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Identification of *Streptomyces* sp. Producing Antibiotics Against Phytopathogenic Fungi, and Its Structure

JEONG, DO HYEON[1], KI DUK PARK[1], SUNG HAN KIM[1], KYUNG RAE KIM[2], SUNG WON CHOI[3], JI TAE KIM[3], KI HYUN CHOI[3], AND JUNG HAN KIM[1]*

[1]*Department of Biotechnology and Bioproducts Research Center, Yonsei University, Seoul 120-749, Korea*
[2]*Department of Pharmacy, Sunggunkwan University, Suwon 440-746, Korea*
[3]*R&D Center Greenbiotech Co., Ltd., Gyeonggi-do 413-835, Korea*

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Abstract In order to develop a biocontrol agent that can effectively control Fusarium wilt on *Cymbidium* genus, the effectiveness of antagonistic microbes against the cause pathogen was screened. The selected microbe showed a broad spectrum of antifungal activity, and the culture broth of this microbe had better preventive effect on Fusarium wilt than the commercial chemical agent in the pot assay. This isolated strain, GBA-12, was identified as *Streptomyces kasugaensis*, and the antifungal substance was purified from a broth culture of GBA-12. This purified substance was identified as a polyene macrolide (YS-822A) that was newly discovered from *Streptomyces kasugaensis*, and it exhibited antifungal activity against several phytopathogenic fungi.

Key words: Antifungal antibiotics, *Streptomyces kasugaensis*, polyene macrolide, *Fusarium oxysporum*

More than 100 diseases have been reported originating from *Cymbidium*, such as black rot, root rot, and Fusarium wilt [17]. Fusarium wilt is especially difficult to control with chemicals [11, 20], and therefore, inflicts a significant loss on the farmers. Thus, a new strategy to control the pathogen causing Fusarium wilt is urgently needed. The most desirable method of control would be the use of a biological agent, and attempts to develop biological controls of plant pathogens have attracted an increasing number of scientists over the last 25 to 30 years [3, 5, 6, 9].

Since streptomycin was discovered from the culture broth of *Streptomyces griseus* by Waksman in 1944 [22], numerous antibiotics have been isolated from actinomycetes, which are still believed to be the richest source of microorganisms for antimicrobial agents. In addition, many agricultural antibiotics were discovered from *Streptomyces* sp., and some of them have been commercialized, such as kasugamycin [26] and blastadin S [13].

In a preceding paper [10], we isolated the pathogens from infected leaves and bulbs of *Cymbidium* 'Anmitsu Hime.' It is understood that *Fusarium oxysporum* f. sp. *cattleyae* displays a strong pathogenicity against *Cymbidium* 'Anmitsu Hime,' and the phytopathogen is the major cause of Fusarium wilt on *Cymbidium* genus. In the course of screening antagonistic microbes against *Fusarium oxysporum* f. sp. *cattleyae*, GBA-12 was isolated from soil samples collected in Jeju, Korea [10].

This paper reports on the identification of the antifungal bacteria producing a novel biocontrol agent that has antifungal activity against phytopathogen of Fusarium wilt. GBA-12 was selected as the strongest antagonistic microbe against Fusarium wilt on *Cymbidium*, and it was found to produce active compounds with phytopathogenic antifungal activity. GBA-12 was selected based on the extent of antifungal activity as well as its possibility to be developed as an alternative biological agent. Cultural and biochemical characteristics of the selected microorganism were examined for taxonomic identification, and the structure of its antifungal substance was identified by using spectrometric techniques such as UV, IR, MS, and NMR spectroscopies.

The phytopathogenic fungi *Fusarium oxysporum*, *Pyricularia oryzae*, *Rhizoctonia solani*, and *Botrytis cinerea* were kindly provided by the Laboratory of Phytopathology, Jeju University, Jeju, Korea. The plant pathogenic fungi were maintained on a potato dextrose agar (PDA). The organic compounds, antibiotics, and other chemicals were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), which were of the highest purity available. The media or ingredients for the media were procured from

*Corresponding author
Phone: 82-2-2123-2884; Fax: 82-2-362-7265;
E-mail: junghan@yonsei.ac.kr

Table 1. Antagonistic and growth-promoting effects of the isolates on Fusarium wilt pathogenicity in *Cymbidium* cultivars after three months treatment.

Cultivar[a] / Treatments	Suvival rate (%)						Leaf length (cm)						Root number (ea)					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
Control	50	40	30	40	60	60	19c[b]	6d	12.5d	14.2b	7.7c	9.0c	5.3b	4.3c	9.4c	5.9d	5.7e	9.5c
Benlate	70	60	60	70	50	40	16.6d	6.5c	8.7e	13.1c	8.0c	9.5c	5.0c	4.2c	8.3d	6.1d	4.2d	5.8d
GBA-12	100	100	90	100	80	90	24.6a	8.7a	15.4a	16.9a	11.0b	13.7a	8.6a	7.4a	12.5b	10.5bc	4.0b	5.3b

[a]1) *Cym.* nevo-marginatum, 2) *Cym. kanran*, 3) *Cym. goeringii*, 4) *Cym. Lancifolium*, 5) *Cym.* 'Anmitu Hime', 6) *Cym.* 'Marirorencin.'
[b]Mean separation by Duncan's multiple range test at the 5% level.

