

Antibiotic-producing bacteria from stag beetle mycangia

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Summary

The search for new antibiotics or antifungal agents is crucial for the chemotherapies of infectious diseases. The limited resource of soil bacteria makes it difficult to discover such new drug candidate. We, therefore, focused on another bacterial resource than soil bacteria, the microbial flora of insect species. In the present study, we isolated 40 strains of bacteria and fungi from the mycangia of three species of stag beetle, *Dorcus hopei binodulosus*, *Dorcus rectus*, and *Dorcus titanus pilifer*. We identified those species with their ribosomal DNA sequences, and revealed that *Klebsiella* spp. are the most frequent symbiont in the stag beetle mycangia. We examined whether these microorganisms produce antibiotics against a Gram-negative bacterium, *Escherichia coli*, a Gram-positive bacterium, *Staphylococcus aureus*, or a fungus, *Cryptococcus neoformans*. Culture supernatants from 33, 29, or 18 strains showed antimicrobial activity against *E. coli*, *S. aureus*, or *C. neoformans*, respectively. These findings suggest that bacteria present in the mycangia of stag beetles are useful resources for screening novel antibiotics.

Keywords: Antimicrobial agents, Indigenous microorganisms, *Dorcus* species

1. Introduction

The discovery of antibiotics has greatly contributed to the treatment of infectious diseases. Due to the emergence of methicillin-resistant *Staphylococcus aureus* and multi-drug resistant *Pseudomonas*, however, further discovery of antibiotics with novel structures or novel molecular actions is crucial (1). Furthermore, even fewer antifungal agents are available than antibacterial agents and only four lineages of polyenes, azoles, candins, and flucytosines are in clinical use. The search for new antifungal agents is therefore also needed. Soil bacteria are a source for producing antibiotics, but the discovery rate of antibiotics from soil bacteria has decreased over the years (2). Therefore, we continue to search for new microbial sources of novel antibiotics.

Various species of microorganisms are present in

all living organisms. Insect species are various and comprise two-thirds of all animal species, but their indigenous microorganisms as a source of antibiotics have not been fully investigated. Thus, the indigenous microorganisms of insects are promising biologic resources. Raising various species of stag beetles is a popular hobby in Japan and techniques for their maintenance are well established (3), making stag-beetles a promising biological resource. Female stag beetles have a microbe-storage organ, named the mycangium, that contains xylose-fermenting yeasts closely related to *Pichia* spp. (4). Stag beetle larvae eat xylose-rich rotting wood, and the utilization of xylose by *Pichia* yeast is essential for the stag beetle larvae, because stag beetles cannot directly utilize xylose (4). The *Pichia* yeast in the mycangium are thought to be attached to the surface of the eggs, enabling vertical transmission from adult to larvae (4). In *Musca domestica*, bacteria on the surfaces of the fly eggs facilitate fly larvae growth by inhibiting the growth of pathogenic fungi (5). Based on these findings, we hypothesized that microorganisms other than *Pichia* yeast exist in the mycangia of stag beetles, where they produce antibiotics that inhibit the growth of pathogenic microorganisms. In the present study we have revealed the presence of bacteria in the mycangia of stag beetles, and that they produce antimicrobial substances.

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2. Materials and Methods

2.1. Animals

Captive bred adults of *Dorcus hopei binodulosus* were purchased from a breeder and bred. The larvae were raised using a mycelium bottle (TSUKIYONO KINOKOEN Ltd., Gunma, Japan, <http://www.tsukiyono.co.jp>) and the emerged females ($n = 7$) were used for the study. A wild female *Dorcus titanus pilifer* was collected from a dry riverbed at the Suzuka River in the Mie prefecture in Japan in 2009, and its offspring larvae were raised using mycelium bottles. Adult females ($n = 5$) in the second generation were used for the study. *Dorcus rectus* larvae were collected from decayed woods in a dry riverbed at the Suzuka River in the Mie prefecture in Japan in 2011. The larvae were raised to adults and the females ($n = 5$) were used for the study.

2.2. Isolation of microorganisms from mycangia

The stag beetle mycangia were excised according to the method described by Tanahashi *et al.* (4). The tissues were dissected in saline using sterile scissors and the samples were spread onto 1.5% agar plates of potato nutrient broth (PNB; 24 g potato dextrose broth, 8 g nutrient broth, 1 L water). The plates were incubated at 23°C for at least 1 d. The developing colonies were distinguished by their morphologies and then aerobically cultured in liquid PNB for 5 d at 23°C.

