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Genes and molecules involved in *Aspergillus fumigatus* virulence

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Summary

Aspergillus fumigatus causes a wide range of diseases that include mycotoxicosis, allergic reactions and systemic diseases (invasive aspergillosis) with high mortality rates. Pathogenicity depends on immune status of patients and fungal strain. There is no unique essential virulence factor for development of this fungus in the patient and its virulence appears to be under polygenetic control. The group of molecules and genes associated with the virulence of this fungus includes many cell wall components, such as ß(1-3)-glucan, galactomannan, galactomannanproteins (Afmp1 and Afmp2), and the chitin synthetases (Chs; *chsE* and *chsG*), as well as others. Some genes and molecules have been implicated in evasion from the immune response, such as the rodlets layer (*rodA*/*hyp1* gene) and the conidial melanin-DHN (*pksP*/*alb1* gene). The detoxifying systems for Reactive Oxygen Species (ROS) by catalases (Cat1p and Cat2p) and superoxide dismutases (MnSOD and Cu,ZnSOD), had also been pointed out as essential for virulence. In addition, this fungus produces toxins (14 kDa diffusible substance from conidia, fumigaclavin C, aurasperon C, gliotoxin, helvolic acid, fumagilin, Asp-hemolysin, and ribotoxin Asp fl/mitogilin F/restrictocin), allergens (Asp f1 to Asp f23), and enzymatic proteins as alkaline serin proteases (Alp and Alp2), metalloproteases (Mep), aspartic proteases (Pep and Pep2), dipeptidyl-peptidases (DppIV and DppV), phospholipase C and phospholipase B (Plb1 and Plb2). These toxic substances and enzymes seems to be additive and/or synergistic, decreasing the survival rates of the infected animals due to their direct action on cells or supporting microbial invasion during infection. Adaptation ability to different trophic situations is an essential attribute of most pathogens. To maintain its virulence attributes *A. fumigatus* requires iron obtaining by hydroxamate type siderophores (ornitin monooxigenase/SidA), phosphorous obtaining (*fos1*, *fos2*, and *fos3*), signal transductional falls that regulate morphogenesis and/or usage of nutrients as nitrogen (*rasA*, *rasB*, *rhbA*), mitogen activated kinases (*sakA* codified MAP-kinase), AMPc-Pka signal transductional route, as well as others. In addition, they seem to be essential in this field the amino acid biosynthesis (*cpcA* and homoaconitase/*lysF*), the activation and expression of some genes at 37 °C (Hsp1/Asp f12, *cgrA*), some molecules and genes that maintain cellular viability (*smcA*, Prp8, anexins), etc. Conversely, knowledge about relationship between pathogen and immune response of the host has been improved, opening new research possibilities. The involvement of non-professional cells (endothelial, and tracheal and alveolar epithelial cells) and professional cells (natural killer or NK, and dendritic cells) in infection has been also observed. Pathogen Associated Molecular Patterns (PAMP) and Patterns Recognizing Receptors (PRR; as Toll like receptors TLR-2 and TLR-4) could influence inflammatory response and dominant cytokine profile, and consequently Th response to infection. Superficial components of fungus and host cell surface receptors driving these phenomena are still unknown, although some molecules already associated with its virulence could also be involved. Sequencing of *A. fumigatus* genome and study of gene expression during their infective process by using DNA microarray and biochips, promises to improve the knowledge of virulence of this fungus.

Key words *Aspergillus fumigatus*, Virulence, Pathogenesis, Genes, Proteins

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Genes y moléculas implicados en la virulencia de Aspergillus fumigatus

Resumen

Aspergillus fumigatus causa enfermedades muy variadas que incluyen micotoxicosis, reacciones alérgicas y enfermedades sistémicas con altas tasas de mortalidad (aspergilosis invasoras; AI). La patogenicidad parece depender del estado inmune del paciente pero también del aislamiento fúngico.
No parece existir un verdadero factor de virulencia único y esencial para el desarrollo de este hongo en el paciente y la virulencia de *A. fumigatus* parece ser poligénica. Entre las moléculas y los genes que se han relacionado con la virulencia de este hongo se encuentran componentes de la pared celular como ß(1-3)-glucano, galactomanano, galactomanoproteínas (Afmp1 y Afmp2), y enzimas quitina sintetasas (Chs; *chsE* y *chsG),* entre otras. Algunos genes y moléculas se han relacionado con la evasión de la respuesta inmune, como la capa *rodlets*, (gen *rodA*/*hyp1*) y la melanina-DHN (gen *pksP*/*alb1*) de los conidios. También los sistemas de detoxificación de los compuestos derivados del oxígeno (ROS) por parte de catalasas (Cat1p y Cat2p) y superoxidodismutasas (MnSOD y Cu,ZnSOD) entre otras, se han apuntado con frecuencia como esenciales en su virulencia. Así mismo, este hongo produce toxinas (la sustancia difusible de los conidios de 14 kDa, fumigaclavina C, aurasperona C, gliotoxina, ácido helvólico, fumagilina, Asp-hemolisina y la ribotoxina Asp fl/mitogilina F/restrictocina), alérgenos (Asp f1 a Asp f23) y proteínas enzimáticas como serina proteasas alcalinas (Alp y Alp2), metaloproteasas (Mep), aspártico proteasas (Pep y Pep2), dipeptidilpeptidasas (DppIV y DppV), fosfolipasa C y fosfolipasa B (Plb1 y Plb2). Estas sustancias tóxicas y enzimas parecen actuar de forma aditiva y/o sinérgica reduciendo la supervivencia de los animales infectados, bien por afección directa de las células del huésped o bien por favorecer la penetración del microorganismo durante la infección. La capacidad de adaptarse a los cambios a distintas condiciones tróficas es un atributo esencial de muchos patógenos. En el caso de la virulencia de *A. fumigatus* se han apuntado como necesarias las siguientes capacidades y reacciones: obtención de hierro mediante sideróforos de tipo hidroxamato (ornitina monooxigenasa/SidA), obtención de fósforo (*fos1*, *fos2* y *fos3*), cascadas de transducción de señales que regulan la morfogénesis y/o la utilización de nutrientes como el nitrógeno (*rasA*, *rasB, rhbA*), quinasas activadas por mitógenos (MAP-quinasa codificada por el gen *sakA*), ruta de señales de transducción de AMPc-Pka, y otras. Parecen ser también esenciales en este campo la biosíntesis de aminoácidos (*cpcA* y homoaconitasa/*lysF*), la activación y expresión de algunos genes a una temperatura de 37 °C (Hsp1/Asp f12, *cgrA*), las moléculas y genes que mantienen la viabilidad celular de *A. fumigatus* (*smcA,* Prp8, anexinas), etc.
Por otro lado, en los últimos tiempos, el conocimiento de la relación entre este patógeno y la respuesta inmune del huésped ha sido mejorada, y se abren nuevas vías de investigación. Se ha detectado la participación en la infección tanto de células no profesionales (endoteliales, epiteliales traqueales y alveolares) como profesionales (células asesinas naturales o NK y células dendríticas). Los patrones moleculares asociados al patógeno (PAMP) y los receptores que reconocen esos patrones (PRR; como por ejemplo receptores de tipo Toll TLR-2 y TLR-4), pueden influir en la respuesta inflamatoria y el perfil de citoquinas dominante, y por tanto en la respuesta Th contra la infección. Los componentes superficiales del hongo y los receptores de las células del huésped que parecen dirigir estos fenómenos, son todavía desconocidos, aunque pudieran estar implicadas algunas de las moléculas ya asociadas con la virulencia. La secuenciación del genoma de *A. fumigatus* y el estudio de la expresión de genes durante los procesos infecciosos de este hongo mediante el uso de microarray y biochips de ADN, permitirán en un futuro no muy lejano ampliar los conocimientos sobre la virulencia de este hongo.

Palabras clave *Aspergillus fumigatus*, Virulencia, Patogénesis, Genes, Proteínas

Aspergillus fumigatus is a saprophyte fungus which survives and grows over a large variety of organic remains and whose most common ecological niche is on the ground. It is one of the most ubiquitous fungi, due to the ease of dispersion of its conidia [100]. The small size of the conidia, from 2 to 3 µm, means that they can remain in suspension in the environment for a long period of time, and can reach the human pulmonary alveoli, since man is constantly exposed to inhaling them [1]. It is calculated that a person could inhale several hundred conidia of *A. fumigatus* per day [100]. In immunocompetent individuals the inhalation of these conidia rarely has serious adverse effects, since they are efficiently eliminated by innate and acquired immune mechanisms [208]. However, when it concerns an immunocompromised host, such as patients who have undergone transplants, patients with various types of leukaemia or people infected by HIV, these mechanisms may not be sufficient for the elimination

of all the conidia. Although inhalation is the most important transmission route for the acquisition of aspergillosis, the existence of other routes in the hospital environment must not be discounted [195]. The diseases caused by *Aspergillus* are very diverse and include, from mycotoxicosis, allergic reactions and colonisation with restricted invasion in immunocompetent individuals, to systemic diseases with high mortality rates in immunocompromised patients, called invasive aspergillosis (IA) [29]. In these immunocompromised patients, the fungus can invade the lung and practically any other organ, producing severe damage. In fact, the majority of patients who suffer an IA die, despite the aggressive antifungal treatment [109].

In the last ten years, *Aspergillus* spp have gained importance as opportunist pathogens, mainly due to the increase in the number of immunocompromised patients. Among this group of patients, *A. fumigatus* has become the most prevalent fungus causing deadly invasive infections. The importance of this species in infections is reflected in the various reviews of its biology and pathology that have been published in the last few years [10,14,29,30,43,100-102,121,133,194].

Since *A. fumigatus* is an opportunist pathogen which does not normally cause illnesses in the immunocompetent host, the study of its virulence can be complicated. Other species of *Aspergillus* can present with similar characteristics in their conidia, but *A. fumigatus* is the most common cause of disease [100]. Although the spores of *A. fumigatus* are found in a small proportion of all the airborne spores within a hospital (approximately 0.3%), it causes approximately 90% of the systemic infections due to *Aspergillus* [29], which is why the existence of characteristic virulence factors within this species has been postulated. On the other hand, the human tissues appear to provide the ideal conditions for the development of invasive disease due to *A. fumigatus,* reducing the impact by other *Aspergillus* such as *Aspergillus flavus* and *Aspergillus niger* [6]. The higher mortality which is seen in the infections with *A. fumigatus* appears to be due to weakened immune response, to the virulence capacity of the microorganism itself and also, probably to the delay in establishing a diagnosis, which can prevent the success of the treatment [44]. One of the main reasons for the mortality associated to infection by this fungus is the delay in its detection, which allows *A. fumigatus* to achieve such a population size and tissue destruction in the host and which makes recovery impossible in patients despite the treatments employed. The study of the virulence factors can be important in two areas. One of them is the diagnosis of the infection due to *A. fumigatus* and the other is to enable possible use these factors as targets for the design of drugs, vaccines and/or specific treatments against the fungus. The design of tests based on ELISA and PCR which detect specific components of *A. fumigatus* or its genes, has given promising results in the detection of infection [44, 100], although in this field some molecules not directly related with the virulence could also be used. The other approach is more complex, since in spite of all the experiments carried out to date, not one virulence factor has been discovered, whose absence may be sufficient to completely suppress the invasiveness and lethality of this fungus. Latgé [101] has indicated that the virulence of *A. fumigatus* must probably be polygenic and that a true virulence factor, unique and essential for the development of this fungus in the lung, does not exist.

Studying the population structure of *A. fumigatus* in isolated local patient populations, a so-called epidemic population has been observed which shows a unique type of electrophoretic pattern or some very closely related ones [194]. There is some class of selective pressure by which this population distribution is produced. An important factor can be the immune response of the host, which has to be weakened for *A. fumigatus* to cause IA. It has also been shown that some clinical isolates are more virulent than the environmental strains, suggesting that the pathogenicity not only depends on the immune state but also on the fungal isolate [8]. For example, differences have been shown between isolates of *A. fumigates* in elastase activity (which is related to the invasion capacity [25]) and in its inhibition capacity of the phagocytic response [21].

According to Latgé [100,101], the term virulence factor should be applied to the molecules or genes which, on being eliminated by a microorganism, remove its virulence without blocking normal growth or *in vitro* morphogenesis. Some genes which have been associated with virulence in *A. fumigatus* should not always be considered virulence factors. For example the *pabaA* gene which encodes the enzyme para-aminobenzoic acid synthetase. This enzyme catalyses the last step in the biosynthesis of folate, and Brown et al. [31], demonstrated that mutant *pabaA*$^-$ strains were avirulent in the mouse model. These mutants are incapable of synthesising folate, an essential co-factor of DNA synthesis and it appears that the lung is incapable of providing this compound to the fungus, therefore, *in vivo,* it would be incapable of growing. But these mutants are also not capable of growing *in vitro* without this compound. Another similar reduction is shown with the *pyrG* gene whose mutation produces auxotrophic mutants incapable of germinating *in vivo* and *in vitro* in the absence of uridine or uracil [203]. They should not be considered virulence factors, despite these authors stating that they were genes essential for virulence and that these genes may be used in the design of new antifungal agents [100,101].

In this review it is intended to give a general, overview of the genes and proteins which have been associated with fungal virulence in the literature, the activities which they can perform and the importance that they could have in IA. In tables 1 to 6, those genes and proteins are shown. In this text, the genes and proteins nomenclature established during the First *Aspergillus* Meeting in Copenhagen in April 2004, has been used.

Composition of the cell wall and its effect on pathogenesis

The cell wall of *A. fumigatus* is a complex structure mainly composed of polysaccharides and where the majority of the antigens secreted by the fungus during its *in vitro* and *in vivo* growth are located [102]. Some components of the wall are directly associated with the colonisation of the host tissue and others with the damage to these same tissues. In table 1, the genes and proteins associated with the cell wall and virulence are shown. The most abundant polysaccharide in the wall is ß(1-3)-glucan which forms a skeleton over it, and becomes an anchor for polysaccharides such as galactomannan and chitin [210]. ß(1-3)-glucan has different biological activities, triggering the activation of complement and the production of inflammatory mediators such as leukotrienes and TNFα [69]. These authors also indicate that ß(1-3)-glucan is deposited in the organs over a long period, being metabolised slowly by phagocytes, as opposed to a more rapid metabolism of mannan. It is detected in patients with deep mycosis and it reflects the inflammatory reactions and the immune response of the host. The detection of this component by means of a Limulus G test is used in Japan for the diag-

Table 1. Genes and proteins related with cell wall structure related with virulence.

