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Verrucosispora fiedleri sp. nov., an actinomycete isolated from a fjord sediment which synthesizes proximicins

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Running title: *Verrucosispora fiedleri* sp. nov.

The GenBank accession number for the 16S rRNA gene sequence of *Verrucosispora fiedleri* MG-37^T is JQ423921.

Abstract

A novel filamentous actinobacterial strain, designated MG-37^T, was isolated from a Norwegian fjord sediment, and examined using a polyphasic approach. The organism had chemotaxonomic and morphological properties consistent with its classification in the genus *Verrucosispora* and formed a distinct phyletic line in the 16S rRNA *Verrucosispora* gene tree. It was most closely related to *Verrucosispora* maris DSM 45365^T (99.5% 16S rRNA gene similarity) and *Verrucosispora gifhornensis* DSM 44337^T (99.4% 16S rRNA gene similarity), but was distinguished from these strains based on low levels of DNA:DNA relatedness, (∽ 56 and ∽ 50%, respectively). It was readily delineated from all of the type strains of *Verrucosispora* species based on a combination of phenotypic properties. Isolate MG-37^T (= NCIMB …… = NRRL-B ……) should therefore be classified as the type strain of a novel species of *Verrucosispora* for which the name *Verrucosispora fiedleri* is proposed.

Key words: *Verrucosispora fiedleri*. Polyphasic taxonomy. Marine sediment. Actinomycetes.

Introduction

The genus *Verrucosispora* forms a distinct branch in the 16S rRNA *Micromonosporaceae* gene tree, and can be distinguished from other genera classified in this family by using chemotaxonomic and morphological features (Goodfellow et al. 2012; Xi et al. 2012) and genus-specific primers (Xie et al. 2011). The taxon currently encompass six species, *Verrucosispora gifhornensis* (Rheims et al. 1998), the type species, *Verrucosispora lutea* (Liao et al. 2009), *Verrucosispora maris* (Goodfellow et al. 2012), *Verrucosispora qiuiae* (Xi et al. 2012), *Verrucosispora sediminis* (Dai et al. 2010) and *Verrucosispora wenchangensis* (Xie et al. 2012), the single members of which can be separated using a combination of

genotypic and phenotypic procedures (Goodfellow et al. 2012; Xi et al. 2012; Xie et al. 2012). The type strains of these species were isolated from a peat bog (*V. gifhornensis*), mangrove soils (*V. lutea*, *V. qiuiae* and *V. wenchangensis*) and deep-sea sediments (*V. maris* and *V. sediminis*).

Verrucosispora strains are the focus of considerable interest as they are the source of new bioactive compounds, as shown by the discovery of the diterpines, gifhornenoles A and B from *V. gifhornensis* (Shirai et al. 2010), the polycyclic polyketides, abyssomicins A to H (Bister et al. 2004; Riedlinger et al. 2004; Keller et al. 2007a, b) from *V. maris* (Goodfellow et al. 2012) and aminofuran antibiotics, proximicins A to C from *Verrucosispora* strain MG-37 (Fiedler et al. 2008). Proximicin A was detected in parallel from the type strain of *V. maris* (Schneider et al. 2008). The characteristic structural element of the proximicins is 4-amino-furan-2 carboxylic acid, a previously unknown γ-amino acid. Proximicins show weak antibacterial activity but have a strong cytostatic effect on various human tumor cell lines (Fiedler et al. 2008). The whole genome sequence of *V. maris* AB-18-032^T contains around 23 biosynthetic gene clusters that encode for the production of known or predicted secondary metabolites (Roh et al. 2011).

Partial characterization of strain MG-37^T showed that it had chemotaxonomic and morphological properties characteristic of the genus *Verrucosispora,* was most closely related to the type strain of *V. gifhornensis*, but could be distinguished from the latter using a few phenotypic properties (Fielder et al. 2008). The aim of the present study was to build upon these initial results by comparing isolate MG-37^T with the type strains of *Verrucosispora* species in a polyphasic taxonomic analysis. The resultant dataset showed that the isolate

represented a new centre of taxonomic variation in the genus for which the name *Verrucosispora fiedleri* sp. nov. is proposed.

Materials and methods

Strains and cultural conditions

Strain MG-37^T was recovered from sediment collected from the Raune Fford, Norway (N60^o 15. 398, E 5^o 08237) at a depth of 250 metres, as described previously (Fiedler et al. 2008). The organism was isolated on a …… plate which had been inoculated with a suspension of the sediment sample then incubated at 30^{o}C for 14 days. The isolate and the type strains of *Verrucosispora* species (Table 2) were maintained on yeast extract-malt extract agar (ISP medium 2; Shirling and Gottlieb 1966) at 28^{o}C and as suspensions of hyphal fragments in glycerol (20%, v/v) at -20^{o}C and -80^{o}C. Biomass for the chemotaxonomic and molecular systematic studies carried out on the isolate and *V. gifhornensis* DSM 44337^T was prepared as described earlier (Goodfellow et al. 2012), as was the biomass of *V. maris* DSM 45365^T needed for the DNA:DNA relatedness study.