2.3. Extraction of genomic DNA

Microbial cells in 2 ml of liquid culture were collected by centrifugation at 204,00×g for 5 min. The cells were lysed by vigorous vortex in lysis buffer (5 mM Tris-HCl [pH8.0], 0.5 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaCl, 1% Triton-X 100, 0.5% sodium dodecyl sulfate, 100 mg/mL glass beads, 50% buffered phenol). The samples were centrifuged at 204,00×g for 5 min and each aqueous supernatant was mixed with 1 volume of 2-propanol and 0.1 volume of 3M sodium acetate. The samples were then centrifuged at 204,00×g for 10 min and the precipitates were washed with 70% EtOH. The precipitated DNA samples were dried and dissolved in Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

2.4. Identification of microbial species

DNA fragments containing 16S rDNA were amplified by PCR using oligonucleotide primers (Table 1) and genomic DNAs was used as the template according to a previous method (4). The PCR reaction mixture was incubated at 94°C for 2 min and then for 30 cycles (94°C, 15 s; 55°C, 30 s; 68°C 2 min). When the DNA

fragments were not amplified, another amplification for eukaryotic 18S rDNA was performed according to a previous method (6). Each amplified DNA fragment was sequenced and similar sequences were searched using NCBI BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The rDNA sequences have been deposited in GenBank (accession numbers; AB862496 to AB862537).

2.5. Measurement of antimicrobial activity

S. aureus RN4220, *E. coli* JM109, and *C. neoformans* H99 were cultured in Luria-Bertani broth, tryptic soy broth, and potato dextrose broth, respectively. The overnight cultures were diluted 5000 times with PNB and poured into microplates. Two-fold serial dilutions of the culture supernatants of the microorganisms isolated from the mycangia were filtered using 0.22-μm polyvinylidene difluoride membranes and poured into the microplates. The plates were incubated overnight at 37°C and the growth of *S. aureus*, *E. coli*, or *C. neoformans* was examined.

Table 1. Primers used in this study

Target	Name of primer	Sequence
16S rDNA	U1	CCAGCAGCCGCGTAATACG
	U2	ATCGGCTACCTTGTACGACTTC
	9F	GAGTTTGATCCTGGCTCAG
	1541R	AAGGAGGTGATCCAGCC
	U515F	GTGCCAGCMGCCGCGGTAA
	E1541R	AAGGAGGTGATCCANCCRCA
	U1510R	GGTTACCTTGTACGACTT
18S rDNA	ITS4	TCCTCCGCTTATTGATATGC
	ITS5	GGAAGTAAAAGTCGTAACAAGG
	LS1	AGTACCCGCTGAACCTAAG
	NL4	GGTCCGTGTTTCAAGACGG

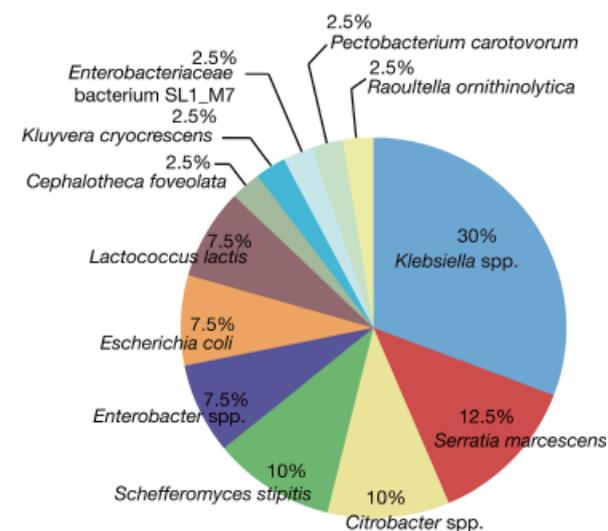


Figure 1. Microbial species isolated from stag beetle mycangia. Proportions of 40 microbial strains isolated from stag beetle mycangia are presented. Numbers in graph represent percentages against whole strains.

3. Results

3.1. Identification of microbial species from the mycangia of *Dorcus hopei binodulosus*, *Dorcus rectus*, and *Dorcus titanus pilifer*

Mycangia of female *Dorcus hopei binodulosus*, *Dorcus rectus*, and *Dorcus titanus pilifer* were excised and their suspensions were scattered in saline. The samples were spread on PNB agar plates and incubated at 23°C.

The appeared colonies were distinguished by their morphologies, cultured in liquid PNB, and then their genomic DNA was extracted. DNA fragments containing 16S rDNA or 18S rDNA were amplified by PCR and sequenced. The most frequent bacterial species was *Klebsiella* sp. and the second most frequent species was *Serratia marcescens* (Figure 1). Other species were in the genres of *Enterobacter*, *Escherichia*, *Lactococcus*, *Citrobacter*, *Kluyvera*, *Raoultella*, *Pectobacterium*, or *Scheffersomyces* (Table 2). *Scheffersomyces* is a

Table 2. Antimicrobial activities in the culture supernatants of microbes isolated from stag beetle mycangia

