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ORIGINAL ARTICLE

Streptomyces oryzae sp. nov., an endophytic actinomycete isolated from stems of rice plant

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An actinomycete strain S16–07^T, isolated from surface-sterilized stems of rice plant (*Oryza sativa* L.), was characterized using a polyphasic approach. Phylogenetic analysis of 16S rRNA gene sequences indicated affiliation of the strain belonged to the genus *Streptomyces*. The highest levels of sequence similarity were found with *Streptomyces smyrnaeus* SM3501^T (97.7% similarity), *S. abikoensis* NBRC 13860^T (97.6% similarity) and *S. thermocarboxydovorans* NBRC 16324^T (97.5% similarity). The cell wall of strain S16–07^T contained LL-diaminopimelic acid. The predominant menaquinones were MK-9(H_6) and MK-9(H_8). Phospholipids detected were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, hydroxy-phosphatidylethanolamine, hydroxy-phosphatidylmonomethylethanolamine and phosphatidylinositol mannosides. The major cellular fatty acids were ai-$C_{15:0}$, i-$C_{16:0}$ and ai-$C_{17:0}$. The G+C content of strain S16–07^T was 70.4 mol%. On the basis of the phylogeny of the isolate and its differences from the most closely related species, the isolate S16–07^T represents a novel species for which the name *S. oryzae* sp. nov. is proposed. The type strain is S16–07^T (= BCC 60400^T = NBRC 109761^T).
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INTRODUCTION

The genus *Streptomyces* was first proposed by Waksman and Henrici[1] and was classified in the family *Streptomycetaceae*, order *Actinobacteria*.[2] This genus currently contains more than 650 recognized species with validly published names.[3] Members of the genus *Streptomyces* are aerobic, Gram-stain-positive, chemo-organotrophic actinomycetes and form an extensively branched substrate mycelium with rarely fragment. The aerial mycelium forms chains of three to many spores at maturity. Chemotaxonomic characteristics show lack of mycolic acids, type I cell wall containing LL-diaminopimelic acid but no characteristic sugars and contain major amounts of saturated, *iso*- and *anteiso*-fatty acids. The major menaquinone is either hexa- or octahydrogenated with nine isoprene units. The phospholipid patterns typically contain diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. The DNA G+C content is in the range 66–78 mol%.[4] During study the diversity of endophytic actinomycetes from stems and roots of rice plant, an actinomycete designated S16–07^T, was isolated and assigned to the genus *Streptomyces*. The aim of the present study was to determine the taxonomic status of the strain S16–07^T using genotypic and phenotypic characteristics. The resultant data indicated that the organism should be classified as a novel species of *Streptomyces*, for which the name *Streptomyces oryzae* sp. nov. is proposed.

MATERIALS AND METHODS

Strain S16–07^T was isolated from surface-sterilized stems of rice plant (*O. sativa* L.) collected from Petchaburi province, Thailand. Stem samples were surface-sterilized according to the method as described by Mingma *et al.*[5] and crushed with sterile glass rod in 1/4 strength Ringer's solution. Crushed plant tissue suspensions were spread on starch casein agar[6] supplemented with ketoconazole (100 μg ml^{-1}), nystatin (50 μg ml^{-1}) and nalidixic acid (25 μg ml^{-1}) and incubated at 28 °C for 21 days. Pure colony of strain S16–07^T was kept on glucose yeast extract agar (containing glucose 1.0% (w/v), yeast extract 1.0% (w/v) and agar 1.5% (w/v)). Spore and cell suspensions were stored as lyophilized ampules at 4 °C and in 20% (v/v) glycerol at − 20 °C.