Genes	Proteins	Characteristics	Pathogenesis related activities	Use
	ß(1-3)-glucan synthase complex			
*fks*1	Fks1p	218 kDa (Catalytic subunit)	Essential for fungal growth	Equinocandin target Diagnosis
*rho*1	Rho1p	28.5 kDa (GTP-binding subunit)		
*rho*2, *rho*3 and *rho4*	Rho2p, Rho3p and Rho4p	(GTP-binding subunit)		
	Putative ABC[a] protein	160 kDa (ß(1-3)-glucan export)		
	Putative membrane ATPase	100 kDa		
*gel*1	Gel1p (Glucanosyltransferase)	48.1 kDa (with GPI[b])	Cell wall assembly and morphology	New antifungal drug target
*gel*2 and *gel*3	Gel2p and Gel3p	(with GPI[b])	Cell wall assembly and morphology	
*engl*1	ß(1-3)-endoglucanase	74 kDa	Cell wall assembly and morphology	
*afmp*1/*asp f*17	Afmp1p (Galactomannoprotein)	31.4 kDa (with GPI[b])	Extracellular antigen Immunomodulator Recognition and cellular adhesion	Diagnosis Platelia Aspergillus® supplement?
*afmp*2	Afmp2p (Mannoprotein)	51.5 kDa	Extracellular antigen Cell wall assembly	Diagnosis Platelia Aspergillus® supplement?
*chs*E and *chs*G	Chitin synthase (class V and III)	167 and 110 Kda (respectively)	Cell wall assembly Antigens Immunomodulator molecules generation (Chito-Oligosaccharides)	New antifungal drug target?
acs	α(1-3)-glucan synthase		Cell wall assembly and morphology	New antifungal drug target?

[a] ATP-binding casette (ABC) class transporter family.
[b] Glycosyl-phosphatidyl-inositol (GPI) membrane anchorage motif.

nosis of fungal infections [69], and its kinetics correlate very well with that of galactomannan in patients with IA [148]. The synthesis of ß(1-3)-glucan has been reviewed by Douglas [46]. The enzyme glucan synthase is a transmembrane protein complex formed by several proteins: Rho1p, Fks1p and another two proteins, a homolog to a 100 kDa membrane ATPase and another homolog to an ABC (ATP-binding cassette) glucan bacterial transporter of 160 kDa. There are four *rho* genes (denominated *rho1* to *rho4*), of which the most studied is *rho1* which encodes a protein of 21.5 kDa for the binding and hydrolysis motifs of GTP. The *fks1* gene encodes a transmembrane protein which is the catalytic subunit of ß(1-3)-glucan-synthetase and its protein has a molecular weight of 218 kDa [13,49]. This protein has been detected in the apex of the conidial germ tube where ß(1-3)-glucan is actively being synthesised [13]. It has been demonstrated that reductions in the expression of this gene are sufficient to inhibit normal growth [130]. Fks1p is an enzyme essential for the growth of *A. fumigatus* and has been the basis of the development of a new family of antifungals, the echinocandins, whose antifungal mechanism of action is to interfere with the synthesis of glucan by competitive inhibition. Also, in the wall, other enzymes are found to a lesser extent, capable of participating in the extracellular elongation and modification of this polysaccharide and thus in the growth of the fungus. Three glucanosyltransferases enzymes exist for the elongation of glucan (Gel), Gel1p, Gel2p and Gel3p, which are encoded by the *gel*1, *gel*2 and *gel*3 genes [132]. All of these belong to the family of glycoside hydrolases and contain the anchoring motif to membrane of the glycosyl-phosphatidyl-inositol (GPI). Gel1p appears to be required in the assembly and morphology necessary for the cell wall [34]. These enzymes could be used as targets for the development of new antifungals. Recently a ß(1-3)-endoglucanase of 74 kDa, belonging to a new family of ß(1-3)-glucanases, has been isolated in *A. fumigatus* [131]. This enzyme is encoded by the *engl1* gene, but it has been observed that its disruption does not lead to any phenotype different from the parent strain, thus it does not appear to play an essential role in the constituent cell growth.

Galactomannan has been detected in the walls of hyphae and conidia of *A. fumigatus*. Some authors indicate that, in the conidia, this galactomannan possesses substitutions with sialic acid motifs which could be related to its binding to fibronectin and laminin [199]. Galactomannan can be detected in the supernatants of the cultures of this fungus and is the principal exoantigen released during tissue invasion [100]. Galactomannan is the most useful diagnostic marker which can be detected in serum, urine, cerebro-spinal fluid, etc., of patients with IA, although its concentration can fluctuate. Two commercialised tests have been developed for its detection in patients with IA, Pastorex Aspergillus (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France), based on latex particle agglutination, and Platelia Aspergillus® (Bio-Rad, Marnes-La-Coquette, France), based on the ELISA technique. This latter technique is much more sensitive than the first in detecting this marker [44], and although it can give false positives [57], it is very useful in the diagnosis of IA in neutropenic patients [73,117,125,147,168].

The *afmp*1 gene encodes an *A. fumigatus* antigenic cell wall galactomannanprotein (Afmp1p) which possesses 284 aminoacids and a predicted molecular mass of 31.4 kDa. It contains in its sequence a region rich in serine/threonine for O-glycosylation, a signal peptide and a signal GPI, which in many proteins is used for anchoring to the eukaryotic membrane. It seems to have important functions in cell recognition and adhesion, acting like a receptor for the transport of ions and nutrients. Specific antibodies against Afmp1 are only developed in patients infected with *A. fumigatus*. This makes this protein useful in the serological diagnosis of aspergilloma and IA [210]. Likewise, Afmp1p can be useful as a potential immunomodulatory glycopeptide by its use in nasal immunisation systems which could stimulate the production of specific IgAs. Since the fungus is acquired by inhalation, these specific IgAs could prevent the adherence of the fungus to the lung, increase the stimulation of the complement pathway and facilitate its phagocytosis, thus preventing infection. A new protein of this super family of antigenic mannoproteins has recently been discovered. The *afmp*2

gene encodes the Afmp2p protein, which has 510 aminoacid residues and a molecular mass of 51.5 kDa. It has an N-terminal signal peptide, a putative GPI C-terminal and regions of O-glycosylation. This protein appears to be involved in the assembly of the cell wall, also being secreted in the culture supernatants of this microorganism [39]. As in the case of Afm1p, this protein is immunogenic and antibodies are developed against it. The fact that this is an abundant secretory protein, its minimal cross reactivity with Afmp1p and the presence of antibodies against Afmp2p in patients with infections due to *A. fumigatus*, suggests that it is a good candidate to complement Afmp1p in the sero-diagnosis of these infections [39], and perhaps enabling the improvement of the commercialised systems.

Chitin is the third polysaccharide component of the wall and several chitin synthases (Chs) have been detected in *A. fumigatus*. Several classes of these enzymes (class I to VI) are reported in the literature. The family of *chs* genes in *A. fumigatus* include at least seven different genes, called *chsA* (class I), *chsB* (class II), *chsC* (class III), *chsD* (class VI), *chsE* (class V), *chsF* (class IV) and *chsG* (clase III). Of all of them, the only genes in which disruption leads to an altered phenotype in *A. fumigatus* are *chsE* and *chsG* [9,115,116]. The *chsE*$^-$ mutant has reduced levels of chitin in the mycelium, swellings throughout the length of the hypha and reduced conidiation [9]. The strains with *chsG*$^-$ mutations showed a reduced activity of the chitin synthetase enzyme, reduced radial growth and highly branched hyphae, suggesting that the function of the enzyme is in the apex of the hypha [115]. The *chsE*$^-$/*chsG*$^-$ double mutation is not lethal for this fungus, and the strains that possess it show some characteristics which are the addition of characteristics of each mutation, and should indicate that these enzymes are related to different, non-interconnected morphogenic routes. The data indicates a participation of the Chs in the virulence of the fungus, perhaps due to stabilisation of the cell wall with chitin, and makes those genes and enzymes possible antifungal targets. For example, the mortality rate of mice infected by *chsG*$^-$ or chsC$^-$/chsG$^-$ mutant strains was reduced [115]. The mutants in the Chs are viable and pathogenic despite the phenotype changes since the fungus has compensation systems for the loss of these Chs activities and replaces the chitin lost by other polysaccharide components. For example, the *chsE*$^-$/*chsG*$^-$ double mutants have a cell wall highly enriched with α-glucan [116]. These data would make studying of the *acs* genes which encode the α(1-3)-glucansynthetase enzymes interesting. Some data show that the disruption of chitin synthesis with inhibitors such as mycomycin are associated with a greater sensitivity and synergic effects with echinocandins and itraconazole, perhaps due to a greater permeability across the wall of these components. A new possibility for treatment would be to study these synergic effects between inhibitors of the cell wall, which may be incapable by themselves to destroy the fungus [116].

Resistance to the innate immune response

The conidia are the infectious particles in aspergillosis [21]. When the fungus is inhaled by the host, the conidia have to reach the pulmonary alveoli and adhere to the proteins of the basal lamina to be able to start the infection, this being the key step in the process [100]. In immunocompetent persons, the innate immune mechanisms appear to be capable of completely eliminating the inhaled conidia and for this reason the infection by *A. fumigatus* is very rare in this population. When these functions are weakened or in times of stress, the fungus can colonise and even invade the tissues of the host, but although the majority of infections are produced in immunocompromised patients, they can also give infections to immunocompetent patients. As in other pathogenic microorganisms, the surface of these conidia appears to be associated with adhesion and initial contact with the immune system. The next step is its germination, the invasion by hyphae of the bronchial wall and dissemination by the blood vessels which carry them to colonise other organs. Among the active mechanisms of the host, the ciliary movement efficiently eliminates many of the inhaled conidia. On the other hand, the alveolar macrophages also appear to be very efficient in the elimination of these conidia and preventing their germination. Phagocyte cells, as well as humoral components (e.g.: complement) participate. Even so, the recognition of these conidia for their elimination can be carried by receptors different to those of complement or the Fc region of the antibodies, since these components are scarce in the lung. It has been suggested that the surfactant proteins of the lung can be associated with the opsonisation of the conidia [2,3]. The alveolar macrophage activity in the destruction of the conidia has been frequently shown to be critical in the resistance of the host to aspergillosis [72]. Some of the genes and molecules which have been associated with this evasion of the immune response by *A. fumigatus* are set out in table 2.

The negatively charged carbohydrates on the surface of the conidia of *A. fumigatus* can mediate the adherence to fibronectin and laminin [196]. On the other hand, the surface of the conidia of *A. fumigatus* is covered by a layer of hydrophobins, called the rodlet layer, which is not present in the mycelium [66]. In the conidium, this external layer of the wall contains the hydrophobins RodAp and RodBp, which are found forming highly insoluble complexes [145]. The *rodA*/*hyp1* gene was characterised by Thau et al. [187] and is a homologue of *rodA* gene of *A. niger*. The protein encoded by this gene, RodAp, has a low molecular weight (16 kDa), is moderately hydrophobic and is rich in cysteines. The *rodB* gene, which shows a high similarity to *rodA*, also encodes a hydrophobin, RodBp, which has a molecular weight of 14 kDa [145]. Mutant strains of *rodA*$^-$ have conidia with a rough surface and deficiencies in binding with collagen and bovine serum albumin, but not in its binding to laminin and to the fibrinogen of pneumocytes. The *rodB*$^-$ mutants behave like their parent strains and do not appear to have any modification in the structure of the rodlets layer. Despite its similarity to RodAp, RodBp does not seem to be a protector in the destruction by alveolar macrophages. In fact, the mutants in the *rodA* gene, like *rodA*$^-$ and *rodA*$^-$*rodB*$^-$, show a high sensitivity to the destruction by macrophages, which is not shown by *rodB*$^-$ mutants. For this reason, RodAp appears to protect the conidia from destruction by the alveolar macrophages. It has been pointed out that the rodlets layer could convert into a barrier for the hydrophilic molecules, like the oxidant components of the phagocytes, or that these molecules could be sequestrated by RodAp, as has been suggested for melanin [70]. However, mutants in these proteins continue being pathogens and do not lose their virulence capacity in mice, but although the mortality rate is similar, the rodletless mutants produce limited lung lesions and weaker inflammatory responses than the parent strains [183].

From the recent literature, the idea is starting to emerge that pigments, such as melanin and derivatives, are important factors of virulence in both plant and human pathogenic fungi [189]. Among the functions proposed for the pigments are included, protection against UV light,

Table 2. Genes and proteins related to the innate immune response resistance.

Genes	Proteins	Characteristics	Pathogenesis related activities	Use
*rodA/hyp*1 and *rodB*	RodAp and RodBp (Hydrophobins)	16 kDa and 14 kDa (respectively)	Immune response protection Adhesion	
Gene cluster	DHN-melanin[a]		ROS[b] protection Prevents C3 deposit	
*pksP/alb*1	Polyketide synthase (DHN-melanin synthesis)	2,343 kDa	Toxic substances production Lysosome fusion inhibition	
*arp*1	Scytalone dehydratase (DHN-melanin synthesis)	19.7 kDa		
*arp*2	Hydroxynaphthalenes reductase (DHN-melanin synthesis)	273 amino acids		
*abr*1	Putative iron multicopper oxidase (DHN-melanin synthesis)	664 amino acids		
*abr*2	Putative laccase (DHN-melanin synthesis)	587 amino acids		
*ayg*1	(DHN-melanin synthesis)	406 amino acids		
catA	Conidial-specific catalase	84.5 kDa (non-glycosylated)	ROS protection	
*cat*1/*catB*	Cat1p, CatB (hyphae catalase)	90 kDa subunit (Glycosylated tetrameric enzyme)	ROS protection	
*cat*2	Cat2p (hyphae catalase)	82 kDa (Non-glycosylated monomeric enzyme)	ROS protection	
	Other four detected catalases		ROS protection	
sod/Asp f6	MnSod/Asp f6 (Superoxide dismutase)	26.5 kDa	ROS protection Type I hypersensitivity Autoimmunity Antigen	Diagnosis
	Cu,ZnSod (Superoxide dismutase)	15.9 kDa	Protection against ROS Antigen	
*mdr*1, *mdr*2, *atrF*, and *mdr*4	ABC[c] transporters		Toxic molecule expulsion	
*mdr*3	MFS[d] transporter		Toxic molecule expulsion	

[a] 1,8-dihydroxynaphthalene-melanin biosynthetic pathway.
[b] Reactive oxygen species (ROS).
[c] ATP-binding cassette (ABC) class transporter family.
[d] Major facilitator superfamily (MFS) class transporter.

enzymatic lysis and oxidants, and sometimes protection against extreme temperatures. A review has recently been carried out on the synthesis of melanin in pathogenic fungi and its importance [99]. The conidia of *A. fumigatus* possess a greyish-green pigment, absent in hyphae [211], which is an important component of them, contributing to their survival and longevity in the environment [205]. Its synthesis is regulated by the development and it seems to be produced in the synthesis route of melanin-1,8 dihydroxynaphthalene (DHN-melanin). This pigment appears adhered to the cell wall of the conidia of *A. fumigatus,* coming into direct contact with the immune system of the host [71,97]. The presence of DHN-melanin on the surface of the conidium appears to protect the fungus from reactive oxygen species (ROS), which uses alveolar macrophages and polymorphonuclear phagocytes for its destruction [100]. The wild strains with pigment have a 10 to 20 times greater resistance against ROS than the white mutant strains, presumably due to their capacity to sequestrate and detoxify these ROS. In fact, the absence of pigment produces white conidia, decreases their virulence and makes them more sensitive to the action of H_2O_2 and sodium hypochlorite, and more susceptible to phagocytosis and to damage by macrophages *in vitro* [70,97,189].

