Spinactinospora alkalitolerans gen. nov., sp. nov., an actinomycete isolated from marine sediment

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A novel marine actinomycete, designated CXB654^T, was isolated from marine sediment collected at a depth of 17.5 m near the Yellow Sea Cold Water Mass, China. Optimal growth occurred at 37.0 °C, at pH 7.0–8.0 and in 3–8 % (w/v) NaCl. Strain CXB654^T formed branched substrate mycelium without fragmentation. Abundant aerial mycelium differentiated into long or short chains of spores and spores were elliptical and cylindrical with spiny surfaces. Strain CXB654^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid, and ribose and glucose as the major whole-cell components. Phospholipids were diphosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol and phosphatidylinositol. MK-10(H₈), MK-10(H₆) and MK-9(H₈) were the predominant menaquinones. The major fatty acids were *i*-C_{16:0} (24.46 %), *ai*-C_{17:0} (20.66 %) and C_{18:0} (20.14 %). The DNA G + C content was 71.1 mol%. Comparative analysis of 16S rRNA gene sequences showed that the novel strain was most closely related to genera within the family *Nocardiopsaceae*, but formed a separate lineage. Highest sequence similarities were to *Murinocardiopsis flavida* DSM 45312^T (96.6 %), *Thermobifida halotolerans* YIM 90462^T (96.5 %) and *Marinactinospora thermotolerans* DSM 45154^T (96.1 %). On the basis of phenotypic, chemotaxonomic and phylogenetic distinctiveness, strain CXB654^T was considered to represent a novel species in a new genus in the family *Nocardiopsaceae*, and the name *Spinactinospora alkalitolerans* gen. nov., sp. nov. is proposed; the type strain is CXB654^T (=DSM 45414^T=LMG 25485^T).

Actinomycetes are Gram-positive bacteria with filamentous growth and high DNA G + C content (60–78 mol%) that are widely distributed in various ecological habitats. The family *Nocardiopsaceae* was created, with *Nocardiopsis* as the type genus, by Rainey and co-workers in 1996 based on data from polyphasic studies (Rainey *et al.*, 1996). At present, the family *Nocardiopsaceae* contains six genera, namely, *Nocardiopsis* (Meyer, 1976), *Thermobifida* (Zhang *et al.*, 1998), *Streptomonospora* (Cui *et al.*, 2001), *Haloactinospora* (Tang *et al.*, 2008), *Marinactinospora* (Tian *et al.*, 2009) and *Murinocardiopsis* (Kämpfer *et al.*, 2010). Most strains of the genus *Nocardiopsis* have been isolated from hypersaline soils (Li *et al.*, 2003, 2004, 2006; Chen *et al.*, 2008; Yang *et al.*, 2008b), but some strains have been isolated from marine sediment (Sabry *et al.* 2004; Kroppenstedt & Evtushenko, 2006; Tian *et al.*, 2009); most *Nocardiopsis* strains are strictly

halophilic. All four *Thermobifida* species were isolated from manure heaps, composts, damp stored hay or salt mines and have outstanding cellulose decomposition abilities (Yang *et al.*, 2008a). Members of the two genera *Streptomonospora* and *Haloactinospora* are strictly halophilic. *Marinactinospora* and *Murinocardiopsis* both contain only a single species, isolated from marine sediment and indoor walls, respectively. 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. A novel strain belonging to this family, designated CXB654^T, was isolated recently from marine sediment and found to be phylogenetically closely related to members of the genera *Murinocardiopsis*, *Thermobifida* and *Marinactinospora*. In the present study, the taxonomic position of strain CXB654^T was determined by a polyphasic taxonomic approach.

Samples were collected from sediment near the Yellow Sea Cold Water Mass (32° 00.398' N 122° 09.722' E), China, in July 2008. The surface layer of the sediment, about 40 cm in depth, was collected by a grab bucket and samples from the top 5–10 cm layer were obtained aseptically and stored in sterile 50 ml conical tubes at 4 °C. Samples were

Abbreviations: DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; ISP, International *Streptomyces* Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CXB654^T is GU112453.