Strain S16–07^T and the type strains of *S. abikoensis* NBRC 13860[7], *S. thermocarboxydovorans* NBRC 16324[8] and *S. lilacinus* ISP 5254[9] were studied together for biochemical, cultural and physiological characteristics. *S. smyrnaeus* SM3501^T has recently been described by Tatar *et al.*[10] and showed the highest similarity with the strain S16–07^T. At this time of writing, *S. smyrnaeus* SM3501^T was just published in *International Journal of Systematic and Evolutionary Microbiology*. Therefore, the cultural and physiological properties of *S. smyrnaeus* SM3501^T were obtained from Tatar *et al.*[10] for comparison purpose. Morphological observations of spores and mycelia of strain S16–07^T were examined after incubation at 27 °C for 14 days by light microscope and scanning electron microscope (JEOL–JSM 5600 LV, Tokyo, Japan). Cultural characteristics were determined from the growth on ISP (International *Streptomyces* Project) media[11] 2, 3, 4 and 5, potato dextrose agar (PDA), Czapek's agar and nutrient agar.[12] The characteristics were recorded after 14 days of incubation at 27 °C. The Color Harmony Manual Charts[13] were used to determine color designations. The utilization of carbohydrates as sole carbon

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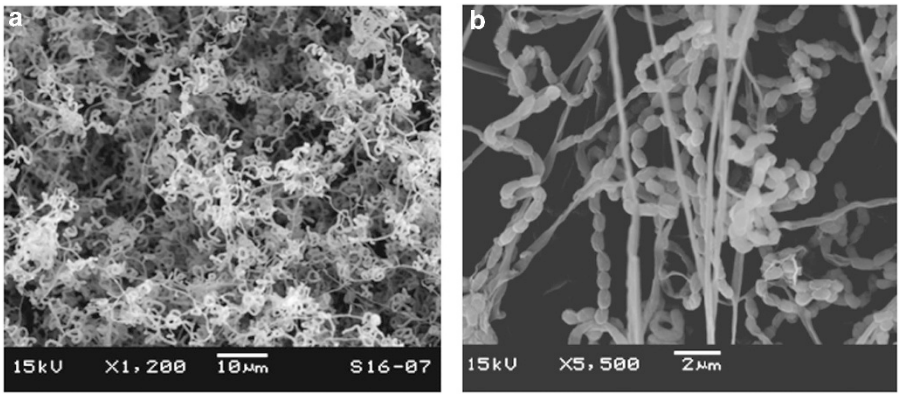

Figure 1 Scanning electron micrographs of the retinaculum apertum (RA) spore chains and smooth-surfaced spores of *Streptomyces oryzae* S16–07^T after cultivation on ISP medium 4 (**a**) and ISP medium 5 (**b**) at 27 °C for 2 weeks.

Table 1 Growth and cultural characteristics of the strain S16–07^T

Agar media	*Growth*	*Aerial mycelium colour; abundance*	*Substrate mycelium color*	*Soluble pigment color*
ISP medium 2	Good	White; moderate	Yellow	Yellow
ISP medium 3	Moderate	White; moderate	Pale yellow	Pale yellow
ISP medium 4	Moderate	White; moderate	Pale yellow	Pale yellow
ISP medium 5	Good	White; moderate	Pale yellow	None
Nutrient agar	Moderate	White; sparse	Pale yellow	None
Potato dextrose agar	Moderate	White; sparse	Pale yellow	Pale yellow
Czapek's agar	Good	White; sparse	White	None

source was investigated on ISP medium 9.[11] Tolerance of sodium chloride (0, 1, 2, 3, 4, 5, 10 and 15%, (w/v)) was tested using ISP medium 2. To determine the optimal temperature and pH for growth, strain S16–07^T was incubated for 14 days on ISP medium 2 at temperatures of 5–50 °C, and at pHs ranging from 3.0 to 11.0 (at intervals of 1.0 pH unit). Enzyme activity profiles were carried out using the API ZYM (bioMérieux) test kits.[14] Melanin pigment was examined on ISP medium 6 and ISP medium 7.[11] The production of hydrogen sulfide was detected using lead acetate strips. Hydrolysis of adenine, casein, cellulose, chitin, guanine, hypoxanthine, tyrosine, starch, xanthine and urea was examined by following the methods of Gordon and Mihm[15] and Gordon *et al.*[16]