Although the synthesis route of DHN-melanin is shared by other fungi, in *A. fumigatus* it has some important differences [99]. The enzymes associated with its synthesis are encoded by a cluster of six genes, identified by two investigation groups in parallel [30,33,55,97,189,190,192]. The six genes are *pksP/alb1*, *ayg1*, *arp1*, *arp2, abr1* and *abr2*. Of them all, the most interesting, from the point of view of virulence, is the *pksP/alb1* gene which encodes a polyketide synthase and catalyses the first step in this pathway. The deletion in other genes of the route produce conidia with different coloration, but they do not have any obvious effects on the virulence [192]. The genes which encode these polyketide synthase enzymes in fungi are type 1 and have been associated with synthesis of pigments and/or mycotoxins [99]. The *pksP/alb1*$^-$ mutant strains show a smooth conidium with a white surface and in mouse models they have a reduced virulence. The greater sensitivity of these mutants could be due to the existence of, at least, two C3 complement binding proteins of 54 and 58 kDa, respectively, which in wild conidia appeared to be masked by the pigments deposited on the surface. The disappearance of these pigments should make the surface accessible to the deposit of complement. In the *alb1*$^-$ strains, an increase is seen in C3 bound to the surface of the conidium and also of the phagocytosis of these mutants, five times greater than in wild strains [72]. The *arp1* and *arp2* genes could also be associated with this phenomenon, since the *arp1*$^-$ and *arp2*$^-$ mutants have a larger deposit of C3 in their conidia [191,192]. The pigmentation, therefore, should frustrate, at least partially, the phagocyte-complement axis of the defence against aspergillosis, preventing the deposit of C3, changing the superficial structure and the activation pattern of the immune system and sequestrating the ROS of the phagocytes. Despite all this, it does not explain why the conidia of *A. fumigatus* are pathogens and rarely so in those of *A. nidulans,* whose surface pigments and their behaviour are similar [70]. It is improbable, therefore, that

the sequestration of the ROS is the principal determinant of the virulence dependent on the *pksP/alb1* gene and it is thought that this gene can produce, by its activity, different substances and polyketide derivatives with a toxic potential over the effector cells [98]. Initially, the biosynthetic route would be activated during sporulation [189], but it appears that this *pksP/alb1* gene could also be expressed during the infection producing inhibitor products which, for example, could be associated with the detected decrease of the fusion of the phagosomes and the lysosomes [72,99]. This would bring about a higher survival of the parent strains within the phagocytes than the strains with mutations in this gene.

During infection due to *A. fumigatus*, the alveolar macrophages and the polymorphonuclear cells, components of the innate defence of the lung, co-operate in the control and elimination of the fungus [146]. The macrophages eliminate the conidia by phagocytosis and the polymorphonuclear phagocytes mediate in the protection against the hyphae by means of a zip binding system and secretion of toxic components [171]. On the other hand, some authors indicate that non-activated macrophages from different anatomic locations, such as the peritoneal macrophages, are incapable of destroying or inhibiting the germination of the *A. fumigatus* conidia [172]. For the destruction of these phagocytosed conidia, the binding of the lysosomes and the acidification of the phagolysosomes, which have been associated to the activation of the hydrolytic enzymes, for example, D-cathepsin, chitinase, etc., seem indispensable [67]. The ROS, such as H_2O_2 or the superoxide ion also appear to be involved in the destruction of the spores [150]. As we have pointed out, these ROS appear to play an essential role in this protection. The detoxifying systems of these ROS can be considered essential in the virulence of the fungi [61]. For this reason, the enzymes which help to control these ROS, such as catalases, peroxidases and superoxide dismutases are considered virulence factors in *A. fumigatus*. The corticoids would inhibit the production of ROS and would play a part in the development of an IA in immunodepressed mice. The enzyme NADPH oxidase which produces ROS is associated with the conidicidal activity of the macrophages, but not so the inducible nitric oxide synthetase enzyme. This enzyme generates nitric oxide and its nitrite and peroxynitrite derivatives. It has been demonstrated *in vitro* that nitric oxide and the peroxynitrite could have some effect on the survival of the conidia and/or its germination, mainly depending on the composition of the media where the test is carried out [87, 88].

Three active catalases, have been detected in *A. fumigatus,* encoded by the *catA* genes, which encode the catalase enzyme present in the conidium [146], and the *cat1/catB* [33] and *cat2* [146] genes which encode the mycelial catalases. The *catA* gene produces a homodimeric enzyme composed of two sub-units of 84.5 kDa. This enzyme is not glycosylated, it is resistant to heat, to ionic metals and detergents, and is only expressed in the conidium. The *catA*$^-$ mutants are more sensitive to treatment with H_2O_2 than the wild strains. Therefore, this enzyme protects the conidia against the effects of environmental H_2O_2, but according to investigations carried out it does not protect the conidia against the respiratory destruction by the alveolar macrophages [146]. This may be because the H_2O_2 is sequestrated by melanin as we have indicated, or it may not be the main ROS in the intracellular destruction of the conidia by the alveolar macrophages [146, 150, 172]. This enzyme has not been considered, therefore, a virulence factor [146]. Cat1p/CatB is one of two catalase enzymes produced by the hyphae of *A. fumigatus* and is made up of four identical sub-units of 90 kDa glycosylated with mannose residues and a signal peptide of 15 aminoacid residues. Cat2p is the second mycelial catalase and is a non glycosylated, monomeric protein, with high sensitivity to heat and with catalase-peroxidase activity. It is assumed, as in the previous case, that these enzymes protect the fungus from ROS, but the mutants with deletion in *cat1*$^-$ do not show any differences in sensitivity to H_2O_2 or virulence compared to the parent strains. Mutants with deletion in *cat2*$^-$ do not show changes in phenotype nor did the deletion affect their virulence. However, the double mutant, *cat1*$^-$*cat2*$^-$, showed less resistance to H_2O_2 and a delay in infection models in the rat. Despite this, the rate of destruction of these double mutants by polymorphonuclear neutrophils did not appear altered. These data imply that the mycelial catalases are not sufficiently protective against polymorphonuclear cells of immunocompetent humans and only give rise to partial and transitory protection of the fungus against respiratory destruction in the experimental rat infection model [146]. It could be that there are other catalases expressed specifically in the infection, since another four catalases have been detected in the genome of *A. fumigatus* which are homologues to Cat1p/CatB and Cat2p.

Another hypothesis, as we have pointed out before, is that other ROS may be associated with the destruction of the hypha. This could indicate that other enzymes such as superoxide dismutase (SOD) could be more efficient in the protection of the mycelium against other ROS. The SOD enzymes are metallo-enzymes which cause dismutation of the toxic superoxide anion. These enzymes are of two types: the ones called MnSOD, which contain Fe and Mn, and others called Cu,ZnSOD, not related to the former and which carry Cu and Zn. The two types of SOD have been detected in *A. fumigatus* [52,64,65]. Holdom et al. [65] have studied an extracellular and antigenic Cu,Zn SOD protein of *A. fumigatus.* This protein has 154 aminoacid residues and three areas almost completely conserved: the interface sub-unit, the metal binding ligand and the residues responsible for the gradient in the electric field, which canalized superoxide dismutase to the Cu(II) active site. However, it shows superficial heterogenicity, which has a profound effect on its antigenicity, and by the use of immunochemical techniques its presence has been demonstrated on the cell walls of the hyphae. The majority of patients infected by *A. fumigatus* produce antibodies against it, which has raised the possibility of its use in the diagnosis of aspergillosis. In fact, some authors have indicated that this protein is more useful in the detection of specific IgA antibodies [36] than galactomannan. Other authors have studied a homotetrameric MnSOD which reacts with IgE, acting like an allergen (Asp f6) and whose structure can have cross reactivity with an enzyme with human origin, participating in auto-reactivity phenomena [52]. As we have indicated, these enzymes can protect the fungus from the oxidative mechanisms of the phagocytes, being more efficient than the catalases.

One hypothesis to take into account, is the use, by the fungus, of efflux pumps associated with the membrane to detoxify components of the immune system, in a similar way to its participation in the resistance to antifungals. The over-expression of these efflux pumps is correlated with multi-resistance to drugs (MDR). There are two classes of efflux transporters, class ABC and class MFS (major facilitator superfamily). In *A. fumigatus,* four genes which encode ABC type transporters have been detected, the *mdr1, mdr2, atrF* and *mdr4* genes and a gene which encodes a protein of the MSF class, the *mdr3* gene [96,134,183].

Direct attack molecules to the host

As part of its virulence attributes, *A. fumigatus* produces a group of pathogenic factors, such as toxins, enzyme proteins which facilitate the adherence and hydrolysis of the components of the cells of the host [100,188], and allergens which prevent an efficient immune response from the host and which generate allergic reactions [54]. Among the degrading enzymes are included proteases [120] and probably phospholipases. The genes and molecules involved are set out in tables 3, 4 and 5. The participation of these molecules in the infection is not always clear, for example, in some cases the environment can facilitate or impede the production of mycotoxins, whose detection *in vitro* does not imply that they are produced in the human organism. We have tried to reflect only those molecules whose participation during infection is much clearer. On the other hand, it has to be taken into account that the effect of these putative factors of virulence can be different when they are studied individually or in combination with other molecules of *A. fumigatus,* which have different activities that may stimulate or interfere with the response of the host [100].

Toxins

Some authors have pointed out that the virulence *A. fumigatus* is due to the release, by the conidia, of toxins which, among other things, prevent their phagocytosis. Of the toxins detected in *A. fumigatus* some have been associated with virulence (Table 3). A diffusible substance from the conidia has been shown to affect the competent macrophages, inhibiting the respiratory burst, phagocytosis and the expression of cytokines by macrophages [21,118]. This substance is stable to heat, has a size less than 14 kDa and its effect is reversible, therefore it does not appeared to be related to other toxins derived from the hypha, such as gliotoxin, fumagilin, helvolic acid and others [118]. This component has still not been identified, but it can be rapidly extracted from the surface of the conidium and can exercise its effect by diffusion into the pulmonary fluid, allowing the fungus to remain in the lung and express its pathogenic effects. That not all the strains of *A. fumigatus* produce this component has been associated with the different pathogenicity observed between strains [21].

Other toxins associated with conidia are, for example, fumigaclavine C (an alkaloid metabolite which is a potent inhibitor of DNA synthesis) and aurasperone C (nerve dysfunction agent) [51, 118], but it has not been reported that they directly affect macrophages. As in the previous case, not all the strains studied produced conidia with these components. Recently, it has been shown that fumigaclavine C affects the T lymphocytes by inhibiting their activation, proliferation and adhesion to extracellular matrices and reducing their production of TNFα [213].

The filtrate of the cultures of *A. fumigatus* appear to contain a series of toxic substances which act in an additive and/or synergic way on the phagocyte cells and reduce the survival of the infected animals [201]. The hyphae of *A. fumigatus* release a number of low molecular weight toxins, particularly gliotoxin (GT), helvolic acid and fumagillin. The greater toxicity was observed for GT, a well characterised fungal metabolite. This GT also appears to be detected in some aerial conidia of *A. fumigatus* [174], but not all strains isolated from the environment produce it, only approximately 11% [45]. This mycotoxin belongs to the group of epipolythiodioxopiperazines (ETP), a class of toxins produced by fungi pathogenic to plants and mammals [58]. The data indicates that GT is produced by a group of genes which appear to be activated when secondary metabolism begins [158]. GT has several powerful bioactivities, in particular the cytotoxicity for mouse macrophages [201] and the inhibition of the activation of NADPH oxidase of polymorphonuclear leucocytes [193]. In vitro, in micromolar concentrations, it induces apoptosis and inhibits the release of IFNγ and IL-4 by human blood cells, and whereas in nanomolar concentrations it impedes the function of the ciliary cells and damages the respiratory epithelium [4,23]. Nevertheless, it has not been

Table 3. Toxins related to the direct attack to the host organism.

