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Fungichromin: A Substance from *Streptomyces padanus* with Inhibitory Effects on *Rhizoctonia solani*

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Streptomyces padanus strain PMS-702 is an antagonist of *Rhizoctonia solani* AG-4, the causal agent of damping-off of cabbage. Treatment of cabbage seeds with the culture filtrate of *S. padanus* strain PMS-702 was effective in reducing the incidence of damping-off of cabbage. The major active ingredient from the culture filtrate of *S. padanus* strain PMS-702 was purified by silica gel column chromatography and identified as the polyene macrolide, fungichromin, by NMR and mass spectral data. Bioassay studies showed that fungichromin had a strong antifungal activity against *R. solani* AG-4, and its minimum inhibitory concentration (over 90% inhibition) was found to be 72 μg/mL. This is the first report of fungichromin from *S. padanus* as an active ingredient for the control of *Rhizoctonia* damping-off of cabbage.

KEYWORDS: Fungichromin; *Streptomyces padanus*; *Rhizoctonia solani*; biocontrol; antifungal substance; polyene macrolide

INTRODUCTION

Damping-off caused by *Rhizoctonia solani* Kühn is a serious seedling disease of numerous crops. It causes severe losses on annual plants such as vegetables and flowers as well as perennial plants such as turf grasses and trees grown in nurseries, glasshouses, and gardens (*1*). In Taiwan, *Rhizoctonia* damping-off has become a major problem for the commercial production of vegetable seedlings grown in cell-plug systems, including cabbage (*2*, *3*). The use of chemical fungicides for control of damping-off is not an ideal option because of problems associated with environmental pollution and the danger of development of fungicide resistance in plant pathogens. Biocontrol has long been recognized as an environmentally sound method for management of plant diseases. Control of plant pathogens by biocontrol agents such as *Streptomyces* spp., *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Trichoderma* spp. can be achieved through the competition for nutrients and the production of cell-wall-degrading enzymes or other toxic metabolites (*4*−*9*). The toxicity of some microbial metabolites is highly selective. For example, phenazine-1-carboxylate produced from *P. fluorescens* strain 2-79 is toxic to the take-all pathogen, *Gaeumannomyces graminis* var. *tritici*, in the rhizosphere of wheat (*10*). The metabolites of soil inhabitant antagonists were easily degraded and safety compared to chemical pesticides, which were used for controlling the soil-borne pathogens (*11*). Numerous examples of biocontrol studies showed that one or more antibiotics produced by biocontrol agents were involved in the suppression of plant pathogens (*12*−*15*).

Antibiotics produced by Actinomycetes, such as genus *Streptomyces*, have been used in agriculture and medicine during the past century. Rothrock and Gottlieb (*5*) applied *Streptomyces hygroscopicus* var. *geldanus* for control of root rot of pea caused by *R. solani*. Using bioautography of thin-layer chromatograms (TLC) as an assay method, the antibiotic geldanamycin was detected in soil treated with *S. hygroscopicus* var. *geldanus*. It was concluded that the antibiosis played an important role in antagonism of *S. hygroscopicus* var. *geldanus* against *R. solani*. A preliminary report by Shih and Huang (*16*) showed that *Streptomyces padanus* strain PMS-702 was antagonistic to *R. solani* AG-4. The objectives of this study were to determine the efficacy of cultural filtrate of *S. padanus* strain PMS-702 for control of *Rhizoctonia* damping-off of cabbage and to identify the key chemical substance in the cultural filtrate responsible for the control of *R. solani* AG-4.

