231.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007).** MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24, 1596–1599.
- Tang, S.-K., Tian, X.-P., Zhi, X.-Y., Cai, M., Wu, J.-Y., Yang, L.-L., Xu, L.-H. & Li, W.-J. (2008).** *Haloactinospira alba* gen. nov., sp. nov., a novel halophilic filamentous actinomycete of the family *Nocardiopsaceae*. *Int J Syst Evol Microbiol* 58, 2075–2080.
- 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.
- Vickers, J. C., Williams, S. T. & Ross, G. W. (1984).** A taxonomic approach to selective isolation of streptomycetes from soil. In *Biological, Biochemical and Biomedical Aspects of Actinomycetes*, pp. 553–551. Edited by L. Ortiz-Ortiz, L. F. Bojalil & V. Yakole. London: Academic Press.
- Waksman, S. A. (1961).** *The Actinomycetes*, vol. II. *Classification, Identification and Description of Genera and Species*. Baltimore: Williams & Wilkins.
- Yang, L. L., Tang, S. K., Zhang, Y. Q., Zhi, X. Y., Wang, D., Xu, L. H. & Li, W. J. (2008).** *Thermobifida halotolerans* sp. nov., isolated from a salt mine sample, and emended description of the genus *Thermobifida*. *Int J Syst Evol Microbiol* 58, 1821–1825.
- Zhang, Z., Wang, Y. & Ruan, J. (1998).** Reclassification of *Thermomonospora* and *Microtetraspora*. *Int J Syst Bacteriol* 48, 411–422.