PCR amplification using genus-specific primers

Isolate MG-37^T and the type strains of *Verrucosispora* species were examined for their ability to generate diagnostic amplification products when probed with the genus-specific 16S rRNA primers S-G-Verr-0195-a-S-20 and S-G-Verr-1152-a-A-18 as described by Xie et al. (2011) albeit with the annealing temperature adjusted to 64^{o}C.

16S rRNA gene sequencing analyses

Isolation of chromosomal DNA, PCR amplification and direct sequencing of PCR products of isolate MG-37^T were carried out, as described by Kim et al. (2002). The resultant almost complete 16S rRNA gene sequence (1429 nucleotides [nt]) was aligned manually with corresponding gene sequences of the type strains of *Verrucosispora* species and the type strains of the type species of representative genera classified in the family *Micromonosporaceae*, retrieved from the DDBJ/EMBL/GenBank databases, using the PHYDIT program (http://plaza.snu.ac.kr/~jchun/phydit/). Phylogenetic trees were inferred using the maximum-likelihood (Felsenstein 1981), maximum-parsimony (Kluge and Farris 1969) and neighbor-joining (Saitou and Nei 1987) tree-making algorithms from the PHYLIP package (Felsenstein 1993).

Chemotaxonomy and morphology

The isolate was examined for chemotaxonomic and morphological properties characteristic for the genus *Verrucosispora* (Goodfellow et al. 2012; Xi et al. 2012). The arrangement of hyphae and spores were examined on an oatmeal agar (ISP medium 3; Shirling and Gottlieb 1966) plate which had been incubated at 28^{o}C for 2 weeks. Spore morphology and ornamentation were observed by scanning gold-coated dehydrated specimens taken from the oatmeal agar plate and examined using a scanning electron microscope (Cambridge Stereoscan 240 instrument), as described by O'Donnell et al. (1993). Cultural properties were determined using ISP media (Shirling and Gottlieb 1966) after incubation at 28^{o}C for 14 days. Standard procedures were used to determine the isomers of diaminopimelic acid (A_2pm; Staneck and Roberts 1974), the acyl type of murein (Uchida et al. 1999),

menaquinones (Minnikin et al. 1984), sugars (Schaal 1985) and polar lipids (Minnikin et al. 1984) and to establish if it contained mycolic acids (Minnikin et al. 1975), in all cases using appropriate controls. The DNA base composition of the isolate was determined after Gonzalez and Sait-Jimenez (2002).

DNA:DNA pairing

The DNA:DNA relatedness value (Δ T*m*) between isolate MG-37^T and *V. maris* AB-18-032^T and *V. gifhornensis* DSM 44337^T, its nearest phylogenetic neighbours, was determined using a fluorimetric method (Gonzalez and Saiz-Jimenez 2005): the optimum temperature for renaturation (Tm) was calculated using TOR-0.51 (% GC) + 47. The melting temperature (Tm) at which 50% of the initial double stranded denatured into single-stranded DNA for isolate MG-37^T g DNA and the isolate MG-37^T / *V. maris* and MG-37^T *V. gifhornensis* hybrid DNA preparations was compared and the differences (Δ Tm) calculated.

Phenotypic tests

Isolate MG-37^T and the type strains of the *Verrucosispora* species were examined, in duplicate, for a broad range of phenotypic tests, as described previously (Goodfellow et al. 2012). All of the media were incubated at 28^{o}C for 2-3 weeks, apart for the temperature tests, following the addition of a standard inoculum equivalent to 2.5 on the McFarland scale.

Results

16S rRNA sequencing and DNA:DNA relatedness studies

It can be seen from Fig. 1 that isolate MG-37^T was recovered in the *Verrucosispora* 16S rRNA gene clade, a result that is underpinned by all of the tree-making algorithms and by a 99% bootstrap value. The isolate formed a subclade in the 16S rRNA gene tree together with the type strains of *V. gifhornensis* and *V. maris*; a relationship which was supported by all of the tree-making algorithms and by a 96% bootstrap value. The organism shared its highest 16S rRNA similarity with *V. maris* DSM 45365^T, namely 99.5%, a value which corresponded to 7 nt differences at 1414 locations; 1 nt difference was in variable region V1, 2 in variable region V2, 1 in variable region V6, and the other 3 nt in non-variable regions. The corresponding 16S rRNA similarity with *V. gifhornensis* DSM 44337^T was 99.4%, which was equivalent to 8 nt differences at 1406 sites; 2 nt differences were in variable region V1, 3 in variable region V2, 1 in the variable region V6, 1 in the variable region V9, and the final one 1 in the non-variable region. The 16S rRNA similarities with the remaining *Verrucosispora* type strains fell within the range 96.1 to 99.0 %, values corresponding to between 56 and 14 nt differences respectively. The lowest similarity was shown against the type strain of *V. qiuiae* which fell outside the *Verrucosispora* clade.

The Δ Tm between isolate MG-37^T g DNA and MG-37^T *V. maris* hybrid DNA, and MG-37^T *V. gifhornensis* hybrid DNA were 8.6^{o}C and 6.6^{o}C respectively (Fig. 2); equivalent to DNA:DNA relatedness values of about 56 and 50%, respectively (Gonzalez and Saiz-Jimenez 2005), a recording well below the 70% cut-off point for the circumscription of bacterial species according to Wayne et al. (1987).

