Fungal-Sporulation Suppressing Substances Produced by *Pseudomonas aeruginosa* KMCS-1.

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Among the bacteria isolated from compost piles of cattle excretion in a pasture located at the suburbs of Chunchon city, *Pseudomonas aeruginosa* KMCS-1 was selected for the test of antifungal substances produced. Six fractions were separated by silica gel column chromatography, and then the antifungal activity of each fraction was assayed against *Escherichia coli, Bacillus subtilis, Candida albicans, Rhizopus* sp., *Aspergillus nidulans, Coprinus cinereus*, and *Pyricularia oryzae* by paper disc method. Two fractions showed significant suppressive activities against *A. nidulans, C. cinereus*, and *P. oryzae*; however, their mycelial growth was not affected by neither of these fractions. Inhibitory activities of these fractions to sporulation was assayed at the concentration of 50, 25, 12.5, and 6.25 μg/ml and the average inhibition rates against sporulation of *A. nidulans, C. cinereus*, and *P. oryzae* were 94.0, 98.3, and 77.9%, respectively. Further purification and analysis of active substances are now being conducted.

Key words: *Pseudomonas aeruginosa*, antifungal substance, asexual sporulation, silica gel chromatography, bioassay

Occurrences of new types of diseases in human, animals, and plants which are often caused by the pathogenic microorganisms have been serious problems. Especially, infection of the AIDS (Acquired Immunodeficiency Syndrome) patients with yeast type pathogenic fungi such as *Candida albicans, Coccidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum, Paracoccidioides brasiliensis* are being increased and their infection frequently results in fatal death (6, 21, 23, 26).

Uncontrolled and unselected uses of organic compounds with antimicrobial activity for the therapy of human diseases or for the control of plant diseases caused serious and unexpected problems, i.e., contamination of environment, appearance of resistance, and non-target effects such as toxic or harmful effects on the health of human or domestic animals (1, 3, 23, 24). To solve such problems caused by the uses of synthetic organic compounds, natural products or microbial agents have been actively pursued, developed and used for the preventive or therapeutic purposes (2, 10, 17, 18). Among such natural products, chitinase from barley and maize (5, 19), zeamatin from maize (20), polyene derivatives from garlic (22), and others have been reported. The uses of microbial agents for the preventive or therapeutic purposes of diseases are categorized into three ways: (i)use of microbes themselves, (ii)use of microbial metabolites, (iii)use of antimicrobial substances as a leading compound for the syntheses of new organic compounds (10, 17).

Recently, screening and development of antifungal substances including mass production of antifungal substances produced by microbes (4, 7, 15), cyclic peptide (9), aromatic compound derivatives, and antifungal substances belongs to imidazole group (16) are being actively studied. In this study, we tried to find leading compounds from the microorganisms for the development of new antifungal agents. We screened *Pseudomonas aeruginosa* KMCS-1, appears to have sporulation-suppressing activities against several different groups of fungi (13), and the preliminary results from the studies on the fungal-sporulation suppressing substances produced by this bacteria are reported.

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Materials and Methods

Species tested

Pseudomonas aeruginosa KMCS-1 was isolated from the compost piles of cattle excretion in a pasture located at the suburbs of Chunchon city, Kangwon-Do, Korea.

Culture media

Cultures of *Pseudomonas aeruginosa* KMCS-1 was routinely grown in YEPD medium (Yeast extract 10 g, Bactopeptone 20 g, Dextrose 20 g, distilled water 1,000 ml) at 37°C for 48 hours.

Extractions of antifungal substances

Extractions were made from the culture broth Maltose minimal medium(maltose 10 g, $(NH_4)_2SO_4$ 3 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, $CaCl_2$ 0.1 g, distilled water 1,000 ml, pH 6.0, supplemented with 5 mM L-proline) after bacterial cells were removed initially by centrifugation, then filtered through nitrocellulose membrane filter(pore size: 0.45 μm, Sigma). Forty liters of filtrate were harvested, and methanol filtrate from XAD-2 column was rotary evaporated. The residue was dissolved in distilled water, and then extracted three times with equal volumes of ethyl acetate. The ethyl acetate extract was rotary evaporated until the samples were concentrated.

