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An Antifungal Antibiotic Purified from *Bacillus megaterium* KL39, a Biocontrol Agent of Red-Pepper *Phytophthora*-Blight Disease

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Abstract *Bacillus megaterium* KL39, an antibiotic-producing plant growth promoting rhizobacterium (PGPR), was selected from soil. The antifungal antibiotic, denoted KL39, was purified from culture filtrate by column chromatography using Dion HP-20, Silica gel, Sephadex LH-20, and prep-HPLC. Thin layer chromatography, employing the solvent system of ethanol:ammonia:water=8:1:1, showed the R_f value of 0.32. The antibiotic KL39 showed a negative reaction with ninhydrin solution, positive with iodine vapor, and also positive with Ehrlich reagent. It was soluble in methanol, ethanol, butanol, and acetonitrile, but insoluble in chloroform, toluene, hexane, ethyl ether, or acetone. Its UV spectrum had the maximum absorption at 208 nm. Amino acid composition, FAB-mass, H-NMR, ^{13}C-NMR, and atomic analyses showed that the antibiotic KL39 (MW=1,071) has a structure very similar to iturin E. The antibiotic KL39 has a broad antifungal spectrum against a variety of plant pathogenic fungi including *Rhizoctonia solani*, *Pyricularia oryzae*, *Monilinia froeticola*, *Botrytis cinenea*, *Altenaria kikuchiana*, *Fusarium oxysporum*, and *F. solani*. An MIC value of 10 μg/ml was determined for *Phytophthora capsici*. Macromolecular incorporation studies with *P. capsici* using radioactive [^{3}H-adenine] as the precursor, indicated that the antibiotic KL39 strongly inhibits the DNA biosynthesis of the fungal cell. Microscopic observation of the antifungal action showed abnormal hyphal swelling of *P. capsici*. The purified antibiotic KL39 was very effective for the biocontrol of *in vivo Phytophthora*-blight disease of pepper.

Key words: Antifungal antibiotic, biocontrol, iturin E, *phytophthora*-blight, red-pepper

Chemical fertilizers and agricultural chemicals have been used in the world to increase the product yield for several decades. There is no doubt that they are effective in crops protection against various soilborne diseases and for pest control and have contributed to increase in crops production. However, their continuous and repetitive uses have brought about the destruction of the ecosystem by decreased natural antagonism and environmental pollution, hence threatening human health. Consequently, biological control and microbial pesticides have been introduced as an effective means, to substitute synthetic agrochemicals. Microbial pesticides are mainly composed of purified compounds, such as antifungal antibiotics, or antagonistic microorganisms.

Various soil antagonistic microorganisms have been used to control soilborne plant pathogens. For example, *Actinomyces* sp. was first applied for the biocontrol against potato scrab in 1927 in the United Sates. White rot of sweet potato was controlled by cross protection, which made use of fungi, in 1951. Subsequently, biological control agents such as *Trichoderma* [27] and *Gliocladium* sp. [12] had been reported to control a variety of fungal pathogens, including *Rhizoctonia*, *Pythium*, *Sclerotinia*, *Sclerotium*, and *Fusarium* sp. [4, 12]. Many microorganisms including *Bacillus thuringiensis* [14] produce Bt toxin, and *Agrobacterium radiobacter* K821 [22], *Chaetomium globosum* [22], *Penicillium oxalicum* [30], and *Pseudomonas* sp. and *Bacillus* sp. [4] have been applied in various agricultural farms. Purified antifungal antibiotics from these antagonistic microorganisms have also been used to control plant blight or rotting diseases. After blastin S [30] was applied to rice blast caused by *Magnaporthe grisea*, polyoxin [6], kasugamucin [10], validamycin [16], and mildiomycin were developed.

B. subtilis is also known as a producer of antifungal peptide antibiotics such as iturin, fengymycin [19], and eumycin [10]. *Pseudomonas* spp. produce aspyrrolnitrin [5] and pyoluteorin [24], and these compounds suppress the plant blight caused by soilborne fungi. It is also applied to agricultural plants as seed inoculants. These antibiotics have target specificities against plant pathogens, and can be

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decomposed quickly after use and do not remain toxic in soil. In Korea, Son *et al.* [28] announced that KRF-001 produced from *Bacillus* sp. has antifungal activity against several plant pathogens. Other antifungal antibiotics have also been studied and developed by several research groups and private companies [10]. Furthermore, a few biocontrol agents have come into the market and are now in circulation

In this study, as an on-going effort to develop biocontrol agents, the antifugal antibiotic of an antagonistic strain, which was isolated from the local soil of Gyeongbuk, Korea, was purified, and the antagonistic mechanism and structural characteristics of the purified antibiotic were investigated. Possible application of the antagonistic bacteria as a microbial fungicide and its antibiotic substances for the biological control of red-pepper phytophthora blight disease were also tested by *in vivo* pot experiment.

MATERIALS AND METHODS

Strain and Production of Antifungal Antibiotic

Previously shown cellulase-positive *Bacillus megaterium* KL39 [9] was used to produce and determine the characteristics of the antibiotic substance with antifungal activity toward *Phytophthora capsici*, which causes a red-pepper phytophthora blight. To produce the antibiotic, *B. megaterium* KL39 was cultivated for 48 h in 0.4% fructose, 0.3% yeast extract, and 5 mM KCl at 30°C with constant shaking at 180 rpm. After the cell pellet was removed by centrifugation at 6,520 ×*g* for 30 min, the antibiotic-containing supernatant was subjected to the further purification step described below.

