Streptomyces sundarbansensis sp. nov., an actinomycete that produces 2-allyloxyphenol

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A novel actinomycete producing 2-allyloxyphenol, designated strain MS1/7^T, was isolated from sediments of the Sundarbans mangrove forest, India. Growth on International *Streptomyces* Project (ISP) media 2, 3, 4, 5 and 7 produced olive green to grey aerial hyphae that carried smooth-surfaced spores in a flexuous (*Rectiflexibiles*) arrangement. The strain contained LL-diaminopimelic acid, but no diagnostic sugars in whole-cell hydrolysates. Hexa-, octa- and a minor amount of tetra-hydrogenated menaquinones with nine isoprene units [MK-9 (H_4, H_6, H_8 and H_{10})] were present as isoprene analogues. Diagnostic phospholipids were phosphatidylethanolamine and diphosphatidylglycerol. The predominant fatty acids were anteiso-$C_{15:0}$ (34.80 %), iso-$C_{16:0}$ (16.45 %), C_{16} (10.53 %) and anteiso-$C_{17:0}$ (10.92 %). The strain showed greater than 99 % 16S rRNA gene sequence similarity to the type strains of several recognized species of the genus *Streptomyces*, but in the phylogenetic tree based on 16S rRNA gene sequences it formed a distinct phyletic line and demonstrated closest relationships to viomycin-producers (*Streptomyces californicus* NRRL B-1221^T, *Streptomyces floridae* MTCC 2534^T and *Streptomyces puniceus* NRRL B-2895^T). However, strain MS1/7^T could be distinguished from these and other closely related species based on low levels of DNA–DNA relatedness (<44 %) and disparate physiological features, principally amino acid utilization and growth in NaCl. Strain MS1/7^T is therefore suggested to represent a novel species of the genus *Streptomyces*, for which the name *Streptomyces sundarbansensis* sp. nov. is proposed. The type strain is MS1/7^T (=MTCC 10621^T=DSM 42019^T).

Streptomycetes remain a rich source of novel bioactive compounds and, on the premise that poorly researched habitats can offer better prospects for discovering new natural products, actinomycetes from such habitats are currently the focus of considerable scientific interest. Poorly explored ecosystems such as estuarine mangroves have the potential to become a new resource for biological and chemical diversity, should salinity be a determinant of bacterial diversity (Hong *et al.*, 2009). Our group (Saha *et al.*, 2006) has described the purification of a bioactive compound (relative molecular weight 300.2 Da, predicted molecular formula $C_{20}H_{28}O_2$) from strain MS1/7^T isolated from the Sundarbans mangrove forest (the world's largest), India. To the authors' knowledge, the natural product 2-allyloxyphenol was obtained from this strain for the first time in any living organism (Arumugam *et al.*, 2010). A study, based on a combination of genotypic and phenotypic methods, is reported here to determine the taxonomic position of strain MS1/7^T.

Sediments from the Lothian Island of the Sundarbans mangrove forest (20° 50′ N 88° 19′ E) were collected in February 2001. Isolation was carried out by the standard dilution-plating technique on medium containing (per litre): 10.0 g starch, 3.0 g casein, 1.0 g peptone, 10.0 g malt extract, 0.5 g K_2HPO_4, 1.0 g yeast extract, 15.0 g agar, 500 ml natural seawater and 500 ml distilled water (pH 7.3). Strain MS1/7^T was maintained in the same medium and subcultured every month. Biomass for molecular systematic and most of the chemotaxonomic studies was obtained after incubation in shake flasks at 30 °C for 96 h in medium containing (per litre): 2.0 g starch, 2.0 g glucose, 2.0 g soybean meal, 0.5 g yeast extract, 0.25 g NaCl, 0.32 g $CaCO_3$, 0.005 g $CuSO_4$, 0.005 g $MnCl_2$, 0.005 g $ZnSO_4$, 500 ml natural seawater and 500 ml distilled water (pH 7.3).

Spore-chain morphology and spore-surface ornamentation of strain MS1/7^T were studied by examining gold–palladium-coated dehydrated specimens of 14-day cultures grown on International *Streptomyces* Project (ISP) 2 agar by scanning

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Abbreviations: ISP, International *Streptomyces* Project; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MS1/7^T is AY550275.

electron microscopy in an FEI Quanta 200-MK2 electron microscope. The coverslip technique (Zhou *et al.*, 1998) was used to observe hyphae and spore-chain characteristics by light microscopy (×1000). Aerial spore mass, substrate mycelial colour, utilization of sugars and the production of diffusible pigments by strain MS1/7^T were investigated on five ISP agar media following incubation at 28 °C for 14 days (Shirling & Gottlieb, 1966).

