Xp-1

Contributions of gliotoxin to *Aspergillus fumigatus* virulence

JinWoo Bok[1], DaWoon Chung[1], Arunmozhi Balajee[2], Kieren Marr[2], David Andes[1], Jens Frisvad[3], Kristian Fog Nielson[3], Nancy P Keller[1*]

[1] University of Wisconsin-Madison, Madison, WI, U.S.A
[2] University of Washington, Seattle WA, U.S.A.
[3] Center for Microbial Biotechnology, Technical University of Denmark, Lyngby, Denmark

Gliotoxin is a non-ribosomal peptide produced by *Aspergillus fumigatus* and other fungi. This compound has been proposed as an *A. fumigatus* virulence factor due to its cytotoxic, genotoxic and apoptosis stimulating properties. Recent identification of the gliotoxin gene cluster identified several genes (e.g. gligenes) likely involved in gliotoxin production including gliZencoding a putative C2H2 zinc binuclear transcription factor. Both silencing of gliZexpression using RNAi technology and replacement of gliZwith a marker gene decreased gliotoxin production and gene expression of other glicluster genes. However, production of other metabolites was also affected by gliZdisruption. Placement of multiple copies of gliZin the genome increased gliotoxin production. The murine pulmonary model indicated a decrease in virulence and loss of gliotoxin production in gliZmutant. Supernatants of the gliZmutant and wildtype showed similar rates of necrotic kill of neutrophil cells, however it appeared that the gliZmutant had lost the ability to trigger apoptosis in these cells. This supports a role for gliotoxin in apoptosis but also indicates other *A. fumigatus* factors, possibly additional toxins, contributed to death by necrosis.

Comparative proteomics of the human-pathogenic fungus *Aspergillus fumigatus*

Olaf Kniemeyer[1*], Daniela Albrecht[2], Olaf Scheibner[3], Reinhard Guthke[2], Axel A. Brakhage[1]

[1] Department of Microbiology and Molecular Biology, Friedrich-Schiller-University Jena and Department of Molecular and Applied Microbiology, Leibniz-Institute for Natural Product Research and Infection Biology (HKI), 07745 Jena, Germany
[2] Department of Molecular and Applied Microbiology, Leibniz-Institute for Natural Product Research and Infection Biology (HKI), 07745 Jena, Germany
[3] Department of Biostructure Chemistry, Leibniz-Institute for Natural Product Research and Infection Biology (HKI), 07745 Jena, Germany

Aspergillus fumigatus is the most important airborne fungal pathogen causing life-threatening infections in immunosuppressed patients. During the infection process *A. fumigatus* has to cope with dramatic changes of the environmental conditions. An interesting questions is whether *A. fumigatus* possesses a higher stress resistance and better adaptation mechanisms compared with other filamentous fungi. To obtain a comprehensive overview about the proteins produced at different physiological conditions that are related to the infectious process a proteomic approach has been applied. Firstly, 2-D gel electrophoresis for filamentous fungi was optimised concerning removal of interfering compounds, protein extraction and separation methods. In a first approach, up- and down-regulated proteins related to heat stress were analysed by applying the 2D-fluorescence difference gel electrophoresis technique (DIGE). During a temperature shift from 30 °C to 48 °C many stress and heat shock proteins were up-regulated. These data were compared with microarray data from a public database [1]. In addition, a database for integrated storage and visualisation of genome, transcriptome and proteome data of *Aspergillus fumigatus* was established based on Protecs (Decodon GmbH, Greifswald). The database enables an integrated analysis of both transcriptome and proteome time series data. The time series data were also investigated by an optimised reverse engineering method [2] to reconstruct the underlying gene regulatory network.

[1] http://www.ebi.ac.uk/arrayexpress; Acc.-No. E-MEXP-332
[2] Guthke R *et al.* (2005): *Bioinformatics*, 21, 1626-1634.

