

MMRC



**ANNUAL REPORT OF MEDICAL MYCOLOGY
RESEARCH CENTER, CHIBA UNIVERSITY 2013**

千葉大学 真菌医学研究センター 報告

17



目 次

Content

はじめに Preface	
病原機能分野 川本 PI (分子細胞シグナリング解析) プロジェクト Project for Molecular Signaling Analysis	3
病原機能分野 知花 PI (カンジダ・グラブラータ フェノーム) プロジェクト <i>Candida glabrata</i> Phenome Project	7
感染免疫分野 米山 PI (感染応答) プロジェクト Project for Immune Response in Infection Diseases	10
感染免疫分野 西城 PI (サイトカイン) プロジェクト Project for Cytokine Research	13
臨床感染症分野 亀井 PI (臨床感染症) プロジェクト Project to Link Basic Sciences and Clinical Medicine	15
微生物資源分野 五ノ井 PI (真菌・放線菌と宿主の分子相互作用研究) プロジェクト Project for Host Pathogen (fungi/actinomycetes) Molecular Interaction	22
微生物資源分野 高橋 PI (微生物創生) プロジェクト Project for Systems Biology of Microorganisms	33
微生物資源分野 バイオリソース管理室 Management Unit of Microbiological Resources	35
文部科学省 ナショナルバイオリソースプロジェクト「病原微生物」 Ministry of Education, Culture, Sports, Science and Technology National BioResource Project "Pathogenic Microorganisms"	38
地球規模課題対応国際科学技術協力事業 (JST & JICA) SATREPS Project (Science and Technology Research Partnership for Sustainable Development)	39
長崎大学熱帯医学研究拠点特定領域共同研究 Cooperative Research of Priority Areas with NEKKEN, Nagasaki University	40
アスペルギルス症を中心とした新興真菌症制圧プロジェクト The Project on Controlling Aspergillosis and the Related Emerging Mycoses	41
平成 24 年度 共同利用・共同研究報告 2012 Fiscal Year Cooperative Research Program Report	42
平成 24 年度 共同利用・共同研究研究会報告 2012 Fiscal Year Cooperative Research Meetings Report	48
2013 年講演会 2013 Scientific Meetings & Seminars	49

はじめに

千葉大学真菌医学研究センターは、真菌感染症の基盤・臨床研究、共同利用・共同研究拠点事業、真菌バイオリソース事業を活動の3本柱とする、我が国で唯一の真菌感染症の研究・教育に特化した公的な研究センターです。

本センターでは、限られた人員で最大限の成果を出すべく、平成21年に着任された野本明男前センター長のもとで、組織を含め様々な改革に取り組んできました。具体的には、センター全体を1部門4分野とし、新たに研究プロジェクト制を導入しました。また、各プロジェクトを担う7名のプロジェクトリーダーPI (principal investigator) を任命し、研究レベルの向上と新たな研究分野の導入を図りました。さらに平成22年には、文科省より共同利用・共同研究拠点としての認定を受け、「真菌感染症研究拠点」のプラットフォームとして全国の真菌研究者との連携を強化しました。したがって、本センターでは、PI個人のプロジェクト研究とともに、共同利用・共同研究拠点事業を通じて、全国の病原真菌研究者と連携した多様な共同研究を活発に行っています。さらに本センターでは、海外との国際共同研究にも積極的に参加して大きな成果をあげています。

昨年12月には、PIプロジェクト及び真菌バイオリソース事業報告会を行い、真菌感染と自然免疫応答を含め幾つかの興味ある研究成果が報告されました。一方、共同利用・共同研究拠点では、成果が大いに期待される重点課題として8課題を採択しました。今回、その一部について、3月に東京大学医科学研究所と合同で成果発表会を行います。26年度は、真菌感染症を含めた難治性感染症の研究とそれらの臨床への応用を目指して、臨床および異分野との連携をさらに積極的に進める予定です。

さて、昨年7月本センターは、文科省から共同利用・共同研究拠点としての中間評価を受けました。本センターでは平成21年以来多くの改革に取り組みましたが、中間評価では大変厳しい評価を頂きました。具体的には、研究実績、大学院教育への貢献、共同研究事業のいずれもが不十分であり、センターの研究機能強化をさらに押し進めることが要望されました。本センターではこの評価結果を真摯に受け止め、これら課題克服へ向けたさらなる取り組みを行う所存です。

10月24日、25日には、本センターが当番校として、国立大学附置研究所・センター長会議第2部会（医学・生物学系）のメンバーが、千葉市内に一同に集まり、合同会議を開催しました。会議二日目には、「超高齢社会に忍び寄るカビの脅威」と題して、公開シンポジウムを行いました。本シンポジウムに先立ち、新聞でもシンポジウムについて紹介され、当日は一般市民を含む多数の参加者があり、活発な討論が行われました。また11月30日には、本センターと山本友子薬学部教授が共催して、「感染症グローバルネットワークフォーラム2013」を薬学部120周年記念講堂で開催しました。本フォーラムでは、国内外、千葉大学医学研究院、薬学研究院、附属病院から研究者を招き、感染症の基礎、臨床、創薬に及ぶ最新の知見を紹介していただきました。さらに10月10日には、本センターの研究が、日本経済新聞「大学、知の明日を築く」という連載特集記事で詳しく紹介されました。昨年は、これら一連の出来事を通じて、本センターに対する、社会、大学、研究コミュニティの期待が極めて大きいことを改めて認識させられました。

超高齢社会に突入した我が国では、真菌をはじめとする難治性感染症の基盤研究を推進し、それら研究成果を臨床や創薬開発へ繋げてゆくことが喫緊の課題となっています。一方、世界規模では、高度病原体による感染症の脅威に加えて、多剤耐性病原体の拡散、あるいは高度医療の普及に伴う難治性感染症の増加が今後大きな問題となります。本センターでは、これらの現状を踏まえて、世界に貢献できる研究センターを目指したいと願っております。

平成26年1月

千葉大学真菌医学研究センター長

笹川千尋

Preface

It is with great pleasure that I present our Annual Report 2013 for the Medical Mycology Research Center (MMRC) at Chiba University. MMRC was originally founded in 1946 as The Institute for Food-Microbiology Chiba Medical College. The Institute was renovated and renamed the Research Institute for Chemobiodynamics in 1973. Four years later, it was again renamed as MMRC to reflect a shift in research focus to pathogenic fungi and associated infectious diseases. In 2001, a division of fungal resources and development was created, which was shortly followed by the certification of MMRC to serve as a National University Cooperation in 2004. In order to further strengthen the research activities at MMRC, it was reorganized in 2010 to a single department consisting of four research divisions – molecular biology of pathogenic fungi, molecular immunology, clinical research, and bioresources. MMRC was certified as a Joint/Research Center this year because of its central role in leading basic science and clinical mycology research in Japan.

The primary focus of MMRC is to achieve and maintain the highest level of research by employing multidisciplinary approaches to infection biology including concepts and methodologies of molecular genetics, bioinformatics, immunology, cell biology, protein chemistry, and clinical research. Currently, MMRC is going to great lengths to promote translational research that is geared towards the prevention, diagnosis, and control of fungal infectious diseases through joint research projects with the School of Medicine, Hospital, and Faculty of Pharmaceutical Sciences at Chiba University.

This annual report summarizes our scientific achievements during 2013. My hope is that this report can be used to promote domestic as well as worldwide collaborations with our scientists, which should ultimately result in substantial contributions to medical fungal research and medicine.

January 22, 2014

Chihiro Sasakawa

Director of MMRC

川本 PI (分子細胞シグナリング解析) プロジェクト

Project for Molecular Signaling Analysis

研究概要 (Summary)

生化学・分子生物学・細胞生物学等の手法を用い、病原酵母・糸状菌の分子細胞研究を行い、病原機能などに関連するシグナリング解析を進め、抗真菌薬シーズ創出など真菌症の分子制御に向けた分子細胞医真菌学への貢献を目指す。

We are conducting basic research on the molecular and cellular biology of pathogenic fungi using biochemistry and molecular biology methods based on gene and protein science as well as ultrastructural morphology and cell biology methods such as electron microscopy.

教 授	川 本 進	Professor	Susumu Kawamoto
准 教 授	山 口 正 視	Associate Professor	Masashi Yamaguchi
准 教 授	横 山 耕 治	Associate Professor	Koji Yokoyama
助 教	清 水 公 徳	Assistant Professor	Kiminori Shimizu
技 術 職 員	大 楠 美 佐 子	Research Technician	Misako Ohkusu
客 員 教 授	東 江 昭 夫	Guest Professor	Akio Toh-e
特 任 助 教	萩 原 大 祐	Research Assistant Professor	Daisuke Hagiwara
技 術 補 佐 員	中 野 百 実 子	Research Promotion Technician	Yumiko Nakano

1. The *CRZI/SPI*-like gene links survival under limited aeration, cell integrity and biofilm formation in the pathogenic yeast *Cryptococcus neoformans*.

Zuzana Moranova^{1,2}, Eric Virtudazob, Kristyna Hricova¹, Misako Ohkusu², Susumu Kawamoto², Vendula Husickova¹, Vladislav Raclavsky¹

¹Department of Microbiology, Faculty of Medicine and Dentistry, Palacky University Olomouc, Czech Republic

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Aims. Limited aeration has been demonstrated to cause slowdown in proliferation and delayed budding, resulting eventually in a unique unbudded G2-arrest in the obligate aerobic pathogenic yeast *Cryptococcus neoformans*. Also, the ability to adapt to decreased oxygen levels during pathogenesis has been identified as a virulence factor in *C. neoformans*. The

aim of this study was to identify and characterize genes that are necessary for the proliferation slowdown and G2-arrest caused by limited aeration.

Methods. Random mutants were prepared and screened for lack of typical slowdown of proliferation under limited aeration. The CNAG_00156.2 gene coding for a zinc-finger transcription factor was identified in mutants showing most distinctive phenotype. Targeted deletion strain and reconstituted strain were prepared to characterize and confirm the gene functions. This gene was also identified in a parallel studies as homologous both to calcineurin responsive (*Crz1*) and PKC1-dependent (*SPI*-like) transcription factors.

Results. We have confirmed the role of the cryptococcal homologue of *CRZI/SPI*-like transcription factor in cell integrity, and newly demonstrated its role in slowdown of proliferation and survival under reduced aeration, in biofilm formation and in susceptibility to fluconazole.

Conclusions. Our data demonstrate a tight molecular link between slowdown of proliferation during hypoxic adaptation

and maintenance of cell integrity in *C. neoformans* and present a new role for the *CRZI* family of transcription factors in fungi. The exact positioning of this protein in cryptococcal signalling cascades remains to be clarified.

2. A new F-actin structure in fungi: actin ring formation around the cell nucleus of *Cryptococcus neoformans*.

Marie Kopecká¹, Susumu Kawamoto², Masashi Yamaguchi²

¹Department of Biology, Faculty of Medicine, Masaryk University, Brno 62500, Czech Republic and ²Medical Mycology Research Centre, Chiba University, Chiba, Japan

The F-actin cytoskeleton of *Cryptococcus neoformans* is known to comprise actin cables, cortical patches and cytokinetic ring. Here, we describe a new F-actin structure in fungi, a perinuclear F-actin collar ring around the cell nucleus, by fluorescent microscopic imaging of rhodamine phalloidin-stained F-actin. Perinuclear F-actin rings form in *Cryptococcus neoformans* treated with the microtubule inhibitor Nocodazole or with the drug solvent dimethyl sulfoxide (DMSO) or grown in yeast extract peptone dextrose (YEPD) medium, but they are absent in cells treated with Latrunculin A. Perinuclear F-actin rings may function as 'funicular cabin' for the cell nucleus, and actin cables as intracellular 'funicular' suspending nucleus in the central position in the cell and moving nucleus along the polarity axis along actin cables.