Strain ID	Source ¹	Microbial species	Similarity ² (%)	Growth inhibitory activity ³		
				<i>E. coli</i>	<i>S. aureus</i>	<i>C. neoformans</i>
DCBM1	<i>Dorcus hopei binodulosus</i>	<i>Klebsiella oxytoca</i>	100	-	-	2
DCBM2	<i>Dorcus hopei binodulosus</i>	<i>Serratia marcescens</i>	99	-	4	4
DCBM3	<i>Dorcus hopei binodulosus</i>	<i>Scheffersomyces stiptis</i>	99	-	-	-
DCBM4	<i>Dorcus hopei binodulosus</i>	<i>Citrobacter koseri</i>	100	> 8	> 8	-
DCBM5	<i>Dorcus hopei binodulosus</i>	<i>Scheffersomyces stiptis</i>	100	-	-	-
DCBM6	<i>Dorcus hopei binodulosus</i>	<i>Serratia marcescens</i>	100	-	2	4
DCBM7	<i>Dorcus hopei binodulosus</i>	<i>Enterobacteriaceae bacterium SL1_M7</i>	99	4	2	-
DCBM8	<i>Dorcus hopei binodulosus</i>	<i>Serratia marcescens</i>	100	8	2	-
DCBM9	<i>Dorcus hopei binodulosus</i>	<i>Enterobacter aerogenes</i>	99	2	2	-
DCBM10	<i>Dorcus hopei binodulosus</i>	<i>Cephalotheca foveolata</i>	99	2	2	-
DCBM11	<i>Dorcus hopei binodulosus</i>	<i>Klebsiella oxytoca</i>	99	8	4	-
DCBM12	<i>Dorcus hopei binodulosus</i>	<i>Klebsiella oxytoca</i>	100	16	2	-
DCBM13	<i>Dorcus hopei binodulosus</i>	<i>Klebsiella oxytoca</i>	99	8	4	-
DCBM14	<i>Dorcus hopei binodulosus</i>	<i>Klebsiella oxytoca</i>	99	8	4	-
DCBM15	<i>Dorcus hopei binodulosus</i>	<i>Enterobacter asburiae</i>	100	4	2	-
DCBM16	<i>Dorcus hopei binodulosus</i>	<i>Escherichia coli</i>	99	2	2	-
DRM1	<i>Dorcus rectus</i>	<i>Lactococcus lactis</i>	100	ND	4	-
DRM2	<i>Dorcus rectus</i>	<i>Citrobacter sp.</i>	99	2	4	-
DRM3	<i>Dorcus rectus</i>	<i>Pectobacterium carotovorum</i>	99	8	-	-
DRM4	<i>Dorcus rectus</i>	<i>Scheffersomyces stiptis</i>	99	8	2	-
DRM5	<i>Dorcus rectus</i>	<i>Lactococcus lactis</i>	100	2	-	-
DRM6	<i>Dorcus rectus</i>	<i>Citrobacter sp. A9IG</i>	99	4	2	-
DRM7	<i>Dorcus rectus</i>	<i>Klebsiella oxytoca</i>	99	32	-	2
DRM8	<i>Dorcus rectus</i>	<i>Klebsiella sp.</i>	99	8	-	4
DRM9	<i>Dorcus rectus</i>	<i>Klebsiella oxytoca</i>	100	8	-	4
DRM10	<i>Dorcus rectus</i>	<i>Escherichia coli</i>	99	4	2	-
DRM11	<i>Dorcus rectus</i>	<i>Enterobacter gergoviae</i>	98	16	2	4
DRM12	<i>Dorcus rectus</i>	<i>Escherichia coli</i>	99	4	2	2
DRM13	<i>Dorcus rectus</i>	<i>Lactococcus lactis</i>	99	ND	2	2
DTPM1	<i>Dorcus titanus pilifer</i>	<i>Klebsiella oxytoca</i>	100	8	-	4
DTPM2	<i>Dorcus titanus pilifer</i>	<i>Citrobacter farmeri</i>	99	16	2	4
DTPM3	<i>Dorcus titanus pilifer</i>	<i>Serratia marcescens</i>	99	16	2	4
DTPM4	<i>Dorcus titanus pilifer</i>	<i>Kluyvera cryocrescens</i>	100	4	4	64
DTPM5	<i>Dorcus titanus pilifer</i>	<i>Klebsiella oxytoca</i>	100	4	2	4
DTPM6	<i>Dorcus titanus pilifer</i>	<i>Klebsiella oxytoca</i>	100	8	-	4
DTPM7	<i>Dorcus titanus pilifer</i>	<i>Klebsiella oxytoca</i>	99	8	4	-
DTPM8	<i>Dorcus titanus pilifer</i>	<i>Serratia marcescens</i>	99	8	2	4
DTPM9	<i>Dorcus titanus pilifer</i>	<i>Serratia marcescens</i>	99	16	2	4
DTPM10	<i>Dorcus titanus pilifer</i>	<i>Raoultella ornithinolytica</i>	99	2	4	2
DTPM11	<i>Dorcus titanus pilifer</i>	<i>Scheffersomyces stiptis</i>	99	2	-	-

¹"Source" indicates the stag beetle species used for isolating microorganisms. ²"%" indicates the percent similarity of the isolated microorganisms to the microbial species listed in "Microbial species". ³Antimicrobial activities against *S. aureus*, *E. coli*, and *C. neoformans* were measured. Numbers in the right three columns are reciprocals of dilutions of the culture supernatants that inhibited microbial growth. "-" means not detected. ND means not determined.