Physiological characteristics typical of the genus *Streptomyces* were determined following standard methods. Unless otherwise stated, all cultures (strain MS1/7^T and reference strains) were grown at 28 °C. Data on ISP characteristics were obtained following Shirling & Gottlieb (1966) and confirmed from the literature (Küster, 1972; Shirling & Gottlieb, 1968a, b, 1969, 1972). Carbohydrate utilization was studied on ISP 9 by incorporating 1 % (w/v) each of arabinose, D-fructose, galactose, glucose, inositol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, sorbitol, sucrose and D-xylose as sole carbon source as well as the amino acids arginine, asparagine, histidine, leucine, methionine, tyrosine and valine as sole carbon/nitrogen sources and incubating the plates for 21 days (Williams *et al.*, 1983). Growth at pH 4–11 (0.5 pH unit intervals), at 20–45 °C (2.5 °C intervals), with phenol (0.1 %) and in the presence of different concentrations of sodium azide was determined following Goodfellow (1971) on ISP 2 with incubation for 14 days. Growth in the presence of 5–20 % (w/v) NaCl (1 % intervals) was investigated according to Tresner *et al.* (1968) by incubating slants of 14-day ISP 2-grown washed saline suspensions for 10 days. Sensitivity/resistance to penicillin, rifampicin and streptomycin were determined following Williams *et al.* (1983) at antibiotic concentrations recommended by Locci (1989) and incubating the plates for 7 days. Catalase activity was tested on 7-day colonies grown on modified Bennett agar (MBA), while nitrate reduction and H_2S production were tested on sloppy nitrate medium following 14 days of incubation (Williams *et al.*, 1983). Degradation tests for adenine, casein, gelatin, starch and L-tyrosine were performed on cultures grown on MBA plates for 21 days according to Williams *et al.* (1983) and Gordon *et al.* (1974). Degradation of aesculin was determined by using defined medium (Williams *et al.*, 1983) after 21 days of incubation. Degradation of cellulose strips in yeast extract broth was observed after 6 weeks of incubation (Goodfellow, 1971). Antimicrobial compound production by strain MS1/7^T was confirmed following the methods described by Arumugam *et al.* (2010) and Saha *et al.* (2006). Physiological characteristics that were obtained reproducibly at least three times are presented.

Chromatographic procedures were used to extract and analyse the isomeric forms of diaminopimelic acid (Hasegawa *et al.*, 1983). Isoprenoid quinones and phospholipids were examined by two-dimensional TLC following Minnikin *et al.* (1984), while whole-cell sugars were identified according to Staneck & Roberts (1974). Fatty acids were extracted, methylated and estimated by GC with the standard Sherlock MIDI (Microbial Identification) system (Kämpfer & Kroppenstedt, 1996). The DNA G+C content of strain MS1/7^T was ascertained by using the thermal denaturation method of Marmur & Doty (1962). The T_m value was measured by UV spectroscopy (UV/visible spectrophotometer; Amersham Pharmacia Biotech).

Isolation of chromosomal DNA and PCR amplification of the 16S rRNA gene were carried out according to Chun & Goodfellow (1995). Sequencing of the PCR product was performed as described by Gu *et al.* (2006). Pairwise levels of similarity of the nearly complete 16S rRNA gene sequence of strain MS1/7^T were determined on the EzTaxon server by using identity analysis (Chun *et al.*, 2007). Reference strains for phylogenetic and phenotypic analyses were chosen on the basis of the top hits from this analysis (http://www.eztaxon.org). The nearly complete 16S rRNA gene sequence of strain MS1/7^T was aligned with sequences of the type strains of related species of the genus *Streptomyces* by using the MUSCLE program, version 1.7 (Edgar, 2004). Phylogenetic analyses were performed according to the neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) methods by using PAUP* version 4b10 (Swofford, 2002). The evolutionary model of substitution was evaluated by using the program jModelTest, version 0.1.1 (Posada, 2008). The (GTR+I+G) evolutionary models of substitution were found to be the best fit for the data. Parameters (base frequencies, rate matrix of substitution and types and shapes of gamma distribution) were estimated from the data. To determine the support for each clade, bootstrap analysis was performed with 1000 resamplings. Trees were rooted by using TreeGraph2 (Stöver & Müller, 2010). Tree topologies were compared by using the online algorithm described by Nye *et al.* (2006) (http://www.mrc-bsu.cam.ac.uk/personal/thomas/phylo_comparison/comparison_page.html).