Genes	Proteins	Characteristics	Pathogenesis related activities	Use
	Conidium toxin diffusible substance	14 kDa	Reversible macrophage inhibition	
	Fumigaclavine C (Alkaloid metabolite)		DNA synthesis inhibition Effect on macrophages? Lymphocyte T inhibition	
	Aurasperone C		Nervous system dysfunction Effect on macrophages?	
	Gliotoxin (epipolythio-dioxopiperazine family, secondary metabolism)		Cytotoxicity (macrophages and respiratory epithelium) NADPH oxidase inhibition (neutrophils) Ciliostasis	Diagnosis
	Fumigacin, helvolic acid (Fusidane, steroidal antibiotic family, secondary metabolism)		Macrophage inhibition (respiratory burst) Ciliostasis and respiratory epithelium damage	
	Fumagillin (Antitumoral antibiotic; secondary metabolism)		Ciliostasis Inhibition of endothelial cell proliferation	
mitF/asp f1/res	Mit F/Asp f1/Res, mitogillin, ribotoxin or restrictocin (RNAase affecting 28S rRNA)	18 kDa	Ribotoxin, protein synthesis inhibition Type I hypersensitivity Cytotoxin	Diagnosis
aspHS	AspHS, Asp-hemolysin	14 kDa (Similar dominio to the low density lipoprotein receptor)	Hemolytic and cytotoxic activity (erythrocytes, macrophages and endothelial cells) Cytokine production induction	
	Aflatoxin B1 and G1		Production on infection?	
	Verruculogen		Production on infection?	

considered a virulence factor because of its low production *during* the normal growth of *A. fumigatus* [201]. However, it has been shown that conditions of high aeration favour the rapid production of this compound, so it could possibly play a role in respiratory infections caused by A. fumigatus [200,202]. According to these authors, *A. fumigatus* could confront the innate immune responses, such as the phagocyte response, with GT. Likewise, it has recently been shown that treatment with amphotericin B, an antifungal agent used in the treatment of IA, can induce its synthesis. This antifungal could activate the secondary metabolism or produce the release of GT to the external medium of the fungus, by altering the permeability of its cell wall [158]. That is to say, that this treatment could exacerbate the effects of the toxin despite its antifungal effect and, surprisingly, facilitate the fungal invasion of the lung. Some authors have proposed to use this toxin in the diagnosis of aspergillosis, by detecting specific antibodies [54] or the antigen itself in serum or urine.

Of the other two important toxins which some strains of *A. fumigatus* can produce, helvolic acid or fumigacin, belongs to a small family of natural steroid antibiotics known as fusidanes. In much higher concentrations than those of GT, it can affect the oxidative burst of the macrophages [118], the metabolism of oxidised low density lipoprotein [181] in vitro, and also causes a complete ciliostasis and rupture of epithelial cells [4]. Fumagillin, the other important toxin, is an anti-tumour antibiotic which is a potent inhibitor of angiogenesis. As reported by Bunger et al. [32], it directly inhibits the proliferation of endothelial cells and in vitro it causes the inhibition of the ciliary functions of the human respiratory epithelium. Its target is the enzyme methionine aminopeptidase 2, to which it forms an irreversible covalent bond. The active concentrations are also rather higher than in the case of GT. Due to secondary metabolism of these latter two toxins, it is not clear what their role is in the in vivo pathogenesis, if they are produced in effective quantities, but by their activities a participation in this can be postulated.

The ribotoxins are a family of ribosome inactivator proteins which have a highly specific activity against the phosphodiester bond of the sarcin/ricin domain universally preserved in 28S ribosomal RNA [74,75]. They are all very similar, with around 85% of identity in the aminoacid sequences [113], and all must enter into the target cell to exercise their toxic action, a step which determines their resistance differential between cells. These types of toxin were discovered in the *Aspergillus* species in the 1960's and their production appears to be associated with the formation of conidiophores. In nature it can be a defence mechanism against insects. In *A. fumigatus* a protein of 18 kDa has been discovered which is a principal allergen/antigen/cytotoxin of this fungus. This protein has been associated with the allergic processes produced by this fungus, since it is one of the immunodominant antigens in patients with allergic aspergillosis. It is also a protein secreted by the fungus and is detected in the urine of infected patients [95,102], thus it is believed that it has some cytotoxic activity in IA, being considered a possible virulence factor [7,100]. This allergen, a member of the family of ribotoxins has received the names Asp f1, mitogillin and in some cases, restrictocin. It is encoded by the *Asp fI/mitF/res* gene, and the detailed study of its structure [153,169] has allowed its application in the diagnosis of the diseases caused by this fungus and it is found among a group well defined antigens, called SDA [110], relevant in the diagnosis of *A. fumigatus*. However, there is controversy between those who find it useful in the diagnosis [204] and those who do not [207].

A. fumigatus also appears to produce a hemolysin, encoded by *asp-HS* and called Asp-HS or Asp-hemolysin. It is composed of 131 aminoacid residues and has a molecular weight of 14 kDa [48]. This molecule has haemolytic activity on sheep and rabbit erythrocytes and cytotoxic effects on macrophages and endothelial cells *in vitro* [85]. These authors have also demonstrated that it acts by inducing the expression of cytokines in macrophages *in vitro*. The hemolytic activity is inhibited by its characteristic binding to apolipoprotein B and low density lipoproteins [56,83,84], since haemolysin has a negatively charged domain similar to the receptor of these low density lipoproteins [100]. This toxin can be detected *in vivo* during infection by *A. fumigatus* and it could therefore contribute to pathogenesis, but experimentation on this is lacking.

Many other toxins have been detected even in aspergilloma isolates, as well as the production of aflatoxin B1 and G1 [149], verruculogen, etc., but their classification as virulence factors depends on their demonstration in the infection. For example, the production of the secondary metabolite aflatoxin appears to be associated with up to 22 highly regulated genes [138], which makes its production *in vivo* unlikely.

Allergens

In immunocompetent patients *Aspergillus* can produce allergic bronchopulmonary aspergillosis (ABPA), allergic rhinosinusitis, asthma, and aspergilloma. A review on the fungal allergy has been published by Kurup et al. [92]. *A. fumigatus* produces a significant number of allergenic molecules which show a reaction with IgE in asthmatic patients and with ABPA [79]. In table 4 are set out some known characteristics of these allergens. Although 23 allergens, which have been given names from Asp f1 to Asp f23 [94,155,170], have been detected and proposed up until now, not all are accepted as recognised allergens. Their production during the growth of the fungus in IA is not clear. These allergens have been characterised and their epitopes studied for their application to improve the detection of diseases associated to this fungus [11,12,86, 94,110,153,155,156,169,177]. Some of these allergens have a high homology in their sequences with known functional proteins and enzymes, but others do not. All these allergens appear to activate an immune response of Type I hypersensitivity with production of high affinity antibodies of IgG and IgE type. Immunocompetent individuals affected regularly have high levels of these antibodies and although they are not always protective against invasive infection, they can be useful for diagnostic purposes. These types of allergens, as well as their recognised biological activity, can serve to redirect the immune response to the fungus by the activation of the Th2 lymphocytes with a high production of IL-4 [78]. These types of responses do not appear to be efficient in the elimination of this fungus [62].

Asp f1 or mitogillin is the RNAse or ribotoxin previously reviewed which reacts with the IgE of patients with ABPA [91]. Asp f2 has a biological activity associated with binding to laminin [92], thus it can participate in the colonisation of the lung. Its production appears to depend on the medium where the microorganism is developed, since the biosynthesis of Asp f2 is, as in other molecules, Zn^{2+} regulated [175]. Asp f6 is the MnSOD of 26.5 kDa whose activity was reviewed earlier. Some allergens have enzymatic activity which has been associated with the invasion of the host, for example Asp f5/Mep has a metalloprotease activity, Asp f10/Pep appears to be a

Table 4. Allergens related with activation of Type I hypersensitivity.

Genes	Proteins	Characteristics	Pathogenesis related activities	Use
asp f1/mitF/res	Mit F/Asp f1/Res, mitogillin, ribotoxin or restrictocin (RNAase affecting 28S rRNA)	18 kDa	Ribotoxin, protein synthesis inhibition Type I hypersensitivity Citotoxin	Diagnosis
asp f2	Asp f2, Asp fII (Secreted or cell associated glicoprotein, ZPS1 family)	37 kDa	Binding to laminin and fibrinogen Type I hypersensitivity	Diagnosis
asp f3	Asp f3, PMP20 (Peroxisomal-like protein, peroxiredoxin 2 family)	19 kDa	Type I hypersensitivity	Diagnosis
asp f4	Asp f4	30 kDa	Type I hypersensitivity	Diagnosis
asp f5/mep	Asp f5/Mep (Glycosylated metalloprotease, ZnMep super family)	42 kDa	Tissue destruction/Invasion Type I hypersensitivity	Diagnosis
asp f6/sod	Asp f6/MnSod (Mitochondrial superoxide dismutase)	26.5 kDa	Protection against ROS Type I hypersensitivity Autoimmunity Antigenic	Diagnosis
asp f7	Asp f7	11.6 kDa	Type I hypersensitivity	Diagnosis
asp f8	Asp f8 Ribosomal P2Protein (60S, phosforylated)	11 kDa	Type I hypersensitivity	Diagnosis
asp f9	Asp f9	33.7 kDa	Type I hypersensitivity	Diagnosis
asp f10/pep	Asp f10/Pep, aspergillopepsin F (Secreted aspartic protease, pepsin familiy)	34 kDa	Tissue destruction/Invasion Type I hypersensitivity	Diagnosis
asp f11	Asp f11, cyclofilin (Dipeptidyl-prolyl-isomerase)	24 kDa	Type I hypersensitivity	Diagnosis
asp f12/ hsp1	Asp f12/Hsp1 (Heat shock protein, family Hsp90, ATPase activity)	65 kDa	Chaperone activity and protein transport in growth at 37 °C Stress response during inflammation Autoimmunity Type I hypersensitivity	Diagnosis
asp f13/alp2	Asp f13/Alp2 (Alkaline serine protease, subtilisin family)	34 kDa	Tissue destruction/Invasion (elastin) Type I hypersensitivity	Diagnosis
asp f15	Asp f15 (Asp f13 precursor)	16 kDa	Type I hypersensitivity	Diagnosis
asp f16	Asp f16 (High homology with Asp f9)	43 kDa	Type I hypersensitivity	Diagnosis
asp f17/mp1	Asp f17/Mp1 (Wall cell mannoprotein, relation with Afmp1?)	19.4 kDa	Type I hypersensitivity	Diagnosis
asp f18	Asp f18 (Vacuolar serine protease, subtilisin family, relation with *alp2*?)	34 kDa	Tissue destruction/Invasion (elastin) Type I hypersensitivity	Diagnosis
asp f22	Asp f22 (Enolase)	47 kDa	Type I hypersensitivity	Diagnosis
asp f23	Asp f23 (Ribosomal L3 protein)	44 kDa	Important at growth Type I hypersensitivity	
aspfx	Asp fx	19 kDa	Type I hypersensitivity	

aspartic protease, Asp f11 or cyclophilin has a dipeptidyl-prolyl-isomerase activity, Asp f13/Alp2 possesses alkaline serine protease activity, and Asp f18 is a vacuolar serine protease. The vacuolar serine protease has been shown that it is a principal allergen, despite its location away from the cell wall [177]. It has been pointed out that the enzymes with serine protease activity are important allergens in asthma [179]. Asp f12/Hsp1 is a heat shock protein of the Hsp90 family, implicated in the stability of the proteins during growth at high temperature, which is commonly associated with the virulence of microorganisms. Asp f22 is an enolase of *A. fumigatus* [94]. In other microorganisms, such as *Candida albicans,* this enzyme has been associated with virulence in the patient with invasive disease, but in the case of *Aspergillus* this is not so clear. Some of these components of the fungus have demonstrated a synergic activity in the activation of the immune system, for example, Asp f13 and Asp f2 boost lung inflammation when they are present together [93]. Some of the allergens, whose degradative enzymatic activity has been associated with IA of the host, will be studied in the next section. The last allergen recognised, Asp f23, appears to be the ribosomal protein L3 of the 60S subunit, with a molecular weight of 44 kDa, with a probable role in the resistance to antifungals and the possible generation in the host of autoimmune responses [170].

Degradative enzymes

The environmental fungi which take part in the recycling of organic materials in the environment, such as *A. fumigatus*, are capable of producing multiple extracellular enzymes to accomplish this function. The penetration in the pulmonary epithelium is a key step in the infection process [80], and as has been recorded by Latgé [100], the pathogenic filamentous fungi use the extracellular enzymes to degrade the structural barriers of the host. In animal tissues these barriers are composed of proteins, therefore the fungus requires proteases to invade them. It is logical to assume that these enzymes could act by making this tissue invasion easier, but they could also participate in the infection by eliminating some mechanisms of the immune defence and/or helping in the obtaining of nutrients. There are old data pointing to the possible relationship of proteases with pathogenesis in animal models and that clinical isolates produce higher quantities of proteases. In some studies it has been shown that protease negative mutant strains are less pathogenic in animal models than their parent strains [81].

The principal components of the lung matrix are elastin [185] and collagen. Therefore enzymes with elastinolytic and collagenolytic activity can have a key role during the infection and are normally considered virulence

factors of these microorganisms. Some authors have carried out a study of the elastase activity index in different strains of *A. fumigatus*, finding differences between strains [25,139] and a clear relationship between this activity and pathogenicity [25]. Strains isolated from patients with IA show high levels of elastase activity. Even though the significance of this activity in environmental strains is still unknown, it has been observed that hospital isolates tend to have a higher elastase activity, which could explain why IA is a nosocomial problem. The cytoskeleton of the alveolar epithelial cells, chiefly actin, suffers the main changes during infection by *A. fumigatus* and it appears that these are mediated by alkaline serine proteases since they are inhibited by antipain, which inhibits the action of these proteases [80]. The data from these authors suggest that *A. fumigatus* opens breaches in the alveolar epithelial cell barriers, secreting proteases that disorganise the actin cytoskeleton and destroy cellular adhesion to the substrate [80]. On the other hand, in allergic reactions, the proteases *A. fumigatus* appear to help the transport of antigens across the layers of epithelial cells. These proteases damage the integrity of the epithelium and bind to the cell surface receptors of the alveolar macrophages, inducing the production of inflammatory chemokines and cytokines [78,154]. The role of the proteases in the virulence of *A. fumigatus* has been extensively studied. At least three types of proteases have been described to be secreted by the fungus in vivo. An alkaline serine protease (Alp) [81,124,159], a metalloprotease (Mep) [112,122] and an aspartic protease (Pep) [162,163] are among the most important extracellular proteases.

