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研究課題名（和文）Ecological function of wood insect associated microbes

研究課題名（英文）Ecological function of wood insect associated microbes

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研究成果の概要（和文）：本研究では、 欧米で甚大な森林被害をもたらしている事が報告されている森林害虫キバチ（Sirex noctilio）の近縁種であり日本に生育するアカアシクビナガキバチを捕獲し、共生する菌類・バクテリアを含めた微生物相による多糖・リグニン分解機構の解明と、そのバイオマス変換技術への応用を目指した研究を行った。捕獲したアカアシクビナガキバチ共生微生物の単離および生化学的解析を行ったところ、子嚢菌門に属するDaldinia属の単離、および本菌による高い木質成分分解酵素の分泌が確認できた。培養上清プロテオミクス解析から本菌の分泌する木質成分分解酵素を網羅的に同定した。

研究成果の概要（英文）：In this study, we attempted to identify the woodwasp symbiont microbes from Hokkaido forest. We captured two wood wasp species, Sirex nitobei and Xiphydria cannelus. One bacteria and one fungus were isolated from a mygcangia of X. canelus, and were identified as Rahnella spp., and Daldinia decipiens species by 16S rRNA and ITS sequencing, respectively. Furthermore, we determined the hemicellulose-degrading activity in the supernatant when D. decipiens was cultured in the liquid medium containing polysaccharides such as cellulose, hemicellulose, wood biomass as sole carbon source, and glucose was used as a control culture. By utilizing proteomics, we were able to determined multiple glycoside hydrolase family enzymes, which are predicted to break down various polysaccharides present in woody biomass.

研究分野：　生化学・分生生物学

キーワード：　木質成分分解性微生物　プロテオミクス

１．研究開始当初の背景

The need for development and improvement of sustainable biofuels technology has been rapidly increased due to the current large demands on liquid fuels and diminishing finite fossil resources in nature. In the bioethanol production, plant biomass requires an extensive chemical pretreatment followed by enzyme hydrolysis to produce fermentable sugars. However, these chemical and enzymatic processes are not economically feasible because of the highly complex cell wall structure formed by recalcitrant cellulose, heterogeneous hemicellulose, and other phenolic compounds. Especially the enzymatic hydrolysis is a technically challenging and expensive process, and the complete enzyme set to effectively deconstruct plant biomass is not available. Although the improvement of several model cellulolytic microorganisms has been well studied, it is beginning to be apparent that the enzyme cocktail prepared from one microorganism is not adequate for complete biomass deconstruction. While in natural cellulosic environment, microbial communities might efficiently utilize plant biomass. Thus, it is essential to understand how natural microbial communities cleverly convert cellulosic plant biomass into simple sugars.

A wood-boring wasp, siricidae insect, is one of the major threats of forest devastation in the north America, Canada, Japan and elsewhere, and various cellulolytic microbes were partially determined by the 16s rRNA sequencing from their mycangia, an internal base organ of female. The newly isolated bacterium from the siricidae *Sirex noctilio*, *Streptomyces* sp. SirexAA-E was identified to possess high cellulose and hemicellulose-degrading potential [Takasuka et al., *Sci Rep*, 2013]. This study and others suggest that there is a large pool of underrepresenting cellulolytic microbes available in natural cellulosic environments and possibly we can discover novel cellulolytic microbial species and their enzymes. In Hokkaido, siricidae insects and others are reported to cause severe forest devastation (Price and Ohgushi, *Res. Popul. Ecol.*, 1995). Like other siricidae insects, I hypothesize that the siricidae insects from Hokkaido forest associate with a vigorous cellulolytic microbial community in their mycangia.

２．研究の目的

My research focus on the discovery of cellulolytic symbiont microbes in the wood devastating wood wasp in Hokkaido forest. A repertoire of plant biomass degrading enzymes, which is utilized by microbe symbiont are of great interest, and proteomic and biochemical analyses will be carried out to determine those enzymes. Additionally, we sought to understand the microbe-insect community present in those invasive insects.

３．研究の方法

Materials.

Glucose, cellobiose, cellulose, and CMC were purchased (Sigma-aldrich, USA). Pectin, oat xylan, lichenan, beech xylan xyloglucan, arabinan and mannan were purchased (Megazyme, Ireland). Poplar and larch were courtesy gift from Dr. Chiaki, Hori, Research faculty of Engineering, Hokkaido University, Sapporo, Hokkaido.

Isolation of symbiont microbes from wood wasps.

Invasive insect species such as siricidae insects were collected from Hokkaido forest in collaboration with Dr. Hideho Hara, Hokkaido Research Organization, Forest Research Department, Forestry Research Institute, Bibai city, Hokkaido. We collected several wood wasps at Bibai city at Hokkaido such as *Xiphydria camelus* and *Sirex nitobei*. Organs of the *X camelus* woodwasp were dissected, and a mycangia was used to isolate microbes. A mycangia was washed in PBS and grounded.

Several different culture medium were used to isolate microbes, for example, potato dextran agar for fungal isolates and m63 minimum medium for bacteria. After a week of cultivation at room temperature, single colonies were inoculated in liquid medium.

Isolation of genomic DNA and determination of species

Isolates were grown in liquid medium, and genomic DNAs were extracted by using the genomic DNA extraction kit (Qiagen, USA). In order to determine species, we performed 16s rRNA sequencing for bacteria and ITS sequencing for fungi isolates. Resultant sequences were blast searched (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

Screening of isolates toward plant biomass deconstruction.

