Original Article

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Isolation of Soil Actinobacterial Strains Showing Antifungal Activity against *Aspergillus flavus*, a Causative Agent of Stonebrood Disease in Honeybee

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Abstract

Stonebrood is a well known fungal disease of honeybee. *Aspergillus flavus* is the most common agent of stonebrood. In this study, we isolated more than 2,000 actinobacterial strains and screened them for antifungal activity against *A. flavus* by inhibition zone measurement. As a result, we found that 4 *Streptomyces* spp. and 1 *Cupriavidus* sp. showed relatively high antifungal activities with the diameters of up to 21 mm inhibition zone. Therefore, this study may play a role to control stonebrood of honeybee in the near future.

Key words: Stonebrood, *Aspergillus flavus*, Actinobacteria, *Streptomyces*, *Cupriavidus*, Antifungal activity

INTRODUCTION

Honeybee is a socially important insect to participate 25% of pollination as a major pollinator for a variety of crops (Evans and Hung, 2000; Morse and Calderone, 2000). Because honeybees tend to make a group to live together with around several ten thousand individuals in a small space, they can provide a proper inhabiting environment for many microorganisms that cause high possibility of various diseases (Morse, 1978).

Chalkbrood disease and stonebrood disease are the most well-known fungal diseases (Lee *et al*., 2004). Stonebrood, first reported by Maassen (1916) in German, is a fungal disease occurred in *Apis millifera* in the state of lava or adult by infection of *Aspergillus* spp., mainly developed at the brood stage (Tanada and Kaya, 1993). This disease is most often infected by *Aspergillus flavus* among *Aspergillus* species (Gilliam and Vandenberg, 1990), and the color of dead larval body is yellowish green due to the spores of *A. flavus*. If they are infected by *A. fumigatus* and/or *A. nidulans*, the green spores can be found in the dead larval body. Since stonebrood has a similar symptom as in chalkbrood, general beekeepers have been recognized as the same disease. However, they can distinguish the two diseases through different body colors, yellowish green-green in stonebrood versus white/black in chalkbrood. Infection occurs by entering of the spores into the digestive gut, which are germinated to be the hyphae. Then the hypae penetrate into the tissues, grow and kill the larvae that can be discovered as hard stones in shape (Lee *et al*., 2004).

Stonebrood disease has been accepted in general beekeepers as a disease that gives less damage than bacterial diseases, but it is reported that stonebrood disease is known as the most serious honeybee disease damaging widely in beekeeping farmers of Gangwon-do province in Korea (Yoon, 2001). In addition, in many cases, the infected larvae with stonebrood disease are very high risky in mixed

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infections with Jose horsemen chalk disease and viral diseases, which cause more serious problems (Yoon, 2001), and in chronic damage for many years within the hive.

Actinobacteria are Gram-positive, chain-forming, and widely distributed in soil and share common characteristics partially with bacteria and fungi. So they vary in morphological and physiological properties, with which actinobacteria produce various secondary metabolites useful as antibiotics, and greatly influenced by culture conditions and the composition of media (Wiens *et al*., 2009). In addition, actinobacteria not only contribute in matter cycle of ecosystems through the degradation of a wide range of substrates by enzymes, enzyme-inhibitors, immunoregulatory molecules, etc. (Jones, 1985; Beppu and Horinouchi, 1991; Shin, 1991), but they also are very valuable in a research field for industrial applications (Vacelet, 1975). Out of around 10,000 antibiotics found from microbial metabolites, actinobacteria produces over 75%, among which a genus *Streptomyces* occupies 74% (Datta *et al*., 2000).

In this study, we tried to isolate actinobacterial strains from various soil samples and tested their antifungal activity against *Aspergillus flavus*, an agent of stonebrood disease.

MATERIALS AND METHODS

Sample collection

Various soil samples were collected from all over the country, and the collected samples were freshly maintained in the plastic bags during transfer and kept them in the refrigerator at 4°C until use (Lee and Kim, 2014).

Isolation of actinobacteria

Across the country, we collected soil samples for finding actinobacterial strains living in soil and for extracting the nutrients to be used as soil extract. The small particles were removed through sieving, and then the suitable soils were placed in each well in a 6-transwell plate with addition of sterilized distilled water for moisturizing. After the membrane filter inserts were placed on soil layer in each well, the five liquid media were poured into each insert, respectively. These media were humic acid-vitamin medium, brain heart infusion (BHI) medium, R2A medium, Bennett's medium and Benedict's modification of the Lindenbein medium (Lee and Kim, 2014).

Certain supernatant from soil-mixed solution including microbes was used as an inoculum. Cultivation was done in a shaking incubator at 120rpm and 28°C for 2 weeks with refill of the liquid media periodically to avoid evaporative loss. A serial dilution was made like 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. 100μL of dilution was used for spreading on the agar plate. After incubation at 28°C for 5 days, all the different types of colonies were picked up, streaked individually on each agar plate and repeated until getting pure colonies. All the obtained pure colonies were conserved in 20% glycerol stocks at −20°C (Nguyen *et al*., 2013).