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MATERIALS AND METHODS

Organisms and Media. *Streptomyces padanus* strain PMS-702 was isolated from spent forest mushroom compost and deposited at the Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan, as CCRC910179. *Rhizoctonia solani* AG-4, strains RST-02 and RST-04, were isolated from a seedling of Chinese kale (*Brassica alboglabra* Bailey) showing damping-off symptoms. Other strains of a different anastomosis group of *R. solani* used in this study were stored at the Department of Plant Pathology, National Chung Hsing University, Taichung, Taiwan. All cultures of *R. solani* were grown on potato dextrose agar (PDA) (200 g of potato infusion, 20 g of glucose, and 20 g of agar in 1 L of water) slants. Solid medium for sporulation of PMS-702 used in this study was International *Streptomyces* Project (ISP) Medium 4 (Bacto ISP Medium4, Difco Co.). The composition of ISP Medium 4 was 10 g of Bacto-soluble starch, 1 g of potassium phosphate, 1 g of magnesium sulfate, 1 g of sodium chloride, 2 g of ammonium sulfate, 2 g of calcium carbonate, 0.001 g of ferrous sulfate, 0.001 g of manganous chloride, 0.001 g of zinc sulfate, 20 g of Bacto agar (pH 7.2), and 1 L of H_2O (*17*). The liquid medium used for PMS-702 was soybean meal−glucose broth (SMGB). The composition of SMGB was 5.0 g of soybean meal, 5.0 g of glucose, and 0.4 g of $CaCO_3$, stirred with distilled water to 1 L (final pH of the medium was adjusted to 7.9−8.1 with 1 N NaOH).

In Vitro Assay for Antagonism. An in vitro plate assay technique was developed to test the inhibitory effects of PMS-702 on strains RST-01 through RST-07. Tests for inhibitory activity were made on PDA in Petri dishes. PMS-702 was inoculated by streaking on PDA at 1.5 cm from the edge of the Petri dish. After incubation for 6 days at 28 °C, the PDA plate was inoculated with each isolate of *R. solani* by placing a PDA plug (0.8 cm diameter) containing mycelial mats removed from 4-day-old culture at 5 cm from PMS-702. The inoculated plates were placed in an incubator at 28 °C for 4 days. The inhibition zone was determined by measuring the distance between *R. solani* and PMS-702 in dual cultures.

Preparation of Inoculum. A spore suspension of PMS-702 was prepared from cultures grown on ISP Medium 4 at 28 °C for 10 days. The suspension was added to SMGB in each 500-mL Erlenmeyer flask at a rate of 10^8 colony-forming units (CFU) per 100 mL of liquid medium. Cultures were kept on a shaker at 120 rpm at 30 °C for 50 h and used as seed stocks.

Fermentation. For large production of culture filtrates, PMS-702 was grown in a 5-L fermentor (BTF-600; Bio-Top, Taiwan) containing 3 L of SMGB and 30 mL of defoamer polyoxyalkylene ether (Adekanol LG109; Asahi-denka Ltd., Japan), aerated at 3 L/min and stirred at 120 rpm at 30 °C for 4 days. The 4-day-old cultures were filtered under vacuum, and the culture filtrates were lyophilized and stored at 4 °C in the dark until used for antibiosis studies and chemical analysis.

Fractionation and Purification of Antibiotic. The culture filtrate and mycelial mats of PMS-702 were separated by centrifugation at 10000*g* for 20 min and extracted with ethyl acetate. Both ethyl acetate extracts of liquid filtrate and mycelial mats showed antifungal activity, and hence they were combined. The whole extract (3 L) was concentrated to 200 mL under vacuum at 37 °C. The crude concentrated solution was adsorbed over silica gel (500 g) and fractionated with a Soxhlet extraction apparatus using hexane (1 L) and ethyl acetate (2 L) solvents. The hexane and ethyl acetate extracts were tested for their activity against RST-04. The ethyl acetate extract was found to be active, and this extract was then subjected to bioassay-guided separation through column (50 × 7.5 cm) chromatography over silica gel (400 g), eluted with $CHCl_3$−MeOH (100:0, 95:5, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50, 0:100). About 200 mL of each fraction was collected. Purifications were performed by column chromatography over silica gel (finer than 200 mesh and 100−200 mesh). The fractions were combined and made into four pooled fractions on the basis of their TLC behavior. The pooled fractions, PM-1 (eluted with chloroform and methanol 95:5 to 90:10), PM-2 (eluted with chloroform and methanol 90:10 to 50:50), PM-3 (eluted with chloroform and methanol 50:50 to 60:40), and PM-4 (eluted with chloroform and methanol 60:40 to 70:30) were screened again for their antifungal activity against RST-04 using the paper disk method (*5*). The fractions PM-3 and PM-4 were most active against RST-04 and they showed one major spot having the same R_f value (0.48, chloroform/methanol/water 7:2.8:0.2) in TLC.

