J. Microbiol. Biotechnol. (2001), **11**(6), 1120–1123

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Streptomyces Showing Antifungal Activities against Six Plant Pathogenic Fungi

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Received: September 28, 2001
Accepted: December 3, 2001

Abstract Screening tests against six plant pathogenic fungi were performed in order to develop biopesticides. Actinomycetes were used to discriminate *Bacillus thuringiensis* for wide use as a microbial pesticide. From more than 100 actinomycetes tested, twelve strains showed potent antifungal activities. We report *in vivo* screening results from fermentation broths of these twelve strains and identification of the strain taxa.

Key words: *In vivo* anti-fungal activities, *Magnaporthe grisea*, *Corticium sasaki*, *Botrytis cinerea*, *Pehytophthora infestans*, *Puccinia recondite*, *Erysiphe graminis*, *Streptomyces*

Fungal infestation of crops causes a significant loss in production. Many fungicides have been developed to combat this problem. However, since living organisms can change their survival abilities according to changes in their environment, the appearance of fungi that are resistant to fungicides is an increasingly serious problem. It is, therefore, necessary to develop new fungicides. Due to consumer rejection of chemical synthetic pesticides, we have focused on development of biopesticides.

Screening tests were used to investigate natural microbial pesticides against six plant pathogenic fungi. To discriminate *Bacillus thuringiensis* for wide use as a microbial pesticide, we screened actinomycetes isolated from soil samples from Cheju Island. The broth of each strain was used for screening instead of the microorganisms themselves. Therefore, secondary metabolites exhibited antifungal activities [3, 5, 6].

Soil samples were collected during 2001 at several sites near Hanla Mountain in Cheju Island, Korea. Samples were used as substrates for isolation of actinomycetes that exhibited antifungal activities. Starch-casein agar was used as an isolation medium. Cyclohexamide was autoclaved and added to the isolation medium at a concentration of 50 μg/ml for inhibition of fungal growth. Heat-labile nystatin sterilized by filtration through filter paper (ϕ 0.2 μm, Millipore, Watertown, MA, U.S.A.) was also added at a concentration of 50 μg/ml. The final pH of the medium was adjusted to 7.0–7.2 for isolation of actinomycetes. Approximately 1 g of soil was placed in a petri dish, then ground and heated at 60°C for 90 min under a dry oven. Dried soil samples were transferred to sterilized bottles after which 10 ml of sterile distilled water was added. Soil suspensions were mixed vigorously and left to stand for 30 min. One-tenth ml portions of the supernatant were innoculated into starch-casein agar. Inoculated plates were incubated at 28°C for 14 days. Colonies showing the typical characteristics of actinomycetes were selected from the plates. Colonies were then transferred to Bennet agar medium adjusted to pH 7.2 and cultured for 10 days at 28°C. Samples of each colony from the agar were inoculated into 30 ml of Bennet broth in a 100 ml capped tube and cultured in a shaking incubator at 28°C for 30 days. Fermentation broths were mixed with isopropanol and centrifuged. Supernatants were concentrated under reduced pressure and filtered. Remnants were dissolved in dimethyl sulfoxide and final concentrations were adjusted to 50 μg/ml.

Samples were tested for *in vivo* antifungal activities against six pathogenic fungi, including rice blast (*Magnaporthe grisea* (Hebert) Barr), rice sheath blight (*Corticium sasaki* Matsu), tomato grey mould (*Botrytis cinerea* Pers ex Fr), tomato late blight (*Pehytophthora infestans* (Mont) de Bary), wheat leaf rust (*Puccinia recondite* Rob ex Desm),

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and barley powdery mildew (*Erysiphe graminis* DC f sp *hordei* Marchal). Crops used for testing included rice (*Oryza saliva* L, cv Nakdong), tomato (*Lycopersicon esculentum* Mill, cv Seokwang), barley (*Hordeum sativum* Pers, cv Dongbori), and wheat (*Triticum aestivum* L, cv Chokwang). Test crops were grown in vinyl pots in a greenhouse at 25 (±5)°C for 1–4 weeks. Potted crop seedlings were sprayed with broth dissolved in water + methanol (95:5 by volume) containing Tween 20 (250 mg/l) as a wetting agent, and allowed to stand for 24 h [2].

For development of pathogenic fungi, treated crop seedlings were inoculated by spraying with fungal spore suspensions (10^7–10^9 spores/l). After incubation of the seedlings in the dark for one day at 25 (±2)°C and 100% RH, seedlings were transferred to a growth chamber and maintained at 25 (±2)°C and 70–80% RH with 12 h of light per day. Disease severity was determined as a percentage of the infected leaf area [2].