Purification of Antifungal Antibiotic

To extract the antibiotic, equal volume of n-butanol was added to the supernatant and the upper layer was collected. The antibiotic substance containing the organic phase was concentrated in a rotary evaporator at 50°C, and the final pellet was dissolved in methanol. The crude antibiotic solution was loaded on a Dion HP-20 (2.5 cm×70 cm) column and eluted with a stepwise gradient of 0, 50, and 100% methanol. The fractions showing antibiosis toward *P. capsici* were concentrated by evaporation and subjected to silica gel column chromatography (2.5 cm×70 cm) using the running phase of ethanol:ammonia:water (8:1:1). The antibiotic was further purified by gel filtration on Sephadex LH-20 using 100% methanol as a running phase.

To confirm the antifungal activity, 100 µl of each fraction was applied onto 5 mm paper disc and the disc was placed on Potato dextrose agar (PDA) where zoospores of *P. capsici* were inoculated. After the plate was incubated at 28°C for 4 days, antibiosis was determined by measuring the zone of fungal growth inhibition. To analyze the characteristics of the antibiotic, the purified antifungal substance was dissolved in acetonitrile and injected into reverse-phase HPLC using a C18 column (Microsorb C18 semi-preparative column, 21.4×250 mm, Varian, Inc., Palo Alto, CA, U.S.A.). The elution of the active fraction with 70% acetonitrile at a flow rate of 1 ml/min was monitored at 265 nm.

Chemical Properties of the Antibiotic KL39

Purified antibiotic was analyzed by thin-layer chromatography (TLC) on Silica gel 60 F_{254} (20×20 cm^2 20 µg particle, Merk Darmstadt, Germany) with ethanol:ammonia:water (8:1:1) as a solvent system. By spraying with six reagents (Ehrlich reagent, 10% H_2SO_4, iodine vapor, hydrochloric acid, BPB-boric acid, ninhydrin) or by heating, the chemical properties of the antifungal antibiotic were observed by the color changes. The solubility of the purified antibiotic was tested using various organic solvents; after removing the insoluble fraction by centrifugation, the antifungal activity was confirmed by measuring zone of fungal growth inhibition as previously described. The UV spectrum of the purified antibiotic in methanol was obtained by spectrophotometery (Hitachi, U-2000, Hitachi, Tokyo, Japan).

Assay for Antifungal Activity

To determine the minimum inhibitory concentration (MIC) of the antifungal compound, the antibiotic dissolved in methanol was serially diluted from 0 to 1,000 µg/ml and loaded on paper discs. The discs were placed on PDA inoculated with zoospores of *P. capsici* and incubated at 28°C for 3 days. The MIC was estimated in µg/ml, when no visible germination of zoospores was observed.

Structural Characteristics of the Antifungal Antibiotic KL39

To study the structural properties of the purified antibiotic KL39, ^{1}H-NMR and ^{13}C-NMR spectra were obtained using FT-NMR (BRUKER, ARX300, Germany) at 300 MHz. All chemical shifts were quoted in ppm. The amino acid composition was determined using the Amino Acid Analyzer (Hitachi, L8800), employing UV and fluorometric detector, after the antibiotic had been hydrolyzed with 6 N hydrochloric acid for 24 h at 110°C. High-resolution mass spectra were obtained with a Mass Spectrometer (Autospec, MassLynx4.0, Micromass Ltd., England), employing fast-atom-bombardment (FAB) operated in the positive-ion mode. The solvent was methanol.

Antifungal Spectrum of the Antibiotic KL39

The antibiotic spectrum was tested by culturing pathogenic fungi with the purified antibiotic. Pathogenic fungi were placed at the center of each plate, and the antibiotic was incorporated in paper disc, which was placed at 15 mm

distance from the edge of the fungi colony. The plates were incubated up to 5 days at 28°C, and the growth inhibition by the antibiotic was monitored. The plant pathogenic fungi tested were *Rhizoctonia solani*, *Pyricularia oryzae*, *Monilinia froeticola*, *Botrytis cinenea*, *Altenaria kikuchiana*, *Fusarium oxysporum*, and *Fusarium solani*. The diameters of mycelial growth were measured from 3 replica plates.

Effect of the Antibiotic KL39 on the Mycelial Growth of *Phytophthora capsici*

To investigate the morphological change of the fungal mycelial structure, *P. capsici* and the paper disc containing the antibiotic were placed side by side on a PDA plate and incubated at 28°C for 3 days. The hyphal strands at the edge of fungal growth were removed and examined under a microscope to observe the effect of the antifungal antibiotic.

Antifungal Mechanism of the Antibiotic KL39 on Macromolecular Incorporation

To investigate the influence of the antibiotic on the macromolecular synthesis in fungi, 0.1 ml of *P. capsici* zoospores was cultured in 0.85% PDB medium mixed with 30 μl of crude antibiotic, using 20 μl of radioactive [^{3}H] adenine, [^{14}C] glucose, or [^{3}H] leucine as precursors of cell structural components. Cultures were incubated 24 h with shaking. *P. capsici* cultured without the antibiotic served as control.