Two supplementary figures are available with the online version of this paper.

transported to the laboratory and processed for cultivation experiments. A 2 g wet sample was suspended in 18 ml sterile seawater and 0.1 ml aliquots of the suspension were spread on sodium propionate–aspartic acid agar plates (0.1 g aspartic acid, 2.0 g peptone, 4.0 g sodium propionate, 0.05 g $K_2HPO_4 \cdot 3H_2O$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 20.0 g agar, 1.0 l 2- to 3-week old seawater, pH 7.2–7.4). After incubation at 28 °C for 3 weeks, colonies were isolated, purified and maintained on International *Streptomyces* Project (ISP) medium 2 agar (Shirling & Gottlieb, 1966) modified with 2- to 3-week-old seawater instead of distilled water. Purified strains were suspended in sterile 0.9 % (w/v) saline supplemented with 15 % (v/v) glycerol and stored at –80 °C.

All media were supplemented with seawater for observation of growth. Strain CXB654^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 ISP 2 agar, nutrient agar (Difco) and potato agar, and yellow–white substrate mycelia grew well on all media. By comparing the cultures with 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 tested. Micromorphology was observed by light microscopy (model BH2; Olympus) and scanning electron microscopy (JSM5600LV; JEOL) using cells incubated for 7, 14, 21 and 28 days on modified ISP 2 agar. After 14 days, the white aerial mycelia differentiated into short or long spore chains. Spore surfaces were spiny (Fig. 1).

Physiological characteristics, including temperature and pH ranges for growth and NaCl tolerance, were tested using ISP 2 as the basal medium. Growth was tested at 4, 16, 20, 28, 37, 40, 42, 44 and 45 °C on ISP 2 agar modified with seawater instead of distilled water. For tolerance experiments involving NaCl concentrations of 0–16 % (at intervals of 1 %), ISP 2 was used as the basal medium. The pH range for growth was investigated between pH 4.0 and 11.0 at intervals of 0.5 units using the buffer system described by Xu *et al.* (2005). 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 peptonisation, and production of H_2S and melanin were performed in 3 % (w/v) NaCl 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. *Thermobifida alba* DSM 43795^T (type species of the genus *Thermobifida*) and *Marinactinospora thermotolerans* DSM 45154^T (the type species of the genus *Marinactinospora*) obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH, Braunschweig, Germany, were used as reference strains under the same culture conditions. Detailed physiological properties of the isolate are given in the genus and species descriptions (see below) and Table 1.

Fig. 1. Scanning electron micrographs showing the long or short spore chains and spores of *Spinactinospora alkalitolerans* gen. nov., sp. nov. CXB654^T after incubation on modified ISP 2 for 14 days at 28 °C. Bars: (a) 10 µm; (b) 10 µm; (c) 1 µm.

Biomass for chemotaxonomic studies was obtained by centrifugation after cultivation in modified ISP 2 broth at 28 °C for 3 days. For analysis of sugars, amino acids and peptides in the cell-wall hydrolysate, wet biomass was thoroughly suspended in isopropanol/water (1:1, v/v). For analysis of menaquinones, phospholipids and fatty acids, wet biomass was freeze-dried. Analysis of whole-cell sugars, amino acids and peptides in the cell-wall hydrolysate and menaquinones, phospholipids and fatty acids were carried out by the Identification Service of the DSMZ and B. J. Tindall, (DSMZ). Whole-cell hydrolysates of strain CXB654^T contained *meso*-diaminopimelic acid and whole-cell sugars were ribose, glucose and traces of galactose and fucose. The menaquinones consisted of MK-10(H_8) (44 %), MK-10(H_6) (20 %), MK-9(H_8) (14 %), MK-10(H_4) (5 %) and MK-10(H_2) (3 %), in addition to some long-chain minor components, which could not be identified. Phospholipids (see Supplementary Fig. S1 available in IJSEM Online) comprised diphosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol and unknown phosphoglycerolipids. The fatty acid profile contained mainly