Cells for the chemotaxonomic studies were obtained after incubation of the strain in ISP medium 2 broth at 27 °C for 2 weeks in shake flasks. The cells were harvested using centrifuged and washed three times with distilled water before freeze-drying. The isomer of diaminopimelic acid was identified using the method of Becker *et al.*[17] and Hasegawa *et al.*[18] The sugar compositions of whole-cell wall were determined by chromatography as described by Lechevalier and Lechevalier.[19] The acyl type of the cell wall was analysed by using the method of Uchida and Aida.[20] Polar lipids were examined using two-dimensional TLC and identified by the method of Minnikin *et al.*[21] The presence of mycolic acid was detected by TLC according to the method of Tomiyasu.[22] Menaquinones were extracted from freeze-dried biomass using the procedure of Collins *et al.*[23] and subsequently analyzed by LC/MS (JMS–T100LP, JEOL) with PEGASIL ODS column (2ø × 50 mm) using methanol/2-propanol (7:3). Fatty acid methyl esters were prepared and separated using a previously described by Sasser[24] and identified using with the MIDI Sherlock Microbial Identification System (Microbial ID; MIDI Version 6.1). The fatty acid analysis was performed at the Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL), Thailand.

Genomic DNA was extracted as described by Kieser *et al.*[25] and used as templates for PCR amplification and sequencing according to the procedure of Mingma *et al.*[5] The resultant 16S rDNA sequence was aligned with closely related 16S rRNA gene sequence from the EzTaxon–e server (http://eztaxon–e.ezbiocloud.net/).[26] Multiple sequence alignments were performed using the CLUSTAL W program integrated in the Molecular Evolutionary Genetics Analysis (MEGA) version 5.0.[27] For phylogenetic analysis, reference strains were chosen according to the highest pairwise similarity among the top 22 BLASTN hits against the EzTaxon–e database. Phylogenetic tree was constructed by the neighbor-joining,[28] maximum likelihood[29] and maximum parsimony[30] methods with the MEGA 5.0 software package. A phylogenetic tree and distance matrix were reconstructed by using the neighbor-joining method and generated using the models by Jukes and Cantor[31]. The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein[32] with 1000 replicates. Genomic DNA for hybridization was prepared according to the method described by Saito and Miura.[33] DNA–DNA relatedness was measured fluorometrically using the microplate hybridization method.[34] The G+C content (mol%) of the DNA was determined by HPLC according to the method of Tamaoka and Komagata.[35]

RESULTS AND DISCUSSION

Strain S16–07^T produced branched, nonfragmented substrate mycelium. The aerial mycelia harbored spore chains of hooks, open loops or primitive spiral, which belong to retinaculum apertum type of morphology that consisted of 10 or more spores per chain. Spore chain morphology of strain S16–07^T was different from other closely related species as follow: *S. smyrnaeus* SM3501^T produced spiral spore chains;[10] *S. abikoensis* NBRC 13860^T produced straight spore chains;[7] *S. thermocarboxydovorans* NBRC 16324^T formed spores in long straight to flexuous[8] and *S. lilacinus* ISP 5254^T produced verticillate spore chain.[9] The spores of strain S16–07^T were oval to rod shaped and 0.5 × 1.0 µm in size. Spore surface was smooth (Figure 1). The cultural characteristics of strain S16–07^T on different kinds of media are presented in Table 1. Strain S16–07^T showed good growth on ISP medium 2, ISP medium 5 and Czapek's agar and moderate growth on several media including ISP medium 3, ISP medium 4, nutrient agar and PDA. The substrate mycelium of strain on most media tested was white to pale yellow with whitish aerial spore mass. Pale yellow to yellow diffusible pigment was detected when the strain was cultured on ISP medium 2, ISP medium 3, ISP medium 4 and PDA. Melanin pigment was not observed on both ISP medium 6 and ISP medium 7.

