

## *Verrucosipora sediminis* sp. nov., a cyclodipeptide-producing actinomycete from deep-sea sediment

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An actinomycete, designated MS426<sup>T</sup>, the culture broth of which showed potent antimicrobial activity, was isolated from a deep-sea sediment sample of the South China Sea. An almost-complete sequence of the 16S rRNA gene of strain MS426<sup>T</sup> was determined and aligned with those of representatives of the family *Micromonosporaceae* available in public databases. Phylogenetic trees were inferred by using three algorithms. Strain MS426<sup>T</sup> formed a branch adjacent to *Verrucosipora lutea* YIM 013<sup>T</sup> in a distinct cluster occupied only by strains of the genus *Verrucosipora*. Strain MS426<sup>T</sup> was distinguishable from the type strains of the two described *Verrucosipora* species by using a combination of chemical and morphological markers and by DNA–DNA relatedness. On the basis of these genotypic and phenotypic differences, the novel antimicrobial strain with pharmaceutical potential represents a novel species, for which the name *Verrucosipora sediminis* sp. nov. is proposed. The type strain is MS426<sup>T</sup> (=CGMCC 4.3550<sup>T</sup> =JCM 15670<sup>T</sup>).

The exploration of microbial diversity in marine environments has become one of the most popular research areas now that the ocean has been recognized as a microbiological ‘rainforest’. Novel microbes and their associated bioactive compounds have been isolated (Demain & Zhang, 2005). Since the description of the marine genus *Salinispora* and its bioactive secondary metabolites, marine actinomycetes, especially members of the family *Micromonosporaceae*, have received attention from both taxonomists and chemists (Maldonado *et al.*, 2005; Laatsch, 2006; Lam, 2006; Knight *et al.*, 2003; Zhang *et al.*, 2005). The discovery of abyssomicin, an inhibitor of *p*-aminobenzoic acid biosynthesis, in the marine strain AB-

18-032 of the genus *Verrucosipora* has led to this genus receiving special attention (Riedlinger *et al.*, 2004).

The genus *Verrucosipora* was established by Rheims *et al.* (1998) and, to date, only two species have been described, namely *Verrucosipora giffhornensis* (Rheims *et al.*, 1998) and *Verrucosipora lutea* (Liao *et al.*, 2009). The genus *Verrucosipora* is characterized by branching hyphae that form a well-developed septate mycelium, absent or sparse aerial mycelium, non-motile, singly borne spores with smooth, warty or hairy surfaces, the presence of 16S rRNA gene signature nucleotides specific to the family *Micromonosporaceae*, a unique nucleotide signature (5'-CAAUUCGGUUG-3') between positions 1132 and 1143 (*Escherichia coli* numbering), DNA G + C content of 69.3–70.0 mol%, peptidoglycan of type A1 $\gamma$  (directly cross-linked, presence of glycine, alanine, glutamic acid and *meso*-diaminopimelic acid in the peptide side-chain), lack of arabinose in whole-cell sugars, a polar lipid composition

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MS426<sup>T</sup> is EU870859.

A scanning electron micrograph of strain MS426<sup>T</sup> is available as supplementary material with the online version of this paper.

of phospholipid type II, MK-9(H<sub>4</sub>) as the major respiratory lipoquinone and a glycolyl type of muramic acid.

The main interest of our joint research project is to build up a high-quality microbe-originated library of natural products and to screen for bioactive metabolites in a high-throughput manner (Bian *et al.*, 2009). The identification of beauvericin as an antifungal potentiator was a good example of the use of the library for drug discovery (Zhang *et al.*, 2007; Zhang, 2005; Demain & Zhang, 2005). Strain MS426<sup>T</sup> was recognized for its antifungal and antibacterial activity, but it did not inhibit the *p*-aminobenzoic acid biosynthesis pathway of methicillin-resistant *Staphylococcus aureus*. The isolation of natural products was performed after a small-scale fermentation. Eight types of cyclodipeptides and two types of nocardamine-like structures were isolated from the active broth, but no abyssomycins were found (unpublished data).

