Streptomyces pharmamarensis sp. nov. isolated from a marine sediment

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A Gram-stain-positive actinobacterium, strain PM267^T, was isolated from a marine sediment sample in the Mediterranean Sea. The novel strain produced extensively branched substrate and aerial hyphae that carried spiral spore chains. Substrate and aerial mycelia were cream-white and white, respectively. Diffusible pigments were not observed. 16S rRNA gene sequence analysis revealed that strain PM267^T belonged to the genus *Streptomyces* and shared a gene sequence similarity of 97.1 % with *Streptomyces artemisiae* YIM 63135^T and *Streptomyces armeniacus* JCM 3070^T. Values <97 % were obtained with other sequences representing members of the genus *Streptomyces*. The cell wall peptidoglycan contained LL-diaminopimelic acid. MK-9(H₈) was the major menaquinone. The phospholipid pattern included phosphatidylethanolamine as diagnostic lipid (type II). Major fatty acids found were iso- and anteiso- fatty acids. The G + C content of the DNA was 71.2 mol%. The strain was halotolerant and was able to grow in the presence of 9 % (w/v) NaCl (with an optimum of 2 %). On the basis of these results and additional physiological data obtained in the present study, strain PM267^T represents a novel species within the genus *Streptomyces* for which the name *Streptomyces pharmamarensis* sp. nov. is proposed (type strain PM267^T = CECT 7841^T = DSM 42032^T).

The genus *Streptomyces* (Waksman & Henrici, 1943) encompasses aerobic, highly branched filamentous actinomycetes with LL-diaminopimelic acid in their cell walls. Strains of the genus *Streptomyces* attract much interest due to their importance in biotechnological processes and their ability to produce many useful compounds. Although at the time of writing there are more than 500 validly published names (Euzéby, 2011; <http://www.bacterio.cict.fr/s/streptomycetes.html>), the genus is still considered a rich microbial source for new natural compounds and isolation of novel strains is still part of screening programs in pharmaceutical companies. Furthermore, the diversity of marine streptomycetes is still poorly studied and the marine environment is considered a relatively untapped niche.

Strain PM267^T was isolated from a marine sediment sample collected at a depth of 43 m in the Mediterranean Sea (Italy). The micro-organism was isolated from a suspension of the sediment sample spread on nutrient agar plates of the following composition (g l⁻¹): L-asparagine, 2.5; glycerol, 20; NaCl, 5.34; KCl, 0.15;

MgSO₄·7H₂O, 0.1; FeSO₄·7H₂O, 0.12; Na₂SO₄, 7.5; MgCl₂·6H₂O, 2.4; CaCO₃, 0.1; agar, 20; supplemented with cycloheximide (0.71 mM) and nalidixic acid (0.79 mM). The plates were incubated at 28 °C for one month under atmospheric pressure. After this period, colonies were picked and transferred onto agar plates of ATCC Medium 172 (ATCC, 1984). The purified strain was stored as a glycerol suspension (20 % v/v) at -80 °C for long-term maintenance. Morphological features of isolate PM267^T were observed by light microscopy (Leica; CTR MIC) using 5- and 7-day-old cultures grown on ISP 2 (International *Streptomyces* Project medium 2; Shirling & Gottlieb, 1966) agar supplemented with 2 % (w/v) NaCl at 28 °C. Spore arrangement and ornamentation were observed by scanning electron microscopy (DSM 940; Zeiss) using 4-week-old cultures grown on the same medium at 28 °C. The Gram-stain reaction (Doetsch, 1981) was performed on a 4-day-old culture.

Strain PM267^T produced extensively branched substrate and aerial hyphae that carried spiral spore chains (*Spirales*) with multiple turns containing more than 10 spores (Fig. S1, available in IJSEM Online). Spores (1.0–1.2 µm) were oval, non-motile and had a smooth spore-surface. Strain PM267^T was Gram-stain-positive.

The 16S rRNA gene of strain PM267^T was amplified and directly sequenced using the REExtract-N-Amp Plant

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PM267^T is FR693804.