To analyze the amount of protein synthesis, the culture broth generated with labeled leucine was mixed with 1 volume of 10% TCA, heated at 90°C for 15 min, and the insoluble materials were collected. The radioactivity in the hot TCA-insoluble material was measured by a liquid scintillation counter (BECKMAN LS 6500 Fullerton, CA, U.S.A.). The amount of DNA and RNA synthesis in *P. capsici* was determined by measuring the radioactivity in the cold TCA-insoluble materials (RNA and DNA) purified from cultures produced with labeled adenine; the cold TCA-insoluble material was prepared by adding 1 ml of 10% cold TCA to the culture and passing through a glass filter. Another cold TCA material was also prepared by adding 1 ml of 1 N KOH to the above cold TCA-insoluble material, heating at 60°C for 2 h, and mixing with 1.8 ml of 20% cold TCA. The final mixture was left overnight at 0°C before passing through the glass filter. To measure the incorporation of [^{14}C] glucose in the cell wall, the radioactivity in the insoluble materials was counted. The insoluble materials were produced by treating culture with 1 ml of 1 N NaOH incubated at 100°C water bath for 20 min, and was collected by passing the mixture through a glass filter.

In Vivo Pot Test of *Phytophthora*-Blight of Red-Pepper

The composition of the soil mixture employed was regular ground soil:perlite:sand=3:1:1 by volume. The soil mixture was autoclaved at 121°C for 20 min for all biocontrol

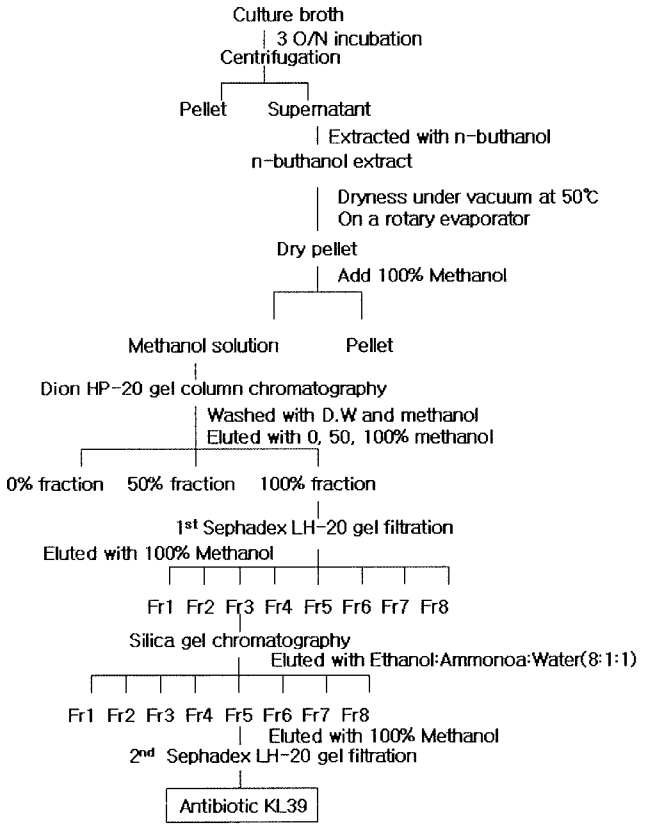

Fig. 1. Purification scheme of the antibiotic KL39 produced from *B. megaterium* KL39.

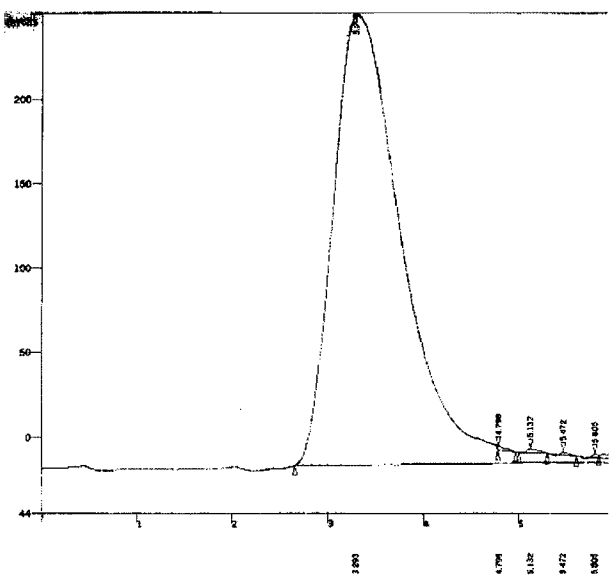

Fig. 2. HPLC spectrum of the antibiotic KL39 purified from *B. megaterium* KL39.