Since they were identical, we combined these fractions and purified them by repeated chromatography over a silica gel column, followed by recrystallization in a mixture of hexane and chloroform (9:1). This yielded the active compound **1**. The structures of the active metabolite have been identified using NMR and mass spectral data. Purification of PM-1 and PM-2 resulted respectively in compounds **2** and compound **3**. The structures of compounds **2** and **3** were confirmed by comparison with authentic samples.

Structure Elucidation of the Antibiotic. The melting point of compound **1** was determined on a Buchi-540 melting point apparatus. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, IR spectra on a Perkin-Elmer 1 spectrometer, ^1H and ^{13}C NMR spectra on a Bruker DRX 500 spectrometer, and EI-MS and FAB-MS respectively on a Hewlett-Packard 5989 B and a Finnigan/Thermo Quest Mat 95 XL mass spectrometer.

Greenhouse Experiments. The inoculum of RST-04 was prepared by growing the organism on a sterile potato chip at 24 °C for 7 days. The artificial growth medium, BVB No. 4 peat moss (Bas Van Burren, Maasland, The Netherlands), was infested with *R. solani* at the rate of 1:1000 (w/w), placed in plastic bags, incubated at 24 °C for 7 days, and used for the experiments. The moisture content of *R. solani*-infested BVB No. 4 medium was adjusted and maintained at 50% (v/v) of water holding capacity.

Seeds of cabbage (*Brassica oleracea* L. var. *capitata* L.) cv. K-1 (Known-You Seed Co., Ltd., Taiwan) were treated with the cultural filtrate of PMS-702. Two polyelectrolytes [poly(acrylamide/dimethylamino ethyl−methacrylate cationic monomer)], FO4240SH and FO4490SH (SNF, St-Etienne, France), were diluted to 200 ppm. These polyelectrolytes were applied as the adjuvant to mix individually with the cultural filtrate at the rate of 1:10 (v/v). Seeds soaked in distilled water for 5 min were used as a control. Twenty seeds per treatment were air-dried and sown immediately in the plastic flat (45 × 35 × 15 cm, $L \times W \times H$) containing *R. solani*-infested BVB No. 4 medium. The experiment was repeated twice, and for each experiment, there were three replicates per treatment.

In another experiment, *R. solani*-infested BVB No. 4 medium was drenched with cultural filtrate of PMS-702 at 300-fold dilution one week before planting, 80 mL of filtrate/320 mL of medium. Twenty cabbage seeds were planted in each of three replicates, and the plants were grown in the greenhouse at 24−28 °C. Incidence percentage of pre-emergence and post-emergence damping-off of cabbage seedlings was determined 14 days after planting. The experiment was repeated twice.

Bioassay of Antibiotic. Supernatants of PMS-702 from SMBG liquid cultures, the extracted fractions, or purified compounds were tested for antibiosis against RST-04 using the paper disk method. Two pieces of 8-mm sterile paper disks (Advantec, Toyo Roshi Kaisha, Ltd., Japan) were respectively soaked in culture filtrate (40 μL), crude extract (40 μL), and each of three purified compounds (0.25 mg/40 μL) for 2 min. The air-dried disks were placed on a PDA plate for dual culturing with RST-04. Each plate was then inoculated with an agar block (8 mm diameter) containing mycelial mats of RST-04 in the center of the plate. The paper disks were 2.2 cm from the pathogen. Inhibition percentage was obtained 4 days after treatment at 28 °C from the equation as follows:

$$\text{inhibition (\%)} = [(\text{growth diameter in untreated control} - \text{growth diameter in treatment}) \times 100]/\text{growth diameter in untreated control}$$

Each treatment consisted of three replicates. The experiment was repeated twice.