Genus-specific primers

Isolate MG-37^T and all of the *Amycolatopsis* type strains, apart from *V. qiuiae* R11147^T, gave the ≈960 base pair amplification produce with primers S-G-Verr-0195-a-S-20 and S-G-Verr-

1152-a-A-18. Corresponding *in-silico* testing with CLUSTAL X 1.81 showed that neither of the primers matched with the appropriate sections of the 16S rRNA gene of the *V. qiuiae* strain indicating that the V2 and V6 variable region of this organism were different from those of the other tested strains.

Chemotaxonomic, cultural, morphological and phenotypic characteristics

Isolate MG-37^T formed an extensively branched, orange substrate mycelium which carried single, warty ornamented spores on long sporophores on oatmeal agar, as exemplified in Fig. 3. It contained a mixture of *meso*- and hydroxyl A_2pm in whole-organism hydrolysates; N-glycolyl muramic acid in the wall peptidoglycan; mannose and xylose as diagnostic sugars; MK-9 (H_4), MK-9 (H_6) and MK-10 (H_4) as predominant isoprenologues in a ratio of 27: 10: 2 (Fig. S1); diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol mannosides as major polar lipids (Fig. 4), and trace amounts of phosphatidylinositol, phosphatidylserine and an unknown polar lipid; but lacked mycolic acids. The G+C content of the DNA was 72.0 mol%, using the fluorimetric method (Fig. 5). In addition, like its closest phylogenetic neighbors, the isolate formed light to dark coloured orange pigments on ISP media, it grew particularly well on tryptic- yeast extract and yeast extract-malt extract agars (Table 1).

Identical results were recorded for the duplicated phenotypic tests. It can be seen from Table 2 that isolate MG-37^T can be distinguished readily from all of the type strains of *Verrucosispora* species by a broad range of phenotypic properties. All of the organisms degraded adenine and xanthine; grew on dextran, D-fucose, D-maltose, D-mannose, D-melezitose, D-sucrose, D-trehalose and xylitol as sole carbon sources; used gelatin, L-

leucine, DL-methionine, L-ornithine, DL-phenylalanine, L-proline, L-threonine, L-tyrosine and urea as sole carbon and nitrogen sources; L-aspartic acid as a sole nitrogen source, and grew at 30 and 37^{o}C, from pH 7.0 to 9.0 and in the presence of novobiocin (8 μg ml^{-1}). In contrast, none of the strains degraded casein, gelatin or hypoxanthine; used *meso*-inositol as a sole carbon source; L-glutamic acid or L-norvaline as carbon and nitrogen sources; grew at 10 or 45^{o}C, at pH 6.0 or in the presence of impenem (8 μg ml^{-1}), neomycin (8 μg ml^{-1}), streptomycin (54 μg ml^{-1}), tetracycline (8 μg ml^{-1}), tyloxin (8 μg ml^{-1}) or vancomycin (2 μg ml^{-1}).

Discussion

The results of the present study confirm and extend those reported by Fiedler et al. (2008) in showing that the proximicin-producing strain MG-37^{T} has chemotaxonomic, molecular and morphological properties in line with its classification in the genus *Verrucosispora* (Rheims et al. 1998; Goodfellow et al. 2012; Xi et al. 2012). The genotypic and phenotypic data show that the organism can be delineated readily from the type and only representative strains of *Verrucosispora* species, notably from its nearest phylogenetic neighbours, *V. gifhornensis* DSM 44337^{T} and *V. maris* DSM 45365^{T}. It is, therefore, proposed that strain MG-37^{T} be recognised as a new *Verrucosispora* species, *Verrucosispora fiedleri* sp. nov. Further comparative studies are needed to determine the taxonomic status of *V. qiuiae* as the type and only representative of this organism falls outside the *Verrucosispora* gene clade and does not give the genus-specific amplification product with the *Verrucosispora* primers.

Description of *Verrucosispora fiedleri*

Verrucosispora fiedleri (fi. ed. le' ri. N.L. gen. masc. *fiedleri*, after Hans-Peter Fiedler in recognition of his contributions to the search and discovery of new antibiotics from actinomycetes).

The description is based on data taken from this and an earlier study (Fiedler et al. 2008). Aerobic, Gram-positive, non-acid-fast actinomycete which forms an extensively branched light to dark orange pigmented substrate mycelium on ISP media. Neither aerial hyphae nor spore vesicles are formed. Single, non-motile, spores with warty surfaces are borne on long sporophores on oatmeal agar. Grows at 28 and 37^{o}C, from pH 7 to 10, and in the presence of up to 2.5%, w/v NaCl. Additional phenotypic properties are cited in the text and in Table 2. The peptidoglycan is rich in *meso*- and hydroxyldiaminopimelic acid and contains N-glycolated muramic acid. Mannose and xylose are the characteristic sugars in whole organism hydrolysates and tetrahydrogenated menaquinone with nine isoprene units is the major isoprenologue. The phospholipid pattern contains diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol as major components. The G+C content of the DNA is 72.0%. Produces proximicins A, B and C.

The type and only strain, MG-37^{T} (NCIMB …… = NRRL B …… was isolated from a sediment sample collected from the Raune fjord in Norway.