Table 1. Differential phenotypic and chemotaxonomic characteristics of strain CXB654^T and genera of the family Nocardiopsaceae

Taxa: 1, strain CXB654^T; 2, *Murinocardiopsis* [data from Kämpfer *et al.* (2010) and this study]; 3, *Thermobifida* [data from Yang *et al.* (2008a) and this study]; 4, *Marinactinospora* [data from Tian *et al.* (2009) and this study]; 5, *Nocardiopsis* (Kroppenstedt & Evtushenko, 2006; Yang *et al.*, 2008b; Chen *et al.*, 2009); 6, *Haloactinospora* (Tang *et al.*, 2008); 7, *Streptomonospora* (Cui *et al.*, 2001; Cai *et al.*, 2008). Cell walls of all the taxa contain meso-diaminopimelic acid.

Characteristic	1	2	3	4	5	6	7
Number of species	1	1	4	1	38	1	5
Isolation source	Marine	Indoor walls	Terrestrial	Marine	Terrestrial or marine	Salt lake	Terrestrial or salt lake
Spore surface	Spiny	No aerial mycelium	Smooth	Wrinkled	Smooth	Smooth	Wrinkled
pH range for growth	6.0–10.5	5–9	7–9	6–9	6–14	6–9	5–9
NaCl concentration range for growth (% w/v)	1–15	0–11	0–5	0–5	0–20	9–21	5–25
Growth temperature (°C):							
Range for optimum growth	37	28	40–45	28	25–40	37	28–37
Maximum	44	34	55	55	45	45	45
Diagnostic sugars*	Rib, Glu	None	None	None	Gal	Gal, Rib	Gal
Predominant menaquinones	MK-10(H ₆), MK-10(H ₈), MK-9(H ₈)	MK-10(H ₄), MK-11(H ₄), MK-12(H ₂), MK-10(H ₈)	MK-10(H ₆), MK-10(H ₈)	MK-11(H ₈ , H ₁₀), 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 ₈ , H ₆)
Diagnostic phospholipids†	PG, PC, DPG, PI, PL	PC, PG, PI, DPG, PL1, PL2, GL	DPG, PME, PC, PI, PG, PL	DPG, PC, PG, PIM, PI, PL	PC, PME	DPG, PG, PC, PIM	PE, PG, PI
Major fatty acids (>10 %)	i-C _{16:0} , ai-C _{17:0} , C _{18:0}	i-C _{16:0} , ai-C _{17:0} , C _{18:1} ω9c	i-C _{16:0} , ai-C _{17:0}	i-C _{16:0} , i-C _{16:1} G, 10-methyl-C _{18:0}	i-C _{15:0} , i-C _{16:0} , i-C _{17:0}	i-C _{16:0} , ai-C _{17:0}	i-C _{15:0} , i-C _{16:0} , i-C _{17:0}

*Rib, Ribose; Glu, glucose; Gal, galactose.

†DPG, Diphosphatidylglycerol; GL, glycolipid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PIM, phosphatidylinositol mannoside; PME, phosphatidylmethylethanolamine; PL, unknown phospholipids; GL, glycolipid.

i-C_{16:0} (24.46 %), ai-C_{17:0} (20.66 %) and C_{18:0} (20.14 %); minor components were ai-C_{15:0} (7.12 %), 10-methyl-C_{18:0} (6.29 %), i-C_{18:0} (5.21 %), i-C_{17:0} (2.89 %), C_{16:0} (2.21 %), i-C_{14:0} (2.13 %), C_{17:0} (1.58 %), 10-methyl-C_{17:0} (1.49 %), i-C_{15:0} (1.18 %), C_{18:1}ω9c (1.09 %), ai-C_{19:0} (1.03 %), C_{19:0} (0.46 %), i-C_{19:0} (0.45 %), C_{20:0} (0.44 %), ai-C_{13:0} (0.17 %) and C_{14:0} (0.17 %).