Test Strains

The test strain, *A. flavus*, was isolated from stonebrood disease-infected bees obtained from Korea Honeybee Disease Institute of Kyonggi University. To isolate *A. flavus*, the infected honeybees were grinded, the grinded material was spread on PDA (potato dextrose agar) plates, and the colonies were checked everyday for 5 days. The isolate was further cultivated on SDA-Y agar plate for 5 days, and then onto SDA-Y + R2A (1:1) agar plate for the inhibition zone test.

Measurement of inhibition zone

A. flavus was inoculated in SDA-Y liquid medium and cultivated in a shaking incubator for 1 day. After the measurement of OD value, the spreading on SDA-Y + R2A (1:1) agar plate was performed. Then 4 different actinobacterial isolates were inoculated with four spots on the fungal lawn plate, respectively. After the incubation for 3~5 days at 25°C, each inhibition zone (diameter) was measured by using a ruler.

Identification of microbes

Genomic DNA was extracted using an InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA), and the 16S rRNA gene was amplified by PCR using the universal primer set for bacteria: 27F (5´-AGA GTT TGA TCM TGG CTC AG-3´) and 1492R (5´-TAC GGY TAC CTT GTT ACG ACT T-3´). The PCR product was purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA) and the sequencing reaction was prepared with the PRISM BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The reaction was incubated at 95°C for 5 min, cooled on ice for 5 min, and then analyzed with an ABI Prism 3730XL DNA Analyzer automated DNA sequencing system (Applied Biosystems). The near-complete sequence of the 16S rRNA gene was identified using the EzTaxon server (Kim *et al*., 2012).

To identify *A. flavus*, a primer set was used: Asp 18S-F (5´-ATC GGG CGG TGT TTC TAT G-3´) and Asp 18S-R (5´-ACC GGG CTA TTT AAG GGC CG-3´) to get 312 bp PCR products. Then the PCR products were checked through electrophoresis (1×TAE, 1.5% agarose gel).

RESULTS AND DISCUSSION

Identification of microorganisms

Aspergillus flavus was identified by checking the specific gene size through PCR reactions with the primers and electrophoresis. Among the tested three isolates, the lane 1 was matched with the size of the gene was the same size as the positive control (lane P: *A. flavus*), showing 300 bp (Fig. 1).

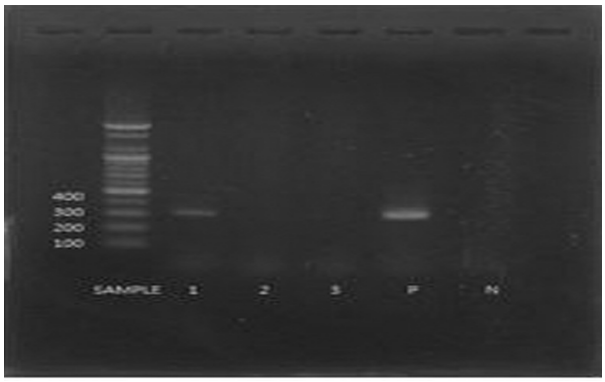


Fig. 1. PCR test result to identify *A. flavus* on electrophoresis gel (lane 1~3: isolates from the larvae with stonebrood; P: positive control; N: negative control).

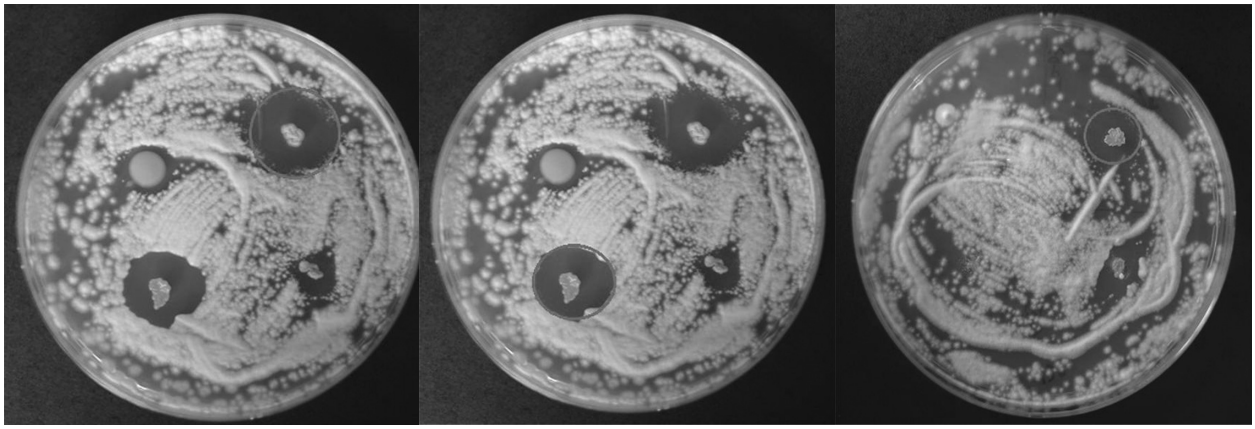
Five isolated strains having antifungal activity were identified as four *Streptomyces* spp. and one *Cupriavidus* sp. as shown in Table 1 by using 16S rRNA gene through EzTaxon. In particullar, *Cupriavidus* sp. L-1 can be a potential new species (98.25% similarity in 16S rRNA gene sequence) that has the antifungal activity as reported for the first time in this study.

