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Identification of *Streptomyces* sp. Producing New Polyene Antibiotics and *In Vivo* Antimicrobial Activity of Tetrin C Against Phytopathogenic Fungi

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Abstract A *Streptomyces* sp. isolated from a soil sample collected in Taejeon, Korea has previously been found to produce two new polyene antibiotics. The two new antibiotics were named "16-methyloxazolomycin (antibacterial)" and "tetrin C (antifungal)", and their chemical structures are presented elsewhere [10, 11]. In the current study, chemotaxonomy, numerical taxonomy, and ISP methods were all employed for the taxonomic study. The spore chains were *spirales* and the spore surface was smooth. The spore mass was a gray series and no melanin pigment was produced. On the basis of the morphological and physiological properties, the microorganism was identified to be *Streptomyces erumpens*, belonging to the gray series of category IV, as defined by Bergey's Manual. Tetrin C at the concentration of 20 µg/ml demonstrated a potent *in vivo* (pot test) preventive effect against rice blast, rice sheath blight, cucumber gray mold, wheat powdery mildew, and barley leaf rust.

Key words: *Streptomyces erumpens*, taxonomy, tetrin C, phytopathogenic fungi, preventive effect

Since the discovery of streptomycin from the culture broth of *Streptomyces griseus* by Waksman [17] in 1944, a large number of antibiotics have been isolated from actinomycetes, which are still believed to be the richest source of microorganisms for antimicrobial agents. However, the continued development of new antibiotics has required taxonomists to assign new species names to prove the novelty of an antibiotic, thereby resulting in the overclassification and proliferation of a species or genus.

As such, one of the most remarkable developments during the past two decades is the development of numerical taxonomy for actinomycete genera classification [2, 19, 20]. However, it is still not easy to identify species with the aid of information available from Bergey's Manual and/or numerical taxonomy. Until the latest revision of "Bergey's Manual of Systematic Bacteriology [18]" in 1989, the identification of actinomycetes was mostly achieved by the method described in "International Streptomyces Project [ISP]". Although the morphological and physiological characteristics described in ISP still play an important role in the identification of *Streptomyces* and its related genus [12-16], the ISP method seems to be inadequate for the beginner taxonomist to identify the organism of interest. Nevertheless, the main chemotaxonomical marker such as the diaminopimelic acid isomer in the cell wall can provide crucial information for identifying a genus. Sometimes, the identification of the active ingredient(s) plays a key role in the identification of the organism of interest. In this respect, a combination of these methods would be more reliable for the characterization of microorganisms.

A variety of bioactive 26-membered polyene macrolide antibiotics, including aminosugar mycosamine, have been produced from *Streptomyces* spp. since the late 1950s. Triene antibiotics containing β-lactone-γ-lactam bicyclospiro and oxazole rings such as oxazolomycin [7] and curromycins [8, 9] are unique secondary metabolites produced by *Streptomyces* spp. In the course of antibiotic screening, *Streptomyces* GK9244, isolated from a soil sample collected in Taejon, Korea was found to produce active compounds with antimicrobial activity against bacteria and fungi, including *in vitro* cytotoxicity against cancer cell lines. The isolation, purification, and structural determination of the active compounds yielded 2 new polyene antibiotics, which were named "16-methyloxazolomycin, an antibacterial agent [10]" and "tetrin C [11], an antifungal agent".

The current study describes the morphological and physiological characteristics of the isolate carried out using numerical taxonomy, the ISP method, and chemotaxonomic approach by determining the cell wall DAP isomer. Results

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showed the isolate to be *S. erumpens*. In addition, tetrin C was found to be a potent *in vivo* (pot test) preventive of rice blast, rice sheath blight, cucumber gray mold, wheat powdery mildew, and barley leaf rust at the concentration of 20 μg/ml.

MATERIALS AND METHODS

Chemicals and Media

The purified chitin was prepared from coarse crab shell flakes of practical grade using the method of Hsu [4]. The organic compounds, antibiotics, and other chemicals were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and were of the highest purity available. The media or ingredients for the media were procured from Difco (Detroit, MI, U.S.A.). The Cefinase disc for the β-lactamase inhibitor production assay was purchased from BBL (Cockeysville, Maryland, U.S.A.).