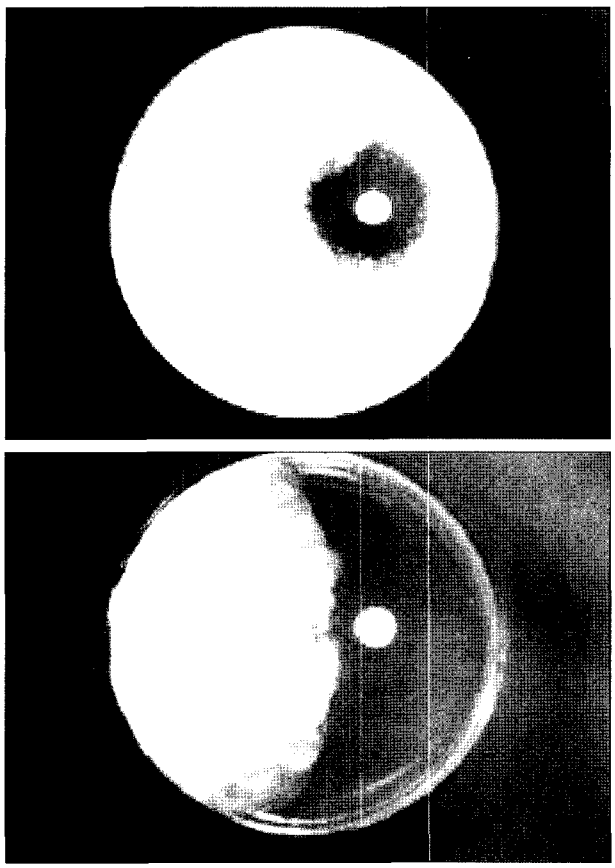

Fig. 3. Antifungal activity of the purified antibiotic KL39 against red-pepper blight-causing *P. capsici* on PDA.
Top, Inhibition of zoospore germination of *P. capsici* on PDA by purified antibiotic KL39; Bottom, Inhibition of the mycelial grown of *P. capsici* on PDA by purified antibiotic KL39.

experiments. Each pot contained 350 ml of zoospores of *P. capsici* to artificially induce Phytophthora blight. Pots were incubated at 28°C and 68% humidity for 3 days before adding 1 ml of *B. megaterium* KL39 (2.3×10^6 CFU) or 1,000 μg/ml of the purified antibiotic KL39. Pots were further incubated at the same condition for another 3–4 days to observe the diseased plants. Experiments were repeated three times.

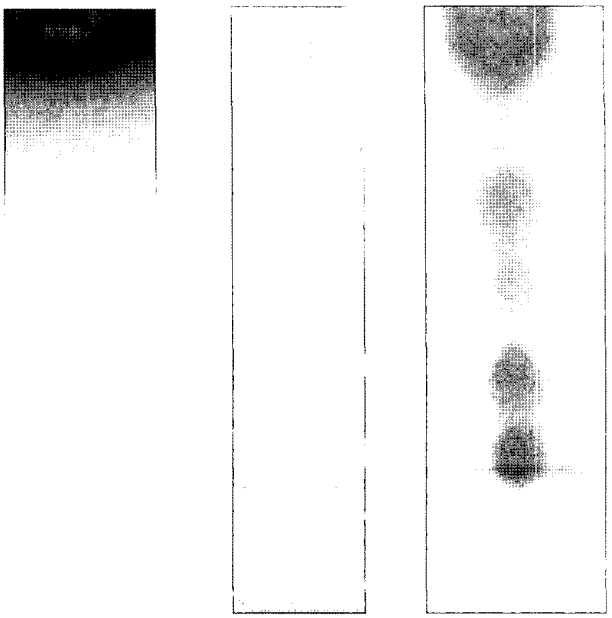

Fig. 4. Thin layer chromatography of the purified antibiotic KL39.
Left, Detection of the antibiotic KL39 on TLC under iodine vapor (R_f=0.34 with Ammonia:Water:Ethanol=1:1:8); Center, Reaction with ninhydrin reagent before the hydrolysis of antibiotic KL39; Right, Reaction with ninhydrin reagent after the acid hydrolysis of antibiotic KL39.

RESULTS AND DISCUSSION

Purification of the Antifungal Antibiotic KL39 from *B. megaterium* KL39

The butanol fraction extracted from the culture broth of *B. megaterium* KL39 was concentrated to dark brown solid by evaporation. It was eluted with 100% MeOH. The soluble material of crude antibiotic had an antifungal activity against red-pepper blight-causing *P. capsici*. On Dion and LH-20 column chromatography, the active fraction was shown to be a mixture of three compounds. Therefore, silica gel column chromatography was carried out to further purify the mixture. The active fraction from silica gel chromatography [ethanol:ammonia:water (8:1:1)] appeared to be a mixture of two components. The components were

Table 1. Characteristics of the antifungal antibiotic KL39 on Silica gel 60 plate.

Detecting reagent	Detecting group	Positive color	Antibiotic KL39
Hydrochloric acid	Glycol	Pink spot on white backgound	-
BPB-boric acid	Sugar alcohol	Yellow spot on blue background	-
Ehrlich reagent	Indole	Puple	-
	Hydroxyindole	Blue	-
	Aromatic amine and urea	Yellow	+
Nihydrin	Amino acid amines and other substances	Pink to puple spot on white background	-
10% H_2SO_4	Organic compound	Black spot on white background	+
Iodine vapor	Organic compound	Yellow spot on white background	+

*All experiments were carried out using ethanol:water:ammonia (8:1:1) as the solvent.

Table 2. Solubility of the antifungal antibiotic KL39 in various solvents.

Solubility	Organic solvents
Good	Methyl alcohol, Ethyl alcohol, n-Butanol, Acetonitrile
Poor	Ethyl acetate, Chlorform, n-Hexane, Toluene, Benzene, Water

*500 μg/ml of the antibiotic KL39 was resuspended in each solvent.