The aim of the present study was to establish the taxonomic position of strain MS426<sup>T</sup>. The strain was isolated from a deep-sea sediment sample, collected from the South China Sea (17° 54' N 114° 34' E, depth of 3602 m) with a grab bucket as described previously (Tian *et al.* 2009; Bian *et al.* 2009), after incubation on Gauze's medium no. 1 (DSMZ medium no. 1048) at 22 °C for 4 weeks. The isolate was maintained on medium 6B agar slants (CGMCC medium no. 0250: 5.0 g yeast extract, 50.0 g glycerol, 5.0 g N-Z amine type A, 10.0 g glucose, 20.0 g soluble starch, 1.0 g CaCO<sub>3</sub> and 15.0 g agar in 1000 ml distilled water; pH 6.0–6.5 at 4 °C) and as suspensions of mycelial fragments in 25% (v/v) glycerol at –70 °C.

Biomass for chemical and molecular studies was obtained by cultivation in medium 6B in shaking flasks (200 r.p.m.) at 28 °C for 1 week. Cultural characteristics were determined after growth at 28 °C for 3 weeks using methods from the International *Streptomyces* Project (ISP; Shirling & Gottlieb, 1966) and modified Bennett's agar (Jones, 1949) and medium 6B agar. Colour determination was performed by comparisons with chips from the ISCC-NBS colour charts (Kelly, 1964). Morphological features of spores and mycelia were observed by light microscopy (Olympus microscope BH-2) and scanning electron microscopy (Quanta 200 FEG) after growth on medium 6B for 2 weeks. Growth at various temperatures, at pH 3.0–10.0 (at intervals of 0.5 pH units) and with 0–10% (w/v) NaCl (at intervals of 0.5% NaCl) was determined using modified Bennett's agar as the basal medium. The media and procedures used to determine physiological features and carbon source utilization have been described by Smibert & Krieg (1981) and Williams *et al.* (1989).

Strain MS426<sup>T</sup> grew well on most media tested, especially on medium 6B supplemented with seawater. Its substrate mycelium was well developed and branched and warty-surfaced spores were formed singly and in clusters, but aerial mycelium was sparse (Supplementary Fig. S1, available in IJSEM Online). Good growth occurred at 28–

37 °C and at pH 5.0–7.5 on medium supplemented with seawater salts. The differential characteristics of strain MS426<sup>T</sup>, *V. lutea* YIM 013<sup>T</sup> and *V. gifhornensis* DSM 44337<sup>T</sup> are given in Table 1 and the detailed characteristics of strain MS426<sup>T</sup> are given in the species description.

The isomer of diaminopimelic acid and whole-cell sugars were analysed according to the procedures developed by Hasegawa *et al.* (1983). Polar lipids were extracted, examined by two-dimensional TLC and identified using previously described procedures (Minnikin *et al.*, 1984). Determination of the *N*-acyl type of muramic-acid residues followed the procedure described by Uchida *et al.* (1999). Menaquinones were isolated according to the methods of Minnikin *et al.* (1984) and separated by HPLC (Kroppenstedt, 1982). Biomass for quantitative fatty acid analysis was prepared by scraping growth from TSB agar plates [3%, w/v, trypticase soy broth (BBL) and 1.5%, w/v, Bacto agar (Difco)] after incubation at 28 °C for 3 days. The fatty acids were extracted, methylated and analysed using the standard Microbial Identification system (MIDI; Sasser, 1990). Genomic DNA for the determination of the G+C content was prepared according to the method of Marmur (1961). The DNA G+C content was determined by the thermal denaturation (*T*<sub>m</sub>) method (Mandel & Marmur, 1968) using a Lambda 25 UV/Vis spectrophotometer (Perkin-Elmer).