Identification of Isolate GK9244

The isolate was obtained from a soil sample collected in Taejeon, Korea. The identification of the isolate was carried out principally according to the methods of Williams *et al.* [20] and ISP [12]. The ability of the isolate to use 25 different carbon sources was examined in a carbon utilization agar (ISP medium 4), as recommended by Shirling and Gottlieb [12]. The utilization of nitrogen sources was determined on the basal medium as described by Williams *et al.* [20]. The colors of the mycelial and diffusible pigments were determined by matching the colors to the Inter-Society Color Council-National Bureau of Standards (ISCC-NBS) Centroid Color Charts [5]. Detailed information on the preparation of the inoculum and the investigation of each unit character has already been described by Kim *et al.* [6].

Microbial Strain

The test microorganisms used for the antimicrobial test were obtained from the Institute of Fermentation, Osaka (IFO), Institute of Applied Microbiology (IAM), Tokyo University, Japan Collection of Microorganisms (JCM), RIKEN, Japan, and the American Type Culture Collection (ATCC).

Diaminopimelic Acid

The isolate GK9244 was cultured in a yeast-malt extract broth for 48 h and pelleted by spinning. The pelleted cell was treated with sodium dodecylsulfate (SDS) to obtain the crude cell wall, which was then hydrolyzed in 6 N HCl at 121°C for 15 min. The crude cell wall hydrolysate was concentrated under reduced pressure and then resolved on cellulose TLC with MeOH/H_2O/5 N HCl/pyridine (80:15:5:10) as the developing solvent [3].

Submerged Culture of Isolate for Antibiotic Production

The isolate was cultured in 100 ml of seed medium consisting of glucose 2%, starch 1%, soybean flour 2.5%, yeast extract 0.4%, NaCl 0.2%, K_2HPO_4 0.005%, and beef extract 0.1% (adjusted to pH 7.3 before sterilization) in a rotary shaker (250 rpm) at 28°C for 24 h in 500-ml Erlenmeyer flasks. The seed culture (300 ml) was then inoculated into a 50-l jar fermentor containing 30 l of an antibiotic production medium (antifoam 0.08%). The fermentation was carried out at 27°C for 96 h with aeration (30 l/min) under constant agitation (250 rpm).

In Vivo (Pot Test) Biological Activity of Tetrin C

Tetrin C was isolated as described previously [11]. The whole culture broth (30 l) of the isolate was centrifuged to separate the mycelial cake and the broth filtrate. The mycelial cake was stirred overnight in 70% aqueous acetone (5 l) and filtered. The acetone extract was concentrated *in vacuo* and pooled with the broth filtrate. It was then passed through a Diaion HP-20 column (7 l) and washed with H_2O followed by MeOH elution. The MeOH eluate was partitioned between *n*-BuOH and H_2O. The *n*-BuOH fraction was fractionated by ODS flash chromatography with aqueous MeOH. The 70% MeOH fraction was gel-filtered on Sephadex LH-20 with MeOH to yield an active fraction. The active fraction was finally purified by reversed-phase HPLC with 63% MeOH. Tetrin C for *in vivo* test was dissolved in 10% aqueous acetone to give the final concentration of 20 μg/ml.

The *in vivo* biological activity of the Tetrin C was investigated against phytopathogenic fungi in green house. The tested fungi were cultured in rice or wheat bran media to allow them to fully produce spore mass and/or sclerotia. *Puccinia recondita* was grown on its host plants, dried, and stored at −20°C until use. *Rhizoctonia solani* was homogenized to slurry with wheat bran and sterilized distilled water. The phytopathogens were diluted to a final concentration of 10^5 spores/ml and sprayed onto the host plant. The tetrin C solution was sprayed onto the host plants 24 h before the inoculation of the pathogens. The preventive effect of the broth filtrate was calculated according to the following formula;

Preventive effect (%)

$$= \left(1 - \frac{\text{No. of necrotic spots on treated plants}}{\text{No. of necrotic spots on control}}\right) \times 100$$

RESULTS AND DISCUSSION

Morphological and Physiological Characteristics of Isolate GK9244

The spores and aerial mycelium were observed, and the spore chain was determined to be *spirales* type (Table 1).

Table 1. Morphological and physiological characteristics of isolate GK9244.