Xp-3

Small molecules with big impacts: roles of fungal siderophores in virulence, asexual and sexual development

Shinichi Oide[1*], Stuart Krasnoff[2], Hubertus Haas[3], Donna Gibson[2], Gillian Turgeon[1]

[1] Dept. of Plant Pathology, 334 Plant Science Bldg., Cornell Univ., Ithaca, NY, 14853, USA
[2] Plant Protection Research Unit USDA-ARS, Tower Road, Ithaca, NY, 14853, USA
[3] Dept. of Molecular Biology, Medical Univ. Innsbruck, A-6020, Innsbruck, Austria

Iron is an essential nutrient for virtually all organisms. In this study, we report the diverse roles that siderophores (microbial iron-chelators) play in fungal developmental biology. *NPS6*, encoding a non-ribosomal peptide synthetase (NRPS) is a virulence determinant in the corn pathogen *Cochliobolus heterostrophus* (*Ch*; Lee *et al.*, 2005). Deletion of *ChNPS6* causes reduction in virulence and hypersensitivity to H2O2. *ChNPS6* is also specifically up-regulated under iron-depleted conditions, and the *Chnps6*-deletion strain shows hypersensitivity to iron depletion, suggesting a role in iron metabolism. MS/HPLC analyses demonstrated that *ChNPS6* is responsible for biosynthesis of the extracellular siderophore, coprogen. Deletion of *NPS6* orthologs in the crucifer pathogen, *Alternaria brassicicola* (*Ab*) and the wheat pathogen, *Fusarium graminearum* (*Fg*) result in the same suite of phenotypic defects. Phylogenetic analyses of fungal NRPSs predict another *Ch* NRPS-encoding gene, *ChNPS2*, is involved in siderophore biosynthesis (Lee *et al.*, 2005). In contrast to *ChNPS6*, deletion of *ChNPS2* does not affect virulence, sensitivity to H2O2 or to iron depletion. The Chnps2Chnps6 double-deletion strain, however, shows further attenuated virulence, increased sensitivity to H2O2 and to iron depletion, compared to *Chnps6*-deletion strain, indicating that *ChNPS2* also plays a role in iron metabolism and virulence. Similar phenotypic observations are found for the *Abnps2*-deletion and the *Abnps2Abnps6* double-deletion strains. MS/HPLC analyses showed that *ChNPS2* is responsible for production of the intracellular siderophore, ferricrocin. In addition to a virulence role, these siderophores play key roles in sexual/asexual development. The *Chnps6*- and *Abnps6*-deletion strains show reduction in asexual sporulation under nutrient poor conditions. Deletion of *NPS2* in *Ch* and *Fg* causes defects in ascospore, but not pseudothecium/perithecium development. Although deletion of *ChNPS6* alone does not affect fertility, double deletion of *ChNPS2* and *ChNPS6* leads to absence of pseudothecia. Exogenous application of iron or ferricrocin restores WT fertility, demonstrating the essential role of iron and siderophores in sexual development. To confirm our findings, we characterized the *SidA* gene encoding L-ornithine-*N*-monooxygenase, responsible for the first committed step of fungal siderophore biosynthesis. *ChSidA1* deletion leads to hypersensitivity to H2O2 and iron depletion, and loss of pseudothecia. Unexpectedly, the *ChsidA1*-deletion strain is similar in virulence to the *Chnps6*-deletion strain, rather than the *Chnps2Chnps6* double-deletion strain, and the latter is slightly more sensitive to iron depletion than the *ChsidA1*-deletion strain. This inconsistency led to discovery of an, as yet uncharacterized, second *SidA*-like gene (*ChSidA2*) in *Ch*. Overall, our data demonstrate the impact of siderophores on fungal development, including pathogenicity, sexual and asexual reproduction.

Identification of novel genes that are differentially expressed in the thermally dimorphic human pathogen *Penicillium marneffei*

David Canovas*, Luke Pase, Alex Andrianopoulos

Department of Genetics, University of Melbourne, Victoria 3010, Australia

Penicillium marneffei is capable of alternating between a hyphal and a yeast growth forms, known as dimorphic switching, in response to a temperture signal. At 25ºC, *P. marneffei* grows in a filamentous form which resembles other saprophytic *Penicillium* species. Hyphal cells grow by apical extension and branching, they are separated by incomplete septa, and are usually multinucleated. A shift to 37ºC triggers the dimorphic switch. The process, known as arthroconidiation, involves the coupling of nuclear division with cell division to yield shorter, uninucleate cells which are separated by double septa. Subsequently, the cell wall material between the double septa is degraded and unicellular yeast cells released. At 37ºC, the growth form is an elongated unicellular and uninucleated yeast, which divides by fission. In addition to dimorphic switching, *P. marneffei* can also undergo asexual sporulation (conidiation) at 25ºC whereby conidia are produced by multicellular conidiophores. The different cell types produced during the two developmental programmes are strictly controlled. A number of genes, including transcriptional regulators, small GTPases and heterotrimeric G-proteins have been found to play a role during conidiation and/or dimorphic switching in *P. marneffei*. In order to identify new genes involved in these two processes, we have screened for genes which are differentially expressed in the various stages of these developmental programmes by using DNA microarrays. We have constructed slides containing an array of 5,000 clones originated from a random genomic DNA library. To identify genes expressed at the different stages of those developmental programmes, target cDNAs from vegetative growth at 25ºC, conidiation at 25ºC and yeast growth at 37ºC have been made. In addition, cDNA representing shorter time points during the temperature shifts (from 25 to 37ºC and viceversa) will allow the identification of early regulators in the process of dimorphic switching. Those clones that are differentially expressed are further identify by DNA sequencing.