Separation and purification of antifungal subastances

Antifungal substances were separated by silica gel flash column chromatography and thin layer chromatography. Crude preparation was dissolved in ethyl acetate, and then applied to a 3×20 cm column(silica gel 60, 230~400 mesh, Merck). Fractions were eluted with 200 ml of hexane-EtOAc(1 : 2) and 600 ml of hexane-EtOAc(1 : 3). Twenty milliliters of each fraction were collected and chromatographed on thin layer chromatography(TLC) plates (silica gel 60 F_{254}, layer thickness 0.2 mm, Merck) with hexane-EtOAc(1 : 2). Plates were examined under ultraviolet light(254 nm and 365 nm) or observed after exposure to anisaldehyde-sulphuric acid. Fractions which showed similar Rf value were pooled and concentrated. Six fractions were finally assayed for antimicrobial activity.

Assay for antifungal activity

Six fractions from crude extract were assayed for the antimicrobial activity against *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, *Rhizopus* sp., *Aspergillus nidulans*, *Coprinus cinereus* 18065 (monokaryon), and *Pyricularia oryzae* P_2 at the concentration of 10 μg/ml by paper disc method. Fractions which showed antifungal activity were selected, and the effects of these fractions on mycelial growth and sporulation were tested against *C. cinereus* 18065, *A. nidulans*, and *P. oryzae* P_2 at the various concentrations. *C. cinereus* 18065, *A. nidulans*, and *P. oryzae* P_2 were cultured on PDA, and then spores were harvested by adding sterilized distilled water. 0.1 ml of spore suspension at 1×10^5 spores/ml was inoculated on the PDA plate. Sterilized paper discs(dia. 1/2 inch) were placed on the center of solidified PDA and each fraction of antifungal substances were loaded at the concentration of 50, 25, 12.5, and 6.25 μg/ml. The effects of each fraction on mycelial growth and sporulation of fungi tested were evaluated after 48 or 72 hours.

Results and Discussion

Separation and purification of antifungal substances

Examination of developed TLC chromatograms of six fractions from *P. aeruginosa* KMCS-1 culture extract under ultraviolet light(254 nm) was shown(Fig. 1). Two fractions, 4 and 5, were thought to be the mixtures of several compounds.

Assay for antifungal activity

Among six fractions assayed, four fractions, 1, 2, 3, and 6, did not show any antimicrobial activity against microorganisms tested. Two fractions, 4 and 5, however, caused visible changes to the growth of some fungi, but not to any of bacteria tested. Antimicrobial spectrum of

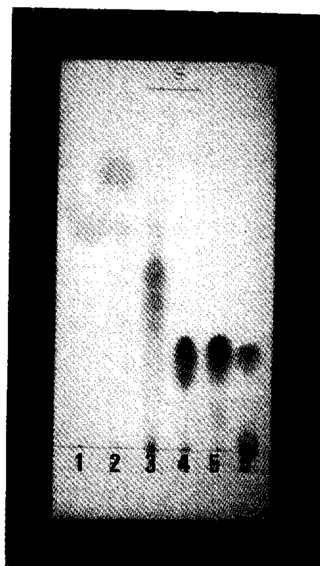

Fig. 1. Thin layer chromatogram of six fractions from *P. aeruginosa* KMCS-1 culture extract. Chromatograms were examined under ultraviolet light(254 nm).

fraction 4 and 5 was almost similar. Antifungal activity of fraction 5, however, appears to be stronger than that of fraction 4 (Table 1).

Table 1. Microorganisms used in assay for antimicrobial activity and their responses to six fractions from *Pseudomonas aeruginosa* KMCS-1 culture extract by thin layer chromatography

Microoragnisms		Fractions					
Classfication	Species	1	2	3	4	5	6
Bacteria	*Escherichia coli*	-	-	-	-	-	-
Bacteria	*Bacillus subtilis*	-	-	-	-	+	-
Yeast	*Candida albicans*	-	-	-	-	-	-
Zygomycetes	*Rhizopus* sp.	-	-	-	+	++	-
Deuteromycetes	*Aspergillus nidulans*	-	-	-	±	+	-
Basidiomycetes	*Coprinus cinereus*	-	-	±	++	++	-
Ascomycetes	*Pyricularia oryzae*	-	-	-	+	++	-

Each fraction was applied at the concentration of 10 μg/ml. Degrees of inhibition in terms of visible changes in pigmentation or texture of colonies were indicated as follows. ++ : very strong, + : strong, ± : weak, - : no visible inhibition.