Unit character	Characteristics
Morphology	Presence of spores and aerial mycelium
Spore chain morphology	*Spirales*
Spore chain ornamentation	smooth, aerial spore mass (+)
Color of aerial spore mass	gray to white series
Melanoid pigment production in	(−); tryptone-yeast Ext. broth, tyrosine agar
Cell wall DAP composition	LL-DAP
Gelatin liquefaction (27°C)	(+); glucose-peptone gelatin, gelatin
Skim milk (27°C, 30°C)	(+); peptonization, (−); coagulation
Cellulose decomposition	Negative
Antimicrobial activity against	(+); *B. subtilis*, *C. albicans*, *Strep. murinus*, *Asp. niger*
	(−); *P. fluorescens*, *M. luteus*, *S. cerevisiae*
Enzymatic activity	(+); proteolysis, lipolysis, H_2S production, hippurate hydrolysis, β-lactamase production on YPG agar and Beecham's FS agar
	(−); lecithinase, pectin hydrolysis, chitin hydrolysis, production of *Klebsiella* β-lactamase inhibitor
Organic compound degradation	(+); hypoxanthine, elastin, L-tyrosine, adenine, DNA, RNA, tween 80, starch, casein, testosterone, urea, gelatin, aesculin, arbutin
	(−); guanine, xanthine, xylan, allantoin
Resistance to antibiotics (μg/ml)	(+); streptomycin, rifampicin (50)
	(−); gentamicin (100), neomycin (50), tobramycin (50), vancomycin (50), demethylchlorotetracycline (500), oleandomycin (100), lincomycin (100), penicillin G (10 IU)
Viable temperature and pH tolerance	(−); 4, 10, 37, and 45°C
	(−); pH 4.3
Tolerance against chemical inhibitors (%)	(+); sodium chloride (4), phenylethanol (0.1, 0.3), potassium tellurite (0.001, 0.01), thallus acetate (0.001)
	(−); sodium chloride (7, 13), sodium azide (0.01, 0.02), phenol (0.1), thallous acetate (0.01), crystal violet (0.0001)

(+); positive, (−); negative.

The spore surface of the isolate was shown to be smooth (Fig. 1), while the color of the spore mass was a gray series. No melanoid pigment was produced in the media and the cell wall diaminopimelic acid was found to be LL-type. From these results, the isolate was placed in the *Streptomyces* genus. The isolate was able to liquefy gelatin in the media. GK9244 was found to peptonize but not coagulate skim milk. Cellulose was not degraded by the isolate.

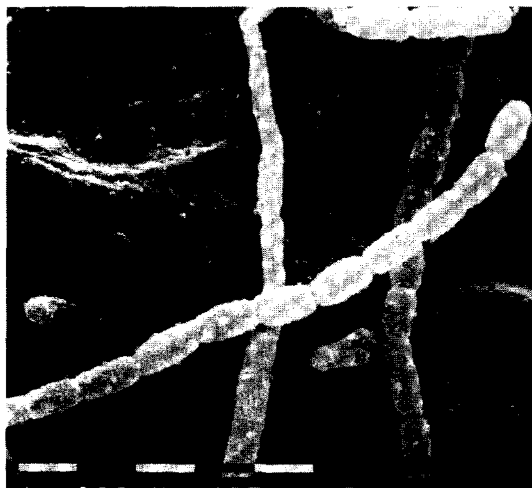

Fig. 1. Spore surface ornamentation of isolate GK9244.

The enzyme activities such as proteolysis, lipolysis, H_2S production, hippurate hydrolysis, and β-lactamase production in YPG agar and Beecham's FS agar were positive. The isolate exhibited antimicrobial activity against *Bacillus subtilis*, *Candida albicans*, *Streptococcus murinus*, and *Aspergillus niger*. Guanine, xanthine, xylan, and allantoin were not decomposed, while other organic compounds were degraded by the isolate. The isolate was susceptible to gentamicin, neomycin, tobramycin, and vancomycin at concentrations of 50 or 100 μg/ml. The isolate was most sensitive to vancomycin, yet much less susceptible to gentamicin, neomycin, and tobramycin. The isolate did not grow at certain pH and temperature. The growth of the isolate was inhibited by sodium chloride (7, 13%), sodium azide (0.01, 0.02%), phenol (0.1%), thallous acetate (0.01%), and crystal violet (0.0001%).

Utilization of Carbon and Nitrogen Sources by Isolate GK9244

The isolate was found to utilize all 11 different kinds of amino acids employed for its growth. The isolate also

Table 2. Utilization of carbon and nitrogen sources by isolate GK9244.

