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Identification and Fermentation of a *Streptomyces* Producing Aurodox Group Antibiotics

KIM, SI-KWAN*, WOON-HYUNG YEO AND SANG-SEOCK KIM

Korea Ginseng and Tobacco Research Institute, Shinseong-Dong Yousong-Gu, Taejon 305-345, Korea

An isolate, 90-GT-129 was found to produce antibiotics with a selective inhibitory activity against *Streptococcus pyogenes* and *Xanthomonas* sp. The isolate formed a gray spiral aerial spore mass with smooth surface. Analysis of the cell wall acid hydrolysate of the isolate revealed presence of LL-diaminopimelic acid, which indicates that the isolate belongs to a cell wall type I actinomycetes. Cultural and physiological characteristics of the isolate placed it in *Streptomyces rochei* synonym cluster. A comparison of the isolate with 26 reference strains of *Streptomyces rochei* synonym demonstrated differences in physiological and cultural characteristics.

Streptococcus pyogenes is the leading cause of streptococcal pharyngitis, so called "strep throat". *S. pyogenes* can also cause related infections of the inner ear (otitis media), the tonsils (tonsilitis), the mammary glands (mastoditis), and infections of the superficial layer of the skin (impetigo). About half of the clinical cases of severe sore throat turn out to be due to *S. pyogenes* (2). Fortunately, most respiratory bacterial pathogens, thus far, had responded readily to antibiotic therapy, such as cephaloridin, penicillin G and ribostamycin. However, *S. pneumonia, S. pyogenes* and staphylococci, organisms that cause respiratory and cutaneous infections are now resistant to virtually all of the older antibiotics (10).

Citrus canker is a bacterial disease causing necrotic spots on fruits, leaves and stems. It is induced by *Xanthomonas campestris* pv. *citri*. This disease has at least 3 distinct forms, based on geographic distribution and different susceptibility of citrus hosts to the pathogen (13). Canker A (Asiatic canker) is the most widely distributed form and is endemic in Asia, Africa, Oceania and South America. Canker A affects many rutaceous hosts and has the broadest host range among the three forms. Canker B (cancrosis B) primarily affects lemon in Argentina, Uruguay and probably Paraguay. Canker C (Mexican lime cancrosis) affects only *Citrus aurantifolia* (Christm.) Swingle Mexican in Brazil.

In the course of our screening program for antibiotics with a selective toxicity, an isolate, 90-GT-129, was found to produce antibiotics with a narrow antimicrobial spectrum against *S. pyogenes* and *Xanthomonas* sp. Antibiotics produced by the isolate were clearly different from ascamycin (11), xanthostatin (3) and xanthocidin (1), *Xanthomonas*-specific antibiotics. Cultural and physiological characteristics, and fermentation of isolate 90-GT-129 are described in this paper. Isolation, physicochemical properties and biological activity of the active compounds produced by the isolate are mentioned elsewhere in the accompanying publication (9).

*Corresponding author
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MATERIALS AND METHODS

Chemicals and Media

Purified chitin was prepared from the practical grade coarse crab shell flakes by the method of Hsu (5). Organic compounds, antibiotics and other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA and were of the highest purity available. Media or ingredients for media were procured from Difco. Cefinase disc for β-lactamase inhibitor production assay was purchased from BBL, Cockeysville, Maryland, USA.

Isolation of Actinomycetes

Modified starch-casein agar (18) and colloidal chitin agar (4) media (pH 7.0-7.5) with or without pine pollen bait were employed for the preferential isolation of actinomycetes. Casein was dissolved in H_2O beforehand by the dropwise addition of 0.05 N NaOH and millipore-filtered cycloheximide was added to the sterilized media at the final concentration of 25 mg/ml. Soil samples were heat-treated at 80°C for 24 h, inoculated in the media according to the conventional pour plate method, and the plates were incubated at 27°C for 5 to 10 days. Pure isolates were cultured on modified Bennett's agar

slant and stored at 4°C for future use. Isolate 90-GT-129 was isolated from the soil sample collected in Mt. Sorak, Kangwon Province.

Identification of the Isolate

Identification of the isolate was carried out principally according to the methods of Williams *et al.* (17) and International Streptomyces Project (ISP) (14). The ability of the isolate to use 25 different sole carbon sources was examined on carbon utilization agar (ISP medium 4), as recommended by Shirling and Gottlieb (14). Utilization of nitrogen sources was determined on the basal medium of Williams *et al.* (18). Colors of mycelial and diffusible pigments were determined on the basis of the Inter-Society Color Council-National Bureau of Standards (ISCC-NBS) Centroid Color Charts (6). Detailed information for the preparation of inoculum and the investigation of each unit character has been described by Kim *et al.* (8).