Isolates were grown in the defined medium containing each carbon source including glucose, cellobiose, cellulose, oat xylan, mannan, pectin, poplar powder and larch powder (5% w/w). The growth was monitored at OD_{600}.

Preparation of the culture supernatant.
Culture supernatant was collected after 5 to 10 days cultivation of above medium containing different carbon sources. Culture was centrifuged for 10 min at 1,500 x g, and the supernatant was filtered (3,000 cut-off membrane, EMD Millipore, USA). Protein concentration was measured by the Bradford assay and sample was visualized by the SDS-PAGE.

Measurement of specific activities of the secretome for different polysaccharides.
The culture supernatants were tested for following substrates, cellulose, CMC, lichenan, oat xylan, beech xylan, xyloglucan, mannan, pectin, and arabinan. Reaction was carried out for 20 hours at 30 ˚C, then halted by heat inactivation. The activity of the culture supernatants were measured by using the 3,5-dinitrosalicylic acid (DNS) assay.

Secretome analysis.
To determine secreted proteins in the culture supernatant, samples were denatured, reduced and trypsin digested followed by the ziptip purification (Ziptip, EMD Millipore, USA). The Orbitrap LC-MS/MS (Q-executive plus, Thermo Scientific, IL USA) was used for proteomic analysis. *D. eschscholzii* genome sequence (http://genomeportal.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=fungi) was utilized in order to assign peptides.

4．研究成果

Female *Xiphydria camelus* was captured at Bibai city, Hokkaido, and one bacteria and one fungus were isolated (**Fig. 1**). Bacteria was determined to be *Rahnella sp.* based on 16s rRNA. By ITS sequence, the isolated fungus was identified as *Daldinia decipiens*. Neither of identified species was previously reported as insect-symbiont.

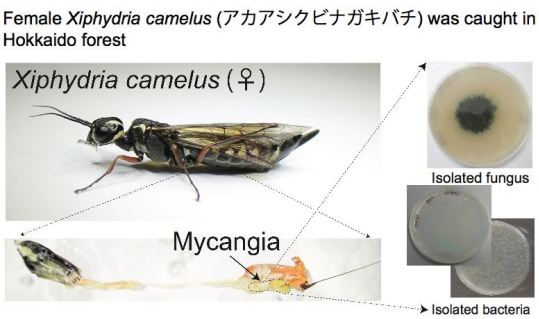

Fig. 1. Female *Xiphydria camelus* was caught in Hokkaido forest at Bibai city Hokkaido, and was dissected to extract the organ called mycangia (dashed circle). One fungus and one bacteria wereisolated on the potato dextran agar

We grew them in the presence of different carbon source such as glucose, cellobiose, xylan, mannan, pectin, poplar powder and larch wood powder in order to test whether those microbes could utilize polysaccharides and plant biomass in culture medium. *D. decipiens* was shown to grew well with various polysaccharides, and thus we decided to focus on analyzing the cellulolytic capability of *D. decipiens*. The supernatants of different carbon source medium after 3 days cultivation at room temperature were collected. Total secreted proteins and protein composition were analyzed by the Bradford and SDS-PAGE analyses, respectively. When cellulose and other polysaccharides (xylan, mannan, pectin, poplar, and larch wood) were used as a carbon source in the medium, secreted proteins were estimated to be more than 5-fold, compared to the medium containing glucose. Additionally, the protein composition varied depending on the carbon sources used in the medium by the SDS-PAGE analysis (**Fig. 2**). To assess plant biomass-degrading activity of each culture supernatant (secretome), the specific activity (U/mg) was measured for cellulose analogues (cellulose, CMC, and lichenan), xylan analogues (oat xylan, beech xylan and xyloglucan), and other pure polysaccharides (D-mannan, pectin and arabinan). Results showed that *D. dicipiens* secretes enzymes with different activity depending on the carbon sources present in the culture medium, and the secretome from poplar or larch showed the most broad activities (manuscript in preparation).

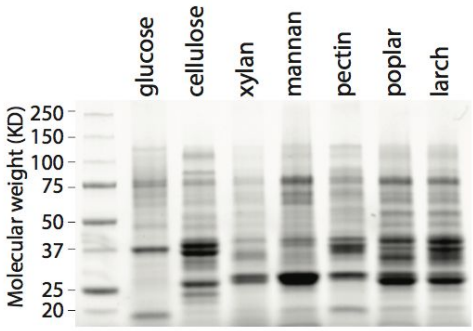

Fig. 2. Secreted enzymes from the culture supernatant of *D. dicipiens* were prepared, which derived from the culture containing indicated carbon source, and analyzed by PAGE. Molecular weights (KD) were indicated.

The secretome analyses were performed for the seven secretomes that were prepared from glucose, cellulose, xylan, mannan, pectin, poplar or larch wood culture. We determined

various putative glycoside hydrolase family enzymes in each secretome, and they were available carbon source dependent. In addition, we determined highly secreted proteins, which functions are currently unknown (manuscript in preparation). It is possible that those proteins play key roles in plant biomass deconstruction, and their protein functions and structures will be studied in the future research.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　1　件）
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〔学会発表〕（計　3　件）
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