Source	Utilization
Nitrogen source (0.1%)	(+); DL-α-amino-n-butyric acid, potassium nitrate, L-cysteine, L-valine, L-threonine, L-serine, L-phenylalanine, L-methionine, L-histidine, L-arginine, L-hydroxyproline
Carbon source (1%)	(+); L-arabinose, D-xylose, *meso*-inositol, mannitol, L-rhamnose, raffinose, D-mannose, D-lactose, adonitol, salicin, trehalose, D-melezitose, D-galactose, cellobiose, sodium citrate (0.1%), sodium propionate (0.1%), sodium pyruvate (0.1%)
	(−); sucrose, D-fructose, D-melibiose, inulin, dextran, xylitol, sodium acetate (0.1%), sodium malonate (0.1%)

(−); negative, (+); positive.

utilized all the carbon sources very well for growth, with the exceptions of sucrose, D-fructose, D-melibiose, inulin, dextran, xylitol, and sodium malonate.

Cultural Characteristics of Isolate GK9244

The growth of isolate GK9244 on the ISP media was good with exception of peptone-yeast extract iron agar and tyrosine agar media. The growth on sucrose-nitrate, nutrient, and Bennett's agar media was moderate. The spore mass color was a gray to white series, while the reverse side color (substrate mycelium color) was yellow to gray depending on the medium employed. No soluble pigment was produced in almost any the agar media. The soluble color in the tyrosine and glucose asparagine agar media remained unchanged when the medium was flooded with acidic or alkaline solution.

In the case of isolate GK9244, the identification of the genus was easily achieved by determining the cell wall diaminopimelic acid isomer (DAP). The cell wall composition of LL-DAP indicated the isolate as part of the *Streptomyces* sp. In addition, the morphological and physiological characteristics suggested that the isolate belonged to Category IV, as defined by Bergey's Manual [18]. Further investigation of Bergey's Manual of Systematic Bacteriology verified the isolate GK9244 to be *S. erumpens*. The characteristics of the spore chains (*spirales*), spore surface ornamentation (smooth), spore mass color (gray), and inability to produce melanin pigment of isolate GK9244 all matched to the characteristics of *S. erumpens*. In addition, the sole carbon source utilization properties of the two strains were consistent, with the exceptions of salicin and D-fructose [1]. It was also found that *S. erumpens* strain had been reported to produce tetrin A and B. However, other information on the morphological and physiological characteristics of *S. erumpens* was unavailable from ISP [12-16] and Bergey's Manual [18]. Although there was no comparison between the characteristics of GK9244 and a reference strain, it was still concluded that the GK9244 isolate was *S. erumpens*. Moreover, since the spore mass color of *S. hygroscopicus*, another organism producing polyene curromycins, is black and its spore surface is rugose type, it was also eliminated from any further comparison.

Preventive Effect of Tetrin C on Fungal Phytopathogens

The *in vitro* antimicrobial and cytotoxic activities (MIC) of 16-methyloxazolomycin and tetrin C are presented elsewhere [10, 11]. The *in vivo* (pot test) preventive effect of tetrin C on rice blast (*Pyricularia oryzae*), rice sheath blight (*Rhizoctonia solani*), cucumber gray mold (*Botrytis cinerea*), wheat powdery mildew (*Erysiphe graminis*), and barley leaf rust (*Puccinia recondita*) accounted for 100, 95, 90, 95, and 95%, respectively. However, the active compounds were found to be unstable in the light as is

Table 3. Cultural characteristics of isolate GK9244 in different media.

Agar medium	Growth	Spore mass color	R.S.C.*	S.P.**
Yeast ext.-malt Ext. (2)#	good	white	P.Y.	none
Oatmeal (3)#	good	I. gray	P.Y.	none
Inorganic salts-starch (4)#	good	Y. white	P.Y.	none
Glycerol-asparagine (5)#	good	grayish white	P.Y.	none
Peptone-yeast Ext. iron (6)#	moderate	none	P.Y.	none
Tyrosine (7)	poor	I. gray	m.o.	S.Br.
Sucrose-nitrate	moderate	I. gray	I. gray	none
Glucose asparagine	good	I. gray	I.Y.	P.Y.
Nutrient	moderate	Y. white	P.Y.	none
Bennett's	moderate	I. Gray	P.Y.	none
Starch	good	Y. white	Y. white	none

#ISP medium number, *reverse side color, **soluble pigment. The color was assigned by matching the color to ISCC-NBS Centroid Color Charts [5].

often the case with polyene antibiotics. Consequently, it would appear that the application of these new antibiotics to pesticides is improbable.

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