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Table 1. Growth and cultural characteristics of isolate MG-37^T and the type strains of the most closely related *Verrucosispora* species

Medium	Strains		
	Isolate MG-37^T	*V. gifhornensis*† DSM 44337^T	*V. maris*† DSM 45365^T
Glycerol-asparagine agar (ISP medium 5)			
Growth	+	++	++
Colour of substrate mycelium	Light orange	Light orange	Light orange
Inorganic salts-starch agar (ISP medium 4)			
Growth	+++	++	+++
Colour of substrate mycelium	Orange	Light orange	Orange
Oatmeal agar (ISP medium 3)			
Growth	+++	++	+++
Colour of substrate mycelium	Orange	Orange	Orange
Peptone-yeast extract agar (ISP medium 6)	++ Orange	+ Orange	+ Orange
Tryptic-yeast extract agar (ISP medium 1)			
Growth	+++	+++	+++
Colour of substrate mycelium	Orange	Orange	Dark orange brown
Tyrosine agar (ISP medium 7)			
Growth	++	++	+
Colour of substrate mycelium	Orange	Orange	Orange
Yeast extract-malt extract agar (ISP medium 2)			
Growth	+++	+++	+++
Colour of substrate mycelium	Dark orange	Orange	Orange

†, Data taken from Goodfellow et al. (2012).
None of the strains formed aerial hyphae or produced diffusible pigments.
Key: +++, abundant growth; ++, moderate growth; +, poor growth.

Table 2. Phenotypic properties which distinguish between isolate MG-37^T and the type strains of *Verrucosispora* species

Characteristic	Isolate MG-37^T	*V. gifhornensis* DSM 44337^T	*V. lutea* YIM 013^T	*V. maris* DSM 45365^T	*V. sediminis* MS 426^T	*V. qiuiae* RtIII47^T	*V. wenchangensis* 234402^T
Spore arrangement	Single	Single, pairs, clusters*	Single, pairs clusters*	Single clusters+	Single clusters*	Single*	Single*
Spore ornamentation	Warty	Hairy, smooth, warty*	Smooth*	Warty*	Warty*	Warty*	Warty†
Biochemical tests:							
Aesculin hydrolysis	-	+	-	-	-		-
Allantoin hydrolysis	+	+	+	+	+		-
Arbutin hydrolysis	-	+	-	+	+		-
H_2S production	-	-	+	-	+		+
Nitrate production	-	-	+	-	+	-	+
Urea hydrolysis	+	+	-	-	-		+
Degradation tests:							
Elastin	+	-	+	-	-		+
Guanine	_	+	-	+	+	+	+
Starch	-	+	-	+	+		-
L-Tyrosine	+	+	-	+	+	-	+
Xylan	-	-	-	-	+		+
Growth on sole carbon soures:							
Adonitol, D-arabitol	-	-	+	+	+		+
Amygdalin	-	+	-	+	-		+
L-Arabinose	_	+	+	-	-		-
Arbutin, erythritol, maltitruse	-	+	-	+	-		-
Cellobiose, ribose	-	-	+	-	+		-

Characteristic	Isolate MG-37^T	*V. gifhornensis* DSM 44337^T	*V. lutea* YIM 013^T	*V. maris* DSM 45365^T	*V. sediminis* MS 426^T	*V. qiuiae* RtIII47^T	*V. wenchangensis* 234402^T
Dulcitol	-	+	+	+	-		-
Ethanol	+	+	-	+	-		-
Fructose	-	-	+	-	-	+	+
L-Fucose, glycerol, lactose	+	-	+	+	+	-	+
Galactose, rhamnose	-	-	+	-	+	+	+
Mannitol	-	-	+	+	+	+	-
Melibiose, turanose	+	-	+	+	+		+
α-Methyl-D-glucoside	-	+	+	+	+		-
Raffinose	+	+	-	-	+		+
Salicin	+	-	-	-	+		+
Sorbitol	-	-	+	-	+		+
Trehalose	+	+	+	+	-	+	+
Xylose	+	+	-	+	+	+	-
Growth on sole nitrogen sources:							
L-Aalanine	+	-	-	+	+	+	+
L-Arginine	+	-	+	+	+		+
L-Glutamic acid	-	+	-	-	-		-
L-Histidine	+	+	+	+	+		+
L-Methionine	+	+	-	+	+		-
L-Phenylalanine	+	-	-	+	+	-	-
L-Serine	-	+	-	-	-		-
L-Valine	+	+	-	+	+		-

Characteristic	Isolate MG-37^T	*V. gifhornensis* DSM 44337^T	*V. lutea* YIM 013^T	*V. maris* DSM 45365^T	*V. sediminis* MS 426^T	*V. qiuiae* RtIII47^T	*V. wenchangensis* 234402^T
Growth on sole carbon and nitrogen sources:							
Acetamide	+	+	-	+	-		-
L-Asparagine	+	+	+	+	-		+
L-Cysteine	+	+	-	+	-		-
Glycine	+	+	-	+	+		-
L-Histidine	+	+	+	+	-		+
L-Isoleucine	+	+	-	+	-		-
Sensitivity to antibiotics (Γ g ml):							
Ampicillin (8), Chloramphenicol (8)	+	+	-	+	-		-
Cephaloridine (4)	+	+	+	+	-		
Ciprofluxacin (4)		+	+	+	-		+
Clindamycin (8), Lincomycin (8)	-	-	+	+	+		-
Erythromycin (10)	+	+	+	+	+		-
Gentamicin (8)	-	-	+	-	-		+
Oxytetracycline (16)	-	-	-	-	+		-
Rifampicin (16)	+	+	+	+	-		-
Tolerance tests:							
Growth in presence of 5%, w/v NaCl		+	-	+	+	+	+
pH range for growth		7-9	7-10	7-10	7-10	0-10	7-10
DNA G+C content ml%		72.0[a]/70.0[b]	69.3[c]	69.5[a]/70.9[c]	66.8[d]	72.0[e]	69.2[f]