We have already pointed out the important role of the alkaline serine proteases in allergic reactions and the fact that almost all the enzymes of this type appear to be antigens, since specific antibodies have been detected in infected patients. The main protease secreted, which is produced when the fungus is incubating with protein (for example, soluble elastin) or protein hydrolysates at neutral pH, is an alkaline serine protease (Alp) with elastolytic activity encoded by the *alp* gene. Its molecular weight is 33 kDa and it appears to be produced by *A. fumigatus* and to a lesser extent *A. flavus* [81]. It appears to be related to the family subtilisins and as well as elastin, it degrades collagen, fibrinogen and casein, also having activity, to a lesser extent, on haemoglobin and serum albumine [102,159]. Structural studies appear to detect this enzyme being secreted in the germinal tube and in the apex of the hypha during the infection [129]. Other serine proteases have been detected in this fungus, such as Alp2, associated with the cell wall and what appears to be the Asp fl3 allergen [160], and a vacuolar serine protease of 34 kDa which is the Asp f18 allergen [179]. The proteases associated to the cell wall could help the hyphae penetrate the connective tissue layers of the host [120]. Disruption studies of the principal gene, producing a*lp*$^-$ mutants, are contradictory since although initially some authors were indicating that these mutants produced less mortality in animals [81], more recent studies do not detect differences in virulence compared to the parent strains [123,180,184,186]. The *alp2*$^-$ mutants have a slightly reduced growth and an 80% decrease in sporulation, thus this gene could be of interest in the study of morphogenesis phenomena and pathogenesis in *A. fumigatus,* as well as its interest in allergic asthma. Several authors have detected other alkaline serine proteases produced by *A. fumigatus*, of which some are extracellular. For example, the Kunert group detected up to six bands of proteolytic activity with different isoelectric points, a principal one which corresponds to Alp and another five lesser ones which seemed to be post-transcriptional modifications rather than isoenzymes and whose significance is unknown [89,90]. Iraneta et al. [68] detected, in extracts of *A. fumigatus,* other alkaline proteases with molecular weights of 180, 84 and 70 kDa which are immunoreactive with IgE. Reichard et al. [159] detected a second protease with extremely alkaline activity, which is called Exalp. Nigam et al. [137] also detected a new secreted allergen or antigen of 56 kDa (Gp56) with serine protease activity. It is possible, therefore, that the elimination and mutation of one of them is compensated for by the production of the rest during the infection or by the production of other proteases which act by substituting the function of these enzymes.

An extracellular collagenolytic activity (30% of the total extracellular collagenase activity) due to a metalloprotease (Mep) of 43 kDa, has been reported in the literature [112,182]. This Mep is not glycosylated, has an elastinolytic activity (less than that possessed by Alp), and concentrations of Zn^{2+} and Co^{2} do not affect it, and it is even induced by Zn^{2+} [112]. Mep does not have homologues with other known metalloproteases, but by its conserved motif it appears to belong to the super family of metalloproteases which contain Zn [119, 182], and within that the family of thermolysins, enzymes which have been associated with the pathogenesis of different bacteria, and is also the Asp f5 allergen. Its production by *Aspergillus* has been observed *in vivo,* which is not surprising since in the biological systems, Zn^{2+} is only surpassed by the presence of iron. A second extracellular glycosylated metalloprotease has been detected in *A. fumigatus* which does not hydrolyse elastin and is inhibited by ions such as Zn^{2+} and Co^{2+} [122]. Other metalloproteases in *A. fumigatus* have been reported in the literature. For example, a metalloprotease of 23 kDa encoded by *mep20* gene has been detected in *A. fumigatus* and *A. flavus* [157]. An intracellular metalloprotease of 82 kDa, encoded by the *mepB* gene, has also been detected, which appears to be associated with the degradation of small cytoplasmic peptides and belongs to a family of thimet oligopeptidases associated with Zn [66]. Studies carried out by different authors into the loss by mutation of these metalloproteases have determined that the *mep*$^-$ mutant strains and *alp*$^-$*mep*$^-$ double mutants retain their virulence [59, 100].

The third large group of extracellular enzymes of *A. fumigatus* produced during infection are the aspartic proteases which appear to belong to the family of pepsins and are called aspergillopepsins [120]. In *A. fumigatus* two aspartic proteases have been detected, one secreted (Pep) and another associated to the cell wall of the fungus (Pep2). Pep, Asp f10 or aspergillopepsin F, is an allergen which has a molecular weight of 34-38 kDa, an optimum enzyme activity pH of 5, is encoded by the *pep* gene, and has been detected during infection by this fungus [103]. This enzyme is secreted by the germinal tubes and the penetrating hyphae, but it is not induced by lung components and its loss by mutation does not decrease the virulence as compared to the parent strain [162]. Pep2 has a molecular mass of 39 kDa, is not secreted and appears joined to the cell wall of the fungus, having a wide range of activities from pH 2 to 7 [161]. Mutants of *pep2*$^-$ do not present changes in the phenotype. Their possible function in the pathogenesis has not been studied, although, as in the case of Alp2, they could facilitate the penetration of the hyphae into the layers of the connective tissues of the host [120].

Gifford et al. [59] have demonstrated that the presence of human serum stimulates the secretion of protease by more than 100 times and that these secreted enzymes degrade the human pulmonary basal lamina which should

Table 5. Extracellular enzymes related to direct attack to the host organism.

Genes	Proteins	Characteristics	Pathogenesis related activities	Use
alp	Alkaline serine protease (subtilisin family)	33 kDa	Tissue destruction/Invasion (elastin, collagen, fibrinogen and casein)	
alp2/asp f13	Alp2/Asp f13 (Extracellular serine protease)	34 kDa	Tissue destruction/Invasion (elastin) Type I hypersensitivity	Diagnosis
asp f18	Asp f18 (Vacuolar serine protease, subtilisin family, relation with *alp2*?)	34 kDa	Tissue destruction/Invasion (elastin) Type I hypersensitivity	Diagnosis
	Other serine proteases	180, 84, 70, and 56 kDa	Tissue destruction/Invasion Type I hypersensitivity? (IgE reactivity)	
	Non-glycosylated metalloprotease (neutral metalloproteases family I)	40 kDa	Tissue destruction/Invasion (collagen)	
mep/asp f5	Mep/Asp f5 (Glycosylated metalloprotease, ZnMep super family)	42 kDa	Tissue destruction/Invasion (collagen) Type I hypersensitivity	Diagnosis
mep20	Metalloprotease	23 kDa	Tissue destruction/Invasion	
mepB	Intracellular metalloprotease	82 kDa	Tissue destruction/Invasion	
pep/asp f10	Pep/Asp f10, aspergillopepsin F (Secreted aspartic protease, pepsin family)	34-38 kDa	Tissue destruction/Invasion Type I hypersensitivity	Diagnosis
pep2	Pep2 (wall-associated aspartic protease)	39 kDa	Tissue destruction/Invasion	
dppIV	DppIV (glycoprotein, dipeptidyl-peptidase activity)	95 kDa	Protein degradation (collagen, hormones and cytokines) T_{CD4+} lymphocyte activation	
dppV	DppV (glycoprotein, dipeptidyl-peptidase activity)	88 kDa	Protein degradation (collagen, hormones and cytokines)	Diagnosis
plb1, plb2 and plb3	Plb1, Plb2 and Plb3 (Phospholipases B)	633, 588, and 630 aminoacids respectively	Tissue destruction/Invasion (lecithin)	
	Phospholipase C		Tissue destruction/Invasion Phosphorus acquisition	

lead to participation in the virulence. Although these proteases have frequently been associated with virulence, the truth is that none of the three principal secreted enzymes are clearly related with the invasiveness of this fungus, as it is shown that the loss of one or several of these genes does not decrease the virulence [59,100]. It is possible that these proteases are not really virulence factors since other species of *Aspergillus* also secrete protease homologues and rarely cause IA. Perhaps the degradation of tissue components by proteases is only a medium to obtain nutrients and successfully compete with normal microbiota [100]. On the other hand, *A. fumigatus* could compensate the loss of these enzymes by secreting others. In fact it is known that the regulation of the secretion of proteases in *A. fumigatus* is complex and the secretion patterns are highly dependent on the composition of the media [59]. The increased secretion due to the presence of serum could indicate that other proteases may be secreted during the infection. This could indicate that a successful study of these enzymes as virulence factors would require multiple disruption systems.

In culture supernatants of *A. fumigatus,* two members of a family of dipeptidyl-peptidases (Dpp), which split dipeptides of the extreme N-terminal of peptides and proteins, have also been detected. Within this family, the enzymes are divided into different classes depending on the nature of the dipeptides produced and their cellular location [16]. These Dpp play an essential role in human physiology, but up until now they have not been considered putative virulence factors of human fungal pathogens. They also belong to the group of alkaline serine proteases [120], and in *A. fumigatus* two, DppIV and DppV, have been detected. DppIV is a glycoprotein of 95 kDa whose specificity is to split the Xaa-Pro or Xaa-Ala dipeptide terminals at neutral pH and in its sequence of aminoacids is found the consensus motif of a serine hydrolase (Gly-X-Ser-X-Gly) [16]. DppV is also a glycoprotein with a molecular mass of 88 kDa and its enzymatic specificity is the release of Ala-Ala, His-Ser and Ser-Tyr dipeptides at neutral pH [15]. Beauvais et al. [15, 16] indicate that these enzymes are secreted in large quantities when the medium contains only proteins and their hydrolysates, the same conditions which favour the secretion of *A. fumigatus* proteases. It is possible that they participate in the degradation of the hydrolysis products of these proteases, and the dipeptides generated are used for the fungus as a source of aminoacids for its growth. These enzymes are able to bind with collagen and also human proteins, such as hormones and cytokines, and degrading them to generate inactive molecules. It has been seen that DppIV and V, as well as their proteolytic activity which can damage the host, participate in the activation of T lymphocytes. For example, DppIV has homology with the CD26 receptor which directly, or by means of other molecules, activates $CD4^+$ T cells. DppV is identical to one of the principal antigens used in the diagnosis of aspergillosis, the so-called chymotryptic antigen. On the other hand, DppV could be a molecule capable of generating protector responses, and it has been seen that mouse survivors of aspergillosis studies have mono-specific antibodies which recognise this protein [15].

The phospholipases are an heterogeneous group of enzymes which are capable of hydrolysing one or more ester bonds in phosphoglycerides. Phospholipase activity can destabilise the membranes of the host, provoking lysis of the cells and releasing secondary lipid messengers. In many microorganisms they have been considered virulence factors. The study of the extracellular phospholipases activity indicates that all the isolations produce them and they include activities of phospholipase C, phospholipase acyl-hydrolase (phospholipase A and/or B) and phospholipase D [24]. The phospholipase B enzymes show phospholipase, lysophospholipase and lysophospholipase transacylase activities, forming a family of enzymes. In *A. fumigatus* these enzymes are encoded by three genes called *plb1, plb2* and *plb3* which encode proteins with

633, 588 and 630 aminoacid residues, respectively [38,42,178]. They are all expressed at 37°C, but only two of the enzymes, Plb1 and Plb2, have a signal peptide and are secreted, the other enzyme being cytoplasmic. The expression of the secreted enzymes is induced by lecithin, a constituent of human lung surfactant [178]. In other fungi such as *Cryptococcus neoformans,* mutants in the *plb1* gene have a reduced virulence, and in this fungus and in *Candida albicans,* these enzymes have been considered virulence factors. Their lower production in clinical isolations than in environmental isolations of *Aspergillus* does not make its activity very clear in the pathogenesis, although it cannot be discounted. This is, perhaps, due to that, in other fungi, Plb is the only phospholipase secreted, while in *A. fumigatus* phospholipase C is also secreted. Comparing the activity between the clinical and environmental isolations, it is observed that the clinical isolations produce more extracellular phospholipase C than the environmental ones [23], but the latter had a higher acyl-hydrolase activity than the clinical ones. This data seems to point to a greater importance of phospholipase C activity in the virulence of *A. fumigatus*. At the moment no studies have been carried out with mutants of these genes in *A. fumigatus*.

Other genes and putative molecules of virulence associated with viability and growth

The rest of the genes and molecules, which have been reported in the literature in relation to the virulence of *A. fumigatus*, are presented in table 6. The capacity to adapt to the changes in the availability of nutrients is an essential attribute of many successful pathogens. The survival of *A. fumigatu*s in the blood stream during IA indicates that this fungus possesses mechanisms to obtain nutrients essential for its growth and reproduction in this environment. The serum of mammals inhibits the growth of many microorganisms, including some of the most common fungal pathogens [59]. It has been seen that the presence of serum activated the production of proteases, which can degrade blood components and endothelial layers to facilitate the growth of the fungus. But the inhibitor effect of serum is in part, associated with a very low free iron within it (10^{-8} M), since it is bound to molecules such as transferrin [59,63]. As pointed out by Gifford et al. [59] the hydrolysis of transferrin would be a potential medium for obtaining iron in the serum, but the secretion of proteases is not detected until the culture begins the logarithmic growth phase. Schrettl et al. [173] indicated that *A. fumigatus* has assimilation systems of reduced iron and of the mobilisation of iron by siderophores. The siderophores are molecules whose molecular weight is greater than 10 kDa and *A. fumigatus* can produce at least six different, hydroxamate type siderophores, the two most prominent are triacetylfusarinine C and ferricrocin. They are rapidly synthesised in the presence of serum, before the proteases are released, and they are required for the beginning of growth. Up until now, they have not been examined in detail, but recently Schrettl et al. [173] have carried out the first tests on the influence of the different systems of obtaining iron in the virulence of *A. fumigatus*. These authors indicate that the inhibition of FtrA, a permease with a high affinity for iron, does not appear to have effect in the virulence in the IA rat model. In contrast to this, it was detected that the enzyme ornithine monooxygenase (SidA) which catalyses the first step of the biosynthesis of hydroxamate type siderophores, is an absolutely essential attribute of virulence [173]. It is very interesting data, since it could be one of the first essential virulence systems demonstrated of this fungus. Combining the absence of a *sidA* ortholog gene and siderophore systems in mammals, these biosynthetic routes could be promising targets for the development of antifungals.

Phosphate is also essential for the growth of fungi. *A. fumigatus* possesses an enzyme arsenal for using inorganic phosphorous (Pi) in its environment. Among them is found a phytase, an acid phosphatase (PhoAp) and phospholipase C. The phytase enzyme is a histidine acid phosphatase which is associated with the degradation of phytate (myoinositol hexaphosphate, the main storage source of phosphate in plants). The *phoA* gene [20] encodes a glycoprotein of 80 kDa molecular mass which is active on phosphate monoesters and diesters. It is an acid phosphatase which is able to be reprimed (reprimable) by phosphate which possesses an GPI anchor sequence and is bound to ß-glucans in the cell wall. It does not appear to participate in the organisation of the cell wall and its role as a virulence factor or possible antifungal target has not been studied. It seems that in media rich in Pi it disappears from the cell wall without affecting the growth and organisation of this structure.