3. Ordered Kinetochores Assembly in the Human-Pathogenic Basidiomycetous Yeast *Cryptococcus neoformans*.

Lukasz Kozubowski^{1,2}, Vikas Yadav³, Gautam Chatterjee³, Shreyas Sridhar³, Masashi Yamaguchi⁴, Susumu Kawamoto⁴, Indrani Bose⁵, Joseph Heitman², Kaustuv Sanyal³

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Kinetochores facilitate interaction between chromosomes and the spindle apparatus. The formation of a metazoan trilayered kinetochore is an ordered event in which inner, middle, and outer layers assemble during disassembly of the nuclear envelope during mitosis. The existence of a similar strong correlation between kinetochore assembly and nuclear envelope breakdown in unicellular eukaryotes is unclear. Studies in the hemiascomycetous budding yeasts *Saccharomyces cerevisiae* and *Candida albicans* suggest that an ordered kinetochore assembly may not be evolutionarily conserved. Here, we utilized high-resolution time-lapse microscopy to analyze the localization patterns of a series of putative kinetochore proteins in the basidiomycetous budding yeast *Cryptococcus neoformans*, a human pathogen. Strikingly, similar to most metazoa but atypical of yeasts, the centromeres are not clustered but positioned adjacent to the nuclear envelope in premitotic *C. neoformans* cells. The centromeres gradually coalesce to a single cluster as cells progress toward mitosis. The mitotic clustering of centromeres seems to be dependent on the integrity of the mitotic spindle. To study the dynamics of the nuclear envelope, we followed the localization of two marker proteins, Ndcl and Nup107. Fluorescence microscopy of the nuclear envelope and components of the kinetochore, along with

ultrastructure analysis by transmission electron microscopy, reveal that in *C. neoformans*, the kinetochore assembles in an ordered manner prior to mitosis in concert with a partial opening of the nuclear envelope. Taken together, the results of this study demonstrate that kinetochore dynamics in *C. neoformans* is reminiscent of that of metazoans and shed new light on the evolution of mitosis in eukaryotes.

IMPORTANCE Successful propagation of genetic material in progeny is essential for the survival of any organism. A proper kinetochore-microtubule interaction is crucial for high-fidelity chromosome segregation. An error in this process can lead to loss or gain of chromosomes, a common feature of most solid cancers. Several proteins assemble on centromere DNA to form a kinetochore. However, significant differences in the process of kinetochore assembly exist between unicellular yeasts and multicellular metazoans. Here, we examined the key events that lead to formation of a proper kinetochore in a basidiomycetous budding yeast, *Cryptococcus neoformans*. We found that, during the progression of the cell cycle, nonclustered centromeres gradually clustered and kinetochores assembled in an ordered manner concomitant with partial opening of the nuclear envelope in this organism. These events have higher similarity to mitotic events of metazoans than to those previously described in other yeasts.

4. Nika/TcsC histidine kinase is involved in conidiation, hyphal morphology, and responses to osmotic stress and antifungal chemicals in *Aspergillus fumigatus*.

Daisuke Hagiwara¹, Azusa Takahashi-Nakaguchi¹, Takahito Toyotome¹, Akira Yoshimi², Keietsu Abe², Katsuhiko Kamei¹, Tohru Gono¹, Susumu Kawamoto¹

¹Medical Mycology Research Center, Chiba University, Chiba, Japan, ²New Industry Creation Hatchery Center, Tohoku University, Sendai, Japan

The fungal high osmolarity glycerol (HOG) pathway is composed of a two-component system (TCS) and Hog1-type mitogen-activated protein kinase (MAPK) cascade. A group III (Nik1-type) histidine kinase plays a major role in the HOG pathway of several filamentous fungi. In this

study, we characterized a group III histidine kinase, Nika/TcsC, in the lifethreatening pathogenic fungus, *Aspergillus fumigatus*. A deletion mutant of *nikA* showed low conidia production, abnormal hyphae, marked sensitivity to high osmolarity stresses, and resistance to cell wall perturbing reagents such as congo red and calcofluor white, as well as to fungicides such as fludioxonil, iprodione, and pyrrolnitrin. None of these phenotypes were observed in mutants of the SskA response regulator and SakA MAPK, which were thought to be downstream components of Nika. In contrast, in response to fludioxonil treatment, Nika was implicated in the phosphorylation of SakA MAPK and the transcriptional upregulation of *catA*, *dprA*, and *dprB*, which are regulated under the control of SakA. We then tested the idea that not only Nika, but also the other 13 histidine kinases play certain roles in the regulation of the HOG pathway. Interestingly, the expression of *fos1*, *phkA*, *phkB*, *fhk5*, and *fhk6* increased by osmotic shock or fludioxonil treatment in a SakA-dependent manner. However, deletion mutants of the histidine kinases showed no significant defects in growth under the tested conditions. Collectively, although the signal transduction network related to Nika seems complicated, Nika plays a crucial role in several aspects of *A. fumigatus* physiology and, to a certain extent, modulates the HOG pathway.

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知花PI (カンジダ・グラブラータ フェノーム) プロジェクト

Candida glabrata Phenome Project

研究概要 (Summary)

病原性酵母カンジダ・グラブラータの全遺伝子改変株を作製し、病原性に関する遺伝子の特定と機能解析ならびに抗真菌薬の標的探索を行う。

Using the pathogenic yeast *Candida glabrata*, we are systematically constructing mutants for gene identification and functional analyses working on the pathogenicity and for screening of anti-fungal drug targets.

准 教 授	知花 博治	Associate Professor	Hiroji Chibana
技 術 補 佐 員	大岩 真理	Research Promotion Technician	Mari Ohiwa
技 術 補 佐 員	相田 優子	Research Promotion Technician	Yuko Aida

1. *Candida glabrata* drug: H⁺ antiporter CgQdr2 confers imidazole drug resistance, being activated by transcription factor CgPdr1.

Catarina Costa^{1,2}, Carla Pires^{1,2}, Tânia R. Cabrito^{1,2}, Adeline Renaudin^{1,2}, Michiyo Ohno³, Hiroji Chibana³, Isabel Sá-Correia^{1,2} and Miguel C. Teixeira^{1,2}

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The widespread emergence of antifungal drug resistance poses a severe clinical problem. Though predicted to play a role in this phenomenon, the drug: H⁽⁺⁾ antiporters (DHA) of the major facilitator superfamily have largely escaped characterization in pathogenic yeasts. This work describes the first DHA from the pathogenic yeast *Candida glabrata* reported to be involved in antifungal drug resistance, the *C. glabrata* QDR2 (CgQDR2) gene (ORF CAGL0G08624g). The expression of CgQDR2 in *C. glabrata* was found

to confer resistance to the antifungal drugs miconazole, tioconazole, clotrimazole, and ketoconazole. By use of a green fluorescent protein (GFP) fusion, the CgQdr2 protein was found to be targeted to the plasma membrane in *C. glabrata*. In agreement with these observations, CgQDR2 expression was found to decrease the intracellular accumulation of radiolabeled clotrimazole in *C. glabrata* and to play a role in the extrusion of this antifungal from preloaded cells. Interestingly, the functional heterologous expression of CgQDR2 in the model yeast *Saccharomyces cerevisiae* further confirmed the role of this gene as a multidrug resistance determinant: its expression was able to complement the susceptibility phenotype exhibited by its *S. cerevisiae* homologue, QDR2, in the presence of imidazoles and of the antimalarial and antiarrhythmic drug quinidine. In contrast to the findings reported for Qdr2, CgQdr2 expression does not contribute to the ability of yeast to grow under K⁽⁺⁾-limiting conditions. Interestingly, CgQDR2 transcript levels were seen to be upregulated in *C. glabrata* cells challenged with clotrimazole or quinidine. This upregulation was found to depend directly on the transcription factor CgPdr1, the major regulator of multidrug resistance in this pathogenic yeast, which has also been found to be a determinant of quinidine and clotrimazole resistance in *C. glabrata*.

2. The dual role of *Candida glabrata* drug: H⁺ antiporter CgAqr1 (ORF CAGL0J09944g) in antifungal drug and acetic acid resistance.

Catarina Costa^{1,2}, André Henriques^{1,2}, Carla Pires^{1,2}, Joana Nunes^{1,2}, Michiyo Ohno³, Hiroji Chibana³, Isabel Sá-Correia^{1,2} and Miguel C. Teixeira^{1,2}

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Opportunistic *Candida* species often have to cope with inhibitory concentrations of acetic acid, in the acidic environment of the vaginal mucosa. Given that the ability of these yeast species to tolerate stress induced by weak acids and antifungal drugs appears to be a key factor in their persistence and virulence, it is crucial to understand the underlying mechanisms. In this study, the drug: H⁺ antiporter CgAqr1 (ORF CAGL0J09944g), from *Candida glabrata*, was identified as a determinant of resistance to acetic acid, and also to the antifungal agents flucytosine and, less significantly, clotrimazole. These antifungals were found to act synergistically with acetic acid against this pathogen. The action of CgAqr1 in this phenomenon was analyzed. Using a green fluorescent protein fusion, CgAqr1 was found to localize to the plasma membrane and to membrane vesicles when expressed in *C. glabrata* or, heterologously, in *Saccharomyces cerevisiae*. Given its ability to complement the susceptibility phenotype of its *S. cerevisiae* homolog, ScAqr1, CgAqr1 was proposed to play a similar role in mediating the extrusion of chemical compounds. Significantly, the expression of this gene was found to reduce the intracellular accumulation of 3H-flucytosine and, to a moderate extent, of 3H-clotrimazole, consistent with a direct role in antifungal drug efflux. Interestingly, no effect of CgAQR1 deletion could be found on the intracellular accumulation of 14C-acetic acid, suggesting that its role in acetic acid resistance may

be indirect, presumably through the transport of a still unidentified physiological substrate. Although neither of the tested chemicals induces changes in CgAQR1 expression, pre-exposure to flucytosine or clotrimazole was found to make *C. glabrata* cells more sensitive to acetic acid stress. Results from this study show that CgAqr1 is an antifungal drug resistance determinant and raise the hypothesis that it may play a role in *C. glabrata* persistent colonization and multidrug resistance.

3. The *Candida glabrata* sterol scavenging mechanism, mediated by the ATP-binding cassette transporter Aus1p, is regulated by iron limitation.

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During disseminated infection by the opportunistic pathogen *Candida glabrata*, uptake of sterols such as serum cholesterol may play a significant role during pathogenesis. The ATP-binding cassette transporter Aus1p is thought to function as a sterol importer and in this study, we show that uptake of exogenous sterols occurred under anaerobic conditions in wild-type cells of *C. glabrata* but not in AUS1-deleted mutant (aus1 Δ) cells. In aerobic cultures, growth inhibition by fluconazole was prevented in the presence of

serum, and AUS1 expression was upregulated. Uptake of sterol by azole treated cells required the presence of serum, and sterol alone did not reverse FLC inhibition of growth. However, if iron availability in the growth medium was limited by addition of the iron chelators ferrozine or apo-transferrin, growth of wild-type cells, but not *aus1Δ* cells, was rescued. In a mouse model of disseminated infection, the *C. glabrata aus1Δ* strain caused a significantly decreased kidney fungal burden than the wild-type strain or a strain in which AUS1 was restored. We conclude that sterol uptake in *C. glabrata* can occur in iron poor environment of host tissues and thus may contribute to *C. glabrata* pathogenesis.

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米山 PI (感染応答) プロジェクト

Project for Immune Response in Infections Diseases

研究概要 (Summary)

感染に対する我々の生体防御は、自然免疫と獲得免疫によって協調して行われている。本プロジェクトでは、特にウイルス感染に応答した自然免疫誘導に注目し、感染センサー分子によるウイルス由来の非自己核酸検知の分子機構の解明と、それによって引き起こされる免疫応答の生理機能を解析することにより、ウイルス感染症に対する新たな治療戦略の開発を目指した解析を行っている。

Innate immune system plays an essential role for self-defense against infection of a variety of pathogens. In this project, we focus on antiviral innate immunity, especially molecular machinery for detection of viral infection and subsequent immune responses. The observations obtained from this study will help us to establish a novel therapeutic or preventive strategy against infectious diseases by viruses.

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特任研究員	平井 玲子	Project Researcher	Reiko Hirai
非常勤技術職員	滝澤香代子	Adjunct Research Technician	Kayoko Takizawa
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1. Identification of regulatory molecule(s), which is responsible for formation of antiviral stress granule (avSG).

Koji Onomoto, and Mitsutoshi Yoneyama

Recently, we investigated intra-cellular localization of viral RNA sensor, retinoic acid-inducible gene I (RIG-I), in influenza A virus (IAV)-infected cells, and demonstrated that infection of IAV induces RIG-I to accumulate in cytoplasmic granular-like structure, which we term antiviral stress granule (avSG). Although we revealed that avSG plays a critical role as platform for RIG-I-mediated antiviral signaling, it remains unclear how viral infection activates the signal via avSG formation. To address this issue, we are trying to identify regulatory molecule(s), which is important to form avSG in response to viral infections, using biochemical approaches.

2. Encephalomyocarditis virus disrupts stress granules, the critical platform for triggering antiviral innate immune responses.

