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ROLE OF COMPONENTS OF G PROTEIN MEDIATED SIGNALING IN THE GERMINATION OF CONIDIA OF THE FILAMENTOUS FUNGUS ASPERGILLUS NIDULANS

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Our previous studies have shown that cAMP signaling mediates the response to the environmental carbon source during the early events of conidial germination in the filamentous fungus A. nidulans. In an effort to identify the cellular components involved in the activation of adenylate cyclase, we have focused our interest on the role of heterotrimeric G proteins. In A. nidulans, 3 G_α subunits have been identified, namely FadA, GanA and GanB. Phenotypic analyses performed with fadA, ganA and ganB null mutants show that only the ΔganB mutant is defective for trehalose breakdown and kinetics of germ tube formation. Moreover, the dominant activating mutant $ganB^{Q208L}$ is able to germinate in the absence of carbon source. This suggests a direct role of GanB in the activation of adenylate cyclase in response to carbon source. Interestingly, the unique G_β and G_γ subunits identified in the A. nidulans genome are also involved in the early events of germination; indeed, ΔG_β and ΔG_γ null mutants show defects in both germination and trehalose breakdown kinetics, similar to those observed with the ΔganB mutant. The $G_{\beta\gamma}$ heterodimer could function by segregating the G_α subunit GanB closed to the plasma membrane in a seven transmembrane domain receptor/G protein complex and/or by direct activation of adenylate cyclase. This is investigated with the construction and phenotypic analysis of the double mutant $ganB^{Q208L};\Delta G_\beta$. Additionally, we have demonstrated that RgsA, a regulator of G protein signaling similar to S. cerevisiae Rgs2, negatively controls germination probably via stimulation of the intrinsic GTPase activity of GanB as loss of RgsA function leads to the same phenotypes as activation of GanB signaling.

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PROTEOMIC AND GENOMIC BASED APPROACHES TO IDENTIFY PUTATIVE VIRULENCE FACTORS IN ASPERGILLUS FUMIGATUS.

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Aspergillus *sp.* are saprophytic fungi which are found in a wide variety of ecological niches. In cases of human infection A. fumigatus is the main etiological agent, with other species being identified as causative agents in some cases. If an immunocompetent individual is repeatedly exposed to Aspergillus conidia serious allergic complications may occur, however if an immunosuppressed individual becomes exposed and an infection occurs which persists, the outcome is often fatal.
We have employed both proteomic and genomic based approaches to identify factors that may be associated with the virulence of A. fumigatus. Fungal conidia were inoculated into a human epithelial lung cell line (HEL) grown in cell culture media (CCM) (virulent model) and into CCM only (avirulent model). Proteins and RNA were extracted at day 4 and day 5 from the virulent model, and from the avirulent model at day 5 which served as a control. Proteins were analysed following two-dimensional (2-D) gel electrophoresis. A number of differentially expressed proteins were detected. These proteins were subject to MALDI-ToF MS (Matrix Assisted Laser Desorption/Ionisation-Time of Flight Mass Spectrometry) analysis to elucidate their identity. A potential match to a probable SagA protein (cell wall associated protein) was obtained and RT-PCR analysis subsequently confirmed that the *sagA* gene is up-regulated under the virulent growth conditions. Matches to deduced *A. fumigatus* peptide sequences from the Sanger website have also shown potential matches to ubiquitin and to UDP glucose, sterol transferase.
RNA (from the virulent and avirulent models) was also subjected to a subtractive hybridisation RT-PCR, in an effort to identify genes that were preferentially expressed during growth in the virulent model system. A number of differentially preferentially expressed genes were cloned and sequenced. Analysis has shown sequences encoding putative proteins which display significantly similarity to fungal proteins that are associated with stress response, hyphal development and cell wall maintenance; specifically a probable salt responsive protein, a fungal ADP/ATP translocase, a probable autophagy protein, a C-4 sterol methyl oxidase, an amino acid permease and polyubiquitin.

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THE RESPONSE OF FILAMENTOUS FUNGI TO CELL WALL STRESS

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Cell walls not only determine the shape of the cell, but also provide physical and osmotic protection with their rigid structure. In filamentous fungi the response to sub-lethal levels of cell wall stress can result in morphological abnormalities such as swollen apical tips. Some time after exposure to cell wall stress the tip morphology of these fungi returns to normal and growth resumes. The ability of fungi to adapt to cell wall stress is known as cell wall remodelling.

In both Aspergillus niger and Penicillium chrysogenum sub-lethal concentrations of Calcofluor-white (CFW) have a similar effect on morphology (swollen apical tips). At a molecular level, CFW results in increased levels of messenger RNA encoding for glutamine:fructose-6-phosphate aminotranferase (AnGfaA and PcGfaA respectively) which synthesises a precursor of chitin, and alpha-1,3-glucan synthase (AnAgsA and PcAgsB respectively).

