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Taxonomy and Fermentation of *Kitasatosporia kimorexae* Producing New Thiopeptide Antibiotics, Kimorexins

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An isolate, 90-GT-302, was found to produce antibiotics inducing typical mycelial swelling in *Magnaporthe grisea* and *Fusarium solani*. This isolate formed yellow substrate and white *rectiflexbiles* aerial mycelia in the early stages of growth. The aerial mycelium gradually changed its color to white and finally formed a gray spore mass. Analysis of the cell wall acid hydrolysate revealed the presence of LL- and *meso*-diaminopimelic acids, glycine, and galactose, which indicated cell wall type X. This result placed our isolate in genus *Kitasatosporia*. A comparison of isolate 90-GT-302 with reference strains of *Kitasatosporia* spp., which not only demonstrated several differences in their physiological properties but also novelty of the active compounds produced by this isolate, led us to designate the isolate as *Kitasatosporia kimorexae*.

Since the Waksman's discovery (20) of antagonistic interrelationships among the soil microorganisms, numerous biologically active substances have been isolated from microbial origins. Among the microbial genera, actinomycetes, especially *Streptomyces*, has been regarded as the richest source of active substances. However, as the discovery of new active compounds accumulates, chances of finding new ones are becoming very low in *Streptomyces*. One way of coping with this problem is the introduction of unique target-directed screening system and isolation of rare actinomycetes as reviewed by Wellington and Cross (21), and Goodfellow and Williams (3).

Nonomura and Ohara suggested preferential isolation methods for *Streptosporangium* (12), *Microtetraspora* (13), *Microbispora* (14), *Thermomonospora* (15) and *Actinomadura* (16). Makkar and Cross (10) successfully isolated *Actinoplanes* with the aid of baits such as pollen and hair. Orchard *et al.* (17) and Hanka *et al.* (4) developed methods for selective isolation of *Norcardiae* and *Streptoverticillium*, respectively. But in the field of screening for biologically active substances, the isolated microorganism should simultaneously produce new biologically active substances.

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In order to satisfy the above-mentioned prerequisites, we employed a target-directed screening system in combination with the isolation of rare actinomycetes. In the course of screening inhibitors against the fungal cell wall biosynthesis, isolate 90-GT-302 was found to produce antibiotics inducing mycelial swelling in *Magnaporthe grisea* and *Fusarium solani*. This is significant in that few antibiotics have been reported that would induce mycelial swelling in *Fusarium solani*, to date. Thus, an attempt to isolate active compounds produced by this isolate and to identify the producing microorganism was made. Taxonomic studies of the isolate are described in this paper. Isolation, physico-chemical properties and biological activity of the active compounds produced by isolate 90-GT-302 will be mentioned elsewhere in the accompanying publication (25).

MATERIALS AND METHODS

Isolation of Rare Actinomycetes

Modified starch-casein agar (24) and colloidal chitin agar (10) media (pH 7.0~7.5) with or without pine pollen bait were employed for the preferential isolation of rare 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 autoclaved media with a final concentration of 25 μg/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-302 was isolated from a soil sample collected in Goesan, Choongbook Province. A subculture of isolate 90-GT-302 was deposited at Korean Collection for Type Culture and assigned accession code KCTC 0119BP.

Chemicals and Media

Purified chitin was prepared from the practical grade coarse crab shell flakes by the method of Hsu and Lockwood (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, U.S.A.

Microbial Strain

Test microorganisms employed for *in vitro* bioassay 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 fluorescens* 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 isolate 90-GT-302.

Taxonomy of the Producing Microorganism

Identification of the isolate was carried out principally according to the methods of Williams *et al.* (23) and International Streptomyces Project (ISP) (18). Spore surface ornamentation was observed with a scanning electron microscope (Model S-430, Hitachi) and classified by Dietz and Mathew category (2). Colors 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 media, and the investigation of each unit character has been described by Kim *et al.* (9).

Chemotaxonomy

Cell wall chemotype was investigated by the method of Kawamoto *et al.* (7). Isolate 90-GT-302 was cultured in the yeast ext.-malt ext. broth for 48 h and pelleted by spinning. The pelleted cells were treated with sodium dodecyl sulfate to obtain crude cell wall, which was then hydrolyzed in 6 N HCl at 121°C for 15 min. HCl was eliminated by concentrating the hydrolysate under reduced pressure and the hydrolysate was developed on cellulose TLC plate (Sigma) with MeOH-H_2O-5 N HCl-pyridine (80:15:5:10) as a developing solvent system. Sugar composition of the cell wall was investigated by the method of Becker *et al.* (1). The pelleted cells were hydrolyzed with 1 N H_2SO_4 in boiling water and developed on paper chromatography with a solvent mixture of n-BuOH-H_2O-pyridine-toluene (5:3:3:4).