Key: +, positive; -, negative; ND, not determined.
* These data were taken from Rheims et al. (1988), Liao et al. (2009), Goodfellow et al. (2012), Dai et al. (2010), Xi et al (2012) and Xie et al. (2012), respectively.
†, unpublished data (Kui Hong, unpublished data); [a] Determined by fluorimetry and thermal denaturation (Goodfellow et al. 2012); [b.] Determined by whole genome sequencing (Roh et al. 2011); [c.] Determined by HPLC (Liao et al. 2009); [d.] Determined by spectrophotometric method (Dai et al. 2010); [e.] Determined by a modified fluorimetric microwell plate method (Xi et al. 2012); [f.] Determined by using melting profiles in microplates (Xie et al. 2012)

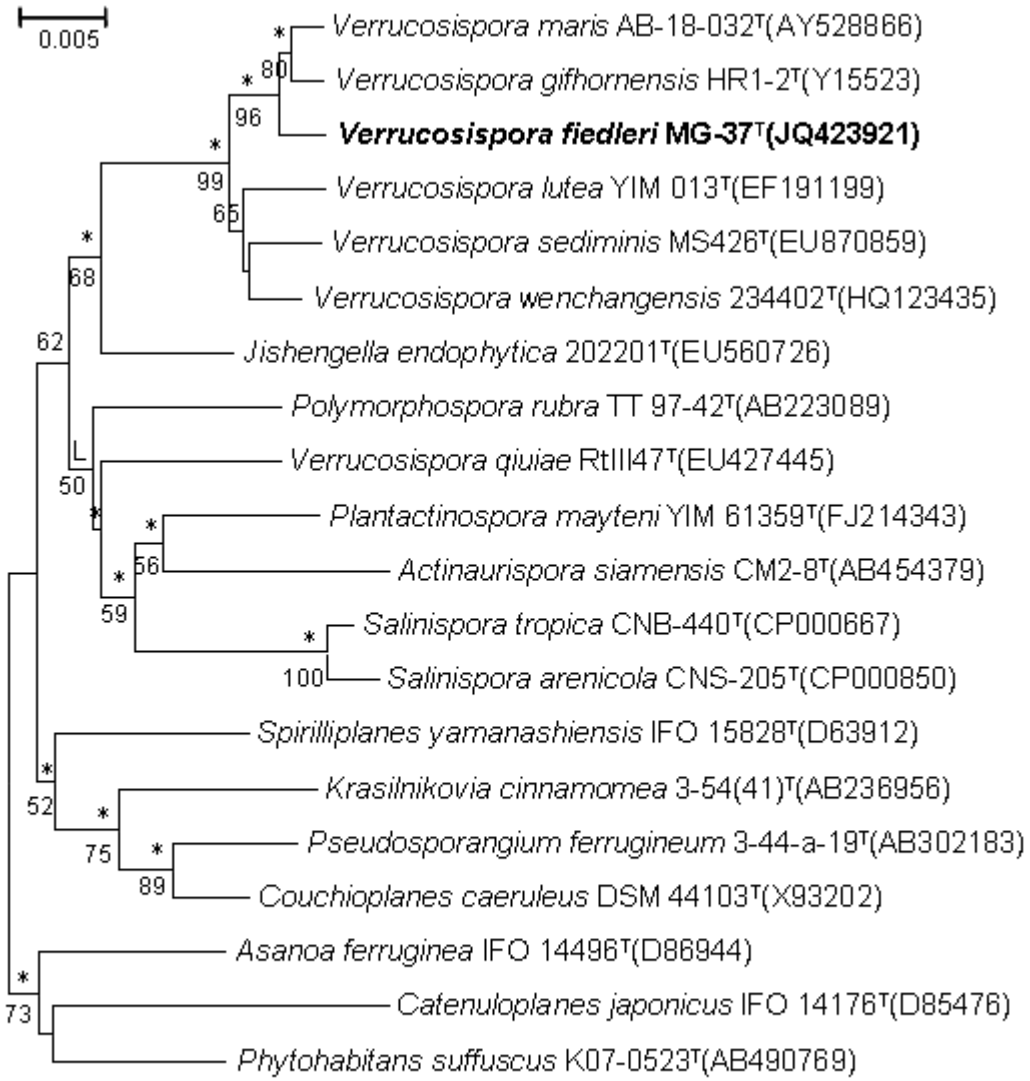

Fig. 1. Neighbor-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between isolate MG-37^T and representatives of genera classified in the family *Micromonosporaceae*. Asterisks indicate branches of the tree that were also found using the máximum-likelihood and máximum-parsimony tree-making algorithms. The L indicates a node that was also recovered in the máximum-likelihood tree. The numbers at the nodes indicate levels of bootstrap support (%) based on a neighbor-joining analysis of 1000

resampled datasets; only values above 50% are given. T, type strain; Bar, 0.005 subtitutions per nucleotide position.