A supplementary table and five figures are available with the online version of this paper.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1320 nt) of isolate PM267^T and phylogenetically related species of the genus *Streptomyces*. Bootstrap values >50% are shown at branch points for 1000 replicates. Asterisks indicate that the corresponding nodes were also recovered in the maximum-likelihood tree. Bar, 5 nt substitutions per 1000 nt.

PCR kit (Sigma) and procedures described previously (Rivas *et al.*, 2003; Trujillo *et al.*, 2007). The contig obtained was compared with those sequences deposited in the public databases and the EzTaxon server (Chun *et al.*, 2007), which was also used to calculate pairwise sequence similarities. The sequences of strain PM267^T and the most closely related taxa were retrieved from GenBank and aligned using the CLUSTAL X program (Thompson *et al.*, 1997). Phylogenetic distances were calculated with Kimura's two-parameter model (Kimura, 1980) and evolutionary trees were inferred using the maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood methods (Felsenstein, 1981). Phylogenetic analyses were performed using the software packages MEGA4 (Tamura *et al.*, 2007) and ARB (Ludwig *et al.*, 2004). Branch support based on 1000 replications was calculated to evaluate the tree topologies.

The 1458 bp sequence corresponding to the 16S rRNA gene of strain PM267^T was compared with sequences deposited in the public databases. The highest levels of sequence similarity were found with sequences related to the genus *Streptomyces*, namely *Streptomyces artemisiae* YIM 63135^T (97.1%), *Streptomyces armeniacus* DSM 43125^T (97.1%), *Streptomyces ferralitis* DSM 41836^T (96.9%) and *Streptomyces cacaoi* subsp. *cacaoi* DSM 40057^T (96.8%). Sequence similarities with other strains of the genus *Streptomyces* were lower than 96.6%. The results of phylogenetic analyses using the maximum-likelihood and maximum-parsimony tree-making algorithms were very similar (Figs S2 and S3). Fig. 1 shows the relationship between strain PM267^T and its closest phylogenetic neighbours. The new isolate formed a cluster with *S. artemisiae* YIM 63135^T, *S. armeniacus* JCM 3070^T and *Streptomyces synnematoformans* S155^T. Branches within the cluster were supported by high bootstrap values between 87 and 100%. These results confirmed the close relationship of strain PM267^T to the genus *Streptomyces*.

Lanoot *et al.* (2004, 2005) reported a good correlation between BOX-PCR profiling and DNA–DNA hybridization in the genus *Streptomyces*, whereby strains showing more than 70% DNA–DNA hybridization values shared almost identical BOX fingerprints. Therefore, this technique was applied to obtain BOX profiles for strain PM267^T and five type strains following the procedure of Trujillo *et al.* (2010) to guarantee differentiation at the genomic level.

A dendrogram based on the Pearson moment coefficient and unweighted-pair group method average (UPGMA) clustering method was reconstructed with the BOX-PCR profiles obtained for the strains studied (Fig. S4). Isolate PM267^T was clearly differentiated from the type strains of *S. artemisiae*, *S. armeniacus*, *S. cacaoi* subsp. *cacaoi*, *S. ferralitis* and *S. synnematoformans*. The G + C content of the DNA determined by the thermal melting point method (Mandel & Marmur, 1968) was 71.2 mol%.

Cultural characteristics of isolate PM267^T and *S. armeniacus* DSM 43125^T, *S. artemisiae* DSM 41953^T, *S. cacaoi* subsp. *cacaoi* DSM 40057^T, *S. ferralitis* DSM 41836^T and *S. synnematoformans* DSM 41902^T were determined on Bennett's (Jones, 1949), oatmeal (ISP 3; Shirling & Gottlieb, 1966), SA1 (Trujillo *et al.*, 2005), yeast-extract malt-extract (ISP 2) and yeast-mannitol (YMA; Vincent, 1970) agars. Media for strain PM267^T were supplemented with 2% (w/v) NaCl.

Strain PM267^T showed abundant growth on SA1 agar, but moderate growth on Bennett's, ISP 2 and ISP 3 and poor growth on YMA. Substrate mycelium colour was cream-white on these media except SA1, on which it was light brown; white aerial mycelium was scantily produced on ISP 2 agar while diffusible pigments were not detected. Differences in cultural characteristics of related type strains are given in Table S1.