To determine the genomic relatedness of strain MS1/7^T, dot-blot hybridization experiments were carried out with digoxigenin-labelled DNA (Dutta & Gachhui, 2007) by using the detection kit from Roche Applied Sciences following the manufacturer's instructions. Strains showing pairwise sequence similarity of 99.32 % and above as obtained from the EzTaxon server were selected for comparison. The genomic DNA probe was prepared from strain MS1/7^T, digested with *Sal*I and separated on a 0.7 % agarose gel. Total DNA digests were transferred from gels to nylon membrane by Southern blotting. Hybridization was performed at 75 °C for 16 h and the membrane was washed under high-stringency conditions (twice with 2×SSC/0.1 % SDS at room temperature for 10 min, once with 0.1×SSC/0.1 % SDS at 75 °C for 15 min). Quantification of band intensities was performed by using Image J (http://rsb.info.nih.gov/ij/index.html) by determining relative intensities in rectangles of equal size.

On ISP media 2, 3, 4, 5 and 7, strain MS1/7^T formed an extensively branched substrate mycelium and olive green to grey aerial hyphae that carried smooth-surfaced spores in a flexuous (*Rectiflexibiles*) arrangement (Fig. 1). The strain contained LL-diaminopimelic acid but no diagnostic sugars

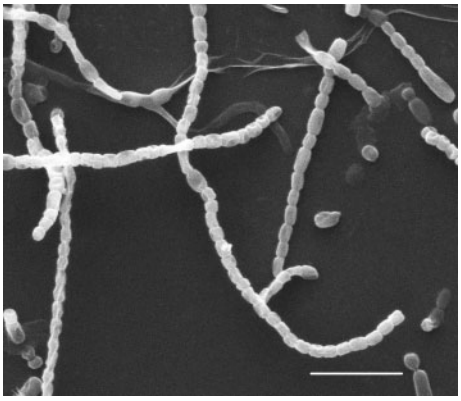

Fig. 1. Scanning electron micrograph showing spore chains and spore surface ornamentation of 14-day-old cells of strain MS1/7^T grown on yeast-malt extract agar (ISP 2) Bar, 5.0 μm.

in whole-cell hydrolysates (cell-wall chemotype I *sensu* Lechevalier & Lechevalier, 1970). It showed a phospholipid pattern consisting of phosphatidylethanolamine and diphosphatidylglycerol (phospholipid type II *sensu* Lechevalier *et al.*, 1977). Hexa-, octa- and a minor amount of tetrahydrogenated menaquinones with nine isoprene units [MK-9 (H_4, H_6, H_8 and H_{10})] as isoprene analogues were detected. The predominant fatty acids detected were anteiso-$C_{15:0}$ (34.80 %), iso-$C_{16:0}$ (16.45 %), C_{16} (10.53 %) and anteiso-$C_{17:0}$ (10.92 %). Thus, strain MS1/7^T exhibited morphological and chemotaxonomic characteristics typical of members of the genus *Streptomyces*.

Table 1 shows the results of similarity analysis performed on the 1490 bp 16S rRNA gene sequence of strain MS1/7^T. The analysis confirmed that strain MS1/7^T belonged to the genus *Streptomyces*, showing greater than 99 % 16S rRNA gene sequence similarity to many members of this genus. The phylogenetic tree based on the NJ algorithm (Fig. 2) shows that strain MS1/7^T formed a distinct phylogenetic line from the cluster containing the type strains of the *Streptomyces* species shown in Table 1. The delineation was supported by significant branch length and a high bootstrap value (100 %). The phylogenetic position of strain MS1/7^T was corroborated by the MP and ML analyses. A similar differentiation of the strain was also found with the MP and ML algorithms and was supported by high bootstrap values (95 % for MP and 90 % for ML) as well as considerable branch lengths. In addition, in all three of the trees, strain MS1/7^T always grouped with *Streptomyces californicus* NBRC 3386^T, *Streptomyces puniceus* NBRC 12811^T and *Streptomyces floridae* NBRC 15405^T, members of the viomycin-producing group of the genus *Streptomyces* (Burkholder *et al.*, 1955), as its closest neighbours, thus confirming the phylogenetic relationship between them. The overall topological scores between the trees were (following Nye *et al.*, 2006): NJ–MP, 83.8 %; NJ–ML, 73.9 %; MP–ML, 77.5 %. This indicated the similarity in general topology. Results from this part of the taxonomic analysis suggested that strain MS1/7^T should be distinguished from the above viomycin-producers as well as other closely related species of the genus *Streptomyces* by using DNA–DNA hybridization.