Fermentation Conditions and Growth Curve

Seed culture was made in 500 ml Erlenmeyer flasks containing 100 ml of medium comprising: 2.0% glucose, 1.0% soluble starch, 0.1% meat extract, 0.4% yeast extract, 2.5% soybean flour, 0.2% NaCl and 0.005% K_2HPO_4. These flasks were incubated at 27°C/250 rpm for 24 h. Flask-cultured seed (300 ml) was transferred to the 50 litre jar fermentor containing 30 litre medium consisted of the same ingredients with the addition of 0.1% antifoaming agents. This medium was prepared with tap water and adjusted to pH 7.3. Fermentation was carried out at 32°C for 96 h. Agitation and aeration were controlled to 50~350 rpm and 5~30 litre/min, respectively, depending upon the growth phase.

Sampling was made every 24 h for 120 h for the investigation of growth profiles of isolate 90-GT-302 in the jar fermentor. Each sample was spinned at 3,000 rpm for 15 min and the pelleted packed cell volume ratio against the whole culture broth volume was measured. pH and antimicrobial activity of the broth supernatant were determined. Pelleted mycelial cake was extracted with 70% aqueous acetone, 1/2 volume (5 ml) of the whole culture broth (10 ml), overnight. *Magnaporthe grisea* was employed for the determination of antimicrobial activity of broth filtrate and mycelial acetone extract, and paper disc agar diffusion method was employed for the monitoring of antibiotic production.

RESULTS AND DISCUSSION

Morphological and Physiological Characteristics of Isolate 90-GT-302

Morphological and physiological characteristics of the isolate are shown in Table 1. Colonies formed on the agar media were large, leathery and similar to those of *Streptomyces* strains. The color of vegetative mycelium in inorganic salts-starch agar medium was yellow and that of aerial mycelium was white in the early stages of growth. Aerial mycelium formed gray spore mass bearing long spore chains of 18-23 *rectiflexibles* spores. The spores were spherical to oval with a smooth surface as observed by the scanning electron microscopy as shown in Fig. 1. Yellow diffusible pigment was produced by this isolate but the color was not changed by pH change. Melanoid pigment was not produced in peptone-yeast ext.-iron agar, tyrosine agar and tryptone-yeast ext. broth (ISP No. 1).

Table 1. Morphological and physiological characteristics of isolate 90-GT-302

Unit character	Characteristics
Morphology	Presence of spores and aerial mycelium
Spore chain morphology	*rectiflexibles*, aerial spore mass (+)
Spore chain ornamentation	smooth
Color of aerial spore mass	gray series
Pigmentation of substrate mycelium	yellow/brown
Melanoid pigment production	(−) : peptone-yeast ext.-iron and tyrosine agar, tyrosine yeast ext. broth (ISP No. 1)
Cell wall DAP composition	LL- and *meso*-DAP type
Gelatin liquefation (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, Str. murinus, A. niger*
Enzymatic activity	(−); nitrate reduction, hippurate hydrolysis, and β-lactamase production on YPG agar, production of *Klebsiella* β-lactamase inhibitor (+); lecithinase, proteolysis, lipolysis, pectin hydrolysis, chitin hydrolysis, H_2S production, β-lactamase production on Beecham's FS agar.
Organic compound degradation	(−); hypoxanthine, guanine, elastin, L-tyrosine, DNA, RNA, allantoin (+); adenine, xanthine, Tween-80, starch, xylan, casein, testosterone, urea, gelatin, aesculin, arbutin
Resistance to antibiotics	(−); gentamicin, streptomycin, tobramycin, vancomycin, demethylchlorotetracycline, oleandomycin (+); neomycin, rifampicin, cephaloridin, lincomycin, penicillin G
Temperature and pH tolerance	(−); 4°C (+); 10°C, 37°C, 45°C, pH 4.3
Chemical inhibitor tolerance (%)	(−); NaCl (7, 10, 13), sodium azide (0.02), thallous acetate (0.001, 0.01), crystal violet (0.0001) (+); NaCl (4), sodium azide (0.01), phenylethanol (0.1, 0.3), phenol (0.1), potassium tellurite (0.001, 0.01)