The stonebrood of honeybee causes larval death by the toxin produced from *A. flavus* which can fill the body fully with hyphal growth after the spore germination in the gut. The hyphae extended from the inside of the body form the conidiospores that have white color in the beginning and

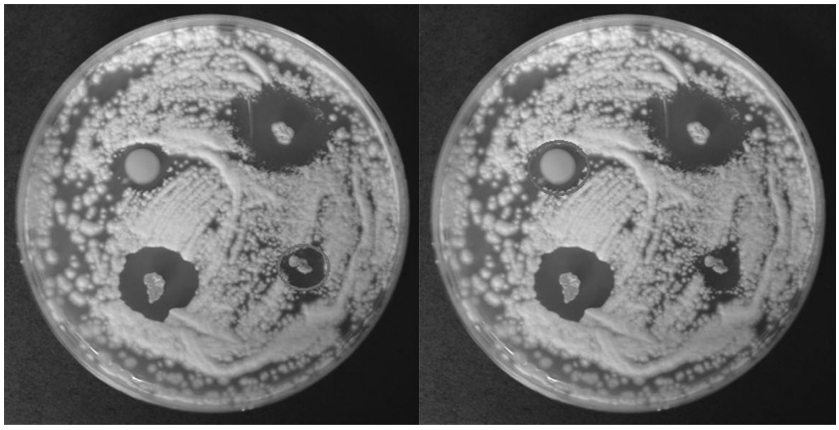
Table 1. List of actinobacterial soil isolates that showed antibacterial activity

No	Strain	Closest cultivated species	Pairwise similarity (%)	Inhibition of zone*	Size (Diameter: mm)
1	A-64	*Streptomyces resistomycificus* NBRC 12814(T)/ AB184166	99.1	++	18
2	T-277	*Streptomyces galbus* DSM 40089(T)/X79852	99.93	+	9
3	T-64	*Streptomyces sporoverrucosus* NBRC 15458(T)/AB184684	99.86	+++	21
4	T-263	*Streptomyces spororaveus* LMG 20313(T)/AJ781370	99.86	++	11
5	L-1	*Cupriavidus basilensis* CCUG 49340(T)/FN597608	98.25	++	12

* +, ++, and +++ indicate the diameters ≤9, 10 to 19, and ≥20mm, respectively.



(a) *Streptomyces sporoverrucosus* (b) *Streptomyces resistomycificus* (c) *Streptomyces spororaveus*



(d) *Streptomyces galbus* (e) *Cupriavidus basilensis*

Fig. 2. Inhibition zone by actinobacterial and other strains against *Aspergillus flavus*.

then different colors according to different species: yellowish green by *A. flavus*, and green by *A. fumigatus* and *A. nidulans* (Samson *et al*., 2000).

Finding of actinbacterial strains having antifungal activity

Up to date, 80% of the antibiotics has been originated from the genus *Streptomyces* (Watve *et al*., 2001). In these days, however, the number of new antibiotics to be founded are not many.

With the goal of finding new antibiotics/compounds from antiobacteria, we have used three methods for the selection of bacteria from soil samples as mentioned in the precious research (Nguyen *et al*., 2013). This study constructed an actinobacterial library including around 2,000 isolates.

Some of actinobacterial isolates in the library were able to inhibit the growth of *A. flavus*, an agent of stonebrood disease. They showed the inhibition zones on *A. flavus* lawn (Fig. 2), which were measured in diameters to compare their antifungal activities against *A. flavus* (Table 1). These isolates are most related with *Streptomyces sporoverrucosus, S. resistomycificus, S. spororaveus, S. galbus,* and *Cupriavidus basilensis*, respectively. Among them, *S.* sp. T-64, *S.* sp. A-64 and *S.* sp. T-263, showed the most effective antifungal activities (Fig. 2 and Table 1). Although *Cupriavidus* sp. L-1 seemed to have enough size of diameter, it was excluded from the most effective strains because the size of spot was too large to consider as

inhibitory area (Fig. 2). However, it is a meaningful isolate since it is a potential new species which may produce a new com-pound to control many antibiotic-resistant infectious pathogens.

Therefore, we expect that stonebrood disease may be controlled by using these actinobacterial cultures or secondary metabolites purified in the near future. Further, we may develop a new drug to control some drug-resistant microbial pathogens.

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