Low levels of genomic DNA relatedness (DNA–DNA hybridization values of less than 44 %) were observed between strain MS1/7^T and the type strains of its phylogenetically closest relatives given in Table 1. Given the recommended threshold of 80 % DNA–DNA relatedness for differentiating *Streptomyces* species as proposed by Labeda (1992), strain MS1/7^T can be distinguished from its closest phylogenetic neighbours.

Table 1. Results of the identity analysis of strain MS1/7^T performed on the EzTaxon server (last accessed 24 August 2010) in relation to the extent of DNA–DNA hybridization with strain MS1/7^T as determined experimentally

Similar species according to rank	NCBI accession no.	Pairwise similarity (%)	Dissimilar nucleotides/total nucleotides	DNA–DNA hybridization (%)*	
				I	II
S. californicus NBRC 3386^T	AB184755	99.451	8/1458	42.7	41.4
S. globisporus NBRC 12867^T	AB184203	99.451	8/1457	37.6	39.3
S. pluricolorescens NBRC 12808^T	AB184162	99.450	8/1454	35.8	40.2
S. rubiginosohelvolus NBRC 12912^T	AB184240	99.449	8/1451	40.5	37.8
S. badius NRRL B-2567^T	AY999783	99.388	9/1470	36.4	42.3
S. anulatus NRRL B-2000^T	DQ026637	99.387	9/1469	33.5	36.7
S. albovinaceus NBRC 12739^T	AB249958	99.383	9/1459	36.7	39.5
S. sindenensis NBRC 3399^T	AB184759	99.383	9/1459	37.4	41.3
S. parvus NBRC 3388^T	AB184756	99.383	9/1459	34.5	38.0
S. puniceus NBRC 12811^T	AB184163	99.383	9/1458	41.8	43.4
S. floridae NBRC 15405^T	AB184656	99.383	9/1458	42.3	40.5
S. fimicarius ISP 5322^T	AY999784	99.320	10/1470	39.5	36.4

*Data for two consecutive tests (I and II) are presented.