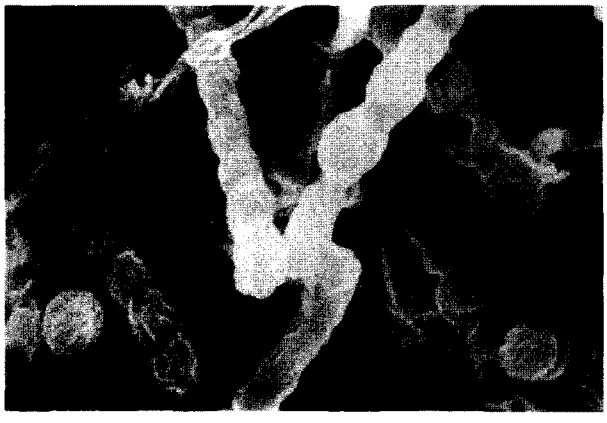

Fig. 1. Spore chain ornamentation of isolate 90-GT-302 (scanning electron micrograph).

Fragmentation of mycelium, sclerotia formation, sporulation on substrate mycelium, sporangium and motile spores were not observed. Glucose-peptone gelatin and single gelatin were liquefied by this isolate. Coagulation and peptonization of the skim milk at 27°C and 30°C were positive not cellulose was not decomposed by this isolate.

Temperature and pH ranges for growth were 10-45°C and pH 4.3~9.0, respectively. Optimum temperature and pH for the growth of isolate 90-GT-302 were 37°C and 8, respectively. Cell wall diaminopimelic acid (DAP) composition showed *meso*- and LL-type. Glycine and galactose were detected also from the cell wall acid hydrolysate.

The isolate showed no antimicrobial activity against 8 test microorganisms defined by Williams *et al.* (8). Nitrate reduction and hippurate hydrolysis were not observed by this isolate. Hydrogen sulfide was produced and lecithinase, proteolysis, lipolysis, pectin hydrolysis and chitin hydrolysis were observed. β-Lactamase was produced by this isolate on Beecham's FS agar media but β-lactamase on YPG and β-lactamase inhibitor were not.

Adenin, xanthine, Tween-80, starch, xylan, casein, testosterone, urea, gelatin, aesculin and arbutin were degraded but the other organic compounds were not. This isolate showed resistance to neomycin, rifampicin, cephaloridin, lincomycin and penicillin G among the 11 antibiotics employed for this test. Growth of the isolate was possible at 10, 37, 45°C, and in pH 4.3 but not at 4°C. Growth of the isolate in the presence of sodium chloride (7~13%), sodium azide (0.02%), thallous acetate (0.01, 0.001%) and crystal violet (0.0001%) was inhibited. The other chemical inhibitors could not inhibit the growth

of the isolate.

Nitrogen Source Utilization

As shown in Table 2, DL-α-amino-n-butyric acid, L-phenylalanine and L-methionine were not utilized, but the other 8 amino acids were utilized by this isolate for growth.

Carbon Source Utilization

Table 2. Nitrogen source utilization of isolate 90-GT-302

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	+

D-Melezitose, adonitol, Jalicin, dextran and xylitol were not used but the other 15 sole carbon sources were utilized for the growth of the isolate (Table 3).

Cultural Characteristics

Cultural characteristics of isolate 90-GT-302 on different kinds of agar media after 3 weeks of cultivation at 27°C are shown in Table 4. Vegetative and aerial mycelia developed well on yeast ext.-malt ext. agar, oatmeal agar, inorganic salts-starch agar, glycerol-asparagine agar, tyrosine agar, Bennett's agar, potato dextrose agar, potato plug and starch agar media. The spore mass color on those media was that of gray series in most of the employed media. The color on the reverse side (vegetative mycelium) was yellow to brown. Phenotype of our isolate was indistinguishable from genus *Streptomyces*. But the presence of LL- and *meso*-DAPs, glycine and galactose clearly placed this isolate in genus *Kitasatosporia*. The other cultural and morphological characteristics of the isolate correspond to those of *Kitasatosporia* with the exception of the formation of a large

Table 3. Carbon source utilization of isolate 90-GT-302

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*	ND
D-Melezitose	−	Sodium citrate*	ND
D-Mannose	+	Sodium malonate*	ND
D-Lactose	+	Sodium propionate*	ND
Inulin	+	Sodium pyruvate*	ND
Adonitol	−		

*0.1% (v/v), ND: not determined.