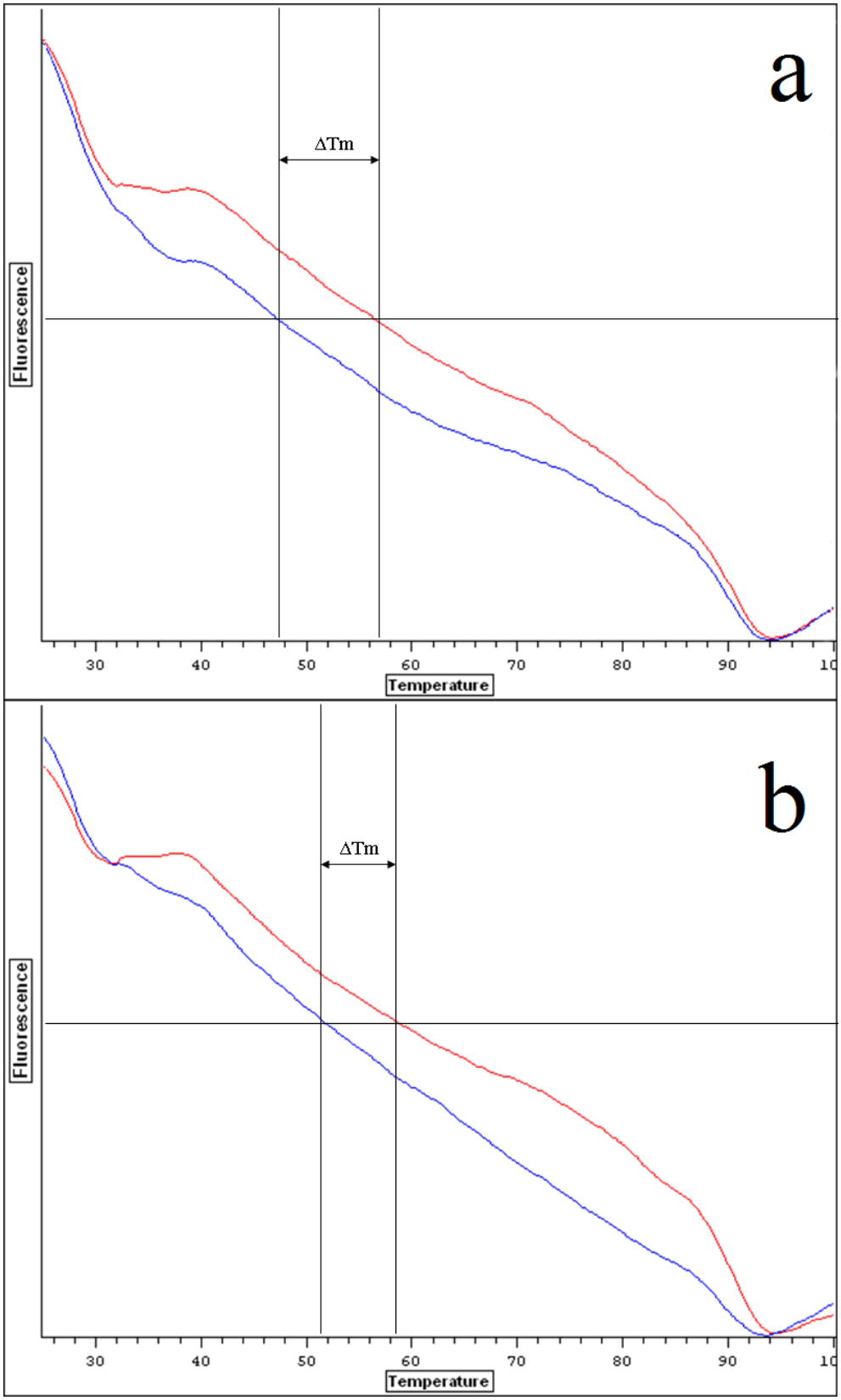

Fig. 2a. Thermal denaturation of genomic DNA from *Verrucosispora* isolate MG-37^T (red line) and the *Verrucosispora* isolate MG-37^T / *V. maris* hybrid DNA mix (blue line). The calculated Δ T*m* is 8.6^{o}C; 2b. Thermal denaturation of genomic DNA from *Verrucosispora* isolate MG-37^T (red line) and the *Verrucosispora* isolate MG-37^T / *V. gifhornensis* hybrid DNA mix (blue line). The calculated Δ T*m* is 6.6^{o}C.