The physiological properties that differentiated strain S16–07^T from *S. smyrnaeus* SM3501^T, *S. abikoensis* NBRC 13860^T, *S. thermocarboxydovorans* NBRC 16324^T and *S. lilacinus* ISP 5254^T are shown in Table 2. Strain S16–07^T utilized adonitol, D(−)rhamnose, raffinose and sucrose, whereas *S. abikoensis* NBRC 13860^T, *S. thermocarboxydovorans* NBRC 16324^T and *S. lilacinus* ISP 5254^T did not. In addition, S16–07^T could use L(+)arabinose, D(+)cellobiose, D(+)

Table 2 Comparison of physiological characteristics of strain S16–07^T and related species of the genus *Streptomyces*

Characteristic	*1*	*2*[a]	*3*	*4*	*5*
Assimilation of sole carbon sources (1.0%, w/v)					
Adonitol	+	+	–	–	–
L(+)Arabinose	+	+	–	+	–
D(+)Cellobiose	+	+	–	++	–
D(–)Fructose	++	+	+	+	–
D(+)Galactose	+	+	–	++	–
Beta-lactose	++	+	–	++	–
D(–)Mannitol	++	+	–	++	–
D(–)Rhamnose	+	ND	–	–	–
Raffinose	+	ND	–	–	–
D(–)Sorbitol	–	+	–	–	–
Sucrose	++	+	–	–	–
Xylitol	–	+	–	–	–
Xylose	+	+	–	+	–
Degradation of					
Adenine	–	+	–	+	–
Casein	+	–	–	+	+
Hypoxanthine	+	ND	–	+	+
Tyrosine	+	ND	+	–	+
Urea	+	–	–	–	–
Growth pH	6.0–9.0	4.0–12.0	5.0–8.0	6.0–11.0	6.0–8.0
Growth in the presence of					
10% NaCl	+	+	–	–	–
15% NaCl	–	+	–	–	–

Strains: 1, *S. oryzae* S16–07^T; 2, *S. smyrnaeus* SM3501^T; 3, *S. abikoensis* NBRC 13860^T; 4, *S. thermocarboxydovorans* NBRC 16324^T; 5, *S. lilacinus* ISP 5254^T. All data were generated in the present study unless otherwise indicated. ++ strongly positive; +, positive; –, negative; ND, not determined.
[a]Data were taken from Tatar *et al.*[10]

galactose, beta-lactose and D(–)mannitol, a property which was negative in *S. abikoensis* NBRC 13860^T and *S. lilacinus* ISP 5254^T. *S. smyrnaeus* SM3501^T utilized D(–)sorbitol and xylitol as the sole carbon source but strain S16–07^T could not. Strain S16–07^T could utilize urea and degrade casein, whereas *S. smyrnaeus* SM3501^T and *S. abikoensis* NBRC 13860^T could not. Strain S16–07^T could tolerate NaCl at concentration up to 10%, whereas *S. smyrnaeus* SM3501^T could tolerate up to 20%. On the other hand, *S. abikoensis* NBRC 13860^T, *S. thermocarboxydovorans* NBRC 16324^T and *S. lilacinus* ISP 5254^T could tolerate only 5% NaCl. Strain S16–07^T utilized fructose, glycerol, *myo*-inositol, maltose, trehalose and xylose as the sole carbon source but not melibiose, D(–)sorbitol and sorbose. Alkaline phosphatase, leucine aminopeptidase, acid phosphatase, phosphoamidase, α-glucosidase and glucosaminidase were detected with the API ZYM enzyme assay, but not chymotrypsin, cystine aminopeptidase, esterase (C4), esterase lipase (C8), α-fucosidase, α-galactosidase, β-galactosidase, β-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase, trypsin and valine aminopeptidase. The temperature range for growth of strain S16–07^T was 14–43 °C, with the optimum temperature 23–32 °C. The pH range for growth was 6.0–9.0.