Chen Seng Ng^{1,2}, Michihiko Jogi, Ji-Seung Yoo^{1,2}, Koji Onomoto, Satoshi Koike³, Takuya Iwasaki³, Mitsutoshi Yoneyama, Hiroki Kato^{1,2} and Takashi Fujita^{1,2}

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In response to stress, cells induce ribonucleoprotein aggregates, SGs. SGs are transient loci containing translation-stalled mRNA, which is eventually degraded or recycled for translation. Infection of some viruses, including

IAV with a deletion of nonstructural protein 1 (IAV Δ NS1), induces SG-like protein aggregates. Previously, we showed that IAV Δ NS1-induced SGs are required for efficient induction of type I interferon (IFN). Here, we investigated SG formation by different viruses using green fluorescent protein (GFP)-tagged Ras-Gap SH3 domain binding protein 1 (GFP-G3BP1) as an SG probe. HeLa cells stably expressing GFP-G3BP1 were infected with different viruses, and GFP fluorescence was monitored live with time-lapse microscopy. SG formations by different viruses was classified into 4 different patterns: no SG formation, stable SG formation, transient SG formation, and alternate SG formation. We focused on encephalomyocarditis virus (EMCV) infection, which exhibited transient SG formation. We found that EMCV disrupts SGs by cleavage of G3BP1 at late stages of infection (> 8 h) through a mechanism similar to that used by poliovirus. Expression of a G3BP1 mutant that is resistant to the cleavage conferred persistent formation of SGs as well as an enhanced induction of IFN and other cytokines at late stages of infection. Additionally, knockdown of endogenous G3BP1 blocked SG formation with an attenuated induction of IFN and potentiated viral replication. Taken together, our findings suggest a critical role of SGs as an antiviral platform and shed light on one of the mechanisms by which a virus interferes with host stress and subsequent antiviral responses.

3. Establishment of *in vitro* reconstitution assay system for RIG-I-mediated signaling.

Reiko Hirai, Michihiko Jogi, Ayaha Koyama and Mitsutoshi Yoneyama

We identified RIG-I as a sensor molecule for viral non-self RNA, however it remains unclear how RIG-I detects viral ribonucleoprotein complex (RNP), which consists of viral genomic RNA and proteins, such as nucleocapsid protein (NP). In this research, we established *in vitro* reconstitution assay system for RIG-I-mediated signaling and examined whether viral RNP could activate RIG-I *in vitro*. Since *in vitro* assay was reported previously (Zeng et al., Cell, 2010), we revised the published methods and

adapted it for RNP. As model RNP, we prepared artificial IAV RNP generated in 293T cells, which are transfected with expression plasmids encoding short viral RNA, His-tagged NP and viral RNA polymerases; PA, PB1 and flag-tagged PB2. The formed IAV RNPs in the cells were purified by affinity chromatography using Ni-conjugated and/or anti-flag antibody-conjugated beads. Now, we are trying to detect RIG-I activation *in vitro* by the artificial IAV RNPs.

4. Functional characterization of domains of IPS-1 using an inducible oligomerization system.

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The innate immune system recognizes viral nucleic acids and stimulates cellular antiviral responses. Intracellular detection of viral RNA is mediated by the RIG-I Like Receptor (RLR), leading to production of type I IFN and pro-inflammatory cytokines. Once cells are infected with a virus, RIG-I and MDA5 bind to viral RNA and undergo conformational change to transmit a signal through direct interaction with downstream caspase recruitment domain (CARD)-containing adaptor protein, IFN- β promoter stimulator-1 (IPS-1, also referred as MAVS/VISA/Cardif). IPS-1 is composed of N-terminal CARD, proline-rich domain, intermediate domain, and C-terminal transmembrane (TM) domain. The TM domain of IPS-1 anchors it to the mitochondrial outer membrane. It has been

hypothesized that activated RLR triggers the accumulation of IPS-1, which forms oligomer as a scaffold for downstream signal proteins. However, the exact mechanisms of IPS-1-mediated signaling remain controversial. In this study, to reveal the details of IPS-1 signaling, we used an artificial oligomerization system to induce oligomerization of IPS-1 in cells. Artificial oligomerization of IPS-1 activated antiviral signaling without a viral infection. Using this system, we investigated the domain-requirement of IPS-1 for its signaling. We discovered that artificial oligomerization of IPS-1 could overcome the requirement of CARD and the TM domain. Moreover, from deletion- and point-mutant analyses, the C-terminal Tumor necrosis factor Receptor-Associated Factor (TRAF) binding motif of IPS-1 (aa. 453-460) present in the intermediate domain is critical for downstream signal transduction. Our results suggest that IPS-1 oligomerization is essential for the formation of a multiprotein signaling complex and enables downstream activation of transcription factors, Interferon Regulatory Factor 3 (IRF3) and Nuclear Factor- κ B (NF- κ B), leading to type I IFN and pro-inflammatory cytokine production.

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西城PI (サイトカイン) プロジェクト

Project for Cytokine Research

研究概要 (Summary)

生体は、多種多様な細胞や組織が互いに時空的に作用することにより恒常性が維持される一つシステムであり、その維持においてサイトカインは中心的な役割を担っている。多くの疾病は単に一つの臓器、組織の異常ではなく、免疫系を始めとする種々のシステムの異常であることから、これらを統合するサイトカインの役割を知ることは非常に重要である。本プロジェクトでは、感染性疾患や炎症性疾患の病態形成におけるサイトカインの役割を解明し、最終的に新たな治療薬の標的分子を見出すことを目的とする。

Cytokines play a central role in maintenance of homeostasis. Because, a disease is not caused by only one problem of an organ, but caused by a systemic disorder, which is regulated by cytokines, it is important to study their functions. We aim to find new therapeutic targets for inflammatory diseases and infectious diseases by investigating the roles of cytokines in pathogenesis.

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技術補佐員 森本 雅子
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1. Dectin-1 and Dectin-2 in innate immunity against fungal infection.

Shinobu Saijo

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Dectin-1 and Dectin-2 are type II transmembrane proteins of the C-type lectin family with single carbohydrate recognition domains (CRDs) in their extracellular region. They are expressed mainly in dendritic cells and macrophages. Dectin-1 recognizes β -glucans with its CRD and transduces signals through its immunoreceptor tyrosine-based activation motif (ITAM)-like motif in the cytoplasmic domain, whereas Dectin-2 recognizes α -mannans and transduces its signal through association with the ITAM-containing Fc receptor γ chain. Upon ligand binding, spleen tyrosine kinase is recruited to the ITAM and activates the caspase recruitment domain family member 9 (CARD9)-nuclear factor- κ B axis,

resulting in the activation of various genes including those encoding pro-inflammatory cytokines. Both β -glucans and α -mannans are major cell wall components of fungi including *Candida albicans* (*C. albicans*) and *Pneumocystis carinii* (*P. carinii*). Recently, we reported that Dectin-1 is important in protection against *P. carinii* by inducing reactive oxygen species, whereas both Dectin-1 and Dectin-2 play important roles in defense against *C. albicans* by preferentially inducing Th17 cell differentiation. We are now working to elucidate molecular mechanisms that underlying anti-fungal immunity.

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亀井 PI (臨床感染症) プロジェクト

Project to Link Basic Sciences and Clinical Medicine

研究概要 (Summary)

アスペルギルス症を当面の最大の目標としつつ、真菌がどうやって人体に侵入するのかという観点から、新しい診断や治療の開発を中心に研究を行っている。またこれと並行して、附属病院における診療活動及び学内外でのコンサルテーションを行っており、学外からの依頼は検査を含めて年間 200-300 件に達している。

実施体制: 教員 2 名 (+ 兼務 1 名) 技官 1 名, 特任助教 2 名, 補助員 2 名で研究及び大学院生 4 名 (博士課程) の教育指導を行なっている。

ACTIVITIES: Our research focuses on the development of diagnostic/therapeutic methods for intractable fungal diseases such as aspergillosis through an investigation into the mechanism of infection. We also take care of patients in the clinic of the University Hospital, while providing consulting services on fungal diseases to physicians/clinical technologists all over the country.

STAFF: Professors (3, including 1 assistant professor working concurrently in the University Hospital), technician (1), research assistant professors (2) and research assistants (2) are working in our group with four graduate school students.

教 授	亀井 克彦	Professor	Katsuhiko Kamei
助 教	田口 英昭	Assistant Professor	Hideaki Taguchi
助 教 (兼任)	渡辺 哲	Assistant Professor (concurrent position)	Akira Watanabe
技 術 職 員	鎗田 響子	Research Technician	Kyoko Yarita
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技 術 補 佐 員	井上 京子	Research Promotion Technician	Kyoko Inoue

1. A double-blind comparative study of the safety and efficacy of caspofungin versus micafungin in the treatment of candidiasis and aspergillosis.

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The safety and efficacy profile of caspofungin and micafungin in Japanese patients with fungal infections were directly compared in this prospective, randomized, double-blind study. The proportion of patients who developed significant drug-related adverse event(s) (defined as a serious drug-related adverse event or a drug-related adverse event leading to study therapy discontinuation) was compared in 120 patients [caspofungin 50 mg, or 50 mg following a 70-mg loading dose on Day 1 (hereinafter, 70/50 mg) group: 60 patients; micafungin 150 mg: 60 patients]. The overall response rate was primarily evaluated in the per-protocol set

(PPS) population. The proportion of patients who developed significant drug-related adverse events was 5.0% (3/60) in the caspofungin group and 10.0% (6/60) in the micafungin group [95% confidence interval (CI) for the difference: -15.9%, 5.2%]. The favorable overall response in the PPS population for patients with esophageal candidiasis, invasive candidiasis, and chronic pulmonary aspergillosis including aspergilloma was 100.0% (6/6), 100.0% (3/3), and 46.7% (14/30) in the caspofungin group, and 83.3% (5/6), 100.0% (1/1), and 42.4% (14/33) in the micafungin group, respectively. In Japanese patients with *Candida* or *Aspergillus* infections, there was no statistical difference in the safety between caspofungin and micafungin. Consistent with other data on these two agents, the efficacy of caspofungin and micafungin was similar.

2. Histopathological Study of Murine Pulmonary Cryptococcosis Induced by *Cryptococcus gattii* and *Cryptococcus neoformans*.

Yoichiro Okubo¹, Megumi Wakayama¹, Hideaki Ohno², Shuhei Yamamoto², Naobumi Tochigi¹, Koichi Tanabe², Yukihiko Kaneko², Satoshi Yamagoe², Takashi Umeyama², Minoru Shinozaki¹, Tetsuo Nemoto¹, Haruo Nakayama³, Daisuke Sasai¹, Takao Ishiwatari¹, Kayoko Shimodaira¹, Yoshiro Yamamoto¹, Katsuhiko Kamei⁴, Yoshitsugu Miyazaki², Kazutoshi Shibuya^{1,5}

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Although *Cryptococcus gattii* can cause life-threatening complications, putative virulence factors of *C. gattii* remain controversial. Therefore, we conducted the present study

to elucidate the virulence factors of the yeast and found that the mortality rate of mice infected with *C. gattii* R265 was significantly higher than that of those infected with *C. gattii* 5815; however, no difference was found in the mortality rates between mice infected with *C. gattii* R265 and *Cryptococcus neoformans* H99. In contrast, we found a significant difference in histopathological findings of the lungs between mice infected with *C. gattii* R265 and *C. neoformans* H99. The former showed alveolar expansion due to yeast proliferation with much lesser macrophage response, whereas the latter showed numerous nodules in the alveolar space consisting of macrophages and multinucleated giant cells. Furthermore, alveolar expansion was more enhanced in mice infected with *C. gattii* R265 than in those infected with *C. gattii* 5815. Our study confirmed that there is a different pathophysiology leading to death during *C. gattii* and *C. neoformans* infections. The result can provide two characteristics of *C. gattii*: one includes some mechanisms to escape from host recognition via macrophage and another includes a high performance of pulmonary structural alteration. These characteristics may be associated with the high virulence of *C. gattii*.

3. Visual analysis of DNA microarray data for accurate molecular identification of non-*albicans* *Candida* isolates from patients with candidemia episodes.

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The performance of a visual slide-based DNA microarray

for the identification of non-*albicans* *Candida* spp. was evaluated. Among 167 isolates that had previously been identified by Vitek 2, the agreement between DNA microarray and sequencing results was 97.6%. This DNA microarray platform showed excellent performance.

4. NikA/TcsC histidine kinase is involved in conidiation, hyphal morphology, and responses to osmotic stress and antifungal chemicals in *Aspergillus fumigatus*.