Minimum Inhibitory Concentration. Fungichromin purified from culture filtrates of PMS-702 was bioassayed on PDA in Petri dishes to determine the minimum inhibitory concentration (over 90% inhibition) of fungichromin against RST-04 (*6*). Fungichromin (2 mg) was dissolved in dimethyl sulfoxide (DMSO, 200 μL), serially diluted in

Table 1. In Vitro Antagonism of *Streptomyces padanus* Strain PMS-702 against *Rhizoctonia solani*

strain of *R. solani*[a]	anastomosis group of *R. solani*	host	inhibition zone (mm) test I[b]	inhibition zone (mm) test II
RST-01	AG-1-IA	rice	24.7 b[c]	24.5 b
RST-02	AG-4	Chinese kale	18.8 d	20.2 d
RST-03	AG-4-HG-I	peanut	20.0 c	20.7 d
RST-04	AG-4	Chinese kale	18.7 de	18.8 e
RST-05	AG-2–1	pea	26.7 a	27.2 a
RST-06	AG-3	potato	20.5 c	22.3 c
RST-07	AG-4-HG-II	sugarbeet	17.8 e	16.8 f

[a] Strains of *R. solani* were respectively cultured on potato dextrose agar. Except for RST-02 and RST-04, all strains were provided by Dr. A. Ogoshi, Hokkaido University, Japan. [b] The inhibition zone was determined by measuring the distance (mm) between the *R. solani* and *S. padanus* strain PMS-702 in dual cultures on a PDA plate for 4 days at 28 °C. Each treatment consisted of three replicates. [c] Means within the same column followed by the same letter are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test.

the same solvent, and added to PDA at 48 °C. Five milliliters of the medium was added in a 5-cm-diameter Petri dish. The final concentrations were 0, 1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, and 120 μg/mL. A 5-mm-diameter plug of RST-04, removed from the margin of a 4-day-old colony on PDA, was placed at 1.5 cm from the edge of the plate. Linear growth of RST-04 at 28 °C was recorded 2 days after treatment. Each treatment consisted of three replicates. Inhibition percentage was obtained from the equation as described above. The experiment was repeated twice.

Data Analysis. Data of greenhouse experiments, and experiments of bioassay of antibiotic effects and minimum inhibitory concentration of PMS-702, were analyzed by SAS/STAT software (SAS Institute, Cary, NC). Means of treatments for each experiment were compared using Duncan's multiple range test ($P \leq 0.05$).

RESULTS

In Vitro Assay for Antagonism. Results of the dual cultures showed that *S. padanus* strain PMS-702 was inhibitory to the growth of several anastomosis groups of *R. solani*, including AG-1, AG-2, AG-3, and AG-4. Among the anastomosis groups of *R. solani* tested, PMS-702 was most inhibitory to the *R. solani* group AG-2-1 (**Table 1**). The inhibition zone of *R. solani* strains RST-01, RST-03, RST-05, and RST-06 was over 20 mm when they were dually cultured with PMS-702 for 4 days at 28 °C.

Greenhouse Experiment. Results of greenhouse experiments showed that the incidence of *Rhizoctonia* damping-off of cabbage was significantly ($P \leq 0.05$) reduced by PMS-702, either applied as seed dressing by the culture filtrate (**Figure 1A**) or drenched by mixing the culture filtrate at 300-fold dilution with *R. solani*-infested growth medium (**Figure 1B**). Adhesion of PMS-702 culture filtrate to cabbage seeds was increased after two kinds of polyelectrolytes, FO4240SH and FO4490SH, were used as an adjuvant of the culture filtrate. Therefore, PMS-702 culture filtrate with an adjuvant to dress seed did show better efficacy of controlling the disease than PMS-702 culture filtrate only in our preliminary tests (data not shown). The incidence of damping-off of cabbage was reduced to 31.3% and 50% respectively by the seeds treated with PMS-702-1 (PMS-702 filtrate amended with FO4240SH) and PMS-702-2 (PMS-702 filtrate amended with FO4490SH), compared to 100% in the control (**Figure 1A**). The incidence of damping-off of cabbage was also significantly reduced by drenching the growth medium with culture filtrate of PMS-702 before planting (**Figure 1B**). The disease incidence was significantly reduced 89.5% by the drenching treatment with culture filtrate of PMS-702 compared to the control.

