ISOLATION OF SOME MICROORGANISMS ASSOCIATED WITH FIVE SPECIES OF AMBROSIA BEETLES AND TWO KINDS OF ANTIBIOTICS PRODUCED BY Xv-3 STRAIN IN THESE ISOLATES

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Introduction

Ambrosia beetles belonging to Scolytidae and Platypodidae have always been found on association with some fungi carried by their mycetangia which are special organs to contain the fungal spores. These beetles have evolved elaborate methods of infecting their tunnels with symbiotic fungi. They bore into host logs, inoculate the tunnels with the spores so that the tunnels are lined with mycelium on which the beetles feed. And, it is well known that the numerous species of these wood-boring beetles cannot survive without ambrosia fungi (including several genera of *Ascomycetes*, "imperfect" fungi and yeasts)[1,2,3,4,5,6,7,8,9,10].

In order to clear the roles of these symbiotes, many investigators have attempted to isolate these specific fungi and to study optimum conditions to grow up for these isolates. Since FRANCKE-GROSMANN[6] had initially succeeded to isolate the symbiotic fungi from some ambrosia beetles, many symbionts were isolated from several species of these beetles. These works were reviewed by FRANCKE-GROSMANN[6,7], BATRA and BATRA[4] and BATRA[3].

However, in many cases the achievment was far from perfect either because of the symbionts cannot be separated without damaging them, or because of the isolates cannot be cultivated at all. Furthermore, the classi-

fication of symbionts seems to be sometimes provisional, since it is impossible to clear the function performed by the symbionts.

In the present study, the isolation of symbiotic microorganisms was done on the five species of the ambrosia beetles, *Xyleborus validus* EICHHOFF, *Xyloterus signatus* FABRICIUS, *Scolytoplatypus shogun* BLANDFORD, *Crossotarsus niponicus* BLANDFORD, *Platypus severini* BLANDFORD. And, we have reported that the yeast, *Endomycopsis platypodis* was commonly isolated and identified from homogenates of whole bodies and galleries of four species of the ambrosia beetles except *S. shogun*, in which *Pichia* sp. was isolated from both sources. Furthermore, one more mold-like fungus was also commonly isolated from both sources of four species of the beetles except *S. shogun*.

And also, we have demonstrated that two kinds of antibiotics, cerulenin and helvolic acid were isolated and identified from a mold-like fungus (Xv-3) associated with *X. validus*.

Materials and Methods

Collection of beetles and their galleries

The ambrosia beetles used on the present study were *X. validus*, *X. signatus* and *S. shogun* as Scolytidae, and *C. niponicus* and *P. severini* as Platypodidae.

Adult beetles and their galleries as samples were collected from pinholed beech logs (*Fagus crenata* BLUME) of about 20 to 25 cm in diameter during the summer of 1975 at Kaminokuni, Hokkaido, and these logs were transported to laboratory and cut into small pieces in order to collect the beetles and their galleries.

Media and culture conditions for isolates

For the isolation of symbiotic microorganisms, nutrient broth, nutrient agar, YM broth and agar (Difco Laboratory, Detroit, Michigan) were used. Media used in shaking cultures were basically similar, but in some cases, 1% beef extract, 0.5% peptone, 6% glucose, 0.3% KH_2PO_4 and 1% NaCl were added.

For the isolation of antibiotics, mold-like fungus, Xv-3 strain isolated from *X. validus* was used. Seven days old cultures on YM agar slant were grown in 200 ml of Difco Peptone broth at 25°C using 500 ml Sakaguchi flasks. Then, 400 ml of the 70 hours culture at 25°C were inoculated onto 25 liters of the same peptone broth using 50 liters big capacity fermentors and reincubated at 25°C for 75 hours until the mycelium growth had reached at optimum conditions for harvest under aerobic condition.