Antifungal activity of two fractions, 4 and 5, were assayed quantitatively by measuring mycelial growth and asexual spore production of *C. cinereus* 18065 (haploid), *A. nidulans*, and *P. oryzae* P_2 after treatment of two fractions. No significant inhibition to the mycelial growth of *C. cinereus* 18065, *A. nidulans*, and *P. oryzae* P_2 was observed by the treatment of two fractions, 4 or 5, (Fig. 3). The average correlation coefficients between no treatment and treatment of fractions against *C. cinereus* 18065, *A. nidulans*, and *P. oryzae* P_2 were 0.9962, 0.9951, and 0.9992, respectively (Pearson correlation coefficient). And maximum differences between treatment with or without fractions in mycelial growth of *C. cinereus* 18065, *A. nidulans*, and *P. oryzae* P_2 were 3.7 mm, 4.3 mm, and 3.2 mm, respectively. These results indicate that both fractions have no inhibiting effect on the mycelial growth of *C. cinereus* 18065, *A. nidulans*, and *P. oryzae* P_2 at the various concentrations for 168 hours of incubation period.

On the other hand, asexual sporulations of *C. cinereus* 18065, *A. nidulans*, and *P. oryzae* P_2 were significantly

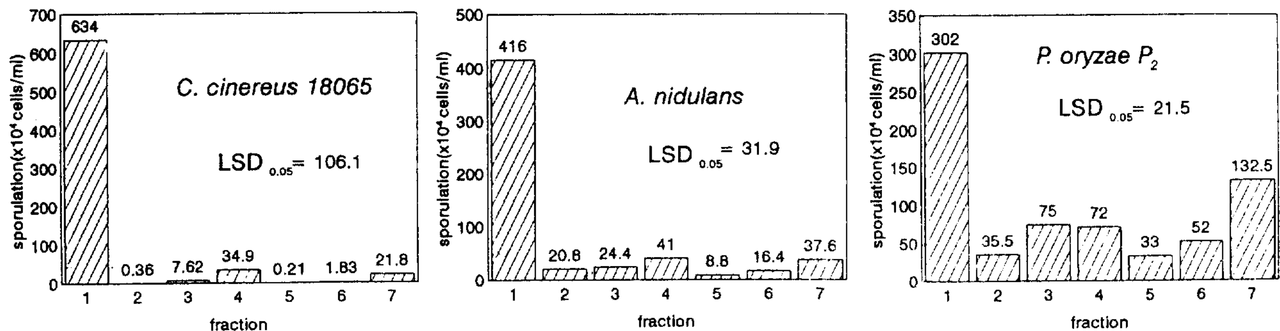

Fig. 2. Effects of the fractions from *P. aeruginosa* KMCS-1 culture extract on sporulation of *C. cinereus* 18065 (left), *A. nidulans* (center), and *P. oryzae* P_2 (hight). 1=No treatment, 2 = fraction 4 (50 μg/ml), 3=fraction 4 (25 μg/ml), 4=fraction 4 (12.5 μg/ml), 5=fraction 5 (25 μg/ml), 6=fraction 5 (12.5 μg/ml), 7=fraction 5 (6.25 μg/ml). Sporulation of *A. nidulans* was evaluated at 48 hrs culture after treatments, while *C. cinereus* 18065 and *P. oryzae* P_2 were done at 72 hrs culture. Each data indicate the mean of three replications. $LSD_{0.05}$ indicate the least significance difference at P=0.05.

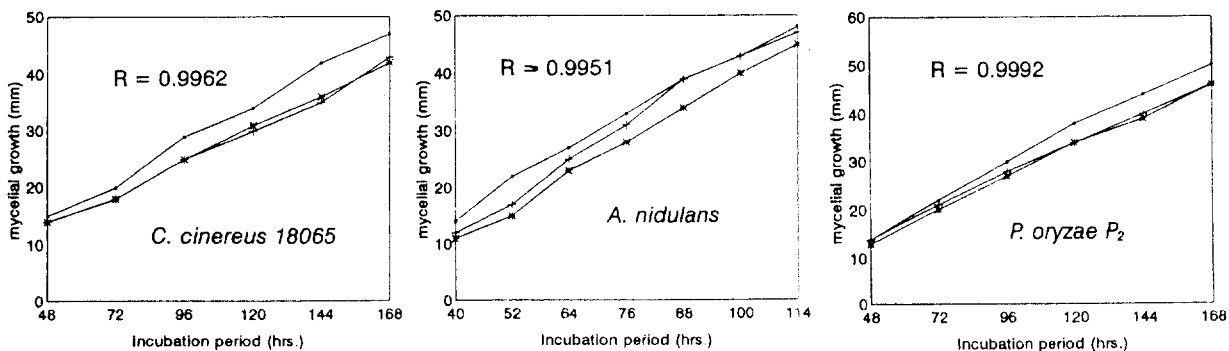

Fig. 3. Effects of the fractions from *P. aeruginosa* KMCS-1 culture extract on mycelial growth of *C. cinereus* 18065 (left), *A. nidulans* (center), and *P. oryzae* P_2 (right). —■—=No treatment, —+—= fraction 4 (12.5 μg/ml), —*—= fraction 5 (12.5 μg/ml). Each data indicate the mean of three replications. R indicate the Pearson correlation coefficients.