In fungi, histidine-kinase enzymes of two components have been associated with response mechanisms to extracellular changes in osmolarity, resistance to fungicides such as dicarboximides and regulation and/or building of the cell wall. The demonstration that three histidine-kinase genes contribute to the virulence of *C. albicans* has shown the importance of signal routes regulated by these enzymes. Several genes have been detected which encode this enzyme class in *A. fumigatus*, *fos1*, *fos2* and *fos3*. The *fos1* gene appears to be expressed remarkably higher *in vivo* than *in vitro* [47]. Its deletion produces a delayed conidiation in comparison with the parent strain [151] and in systemic aspergillosis mouse models, the mutant strains show a significantly reduced virulence [41]. It is therefore a proposed virulence factor and is also an interesting target for the development of new antifungals.

In other fungi, the signal routes regulated by nutrients have been associated with pathogenesis. The signal transduction cascades, which regulate morphogenesis and/or use of nutrients such as nitrogen, are important, since modifications in the genes of these routes can produce less virulent phenotypes. The study of genes overproduced by *A. fumigatus* in cultures with endothelial cells has indicated that several of these genes are implicated in these types of routes. One produces a protein belonging to the family of Ras proteins and another two of them encode the regulator subunit of the cAMP dependent protein kinase [164]. The Ras proteins are small GTPases associated to the membrane related to multiple aspects of cell growth and development. This family of highly conserved proteins controls multiple regulation cascades, and some of the genes that encode them have been associated with virulence. In *A. fumigatus* two proteins of this family have been detected, RasA and RasB, which are only differentiated by about 20 aminoacids absent in RasA [53]. These authors indicate that the inactivation of these *rasA* or *rasB* genes lead to defects in the germination of conidia, although the effect is more profound with *rasA*. The overexpression or different level of expression leads, for example, to the over-production of aerial hyphae, hyperbranching, a reduced conidiation or even an uncoupling of the mitosis times and formation of the germinal tube, which can generate an accumulation of many nuclei in the cells. Therefore, it appears that these genes participate in the germination, *rasA,* and/or in the formation of conidia, *rasB*. The *rasB* gene appears to be overexpressed when the microorganism grows in contact with the cells, which has

Table 6. Other genes related with the pathogenicity of *A. fumigatus*.

Genes	Proteins	Characteristics	Pathogenesis related activities	Use
pabaA	Aminobenzoic acid synthase	91,6 kDa	Folate synthesis and growth	New antifungal drug target
pyrG	Orotidine-5'-monophosphate decarboxylase		Uracil and uridine synthesis	New antifungal drug target
	Triacetylfusarinine C, ferricrocin (and other 4 siderophores)	(hydroxamate siderophores)	Conidium germination and growth (Iron acquisition)	
sidA	Ornithine monooxygenase		Hydroxamate siderophores production	New antifungal drug target
phoAp	PhoAp, cell-wall acid phosphatase (GPI[a] glycoprotein, linked to ß-glucan)	80 kDa	Growth, inorganic phosphorus acquisition Hypothetical role in virulence	
fos1, fos2 and fos3	Histidine kinases		Osmotic change adaptation regulation Virulence factor proposed	New antifungal drug target
rasA and rasB	RasA and RasB (GTPases, Ras family regulatory proteins)		Growth regulation Hypothetical role in virulence	
rhbA	RhbA (Rheb subfamily of the Ras family)	21 kDa	Use of nitrogen sources Growth regulation (nutrient sensor) Hypothetical role in virulence	
areA	GATA transcriptional factor		Growth, use of nitrogen sources	
sakA	SakA (MAP-kinase)		Cellular homeostasis regulation and response to H_2O_2	
cAMP regulation cluster			Multiple regulatory functions (virulence)	
pkaR and pkaC	Regulatory (type II) and catalytic subunits predominant in protein kinase A		Participation in melanin synthesis	
gpaA and gpaB	Alpha subunits of heterotrimeric regulatory G protein		Transduction signals Defence against killing by macrophages	
*acy*A	Adenylate cyclase			
hsp1/asp f12	Hsp1/Asp f12 (Heat shock protein, Hsp90 family)	65 kDa	Chaperone activity and protein transport at 37 °C Stress response during inflammation Autoimmunity Type I hypersensitivity	
thta		141 kDa	Essential for growth at high temperatures	
cgrA	CgrA (Yeast nucleolar protein ortholog)		Ribosome synthesis at 37 °C	New antifungal drug target
smcA	SmcA		Structural maintenance of chromosome (essential for growth)	
prp8	Prp8 (intein)		Critical component of spliceosome (intron removal from pre-mRNA)	New antifungal drug target
anxc4, anxc3.1 and anxc3.2	Annexins (Calcium and phospholipid -binding proteins)		Exocytosis activity and membrane fusions	New antifungal drug target
cpcA	Transcriptional activator (cross-pathway control system of amino acid biosynthesis)		Growth in host	
lysF	Homoaconitase (Lysine biosynthesis)		Growth in host	
	Metacaspases		Apoptosis	New antifungal drug target

[a] Glycosyl-phosphatidyl-inositol (GPI) membrane anchorage motif.

been associated with a hypothetical role in virulence [164]. Panepinto et al. [142] have detected another related gene, *rhbA*. This gene is the only one in the genome of *A. fumigatus* that encodes a protein homolog of the Rheb family, which is a subfamily of Ras. This gene has an ORF (open reading frame) of 561 bp and encoded protein has a predicted molecular weight of 21 kDa. It is expressed during the complete asexual cycle of *A. fumigatus* but its expression is boosted when the fungus is in contact with human tissue or it grows under conditions that lack nitrogen. Its importance in infection has been deduced because *rhbA*$^-$ mutants show a significant reduction in virulence against the parent type in IA models in the mouse [143]. This reduced virulence is also correlated with a lack of growth on sources poor in nitrogen. This data suggests that the versatility of the use of nitrogen can contribute to the growth of this fungus *in vivo* and that the Rhba protein may act as a nutrient sensor. Other data which supports this idea is that the disruption of the GATA transcription factor which regulates the repression by nitrogen catabolites, encoded by the *areA* gene, also produces mutants of reduced virulence in IA animal models.

Different routes and enzyme regulators have been associated with the pathogenesis. The mitogen-activated kinases (Map-kinases) play a central role in the regulation of cell homeostasis and the response to the environmental changes of the eukaryotes. Within *A. fumigatus* there is a MAP-kinase encoded by the *sakA* gene whose homologues in other fungi have been associated with virulence. In the case of *A. fumigatus,* a greater transcription of this gene is observed when the microorganism grows in osmotic stress or in response to H_2O_2 [209]. These authors indicate that *sakA*$^-$ mutants have a hyphae growth stop. However, the germination of the conidia in the mutants is not stopped, only decreased, except when the media have inadequate sources of nitrogen. The *sakA* gene is active in lack of nitrogen conditions and acts by negatively regulating the germination of the conidia.

Another of the known regulation cascades with multiple functions is the regulation by cAMP. The *pkaR, pkaC*1, *gpaA, gpa*B and *acy*A genes encode proteins of the cAMP signal cascade route [104, 106]. The *pkaC1* and *pkaR* genes encode subunits of protein kinase A, where PkaC1 is the predominant catalytic subunit and PkaR is the type II regulator subunit [140]. Both genes are overexpressed during the culture with alveolar epithelial cells. The *gpaA* and *gpaB* genes encode the alpha subunit of the heterotrimeric G protein (regulator) and the *acyA* encodes adenylate cyclase. It is observed that the *gpaB*$^-$ and *acyA*$^-$ mutants show reduced conidiation and the *acyA*$^-$ mutant also a reduced growth, which is not seen in the other mutant. Mutant of *pkaC1*$^-$ and *gpaB*$^-$ are almost avirulent in models of low inhalation doses in the mouse [106]. For this reason, these authors note that the cAMP-Pka signal transduction pathway is required for the virulence of *A. fumigatus* [106]. It has also been proposed that this route can be implicated in the virulence by another pathway, which is its participation in the synthesis of melanin in the conidia [99,106]. Although mutants in some genes of the cAMP signal pathway in other fungi have production deficiencies of melanin, information on *A. fumigatus* is scarce [99]. Liebmann et al. [106] showed that the expression of the *pksP/alb1* in *gpaB*$^-$ mutants is reduced and the death rate of the conidia *gpaB*$^-$ and the *acyA*$^-$ strains by macrophages *in vitro* is significantly higher than in the parental one. For this reason, these authors suggest that cAMP trigger a system, which protects the fungus from the effects of the immune effector cells of the host.

Some authors have studied the activation and expression of genes at a temperature of 37°C, that is, genes which will be activated when the fungus grows at human body temperature. Among these genes may be found the virulence factors developed by the fungus during infection. Kumar et al. [86], found a protein, Hsp1, in a bank of cDNA of *A. fumigatus*, which reacted with the IgE and IgG of patients with ABPA, and coincided with the allergen Asp f12. Its partial sequence revealed that it was a member of the Hsp90 family of heat shock proteins. This family included proteins which act as chaperones and are capable of forming complexes with many proteins transporting them across the cytoplasm, being able to be associated with immunophilins, dyneins and importins, as well as several receptors [152]. This Hsp1 is assumed to play a key role in the virulence of *A. fumigatus*. Chang et al. [37], on the other hand, have identified a thermo-tolerant gene of *A. fumigatus* which encodes a putative protein of 141 kDa of unknown function, the *thta* gene. This gene seems to be essential for the growth of this fungus, but it does not appear to contribute to the pathogenesis. For the growth at 37 °C, Bhabhra et al. [22] indicated that the *cgrA* gene seems to be important, but not at ambient temperature. This gene is the ortholog of the nucleolar protein of yeasts, which function in the synthesis of ribosomes. These authors have detected a loss of virulence of the *cgrA*$^-$ mutants, since they presented with a lower colonisation in the lung tissue of immunocompromised mice. The lower virulence of the *cgrA*$^-$ mutants was not observed in the 25 °C infection model on *Drosophila*. For this reason, it seems that *cgrA* and its products are required for the growth and virulence at 37 °C of wild strains of *A. fumigatus*. A more detailed study of this gene has indicated that this nucleolar protein appears to be conserved between different fungi and can be valuable as an antifungal target [26]. Shankar et al. [176] have identified the expression at 37 °C of 68 new EST (expressed sequence tags) assigning 45 putative functions based on similarity of sequence. The identities of some of these genes suggest that they could be associated with the pathogenesis or autoimmune reactions. These genes can be possible targets in the development of antifungals or be useful in the diagnosis of the infection [176].

Other genes have been associated to the cellular viability of *A. fumigatus* and subsequently with virulence and as possible targets of new antifungals. The *smcA* gene encodes a member of the family of proteins charged with the maintenance of the structure of the chromosome and is essential for the growth and cellular viability of *A. fumigatus* [49]. Other molecules studied are the inteins, whose protein breakdown function is required to obtain and regulate mature proteins of the host. Prp8 is an extremely large conserved protein, with 819 aminoacid residues [107], and is a critical component of spliceosome, which is a large complex of ribonucleoprotein which catalyses the extraction of the introns in pre-mRNA. This protein could be a blank for the development of new antifungals. The annexins have also been studied, which are a family of calcium and phosphorous binding proteins and which have been implicated in a wide range of activities including exocytosis and the fusion of the membranes, as well as the regulation of Ca^{2+} channels [77]. In *A. fumigatus* the expression of three annexin genes has been detected, two classics *anxc3.1* and *anxc3.2* and one new, *anxc4*. These genes, their products and their specific fungal interactions, could be used as new targets for the design of new antifungals, especially *anxc4*, which is completely different from the classics, which are present in animals [77]. The biosynthesis of aminoacids is also essential for the growth of microorganisms. In *A. fumigatus* deletion of the *cpcA* gene produces mutants with decreased virulence [82]. This gene encodes the transcriptional activator of the cross-pathway control system of amino acid biosynthesis. The *cpcA*$^-$ strains showed a lower capacity to survive *in vivo* than in their parent strains, perhaps due to being found in conditions of stress within the host. These authors point out that this system of control could play a significant role in pulmonary aspergillosis. Among these aminoacid biosynthesis pathways, other authors have obtained *lysF*$^-$ mutants with decreased virulence in IA mouse models with low dose [105]. This gene encodes a homoaconitase of aminoacid lysine biosynthesis, and these authors indicate the importance of this functional enzyme in the survival of *A. fumigatus in vivo*, as well as its potential as an antifungal target. Likewise, several genes, which encode components of the mitochondria, are essential in *Aspergillus,* since they are strict aerobes [50].

A. fumigatus and the regulation of the immune system

It has been demonstrated *in vitro* that both the conidia and the hyphae could be phagocytosed by the non-professional phagocytic cells, such as endothelial cells and tracheal and alveolar epithelial cells [108, 144, 197, 198]. The cells thus internalised could survive the immune response and act as a reservoir for the dissemination of these microorganisms. These authors pointed out that in conditions of stress *Aspergillus* could direct their capture by these cells. In fact, a significant number of conidia (30 to 50%) can be internalised by non-professional phagocytes in cultures and in acid conditions they can germinate in their interior [197, 198]. The hyphae can also be internalised by these cells, but to a lesser extent [108]. Once they are internalised, progressive damage has been detected in these cultures in a very short time, about 4 hours, demonstrating that the viable conidia produce more

damage than the hyphae [108]. The surface components of the fungus and the surface receptors present in these cells seems to direct this phenomenon, although it is still not known which molecules are involved. It appears that the angioinvasion and thrombosis caused by this fungus *in vivo* may have their origin in this invasion of the epithelial and endothelial cells.

The decrease in natural killer cells (NK) or their dysfunction is a lateral effect in immunosuppressor treatment associated with IA. The early recruitment of NK cells in infection sites by chemokines such as MCP-1/CCL-2 appears to be important in the efficient defense of the host to the infection by *Aspergillus* [126]. These authors have demonstrated in neutropenic mice that the elimination of NK or preventing its early recruitment in the lung, doubles the mortality obtained due to infection by this fungus. This is not observed in immunocompetent mice since in them, other innate systems are sufficient to eliminate the pathogen. The NK activity only seems important in the defence of immunosuppressed mice and for that reason it has not been able to be detected. This data shows the need for more in-depth studies of these molecules, which activate or suppress the recruitment of these cells by the fungus.