Xp-5

Gene expression analysis of *Trichophyton rubrum* responses to cytotoxic drug challenge

Fernanda Gonzales Paião*, Jeny Rachid Cursino-Santos, Fernando Segato, Nalu Teixeira de Aguiar Peres, Nilce Maria Martinez-Rossi

Departamento de Genética da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo

Cytotoxic agents that interfere with several cellular functions are commonly used to control infections, but their mechanisms of action are often poorly understood. Dermatophytosis is an infectious disease caused by a closely related group of pathogenic molds such as *T. rubrum*, that represents the most prevalent fungus isolated from skin and nail lesions of humans. However, little information is available about the biology of this fungus. In the present study, we investigated the changes in the gene expression profile of *T. rubrum* following exposure to acriflavine, fluconazole, griseofulvin, terbinafine and undecanoic acid, which represent different classes of antifungal agents. The aim of this study was to contribute to a better understanding of the mechanism of action of these agents and to detect genes of *T. rubrum* which might be involved in the general stress response. Using a suppression subtractive hybridization (SSH) approach, we isolated transcripts which were differentially expressed during the exposure of *T. rubrum* to the above mentioned cytotoxic agents. An array of 144 clones from the subtractive library was screened by reverse Northern blotting, using several probes obtained from exposure of *T. rubrum* to each one of the cytotoxic agents under study. Sequence analysis of the 144 clones obtained resulted in 54 unique sequences. BLAST X search showed 30 ESTs without similar proteins in the databases, possibly representing novel genes or *T. rubrum* exclusive genes. 24 ESTs with similar proteins in the database were identified as being involved in different cellular processes such as cell differentiation, cell cycle, metabolism and resistance mechanisms. Most of the ESTs with a deduced identity show similarities with hypothetical proteins whose functions are unknown. This is the first large-scale comprehensive search for *T. rubrum* genes which respond to the exposure to these cytotoxic agents and suggests that a subtractive cDNA library may be very useful in the process of antifungal drug development.

Financial support: FAPESP, FAEPA, CNPq and CAPES

Full virulence of the dermatophyte *Trichophyton rubrum* is dependent on transcription factor PacC

Henrique C.S. Silveira[1*], Mônica S. Ferreira-Nozawa[2], Carlos J. Ono[2], Diana E. Gras[1], Antônio Rossi[2], Nilce M. Martinez-Rossi[1]

[1] *Departamento de Genética, FMRP-USP*
[2] *Departamento de Bioquímica e Imunologia, FMRP-USP, Ribeirão Preto, Brazil*

Dermatophytosis is commonly caused by fungi that parasite the human skin and nails. Although several factors contribute to their virulence, adaptive responses to environmental pH signaling have also been evidenced. The functionality of gene *pacC*, which encodes a protein that is homologous to the PacC/Rim101p family of pH signaling transcription regulators, was examined in *T. rubrum* for the ability of the H6 and *pacC-1* mutant strains to grow on nails *in vitro*. The disruption of gene *pacC* was checked by Southern analysis for the predicted disruption restriction fragments and by Western blot analysis, where the proteolytic processing of the PacC protein at pH 5.0 was shown. Moreover, disruption of gene *pacC* was correlated with a decreased ability of the mutant *pacC-1* to grow on human nail fragments as the only nutrition source. This result is consistent with thefact that keratinolytic proteases are under the regulation of gene *pacC*. Also, *T. rubrum* failed to grow on nail fragments in the presence of the protease inhibitor PMSF, in a clear indication that proteases with keratinolytic activity are indispensable for the utilization of nails as nutrition source. Furthermore, the growth of *T. rubrum* is dependent on the initial pH, with an apparent optimum at pH 4.0, although the pH of the medium changed during cultivation, reaching values that ranged from 8.3 to 8.9. The derepression of keratinolytic and nonspecific proteolytic enzymes with optimum activity at acid pH occurs during the initial stages of infection probably because human skin has an acidic pH. In addition, the metabolization by fungi of some amino acids, such as glycine, for example, causes the alkalinization of the growth medium, raising the pH to values as high as 9.0, an ambient in which most of the known keratinolytic proteases of dermatophytes have optimal enzymatic activity. This suggests that the secretion of keratinases with optimal activity at alkaline pH is required for the dermatophyte to complete its installation and to remain in the host. We therefore suggest that full virulence of *T. rubrum* depends on the action of protein PacC.