The cell-wall diamino acid in the peptidoglycan layer of strain MS426<sup>T</sup> was *meso*-diaminopimelic acid. Glycine, alanine and glutamic acid were also detected. The major whole-cell sugars were glucosamine, glucose and mannose. The muramic acid was in the *N*-glycolylated form. The predominant menaquinone was MK-9(H<sub>4</sub>) and a minor amount of MK-9(H<sub>2</sub>) was detected. The phospholipids consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides and some unknown phospholipids (phospholipid type II; Lechevalier *et al.*, 1977). The major fatty acids (>10% of the total) were C<sub>17:0</sub> (23.0%), iso-C<sub>16:0</sub> (18.6%) and iso-C<sub>15:0</sub> (10.6%); smaller amounts of C<sub>17:1ω9c</sub> (8.1%), C<sub>18:0</sub> (6.8%), anteiso-C<sub>15:0</sub> (6.8%), iso-C<sub>17:0</sub> (5.0%), anteiso-C<sub>17:0</sub> (4.3%), anteiso-C<sub>16:0</sub> (3.7%), iso-C<sub>14:0</sub> (2.5%), C<sub>15:0</sub> (2.5%), C<sub>18:1ω9c</sub> (2.5%) and C<sub>16:0</sub> (1.9%) were also found. The G+C content of the genomic DNA was 66.8 mol%. The chemotaxonomic properties of strain MS426<sup>T</sup> were similar to those of *V. lutea* YIM 013<sup>T</sup> and *V. gifhornensis* DSM 44337<sup>T</sup> [A1γ as the peptidoglycan type, phospholipid type II, fatty acid type 2d (Kroppenstedt, 1985) and MK-9(H<sub>4</sub>) as the predominant menaquinone], which indicated that strain MS426<sup>T</sup> belongs to the genus *Verrucosispora* (Rehms *et al.*, 1998). However, strain MS426<sup>T</sup> could be differentiated from *V. lutea* YIM 013<sup>T</sup> and *V. gifhornensis* DSM 44337<sup>T</sup> on the basis of the composition of whole-cell sugars, phospholipids and major fatty acids (Table 1).

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were performed as described by Li *et al.*

**Table 1.** Phenotypic properties that distinguish strain MS426<sup>T</sup> from type strains of the genus *Verrucosispora*

Strains: 1, *Verrucosispora sediminis* sp. nov. MS426<sup>T</sup>; 2, *V. lutea* YIM 013<sup>T</sup>; 3, *V. gifhornensis* DSM 44337<sup>T</sup>. Data for reference strains were taken from Rheims *et al.* (1998) and Liao *et al.* (2009). +, Positive; w, weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3
Spores			
Arrangement	Single, clusters	Single, pairs, clusters	Single, pairs, clusters
Ornamentation	Warty	Smooth	Smooth, warty, hairy
Aerial mycelium	Sparse	Sparse	Absent
Growth with NaCl (%)			
Range	0–6	0–7	0–4
Optimum	3.5	ND	ND
Growth temperature (°C)			
Range	20–45	20–45	20–40
Optimum	28–37	28	35
Production of:			
Nitrite from nitrate	+	+	–
H <sub>2</sub> S	–	–	ND
Carbon source utilization			
D-Fructose	–	+	–
Lactose	+	+	–
Melibiose	+	+	–
D-Ribose	+	+	–
Trehalose	+	–	+
Dulcitol	–	+	–
Glycerol	+	+	–
Salicin	+	–	+
Nitrogen source utilization			
L-Alanine	+	–	w
L-Glutamic acid	+	–	+
L-Phenylalanine	+	–	+
L-Valine	+	–	w
Major cell-wall sugars*	GlcN, Glu, Man	Xyl, Glc	Man, Xyl
Polar lipids†	PE, DPG, PIM, PI, PL	PE, DPG, PIM, PI, PL	PE, DPG, PIM, PS, PL
Major fatty acids (>10%)‡	C <sub>17:0</sub> , i-C <sub>16:0</sub> , i-C <sub>15:0</sub>	i-C <sub>16:0</sub> , i-C <sub>15:0</sub>	i-C <sub>16:0</sub> , i-C <sub>15:0</sub> , ai-C <sub>17:0</sub>
DNA G + C content (mol%)	66.8	69.3	70

\*Glc, Glucose; GlcN, glucosamine; Man, mannose; Xyl, xylose.

†DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; PS, phosphatidylserine; PL, unknown phospholipid.

‡ai, anteiso-branched; i, iso-branched.