Lastly, other interesting cell populations in the responses against this fungus have come to be studied. One of them is the intervention of dendritic cells (DC) which take part in the appropriate activation of the innate immune system and which also activate the adaptive immunity [165]. It has been established that the recognition of the invading fungus by the immune system is mediated by what are called pathogen associated molecular patterns (PAMP) and the receptors that recognise these patterns (PRR), such as, for example, receptors of the IL-1R/TLR family. Their coordinated action, in a proportion that depends on the fungus, the morphotype and the infection route, differentially activates the immune system [17, 18]. The immature DC, among other cells, such as alveolar macrophages, are found with PAMP in the peripheral tissues. They mature and decode the information associated to the pathogen, acting like antigen presenter cells and producing an inflammatory response and a profile of dominant cytokines, which leads to the development of a response Th crucial for the success against the infection. A review has recently been published on the possibility of using the activation of DC for vaccination against opportunist fungi [27]. In fact, the activation of different patterns of cytokines by components of the fungus or the live conidia themselves could be associated with the survival or death of the infected host [154]. For example, the early activation of high levels of IFN-γ will produce protection in the mouse, and high levels of IL-10 will produce sensitivity to the infections in the mouse [35, 40]. In some fungi, molecules involved in these activations have been recognised, such as aspartic proteases, mannans and phospholipomannans, which are important in the virulence of *C. albicans* [167]. It appears that the DC are activated by molecules of the fungus by means of two Toll-like receptors (TLR), TLR-2 and TLR-4 [28, 166]. These receptors are also essential in the activation of macrophages of the mouse induced by *Aspergillus* [114]. In *A. fumigatus* the activity of these molecules has not been demonstrated, although, as we have seen before, these same type of molecules and others, such as allergens, glucan, etc., could be some of these PAMP. It has been detected that proteins secreted by the fungus and present in the culture supernatant, can activate DC and granulocytes and this activation is mediated by TLR-2 and TLR-4 [28]. According to that reported by Roeder et al. [165] it seems that the conidia activate the two TLR receptors, while the hyphae only activate TLR-2. These authors indicate that other receptors may also be involved in interacting with the TLRs in the recognition of *A. fumigatus*, for example lectins which recognise galactomannans, or dectin 1, a new lectin type receptor for ß-glucans. Recent data suggests that the TLRs offer escape mechanisms to some microorganism pathogens. Particularly, the activation of TLR-2 appears to induce immunosuppression by inducing the release of IL-10 [135, 136]. It is known that the germination of the inhaled conidium is important to initiate and establish the infection and the conidia and hyphae could stimulate the production of cytokines via TLR in the mouse [111]. In this germination the surface layer of the fungus could be altered exposing deeper layers of the cell wall [208] and affecting the PAMP, which could be considered as an evasion mechanism of the immune system. The data appears to indicate that when mice are infected by the conidia of *A. fumigatus* the populations of T_{CD4+} cell producers of IFN-γ are activated by means of the DC, while in animals infected with hyphae the IL-4 producer cells are increased [167].

As we have pointed out, it is not clear which are the active components in the activation system of PAMPs, but Zhang et al. [212] have reported the importance of the surface components. For example, ß-glucan, which can activate the complement system, producing the active deposit of C3 on the surface of yeasts and also appears to activate the production of larger quantity of C3 and the expression of TLR on the human macrophages, mainly TLR-2 and TLR-4. The macrophages appear to be bound and activated by the hyphae of *A. fumigatus* by these receptors, but it is not clear to what the structures of the fungus are bound [111]. This is interesting due to ß-glucan being an important component of the hyphae cell wall of *A. fumigatus* and due to it being released by invasive fungi in the infected tissue. These types of TLR receptors can also activate the polymorphonuclear phagocytes and some authors indicate that the activation by TLR-2 promotes fungicide activity and the release of pro-inflammatory cytokines, while the activation by TLR-4 favours the participation of the azurophil granules and IL-10 [17]. However, some of the virulence factors, such as the allergens, may be able to participate in developing a Th2 response with the production of specific IgE and cytokines inhibitors of the cellular immune response. In fact allergic aspergillosis is a lung complication mediated by Th2 lymphocytes [79]. This Th2 response does not appear to be very efficient, at least on IA. In fact, in these infections the favourable responses to treatments correlate with a higher IFNγ/IL-10 ratio [62]. On the other hand, some allergens have homology with human antigens, therefore an allergic response against them can provoke a response to autoantigens, for example, MnSOD, P2 ribosomal protein and cyclophilin [5].

In *A. fumigatus*, two routes similar to those involved in the apoptosis have been detected. The cell death of the fungus in patients infected by *A. fumigatus* produced by the action of phagocytes or treatment with amphotericin B can be associated with these phenomena and it is the fungus itself which participates in its own death by routes similar to apoptosis [128]. These authors have managed, with low concentrations of H_2O_2 and amphotericin B, to trigger of a mechanism similar to known apoptosis routes. In fact, *A. fumigatus* appears to contain two homologues to metacaspases [127], with a high level of identity to *casA* of *Aspergillus nidulans*. It is possible that one of these metacaspases is activated during the lack of nutrients on entering into the stationary phase, and H_2O_2 and amphotericin B might activate the second.

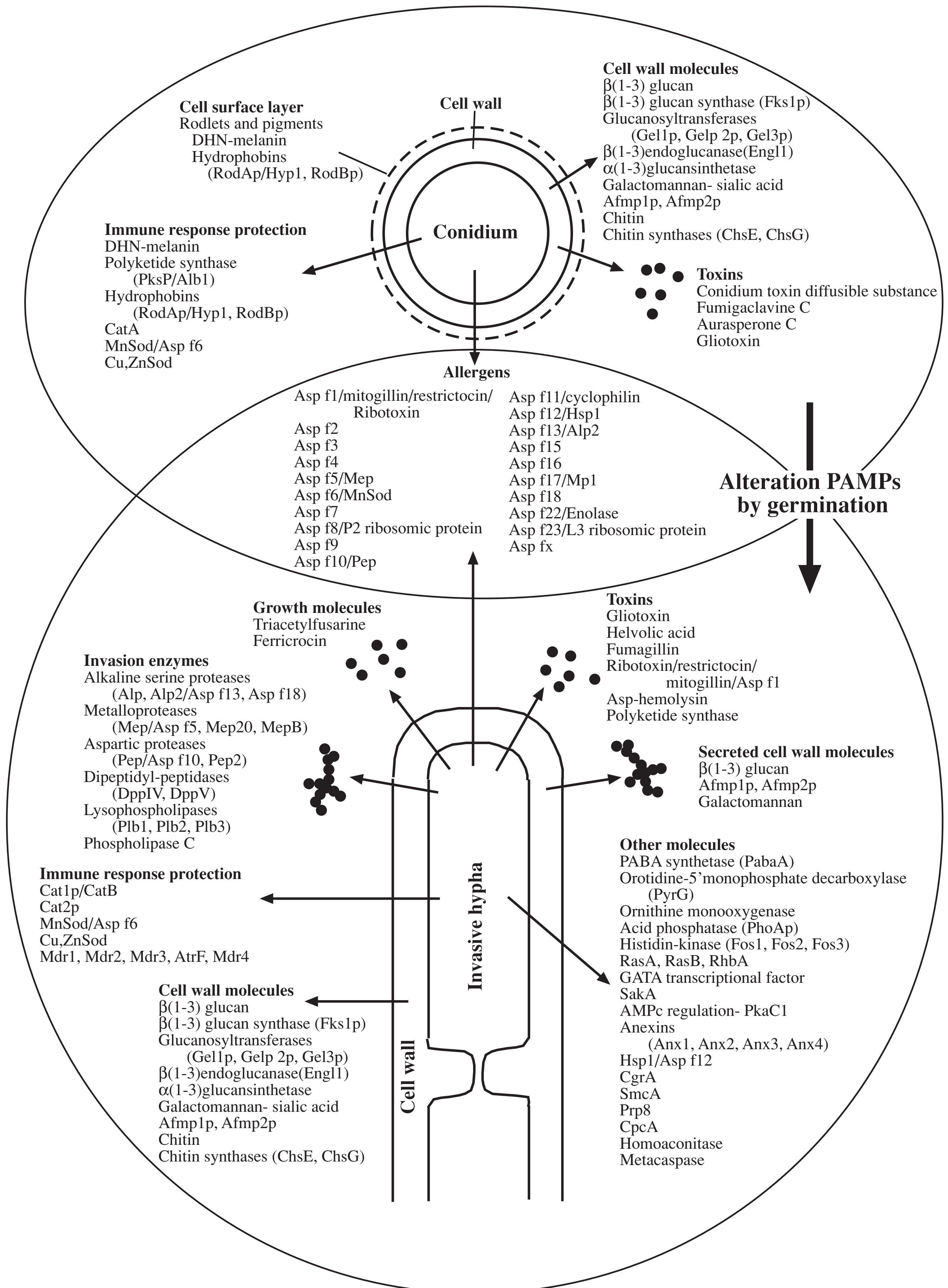

Figure 1. Putative molecules of the conidium and hypha related with virulence.

Conclusions

As has been seen during this review many genes and proteins exist which have been studied and associated with the virulence of *A. fumigatus*. These vary greatly in their structure, activity and mechanisms with which they protect or facilitate infections by this fungus. None of them have been shown to be sufficiently important to fully explain the virulence of *A. fumigatus*, except perhaps *pksP/alb1* and the siderophores during IA and the allergens in patients with ABPA. The effect of these virulence factors has normally been studied by the use of mutants in which that gene or protein has been eliminated. Up until now, the norm has been to see only small decreases in the virulent capacity of the mutant strains or not to be able to state that these factors were essential in the virulence of the fungus. This can be explained by considering that the virulence must be multifactorial by the opportunist nature of the majority of the infections produced by *A. fumigatus*. That is to say, the virulence does not depend on only a single factor and for its investigation multiple mutants are required. Although methods are being developed to obtain and isolate these types of mutants, it is difficult to obtain mutants with more than two mutations in the genome. Also, the pleiotropic effect of some genes can play an important role in this field. Some genes associated with virulence can have more than one function or have important functions even if *A. fumigatus* is not infecting the host. For this reason, these multiple mutants can not only have suppressed virulence, but also their viability can be seen to be affected under laboratory conditions. An example of this would be the *pabaA* gene. On the other hand, some of the losses produced in the mutants of this fungus can be compensated by the production of different molecules with similar activities, as can be the case in the degradative enzymes.

It has to be pointed out that these experiments based on the loss of a function by mutation are not the only ones that provide interesting results. The expression studies of the fungal genes during invasion or the interaction with the immune response of the host can help in the knowledge and understanding of some of the genes of *A. fumigatus* implicated in virulence. Some of them have already been studied and have been reported in this review, particularly overproduction during the infection or in the presence of cells from the host *in vitro*. This is another evidence of its association with pathogenesis. Some genes and putative molecules of virulence of *A. fumigatus* can have their homologues in other fungus pathogens. For this reason it would also be interesting to study the genome of other fungal pathogens to detect other genes of virulence in *A. fumigatus*. To investigate in more detail which genes are expressed during the pathogenesis, the study of the transcriptomes under the different conditions of stress which are found in this fungus during IA, will allow a better understanding of the opportunist pathogenesis of this fungus. On the other hand, the project of sequencing the genome of *A. fumigatus* is being finalised, the sequence is practically done and only requires the detection of the ORFs and their distribution in chromosomes. In the near future this sequencing project will allow obtaining more data on the virulence mechanisms, by the use of microarrays and biochips of complete genome or a subset of virulence related genes. There are already attempts to apply this type of approximation to the study of the virulence of *A. fumigatus* [76,141]. In fact, a commercial firm and the TIGR Institute for Genomic Research already offers a microarray of *A. fumigatus*. This tool will allow the understanding of the pathogenesis of this opportunist microorganism to advance much more rapidly.