Pots were arranged as a randomized complete block with three replicates per treatment. Each pot was assayed for extent of infection by visual estimation of the percentage of leaf area covered by sporulating lesions, or the percentage of chlorotic present with necrotic symptoms on the inoculated foliage or sheaths. Data are the result of two trials. The mean value of six estimates for each treatment was converted into a percentage of fungal control using the equation:

$$\% \text{ Control} = 100\ [(A-B)/A]$$

where A=area of infection (%) on leaves or stems sprayed with Tween 20 solution alone and B = area of infection (%) on treated leaves or sheaths. Analysis of variance was performed with the PROC GLM procedure (SAS Institute, Cary, NC, U.S.A.). If P>F was less than 0.01, means were separated by a test that showed the least significant difference at a level of P=0.05 [2].

Among more than 100 actinomycetes screened against six pathogenic fungi, twelve strains showing activities greater than at least 60% of the fungal control are listed in Table 1. Activities were compared with activities of fungicides used in the field (Table 2). Strains 2-41, 2-42, 2-43, and 2-55 showed potent activities against rice blast (*Magnaporthe grisea*). Strains 2-17, 2-21, and 2-22 showed potent activities against tomato grey mould (*B. cinerea*). Strains 2-15, 2-17, 2-19, 2-21, 2-31, and 2-32 showed potent activities against tomato late blight (*P. infestans*). Strain 2-35 showed a potent activity against wheat leaf rust (*Puccinia recondite*). Since the concentrations of pesticides used as references were different, strain activities could not be compared directly. However, it is clear that the broth fermented from strain 2-17 showed a potent activity against tomato late blight (*P. infestans*). The activity caused by strain 2-17 (96% at a 50 μg/ml concentration) is comparable to the activity of chlorothalonil (50% at the same concentration, used as a reference).

We identified the twelve strains that exhibited anti-fungal activities. 16S rDNA analysis was performed for identification of strain 2-17. The partial nucleotide sequences (500-bps) containing the hypervariable a region of 16S rDNA from DNAs of the strain were analyzed. DNAs were prepared by the modified bead beater-phenol method [1]. A loopful of the culture was suspended in 200 μl of TEN buffer (100 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl; pH 8.0), placed in a 2.0 ml screw capped tube filled

Table 1. The results screened against six pathogenic fungi of the fermentation broths of twelve strains (Concentration: 50 μg/ml).

Strains	RCB	RSB	TGM	TLB	WLR	BPM
2-15	0	0	0	88	7	42
2-17	10	0	84	96	13	0
2-19	0	0	7	71	0	0
2-21	0	0	75	64	3	0
2-22	0	0	50	60	0	0
2-31	0	0	0	82	27	17
2-32	10	0	0	64	13	33
2-35	10	0	0	50	87	0
2-41	85	0	0	0	0	0
2-42	70	0	0	0	0	0
2-43	92	0	0	13	3	0
2-55	60	0	0	0	0	0

RCB, rice blast (*Magnaporthe grisea* (Hebert) Barr); RSB, rice sheath blight (*Corticium sasaki* Matsu); TGM, tomato grey mould (*B. cinerea*); TLB, tomato late blight (*P. infestans*); WLR, wheat leaf rust (*Puccinia recondite* Rob ex Desm); BPM, barley powdery mildew (*Erysiphe graminis* DC f sp *hordei* Marchal).
Unit: % Control.

Table 2. Activities of the pesticides used as references.

Pesticides	Conc. (μg/ml)	RCB	RSB	TGM	TLB	WLR	BPM
Blasticidin-S	1	86					
Tricyclazole	0.5	96					
Validamycin	5		71				
Flutolanil	20		100				
Fludioxonil	5			84			
Dichlorofluanid	40			97			
Dimethomorph	10				88		
Chlorothalonil	50				50		
Flusilazole	10					99	
Mancozeb	10					93	
Flusilazole	0.5						82
Benomyl	1						72

RCB, rice blast (*Magnaporthe grisea* (Hebert) Barr); RSB, rice sheath blight (*Corticium sasaki* Matsu); TGM, tomato grey mould (*B. cinerea*); TLB, tomato late blight (*P. infestans*); WLR, wheat leaf rust (*Puccinia recondite* Rob ex Desm); BPM, barley powdery mildew (*Erysiphe graminis* DC f sp *hordei* Marchal).
Unit: % Control.

Table 3. Identification of twelve strains showing potent activities by 16S rDNA analysis and a fatty acid analysis.