(2007). The 16S rRNA gene sequence of strain MS426<sup>T</sup> (1458 nt) was determined and compared with sequences in the public databases. For an initial taxonomic classification, a combination of the results from BLAST (Altschul *et al.*, 1997; <http://www.ncbi.nlm.nih.gov/blast>), the EzTaxon server version 2.1 (Chun *et al.*, 2007; <http://147.47.212.35:8080/index.jsp>) and the classifier program of the Ribosomal Database Project II (<http://rdp.cme.msu.edu/index.jsp>) was used. For phylogenetic analysis, a similarity matrix for 16S rRNA gene sequences from strain MS426<sup>T</sup> and related taxa in the family *Micromonosporaceae* was generated after multiple alignments with CLUSTAL X (Thompson *et al.*, 1997). Three tree construction methods were employed: neighbour-joining trees (Saitou & Nei,

1987) were calculated by using distances corrected according to Kimura's two-parameter model (Kimura, 1980, 1983) with TREECON version 1.3b (Van de Peer & De Wachter, 1994), a maximum-parsimony tree (Fitch, 1971) was constructed with MEGA version 4.0 (Tamura *et al.*, 2007) and a maximum-likelihood tree (Felsenstein, 1981) was constructed with the online version of PHYML (Guindon *et al.*, 2005). The topologies of the trees were evaluated with bootstrap analysis (Felsenstein, 1985), based on 1000 resamplings for the neighbour-joining and maximum-parsimony trees and 500 resamplings for the maximum-likelihood tree. DNA–DNA hybridization was carried out in duplicate following the procedure described previously (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992).

A preliminary comparison between the almost-complete 16S rRNA gene sequence of strain MS426<sup>T</sup> and sequences in public databases indicated that strain MS426<sup>T</sup> belonged to the branch corresponding to the family *Micromonosporaceae*. The neighbour-joining tree (Fig. 1) indicated that strain MS426<sup>T</sup> was closely associated with *V. lutea* YIM 013<sup>T</sup> and *V. giffhornensis* HR1-2<sup>T</sup>, with sequence similarity of 99.01 and 98.89%, respectively. It is clear from the phylogenetic analyses that strain MS426<sup>T</sup> belongs to the genus *Verrucosispora*. DNA–DNA relatedness between strain MS426<sup>T</sup> and *V. lutea* YIM 013<sup>T</sup> and *V. giffhornensis* DSM 44337<sup>T</sup> was (mean  $\pm$  SD) 53.0  $\pm$  5 and 42.0  $\pm$  5%, respectively, well below the 70% cut-off point recommended for assignment of strains to genomic species (Wayne *et al.*, 1987).

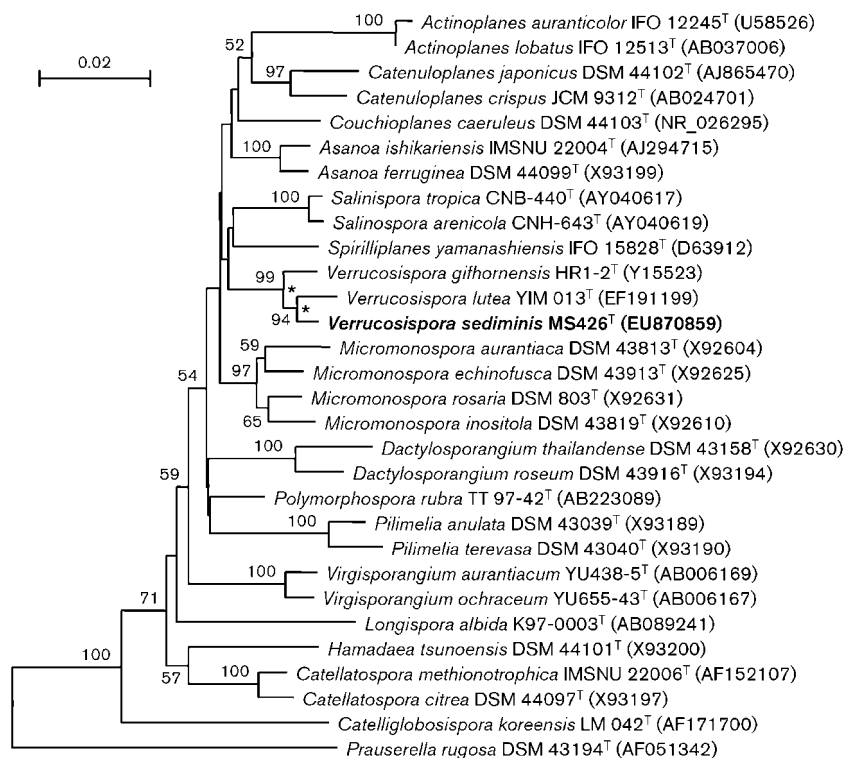
Therefore, on the basis of phenotypic, chemotaxonomic and phylogenetic data and genotypic distinctiveness, strain MS426<sup>T</sup> should be placed in the genus *Verrucosispora* as the type strain of a novel species, for which we propose the name *Verrucosispora sediminis* sp. nov.