Financial support: FAPESP, FAEPA, CAPES and CNPq.

Xp-7

Gene regulation in the emerging fungal pathogen *Trichoderma longibrachiatum* during its growth in the presence of bronchial epithelial cells

Monika Schmoll[1], László Kredics[2*], Christina Kratzer[3], Zsuzsanna Antal[2], Christian P Kubicek[1]

[1] Research Area Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, TU Wien, Getreidemarkt 9-1665, A-1060 Vienna, Austria
[2] Microbiological Research Group, Hungarian Academy of Sciences and University of Szeged, P. O. Box 533, H-6701 Szeged, Hungary
[3] Department of Internal Medicine I, Division of Infectious Diseases and Chemotherapy, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria

The growing importance of human infections by filamentous fungi, many of which are resistant to currently used antimycotic agents, necessitates the investigation of the mechanisms of their pathogenicity and virulence. One species of the genus *Trichoderma*, *T. longibrachiatum* - which is a close relative of *Trichoderma reesei* (*Hypocrea jecorina*) used in biotechnology and agriculture - is also on the list of these potential pathogens. In order to identify genes which are involved in the attack of human cells, and which could serve as a target for design of new antimycotics, we initiated a screen for genes characteristically expressed during *Trichoderma* infection. To this end, an experimental system of simulated infection was developed and the method of Rapid Subtraction Hybridization (RaSH) used for the detection of genes differentially expressed between a clinical *T. longibrachiatum* isolate grown in confrontation with bronchial epithelial cells, and the same strain grown in BEGM (bronchial epithelial growth medium) alone. Consequently, 500 independent and putatively positive clones were screened and the differentially expressed clones sequenced. The close phylogenetic relationship to *T. reesei* is used for the identification of the full-length genes from the *T. reesei* genome sequence database. Among the genes found to be up-regulated under infection conditions there was a cyanoviridin-N-homologue, for homologues of which an antiviral effect has been shown, as well as a putative extracellular DNase, for which bacterial homologues with virulence promoting function are known. We also detected a member of the protein family of mannosyltransferases, which are required for virulence in *Candida albicans* as well as genes putatively involved in ATP/ADP translocation and ferric iron uptake.

Capsule biogenesis in *Cryptococcus neoformans*

Jan Grijpstra*, Hans de Cock

Microbiology, Department of Biology, Faculty of Science, Utrecht University Padualaan 8, 3584 CH Utrecht, The Netherlands.

The opportunistic human pathogen *Cryptococcus neoformans* can cause life-threatening meningo-encephalitis, also called cryptococcosis, because it can grow at 37°C and produces virulence factors. The polysaccharide capsule is one of its main virulence factors and is required to counteract the host-defence system. The capsule mainly consists of two distinct polysaccharides, glucuronoxylomannan (GXM), which makes up approximately 90% of the capsule and galactoxylomannan (GalXM), which, together with some mannoproteins, is the minor constituent of the capsule.

How capsular polysaccharides are synthesized, transported and assembled by the Cryptococ is largely unknown. Synthesis of GXM and GalXM requires a series of specific enzymes for each of these polysaccharides. The route for transport and assembly of GXM and GalXM, however, might be the same for both polysaccharides or, alternatively, be completely independent routes. Analysis of mutants affected in capsular biogenesis, as shown by India ink staining and specific reactions with monoclonal antibodies directed against GXM, might reveal whether the biogenesis of GXM and GalXM share overlapping routes.