Strains	16S rDNA analysis	The highest homology value (%)	Fatty acid analysis	Similarity indexes
2-15	*S. aureofaciens*	97	*S. aureofaciens*	0.363
2-17	*S. halstedii olivaceus*	99.8	*S. halstedii olivaceus*	0.124
2-19	*S. panayensis*	98	*S. panayensis*	0.565
2-21	*No match*	-	*S. septatum*	0.053
2-22	*No match*	-	No match	-
2-31	*S. glaucescens*	99	*S. glaucescens*	0.194
2-32	No match	-	*S. cyaneus chartreusis*	0.186
2-35	No match	-	No match	-
2-41	*S. aureofaciens*	98	*S. aureofaciens*	0.026
2-42	*S. violaceusniger*	98	*S. violaceusniger*	0.021
2-43	*S. olivoreticuli*	97	No match	-
2-55	*S. lavendulae*	99	*S. lavendulae*	0.068

with 100 µl (packed volume) of glass beads (ϕ 0.1 mm; Biospec products, Bartlesville, OH, U.S.A.), and 100 µl of phenol-chloroform-isoamyl alcohol (50:49:1). The tube was oscillated on a Mini-Bead Beater (Biospec products) for 1 min to disrupt the bacteria. Then, the tube was centrifuged (12,000 ×g, 5 min) to separate the aqueous phase. After the aqueous phase was transferred to a clean tube, 250 µl of ice-cold ethanol was added to allow the DNA to precipitate, and the mixture was maintained at −20°C for 10 min. The DNA pellet was washed in 70% ethanol dissolved in 60 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and stored. PCR analysis of 500-bps 16S rDNA was performed according to the method of Ueda *et al.* [6]. The sequences of synthesized oligonucleotides used were: sense primer (16Sf), 5'-TCACGGAGAGTTT-GATCCTG-3'; and antisense primer (16Sr), 5'-GCGGCT-GCTGGCACGTAGTT-3'. The sequence of each primer was selected from the conserved region and corresponded to nucleotide positions 1 to 20 for the sense primer and 481 to 500 of the anti-sense primer of the *S. ambofaciens* rDNA sequences.

PCR was performed in a 20 µl reaction mixture tube (Accupower PCR PreMix; Bioneer, Chungbuk, Korea). The reaction mixture was subjected to 30 cycles of amplification (60 sec at 95°C, 45 sec at 60°C, and 80 sec at 72°C) followed by a 5-min extension at 72°C (model 9600 Thermocycler; Perkin-Elmer, Foster City, CA, U.S.A.). PCR products were electrophoresed on 3% agarose gel and purified using a QIAEX II gel extraction kit (Qiagen, Hilden, Germany). The nucleotide sequences (500-bps) of purified PCR products were directly determined with sense and antisense primers with an Applied Biosystems model 373A automatic sequencer and a BigDye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems; part no. 4303153). The sequencing mixture was prepared according to the supplier's protocol. The mixture was directly sequenced using a PCR amplification of 30 cycles (10 sec at 95°C and 4 min at 60°C). The 16S rDNA sequences (500-bp) of strain 2-17 identified from GenBank by the BLAST program showed the highest homology (99.8% identity) with *Streptomyces halstedii olivaceus* [4]. All strains except four were identified (Table 3).

A fatty acid analysis was performed because some of the highest homology values in 16S rDNA analysis were less than 98%. Whole-cell fatty acid methyl esters between 9 and 20 carbons were analyzed using the MIDI automated Microbial Identification System (MIS, MIDI Inc., Newark, DW, U.S.A.) with gas chromatography (GC) analysis. Strain 2-17 was prepared as described in the MIDI operating manual for the MIS. The sample was saponified with sodium hydroxide in methanol, methylated with hydrochloric acid in methanol, extracted with hexane in methyl *tert*-butyl ether, and cleaned by base wash with sodium hydroxide. Identification of actinomycetes was performed on a Hewlett-Packard (Avondale, PA, U.S.A.) GC system. Searching in the MIS ACTIN1 library version 3.8 found that *Streptomyces halstedii olivaceus* was the closest species. Because the similarity index value of 0.124 was less than the acceptable guide range (0.400) for identical species, strain 2-17 is suspected to be a new taxa related to *S. halstedii olivaceus*. Fatty acid analyses were performed on the other strains. Results are listed in Table 3. Even though all other strains (except strain 2-19) did not exactly correspond to known species, they were all related to *Streptomyces*. Ten strains were identified based on 16S rDNA analysis and a fatty acid analysis. However, strains 2-22 and 2-35 are thought to be new taxa related to *Streptomyces*.

Strain 2-17 exhibited a potent activity against tomato late blight (*P. infestans*). Chlorothalonil (used here as a reference), which is used to control fungal diseases in greenhouses by acting on the enzyme systems of fungi, shows non-target toxicity because it is a broad-spectrum fungicide. Due to the fact that strain 2-17 exhibited a very low activity against all pathogenic fungi except tomato late blight and tomato grey mould (Table 1), use of strain 2-17 as a fungicide would prevent the problems associated with

the broad spectrum activity of Chlorothalonil. In addition, the antifungal activity was caused by a broth. Therefore, strain 2-17 is applicable to water cultures of tomatos. Strain 2-35 was the only strain that showed a potent activity against wheat leaf rust (*Puccinia recondite*). Even though its activity was weaker than the fungicides used as references, it was a new species of *Streptomyces* that should be useful for antifungal treatment of specific target crops where public sentiment will not allow for the use of conventional synthetic chemical fungicides.

Acknowledgment

This work was supported by a grant received from Biogreen 21 project in 2001.

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