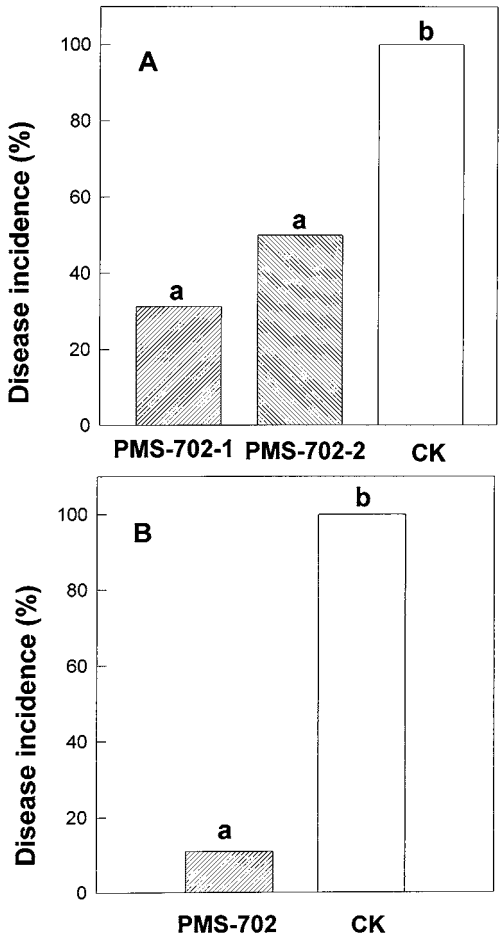

Figure 1. Effect of the cultural filtrate of *Streptomyces padanus* strain PMS-702 on control of damping-off of cabbage caused by *Rhizoctonia solani* strain RST-04. (A) Cabbage seeds dressed with the cultural filtrate of *S. padanus* strain PMS-702. Two kinds of polyelectrolytes, FO4240SH and FO4490SH, were diluted to 200 ppm and applied as the adjuvant of PMS-702-1 and PMS-702-2, respectively. (B) Culture broth of *S. padanus* strain PMS-702 applied to the *R. solani*-infested BVB No. 4 growth medium one week before sowing. Significant differences among treatments are indicated on columns by different letters at $p \leq 0.05$ according to Duncan's multiple range test.

Fractionation and Purification of Antibiotic. The crude extract (180 mg) of PMS-702 was subjected to silica gel column chromatography, and four pooled fractions, PM-1, PM-2, PM-3, and PM-4, were obtained. All the fractions were tested for their activity. Fractions PM-1 and PM-2 did not reduce growth of RST-04. Fractions PM-3 and PM-4 were markedly effective in reducing 57% and 62% growth of RST-04, respectively, and proved to be active ingredients. These active fractions PM-3 and PM-4 were then subjected to repeated chromatography, followed by recrystallization in a mixture of hexane and chloroform (9:1), and then afforded compound **1** (20 mg).

Structure Elucidation of Compound 1. Compound **1** was a pale yellow amorphous powder: mp 205−206 °C; $[\alpha]_D$ −177 (MeOH); UV λ max 356, 337, 340, and 320 nm; IR (KBr) ν_{max} 3400, 2930, 1721, 1082, and 850 cm^{-1}; MS (*m*/*z*, relative intensity) 693 [(M + Na), 12], 677 (2), 649 (2), and 629 (2). The compound was soluble in acetone, acetonitrile, butanol, chloroform, dimethyl sulfoxide, ethanol, methanol, and 2-propanol, while it was insoluble in hexane and water. The compound was identified as the polyene macrolide, fungichromin (**Figure 2**), on the basis of 1D and 2D NMR and

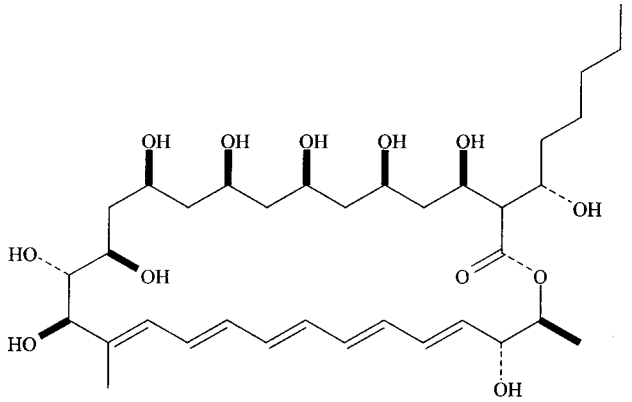

Figure 2. Structure of fungichromin, the active metabolite from *Streptomyces padanus.*

mass spectral data (*18*, *19*). Compounds **2** (50 mg) and **3** (15 mg), obtained from PM-1 and PM-2, were respectively identified as sterol glucoside and daidzein by comparison with their authentic samples.