Difco (Detroit, MI, U.S.A.). After completing the fermentation process, 10 ml of the culture broth was centrifuged at 3,000 rpm for 10 min. The supernatant was assayed for active compound content. The test strain, *Streptomyces* GBA-12 cultivated with YM liquid medium at 27°C for 24 h, was inoculated at the rate of 0.2% in 10 ml of YM agar medium. The activity of culture broth was assayed by using a paper disk method [14, 24]. Nearly 500 bacterial isolates from field soils were screened for antifungal activity against *F. oxysforum*. Several of these antifungal bacteria, including GBA-12, were selected, and GBA-12 exhibited a potent antifungal effect on several major phytopathogens, including *Rhizoctonia solani*, *Pyricularia oryzae*, and *Botrytis cinerea* as well as *F. oxysporum*. The GBA-12 was chosen for further indepth studies. The selected strain GBA-12 was tested for adaptability and preventive power in a small scale. Four species of oriental *Cymbidium* and two species of western *Cymbidium* were tested, and Fusarium wilt pathogen, commercial chemical agent benlate, and isolated GBA-12 culture broth were treated for tests. The results are as follows (Table 1); the survival rate of the controls were 30-60%, the benlate treatments were 40-70%, and the isolated GBA-12 treatments were 80-100%. The survival rate of isolated GBA-12 treatments and the growth-promoting effect were outstanding.

To determine the genus of the actinomycete strain GBA-12, the type of 2,6-diaminopimelic acid (DAP), which is one of the cell wall components of actinomycete mycelia, was analyzed by the methods of the International Streptomyces Project (ISP) that was suggested by Shiriling and Gottlieb, and *Bergey's Manual of Systematic Bacteriology* [27]. The spores and aerial mycelium were observed, and the spore chain was determined to be a spiral type. The spore surface of the isolate was shown to be smooth, while the color of the sporulate was in a series of grey. No melanoid pigment was produced in the media, and the cell wall DAP (diaminopimelic acid) was found to be a LL-type (Table 2). From these results, the isolate was placed in the *Streptomyces* genus. The growth of isolate GBA-12 on the ISP media was substantial (data not shown). Aerial mycelium color was grey or yellow, while the reverse side color was yellow to brown depending on the medium employed. No soluble pigment was produced in any other agar media. Amino acid composition of the cell wall was found to be glutamine, alanine, and glycine.

Table 2. Morphological characteristics of the isolate GBA-12.

Morphological characteristics	GBA-12
Spore chain	Spiral
Spore size	1.2-1.4×2.0 μm
Spore surface	Smooth
Color of sporulated	Grey
DAP	LL-type

To confirm the identification of the isolate, 16S rRNA sequencing was carried out by a slightly modified method that was previously published [12, 21]. Wizard PCR Preps DNA Purification System (Promega Corp., Madison, U.S.A.), GenAmp™PCR System 9700 (Perkin-Elmer, Boston, U.S.A.), ABI PRIBSM™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Boston, U.S.A.), ABI PRIBSM™ 310 Genetic Analyzer (Perkin-Elmer, Boston, U.S.A.), and Phylogenetic Inference Package (PHYLIP version 3.5c) were used for the sequencing analysis. The 16S rRNA sequence of isolated GBA-12 was compared with other *Streptomyces* sp. When the GenBank search was performed, the sequence showed 98.94% similarity with *Streptomyces*

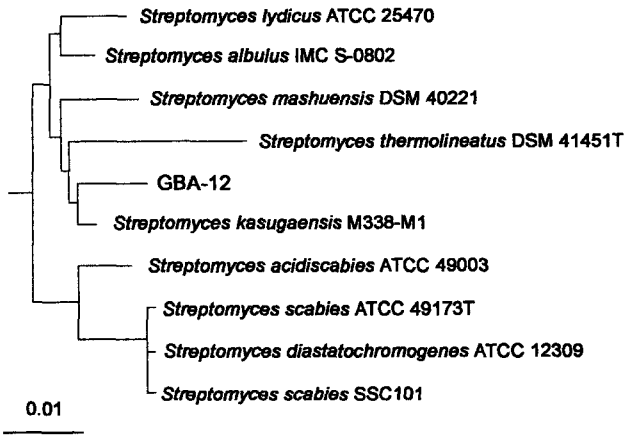

Fig. 1. Dendrogram showing the relationships between GBA-12 and other *Streptomyces* sp.
The rooted tree constructed using the neighbor-joining method; the scale bar indicates 0.01 substitutions per nucleotide position.