Table 3. Antifungal activity of the antibiotic KL39 against *P. capsici* for the determination of MIC.

Antibiotic KL39	Antifungal activity*
100 (μg/ml)	+++
50	++
25	+
10	-
5	-

MIC=10 μg/ml (against *P. capsici*).
*Distance between the margin of paper disc and the inhibition zone was measured.
+, Weakly antagonistic (<1 mm inhibition zone); ++, moderately antagonistic (1-4 mm inhibition zone); +++, strongly antagonistic (>4 mm inhibition zone); -, not antagonistic.

finally resolved on a Sephadex LH 20 gel column with 100% MeOH to yield a single compound (Fig. 1). The analysis of the purified antibiotic by the reversed-phase HPLC with 70% acetonitrile showed patterns of antibiotics whose retention time was 3.2 min (Fig. 2): The purified antibiotic was obtained as a single peak, confirming that the antibiotic was a single compound. The purified antibiotic KL39 had the antifungal ability, which inhibited the mycelial growth and zoospore germination of *P. capsici* (Fig. 3).

Chemical Characteristics of the Antibiotic KL39 on TLC Plate

On silica gel 60 thin-layer plate developed with ammonia: water:EtOH (8:1:1 v/v), the purified antifungal compound migrated as a single spot, and its R_f value was 0.34 (Fig. 4). The antibiotic KL39 gave a positive color reaction with Ehrlich reagent, 10% H_2SO_4, and iodine vapor, but a negative reaction with hydrochloric acid, BPB-boric acid, and ninhydrin. These results showed that the antibiotic does not have glycol, sugar alcohol, or linear peptide structure. When the purified antibiotic was hydrolyzed with 6 N HCl, however, a positive result was detected with ninhydrin solution on TLC (Fig. 4); the result indicated the absence of free amino acid group and the antibiotic seems to have a cyclic amino acid structure. This was in agreement with the result of antibiotic RFK-001 studied by Son *et al.* [28] and YHM-1 by Kim *et al.* [16]. The physicochemical properties of the antibiotic KL39 are summarized in Table 1.

Solubility and UV Spectrum of the Antifungal Antibiotic KL39

Antibiotic KL39 was soluble in polar organic solvents such as methyl alcohol, ethyl alcohol, n-butanol, and acetonitrile, but insoluble in water and nonpolar organic solvents such as ethyl acetate, chloroform, n-hexane, toluene, and benzene (Table 2). Therefore, this antibiotic seems to be a fat compound, which is soluble in polar organic solvents, but not in water. The UV spectrum of the antifungal antibiotic showed a characteristic absorption: Its maximal UV spectrum (in MeOH) was 208 nm (Fig. 5). This result is different from that of the antifungal antibiotic produced from *Bacillus* sp. YJ-63 and reported by Shin [26], but almost identical to the maximal absorption of other lipopeptide antibiotic [11, 25]. This comparison led us to conclude that KL39 might be a lipopeptide.

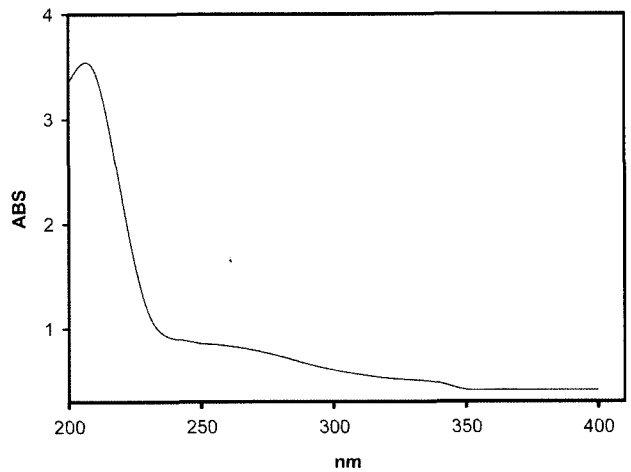

Fig. 5. UV absorbtion spectrum of the antibiotic KL39 in methanol.

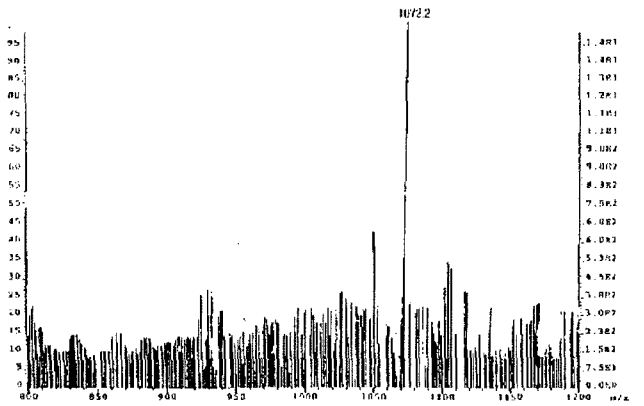

Fig. 6. Positive FAB-mass spectrum of the antibiotic KL39 from *B. megaterium* KL39.

Table 4. Amino acid composition of the antibiotic KL39 produced from *B. megaterium* KL39.