Table 4. Cultural characteristics of isolate 90-GT-302

Medium	Growth	Spore mass color	R.S.*	S.P.**
Yeast ext. malt ext. agar (ISP No.2)	good	med.Gy (265)	m.ol.Br (95)	none
Oatmeal agar (ISP No.3)	good	d.gy.Y (91)	s.Y (84)	none
Inorganic salts-starch agar (ISP No.4)	good	med.Gy (265)	i.OI (106)	none
Glycerol-asparagine agar (ISP No. 5)	good	gy.pink (8)	s.Y (84)	none
Peptone-yeast ext.-iron agar (ISP No. 6)	moderate	none	i.OY (70)	none
Tyrosine agar (ISP No.7)	good	white (263)	s.Y (84)	none
Sucrose-nitrate agar	moderate	white (263)	s.Y (84)	none
Glucose asparagine agar	moderate	med.Gy (265)	deep OY (69)	none
Nutrient agar	moderate	white (263)	p.Y (89)	none
Bennett's agar	good	med.Gy (265)	deep OY (69)	none
Potato dextrose agar	good	med. Gy (265)	v. OY (66)	none
Czapek's agar	poor	none	deep y. Br (75)	none
Carrot plug	moderate	br.pink (3)	none	none
Potato plug	good	p.Y. (89)	none	none
Starch agar	good	i.Oi.GY (112)	s.Y (84)	none

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

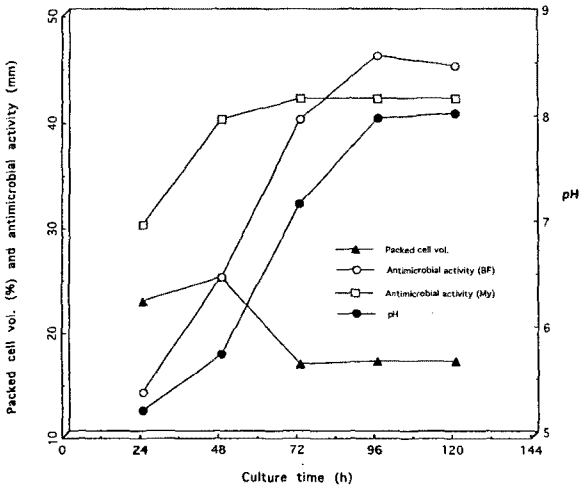

Fig. 2. Time-course growth curve of *Kitasatosporia kimorexae* KCTC 0119BP in 50 litre jar fermentor containing 30 litre medium.

Antimicrobial activity of mycelial cake (My) was performed with 70% acetone extract, 1/2 volume (5 ml) of the broth filtrate (BF, 10 ml), and *Magnaporthe grisea* IFO 5994 as the test microorganism. Initial pH of the medium was 7.3.

colony on those media in which growth was excellent.

There are 7 strains of *Kitasatosporia* thus far reported: *K. cystarginea*, *K. melanogena* (9), *K. papulosa*, *K. grisea*, *K. phosalacinea* (22), *K. griseola*, and *K. setae* (24). The taxonomic characteristics of the isolate were compared with those of type strains of *Kitasatosporia* on the basis of reported description. Among the 7 strains, *K. setae* best resembles the isolate, but *K. setae* has been known to be incapable of utilizing raffinose, D-fructose, L-rhamnose, sucrose, melibiose, D-mannitol and *meso*-inositol unlike the isolate. *K. melanogena* is different in melanoid pigment production from our isolate, and *K. papulosa* is different in skim milk coagulation, gelatin liquefaction, and tyrosine degradation. Furthermore, it is noteworthy that all the *Kitasatosporia* spp. reported to date were found to produce antibacterial agents or herbicidal agents (11). From these results, the isolate was identified as *Kitasatosporia kimorexae* and a subculture of the isolate was deposited at Korea Collection for Type Culture with the accession code KCTC 0119BP. Studies on fatty acid composition, menaquinone composition, phospholipid composition, and nucleic acid will be conducted to further support the results.

Growth Profiles of *Kitasatosporia kimorexae* 0119BP

As shown in Fig. 2, pH and packed cell volume show the typical characteristics of actinomycetes grown in submerged antibiotic production medium. The highest antimicrobial activity of the broth filtrate was observed at 96 h culture. The half of the active compounds was found to be contained in the mycelial cake. Intracellular accumulation of the active compounds occured from the early stages of growth.

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