Disruption of the CFW inducible alpha-1,3-glucan synthase (agsA) gene of A. niger results in a moderate increase in sensitivity to CFW, indicating that other genes must compensate for the loss of agsA. Four additional alpha-1,3-glucan synthase encoding genes have been identified in A. niger. Northern analysis and RT-PCR have been used to identify conditions under which these genes are expressed. The results of these experiments show different patterns of expression for the ags genes.

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THE CROSS-PATHWAY CONTROL SYSTEM CONTRIBUTES TO PATHOGENICITY OF ASPERGILLUS FUMIGATUS

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The filamentous saprophyte Aspergillus fumigatus accounts for the majority of aspergillosis incidents, which represent a severe threat to immunocompromised individuals. Detailed knowledge on factors contributing to pathogenicity of this opportunistic pathogen is scarce with only few determinants being identified. We focus on the Cross-Pathway Control (cpc) system of A. fumigatus, a global regulatory network acting primarily on amino acid biosynthesis to counteract conditions of starvation.
The cpcA locus encoding the transcriptional activator protein of the system was identified. Its gene product represents a highly conserved protein that is the functional orthologue of Gcn4p, the yeast master regulator of gene expression upon starvation and stress. Generation of cpcA deletion mutants revealed its central role for the cpc response of this fungus upon amino acid starvation. Remarkably, cpcA null mutants are attenuated in virulence as revealed in a murine model of invasive aspergillosis, implicating an influential role of this conserved fungal regulatory system in establishing fungal growth upon host infection. Mixed infection experiments show that cpcA mutants are outgrown in vivo by the wild-type competitor, supporting the statement that the cross-pathway control system transcriptional activator is a virulence determining factor in aspergillosis.
By inspection of the A. fumigatus genome sequence, the cpcC locus was identified, which encodes a highly conserved sensor kinase perceiving the cpc-triggering signal. Next steps aim at the targeted replacement of this upstream component by a novel marker module in order to characterise the stress conditions encountered by the fungal pathogen upon host infection and to determine the actual level of CPCA that is required for full virulence of A. fumigatus.

WAX MOTH LARVAE AS A MODEL FOR GENE EXPRESSION STUDIES DURING ASPERGILLUS FUMIGATUS INFECTION

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Aspergillus fumigatus is a significant opportunistic pathogen in the immunocompromised individual, often causing fatal infections. To facilitate the validation of anti-fungal drug targets, the expression levels of selected genes must be determined during A. fumigatus infection. A gene must be expressed during infection for it to be a suitable drug target. Previously, mouse models have been used as models of A. fumigatus infection and expression. The use of wax moth larvae (Galleria melonella) as an alternative model was investigated in the present study. These organisms, currently used for pathogenicity and drug susceptibility studies, are easy to culture and inoculate, inexpensive and results are obtained rapidly. A range of genes was selected for study to compare their expression levels in vitro and during A. fumigatus infection in larvae and mice, using real-time RT-PCR. Using b-tubulin as the housekeeping gene, for each model relative expression levels were calculated for each gene. For example, actin and GAPDH showed similar expression levels for both models whereas pyrG expression was greater in the A. fumigatus-infected larvae than in the infected mouse tissue. Differences in A. fumigatus expression profiles between the models and therefore the validity of using the wax moth larvae as an alternative model for expression studies will be discussed.

APPEARANCE OF APOPTOTIC AND NECROTIC MARKERS IN ASPERGILLUS FUMIGATUS TREATED WITH VARIOUS CONCENTRATIONS OF H_2O_2

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Cell death in the opportunistic fungal pathogen *Aspergillus fumigatus* was associated with an apoptotic-like phenotype. In this study, cell death following treatment with H_2O_2 was investigated using a number of well-characterised biochemical markers of apoptosis, namely; DNA fragmentation using the TUNEL assay, translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasmalemma using annexin V-FITC and caspase activity. Propidium iodide (PI) staining was used as a marker of cell viability. In addition the influence of the broad-spectrum caspase inhibitor Z-VAD-fmk and inhibition of protein synthesis by cycloheximide on the appearance of an apoptotic phenotype was also investigated.
When treated with low but toxic concentrations of H_2O_2, an apoptotic phenotype also developed within 2 h and appeared prior to cell death as followed by PI staining. Higher concentrations induced immediate cell death with no apoptotic phenotype. However, whilst inhibiting protein synthesis blocked the development of an apoptotic phenotype at low concentrations of H_2O_2, the caspase inhibitor had no effect nor was any caspase activity toward substrates for caspase-1, -3 or -8 detected, suggesting either a different caspase was involved or that a caspase independent pathway was operating.