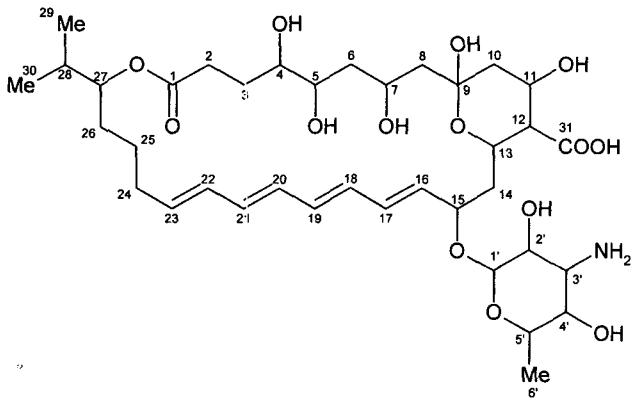

Fig. 2. The structure of purified antifungal compound YS-822A.

kasugaensis. A dendrogram on the basis of 16S rRNA sequences showing the phylogenetic relationship of *Streptomyces kasugaensis* is shown in Fig. 1. Based on the DAP type of cell wall, the morphological characteristics, and the 16S rRNA sequence analysis, GBA-12 was concluded to belong to the *Streptomyces kasugaensis*.

In each step of the purification, the fractions were tested for biological activity against *Fusarium oxysporum* by a paper disk assay procedure. Finally, HPLC was performed by using the Waters HPLC apparatus (Waters, Milford, U.S.A.), and reverse phased column (Luna 5 μm, C_{18} 4.6 mm×250 mm, Phenomenex, Torrance, U.S.A.) was used to confirm the purity. The structure of the purified antifungal material was then examined by using an EI-MS (Micromass Q-TOF2, Micromass, Manchester, U.K.), NMR (AVENCE, BRUKER, Rheinstetten, Germany), FT-IR (IFS 66, BRUKER, Rheinstetten, Germany), and UV (HP8452A, Hewlett Packard, U.S.A.) spectroscopies. According to these analyses, the structure of this compound was identified as YS-822A (Fig. 2), which has already been isolated from *Streptoverticillium eurocidicum* var *asterocidicus* S-822 [1].

Antifungal activity of the purified active compound was measured as MICs by methods described previously [8, 18]. The purified active compound (YS-822A) exhibited a good antibiotic activity against several soilborne fungal phytopathogens (Table 3). The *in vitro* antifungal activities (MICs) of YS-822A, which were measured with cycloheximide as a reference, are as follows; *Fusarium oxysporum* was 4 μg/ml, *Rhizoconia solani* was 2 μg/ml, *Botrytis cinerea* was 2 μg/ml, and *Pyricularia oryzae* was 2 μg/ml.

Table 3. Antifungal activity of YS-822A against several phytopathogenic fungi.

Fungus tested	MICs (μg/ml)*	
	Purified active compound	Cycloheximide
Fusarium oxysporum	4	32
Rhizoctonia solani	2	32
Botrytis cinerea	2	32
Pyricularia oryzae	2	32

*MICs are defined as the minimal concentration of YS-822A and cycloheximide inhibition of fungal growth on PDA media after 7 days.

Generally, the plant disease of soilborne outbreaks occurs in the root of the plant. Because the pathogen exists in the soil and the symptoms of infection are shown in the latter phase of development, the soilborne disease is very difficult to control with chemical agents [2]. The Fusarium wilt occurs mainly in the bulb part of the orchid, and it is the cause of root corruption. In the previous report, the cause pathogen of Fusarium wilt was found to be *Fusarium oxysporum* f. sp. *cattleyae* [10]. The *F. oxysporum* was a phytopathogenic fungi that caused many crop diseases, and many attempts have been made to control this pathogen with biocontrol agents. Representatives of these cases are *Pseudomonas* spp. [7, 15], *Trichoderma* spp. [19], *Penicillium* spp. [16], *Bacillus* spp. [29], *Streptomyces* spp. [4] and nonpathogenic *Fusarium oxysporum* [23, 25, 28].

The *Streptomyces kasugaensis* is well-known for producing kasugamycin, the representative agricultural antibiotic [26]. This is the first report made on *S. kasugaensis* producing a tetraene macrolide antibiotic, YS-822A. The YS-822A has already been isolated from *Streptoverticillium eurocidicum* var *asterocidicus* S-822 as an antibiotic, however, it could not attract a great deal of attention as an agricultural antibiotic because of its lower therapeutic antifungal activity compared to amphotericin B or nystatin, which has similar functional groups [1]. However, according to our studies, the YS-822A has a higher antifungal activity against several phytopathogenic fungi which have not been reported. Although YS-822A has an unstable structure, as is often the case with polyene antibiotics, there is a possibility that it could be developed as a biocontrol agent because of its broad antifungal spectrum against phytopathogenic fungi.

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