Chemotaxonomic analyses were carried out to support the phylogenetic affiliation of strain PM267^T to the genus

Table 1. Cellular fatty acid profiles of strain PM267^T and *Streptomyces artemisiae* DSM 41953^T

Fatty acid	PM267 ^T (ACTIN1)	PM267 ^T (TSBA6)	<i>S. artemisiae</i> DSM 41953 ^T (ACTIN1)	<i>S. artemisiae</i> DSM 41953 ^T (TSBA6)
Saturated straight chain				
14:0				
16:0	1.83	1.83	1.77	1.77
17:0	0.29	0.29		
18:0			0.73	0.73
Unsaturated straight-chain				
16:1 ω 7c	6.16			
17:1 ω 8c	2.43	2.43	0.43	0.43
18:1 ω 9c	0.77	0.77		
18:1 ω 7c				
Saturated branched-chain				
iso-14:0	0.24	0.24	2.73	2.73
iso-15:0	1.70	1.70	2.34	2.34
iso-16:0	10.34	10.34	43.5	43.45
iso-17:0	6.89	6.89	2.40	2.40
iso-18:0			0.6	0.59
anteiso-15:0	1.04	1.04	7.45	7.45
anteiso-17:0	7.78	7.78	12.02	12.02
10-Me 17:0	3.12	3.12		
9?-Me 16:0	25.05		2.4	
Unsaturated branched-chain				
iso-15:1 G	3.62	3.62	0.4	0.4
iso-16:1 G	14.6	14.6	15.60	15.60
iso-18:1 H			1.25	1.25
anteiso-15:1 A	1.12	1.12	0.4	0.4
anteiso-17:1 C	11.9		5.24	
anteiso-17:1 A		11.9		
anteiso-17:1 ω 9c				5.24
Hydroxy				
15:0 2-OH	0.72	0.72		
Summed features*				
3		6.16		0.71
9		25.05		2.4

*Summed feature 3 contains 16:1 ω 7c/16:1 ω 6c; summed feature 9 contains iso-17:1 ω 9c/10-Me 16:0.

Streptomyces. The strain was grown in ISP 2 broth (supplemented with 2 % w/v NaCl) in flasks on a rotary shaker at 120 r.p.m. and 28 °C for 1 week. Biomass was harvested, washed in distilled water and freeze-dried. Isomers of diaminopimelic acid in whole-cell hydrolysates were determined by TLC on cellulose plates using the solvent system of Rhuland *et al.* (1955). Polar lipids and menaquinones were prepared according to the method of Minnikin *et al.* (1984) and analysed by two-dimensional TLC and HPLC, respectively. Methyl esters of cellular fatty acids were prepared from cells grown for 7 days until good growth was obtained on TSBA (trypticase soy broth agar; Becton Dickinson) plates supplemented with 2 % (w/v) NaCl and analysed using the standard MIDI system, Sherlock version 6.1 (Microbial ID). TSBA6 and ACTIN1 databases were used for peak identification. *S. artemisiae*

DSM 41953^T was included for comparison, but this strain was grown for 5 days on TSBA without NaCl.

LL-Diaminopimelic acid was detected in the peptidoglycan of strain PM267^T, which is characteristic for members of the genus *Streptomyces*. The major menaquinone found was MK-9(H₈) (74 %), followed by MK-9(H₆) (9 %), MK-9(H₂) (6 %), MK-10(H₂) (3 %), MK-9(H₄) (0.5 %) and MK-10 (0.5 %). This pattern differs from that of its closest phylogenetic neighbour, *S. artemisiae* YIM 63135^T, where MK-9(H₆) (62.8 %), MK-9(H₈) (31.4 %) and MK-9(H₄) (5.9 %) were reported (Zhao *et al.*, 2010), while MK-9(H₈) was found in *S. ferralitis* (Saintpierre-Bonaccio *et al.*, 2004). Unfortunately, menaquinone data are not available for *S. armeniacus* or *S. cacaoi*. The presence of MK-9(H₈) and MK-9(H₆) is characteristic of strains belonging to the genus

Table 2. Differential characteristics between strain PM267^T and related species of the genus *Streptomyces*

Strains: 1, PM267^T; 2, *S. artemisiae* DSM 41953^T; 3, *S. armeniacus* DSM 43125^T; 4, *S. cacaoi* DSM 40057^T; 5, *S. ferralitis* DSM 41836^T; 6, *S. synnematoformans* DSM 41902^T. All data obtained from the present study except where indicated. +, Positive; −, negative; w, weakly positive; ND, not determined.