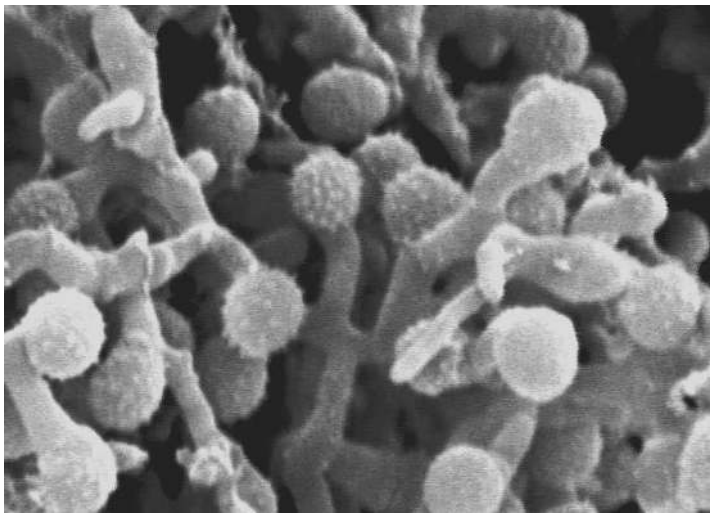

Fig. 3. Scanning electron micrograph of *Verrucosispora* isolate MG-37^T grown on oatmeal agar for 2 weeks at 28^{o}C showing single, ornamented spores borne on long sporophores. Bar, μm.

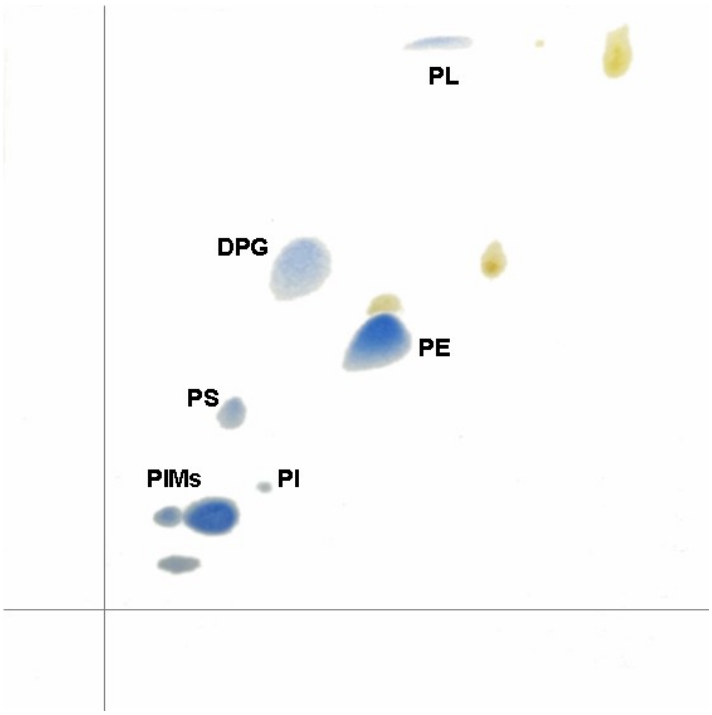

Fig. 4. Two-dimensional thin-layer chromatography of isolate MG-37^{T}. Chloroform: methanol: water (32.5: 12.5: 2.0) was used in the first direction and chloroform: acetic acid: methanol: water (40: 7.5: 6: 2) in the second direction. DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PIMs, phosphatidylinositol mannosides; PS, phosphatidylserine; PI, phosphatidylinositol; and PL, unknown phospholipid.