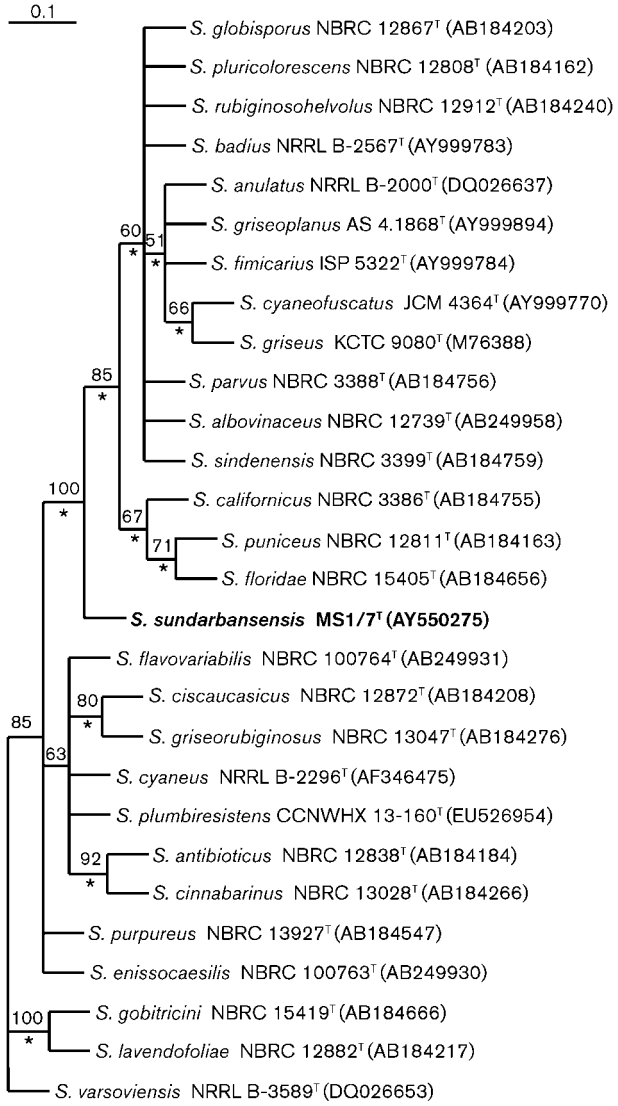

Fig. 2. Unrooted phylogenetic tree based on 16S rRNA gene sequences obtained by the NJ method showing the position of strain MS1/7^T among its phylogenetic neighbours. Numbers at nodes indicate levels of bootstrap support (%) based on a NJ analysis of 1000 resampled datasets; only values >50 % are shown. Asterisks indicate branches that were also recovered using the MP and ML algorithms. GenBank accession numbers are given in parentheses. Bar, 0.1 nt substitutions per site. The sequence of *Streptomyces varsoviensis* NRRL B-3589^T was used as the outgroup.

Table 2 provides a comparison of the phenotypic characteristics of strain MS1/7^T with those of the type strains of its 12 closest relatives based on the EzTaxon search and included in the phylogenetic tree (Fig. 2). It was evident that characteristics such as colour of aerial spore mass and colony, and production of diffusible pigments differed significantly between strain MS1/7^T and the other reference strains. In addition, there were dissimilarities in the utilization of sugars, amino acids, growth with NaCl, degradation of aesculin and resistance/sensitivity pattern to various antibiotics. Strain MS1/7^T produced 2-allyloxyphenol (Arumugam *et al.*, 2010) and 4a,8a-dimethyl-6-(2-methyl-propenyloxy)-3,4,4a,4b,5,6,8a,9-octahydro-1H phenanthren-2-one (Saha *et al.*, 2006) as antimicrobial substances, but not viomycin, in contrast to its three closest phylogenetic neighbours (Fig. 2). Strain MS1/7^T could be differentiated from the viomycin-producing species of the genus *Streptomyces* based on non-utilization of arginine, leucine, methionine and tyrosine as sole nutrients, growth with NaCl, degradation of aesculin and resistance/sensitivity pattern to various antibiotics.

Based on data from the present study conducted using a polyphasic taxonomic approach, strain MS1/7^T is thus considered to represent a novel species of the genus *Streptomyces*, for which the name *Streptomyces sundarbansensis* sp. nov. is proposed.

Description of *Streptomyces sundarbansensis* sp. nov.

Streptomyces sundarbansensis [sun.dar.bans.en′sis. N.L. masc. adj. *sundarbansensis* pertaining to the Sundarbans (local language, Bangla, meaning the beautiful forest), the world's largest tidal mangrove forest, Republic of India, the geographical origin of the type strain].

Aerobic, Gram-positive-staining, non-motile actinomycete. Spores are borne in flexuous (*Rectiflexibiles*) chains of 15–30 smooth-surfaced spores, 0.66–0.86 μm long and 0.52–0.59 μm wide. Grows well on: ISP 2 agar with olive green aerial mycelia and black (reverse side) substrate mycelia; ISP 3 agar with grey to olive green aerial mycelia and brown (reverse side) substrate mycelia; ISP 5 agar with olive green aerial mycelia and brown (reverse side) substrate mycelia; ISP 4 and ISP 7 agar with grey to olive green aerial mycelia and black (reverse side) substrate mycelia. Does not produce diffusible pigments on the above media. No melanoid pigments are produced. Whole-cell hydrolysates contain LL-diaminopimelic acid but no diagnostic sugars. Phosphatidylethanolamine and diphosphatidylglycerol are present as diagnostic phospholipids. The predominant cellular fatty acids are $C_{15:0}$, iso-$C_{16:0}$, C_{16} and anteiso-$C_{17:0}$. Utilizes D-fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sorbitol, sucrose and D-xylose as sole carbon source, but not arabinose, inositol, raffinose or rhamnose. Utilizes asparagine, histidine and valine as sole carbon/nitrogen sources but not arginine, leucine methionine or tyrosine. Positive for catalase activity, but negative for H_2S production and nitrate reduction. Degrades adenine, casein, aesculin, gelatin and starch, but not cellulose or L-tyrosine. Grows in the presence of 15 % (w/v) NaCl (optimally in 3 % NaCl), at pH 4.5–8.5 (optimally at 7.5) and at 25–35 °C (optimally at 30 °C), but not with 0.1 % phenol or different concentrations of sodium azide. Sensitive to streptomycin and rifampicin, but resistant to penicillin. Produces 2-allyloxyphenol and 4a,8a-dimethyl-6-(2-methyl-propenyloxy)-3,4,4a,4b,5,6,8a,9-octahydro-1H phenanthren-2-one as antimicrobial compounds.