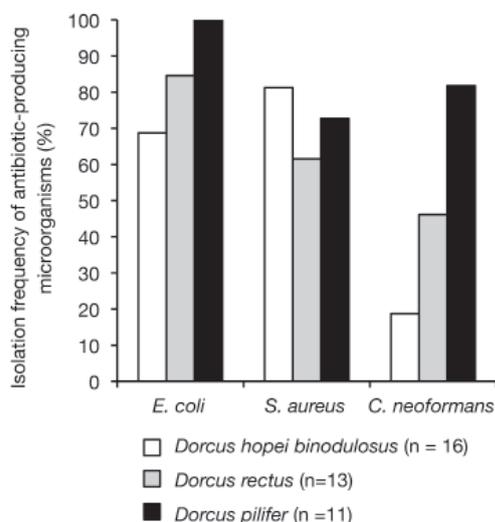


Figure 2. Isolation frequency of antibiotic-producing microorganisms from three different stag beetles. Isolation frequencies of microbial strains with growth inhibitory activity against *E. coli*, *S. aureus*, and *C. neoformans* are shown according to the stag beetle species.

Pichia yeast that is reported to be present in stag beetle mycangia (4).

3.2. Antimicrobial activities in culture supernatants of the mycangial microbes

We measured growth inhibitory activities of culture supernatants of microbes isolated from the stag beetle mycangia against *E. coli*, *S. aureus*, and *C. neoformans*. Of 40 strains isolated from mycangia, 33, 29, or 18 strains showed growth inhibitory activities against *E. coli*, *S. aureus*, or *C. neoformans*, respectively (Table 2). Microbes that inhibit the growth of *E. coli* or *S. aureus* were isolated at almost the same frequency from the three stag beetle species (Figure 2). Many microbes that inhibit the growth of *C. neoformans* were isolated from *Dorcus titanus pilifer*, whereas few were isolated from *Dorcus hopei binodulosus* (Figure 2).

4. Discussion

The present study demonstrated the existence of microbes in stag beetle mycangia that produce antibiotics against *E. coli*, *S. aureus*, and *C. neoformans*. This finding clearly indicates that the microbial flora of stag beetle mycangia comprise a promising source of novel antimicrobial agents.

Antimicrobial activities against *E. coli* or *S. aureus* were observed at almost the same frequency between indigenous microorganisms from *Dorcus hopei binodulosus*, *Dorcus rectus*, and *Dorcus titanus pilifer*. Antifungal activities against *C. neoformans* were frequently observed in microorganisms obtained from *Dorcus titanus pilifer*, but rarely in those from *Dorcus hopei binodulosus*. Larvae of *Dorcus hopei binodulosus*

preferred woods decayed by white-rot fungus, which are often found in high positions of standing dead woods with low moisture (7). Larvae of *Dorcus titanus pilifer*, on the other hand, preferred woods decayed by brown-rot fungus, which are often located in underground positions of standing dead woods or in fallen trees with high moisture (7). Larvae of *Dorcus rectus* preferred both types of decayed woods (7). The humid environment inhabited by larvae of *Dorcus titanus pilifer* is assumed to contain more pathogenic fungi than a drier environment. Therefore, larvae of *Dorcus pilifer* may protect themselves against infection by antifungal agents produced by the indigenous microorganisms.

Antifungal activities were frequently observed in the culture supernatants of *Klebsiella oxytoca* and *Serratia marcescens* isolated from stag beetle mycangia (Table 2). *Klebsiella oxytoca* was present on housefly eggs and inhibited fungal growth to promote fly larvae growth (5). Thus, the role of *Klebsiella oxytoca*, inhibition of fungal growth may be conserved in insects, including houseflies and stag beetles. In addition, *Serratia marcescens* was pathogenic to various species of insects (8,9,10,11). The virulence properties of *Serratia marcescens* against insects differed from strain to strain, and some strains were non-pathogenic (9). Such non-pathogenic *Serratia marcescens* may have a role to protect the host animal from fungal infection by producing anti-fungal agents. The biological significance of the antibiotic-producing indigenous microorganisms in the stag beetle requires further investigation.

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