Isolation of microorganisms from mycetangia of P. severini

On the external skeleton of the caudal half of the pronotum, the female adults of *P. severini* have a large number of small pits (these are mycetangia) which can carry the viable spores. For the isolation of these spores, the external skeletion of the pronotum was removed from insect body and washed with sterile water, and then, spores were picked up from the pits using a micromanipulator (Fonbrane ReF 141 No. 511 CH., Beaudouin, Constr, Paris) after fixation on a nutrient agar slab. The spores isolated were then transferred to YM agar plate and incubated at 25°C up to 7 days.

Isolation of Microorganisms from homogenates of adult beetles

Symbiotic microorganisms were isolated from the adult beetles (male and female) of five different species according to the procedure described below.

The adult beetles of each species were collected from the pinholed beech logs on cheese cloth and washed twice with 200 ml of sterile destilled water. Insect bodies were homogenized gently in 1 ml of YM broth with sterile teflon homogenizer. The homogenates were divided into two portions. One was spread over YM agar plate containing a mixture of penicillin G (200 U/ml, Meiji-Seika Ltd., Tokyo) and streptomycin (0.1 mg/ml, Boehringer Mannheim Gmbh, W-Germany) and the remainder was transferred onto a nutrient agar slab to pick up spores with the aid of a micromanipulator. Both the homogenates on YM agar plate and spores isolated on a nutrient agar slab were incubated at 25°C up to 7 days. Each strain was isolated from the colonies which are apparently showing different morphological characteristics on the agar plates.

Isolation of microorganisms from galleries of the beetles

The fungi which have grown on the galleries of *X. validus*, *X. signatus*, *S. shogun*, *C. niponicus* and *P. severini* were isolated. Pinholed beech logs were cut out into small pieces of about 2 cm^3. After removal of adults and larvae from their galleries, the inner surface of each gallery was washed with 10 ml of sterile distilled water. The washing water was laid on YM and nutrient agar plates containing a mixture of penicillin G and streptomycin as streaks, followed by the incubation at 25°C for 3 days up to 10 days to check the appearance of colonies of slow growing microorganisms.

Isolation of antibacterial and antifungal compounds

a) Antibiotics from culture fluid of a mold-like fungus Xv-3 (Compound I):
A mold-like fungus Xv-3 isolated from galleries and homogenates of *X.*

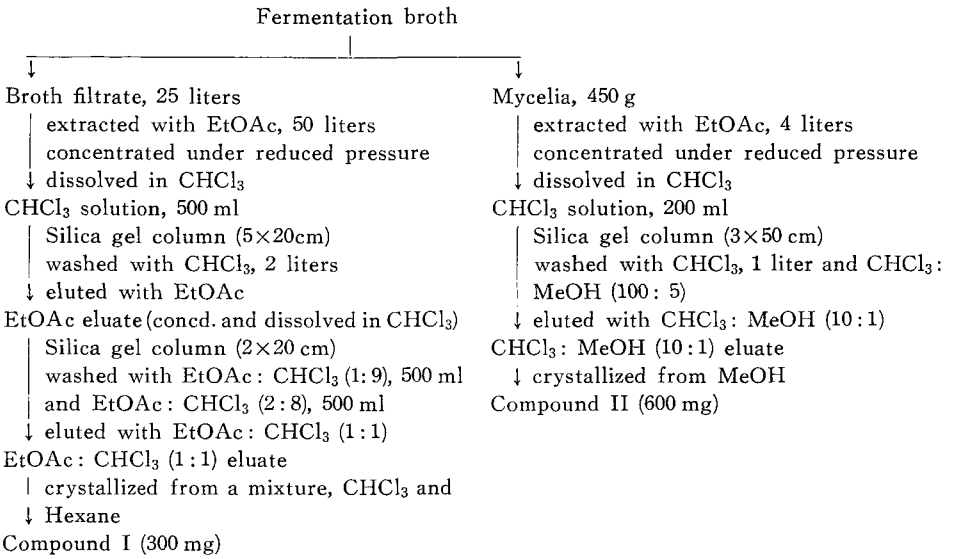


Fig. 1. Procedures for fractionations and purifications of Compound I and II.