Microbial Strain

Test microorganisms employed for antimicrobial test were obtained from the Institute of Fermentation, Osaka (IFO), Institute of Applied Microbiology (IAM), Tokyo University, Japan Collection of Microorganism (JCM), RIKEN, Japan, and the American Type Culture Collection (ATCC). *Bacillus subtilis* IAM 1069, *Pseudomonas fluorescence* IAM 1201, *Escherichia coli* AB 1157, *Micrococcus* luteus JCM 1464, *Candida albicans* IFO 6258, *Saccharomyces cerevisiae*, *Streptomyces murinus* JCM 4333 and *Aspergillus niger* ATCC 9642 were employed for the antimicrobial test of the isolate.

Chemotaxonomy

Cell wall chemotype was investigated by the method of Kawamoto *et al.* (7). Isolate 90-GT-129 was cultured in yeast ext.-malt ext. broth for 48 h and pelleted by spinning. The pelleted cell was treated with sodium dodecyl sulfate to obtain crude cell wall, which was then hydrolyzed in 6 N HCl at 121°C for 15 min. Hydrochloric acid was eliminated by concentrating under reduced pressure and the hydrolysate was developed on cellulose TLC plate with MeOH-H_2O-5 N HCl-pyridine (80 : 15 : 5 : 10) as a developing solvent system.

Fermentation Conditions and Growth Curve

The seed culture was made in 500 ml Erlenmeyer flasks containing 100 ml of medium which consisted of 0.5% glucose, 1.0% soluble starch, 0.5% meat extract, 0.2% yeast extract, 0.3% polypeptone, 0.2% casein, 1% glycerol, 0.2% $CaCO_3$ and 0.001% thiamine·HCl. These flasks were incubated at 27°C/250 rpm for 24 h. One flask volume (100 ml) of the seed was transferred to a 15 liter jar fermentor containing antibiotic production medium (10 litres) which consisted of the same ingredients as seed culture and 0.1% antifoaming agent. This medium was prepared with tap water and adjusted to pH 7.3. Fermentation was carried out at 28°C for 96 h. Agitation and aeration were controlled to 50-350 rpm and 2-10 l/min, respectively depending on the growth phase.

For the investigation of growth and antibiotic production properties of the isolate in a jar fermentor containing the submerged medium, culture was carried out for 120 h and sampling was made every 24 h for 120 h. Each sample was spinned at 3,000 rpm for 15 min and packed cell volume ratio against whole culture broth volume was measured. pH and antimicrobial activity of the broth supernatant was determined. Pelleted mycelial cake was extracted overnight with 70% aqueous acetone, 1/2 volume (5 ml) of the whole culture broth (10 ml). *Xanthomonas campestris* pv. *citri* IFO3781 was employed for the monitoring of antibiotics production in broth filtrate and mycelial cake, and paper disc agar diffusion method was used for the assay.

RESULTS AND DISCUSSION

Morphological and Physiological Characteristics of the Isolate

Spores, aerial mycelium and aerial spore mass were observed (Table 1). Aerial mycelium bore a compact spiral spore chain with a smooth spore surface (Fig. 1). Spore mass color was of gray series. Neither distinctive substrate mycelial pigment nor diffusible pigment was observed on inorganic salts-starch agar medium. Melanin was produced both on peptone-yeast ext.-iron agar and tyrosine agar media. Melanin pigment was produced also in tryptone yeast ext. broth (ISP medium 1). Fragmentation of mycelium, sclerotia formation, and sporulation on substrate mycelium were not observed.

Analysis of the cell wall acid hydrolysate indicated the presence of LL-diaminopimelic acid (DAP). Glucose-peptone-gelatin was liquified by the isolate but single gelatin was not. Neither coagulation nor peptonization of skim milk was observed. Cellulose was not decomposed by the isolate. The isolate showed no antimicrobial activity against all 8 test microorganisms employed for this test. In enzyme assay, only lipolysis on egg yolk agar medium was observed among the 11 unit characters defined for enzymatic activity assay.

Of the 18 organic compounds used for degradation test, all the compounds were degraded by the isolate with the exception of guanine, RNA, xylan, allantoin, and arbutin. The isolate showed resistance to neomycin, tobramycin, rifampicin, oleandomycin and penicillin G at the diagnostic concentrations. The resistance was very weakly positive to gentamicin but variable to streptomycin depending upon the growth condition of the isolate. The isolate was unable to grow in the presence of NaCl at the concentration higher than 7% and thallous acetate. Growth occurred in the presence of other chemical inhibitors tested.