An analysis of whole-cell hydrolysates showed that the strain S16–07^T contained LL-diaminopimelic acid, which was characteristic for the genus *Streptomyces*. The whole-cell sugars were detected as galactose, glucose and ribose. The muramic acid in the peptidoglycan was N-acetylated. Polar lipids were of type II, according to the phospholipid classification of Lechevalier *et al.*[36] and included phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, hydroxy-phosphatidylethanolamine, hydroxy-phosphatidylmonomethylethanolamine and phosphatidylinositol mannosides (Supplementary Figure S1). The major menaquinones found were MK–9(H_6) (49.6%) and MK–9(H_8) (41.6%), with minor amounts of MK–10(H_6) (4.9%) and MK–10(H_8) (3.9%). The fatty acids were ai-$C_{15:0}$ (29.4%), i-$C_{16:0}$ (28.3%), ai-$C_{17:0}$ (17.9%), i-$C_{15:0}$ (3.8%),

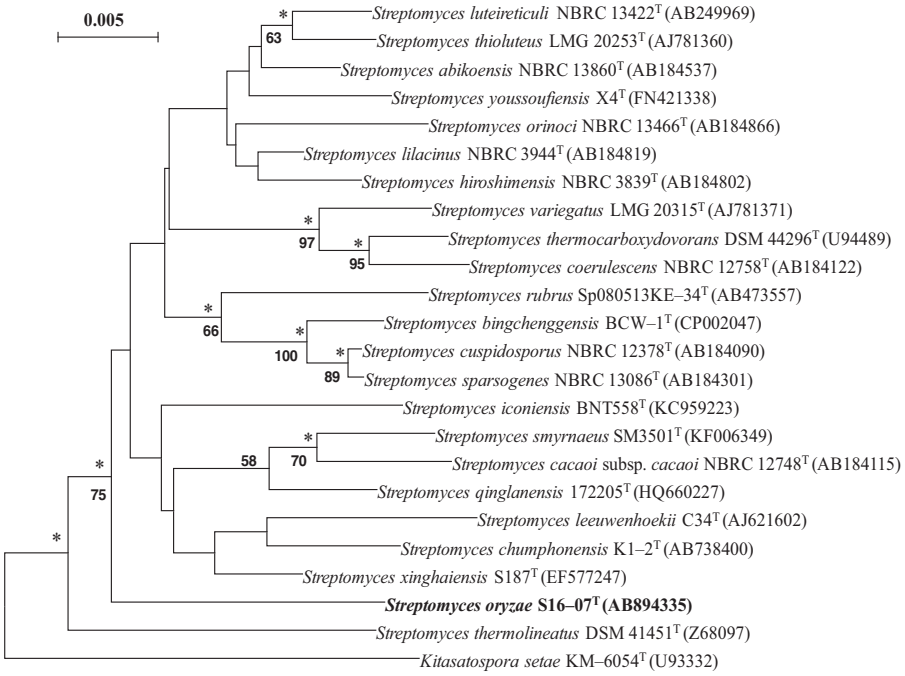

Figure 2 Neighbor-joining phylogenetic tree, based on nearly complete 16S rRNA gene sequences, showing the relationships between strain S16–07^T and strains of related species of the genus *Streptomyces*. Numbers at nodes are bootstrap values based on 1000 resamplings (only values >50% are indicated). Asterisks indicate that the clades are also recovered in maximum-likelihood and maximum-parsimony trees. Bar, 0.005% sequence divergence.

i-$C_{14:0}$ (3.8%), $C_{16:0}$ (3.2%), i-H-$C_{16:1}$ (3.1%), ai-$C_{17:1}$ *w9c* (2.7%), i-$C_{17:0}$ (1.7%), i-$C_{18:0}$ (0.8%), $C_{17:0}$ *cyclo* (0.8%), ai-$C_{13:0}$ (0.7%), 2OH-$C_{17:0}$ (0.5%), $C_{14:0}$ (0.4%), $C_{15:1}$ *w6c* (0.4%), i-$C_{17:1}$ *w5c* (0.4%), $C_{17:0}$ (0.3%), ai-$C_{16:0}$ (0.3%), i-3OH-$C_{16:0}$ (0.2%) and ai-$C_{14:0}$ (0.2%). Mycolic acids were not detected. The G+C content of the DNA was 70.4%.