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The fungal high osmolarity glycerol (HOG) pathway is composed of a two-component system (TCS) and Hog1-type mitogen-activated protein kinase (MAPK) cascade. A group III (Nik1-type) histidine kinase plays a major role in the HOG pathway of several filamentous fungi. In this study, we characterized a group III histidine kinase, NikA/TcsC, in the lifethreatening pathogenic fungus, *Aspergillus fumigatus*. A deletion mutant of *nikA* showed low conidia production, abnormal hyphae, marked sensitivity to high osmolarity stresses, and resistance to cell wall perturbing reagents such as congo red and calcofluor white, as well as to fungicides such as fludioxonil, iprodione, and pyrrolnitrin. None of these phenotypes were observed in mutants of the SskA response regulator and SakA MAPK, which were thought to be downstream components of NikA. In contrast, in response to fludioxonil treatment, NikA was implicated in the phosphorylation of SakA MAPK and the transcriptional upregulation of *catA*, *dprA*, and *dprB*, which are regulated under the control of SakA. We then tested the idea that not only NikA, but also the other 13 histidine kinases play certain roles in the regulation of the HOG pathway. Interestingly, the expression of *fos1*, *phkA*, *phkB*, *fhk5*, and *fhk6* increased by osmotic shock or fludioxonil treatment in a SakA-dependent manner. However, deletion mutants of

the histidine kinases showed no significant defects in growth under the tested conditions. Collectively, although the signal transduction network related to NikA seems complicated, NikA plays a crucial role in several aspects of *A. fumigatus* physiology and, to a certain extent, modulates the HOG pathway.

5. *Scedosporium aurantiacum* brain abscess after near-drowning in a survivor of a tsunami in Japan.

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Many victims of the tsunami that occurred following the Great East Japan Earthquake on March 11, 2011 developed systemic disorders owing to aspiration pneumonia. Herein, we report a case of tsunami lung wherein *Scedosporium aurantiacum* was detected in the respiratory tract. A magnetic resonance image of the patient's head confirmed multiple brain abscesses and lateral right ventricle enlargement. In

this case report, we describe a potential refractory multidrug-resistant infection following a tsunami disaster.

6. *Penicilliosis marneffei* Complicated with Interstitial Pneumonia.

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A 71-year-old man with interstitial pneumonia was hospitalized due to a pulmonary infection. He had been living in Thailand and had returned to Japan three months earlier. Antibiotic therapy initially cleared the infection; however, the patient's condition relapsed. *Pseudomonas aeruginosa* and *Penicillium* sp. were both detected in sputum and bronchial lavage fluid cultures and *Penicillium* sp. was identified to be *P. marneffei*. The infiltration observed on chest radiographs improved following treatment with itraconazole and tazobactam/piperacillin, and no relapse occurred. We herein report the first case of a non-HIV patient with *P. marneffei* infection in Japan.

7. Glucoamylase is a major allergen of *Schizophyllum commune*.

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Background. *Schizophyllum commune* is one of the causative agents of basidiomycosis including disorders such as allergic bronchopulmonary mycosis, allergic fungal sinusitis, and mucoid impaction of bronchi, the incidence of those of which has been increasing. These mycoses are difficult to diagnose because only a limited number of diagnostic tools are currently available. The biggest problem is that no specific antigens of *S. commune* have been identified to enable serodiagnosis of the disease.

Objective. In this study, we attempted to identify a major antigen of *S. commune* to establish a reliable serodiagnostic method.

Methods. We used mass spectrometry to identify an antigen that reacted with the serum of a patient with allergic bronchopulmonary mycosis caused by *S. commune*. The protein was expressed in *Escherichia coli*, highly purified, and the patient sera IgG and IgE titers against the protein were determined by enzyme-linked immunosorbent assay.

Results. The protein identified as a major antigen of *S. commune* was named Sch c 1; it was a homolog of glucoamylase. The IgG and IgE titers against Sch c 1 in patient sera were significantly higher than those in healthy volunteer sera ($p < 0.01$).

Conclusions & Clinical relevance. Sch c 1 is recognized

by the host immune system of patients as an antigen/allergen. The purified glucoamylase Sch c 1 is a promising candidate antigen for the serodiagnosis of *S. commune*-induced mycosis.

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五ノ井 PI (真菌・放線菌と宿主の分子相互作用研究) プロジェクト Project for Host Pathogen (fungi/actinomycetes) Molecular Interaction

研究概要 (Summary)

微生物資源分野では、バイオリソース管理室と協力し、日本国内および海外のヒトや動物に由来する病原真菌・病原放線菌を収集、管理、分譲している。これらの菌株数は、現在約2万に達するが、菌のマーカー遺伝子やゲノムを解析し、また薬剤感受性や電子顕微鏡による形態観察、2次代謝産物の解析などを行い菌株資源、遺伝子資源としての付加価値の向上に努めている。さらなる独自の研究テーマ (PIプロジェクト) については下記『主なテーマ』を参照してください。

主なテーマ (Research Focus)

- 1) ヒト・動物の病原真菌・病原放線菌の収集、分類、系統解析、2次代謝産物の解析、病原因子解析、2次代謝産物生合成遺伝子、ゲノムの解析を行っている。
- 2) 真菌・放線菌のヒトへの感染機構の解明を分子生物学的手法、動物モデル、ゲノム解析などを用いて行っている。特に、近年は、糖鎖と糖鎖受容体を介した菌と宿主の相互作用解明に力を入れている。
- 3) 真菌感染発症と宿主の栄養状態やストレス状態との関連を動物モデルなどを用いて研究している。特に代謝関連分子と免疫関連分子の機能的リンクに興味を持っている。

In cooperation with Bio-Resource management office, we collect pathogenic fungi and actinomycetes in both inside and outside of Japan. We identify pathogenic fungi and actinomycetes as a public service, and analyze their phylogenetic relations. We store fungi and actinomycetes with the support of the National BioResource Projects in Japan, and distribute them upon request. Currently we stock approximately 20,000 strains. We analyze sequences of marker genes and genomes, drug-sensitivities, and observe fine structures using electron-microscopy, to enhance biodiversity values. Other projects are listed below.

- 1) We collect, identify and phylogenetically analyze of human and animal pathogenic fungi and actinomycetes. We also analyze 2nd metabolites and their synthetic enzymes, pathogenic factors, and genomes.
- 2) We analyze infection mechanisms of human pathogenic fungi and actinomycetes using molecular methods, animal models, and genome analysis. In particular, we are trying to understand roles of cell surface glycans and their receptors (lectins) of human and fungi in infection.
- 3) We study effects of diets and mental stresses on fungal infections mainly using animal models and molecular methods. We are trying to clarify yet unknown links between metabolism and immune-related molecules.

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1. Identification of fungal pathogens by visible microarray system in combination with isothermal gene amplification.

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The increasing incidence of infectious diseases caused by fungi in immunocompromised patients has encouraged researchers to develop rapid and accurate diagnosis methods. Identification of the causative fungal species is critical in deciding the appropriate treatment, but it is not easy to get satisfactory results due to the difficulty of fungal cultivation and morphological identification from clinical samples. In this study, we established a microarray system that can identify 42 species from 24 genera of clinically important fungal pathogens by using a chemical color reaction in the detection process. The array uses the internal transcribed spacer (ITS) region of the rRNA gene for identification of fungal DNA at the species level. The specificity of this array was tested against a total of 355 target and non-target fungal species. The fungal detection was succeeded directly from 103 CFU/ml for whole blood samples and 50 fg DNA per 1 ml of serum samples indicate that the array system we established is sensitive to identify infecting fungi from clinical sample. Furthermore, we conducted isothermal amplification in place of PCR amplification and labeling. The successful identification with PCR-amplified as well as isothermally amplified target genes demonstrated that our microarray system is an efficient and robust method for identifying a variety of fungal species in a sample.

2. Lectin-Microarray Technique for Glycomic Profiling of Fungal Cell Surfaces.

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Lectin microarrays are rows of lectins with different carbohydrate-binding specificities spotted on surfaces of glass-slides. Lectin microarray technique enables glycomic analyses of carbohydrate composition of fungal cell walls. We will describe an application of the technique in analyzing cell surface glycome of yeast-form fungal cells in the living state. The analysis reveals genus- and species-dependent complex cell surface carbohydrate structures of fungi, and enabled us, therefore, to suggest that cell walls of yeast cells, which have been considered to have relatively simple structures, actually have a more complex structure containing galactose and fucose. This shows that the technique can be used to find new insights in the study of phylogenetic relations and in the classification of cells in the fungal kingdom based on cell wall glycome.

3. Refeeding with a standard diet after a 48-h fast elicits an inflammatory response in the mouse liver.

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Unhealthy eating behaviors increase the risk of metabolic diseases, but the underlying mechanisms are not fully

elucidated. Because inflammation contributes to the pathogenesis of metabolic diseases, it is important to understand the effects of unhealthy eating on the inflammatory state. The objective of our present study was to address the effects of a fasting-refeeding regime, a model of irregular eating, on the hepatic inflammatory responses in mouse. The animals were fasted for 48 h and then refed either a standard or low-carbohydrate/high-fat diet. Inflammatory gene expression in the liver was then sequentially measured for the first 17 h after initiation of refeeding. To assess the roles of dietary carbohydrates and toll-like receptor 2 (TLR2) in the refeeding-induced inflammatory changes, gene expression levels in mice refed only carbohydrates (α -corn starch and sucrose) at different doses and in TLR2-deficient mice refed a standard diet were also analyzed. Refeeding with a standard diet increased the liver expression of Tlr2, proinflammatory mediators (Cxcl10, Cxcl1, Cxcl2, Icam-1) and negative regulators of TLR-signaling (A20 and Atf3). These increases were attenuated in mice refed a low-carbohydrate/high-fat diet. Refeeding only α -corn starch and sucrose also increased the expression of these inflammatory pathway genes depending on the doses. TLR2 deficiency significantly attenuated the refeeding-induced increase in the liver expression of Cxcl10, Cxcl1, Icam-1 and A20. These findings suggest that an irregular eating behavior can elicit a liver inflammatory response, which is at least partly mediated by TLR2, and that dietary carbohydrates play critical roles in this process. © 2013 Elsevier Inc.

4. Molecular identification and antimicrobial susceptibility of *Nocardia* spp. isolated from bovine mastitis in Brazil.

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Nocardia spp. infections can cause severe damage to the mammary gland due to suppurative pyogranulomatous lesions and lack of clinical cure in response to conventional antimicrobial therapy. Although *Nocardia* infections are considered relatively uncommon in cows, there has been an apparent worldwide increase in the incidence of bovine mastitis caused by *Nocardia* spp, perhaps due to environmental transmission of this ubiquitous pathogen. The objectives of present study were to determine: (i) species distribution of 80 *Nocardia* isolates involved in bovine mastitis (based on molecular methods); and (ii) antimicrobial susceptibility pattern of all isolates from three geographical areas in Brazil. In this study, *Nocardia nova* (80%) was the most frequently isolated species, followed by *Nocardia farcinica* (9%). Additionally, *Nocardia puris*, *Nocardia cyriacigeorgica*,

Nocardia veterana, *Nocardia africana*, and *Nocardia arthritidis* were detected using 16S rRNA sequencing. This is apparently the first report of *N. puris*, *N. veterana*, *N. cyriacigeorgica*, *N. arthritidis* and *N. africana* in association with bovine mastitis. Based on the disk diffusion test, isolates were most frequently resistant to cloxacillin (75%), ampicillin (55%) and cefoperazone (47%), whereas few *Nocardia* spp. were resistant to amikacin, cefuroxime or gentamicin.

5. Visual analysis of DNA microarray data for accurate molecular identification of non-*Albicans Candida* isolates from patients with candidemia episodes.

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The performance of a visual slide-based DNA microarray for the identification of non-*Albicans Candida* spp. was evaluated. Among 167 isolates that had previously been identified by Vitek 2, the agreement between DNA microarray and sequencing results was 97.6%. This DNA microarray platform showed excellent performance.

6. NikA/TcsC Histidine Kinase Is Involved in Conidiation, Hyphal Morphology, and Responses to Osmotic Stress and Antifungal Chemicals in *Aspergillus fumigatus*.

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The fungal high osmolarity glycerol (HOG) pathway is composed of a two-component system (TCS) and Hog1-type mitogen-activated protein kinase (MAPK) cascade. A group III (Nik1-type) histidine kinase plays a major role in the HOG pathway of several filamentous fungi. In this study, we characterized a group III histidine kinase, NikA/TcsC, in the life-threatening pathogenic fungus, *Aspergillus fumigatus*. A deletion mutant of *nikA* showed low conidia production, abnormal hyphae, marked sensitivity to high osmolarity stresses, and resistance to cell wall perturbing reagents such as congo red and calcofluor white, as well as to fungicides such as fludioxonil, iprodione, and pyrrolnitrin. None of these phenotypes were observed in mutants of the SskA response regulator and SakA MAPK, which were thought to be downstream components of NikA. In contrast, in response to fludioxonil treatment, NikA was implicated in the phosphorylation of SakA MAPK and the transcriptional upregulation of *catA*, *dprA*, and *dprB*, which are regulated under the control of SakA. We then tested the idea that not only NikA, but also the other 13 histidine kinases play certain roles in the regulation of the HOG pathway. Interestingly, the expression of *fos1*, *phkA*, *phkB*, *fbk5*, and *fbk6* increased by osmotic shock or fludioxonil treatment in a SakA-dependent manner. However, deletion mutants of the histidine kinases showed no significant defects in growth under the tested conditions. Collectively, although the signal transduction network related to NikA seems complicated, NikA plays a crucial role in several aspects of *A. fumigatus* physiology and, to a certain extent, modulates the HOG pathway.