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SEQUENCING AND ANALYSIS OF ASPERGILLUS FUMIGATUS AF 293

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Aspergillus fumigatus is a filamentous fungus that grows on nonliving organic material in the soil and in human homes. Exposure to *A. fumigatus* can cause an allergic response in sensitive individuals. More importantly, the fungus is an opportunistic pathogen of bone marrow transplant patients, AIDS patients, and other immune compromised individuals. An international group of scientists have collaborated to sequence and analyze the genome of *A. fumigatus*. The Institutions performing the sequencing include TIGR (USA), the Sanger Institute (UK), the Universities of Salamanca (Spain) and Complutense (Spain), and the Centro de Investigaciones Biológicas (Spain). A clinical isolate, Af293, was selected for sequencing. The total genome size based on the sequence is 28.6 Mb. The project currently is in the genome finishing stage. Automated genome annotation of the *A. fumigatus* scaffolds has been accomplished revealing 9,744 genes. Of these, function can be assigned to approximately 4,300 of them. The coding fraction of the genome is 49.1%, the mean gene length is 1,443 bases, and over 75% of the genes have introns, with the mean number being 2.7. The mean intron size is 122 bases. Analysis of the genome is being undertaken by Aspergillus community scientists. A status report on the project is presented.

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CHARACTERIZATION OF ANTIFUNGAL PROTEINS FROM FILAMENTOUS ASCOMYCETES

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Filamentous Ascomycetes secrete small, lysine- and cysteine-rich proteins into the culture supernatant which are potent growth inhibitors of other microorganisms, mainly filamentous fungi, such as the opportunistic human pathogen Aspergillus fumigatus or the plant pathogen Botrytis cinerea. Although these proteins show high homology within their primary amino acid sequence they differ in species specificity and in their site of action[1,2]. We are currently characterizing the properties of the antifungal proteins from Aspergillus (A.) giganteus, A. niger and A. ficuum. We will present data on the species specificity and the minimal inhibitory concentration (MIC). Furthermore, we are performing indirect immunofluorescence studies in order to elucidate their site of action. A better understanding of the function and the influence of these proteins on sensitive target organisms is a prerequisite to consider them as promising candidates for the development of novel antimycotic drugs, for food preservation or for the breeding of plants, which are resistant against phytopathogenic fungi.

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IDENTIFICATION OF SPORE SURFACE COMPONENT(S) FROM ASPERGILLUS FUMIGATUS WHICH POSSESS DNA CLEAVING AND PROTEASE ACTIVITY

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Aspergillus fumigatus diffusate (AfD) is a water-soluble extract produced from a spore suspension, filtered and concentrated. This AfD has several biological activities, including protease activity and the ability to cleave genomic and plasmid DNA. The unfractionated AfD shows DNA scission capability both in the absence and the presence of metals ions. Metal ions, Mg^{2+}, Mn^{2+} and Fe^{3+} stimulated both plasmid and genomic DNA scission. Partial purification by TLC and HPLC suggests the presence of at least two activities which differ in the metal ion dependence. The ability to cleave DNA is non-enzymatic and may be of importance in generating DNA damage 'in vivo' with the possibility of leading to the onset cancers.

Proteases have been identified by liquid culture and were of hyphal origin. For the first time, a protease has been detected from the surface of the fungal spore, isolated within the AfD. This has been partially purified by reversed-phase HPLC. The activity is greatest in the alkaline range and is enhanced at least 10-fold in the presence of Fe^{2+} and Fe^{3+}. On inhalation of the spores, it is hypothesised that the protease will be released into the aqueous surface of the lung, where it may cause damage to the lung epithelial cells and proteins of the extracellular matrix, thus increasing the chances of persistence of the spores and infection in the lung.

SPORE SURFACE COMPONENT(S) FROM ASPERGILLUS FUMIGATUS WHICH INHIBIT THE OXIDATIVE BURST IN RAT ALVEOLAR MACROPHAGE AND HUMAN CELL LINES.

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Aspergillus fumigatus is a saprophytic fungus that is present ubiquitously within the environment. This specific fungus has been implicated as a causative agent of human aspergillosis, and is believed to account for 80-90% of these infections. Aspergillosis normally manifests within the lung in the form of allergic bronchopulmonary aspergillosis (ABPA), aspergilloma or invasive aspergillosis (IA), and ranges in severity from a mild allergic reaction to a life threatening systemic infection. It occurs predominantly amongst the increasing population of patients with an immune deficiency and currently poor diagnosis and treatments are available. The conidia are the infectious agents of the fungi with a diameter of 2-3 μm that allows easy penetration into the lungs. The primary defence mechanism against the inhaled spores are the alveolar macrophage that elicit the oxidative burst generating superoxide. An aqueous spore suspension when incubated for up to 3hours, will allow diffusion of spore surface components into solution. This is termed Aspergillus fumigatus diffusate (AfD). This is a complex mixture of at least 24 proteins/peptides, carbohydrates and other small molecules. The oxidative burst inhibitor has previously been shown to be associated with a carbohydrate component after reversed-phase HPLC purification. The current studies have shown that dialysis of the AfD through a membrane with a MW cut-off of 1000 daltons inactivated the inhibitory effect of components of < 1Kda and > 1Kda. Activity was regained after reconstitution of the two fractions suggesting that the inhibitory activity comprises of at least two components. The inhibitory activity does not appear to be metal ion dependent. Potential mechanisms for this inhibitory effect will be discussed particularly inhibition by AfD of tyrosine phosphorylation and the involvement of protein kinases.