Amino acids	Amount (nmol/ml)	Molar ratio
Aspartate (Asparagine)	280	2.90 (3)
Glutamate (Glutamine)	97	1.00 (1)
Proline	92	0.95 (1)
Serine	90	0.93 (1)
Tyrosine	89	0.91 (1)

Antifungal MIC Assay

The efficacy of the purified antibiotic KL39 at various concentrations was tested for its inhibition activity against red-pepper blight-causing *P. capsici*. As described in Table 3, the MIC of antibiotic KL39 against *P. capsici* was determined to be 10 μg/ml.

Structural Characteristics of the Antibiotic KL39

FAB mass spectrum revealed a molecular weight of 1,071 for the antibiotic KL39, showing a molecular ion peak $[M+H]^+$ at 1,072 (M+H)+ (Fig. 6). The amino acid composition was found to be aspartate (asparagine), glutamate (glutamine), proline, serine, and tyrosine in a molar ratio of nearly 3:1:1:1:1 (Table 4). The chemical shift character of the antibiotic KL39 was studied by its ^{1}H-NMR spectrum (Fig. 7): The 0.76 to 1.04 ppm region indicated a terminal branch structure of aliphatic chain and suggested protons of methyl group or methylene group in 1–4 ppm. Therefore, this result showed that the antibiotic KL39 would be an aliphatic compound, having a methylene group or a methyl group.

Two doublets in 6.0–6.4 ppm indicated an aromatic amino acid. This signal is shown only in tyrosine. The antibiotic KL39 was once more confirmed to have a tyrosine residue. Protons, which were combined at β or γ-carbon of the α-amino acids, such as Asn, Gln, Pro, Ser, and Tyr, appeared near 1.6–3.6 ppm. Signals of 4.0–4.6 ppm were protons bonding at the α carbon of those amino acids. Signals of 7.5–8.0 ppm were protons to indicate Asn, Ser, Tyr, Gln, and β-amino acid. Signals around

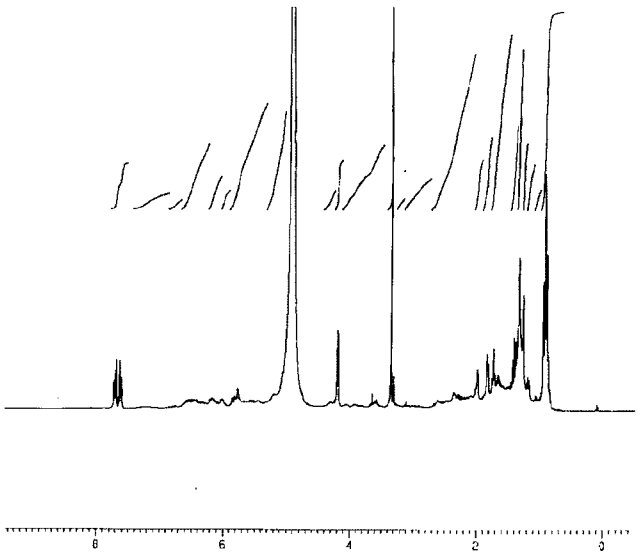

Fig. 7. ^{1}H-NMR spectrum of the antifungal antibiotic KL39 produced from *B. megaterium* KL39.

1.4 ppm were protons at the β-carbon of the β-amino acid side. Signals due to the carbon of aliphatic compound, were shown in 10–60 ppm (Fig. 8). Peaks of 20–31 ppm exposed the long chain as CH_2. In addition, the benzene ring of tyrosine appeared in 120–140 ppm as the aromatic region. Signals in 170–180 ppm were the carbon of carboxylic acid. The ^{13}C-NMR spectrum was also repeated twice, and their signs compositely appeared for the identification of the antibiotic KL39. Its chemical shift by ^{1}H-NMR and ^{13}C-NMR and the results of amino acid analysis and Mass spectrum were compared with the iturin family, and agreed with those previously reported for the iturin family [10, 13, 25, 29, 30]. Therefore, the purified antibiotic KL39 is one of the iturin E derivatives (Fig. 9).

Antifungal Spectrum of the Antibiotic KL39

To examine the antifungal spectrum of the antibiotic KL39, the inhibitory efficacy of the antibiotic against 8 plant

Table 5. Physicochemical properties of the antifungal antibiotic KL 39 produced from *B. megaterium* KL 39.

Appearance		Brown crystal
Fab-Mass (m/z)		1,071
UV absorption (λ^{MeOH} max nm)		208 nm
Molecular formula		$C_{50}H_{77}N_{11}O_{15}$
Solubility	Soluble	MeOH, n-butanol, EtOH, Acetonitrile
	Insoluble	Toluene, Chloroform, Ethyl Ether, Benzene, Acetone
Color reaction	Positive	Iodine vapor, 10% H_2SO_4, Ehrlich reagent
	Negative	Hydrochloric acid, BPB-boric acid, Ninhydrin
Rf value (EtOH:Ammonia:Water)		0.32
MIC (against *Phytophthora capsici*)		10 μg/ml
Reaction time (t_R), HPLC (with 70% acetonitrile at flow of 1 ml/min)		3.2 min

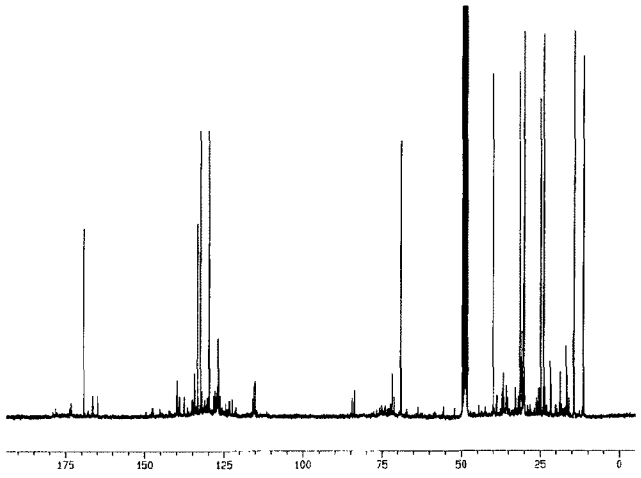

Fig. 8. ^{13}C-NMR spectrum of the antifungal antibiotic KL39 produced from *B. megaterium* KL39.