7. *Aspergillus huiyanae* sp. nov., a teleomorphic species in sect. *Fumigati* isolated from desert soil in China.

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Aspergillus huiyanae, a new teleomorphic species isolated from desert soil in Xinjiang, China, was described and illustrated. *Aspergillus huiyanae* is characterized by its yellowish white to pale yellow cleistothecia, broadly lenticular ascospores with two equatorial crests and irregularly ribbed to slightly reticulate convex surfaces, and subglobose to ovate or broadly ellipsoidal conidia with smooth walls. This species was supported further by the analyses of the β -tubulin, calmodulin and actin gene sequences.

8. Prevalence of hepatitis C virus subgenotypes 1a and 1b in Japanese Patients: Ultra-deep sequencing analysis of HCV NS5B genotype-specific region.

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Background: Hepatitis C virus (HCV) subgenotypes 1a and 1b have different impacts on the treatment response to peginterferon plus ribavirin with direct-acting antivirals (DAAs) against patients infected with HCV genotype 1,

as the emergence rates of resistance mutations are different between these two subgenotypes. In Japan, almost all of HCV genotype 1 belongs to subgenotype 1b. Methods and Findings: To determine HCV subgenotype 1a or 1b in Japanese patients infected with HCV genotype 1, real-time PCR-based method and Sanger method were used for the HCV NS5B region. HCV subgenotypes were determined in 90% by real-time PCR-based method. We also analyzed the specific probe regions for HCV subgenotypes 1a and 1b using ultra-deep sequencing, and uncovered mutations that could not be revealed using direct-sequencing by Sanger method. We estimated the prevalence of HCV subgenotype 1a as 1.2-2.5% of HCV genotype 1 patients in Japan. Conclusions: Although real-time PCR-based HCV subgenotyping method seems fair for differentiating HCV subgenotypes 1a and 1b, it may not be sufficient for clinical practice. Ultra-deep sequencing is useful for revealing the resistant strain(s) of HCV before DAA treatment as well as mixed infection with different genotypes or subgenotypes of HCV.

9. Hyrtimomines D and E, bisindole alkaloids from a marine sponge *Hyrtios* sp.

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Two new alkaloids, hyrtimomines D (1) and E (2), were isolated from an Okinawan marine sponge *Hyrtios* sp. The structures of 1 and 2 were elucidated on the basis of spectroscopic analysis. Hyrtimomines D (1) and E (2) are structurally unique bisindole alkaloids possessing the canthin-6-one skeleton with a hydroxyindole and an imidazolium units. Hyrtimomines D (1) and E (2) exhibited antimicrobial activity.

10. Nagelamides U-W, bromopyrrole alkaloids from a marine sponge *Agelas* sp.

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Three new structurally unique bromopyrrole alkaloids, nagelamides U-W (1-3), were isolated from a marine sponge *Agelas* sp. Nagelamides U (1) and V (2) possess a γ -lactam ring with an N-ethanesulfonic acid and guanidino moieties, while nagelamide W (3) has two aminoimidazole moieties in the molecule. The structures of 1-3 were elucidated on the basis of spectroscopic data. Nagelamides U (1) and W (3) exhibited antimicrobial activity against *Candida albicans*. © 2013 Elsevier Ltd. All rights reserved.

11. Two new species of *Aspergillus* section *Fumigati* isolated from caatinga soil in the state of Pernambuco, Brazil.

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Aspergillus caatingaensis and *A. pernambucoensis*, isolated from semi-desert soil in caatinga area, the State of Pernambuco, Brazil, are described and illustrated. *Aspergillus caatingaensis* is characterized by its white cleistothecia, broadly lenticular ascospores with four equatorial crests and irregularly ribbed to slightly reticulate with aculeate convex surfaces, and ellipsoidal to broadly ellipsoidal conidia with a smooth wall. *Aspergillus*

pernambucoensis is characterized by its, white cleistothecia, lenticular ascospores with two equatorial crests and irregularly ribbed with tuberculate to verrucate convex surfaces, and ovoid to broadly ellipsoidal conidia with a smooth wall. The validation of these new species is supported further by analyses of the β -tubulin, calmodulin and actin gene sequences.

12. Nagelamides X-Z, dimeric bromopyrrole alkaloids from a marine sponge *Agelas* sp.

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Three new dimeric bromopyrrole alkaloids, nagelamides X-Z (1-3), were isolated from a marine sponge *Agelas* sp. Nagelamides X (1) and Y (2) possess a novel tricyclic skeleton consisting of spiro-bonded tetrahydrobenzaminoimidazole and aminoimidazolidine moieties. Nagelamide Z (3) is the first dimeric bromopyrrole alkaloid involving the C-8 position in dimerization. The structures of 1-3 were elucidated on the basis of spectroscopic data. Nagelamides X-Z (1-3) exhibited antimicrobial activity.

13. **Manzamenone O, new trimeric fatty acid derivative from a marine sponge *Plakortis* sp.**

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A new structurally unique trimeric fatty acid derivative, manzamenone O (1), was isolated from a marine sponge *Plakortis* sp. Manzamenone O (1) has a novel skeleton consisting of C-C bonded octahydroindenone and dioxabicyclo [3.3.0]octane moieties and three long aliphatic chains. The structure of 1 was elucidated on the basis of spectroscopic data and conformational analysis. Manzamenone O (1) exhibited antimicrobial activity against *Micrococcus luteus*, *Aspergillus niger*, and *Trichophyton mentagrophytes*.

14. **Identification of distinct ligands for the C-type lectin receptors mincle and dectin-2 in the pathogenic fungus *Malassezia*.**

Ishikawa T¹, Itoh F⁴, Yoshida S⁴, Saijo⁵, Matsuzawa T⁶, Gono T⁶, Saito T^{8,9}, Okawa Y⁴, Shibata N⁴, Miyamoto, T², Yamasaki S^{1,3}

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Various C-type lectin receptors (CLRs), including Mincle and Dectin-2, function as pattern recognition receptors and play a central role in immunity to fungal pathogens. However, the precise structures of the CLR ligands in various pathogenic fungi have yet to be completely defined. Here we report that *Malassezia*, an opportunistic skin fungal pathogen, is cooperatively recognized by Mincle and Dectin-2 through distinct ligands. Solvent-based fractionation

revealed that Mincle and Dectin-2 recognize lipophilic and hydrophilic components of *Malassezia*, respectively. Mass spectrometry and nuclear magnetic resonance (NMR) revealed glyceroglycolipid and unique mannosyl fatty acids linked to mannitol as two Mincle ligands. An O-linked mannanose-rich glycoprotein was identified as a *Malassezia* ligand for Dectin-2. Cytokine production in response to the Mincle ligands and the Dectin-2 ligand was abrogated in Mincle^{-/-} and Dectin-2^{-/-} dendritic cells, respectively. These results demonstrate that Mincle and Dectin-2 recognize distinct ligands in *Malassezia* to induce host immune responses.

15. **Zamamiphidin A, a new manzamine related alkaloid from an Okinawan marine sponge *Amphimedon* sp.**

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A manzamine related alkaloid, zamamiphidin A (1), consisting of a new heptacyclic ring system has been isolated from an Okinawan marine sponge *Amphimedon* sp. The structure of 1 including the relative stereochemistry was elucidated on the basis of the spectroscopic data. Compound 1 showed antibacterial activity against *Staphylococcus aureus* (MIC, 32 µg/mL).

16. **Testicular nocardiosis accompanied by cutaneous lesions in an immunocompetent man.**

Yamaguchi H¹, Sekimoto E¹, Shirakami A¹, Shibata H¹, Ozaki S¹, Shigekiyo T¹, Noda T², Shikiji T², Kanda, K³, Hirose T⁴, Matsuzawa T⁵, Gonoï T⁵

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We herein report the case of a 77-year-old man admitted for an acute cutaneous infection and persistent fever. A physical examination revealed systemic small blisters and scrotal swelling. He was suspected of having complications from chickenpox or bullous impetigo as the initial diagnosis. *Nocardia* was detected on an aspiration biopsy of the small blisters and the surgically removed testis at a later date. Testicular nocardiosis is a rare condition; however, we should consider nocardiosis in the differential diagnosis because delay in providing treatment may worsen a patient's general condition.

17. Manzamenones L-N, new dimeric fatty-acid derivatives from an Okinawan marine sponge *Plakortis* sp.

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Three new polyketides, manzamenones L-N (1-3), have been isolated from an Okinawan marine sponge of the genus *Plakortis*. The structures of 1-3 were elucidated on the basis of spectroscopic data. Manzamenones L-N (1-3) were new dimeric fatty-acid derivatives consisting of a tetrahydroindenone with three carboxy groups and two hexadecanyl chains. Manzamenones M (2) and N (3) showed antimicrobial activity against several bacteria and fungi.

18. Ultra-Deep Sequencing Analysis of the Hepatitis A Virus 5'-Untranslated Region among Cases of the Same Outbreak from a Single Source.

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Hepatitis A virus (HAV) is a causative agent of acute viral

hepatitis for which an effective vaccine has been developed. Here we describe ultra-deep pyrosequences (UDPSs) of HAV 5'-untranslated region (5'UTR) among cases of the same outbreak, which arose from a single source, associated with a revolving sushi bar. We determined the reference sequence from HAV-derived clone from an attendant by the Sanger method. Sixteen UDPSs from this outbreak and one from another sporadic case were compared with this reference. Nucleotide errors yielded a UDPS error rate of < 1%. This study confirmed that nucleotide substitutions of this region are transition mutations in outbreak cases, that insertion was observed only in non-severe cases, and that these nucleotide substitutions were different from those of the sporadic case. Analysis of UDPSs detected low-prevalence HAV variations in 5'UTR, but no specific mutations associated with severity in these outbreak cases. To our surprise, HAV strains in this outbreak conserved HAV IRES sequence even if we performed analysis of UDPSs. UDPS analysis of HAV 5'UTR gave us no association between the disease severity of hepatitis A and HAV 5'UTR substitutions. It might be more interesting to perform ultra-deep sequencing of full length HAV genome in order to reveal possible unknown genomic determinants associated with disease severity. Further studies will be needed.

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高橋 PI (微生物創生) プロジェクト

Project for Systems Biology of Microorganisms

研究概要 (Summary)

当分野では、計算機を駆使して新たな生物学的知見の発見を目指しています。一つは、膨大な実験データを対象にデータ処理技術の開発を通じた生命の理解を目指した「バイオインフォマティクス」の研究を行っています。また、生命を真にシステムとして理解することを目的とした「システムズバイオロジー」の研究も進めています。

Our research areas are Systems Biology and Bioinformatics. Our Bioinformatics approach aims to deeply and clearly understand massive biological experiment data, e.g., sequence data by next generation sequencers. Systems Biology aims to understand how biological systems work and help the experimental design mainly by mathematical modelling approach.

准 教 授 高橋 弘喜

Associate Professor

Hiroki Takahashi

1. Functions of the Hha and YdgT proteins in transcriptional silencing by the nucleoid proteins, H-NS and StpA, in *Escherichia coli*.

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The Hha and YdgT proteins are suggested to modulate the expression of horizontally acquired genes by interacting with H-NS and StpA, which play central roles in the transcriptional silencing of such genes. However, it is also possible that Hha/YdgT repress gene expression independently of H-NS/StpA, as we have not fully understood the molecular mechanism through which Hha/YdgT modulate H-NS/StpA activity. To gain further insight into the basic functions of Hha/YdgT, we analysed the impact of hha/ydgT double inactivation on the transcriptome profile of *Escherichia coli* K-12, and compared the effects with that of hns/stpA double inactivation. In addition, we examined the effects

of hha/ydgT inactivation on the chromosomal binding of H-NS, and conversely the effects of hns/stpA inactivation on the chromosomal binding of Hha. Our results demonstrated that the chromosomal binding of Hha requires H-NS/StpA, and is necessary for the repression of a subset of genes in the H-NS/StpA regulon. Furthermore, the distribution of H-NS binding around Hha/YdgT-dependent and -independent genes suggests that Hha/YdgT proteins modulate formation of the H-NS/StpA-DNA complex.

2. Improved production of secreted heterologous enzyme in *Bacillus subtilis* strain MGB874 via modification of glutamate metabolism and growth conditions.