The chromosomal DNA G+C content was determined according to the methods described by Mesbah *et al.* (1989) using reverse-phase HPLC. The DNA G+C content of strain CXB654^T was 71.1 mol%.

Genomic DNA extraction and PCR amplification of the 16S rRNA gene were conducted as described by Li *et al.* (2007). The 16S rRNA gene sequence was aligned and compared with available sequences in the GenBank/EMBL/DBJ database using BLAST searches. Identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Sequences were aligned using CLUSTAL X 1.8 (Thompson *et al.*,

1997) and edited manually using BioEdit Sequence Alignment Editor version 5.0.9 (Hall, 1999). Phylogenetic trees were constructed using the neighbour-joining and maximum-likelihood methods with Kimura 2-state parameter model analyses implemented in the program MEGA version 4 (Tamura *et al.*, 2007). In each case, bootstrap values were calculated based on 1000 replicates.

BLAST results for the 16S rRNA gene sequence of strain CXB654^T showed that its closest relatives were members of genera *Murinocardiopsis*, *Thermobifida* and *Marinactinospora* in the family Nocardiopsaceae, with the highest gene sequence similarity of 96.6 % with *Murinocardiopsis flavida* DSM 45312^T; similarities were 96.5 and 96.1 % with *Thermobifida halotolerans* YIM 90462^T and *Marinactinospora thermotolerans* DSM 45154^T, respectively. The 16S rRNA gene-based neighbour-joining (Fig. 2) and maximum-likelihood trees (Supplementary Fig. S2 available in IJSEM Online) showed the phylogenetic relationship between strain CXB654^T and members of closely related genera and revealed that strain CXB654^T formed a distinct lineage among members of the family Nocardiopsaceae.

Fig. 2. Phylogenetic dendrogram obtained by neighbour-joining analysis based on 16S rRNA gene sequences showing the position of strain CXB654^T and its phylogenetic neighbours. Numbers at nodes are bootstrap values based on 1000 resamplings (only values $\geq 50\%$ are indicated). Bar, 0.5% sequence divergence.

Strain CXB654^T could be differentiated from members of the phylogenetically most closely related genera *Murinocardioptosis*, *Thermobifida* and *Marinactinospora* as follows. (1) The surfaces of spores of strain CXB654^T were spiny, unlike those of all other genera in the family *Nocardiothermobifidaceae* apart from the genus *Murinocardioptosis*, which has no aerial mycelium. (2) Strain CXB654^T required NaCl for growth, enabling it to be distinguished from members of the genera *Murinocardioptosis*, *Thermobifida* and *Marinactinospora*. (3) The diagnostic sugars of strain CXB654^T were ribose and glucose, but closely related genera had no diagnostic sugar. (4) The predominant menaquinones for strain CXB654^T were MK-10(H₈), MK-10(H₆) and MK-9(H₈), which differed from those of the genera *Murinocardioptosis* [MK-10(H₄) and MK-11(H₄)], *Thermobifida* [MK-10(H₆) and MK-10(H₈)] and *Marinactinospora* [MK-11(H₈) and MK-11(H₁₀)] in that strain CXB654^T possessed MK-9(H₈), but the other genera did not. (5) Diagnostic phospholipids of the genera *Murinocardioptosis*, *Thermobifida* and *Marinactinospora* are glycolipid, phosphatidylmethylethanolamine and phosphatidylinositol mannoside, respectively, which are not found in strain CXB654^T. Other characteristics that differentiate strain CXB654^T from members of the other genera of the family

Nocardiothermobifidaceae are shown in Table 1. Based on data from this polyphasic study, strain CXB654^T represents a novel species in a new genus for which the name *Spinactinospora alkalitolerans* gen. nov., sp. nov. is proposed.

Description of *Spinactinospora* gen. nov.

Spinactinospora (Spin.ac.ti.no'spo.ra. L. n. *spina* spine; Gr. n. *actis actinos* a ray; Gr. n. *spora* a seed; N.L. fem. n. *Spinactinospora* an actinomycete with spiny spores).