Table 2. Differential phenotypic characteristics between strain MS1/7^T and phylogenetically related species of the genus *Streptomyces*

Strains: 1, MS1/7^T; 2, *S. californicus* NRRL B-1221^T; 3, *S. floridae* MTCC 2534^T; 4, *S. puniceus* NRRL B-2895^T; 5, *S. rubiginoshelvolus* NRRL B-5425^T; 6, *S. fimicarius* NRRL ISP-5322^T; 7, *S. globisporus* NRRL B-2872^T; 8, *S. sindenensis* NRRL B-1866^T; 9, *S. albovinaceus* NRRL B-2566^T; 10, *S. badius* NRRL B-2567^T; 11, *S. pluricolorescens* NRRL B-2121^T; 12, *S. parvus* MTCC 322^T; 13, *S. anulatus* NRRL B-2000^T. B, brown; BL, black; G, grey; N, none; OG, olive green; P, pink; PY, pale yellow; R, resistant; S, sensitive; V, violet; W, white; Y, yellow.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Colour of aerial spore mass on ISP media (2, 3, 4, 5, 7)	OG	G	W–V	Y	Y	Y	Y	G	W	Y	Y	Y	Y
Reverse side colony colour on ISP media (2, 3, 4, 5, 7)	BL–B	V	V	V	Y	Y	PY	N	N	N	Y	PY	PY
Production of diffusible pigments in ISP media (2, 3, 4, 5, 7)	N	P	V	V	N	P	Y	N	N	N	N	Y	Y
Growth on sole carbon source (1 %, w/v)													
Arabinose	−	−	+	−	+	+	−	+	+	+	+	+	+
Rhamnose	−	−	−	−	+	+	−	−	+	−	+	+	+
Sorbitol	+	−	−	+	−	+	+	+	−	−	+	−	−
Sucrose	+	−	+	−	−	−	−	−	−	−	−	−	−
Growth on sole amino acids (1 %, w/v)													
Arginine	−	+	+	+	+	+	+	+	+	+	+	+	+
Leucine	−	+	+	+	+	+	+	+	+	+	+	+	+
Methionine	−	+	+	+	+	+	+	+	+	+	+	+	+
Tyrosine	−	+	+	+	+	+	+	+	+	+	+	+	+
Resistant/sensitive to antibiotics													
Penicillin (10 U ml^{-1})	R	R	R	S	S	R	R	R	R	R	S	S	S
Rifampicin (50 μg ml^{-1})	S	S	S	S	R	S	R	R	R	R	R	S	S
Streptomycin (100 μg ml^{-1})	S	R	R	S	S	S	S	S	R	S	R	S	R
Degradation of aesculin	+	−	+	−	−	−	+	−	+	−	+	−	−
Growth with NaCl (15 %, w/v)	+	−	−	−	−	−	−	−	−	−	−	−	−
Production of antimicrobial substances*	2-AP and DMOP	Vio	Vio	Vio	X	Gris	Lan	Act	X	X	Lar	X	X

*Act, actinomycin D (Praveen *et al.*, 2008); 2-AP, 2-allyloxyphenol (Arumugam *et al.*, 2010); DMOP, 4a,8a-dimethyl-6-(2-methyl-propenyloxy)-3,4,4a,4b,5,6,8a,9-octahydro-1H phenanthren-2-one (Saha *et al.*, 2006); Gris, griseorubin (Dornberger *et al.*, 1980); Lan, landomycin (Ostash *et al.*, 2005); Lar, largomycin (Zaheer *et al.*, 1985); Vio, viomycin (Burkholder *et al.*, 1955); X, no antimicrobial substances reported.

The type strain, MS1/7^T (=MTCC 10621^T=DSM 42019^T), was isolated from sediments of the Sundarbans mangrove forest, India. The G+C content of the genomic DNA of the type strain is 71.9 mol%.

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