Table 1. Morphological and physiological characteristics of isolate 90-GT-129.

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 series
Melanoid pigment production in	peptone-yeast ext.-iron and tyrosine agar, tyrosine yeast ext. broth (ISP No. 1)
Cell wall DAP composition	LL-DAP type
Gelatin liquefaction (27°C)	glucose-peptone-gelatin agar (+), single gelatin (−)
Skim milk (27 and 30°C)	coagulation (−), peptonization (−)
Cellulose decomposition	negative
antimicrobial activity	(−): *B. subtilis*, *P. fluorescens*, *E. coli*, *M. luteus*, *C. albicans*, *S. cerevisiae*, *S. murinus*, *A. niger*
Enzymatic activity	(−): lecithinase, proteolysis, pectin hydrolysis, chitin hydrolysis, nitrate reduction, hippurate hydrolysis, β-lactamase and β-lactamase inhibitor production (+): lipolysis, H_2S production.
Orgainic compound degradation	(−): guanine, RNA, xylan, testosterone, allantoin, arbutin (+): hypoxanthine, elastin, L-tyrosine, adenine, xanthine, DNA, Tween-80, starch, casein, urea, gelatin, aesculin
Resistance to antibiotics	(−): gentamicin, streptomycin, cephaloridin, vancomycin, demethylchlorotetracycline (+): neomycin, tobramycin, rifampicin, oleandomycin, lincomycin, penicillin G
Temperature and pH tolerance	(−): 4°C, 45°C (+): 10°C, 37°C, pH 4.3
Chemical inhibitor tolerance (%)	(−): NaCl (7,10,13), thallous acetate (0.001, 0.01) (+): NaCl (4), sodium azide (0.01, 0.02), phenylethanol (0.1, 0.3), phenol (0.1), potassium tellurite (0.001, 0.01), crystal violet (0.0001)

Fig. 1. Spore chain ornamentation of isolate 90-GT-129 observed by scanning electron micrograph.

Utilization of Nitrogen Sources

Strain 90-GT-129 was found to utilize all the nitrogen sources tested (Table 2). Of these, utilization of L-valine, L-threonine, L-serine, L-phenylalanine, L-methionine and L-hydroxyproline was much greater than that of the positive control (L-proline). Furthermore, a significant growth of the isolate was observed in the absence of nitrogen source but the growth was less significant than that of the positive control.

Utilization of Carbon Sources

The isolate was unable to use inulin, adonitol, salicin, dextran, xylitol, or sodium malonate for growth (Table 3). Utilization of *meso*-inositol, raffinose and D-melezitose was not clear. On the other hand, the utilization of sodium acetate, sodium citrate and sodium pyruvate was weakly positive, and the remaining 13 carbon sources were used efficiently by the isolate for growth.

Cultural Characteristics of the Isolate

Aerial and substrate hyphae were developed well on ISP-2, -3, -5, -7, glucose-asparagine and Bennett's agar media but poorly on other media except ISP medium 2 (Table 4). Spore mass color represented gray series. The color of substrate mycelium (reverse side) was brown. Yellowish brown soluble pigment was observed in ISP-2, -3, -6, -7, and Bennett's agar media but it did not change

Table 2. Nitrogen source utilization of isolate 90-GT-129.

Nitrogen source (0.1%, v/v)	Utilization
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	+

Table 3. Carbon source utilization of isolate 90-GT-129.

Carbon source (1.0%)	Utilization	Carbon source (1.0%)	Utilization
L-Arabinose	+	Salicin	−
Sucrose	+	Trehalose	+
D-Xylose	+	D-Melibiose	+
meso-Inositol	−	Dextran	−
Mannitol	+	D-Galactose	+
D-Fructose	+	Cellobiose	+
L-Rhamnose	+	Xylitol	−
Raffinose	−	Sodium acetate*	+
D-Melezitose	−	Sodium citrate*	+
D-Mannose	+	Sodium malonate*	−
D-Lactose	+	Sodium propionate*	+
Inulin	−	Sodium pyruvate*	+
Adonitol	−		

* 0.1%, v/v.

by flooding acidic or alkaline solution.

Growth Profile of the Isolate

Time-course change in pH and packed cell volume showed typical properties of *Streptomyces* growth in a submerged antibiotic production medium (Fig. 2). The antibiotics production reached maximum at 96 h and pH increased near to 8.0 at that time. Intracellular accumulation of antibiotics occured from the early stage of growth and almost half the amount of the total antibiotics was contained in the cell. Packed cell volume of the isolate in the jar fermentor increased until 96 h and antimicrobial activity did not decrease until 120 h of fermenting.