The 16S rRNA gene-based tree, constructed using the neighbor-joining method, showed that the strain S16–07^T formed a separate phyletic line from other representatives of the genus *Streptomyces* (Figure 2), notably from its closely related species *S. smyrnaeus* SM3501^T (97.7%; 33/1414), *S. abikoensis* NBRC 13860^T (97.6%; 34/1403), *S. thermocarboxydovorans* NBRC 16324^T (97.5%; 35/1410) and *S. lilacinus* ISP 5254^T (97.5%; 35/1404). However, it was clear that strain S16–07^T represented a novel subline within the genus *Streptomyces* as its position in the tree was separated from these nearest phylogenetic neighbors. DNA–DNA hybridization tests were carried out between strain S16–07^T and closely related strains selected on the basis of their 16S rDNA sequence similarity. DNA–DNA relatedness values between strain S16–07^T and *S. abikoensis* NBRC 13860^T (3.0%), *S. thermocarboxydovorans* NBRC 16324^T (6.5%) and *S. lilacinus* ISP 5254^T (13.8%) were all significantly lower than 70%, the threshold value for the delineation of genomic species.[37] On the basis of 16S rRNA gene sequence data, DNA–DNA hybridization studies and biochemical properties, as well as physiological properties, it is proposed that strain S16–07^T represents a novel species of genus *Streptomyces*, for which the name *S. oryzae* is proposed. The type strain is S16–07^T.

Description of *S. oryzae* sp. nov.

S. oryzae (o.ry'zae. L. gen. n. *oryzae* of rice, referring to the rice plant where the strain was isolated) is aerobic, Gram-stain-positive, catalase- and oxidase-positive. The substrate mycelium does not fragment. The aerial hyphae bearing smooth-surfaced spores in hooks, open loops or primitive spiral spore chain (retinaculum apertum). White aerial mycelium and white to pale yellow substrate mycelium are produced on most media. A yellow soluble pigment is produced on ISP medium 2, ISP medium 3, ISP medium 4 and PDA. Good growth occurs on ISP medium 2, ISP medium 5 and Czapek's agar. Moderate growth is observed on ISP medium 3, ISP medium 4, nutrient agar and PDA. Melanin pigment is not produced. Growth occurs between 14 and 43 °C and at pH 6.0–9.0. Uses adonitol, L(+)arabinose, D(+)cellobiose, D(−)fructose, D(+)galactose, D(+)glucose, glycerol, *myo*-inositol, beta-lactose, maltose, D(−)mannitol, D(−)rhamnose, raffinose, sucrose, D(+)trehalose and xylose as sole carbon sources, but not melibiose, D(−)sorbitol, sorbose and xylitol. The organism degrades casein, hypoxanthine, starch, tyrosine, xanthine and urea, but does not degrade adenine, cellulose, chitin and guanine. Tests for nitrate reductase and H_2S production are positive. LL-diaminopimelic acid is the diagnostic amino acid in the peptidoglycan and the muramic acid acyl type is acetyl. Galactose, glucose and ribose are found in whole-cell hydrolysates. The predominant menaquinones are MK-9(H_6) and MK-9(H_8). The major cellular fatty acids are ai-$C_{15:0}$, i-$C_{16:0}$ and ai-$C_{17:0}$. The polar lipids include phosphatidylglycerol, diphosphatidylglycerol, hydroxy-phosphatidylethanolamine, hydroxy-phosphatidylmonomethylethanolamine, phosphatidylethanolamine and phosphatidylinositol mannosides. Mycolic acids are absent. The DNA G+C content of the type strain is 70.4 mol%.

The type strain, S16–07^T (=BCC 60400^T=NBRC 109761^T), was isolated from stems of rice plant, *O. sativa* L., collected in Petchaburi province, Thailand.

Accession code

The DDBJ accession number for the 16S rRNA gene sequence of the strain S16–07^T is AB894335.

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