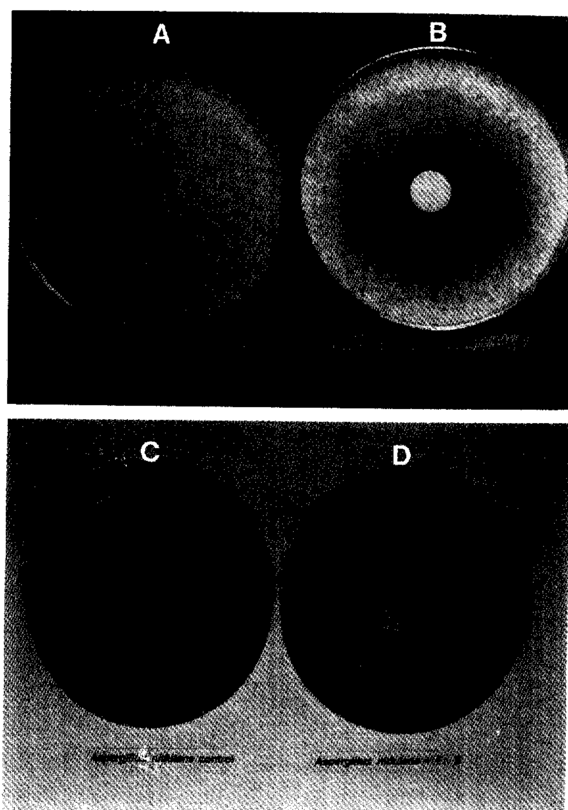

Fig. 4. Effects of the fractions from *P. aeruginosa* KMCS-1 culture extract on sporulation of *C. cinereus* 18065 and *A. nidulans*. A: No treatment on *C. cinereus* 18065 culture, B: fraction 5 (12.5 μg/ml) treatment on *C. cinereus* 18065 culture, C: No treatment on *A. nidulans* culture, D: fraction 5 (12.5 μg/ml) treatment on *A. nidulans*. Photographs were taken 48 hours after treatment.

suppressed by the treatment of fractions, 4 or 5, as compared to those of no treatment (Fig. 2), and, however, there were no significant differences among various concentrations within the treatment of same fraction. The average inhibition rates against sporulation of *C. cinereus* 18065, *A. nidulans*, and *P. oryzae* P$_2$ were 94.0%, 98.3%, and 77.9%, respectively (Fig. 4). Preliminary experiment showed that both fractions also have suppressing effects on the spore germination as well as sporulation of fungi tested (data not included). Based on these results, these fractions seemed to be specifically related to the suppression of asexual sporulation, but not to the mycelial growth of fungi tested. So far, most of efforts to screen antifungal substances had been focused at the inhibiting effects on the mycelial growth of fungal organisms (8, 9, 11, 14, 25), and efforts to find substances with sporulation-suppressing activity were virtually none. Among these, Kim *et al.*(9) reported on the isolation and identification of three substances, phenazine-1-carboxylic acid, oxychlororaphine, and pyoluteorin, from the broth medium of *P. aeruginosa*. Phenazine-1-carboxylic acid and pyoluteorin significantly inhibited the mycelial growth of several fungi, i.e., *Helminthosporium oryzae*, *Phomopsis* sp., and *Phytophthora cactorum* by phenazine-1-carboxylic acid, and *Pythium ultimum* and *Ph. cactorum* by pyoluteorin, no antifungal activity was shown by oxychlororaphine. These results on antifungal activity of the substances against *Aspergillus* spp. and *P. oryzae* are similar to those of our study. However, the effect of antibiotic substances on fungal sporulation had never been investigated. Occasionally, antifungal substances which have a inhibiting effects on fungal sporulation, but not on mycelial growth or vice versa can be existed. Therefore, screening of various compounds for antifungal activity should be evaluated by testing inhibiting effects on sporulation as well as on mycelial growth. For the control of fungal diseases, fungal-sporulation or germination suppressing substances might be more effective than the substances with inhibitory activities to the mycelial growth. Finally, it is necessary that the antifungal spectrum of these two fractions from *P. aeruginosa* should be determined against various groups of fungi including pathogenic fungi. Purification and analysis of both fractions, from now on, should be further carried out and compared with previous works.

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