validus was cultured aerobically in 25 liters of peptone broth culture medium for isolation of antibiotics. The fractionating procedures of compound I and II were carried out as shown on Fig. 1. After removal of the fungal mass by filtration, culture filtrate was extracted with ethyl acetate. An antibacterial activity was observed in the ethyl acetate fraction, which was chromatographed on a silica gel column (Kieselgel 60, Merk Ltd., USA). And then, active fraction was rechromatographed on silica gel column. Finally, the compound I was crystallized as white needles from chloroform-hexane.

b) Antibiotics from cell extract of a mold-like fungus Xv-3 (Compound II):

About 450 g of dried fungal cells from galleries and homogenates of *X. validus* were extracted with ethyl acetate at room temperature. A marked antibacterial activity was observed in the ethyl acetate fraction, which was chromatographed on a silica gel column. Compound II was crystallized from methanol as white needles. All of the antibacterial activity was followed by the agar plate diffusion assay using *Bacillus subtilis* and *Staphylococcus aureus* as test microorganisms.

Physicochemical analysis

Hitach Model RMU-6 chromatography-mass spectrometry system was used for analysis of structure of compounds, and Hitachi-R-20 B NMR was also used for analysis by the nuclear magnetic resonance spectra.

Results

Isolation of microorganisms from the mycetangia of P. severini

Two strains (PsM-1 and PsM-2) of microorganisms were isolated from the mycetangia of *P. severini* using micromanipulator. After PsM-1 and PsM-2 were morphologically investigated, PsM-1 was put into Cn-1 group and PsM-2 was also put into Cn-4 group. Morphological characteristics were described in Table 2 and Fig. 2.

Isolation of microorganisms from homogenates of whole bodies and galleries of five species of the beetles

All of isolates from homogenates of whole bodies and galleries of five species of the beetles were shown in Table 1. Morphological investigations

TABLE 1. Some microorganisms isolated from mycetangia, homogenates of whole bodies and galleries of the ambrosia beetles.

Beetles	Mycetangia	Homogenates of whole bodies	Galleries
Scolytidae			
Xyleborus validus	untested	Xv-1*	XvG-1*
		Xv-2**	XvG-2
		Xv-3****	XvG-3**
Xyloterus signatus	untested	Xs-1*	XsG-1*
		Xs-2**	XsG-2**
		Xs-3	
Scolytoplatypus shogun	untested	Ss-1	SsG-1
		Ss-2	SsG-2
		Ss-3***	
Platypodidae			
Crossotarusus niponicus	untested	Cn-1*	CnG-1*
		Cn-2	CnG-2**
		Cn-3	CnG-3
		Cn-4**	
		Cn-5	
Platypus severini	PsM-1*	Ps-1*	PsG-1*
	PsM-2**	Ps-2**	PsG-2
			PsG-3**
			PsG-4
			PsG-5

* *Endomycopsis platypodis.*
** The same, but unidentified strain; antibacterial substances were slightly produced.
*** *Pichia* sp.
**** Antibacterial substances were highly produced.

TABLE 2. Morphological characteristics in Cn-1

Endomycopsis platypodis (Baker et Kreger-van Rij)
Synonym: *Hansenula platypodis* (Baker et Kreger-van Rij) Fiol 1967
Growth in yeast extract-malt extract: After 3 days at 25°C the cells are spherical and oval, 1.4-4.1-10.1 μ; single or in pair. Elongated budding cells and pseudomycelium are also present. Vegetative reproduction occurs by multilateral budding and by budding on broad base. A sediment and a ring are present. After one month at 25°C a flocculent sediment and a ring are present.
Growth in yeast extract-malt extract agar: After one month at 25°C the streak culture is cream-colored, convex butyrous, smooth dull; the middle part is slightly raised, punctate and cream-colored with a brown tinge; the edge is fringed with mycelium.
Dalmau plate culture on corn-meal agar: True mycelium and pseudomycelium bearing blastspores are abundantly produced. Single, distinct, centrallylocated pore bodies of dolipore type present in the hyphal septa. Anastomoses may occur.
Formation of ascospore: The asci are situated terminally or laterally on the hyphae. They contain usually four, hat-shaped spores. Sporulation was good on corn-meal and YM agar.
Assimilation of potassium nitrate: Positive.