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Background. The *Bacillus subtilis* genome-reduced strain MGB874 exhibits enhanced production of exogenous extracellular enzymes under batch fermentation conditions. We predicted that deletion of the gene for RocG, a bi-functional protein that acts as a glutamate dehydrogenase and an indirect repressor of glutamate synthesis, would improve glutamate metabolism, leading to further increased enzyme production. However, deletion of *rocG* dramatically decreased production of the alkaline cellulase Egl-237 in strain MGB874 (strain 874Δ*rocG*).

Results. Transcriptome analysis and cultivation profiles suggest that this phenomenon is attributable to impaired secretion of alkaline cellulase Egl-237 and nitrogen starvation, caused by decreased external pH and ammonium depletion, respectively. With NH₃-pH auxostat fermentation, production of alkaline cellulase Egl-237 in strain 874Δ*rocG* was increased, exceeding that in the wild-type-background strain 168Δ*rocG*. Notably, in strain 874Δ*rocG*, high enzyme productivity was observed throughout cultivation, possibly due to enhancement of metabolic flux from 2-oxoglutarate to glutamate and generation of metabolic energy through activation of the tricarboxylic acid (TCA) cycle. The level of alkaline cellulase Egl-237 obtained corresponded to about 5.5 g l⁻¹, the highest level reported so far.

Conclusions. We found the highest levels of production of alkaline cellulase Egl-237 with the reduced-genome strain 874Δ*rocG* and using the NH₃-pH auxostat. Deletion of the glutamate dehydrogenase gene *rocG* enhanced enzyme production via a prolonged auxostat fermentation, possibly due to improved glutamate synthesis and enhanced generation of metabolism energy.

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バイオリソース管理室

Management Unit of Microbiological Resources

研究概要 (Summary)

病原真菌・放線菌の「保存・管理・提供」体制を整備し、最新情報が付加された信頼できる菌株の提供を通じて、真菌症ならびにその原因菌の研究・教育の基盤を支援している。

We are developing a system for preservation, management and distribution of pathogenic fungi and actinomycetes. We support the base of research and education of mycoses and their pathogens in order to supply reliable strains that are added with new information.

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助 教	田中 玲子	Assistant Professor	Reiko Tanaka
技 術 職 員	伊藤 純子	Research Technician	Junko Ito
技 術 補 佐 員	長村 由美	Research Promotion Technician	Yumi Osamura

1. *Aspergillus waksmanii* sp. nov. and *Aspergillus marvanovae* sp. nov., two closely related species in section Fumigati described using polyphasic approach.

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Two new and phylogenetically closely related species in *Aspergillus* section Fumigati are described and illustrated. Homothallic *Aspergillus waksmanii* sp. nov. was isolated from New Jersey soil (USA) and is represented by the ex-

type isolate NRRL 179^T (= CCF 4266^T = Thom 4138. HS2^T = IBT 31900^T). *Aspergillus marvanovae* sp. nov. was isolated from water with high boracic acid anions content in Dukovany nuclear power station (Czech Republic). The sexual stage of this species is unknown, but the MAT1-1 locus was successfully amplified suggesting that the species is probably heterothallic and teleomorphic but is represented by only the ex-type isolate CCM 8003^T (= CCF 4037^T = NRRL 62486^T = IBT 31279^T = IFM 60873^T). Both species can be distinguished from all previously described species in section Fumigati based on morphology, maximum growth temperature, sequence data from five unlinked loci and unique secondary metabolites profiles.

2. Genotypes of *Candida albicans* isolated from healthy individuals and their distribution in patients with oral candidiasis.

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For the study of *Candida albicans* genotypes involved in development of candidiasis, *Candida albicans* isolates were collected from healthy volunteers and patients with oral candidiasis and genotyped on the basis of 25S rDNA and microsatellite polymorphisms. In the microsatellite analysis using two microsatellite markers (CDC3 and CAI), 63 healthy volunteer isolates were classified into 35 genotypes (allelic relations to CDC3 alleles 1 : 2/CAI alleles 1 : 2), among which genotypes II (115 : 119/23 : 23), III (115 : 123/18 : 27), and V (123 : 127/32 : 41) were found at frequencies of 12.7%, 7.9%, and 7.9%, respectively. In 68 oral candidiasis isolates classified into 39 genotypes, genotypes II and III were identified in 4.4% and 20.6% of the isolates, respectively. The frequency of genotype III was higher in the candidiasis isolates than in the healthy isolates ($p < 0.05$). These results suggest that genotype III *C. albicans* assigned by CDC3/CAI is related to the development of oral candidiasis.

3. Method for rapid detection and identification of *Chaetomium* and related genera and evaluation of resistance to peracetic acid.

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In a beverage industry, peracetic acid has been increasingly used as a disinfectant for the filling machinery and environment due to merits of leaving no residue, safety to humans and its antiseptic effect against fungi and endospores of bacteria. Recently, *Chaetomium globosum* and *C. funicola* were reported resistant to peracetic acid, however little is known concerning the detail of peracetic acid resistance. Therefore we assessed the peracetic acid resistance of the species of *Chaetomium* and related genera under identical conditions and made a thorough observation of the microstructure of their ascospores by transmission electron microscopy. At the result, *C. globosum* and *C. funicola* showed the high resistance against peracetic acid (the 1D antiseptic effect after 900 s and 3D antiseptic effect after 900 s) and had thick cell walls of ascospores that might impede the action mechanism of peracetic acid. We also developed species-specific primers to identify *C. globosum* and *C. funicola* using polymerase chain reaction (PCR) to amplify the β -tubulin gene. PCR using the primer sets designed for *C. globosum* (Chae 4F/4R) and *C. funicola* (Cfu 2F/2R) amplified PCR products specific for *C. globosum* and *C. funicola*, respectively. PCR using these two primer sets did not detect other fungi involved in food spoilage and environmental contamination. This identification method is rapid and simple with extremely high specificity.

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文部科学省 ナショナルバイオリソースプロジェクト 「病原微生物」

Ministry of Education, Culture, Sports, Science and Technology National BioResource Project “Pathogenic Microorganisms”

文部科学省では 2002 年度からナショナルバイオリソースプロジェクト (NBRP) を開始し、国が戦略的に整備することが重要なものについて体系的に収集、保存、提供などを行うための体制を整備してきた。その後 5 年ごとの見直しを行い、2012 年度より第 3 期が開始された。

NBRP 病原微生物中核機関である千葉大学真菌医学研究センター (病原真菌・放線菌)、大阪大学微生物病研究所および岐阜大学大学院医学研究科 (病原細菌) と長崎大学熱帯医学研究所 (病原性原虫) は、相互の機関の連携を図り、これらの病原微生物株の収集・保存・提供体制を整備して、高度情報を賦与した信頼できる病原微生物株として提供し、感染症と病原体の教育・研究をする人々を支援している。

本プロジェクトは、今後いかなる感染症が発生しても対応できる病原微生物コレクションを目指している。

In FY2002, the Ministry of Education, Culture, Sports, Science and Technology (MEXT) implemented the National BioResource Project (NBRP) to construct the framework for systematic collection, preservation, and distribution of

bioresources, with a focus on those that required strategic development by the national government. After the reviewing the NBRP of every five years, in FY2012, the third phase has started.

Chiba University’s Medical Mycology Research Center (MMRC) is the “NBRP Center” for pathogenic microorganism, and this project is carried out by MMRC (pathogenic fungi/actinomycetes), Osaka University’s Research Institute for Microbial Diseases (pathogenic bacteria), Gifu University’s Graduate School of Medicine (pathogenic bacteria), and Nagasaki University’s Institute of Tropical Medicine (pathogenic protozoa). Working together, they cooperate in various efforts to support education and research pertaining to infectious diseases and pathogens. Specifically, they are developing a system for collection, preservation, and distribution of pathogenic microorganisms, and they supply reliable strains of pathogenic microorganisms that are backed by high-level information.

The project aims to establish a reliable and sufficient at the collection to deal with infectious diseases carried by any pathogenic microorganisms.

地球規模課題対応国際科学技術協力事業 (JST & JICA) 「AIDS 患者及びその他の免疫不全患者における 新規診断法による真菌症対策」プロジェクト

SATREPS project (Science and Technology Research Partnership for Sustainable Development) Diagnostic Approaches in the Management of Fungal Infections in AIDS and Other Immunocompromised Patients

独立行政法人国際協力機構 (JICA) および独立行政法人科学技術振興機構 (JST) の地球規模課題対応国際科学技術協力事業 (SATREPS) により、平成 22 年度よりブラジル連邦共和国サンパウロ州立カンピーナス大学医学部感染症科との間で協力事業を実施し、平成 25 年 3 月で終了した。本プロジェクトの目的は AIDS を始めとした免疫不全患者における真菌症の診断法・治療法の研究を推進・実用化し、その成果をブラジルに提供して両国の医療の向上に役立てることにある。

途中、東日本大震災など予期せぬ事態が生じたが、3 年間の実施期間中に主要なテーマ (独自の DNA チップによる病原真菌の同定及び真菌症の診断、 β -グルカンによる真菌症の診断、リアルタイム PCR 及び LAMP 法による真菌症の診断・原因菌の同定、薬剤最適投与方法の検討等) を無事完了することができた。本プロジェクトの研究成果はブラジルを始めとして周辺中南米諸国およびブラジルの影響下にあるポルトガル語圏アフリカ諸国の医療レベルにも影響を与える大規模なものであり、本プロジェクトは終了時評価で「A+」の高評価を得た。なおこの終了時評価については、<http://www.jst.go.jp/globalhyouka/index.html> にて閲覧可能である。

Increasing numbers of fungal infections caused by yeasts and moulds are a serious threat not only to the Japanese society, but also to many other countries, including Brazil. These infections hamper the patients' quality of life and sometimes claims their lives. In this SATREPS project, sponsored by JST and JICA, we worked together with the Division of Infectious Diseases, University Hospital of UNICAMP (State University of Campinas, Brazil) to develop accurate, rapid, and sensitive diagnostic methods for fungal infections, such as DNA chips for the infection, and applied these methods to clinical cases in Brazil.

The project was performed during FY2010 to FY2012, and the results were very promising. Our project was highly rated by the evaluation committee with the score of A+ on completion of the project term. Since these new technologies can be applied to diagnosis of patients in other countries, particularly in South America and Portuguese-spoken African countries, the fruit of our study is expected to give substantial beneficial ripple effects to these societies.

長崎大学熱帯医学研究拠点特定領域共同研究

「熱帯地域, 特にアフリカおよびベトナムで発生している真菌症・放射菌症の原因菌の収集と形態学的, 生理学的, 分子生物学的解析」プロジェクト

Cooperative Research of Priority Areas with NEKKEN, Nagasaki University

Project for Morphological, Physiological and Molecular Biological Analysis of Pathogenic Fungi and Actinomycetes Collected in Africa and Vietnam.

長崎大学熱帯医学研究所ケニア拠点を中心に, 上記プロジェクトを展開しています. 現在までにケニア全土の主要穀物 (トウモロコシ, 小麦) やミルクなどを汚染するカビ毒 (発がん性アフラトキシン他) とその生産菌の解析を進め, 現地食物の多くが, 世界の安全基準値を大きく上回るカビ毒で汚染されていることを明らかにしました. 結果は昨年度, 現地のマスコミにも取り上げられ, 大きな反響を呼び起こしました. また新たに現地で, エイズ患者の命を奪う主な原因である真菌感染症, 特にクリプトコッカス属菌による感染を中心に疫学的調査を計画しています. 海外での研究は, 現地の研究者や監督官庁と信頼関係を築き, 許可を得るなど多くの問題を解決しなければ前進できません. しかし, 現地の医療に貢献し, 人々の生活の質 (QOL) の向上を図り, さらに日本との友好を深めるために努力を重ねています. 一方これらの研究は地球のグローバル化, 温暖化, 環境・食糧事情の悪化が進む中で, 日本人の医療や QOL の維持にも, 将来大きく貢献するはずで



2012年2月 ケニア国キスム市の病院・研究施設前

Under assistance of Kenya Research Station, Inst. NEKKEN, Nagasaki Univ., we are analyzing toxins contaminating major local grains (maize, wheat) and milks, and also producer fungi. We found the local foods are contaminated by the toxins at concentrations far above the international standards. The result has been announced in newspapers, and received large attention. A new project for epidemiological study of Cryptococcal fungi in HIV-infected patients is launched in collaboration with Kenya Medical Res. Inst. (KEMRI) and doctors from UCSF, USA.