Characteristic	1	2	3	4	5	6
Growth with NaCl (w/v)						
5 %	+	+	+	+	+	−
7 %	+	−	+	−	−	−
9 %	+	−	−	−	−	−
Nitrate reduction	−	−	−	−	−	+
Degradation of:						
Gelatin	+	+	+	+	−	−
Starch	+	+	+	+	−	+
Tween 20	+	+	+	+	−	+
L-Tyrosine	−	−	−	−	+	−
Urea	−	−	+	+	+	−
Xylan	+	−	−	+	+	−
Growth at:						
12 °C	−	+	−	+	−	−
20 °C	+	+	+	+	+	−
37 °C	−	+	−	+	+	−
pH 4.5	−	−	−	−	+	−
pH 5.5	−	−	−	+	+	−
pH 9.0	−	−	−	+	−	−
Carbon sources (1 %, w/v)						
Cellobiose	+	+	−	+	+	−
Galactose	+	+	−	+	+	−
Glucose	+	+	−	+	+	−
L-Arabinose	+	−	w	+	+	+
Maltose	+	−	−	w	+	−
Mannitol	+	−	w	+	+	+
Raffinose	+	−	−	+	+	+
Rhamnose	+	+	+	+	−	−
Sucrose	+	−	−	−	−	+
Trehalose	+	+	−	w	+	−
L-Asparagine	−	+	−	+	−	−
L-Lysine	+	+	−	+	+	+
L-Proline	+	+	−	+	+	−
L-Tyrosine	+	−	−	−	+	−
Enzymic activity						
Acid phosphatase	+	+	−	+	+	−
N-Acetyl-β-glucosaminidase	+	+	−	+	+	+
Pyrazinamidase	−	−	+	−	−	−
Pyrrolidonyl arylamidase	+	−	−	−	−	−
Trypsin	+	+	−	−	−	−
α-Chymotrypsin	+	−	−	−	+	−
α-Fucosidase	+	−	−	−	−	−
α-Glucosidase	−	−	−	+	+	−
α-Mannosidase	+	−	−	−	+	−
β-Galactosidase	+	−	−	+	+	−
β-Glucuronidase	−	−	−	−	−	+
Menaquinone composition*	MK-9(H ₂), MK-9(H ₄), MK-9(H ₆), MK-9(H ₈), MK-10, MK-10(H ₂)	MK-9(H ₄), MK-9(H ₆), MK-9(H ₈)	MK-9(H ₄), MK-9(H ₆)	ND	MK-9(H ₈)	MK-9(H ₄), MK-9(H ₆), MK-9(H ₈)

*Data for taxa 2–6 from Zhao *et al.* (2010); Wellington & Williams (1981); Saintpierre-Bonaccio *et al.* (2004); Hozzein & Goodfellow (2007).

Streptomyces and supports the assignment of strain PM267^T to the genus *Streptomyces*. The phospholipid composition of the novel strain included diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and an unidentified phospholipid. The phospholipid type, PII (Kroppenstedt, 1985), agrees with that reported for the genus *Streptomyces*. TLC plates with the phospholipid profile of strain PM267^T developed with molybdenum blue, ninhydrin and anisaldehyde reagents are shown in Fig. S5.