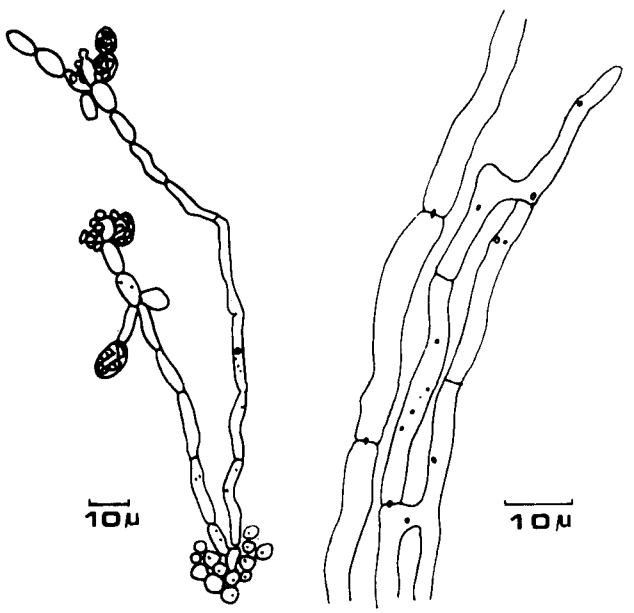


Fig. 2. Cn-1. Pseudomycelium, true mycelium and spores in YM agar slide culture after 4 days at 25°C.