Here, we have investigated whether mutations in the four cap-genes, previously identified by Kwon-Chung *et al.*, affect only GXM or both GXM and GalXM biogenesis by using dot blotting against intact cells with Polyclonal antisera directed against GalXM or mainly against GXM. Wild type H99 serotype A, its four isogenic derivatives with deletions in the cap-genes (*cap10, 59, 60* and *64* (G. Janbon)) and CAP67 serotype D mutant were used.

We show that antisera directed against GalGXMan of *Cryptococcus laurentii* (S. Bystrický) also reacts specifically with purified GalXM of C. neoformans serotype D. No GalXM could be detected on the cell surface of a CAP67 mutant, which is known to produce only GalXM, nor on the H99 Wt and its four D*cap* mutants.

The apparent absence of GXM on the cell surface of all the D*cap* mutants appears not to due to the lack of intact α(1-3)-glucan, the layer previously shown to be required for GXM attachment, since we show that GXM can re-associate to the cell wall of all four cap-mutants.

Xp-9

Functional analysis of essential genes in *Aspergillus fumigatus*

Gethin John Allen[1*], Mike Birch[2], Geoffrey Turner[1]

[1] *Department of Molecular Biology and Biotechnology, University of Sheffield, Firth court, Western Bank, Sheffield. S10 2TN*
[2] *F2G Ltd, PO Box 1, Lankro Way, Eccles, Manchester, M30 0BH*

Essential gene products are seen as potential targets for developing antifungal drugs, and genome wide screening methods to identify such genes have been devised in a number of laboratories. These have made use of random insertion approaches in diploids, Where failure to recover haploid segregants after haploidisation is considered evidence of essentiality.

Genes identified by such a procedure may also include those whose deletion leads to impaired growth, rather than no growth, since the latter could also result in failure to recover haploid mutant segregants.

In many cases bioinformatics can provide information about the probable function of such essential genes. However, often very little is known about apparent orthologuous genes, and orthologs may not have identical functionality in different species. Bioinformatics can be used to analyse the predicted primary sequences of gene products, but this may reveal very little about their functions. Establishing or confirming gene function therefore requires experimental analysis, including mutation. Since haploid mutants are often not viable, other approaches are needed for functional analysis of essential genes.

These include recovery of haploid spores carrying lethal deletions from balanced heterokaryons, use of conditional promoters like the *alcA* promoter (Romero *et al.*(2003) Fungal Genet Biol 40:103–114), and protein tagging.

A selection of putative essential genes have been analysed by downregulation after promoter exchange, and total deletion, the results and limitations of which will be discussed.

The triacetylfusarinine C esterase EstB is involved in utilization of siderophore-bound iron in *Aspergillus fumigatus*

Claudia Kragl*, Markus Schrettl, Beate Abt, Hubertus Haas

Division of Molecular Biology/Biocenter, Medical University Innsbruck, Fritz-Pregl-Str.3, A-6020 Innsbruck, Austria

Under low concentrations of the essential metal nutrient iron, *Aspergillus fumigatus* excretes desferri-triacetylfusarinine C (DF-TafC), a hydroxamate-type siderophore, to mobilize extracellular iron. DF-TafC is a cyclic peptide consisting of three N5-cis-anhydromevalonyl-N5-hydroxy-L-ornithine residues linked by ester bonds in contrast to peptide linkages found in most of the other hydroxamate-type siderophores. Subsequent to binding of iron and uptake, TafC is hydrolyzed, the cleavage products (fusarinines) are excreted, and the iron is transferred to the intracellular siderophore desferri-ferricrocin. We have identified and characterized the gene encoding the TafC-esterase, termed EstB. Expression of *estB* was SreA-mediated iron regulated, indicating a role in iron metabolism. HPLC-analysis demonstrated increased intracellular TafC accumulation and decreased TafC hydrolysis in the esterase-deficient *A. fumigatus* strain *ΔestB*. Cellular extract of *ΔestB* completely lacked TafC-esterase activity. Consistently, His-tagged and in *E. coli* expressed EstB showed specific TafC-esterase activity. However, EstB was not able to hydrolyze fusarinine C, which is distinguished from TafC by lacking three N2-acetyl residues. The *ΔestB* mutant strain showed delayed transfer of TafC-iron to desferri-ferricrocin and, in agreement, delayed iron sensing. Furthermore, *ΔestB* displayed a slightly decreased radial growth rate during iron-depleted conditions. Taken together, these data show that EstB plays a central role in the siderophore-mediated iron uptake and storage in *A. fumigatus*.

This work was supported by Austrian Science Foundation (grant FWF-P15959-B07).