Gram-positive, aerobic, moderately alkalitolerant, filamentous actinomycete. Aerial mycelium forms short or long chains of spores; spore surfaces are spiny. Substrate mycelium is branched with non-fragmenting hyphae. No diffusible pigments are produced. Whole-cell hydrolysates contain *meso*-diaminopimelic acid, ribose and glucose. Predominant menaquinones are MK-10(H₈), MK-10(H₆) and MK-9(H₈). The phospholipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol and unknown phosphoglycolipids. Major fatty acids are i-C_{16:0}, ai-C_{17:0}, and C_{18:0}. The type species is *Spinactinospora alkalitolerans*.

Description of *Spinactinospora alkalitolerans* sp. nov.

Spinactinospora alkalitolerans (al.ka.li.to'le.rans. N.L. n. *alkali* alkali; L. part. adj. *tolerans*, tolerating; N.L. part. adj. *alkalitolerans* alkali-tolerating).

Displays the following properties in addition to those described for the genus. Growth is good on ISP 2, ISP 3, ISP 4 and ISP 5 agars, Czapek solution agar, nutrient agar and potato agar. The colour of the aerial mycelium is white to white–yellow and the substrate mycelium is pale yellow to light yellow or deep yellow. Spores are elliptical and cylindrical with spiny surfaces. No diffusible pigments are produced. Growth occurs between 16 and 44 °C, but not at 4 or 45 °C. Grows at pH 6.0–10.5 and in 1–15% (w/v) NaCl, with optimum growth at pH 7.0–8.0, at 37 °C and in 3–8% (w/v) NaCl. Utilizes dextrin, glycogen, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, D-arabitol, cellobiose, D-fructose, D-galactose, D-gluconic acid, α-D-glucose, maltose, maltotriose, D-mannose, 3-methyl-D-glucose, D-ribose, sedoheptulosan, turanose, xylitol, D-xylose, acetic acid, L-alaninamide, L-alanine, L-asparagine, L-glutamic acid, L-serine, 2,3-butanediol, glycerol and inosine as sole carbon sources, but not mannan, *myo*-inositol, L-fucose, L-arabinose, α-lactose, lactulose, D-mannitol, melezitose, melibiose, sucrose, D-sorbitol, L-rhamnose, raffinose, trehalose or D-alanine. Susceptible to (μg per disc): penicillin (10), ampicillin (100), piperacillin (100), cefalexin (30), cefazolin (30), cefradine (30), cefuroxime (30), ceftriaxone (30), cefoperazone (75), amikacin (30), gentamicin (10), kanamycin (30), neomycin (30), tetracycline (30), doxycycline (30), minocycline (30), erythromycin (15), medecamycin (30), vancomycin (30), chloromycetin (30), clindamycin (2). Resistant to (μg per disc): oxacillin (1), ampicillin (10), ceftazidime (30), norfloxacin (10), ofloxacin (5), profloracin (5), polymyxin B (300), sulfamethoxazole (75) and furazolidone (300). Hydrolysis of starch and cellulose, melanin production, H₂S production, gelatin liquefaction, milk coagulation and milk peptonization are negative, whereas hydrolysis of Tweens 20, 40, 60 and 80, nitrate reduction, utilization of urea, catalase and oxidase are positive. The fatty acid profile contains i-C_{16:0}, ai-C_{17:0}, C_{18:0}, ai-C_{15:0}, 10-methyl-C_{18:0}, i-C_{18:0}, i-C_{17:0}, C_{16:0}, i-C_{14:0}, C_{17:0}, 10-methyl-C_{17:0}, i-C_{15:0}, C_{18:1ω9c}, ai-C_{19:0}, C_{19:0}, i-C_{19:0}, C_{20:0}, ai-C_{13:0} and C_{14:0}.

The type strain is CXB654^T (=DSM 45414^T=LMG 25485^T), isolated from sediment at a depth of about 17.5 m near the Yellow Sea Cold Water Mass, China. The DNA G+C content of the type strain is 71.1 mol%.

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