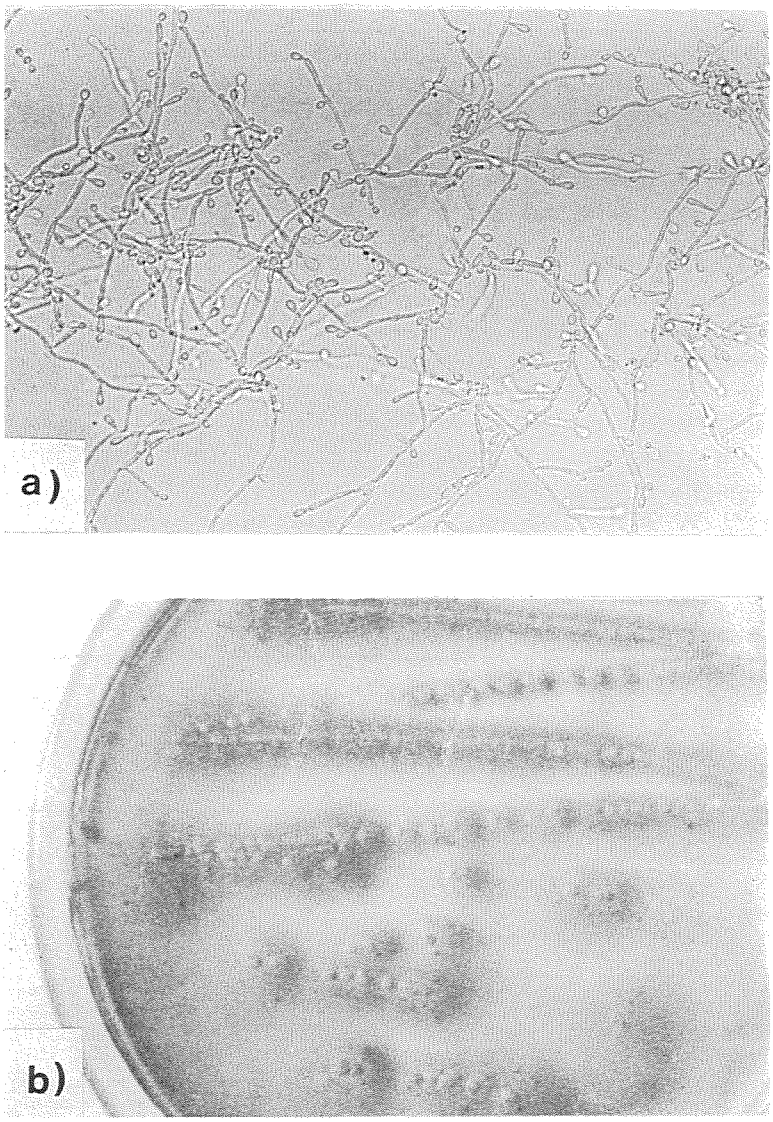


Fig. 3. Mold-like fungus, Cn-4 isolated from homogenates of adult whole bodies of *C. niponicus*.

a) YM agar culture after 3 days at 25°C. (×200).
b) Colonies on YM agar culture after 3 days at 25°C.

in these isolates were carried out. The strains PsM-1, Ps-1, PsG-1, Cn-1, CnG-1, Xv-1, XvG-1, Xs-1 and XsG-1 were classified as same group by morphological and physiological investigations, and identified as *Endomycopsis platypodis*. The descriptions were shown in Table 2 and Fig. 2.

PsM-2, Ps-2, PsG-3, Cn-4, CnG-2, Xv-2, XvG-3, Xs-2 and XsG-2 morphologically occurred with showing same typed aerial hyphae and mycerium, and light brown colored colonies (Figs. 3 a and 3 b). However, these isolates were still unidentified and investigations to identify are continuously done.

The microorganisms (Ss-1, Ss-2 and Ss-3) from *S. shogun* were not

TABLE 3. Morphological and physiological characteristics in Ss-3

Pichia sp.

Growth in YM broth: After 3 days at 25°C, the budding yeast cells are spherical, oval and cylindrical, (1.2-3.5-8.0.)×(1.2-4.4-12.5) μ, and occur singly or in pairs. A sediment and a ring are present. After one month at 25°C, a sediment and a ring are present.

Growth in YM agar: After 3 days at 25°C, the budding yeast cells are spherical to oval, cylindrical, (1.5-3.5-5.0)×(1.5-4.0-10.0) μ, and occur singly or in branched chains. After one month at room temperature the streak culture is cream-colored, dull, butyrous, smooth or slightly wrinkled, with a undulate partly filamentous margin.

Dalmau plate culture on corn-meal agar: A primitive pseudomycelium is abundantly formed. It consists of oval chain cells and of a tree-like appearance.

Formation of ascospore: Asci are oval to long-oval. The spores are hat-shaped, usually four are formed per ascus. They are easily liberated from the ascus. Sporulation is good in YM broth, on YM agar and cornmeal agar.

Fermentation:

Glucose	+	Sucrose	−	Lactose	−
Galactose	−	Maltose	−	Raffinose	−
Trehalose	−				

Assimilation of carbon compounds:

Glucose	+	Trehalose	−	Rhamnose	+
Galactose	+	Lactose	−	Ethanol	+
L-Sorbose	+	Melibiose	−	Mannitol	+
Sucrose	+(weak)	Raffinose	−	Inositol	−
Maltose	+	Xylose	+(weak)		
Cellobiose	−	L-Arabinose	−		

Assimilation of potassium nitrate: Negative

always concurred with them of another species of the beetles. Morphological and physiological characteristics of Ss-3 were shown in Table 3 and Fig. 4, and Ss-3 was identified as *Pichia* sp.

Isolation of antibiotics

Two kinds of antibiotics, compound I and compound II were isolated from culture broth and cell extract of Xv-3, respectively. From culture broth, the fractions containing the antibacterial activity were eluted with enthyl acetate fraction followed by ethyl acetate : chloroform (1 : 1, v/v). Finally, the compound I was crystallized as white needles (mp 80–81°C) in chloroform-hexane mixture solution. The yield of compound I was 300 mg from 25 liters of culture broth. It was determined that molecular weight was 223 and molecular formula was $C_{12}H_{17}NO_3$ by GC-MS, proton- and carbon-NMR analysis.