#### Description of *Verrucosispora sediminis* sp. nov.

*Verrucosispora sediminis* (se.di.mi'nis. L. gen. n. *sediminis* of the sediment).

Aerobic, Gram-positive-staining, non-motile actinomycete with extensively branched substrate hyphae (0.2  $\mu$ m in diameter). Sparse aerial hyphae (deep brown; ISCC-NBS no. 56) are observed on oatmeal agar (ISP 3 medium prepared with seawater). Substrate mycelium (soft orange; ISCC-NBS no. 50) is well developed and forms single or clustered spores. The spore surface is warty and the aerial

spore mass is white (ISCC-NBS no. 263). No diffusible pigments are produced. Good growth occurs on medium 6B agar, ISP 2 agar and modified Bennett's agar; moderate growth occurs on oatmeal agar and inorganic salts-starch agar. On medium 6B agar prepared without seawater, grows at 20–45 °C (optimum 28–37 °C), pH 3.5–10.0 (optimum pH 5.0–7.5) and 0–6% (w/v) NaCl (optimum 3.5% NaCl). Positive for nitrate reduction and negative for H<sub>2</sub>S and melanin production and arbutin hydrolysis. L-Arabinose, arbutin, D-glucose, melibiose, *meso*-erythritol, glycerol, inositol, lactose, melezitose, D-ribose, salicin and trehalose are utilized as sole carbon and energy sources only when seawater is added to the medium. L-Alanine and L-serine are utilized as sole nitrogen sources in the absence of seawater salts, but L-glutamic acid, L-glycine, DL-methionine, L-methionine, L-phenylalanine, L-tryptophan and L-valine are not. Starch and casein are degraded, but elastin, guanine, hypoxanthine and uric acid are not. Growth occurs in the combined presence of ( $\mu$ g ml<sup>-1</sup>) erythromycin (32) and lincomycin hydrochloride (64), but not in the combined presence of cefoxitin (64), cephaloridine hydrochloride (32), chlortetracycline hydrochloride (4), doxycycline hydrochloride (8), erythromycin (64), fusidic acid (16) and rifampicin (32); however, when seawater salts are included in the medium, grows well in the combined presence of cephaloridine hydrochloride (32), chlortetracycline hydrochloride (8) and erythromycin (64). The major whole-cell sugars are glucosamine, glucose and mannose. The predominant menaquinone is MK-9(H<sub>4</sub>). The phospholipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phospho-



**Fig. 1.** Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequences showing the relationships between strain MS426<sup>T</sup> and representatives of the family *Micromonosporaceae*. Bootstrap values (>50%) based on 1000 resamplings are shown at branch nodes. Asterisks indicate that the corresponding nodes were also recovered with the maximum-likelihood and maximum-parsimony algorithms. Bar, 0.02 substitutions per nucleotide position.

tidylinositol mannosides and some unknown phospholipids. The major cellular fatty acids (>10%) are C<sub>17:0</sub>, iso-C<sub>16:0</sub> and iso-C<sub>15:0</sub>. The DNA G+C content of the type strain is 66.8 mol% (T<sub>m</sub>).

The type strain is MS426<sup>T</sup> (=CGMCC 4.3550<sup>T</sup> =JCM 15670<sup>T</sup>), isolated from a deep marine sediment in the South China Sea.

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