Bioassay of Antibiotic. The antifungal activity of different fractions from culture broth of PMS-702 was evaluated using RST-04. The paper disk assay method indicated that fungichromin was most effective in inhibiting the growth of RST-04 by 94%. Both the culture filtrate of PMS-702 and its crude extract as the control also showed respectively 23% and 35% efficacy suppressive to RST-04. However, daidzein and sterol glycoside extracted from culture broth of PMS-702 did not inhibit the growth of RST-04.

MIC of Fungichromin against *R. solani*. The concentration of fungichromin was highly correlated ($R^2 = 0.98$) with the inhibition of mycelial growth of RST-04 in PDA plates. The relationship equation between concentration of fungichromin (x) and inhibition percentage of mycelial growth of RST-04 (y) was $y = 5.61 + 2.18x - 0.014x^2$. The mininum concentration of fungichromin for inhibition of RST-04 was 72 μg/mL. The mycelial growth of RST-04 was completely inhibited by fungichromin at 80−120 μg/mL.

DISCUSSION

The use of antagonistic microorganisms such as *Streptomyces* spp. is an ideal method for control of plant diseases (*20*−*22*). A commercial product, Mycostop, containing of *Streptomyces griseoviridis* was applied through the irrigation system to control important plant pathogens such as *Fusarium oxysporum*, *Botrytis cinerea*, and *Alternaria brassicicola* (*23*−*25*). However, seeds treated with *S. griseoviridis* were not effective in the control of *R. solani* of cauliflower. Recently, Sabaratnam and Traquair (26) reported that *Streptomyces* sp. Di-944 was as effective as the fungicide, oxine benzoate, when applied as drenching for control of *Rhizoctonia* damping-off of tomato. Furthermore, efficacy of *Streptomyces* sp. Di-944 on controlling *Rhizoctonia* damping-off was much better than that of *S. griseoviridis* when both were respectively dressed to tomato seeds. This study revealed that application of culture filtrates from *S. padanus* PMS-702 was effective in protecting cabbage seedling from infection of *R. solani*, either by seed dressing or by mixing with growth medium before seeding.

Previous reports indicated that fungichromin was produced by numerous species of fungi including *S. cellulose*, *S. fradiae*, *S. griseus*, *S. roseoluteus*, and *Streptoverticillium cinnamomeum* subsp. *cinnamomeum* (*27*, *28*). In our study, fungichromin was obtained from culture filtrates of *S. padanus*. It further proved that fungichromin was the key antifungal substance, as evidenced by the fact that the culture filtrate, crude extract, and pure fungichromin from *S. padanus* were all inhibitory to *R. solani*.

S. griseoviridis produced several metabolites which contained an aromatic heptene polyene-like candicidin. The metabolites were inhibitory to the growth of fungi, *Candida albicans*, *Fusarium culmorum*, and *Saccharomyces cerevisiae* (*7*). *S. griseus* had been used to control asparagus root diseases caused by *F. oxysporum* f. sp. *asparagi* and *F. moniliformae*. It was demonstrated that antagonism of *S. griseus* was closely associated with its polyene antibiotic faeriefungin (*30*). In our study, hyphae of *R. solani* treated with culture filtrates of *S. padanus* strain PMS-702 showed signs of necrosis and fractures when examined under a scanning electron microscope (data not shown). This suggests that the antibiotic from *S. padanus* may be related with hyphal collapse of *R. solani*. Polyenes are a group of macrolide antibiotics which selectively damage the permeability of membranes of yeasts, a wide variety of fungi, and other eukaryotic cells (*30*). The results of this study conclude that fungichromin is a major ingredient from culture filtrate of *S. padanus* strain PMS-702 and may play an important role for inhibition of the damping-off pathogen *R. solani*.

ABBREVIATIONS USED

ISP, International *Streptomyces* Project; MIC, minimum inhibitory concentration; PDA, potato dextrose agar; SMGB, soybean meal−glucose broth.

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