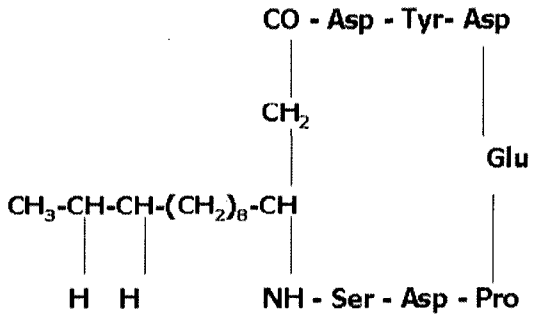

Fig. 9. Proposed structure of the antibiotic KL39 of *B. megaterium* KL39.

pathogenic fungi were determined using the paper disc method on PDA. Each disc was inoculated with 100 μg/ml final concentration of the crude antifungal antibiotic KL39.

The antibiotic KL39 strongly inhibited the mycelial growth of *Rhizoctonia solani*, *Pyricularia oryzae*, *Monilinia froeticola*, *Botrytis cinenea*, *Altenaria kikuchiana*, *Fusarium oxysporum*, and *Fusarium solani*. In particular, mycelial growth of *A. kikuchiana*, *F. oxysporum*, and *F. solani* was inhibited by more than 50%. However, *Rhizoctonia solani* was weakly inhibited. Thus, it had a broad antifungal spectrum and its range was wider than the bafiolmycin produced from *Streptomyces*, known to be effective against *P. capsici* [15]. The inhibition rate of the antibiotic KL39 against each plant pathogens is shown in Table 6.

Morphological Change of *P. capsici* by the Antifungal Antibiotic KL39

To determine the mode of antifungal action of the antibiotic KL39, the crude antibiotic was applied at a spot 15 mm from the margin of the fungal disc. After 24 h of inoculation, microscopic observation indicated that the antibiotic KL39 caused the formation of swellings in the hyphae or at the tips of the hyphal strands, where normal hyphal walls are smooth with no swellings or vacuolation. No lysis of hyphae was observed (Fig. 10). It is well known that the antibiotic substance produced by *Bacillus* sp. can influence the morphology of fungal mycelium [3]. These morphological phenomena due to antibiotics have been shown in *Fusarium solani* by the antibiotic YB70 of *Bacillus subtilis* YB70 [16] and the antibiotic YH-16 from *Bacillus* sp. YH-16 [9]. The antifungal antibiotic of *Enterobacter* sp. 54 against *P. capsici* was also shown to be very similar with the antibiotic KL39 [32]. Iturin class antibiotic is known to pass through the cell wall and disrupts the plasma membrane by formation of small vesicles; therefore, aggregation of intramembrane particle can be seen [20, 21]. Moreover, it can interact with the nuclear membrane and probably with membranes of other cytoplasmic organelles [17]. Its mode of action has been studied on artificial membrane [20]; the formation of conducting pore and increase of the permeability to lipid membrane are apparently due to iturin. Hence, the morphological change in mycelial structure by the antibiotic KL39 is thought to be an alteration of cell permeability.

Antifungal Mechanism by the Antibiotic KL39 in Macromolecules Incorporation

In order to examine the mode of antifungal action, radioactive precursors of biomacromolecules and the antibiotic KL39

Table 6. Antifungal spectrum of the antibiotic KL39 against various plant pathogenic fungi.

Plant pathogen fungi	Main host plant	Plant disease	Inhibition rate (%)*
Alternaria kikuchiana	Pear	Black spot	66
Pyricularia oryzae	Cavara	Rice blast	46
Botryis cinenea, persoon	Berry	Gray mold	72
Fusarium solani	Ginseng	Root-rot	56
Fusarium oxysporum	Cucumber	*Fusarium*-wilt	100
Rhizoctonia solani	Cucumber	Damping off	20
Phytophthora capsici	Red-pepper	Blight	90
Monilinia froeticola	Prunnus	Brown spot	68

*Inhibition rate=[1-(mycelial growth of treatment/ mycelial growth of control)]×100.