Compound II was isolated from cell extracts of Xv-3. A marked antibacterial activity was initially observed in the ethyl acetate extract. And then, after fractionating with aliquot of chloroform and chloroform-MeOH (100 : 5), activity was eluted with chloroform-MeOH (10 : 1). Compound II

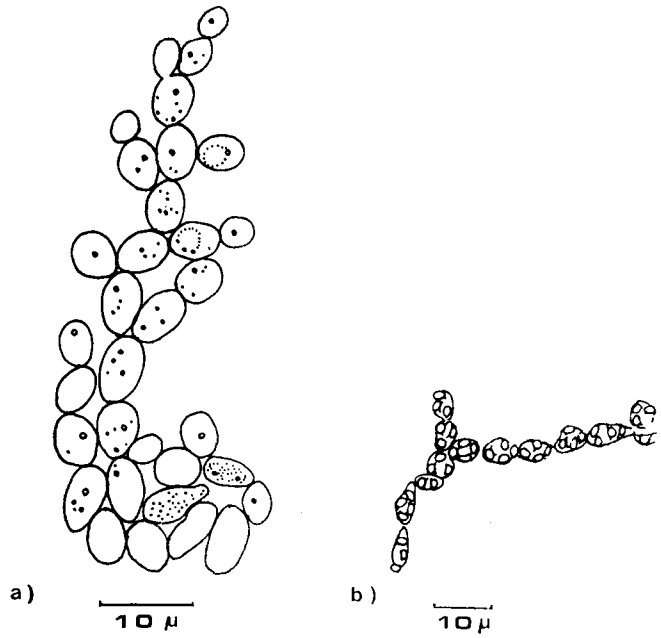
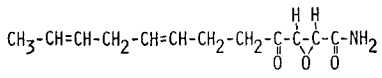


Fig. 4. Ss-3. a) Dalmau plate culture on corn-meal agar after 5 days at 25°C. b) YM agar slide culture after a week at 25°C.

(a) Cerulenin



(b) Helvolic acid

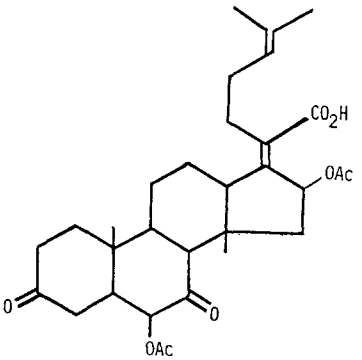


Fig. 5. Antibiotics produced by mold-like fungus, Xv-3 isolated from *X. validus*

was crystallized as white needles (mp 203–205°C) in MeOH. The yield was extracted 600 mg from 450 g of dried cells. It was also determined that molecular weight was 508 from GC-MS and 568 from proton- and carbon-NMR and molecular formula was $C_{33}H_{44}O_8$ by GC-MS and NMR analysis. The discrepancy of the molecular weight between GC-MS and NMR spectrum was apparently due to the removal of acetyl group from the compound during the operation of mass-spectrometer. Both compound I and II were identified with authentic cerulenin and helvolic acid which were generously provided by Prof. S. OMURA, Kitasato University (Figs. 5 a and 5 b).

These antibiotics were also isolated from culture broth and cell extracts of Cn-4, CnG-2, Xv-2, XvG-3, Xs-2, XsG-2, PsM-2, Ps-2 and PsG-3, although yields from them were far less than Xv-3.

Discussion

Ambrosia beetles are known to carry symbiotic microorganisms within their mycetangia located in their body or in their external skeleton to infect their galleries with viable spores.

BATRA[1,2] has proposed his opinion on primary and auxiliary ambrosia fungi, based on species specificity of the fungus to each beetle. He has also suggested that the primary ambrosia fungus of one beetle may sometimes play role of an auxiliary fungus for other species, and that the adult beetles contain their primary ambrosia fungi in their mycetangia predominantly even at the case of feeding on auxiliary or non-ambrosia fungi, in spite of that they seem to be non-specific.