The chemotaxonomic characteristics described above clearly placed the isolate in the genus *Streptomyces*. Identification studies based on the method of Williams *et al.* indicated that isolate 90-GT-129 belonged to *S. rochei* synonym cluster (18). A comparison with the 26 type strains belonging to *S. rochei* synonym indicated that *S. indigoferus* (ISP 1524) (16) and *S. griseomyceni* (ISP 5159) (15) were most compatible with isolate 90-GT-129, but clear differences were observed in melanoid pigment production and aerial mycelium color, respec-

Table 4. Cultural characteristics of isolate 90-GT-129.

Medium	Growth	Spore mass color	R.S.*	S.P.**
Yeast ext.-malt ext. agar (ISP No. 2)	good	I. gray (264)	m. Br (58)	I. y. Br (76)
Oatmeal agar (ISP No. 3)	good	I. gray (264)	I. gy. Br (60)	I. y. Br (76)
Inorganic salts-starch agar (ISP No. 4)	mode-rate	I. br. gy (63)	I. gy. Br (60)	none
Glycerol-asparagine agar (ISP No. 5)	good	I. br. gy (65)	br. pink (33)	none
Peptone-yeast ext.-iron agar (ISP No. 6)	poor	none	gy. Br (61)	m. y. Br (77)
Tyrosine agar (ISP No. 7)	good	I. gray (264)	gy. Br (61)	I. y. Br (76)
Sucrose-nitrate agar	poor	br. pink (33)	I. gy. Br (79)	none
Glucose asparagine agar	good	I. br. gy (63)	I. Br (57)	none
Nutrient agar	poor	none	y. White (92)	none
Bennett's agar	good	I. br. gy (63)	m. Br (58)	I. y. Br (76)
Starch agar	poor	y. White (92)	y. White (92)	none

*Reverse side color, **soluble pigment. Color code was assigned according to a ISCC-NBS Centroid Color Charts.

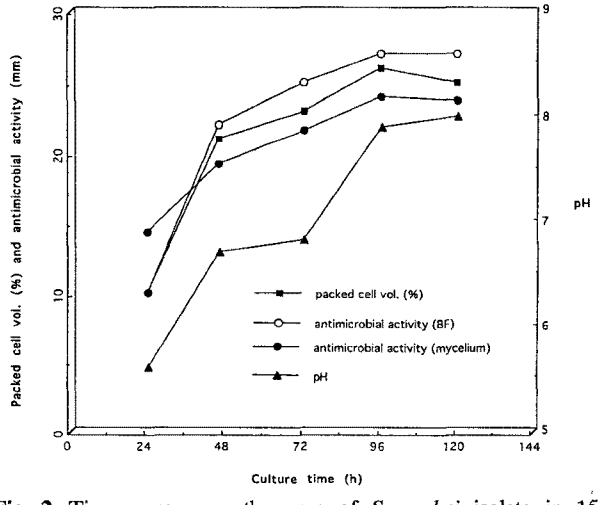

Fig. 2. Time-course growth curve of *S. rochei* isolate in 15 liter jar fermentor containing 10 liter medium.
Antimicrobial activity of mycelial cake was performed with 70% acetone extract, 1/2 volume of the broth filtrate (BF), against *Xanthomonas campestris* pv. *citri*.

tively. *S. griseomyceni* forms green aerial mass. In addition, carbon source utilization pattern of the 2 type strains demonstrated significantly different characteristics from isolate 90-GT-129. From these results, we identified isolate 90-GT-129 to be a *S. rochei* synonym.

The *S. rochei* isolate was found to produce complex antibiotics of aurodox group. The main active compound was identified to be kirromycin (mocimycin)-like aurodox group antibiotic on the basis of FAB mass and ^{1}H and ^{13}C NMR spectroscopic evidences (9). However, the isolate showed different characteristics from *S. collinus* which produced kirromycin only. Melanin production on tyrosine agar medium, utilization of *meso*-inositol and raffinose, and antibiotic production properties were different in both microorganisms.

It has been known that production of aurodox group antibiotics are afflicted by generally low fermentation yields because biosynthesis of aurodox group compounds is regulated by feed-back inhibition (12). However, growth properties of the *S. rochei* isolate in the submerged antibiotic production medium indicated that the isolate was quite resistant to the antibiotics produced by the isolate itself.

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