アスペルギルス症を中心とした新興真菌症制圧プロジェクト

The Project on Controlling Aspergillosis and the Related Emerging Mycoses

真菌症の中でもアスペルギルス症は特に多彩な病型をもち、重症度、経過、治療とも大きく異なっている。その中でも慢性肺アスペルギルス症では、症例ごとに活動性が異なるばかりか、同一症例においても経過中に急性増悪がみられることがあり、これらのさまざまな病原性をもった菌株のゲノムを比較することにより、病原因子や病原機構の解明に結びつくことが期待される。

慢性肺アスペルギルス症（CPA: 菌球型を含む）及び侵襲性肺アスペルギルス症（IPA）のそれぞれ4症例から得られた *Aspergillus fumigatus* について genome sequencer (Illumina, MiSeq) を用いて菌のゲノム配列を比較したところ、ゲノム株 (Af 293) のゲノム配列と比較して 81 826 の SNPs が見つかり、このうち CPA 間で共通の非同義置換は 7 個、IPA では同じく 27 個であった。また機能不明遺伝子 Afu3g05880 に内在する tandem repeat は、CPA では、より少ない一定の繰り返し数を示し、IPA とは異なることが示された。今後、これらの遺伝子の機能を探るとともに、ゲノム配列の解析を進め、菌が CPA と IPA という異なる病態をとる原因に迫りたい。並行して *A. fumigatus* の group sIII histidine kinase (NikA) の機能解析、新しいレクチンの発見 (*A. fumigatus* L-Fucose specific lectin: AFL など)、*A. fumigatus* に感染する mycovirus 等の新しい知見を得た。病原機構の解明、治療法開発に向けて研究を進める予定である。

Aspergillosis is known to take various forms, and the severity, clinical course, and treatments differ significantly depending on the forms. Chronic necrotizing pulmonary

aspergillosis (CNPA) is one example: the activity of the disease varies among the patients, and sometimes an acute exacerbation results in serious outcome. Genetic analyses of the causative fungi isolated from the patients of various forms/activities are expected to help clarify the mechanism of their pathogenicity.

To this end we performed the whole genome sequence (WGS) analysis of eight strains isolated from patients with IPA (invasive pulmonary aspergillosis) and CNPA in Japan with the aid of a next-generation sequencer. 81 826 SNPs were identified by mapping the reads to *A. fumigatus* genome reference strain Af293. Of the identified SNPs, seven and 27 non-synonymous substitutions were specific to strains isolated from patients with IPA and CNPA, respectively. Moreover, the tandem repeat sequence in 5'-UTR of Afu3g05880 was found as a novel microsatellite region for differentiating between *A. fumigatus* strains isolated from patients with IPA and CNPA. We expect comprehensive characterization of genetic variations of strains isolated from patients with IPA and CNPA helps better understanding of molecular mechanisms in aspergillosis.

We also performed molecular and cellular biological research of *A. fumigatus*, and reported in a paper a novel pathway of group III histidine kinase (NikA). We have also obtained new results on lectins of *A. fumigatus*, and mycoviruses which infect the fungus. These results will be published soon.

平成 24 年度 共同利用・共同研究報告

2012 Fiscal Year Cooperative Research Program Report

研究課題 12 - 1

クリプトコックスの低酸素ストレス応答機構解析～低酸素応答遺伝子として見出した「転写因子 A」の分子機能解析を中心として～

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Hypoxia signaling analysis of *Cryptococcus neoformans*

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研究成果

病原酵母 *Cryptococcus neoformans* は、生存に酸素が必須

な偏性好気性真菌であり、本菌は、肺で感染後、脳血液関門を越え脳髄膜へ移行して病原性を発揮して行く際、高酸素環境から低酸素環境への酸素欠乏ストレス条件に打ち勝ってはじめて増殖し、病原性を発揮して行く。すなわち、本菌の低酸素環境ストレス応答は、本菌感染の病原因子の一つと言える。我々は、本菌の細胞周期制御機構を研究中、低酸素環境条件下では細胞周期制御が flexible になるという、本菌のユニークな低酸素ストレス応答現象を見出した。そして、我々は、*Agrobacterium* を用いた、ゲノムランダム挿入遺伝子変異体ライブラリーを構築してスクリーニングし、低酸素応答遺伝子として、これまでに「転写因子 A」を見出して来たが、平成 24 年度には、「転写因子 A」の分子機能解析を更に詳細に進めた。

C. neoformans の低酸素応答遺伝子「転写因子 A」は、カルシニューリン応答 (Crz1) 転写因子、及び、PKC1-依存性 (Sp1) 転写因子とに homologous な分子であり、C 末端近くに、いわゆる、Zinc-finger を 3 つ持つ転写因子の一つ「Crz1/Sp1」であった。また、低酸素環境下、本菌が G2 arrest 状態に入るために必須な遺伝子として同定した本分子は、低酸素環境下での増殖の slowdown の他、本菌の細胞壁の integrity の維持、莢膜合成の抑制的な制御、バイオイラム生成の促進、フルコナゾール感受性、カルシウム感受性など、本菌の感染にも関連した種々の細胞生理機能に重要な役割を持つ分子であることを明らかにした。真菌の低酸素応答シグナリングに転写因子「Crz1/Sp1」が重要な役割を担っていることは、我々が *C. neoformans* で初めて見出し報告したが、更に、本菌における低酸素応答転写因子「Crz1/Sp1」のシグナル伝達下流分子の探索などを行って、*C. neoformans* の低酸素ストレスに対する環境応答シグナリング機構全体の解明を目指すとともに、本菌病原性への本分子の寄与を検討、考察しつつある。

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研究課題 12 - 2

新規抗真菌薬開発を目指したアスペルギルス属糸状菌の薬剤耐性機構とシグナル伝達機構の解析

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Molecular analyses of mechanisms of drug resistance and signal transduction leading to novel antifungal drug discovery in *Aspergillus* fungi

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研究成果

A. fumigatus の薬剤耐性化機構については、薬剤の標的分子や排出系の変異によることが示唆されているものの、遺伝子レベルでの詳細な解析がほとんどなされていない。一方、*A. fumigatus* はヒト粘膜上での感染と生育を示すことから、このような固相表面における生育に関わる環境応答系が重要な機能を持っていると考えられる。そこで、本研究では *A. fumigatus* に対する効果的な新規創薬開発に資するため、薬剤耐性化の分子機構の解明ならびに浸透圧シグナル伝達系の機構解明を目指した。

我々は麹菌においてアゾール系薬剤の排出に関与する ABC トランスポーターの発現を制御する新規 Zn₂Cys₆

型転写因子 AtrR を世界で初めて見出し、*A. fumigatus* においてもこのオーソログが同様にアゾール耐性に関与していることを明らかにした。*A. fumigatus* の *atrR* 破壊株の RNA-seq 解析を行ったところ、AtrR はトランスポーター遺伝子だけでなく、エルゴステロール生合成酵素遺伝子の発現にも関わっていることが示された。非常に興味深いことに、AtrR 制御下のエルゴステロール生合成酵素遺伝子は、すでに報告のある bHLH 型転写因子 SrbA 制御下のものと一致していたことから、エルゴステロール生合成酵素の発現は AtrR と SrbA の 2 種類の転写因子によって協調的に制御されている可能性が強く示唆された。また、アゾール系薬剤耐性に関与していると考えられる ABC トランスポーター (AbcC/Cdr1B) の遺伝子は SrbA 制御下になく、AtrR 単独による制御を受けていると考えられた。

一方、*A. nidulans* の二成分性情報伝達系の生育必須因子であるリン酸基中間因子遺伝子 *ypdA* の発現制御株 (*CypdA*) と、下流 response regulator 遺伝子 *sskA* および *srrA* の欠損を加えた *CypdAsskAΔ*, *CypdAsrrAΔ*, *CypdAsskAΔ srrAΔ* 株を育種した。*ypdA* の発現抑制により、*ypdA* の転写量と蛋白質発現量が野生株比 5% 以下となった。*ypdA* の発現抑制は、フルジオキシニル処理と類似する致死をもたらしたが、*sskAΔ*, *srrAΔ* の導入は生育を部分的に回復させ、*sskAΔ srrAΔ* の導入は生育を完全に回復させた。*CypdAsskAΔ srrAΔ* の生育は回復したが、隔壁間長が長くなる *sskAΔ* 変異の形質を残していた。

研究課題 12 - 3

新規性の高い抗真菌薬標的の探索と阻害剤のアッセイ系の開発

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Searching for anti-fungal drug targets having high novelty and developing of an assay system for screening the repressors

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研究成果

Saccharomyces cerevisiae, *C. albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans* の真菌類およびヒトゲノムのデータベースを用いて、最適な標的の条件として 1) ヒトに相同性がないか、あるいは極めて低いこと、2) ゲノム配列が公開されている病原真菌に高く保存されていること、3) *in vivo* で必須であること、以上の 3 点に条件として薬剤標的に探索を進めている。まず、条件 1) と 2) を満たす遺伝子を *Candida glabrata* のゲノムから抽出した。*C. glabrata* は、病原真菌の中で遺伝子操作が最も容易であるため、我々は本菌を病原真菌の実験モデル生物と位置づけ、ゲノムワイドな遺伝子機能解析を進めている。*C. glabrata* のゲノムから抽出した遺伝子のうち 100 遺伝子について Tet 株を構築することができた。Tet 株は、テトラサイクリンを添加した培地において、Tet promoter が挿入された遺伝子の転写が抑制される。したがって、Tet 株が、テトラサイクリンに対して高い感受性を示すことを基準にすることにより各遺伝子が生育に必須か否かを判定することができる。さらに標的分子あるいはその遺伝子が感染時に *C. glabrata* にとって必須であることを検証することは標的分子を絞り込む上で極めて重要である。Tet 株を感染させた場合には、蚕幼虫

の餌にテトラサイクリンを添加することによって Tet 株中の当該遺伝子の発現を抑制することができた。最終的に抗真菌薬の標的として 5 つに絞ることができた。今後これらの株を用いてマウスを用いた感染実験検証、組換えタンパク等を用いて阻害剤のアッセイ系を開発して行きたい。

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研究課題 12 - 4

真菌感染防御における IL-17 産生機構に関する研究

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Studies on the production of IL-17 in immune responses against fungal infection

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研究成果

IL-17A は主に活性化 CD4⁺T 細胞から産生され、細胞外寄生菌に対する感染防御や自己免疫疾患の発症において中心的な役割を担っている。申請者らは、マウス骨髄由来樹状細胞 (BMDC) を *Candida albicans* (*C. albicans*) で刺激し、その培養上清が naïve T 細胞を Th17 細胞に分化させることを見出した。一方、IL-17A ノック

クアウトマウスに *C. albicans* を感染させると野生型 (WT) マウスと比較し生存率が有意に低下することを明らかにした。しかし最近、CD4⁺細胞以外にも IL-17A を産生する細胞が見出され、Th17 と他の IL-17 産生細胞との機能の違いが注目されている。

そこで、まずマウスに *C. albicans* を感染させ in vivo でも IL-17A が産生されるかどうかを検討したところ、感染後比較的早期に IL-17A が産生されることが明らかとなった。今後は、ノックアウトマウスやノックインマウスと細胞移植の系などを組み合わせ、どの細胞から分泌される IL-17A が感染防御に重要な役割を担っているのかを明らかにする予定である。

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研究課題 12 - 5

真菌性肺炎の発症機序の解明

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研究成果

気道や腸管上皮、また、皮膚表皮では、細胞と細胞の間が様々な接着分子を介して連結され (tight junction), 外来抗原の侵入を防いでいる。その tight junction の破壊に、外来および内在性の protease が関与していることが示されつつある。真菌 *Aspergillus* による肺炎は、*Aspergillus* 自体の感染による免疫応答だけでなく、*Aspergillus* が産生する protease による宿主の気道上皮の破壊が発症及び症状の重症化に関わっている可能性が強く示唆されている。実際に、マウスに *Aspergillus* 由来の protease を吸入させると、組織破壊と好中球の浸潤を伴う強い気道炎症が認められた。さらに、各種欠損マウスを用いた結果、*Aspergillus* protease による好中球性気道炎症は、T 細胞、B 細胞、NKT 細胞、マスト細胞非依存的であることが明らかになった。*Aspergillus* protease による好中球性気道炎症局所では、IL-17, IL-17F, IL-21, IL-23, IL-6, TNF といった Th17 型サイトカインおよび誘導因子の発現の亢進が認められ、これらサイトカインが好中球性気道炎症の誘導に関与することが期待された。しかしながら、これらのサイトカイン欠損マウスでも *Aspergillus* protease による好中球性気道炎症は正常マウスと同程度に誘導されることが明らかになった。現在、これらサイトカイン以外に、好中球性気道炎症の誘導に関わる因子の同定を進めている。

研究課題 12 - 6

真菌による重症喘息発症機構の解明: *Schizophyllum commune* 応答を中心として

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Roles of Fungi in the Development of Severe Asthma: Possible roles of the immune responses against *Schizophyllum commune*

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研究成果

真菌への暴露, 感作は喘息の重症化に関与することが知られている. スエヒロタケ (SC) は喘息, アレルギー性気管支肺真菌症 (ABPM) の発症に関与することが報告されている真菌だが, これまでに SC 特異的抗体の効率的なスクリーニング方法の報告は無く, 喘息患者における SC 感作率は不明である. 本研究では ELISA 法による SC 特異的抗体のスクリーニング方法を確立し, 喘息患者における SC 感作率を検討するとともに, 喘息重症度との相関を明らかにすることを目的とした.