In the present study, two kinds of microorganisms with different mor-

phological characteristics were isolated from the mycetangia of *P. severini.* One of them was identified as *Endomycopsis platypodis,* and the other was unidentified yet. These strains were commonly isolated from homogenates of whole bodies and galleries of four species of the beetles except *S. shogun.*

It is important fact that two kinds of strains have been carried in the mycetangia of *P. severini.* This fact is meaning that these two strains can mutually live in the same mycetangia. It is assumed that both of them are the true primary ambrosia fungi of *P. severini.*

In the three species of ambrosia beetles, *C. niponicus, X. validus* and *X. signatus,* the isolation of microorganisms from mycetangia has not succeeded still now; therefore, it is not clear whether the two strains mentioned above are the true primary symbionts of these beetles or not, although two strains were isolated from all of the three species. It may need further observations to discuss about primary and auxiliary symbionts, and also about species specificity of the fungi.

Two strains of microorganisms were isolated from the bodies and galleries of *S. shogun,* and these strains were different from those of the other four species of the beetles. One of the strains was identified as *Pichia* sp.

In the field condition, it is always found in the new galleries of the beetles that the symbiotic fungi associated with each beetle grow incredibly pure. In the present study, two kinds of antibiotics, cerulenin and helvolic acid, were isolated from a strain associated with *X. validus.* It is very interesting fact for the role of symbiotic microorganisms that contaminations due to another bacteria and fungus seem to be limited by these antibiotics. These antibiotics were also isolated from the unidentified strains mentioned above which associated with *P. severini, C. niponicus* and *X. signatus,* although yields of them were far less than the strain associated with *X. validus.*

It has already been reported that cerulenin and helvolic acid as microbial antibiotics are produced from the strains of *Cephalosporium caeruhen, Helicoceras oryzae, Sartorya* sp., *Aspergillus funigatus, Emericellopsis terricola* and *Acrocylindrium oryzae,* and both of the antibiotics have been isolated from culture broth of each strain under aerobic condition of 27–30°C. However, helvolic acid reported in this paper was not only obtained from culture broth, but also found in cell extracts. These results suggest that the microbial symbiont of ambrosia beetles reported here might be new strain that produces both cerulenin and helvolic acid.

Summary

Isolation of microorganisms was done on the five species of the ambrosia beetles, *Xyleborus validus* EICHHOFF, *Xyloterus signatus* FABRICIUS, *Scolytopratypus shogun* BLANDFORD, *Crossotarsus niponicus* BLANDFORD and *Platypus severini* BLANDFORD.

Two kinds of microorganisms were isolated from mycetangia of *P. severini* by micromanipulator. One of them was identified as yeast, *Endomycopsis platypodis*, and one more strain was morphologically investigated but unidentified yet. It is assumed that both of them are the true primary ambroia fungi of *P. severini*.

Among several microorganisms isolated from homogenates of whole bodies and galleries of four species of the beetles except *S. shogun*, two strains were common. These strains were the same with the strains isolated from the mycetangia of *P. severini*. A strain isolated from *S. shogun* was identified as *Pichia* sp.

It was found that a strain isolated from *X. validus* have produced two kinds of antibiotics, cerulenin and helvolic acid, which have shown highly antibacterial activity to *Bacillus subtilis* and *Staphylococcus aureus*. A little yield of cerulenin and helvolic acid were also found from the unidentified strain isolated from the four species of the beetles. In the new galleries of the beetles, it is always found that the symbiotic fungi grow incredibly pure. Contaminations due to another bacteria and fungus seem to be limited by these antibiotics.

Acknowledgement

Authentic cerulenin and helvolic acid were generously provided to us by Prof. S. OMURA of Kitasato University and we are grateful to his valuable discussions. We also grateful to all members of the Hiyama Forest Experiment Station of Hokkaido University for their aid in collection of pinhold beech logs. We also thank Miss Chie Goto for her technical assistance.

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