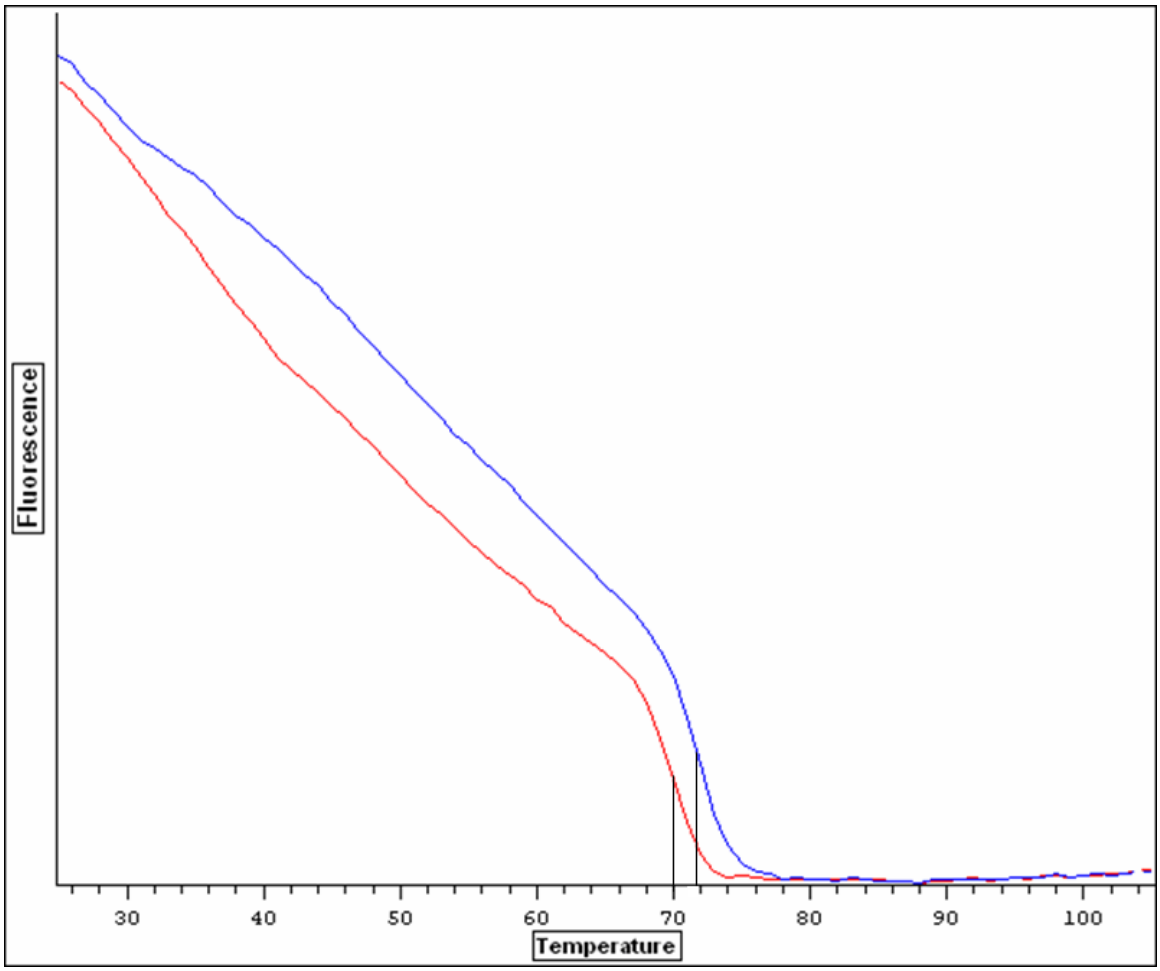

Fig. 5. Fluorimetric estimates of G+C mol% content in *V. maris* (red line) and *Verrucosispora* isolate MG-37^T (blue line). The analysis was carried out after Gonzalez and Saiz-Jimenez (2002). Tm was calculated from the mínimum value of the slope tangent in the melting curve of fluorescence versus temperatura and G+C mol% using the formula % GC = 1.99 Tm – 71.08.

Supplementary Figures and Legends

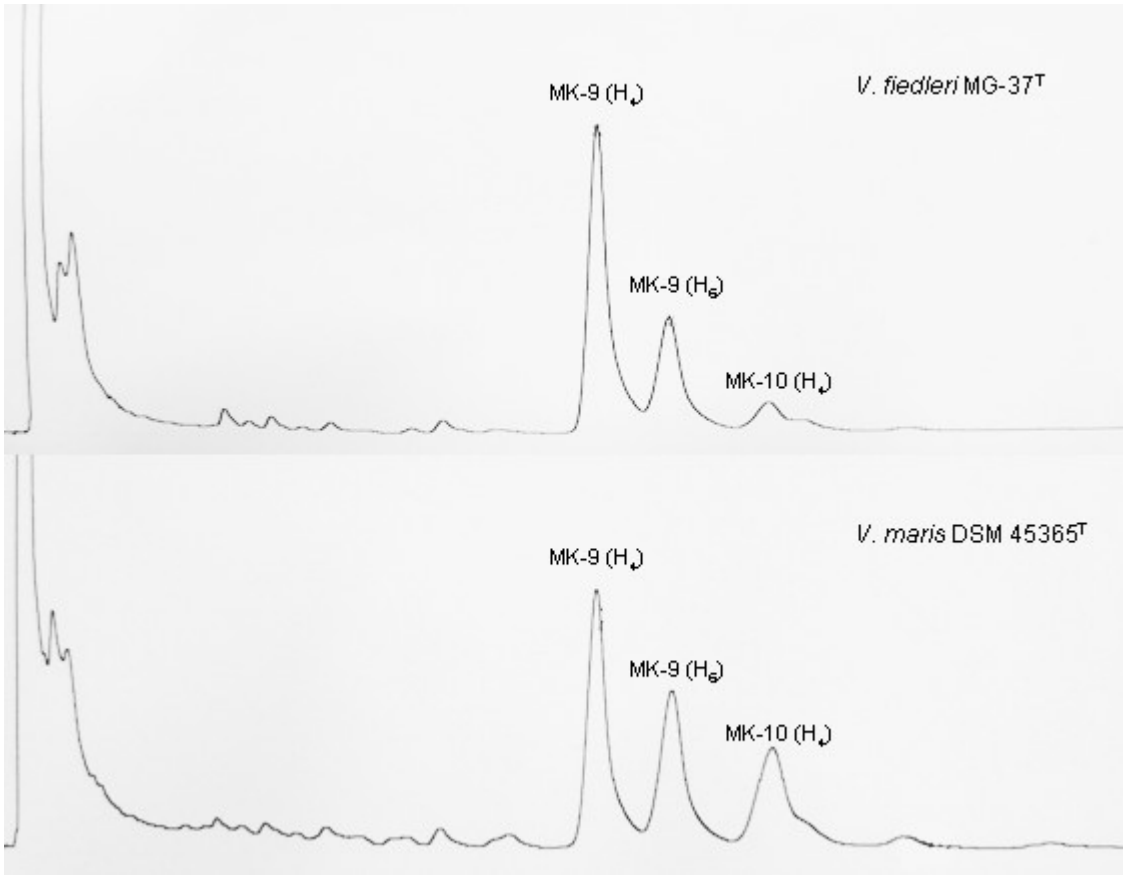

Fig. S1. HPLC profiles of menaquinones of MG-37^T, and *V. maris* was included as a reference. Methonal: isopropanol (3:2) was used as the mobile phase, and the column temperature are 25°C.