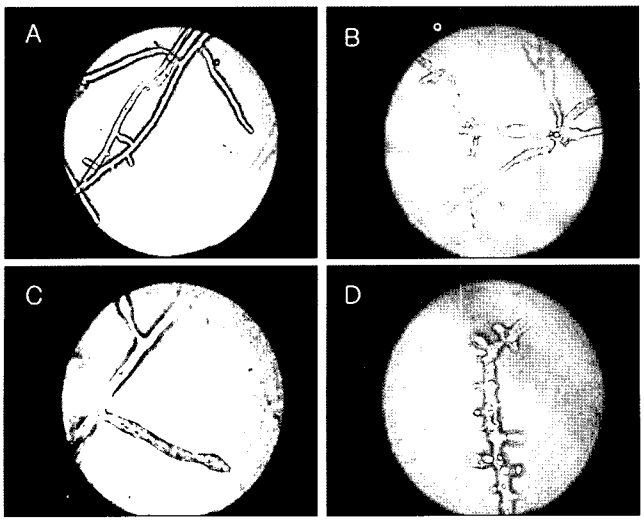

Fig. 10. Microphotography of the antifungal swelling phenomena of *P. capsici* mycelium by the antibiotic KL39 produced from *B. megaterium* KL39.
A, C: Nomal mycelial growth of *P. capsici*. B, D: Abnormal mycelial growth of *P. capsici* caused by the antibiotic of *B. megaterium* KL39.

were added to zoospores of *P. capsici*, cultivated for 24 h, and the radioactivity in germinated zoospores of *P. capsici* was then measured with a liquid scintillation counter. Table 7 shows the drastic reductions of incorporation of radioactive leucine and adenine in the presence of the antibiotic KL39. On the other hand, glucose incorporation was only slightly reduced. Accordingly, the antibiotic KL39 inhibited the synthesis of DNA, RNA, and proteins in macromolecules, but not cell wall. The inhibition of RNA and protein synthesis seems to be caused by inhibition of DNA synthesis (Table 7).

Laurence *et al.* [17] reported that when UV absorbing materials extracted from yeast cells of *Candida albicans* and *Saccharomyces cerevisiae* were treated with iturin A, UV absorption at 260 nm or 280 nm was rapidly diminished with no cell wall damage, suggesting that the iturin class would affect DNA and RNA synthesis. This is in agreement with the mode of antifungal action of the antibiotic KL39 shown by radioprecursors studies. It is known that lipopeptide antibiotic interacts with the nuclear membrane, plasma membrane, and cytoplasmic organelles [17]; therefore, it is likely that DNA and RNA synthesis were inhibited.

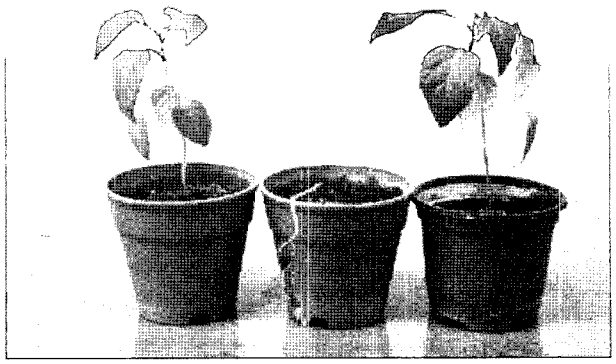

Fig. 11. *In vivo* antifungal activity of *B. megaterium* KL39 on the red-pepper blight caused by *P. capisici.*
Left, *P. capsici vs B. megaterium* KL39; Center, Only *P. capsici* infected; Right, *P. capsici vs* Antibiotic KL39 (1 mg).

In Vivo Pot Test of the Antibiotic KL39 Against *Phytophthora*-Blight Red-Pepper

Suppression of red-pepper *Phytophothora*-blight disease was evaluated in pot experiment. Seeds of red-pepper were sown in a 15-cm diameter pot containing the soil mixture. Pots were then maintained at 28°C under the sunlight lamp of 2,000 Lux. Soil humidity was maintained by watering daily. After three weeks, healthy and uniform plants were selected for experiment, and the experiment was repeated 3 times.

As shown in Fig. 11, treatment with the antibiotic KL39 or cells of *B. megaterium* KL39 suppressed the red-pepper *Phytophthora*-blight, when the pot was inoculated with pathogenic spores of *P. capsici* that caused *Phytophthora*-blight disease.

B. megaterium KL39 colonized successfully in the rhizosphere of red-pepper, and the strain or the antibiotic KL39 suppressed the blight disease in the pot. *In vivo* bioassay, *Pseudomonas* sp., *Streptomyces* sp. producing antibiotic substances, showed antagonistic activity against red-pepper blight caused by *P. capsici.* However, antagonistic activity of the antibiotic from *Bacillus* sp. was only reported against *Rhizoctonia solani* [31] and *Fusarium solani* [1, 10] in pot assays. This result showed that the *B. megaterium* KL39 or its antifungal antibiotic KL39 has the potential to

Table 7. Antifungal mechanism of the antibiotic KL39 in the incorporation of radioactive precursors into macromolecules during the zoospore germination of the *P. capsici.*

Cellular constituents	Labeled precusors	Data (cpm)		Relative value of incoporation (%)
		No antibiotic (cpm)	With antibiotic (cpm)	
DNA and RNA	^{3}H- Adenine	1.55×10^{6}	3.33×10^{3}	2.1
DNA	^{3}H-Adenine	1.43×10^{4}	2×10^{2}	14.7
Protein	^{3}H-Leucine	2.83×10^{5}	2.55×10^{4}	9.3
Cell wall	^{13}C-Glucose	1.77×10^{4}	1.68×10^{4}	100

be used as a biocontrol agent that can be an alternative to chemical fungicides.

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