まず SC による ABPM 患者血清を用いて主要抗原を探索・同定した. この主要抗原を用いて SC 特異的抗体測定 ELISA 法を確立し, 喘息患者における感作率を検討した. 47 名の喘息患者 (Step2 6 名, Step3 29 名, Step4 12 名) 中, 4 名が SC 特異的 IgG 陽性, 6 名が SC

特異的 IgE 陽性であることが明らかとなった. SC 特異的抗体陽性喘息患者はアスペルギルスにも感作されている傾向が見出されたが, SC 特異的抗体価はアスペルギルス特異的抗体価との相関性は認められなかった. これらの結果から喘息患者の一部は SC に感作が成立していることが明らかとなった.

研究課題 12 - 7

病原性担子菌酵母糖タンパク質糖鎖の構造解析

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Structural analysis of glycoprotein sugar chains in pathogenic Basidiomycete yeast.

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研究成果

病原性担子菌酵母を含む真核生物において多くの糖タンパク質には糖鎖が付加しており, その糖鎖構造は多様である. 非病原性モデル子囊菌酵母である出芽酵母や分裂酵母では糖鎖構造および糖鎖生合成関連遺伝子が報告されている. また, 病原性子囊菌酵母である *Candida albicans* および *Aspergillus fumigatus* では糖鎖生合成関連遺伝子の機能不全変異により病原性の低下が報告されている. 一方, 病原性担子菌酵母においては *Cryptococcus neoformans* を除いて, 糖鎖生合成関連遺伝子の解析どころか, 糖鎖構造情報はほとんど蓄積されていない. 前年度までに代表的な病原性担子菌酵母である *C. neoformans*, *Rhodotorula mucilaginosa* および *Malassezia furfur* の糖鎖構造解析に着手し, 特に O-結合型糖鎖について構造情報を得ていた. 本年度はさらに上述の 3 種の病原性担子菌酵母の N-結合型糖鎖の構造解析を行った.

各病原性担子菌酵母を培養後, ヒドラジン分解-ピリジルアミノ化法を用いて, ピリジルアミノ化糖鎖を調

製後、サイズ分画 HPLC、逆相 HPLC および MALDI-TOF-MS による解析を行った。その結果、*C. neoformans* においては Hex₇₋₉PentGlcNAc₂ の糖鎖が主要な糖鎖として検出された。*R. mucilaginosa* については、サイズ分画 HPLC による分析が終了し、Hex₅₋₇GlcNAc₂ の糖鎖が主要な糖鎖として検出された。*M. furfur* についても同様にサイズ分画 HPLC、逆相 HPLC によるピリジルアミノ化糖鎖の分画を行い精製糖鎖を得た後、LC-ESI-MS/MS による解析を行った。主要な糖鎖について、標準糖鎖と両 HPLC にて溶出位置が一致する糖鎖が検出され、LC-ESI-MS/MS の結果を考慮し以下の様に構造決定することができた；Man α 1,2Man α 1,6 (Man α 1,3) Man α 1,6 (Man α 1,2Man α 1,2Man α 1,3) Man β 1,4GlcNAc β 1,4GlcNAc (M8A), Man α 1,2Man α 1,6 (Man α 1,3) Man α 1,6 (Man α 1,2Man α 1,3) Man β 1,4GlcNAc β 1,4GlcNAc (M7A), Man α 1,2Man α 1,6 (Man α 1,2Man α 1,3) Man α 1,6 (Man α 1,2Man α 1,3) Man β 1,4GlcNAc β 1,4GlcNAc (M8B), Man α 1,2Man α 1,6 (Man α 1,2Man α 1,3) Man α 1,6 (Man α 1,2Man α 1,2Man α 1,3) Man β 1,4GlcNAc β 1,4GlcNAc (M9A)。本研究の結果により、本 3 種病原性担子菌酵母糖鎖の基礎情報が取得できた。これらの情報を元に将来的には糖鎖生合成関連酵素遺伝子の同定、本酵素の阻害剤の探索の効率化が達成され、効果的な抗真菌剤の開発へと発展することが期待される。

研究課題 12 - 8

リボソームタンパク質をバイオマーカーとした質量分析法による真菌類の同定・分類法の開発

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((独) 産業技術総合研究所 環境管理技術研究部門)

Development of method for classification and identification of fungi based on the profiling of ribosomal proteins as observed by mass spectrometry

Takashi Yaguchi, Reiko Tanaka

(Medical Mycology Research Center, Chiba University)

Hiroaki Sato, Masaki Torimura

(National Institute of Advanced Industrial Science and Technology)

研究成果

生物種の同定・分類は基準株との比較で行われるため、*A. fumigatus* 基準株 (IFM 47795 = Af293) の精製リボソームタンパク質の質量分析を行い、基準株のリボソームタンパク質の正確な分子量リストを作成することを目的とした。試料菌株をビーズ破砕し、超遠心分離機 (真菌医学研究センター所有) を用いてリボソームを抽出し、マトリックス支援レーザー脱離イオン化質量分析計 (MALDI-TOFMS, 産総研所有) を用いて各リボソームタンパク質のマスペクトルを観測した。各リボソームタンパク質の正確な分子量を解析し、翻訳アミノ酸配列から計算される分子量との差異を比較して翻訳後修飾の有無を明らかにした。その結果、全ゲノム解読された *A. fumigatus* および類縁の *Neosartorya fischeri* の計 3 株について、分類の基準となるリボソームタンパク質の正しい分子量リストを作成した。さらに、ゲノム解読株間で、一部のリボソームタンパク質に変異が生じていることを実証し、*A. fumigatus* 類縁菌の同定及び株レベルでの識別が、本法によって可能であることを見出した。

今後、DNA 解析法で得られた分類結果や形態および薬剤耐性などの特徴と比較することによって、本法の妥当性を実証するとともに、得られたマスペクトルをデータベース化し、真菌の新しい分類指標を提示することが期待できる。

平成 24 年度 共同利用・共同研究研究会報告

2012 Fiscal Year Cooperative Research Meetings Report

研究会 - 1

千葉大学感染症研究ネットワーク 第 1 回研究会

山本友子・高屋明子・佐藤慶治
(千葉大学大学院薬学研究院)
米山光俊・川本 進・亀井克彦
(千葉大学真菌医学研究センター)
北 潔 (東京大学大学院医学研究科)

Chiba University Research Network on Infectious Diseases

Tomoko Yamamoto, Akiko Takaya, Yoshiharu Sato
(Department of Microbiology and Molecular Genetics,
Graduate School of Pharmaceutical Sciences, Chiba
University)
Mitsutoshi Yoneyama, Susumu Kawamoto, Katsuhiko
Kamei
(Medical Mycology Research Center, Chiba University)
Kiyoshi Kita
(Graduate School of Medicine and Faculty of Medicine,
The University of Tokyo)

研究成果

千葉大学の学内には、感染症研究にかかわる医学部、

薬学部、附属病院、融合科学研究科などに数多くの研究者がおり、それぞれが活発に研究を進めている。しかしそれらの連携は意外なほど少なく、ネットワーク化が期待されていた。そこで、これら千葉大学の学内各部局における感染症研究の統合、さらには活性化を進めることを目的として今年度から本センターの共同利用・共同研究会として「千葉大学感染症研究ネットワーク」を企画した。第 1 回目となる今回は平成 24 年 6 月 23 日 (土) に千葉大学薬学部大会議室 (医薬系総合研究棟Ⅱ地下 1 階) にて開催した。学内の感染症研究グループから計 8 題の一般演題が発表され、これに引き続き、北 潔教授 (東大医学研究科) から「感染症研究が結ぶ世界の絆」と題した特別講演をいただいた。この種の学内ネットワークを目指した会合は今回が初めての試みであったが、参加者は 63 名に達した。

全体を通じて非常に活発な議論が行われ、学内各部局における感染症研究の統合、活性化という目的は十分に達成されたものと考えられた。今後更に発展させるべく計画を進めている。

2013 年講演会

2013 Scientific Meetings & Seminars

1) 第 125 回 真菌医学研究センター講演会

Dr. Jan Schmid (Massey University, Palmerston North, New Zealand)

“The biological significance of sex and protein-coding DNA repeats in *Candida albicans*”

日時: 8 月 27 日 (火) 15 時～

場所: 真菌医学研究センター B1 講堂

2) 第 3 回感染免疫応答セミナー (主催: COE スタートアッププログラム「感染免疫応答」, 共催: 博士課程教育リーディングプログラム)

猪原直弘博士

Naohiro Inohara

(University of Michigan Medical School)

“*C. difficile* 感染症重症化を防ぐ薬剤と自然免疫の役割～マウスモデルからの示唆”

日時: 平成 25 年 9 月 20 日 (金) 18 時 30 分～

場所: 医学部本館 2 階 大カンファレンスルーム

3) 平成 25 年国立大学附置研究所・センター長会議 第 2 部会シンポジウム (主催: 国立大学附置研究所・センター長会議 第 2 部会, 後援: 千葉市, 日本感染症学会, 日本医真菌学会)

講演: 亀井克彦教授

Katsuhiko Kamei

“カビによる病気と診断・治療の現状”

西城 忍 特任准教授

Shinobu Saijo

“カビの病気を防ぐ免疫のしくみ”

高橋弘喜准教授

Hiroki Takahashi

“ゲノム情報から読み解くカビの病気”

日時: 平成 25 年 10 月 25 日 (金) 10 時～12 時

場所: 京成ホテルミラマーレ 6 階ローズルーム



4) 第 2 回感染症研究グローバルネットワークフォーラム 2013 (主催: 千葉大学真菌医学研究センター 共同利用・共同研究拠点事業, 特別推進研究「病原細菌の自然免疫克服戦略の解明とその応用」, 基盤研究 A「エフェクターと宿主標的分子間相互作用を基盤としたサルモネラ感染分子機構の解明」)

特別講演:

金城雄樹

(国立感染症研究所 室長)

Yuki Kinjo

“感染免疫における iNKT 細胞の役割”

常世田好司

(German Rheumatism Research Centre Berlin, Department Head)

Koji Tokoyoda

“感染を記憶する骨髄”

飯田哲也

(大阪大学微生物病研究所 教授)

Tetsuya Iida

“次世代 DNA シーケンサを用いた感染症研究”

荒川宜親
(名古屋大学大学院医学系研究科 教授)
Yoshichika Arakawa
“新型多剤耐性グラム陰性菌が獲得した耐性機構と
それらの地球規模での拡散”

一般講演:

石和田稔彦
(千葉大学医学部附属病院 講師)

Naruhiko Ishiwada
“小児細菌性髄膜炎予防ワクチンと日本への導入効果
に関する研究”

西村 基
(千葉大学医学部附属病院/医学研究院 助教)

Motoi Nishimura
“臨床細菌検査の動向－質量分析計による最近同定
など－”

野呂瀬一美
(千葉大学医学研究院 助教)

Kazumi Norose
“トキソプラズマ性網脈絡膜炎の病態解析－ケモカ
インとT細胞の動態－”

星野忠次
(千葉大学薬学研究院 准教授)

Tyuji Hoshino
“2価金属原子対を標的とした抗ウイルス薬の開発”

神田達郎 (千葉大学医学研究院 講師)
Tatsuo Kanda
“B型肝炎ウイルスに対する肝細胞自然免疫応答”

西城 忍
(千葉大学真菌医学研究センター 准教授)
Shinobu Saijo
“C型レクチンと真菌感染防御機構”

西田篤司
(千葉大学薬学研究院 教授)
Atsushi Nishida
“アレルギー性気管支肺真菌症患者から単離された
Schizophyllum commune (和名: スエヒロタケ) が生産
する化学物質”

日時: 平成 25 年 11 月 30 日 (土)

9 時 30 分 ~ 18 時 10 分

場所: 千葉大学薬学部 120 周年記念講堂

5) 第 4 回感染免疫応答セミナー (主催: COE スタート
アッププログラム「感染免疫応答」, 共催: 博士
課程教育リーディングプログラム)

Glen N. Barber 博士
(University of Miami, School of Medicine)
“Cytosolic DNA sensors, STING and Innate immunity”

日時: 平成 25 年 12 月 6 日 (金) 17 時~

場所: 医学部本館 1 階 第 1 講義室



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