The major fatty acids detected in isolate PM267^T were a mixture of unsaturated straight-chain and saturated/unsaturated branched-chain fatty acids. According to the database TSBA6, the major component was summed feature 9 (25.05 %) which contained iso-17:1 ω 9c and/or 10-Me 16:0 followed by iso-16:1 (14.6 %), anteiso-17:1 (11.9 %), iso-16:0 (10.34 %), anteiso-17:0 (7.8 %) and iso-17:0 (6.9 %). When the data were compared against the database ACTIN1 the same fatty acids were identified except for summed feature 9, which was identified as 9-Me 16:0. Therefore, this mixture could not be resolved by the MIDI system. In the case of *S. artemisiae* DSM 41953^T, a similar profile was detected using both databases and the major fatty acids found were iso-16:0 (43.5 %), iso-16:1 (15.6 %), anteiso-17:0 (12.0 %) and anteiso-15:0 (7.5 %); however, summed feature 9 only accounted for 2.4 % of the components. These fatty acids were also reported by Zhao *et al.* (2010) as major components. A detailed profile of both strains is given in Table 1.

Catalase and oxidase activities were determined as described previously (Trujillo *et al.*, 2006). Hydrolysis of various chemical compounds was determined according to Trujillo *et al.* (2005). Carbon source utilization was performed as described by Williams *et al.* (1983). Determination of temperature and pH for growth and tolerance to NaCl was carried out on SA1 agar. API ZYM tests (bioMérieux) were also carried out following the manufacturer's instructions. The type strains *S. artemisiae* DSM 41953^T, *S. armeniacus* DSM 43125^T, *S. cacaoi* subsp. *cacaoi* DSM 40057^T, *S. ferralitis* DSM 41836^T and *S. synnematoformans* DSM 41902^T were included for comparison in all tests. Where appropriate, media were supplemented with 2 % (w/v) NaCl.

Strain PM267^T differed from the five type strains of the genus *Streptomyces* compared by its high tolerance to NaCl (up to 9 %, w/v), inability to reduce nitrate compared to *S. synnematoformans* DSM 41902^T, and its ability to degrade different chemical compounds such as xylan. L-Arabinose, maltose, mannitol, raffinose and sucrose were used as carbon sources by strain PM267^T, but not by *S. artemisiae* DSM 41953^T. Several enzymic substrates were also useful for differentiating strain PM267^T from its closest phylogenetic relatives. These and the results of other tests are provided in Table 2.

Given the differences found at the genotypic and phenotypic level, strain PM267^T should be classified as a novel species in

the genus *Streptomyces*, for which the name proposed is *Streptomyces pharmamarensis* sp. nov.

Description of *Streptomyces pharmamarensis* sp. nov.

Streptomyces pharmamarensis sp. nov. (phar.ma.ma.ren'sis. N.L. masc. adj. *pharmamarensis* pertaining to PharmaMar, the company where the strain was isolated).

Gram-stain-positive halotolerant aerobic actinomycete that forms extensively branched substrate and aerial hyphae that carry spiral spore chains (*Spirales*) with multiple turns containing more than 10 spores. Spores (1.0–1.2 μ m) are oval, non-motile and have smooth surfaces. Substrate mycelium colour is cream–white on several media except SA1, on which it is light brown; white aerial mycelium is scanty produced on ISP 2 agar. Diffusible pigments have not been observed. Cell wall contains L-diaminopimelic acid. Menaquinones found are MK-9(H₈), MK-9(H₆), MK-9(H₂), MK-10(H₂), MK-9(H₄) and MK-10. The phospholipid composition includes diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides (2) and one unidentified phospholipid. The DNA G+C content of the genomic DNA of the type strain is 71.2 mol%. Positive for catalase and oxidase. Grows in the presence of 9 % NaCl (w/v) with an optimum of 2 %. Grows well between 20 and 30 °C, but not at 37 °C. Utilizes L-lysine, melibiose, raffinose, starch, sucrose, L-serine, L-tyrosine, L-valine and xylose as carbon sources, but not L-asparagine, cellulose, citrate, gluconic acid, quinic acid or sorbose. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase and N-acetylglucosaminidase; negative for lipase (C14), α -galactosidase and β -glucosidase. Additional data are given in Table 2.

The type strain PM267^T (=CECT 7841^T =DSM 42032^T) was isolated from a marine sediment sample collected at a depth of 43 m in the Mediterranean Sea off the coast of Italy.

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