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References

1. Abarca ML. Taxonomía e identificación de especies implicadas en la aspergilosis nosocomial. Rev Iberoam Micol 2000; 17: S79-S84.
2. Allen MJ, Harbeck R, Smith B, Voelker DR, Mason RJ. Binding of rat and human surfactant proteins A and D to *Aspergillus fumigatus* conidia. Infect Immun 1999; 67: 4563-4569.
3. Allen MJ, Voelker DR, Mason RJ. Interactions of surfactant proteins A and D with *Saccharomyces cerevisiae* and *Aspergillus fumigatus*. Infect Immun 2001; 69: 2037-2044.
4. Amitani R, Taylor G, Elezis EN, Llewellyn-Jones C, Mitchell J, Kuze F, Cole PJ, Wilson R. Purification and characterization of factors produced by *Aspergillus fumigatus* which affect human ciliated respiratory epithelium. Infect Immun 1995; 63: 3266-3271.
5. Appenzeller U, Meyer C, Menz G, Blaser K, Crameri R. IgE-mediated reactions to autoantigens in allergic diseases. Int Arch Allergy Immunol 1999; 118: 193-196.
6. Araujo R, Rodrigues AG. Variability of germinative potential among pathogenic species of *Aspergillus*. J Clin Microbiol 2004; 42: 4335-4337.
7. Arruda LK, Mann BJ, Chapman MD. Selective expression of a major allergen and cytotoxin, Asp f I, in *Aspergillus fumigatus*. Implications for the immunopathogenesis of *Aspergillus*-related diseases. J Immunol 1992; 149: 3354-3359.
8. Aufauvre-Brown A, Brown JS, Holden DW. Comparison of virulence between clinical and environmental isolates of *Aspergillus fumigatus*. Eur J Clin Microbiol Infect Dis 1998; 17: 778-780.
9. Aufauvre-Brown A, Mellado E, Gow NAR, Holden DW. *Aspergillus fumigatus chsE*: a gene related to CHS3 of *Saccharomyces cerevisiae* and important for hyphal growth and conidiophore development but not pathogenicity. Fungal Genet Biol 1997; 21: 141-152.
10. Banerjee B, Kurup VP. Molecular biology of *Aspergillus* allergens. Front Biosci 2003; 8: s128-s139.
11. Banerjee B, Kurup VP, Greenberger PA, Johnson BD, Fink JN. Cloning and expression of *Aspergillus fumigatus* allergen Asp f 16 mediating both humoral and cell-mediated immunity in allergic bronchopulmonary aspergillosis (ABPA). Clin Exp Allergy 2001; 31: 761-770.
12. Banerjee B, Kurup VP, Greenberger PA, Kelly KJ, Fink JN. C-terminal cysteine residues determine the IgE binding of *Aspergillus fumigatus* allergen Aspf2. J Immunol 2002; 169: 5137-5144.
13. Beauvais A, Bruneau JM, Mol PC, Buitrago MJ, Legrand R, Latgé JP. Glucan synthase complex of *Aspergillus fumigatus*. J Bacteriol 2001; 183: 2273-2279.
14. Beauvais A, Latgé JP. Membrane and cell wall targets in *Aspergillus fumigatus*. Drug Resist Updat 2001; 4: 38-49.
15. Beauvais A, Monod M, Debeaupuis JP, Diaquin M, Kobayashi H, Latgé JP. Biochemical and antigenic characterization of a new dipeptidyl-peptidase isolated from *Aspergillus fumigatus*. J Biol Chem 1997; 272: 6238-6244.
16. Beauvais A, Monod M, Wyniger J, Debeaupuis JP, Grouzmann E, Brakch N, Svab J, Hovanessian AG, Latgé JP. Dipeptidyl-peptidase IV secreted by *Aspergillus fumigatus*, a fungus pathogenic to humans. Infect Immun 1997; 65: 3042-3047.
17. Bellocchio S, Montagnoli C, Bozza S, Gaziano R, Rossi G, Mambula SS, Vecchi A, Mantovani A, Levitz SM, Romani L. The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens *in vivo*. J Immunol 2004; 172: 3059-3069.
18. Bellocchio S, Moretti S, Perruccio K, Fallarino F, Bozza S, Montagnoli C, Mosci P, Lipford GB, Pitzurra L, Romani L. TLRs govern neutrophil activity in aspergillosis. J Immunol 2004; 173: 7406-7415.
19. Bernard M, Latgé JP. *Aspergillus fumigatus* cell wall: composition and biosynthesis. Med Mycol 2001; 39: 9-17.
20. Bernard M, Mouyna I, Dubreucq G, Debeaupuis JP, Fontaine T, Vorgias C, Fuglsang C, Latge JP. Characterization of a cell-wall acid phosphatase (PhoAp) in *Aspergillus fumigatus*. Microbiology 2002; 148: 2819-2829.
21. Bertout S, Badoc C, Mallie M, Giaimis J, Bastide JM. Spore diffusate isolated from some strains of *Aspergillus fumigatus* inhibits phagocytosis by murine alveolar macrophages. FEMS Immunol Med Microbiol 2002; 33: 101-106.
22. Bhabhra R, Miley MD, Mylonakis E, Boettner D, Fortwendel J, Panepinto JC, Postow M, Rhodes JC, Askew DS. Disruption of the *Aspergillus fumigatus* gene encoding nucleolar protein *CgrA* impairs thermotolerant growth and reduces virulence. Infect Immun 2004; 72: 4731-4740.
23. Birch M, Denning DW, Robson GD. Comparison of extracellular phospholipase activities in clinical and environmental *Aspergillus fumigatus* isolates. Med Mycol 2004; 42: 81-86.
24. Birch M, Robson G, Law D, Denning DW. Evidence of multiple extracellular phospholipase activities of *Aspergillus fumigatus*. Infect Immun 1996; 64: 751-755.
25. Blanco JL, Hontecillas R, Bouza E, Blanco I, Pelaez T, Muñoz P, Pérez Molina J, García ME. Correlation between the elastase activity index and invasiveness of clinical isolates of *Aspergillus fumigatus*. J Clin Microbiol 2002; 40: 1811-1813.
26. Boettner D, Huebner N, Rhodes JC, Askew DS. Molecular cloning of *Aspergillus fumigatus CgrA*, the ortholog of a conserved fungal nucleolar protein. Med Mycol 2001; 39: 517-521.
27. Bozza S, Montagnoli C, Gaziano R, Rossi G, Nkwanyuo G, Bellocchio S, Romani L. Dendritic cell-based vaccination against opportunistic fungi. Vaccine 2004; 22: 857-864.
28. Braedel S, Radsak M, Einsele H, Latgé JP, Michan A, Loeffler J, Haddad Z, Grigoleit U, Schild H, Hebart H. *Aspergillus fumigatus* antigens activate innate immune cells via toll-like receptors 2 and 4. Br J Haematol 2004; 125: 392-399.
29. Brakhage AA, Langfelder K. Menacing mold: the molecular biology of *Aspergillus fumigatus*. Annu Rev Microbiol 2002; 56: 433-455.
30. Brakhage AA, Langfelder K, Wanner G, Schmidt A, Jahn B. Pigment biosynthesis and virulence. Contrib Microbiol 1999; 2: 205-215.
31. Brown JS, Aufauvre-Brown A, Brown J, Jennings JM, Arst H Jr, Holden DW. Signature-tagged and directed mutagenesis identify PABA synthetase as essential for *Aspergillus fumigatus* pathogenicity. Mol Microbiol 2000; 36: 1371-1380.
32. Bunger J, Westphal G, Monnich A, Hinnendahl B, Hallier E, Muller M. Cytotoxicity of occupationally and environmentally relevant mycotoxins. Toxicology 2004; 202: 199-211.
33. Calera JA, Paris S, Monod M, Hamilton AJ, Debeaupuis JP, Diaquin M, López-Medrano R, Leal F, Latgé JP. Cloning and disruption of the antigenic catalase gene of *Aspergillus fumigatus*. Infect Immun 1997; 65: 4718-4724.
34. Carotti C, Ragni E, Palomares O, Fontaine T, Tedeschi G, Rodriguez R, Latgé JP, Vai M, Popolo L. Characterization of recombinant forms of the yeast Gas1 protein and identification of residues essential for glucanosyltransferase activity and folding. Eur J Biochem 2004; 271: 3635-3645.
35. Centeno-Lima S, de Lacerda JM, Do Carmo JA, Abecasis M, Casimiro C, Exposito F. Follow-up of anti-*Aspergillus* IgG and IgA antibodies in bone marrow transplanted patients with invasive aspergillosis. J Clin Lab Anal 2002; 16: 156-162.
36. Centeno-Lima S, Silveira H, Casimiro C, Aguiar P, do Rosario VE. Kinetics of cytokine expression in mice with invasive aspergillosis: lethal infection and protection. FEMS Immunol Med Microbiol 2002; 32: 167-173.
37. Chang YC, Tsai HF, Karos M, Kwon-Chung KJ. THTA, a thermotolerance gene of *Aspergillus fumigatus*. Fungal Genet Biol 2004; 41: 888-896.
38. Chen SC, Wright LC, Golding JC, Sorrell TC. Purification and characterization of secretory phospholipase B, lysophospholipase and lysophospholipase/transacylase from a virulent strain of the pathogenic fungus *Cryptococcus neoformans*. Biochem J 2000; 347: 431-439.
39. Chong KT, Woo PC, Lau SK, Huang Y, Yuen KY. AFMP2 encodes a novel immunogenic protein of the antigenic mannoprotein superfamily in *Aspergillus fumigatus*. J Clin Microbiol 2004; 42: 2287-2291.
40. Clemons KV, Grunig G, Sobel RA, Mirels LF, Rennick DM, Stevens DA. Role of IL-10 in invasive aspergillosis: increased resistance of IL-10 gene knockout mice to lethal systemic aspergillosis. Clin Exp Immunol 2000; 122: 186-191.
41. Clemons KV, Miller TK, Selitrennikoff CP, Stevens DA. *fos-1*, a putative histidine kinase as a virulence factor for systemic aspergillosis. Med Mycol 2002; 40: 259-262.
42. Cox GM, McDade HC, Chen SC, Tucker SC, Gottfredsson M, Wright LC, Sorrell TC, Leidich SD, Casadevall A, Ghannoum MA, Perfect JR. Extracellular phospholipase activity is a virulence factor for *Cryptococcus neoformans*. Mol Microbiol 2001; 39: 166-175.
43. Del Palacio A, Cuétara M, Pontón J. La aspergilosis invasora. Rev Iberoam Micol 2003; 20: 77-78.
44. Del Palacio A, Cuétara M, Pontón J. El diagnóstico de laboratorio de la aspergillosis invasora. Rev Iberoam Micol 2003; 20: 90-98.
45. Dos Santos VM, Dorner JW, Carreira F. Isolation and toxigenicity of *Aspergillus fumigatus* from moldy silage. Mycopathologia 2003; 156: 133-138.

46. Douglas CM. Fungal beta(1,3)-D-glucan synthesis. Med Mycol 2001; 39: 55-66.
47. Du C, Li R, Ma S, Wang D. Cloning of *Aspergillus fumigatus* histidine kinase gene fragment and its expression during invasive infection. Mycopathologia 2002; 153: 5-10.
48. Ebina K, Sakagami H, Yokota K, Kondo H. Cloning and nucleotide sequence of cDNA encoding Asp-hemolysin from *Aspergillus fumigatus*. Biochim Biophys Acta 1994; 1219: 148-150.
49. Firon A, Beauvais A, Latgé JP, Couve E, Grosjean-Cournoyer MC, D'Enfert C. Characterization of essential genes by parasexual genetics in the human fungal pathogen *Aspergillus fumigatus*: impact of genomic rearrangements associated with electroporation of DNA. Genetics 2002; 161: 1077-1087.
50. Firon A, Villalba F, Beffa R, D'Enfert C. Identification of essential genes in the human fungal pathogen *Aspergillus fumigatus* by transposon mutagenesis. Eukaryot Cell 2003; 2: 247-255.
51. Fischer G, Muller T, Schwalbe R, Ostrowski R, Dott W. Species-specific profiles of mycotoxins produced in cultures and associated with conidia of airborne fungi derived from biowaste. Int J Hyg Environ Health 2000; 203: 105-116.
52. Fluckiger S, Mittl PR, Scapozza L, Fijten H, Folkers G, Grutter MG, Blaser K, Crameri R. Comparison of the crystal structures of the human manganese superoxide dismutase and the homologous *Aspergillus fumigatus* allergen at 2-A resolution. J Immunol 2002; 168: 1267-1272.
53. Fortwendel JR, Panepinto JC, Seitz AE, Askew DS, Rhodes JC. *Aspergillus fumigatus rasA* and *rasB* regulate the timing and morphology of asexual development. Fungal Genet Biol 2004; 41: 129-139.
54. Fox M, Gray G, Kavanagh K, Lewis C, Doyle S. Detection of *Aspergillus fumigatus* mycotoxins: immunogen synthesis and immunoassay development. J Microbiol Methods 2004; 56: 221-230.
55. Fujii I, Yasuoka Y, Tsai HF, Chang YC, Kwon-Chung KJ, Ebizuka Y. Hydrolytic polyketide shortening by ayg1p, a novel enzyme involved in fungal melanin biosynthesis. J Biol Chem 2004; 279: 44613-44620.
56. Fukuchi Y, Kudo Y, Kumagai T, Ebina K, Yokota K. Oxidized low density lipoprotein inhibits the hemolytic activity of Asp-hemolysin from *Aspergillus fumigatus*. FEMS Microbiol Lett 1998; 167: 275-280.
57. García ME, Durán C, Cruzado M, Andrino M, Blanco JL. Evaluation of molecular and immunological techniques for the diagnosis of mammary aspergillosis in ewes. Vet Microbiol 2004; 98: 17-21.
58. Gardiner DM, Cozijnsen AJ, Wilson LM, Pedras MS, Howlett BJ. The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus *Leptosphaeria maculans*. Mol Microbiol 2004; 53: 1307-1318.
59. Gifford AH, Klippenstein JR, Moore MM. Serum stimulates growth of and proteinase secretion by *Aspergillus fumigatus*. Infect Immun 2002; 70: 19-26.
60. Girardin H, Paris S, Rault J, Bellon-Fontaine MN, Latgé JP. The role of the rodlet structure on the physicochemical properties of *Aspergillus* conidia. Lett Appl Microbiol 1999; 29: 364-369.
61. Hamilton AJ, Holdom MD. Antioxidant systems in the pathogenic fungi of man and their role in virulence. Med Mycol 1999; 37: 375-389.
62. Hebart H, Bollinger C, Fisch P, Sarfati J, Meisner C, Baur M, Loeffler J, Monod M, Latgé JP, Einsele H. Analysis of T-cell responses to *Aspergillus fumigatus* antigens in healthy individuals and patients with hematologic malignancies. Blood 2002; 100: 4521-4528.
63. Hissen AH, Chow JM, Pinto LJ, Moore MM. Survival of *Aspergillus fumigatus* in serum involves removal of iron from transferrin: the role of siderophores. Infect Immun 2004; 72: 1402-1408.
64. Holdom MD, Hay RJ, Hamilton AJ. The Cu,Zn superoxide dismutases of *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, and *Aspergillus terreus*: purification and biochemical comparison with the *Aspergillus fumigatus* Cu,Zn superoxide dismutase. Infect Immun 1996; 64: 3326-3332.
65. Holdom MD, Lechenne B, Hay RJ, Hamilton AJ, Monod M. Production and characterization of recombinant *Aspergillus fumigatus* Cu,Zn superoxide dismutase and its recognition by immune human sera. J Clin Microbiol 2000; 38: 558-562.
66. Ibrahim-Granet O, D'Enfert C. The *Aspergillus fumigatus mepB* gene encodes an 82 kDa intracellular metalloproteinase structurally related to mammalian thimet oligopeptidases. Microbiology 1997; 143: 2247-2253.
67. Ibrahim-Granet O, Philippe B, Boleti H, Boisvieux-Ulrich E, Grenet D, Stern M, Latgé JP. Phagocytosis and intracellular fate of *Aspergillus fumigatus* conidia in alveolar macrophages. Infect Immun 2003; 71: 891-903.
68. Iraneta SG, Duschak VG, Rodríguez SM, Alonso A. Serine proteinases with gelatinolytic activity in an *Aspergillus fumigatus* allergenic extract. J Invest Allergol Clin Immunol 2002; 12: 257-262.
69. Ishibashi K, Miura NN, Adachi Y, Tamura H, Tanaka S, Ohno N. The solubilization and biological activities of *Aspergillus* beta-(1->3)-D-glucan. FEMS Immunol Med Microbiol 2004; 42: 155-166.
70. Jahn B, Boukhallouk F, Lotz J, Langfelder K, Wanner G, Brakhage AA. Interaction of human phagocytes with pigmentless *Aspergillus* conidia. Infect Immun 2000; 68: 3736-3739.
71. Jahn B, Koch A, Schmidt A, Wanner G, Gehringer H, Bhakdi S, Brakhage AA. Isolation and characterization of a pigmentless-conidium mutant of *Aspergillus fumigatus* with altered conidial surface and reduced virulence. Infect Immun 1997; 65: 5110–5117.
72. Jahn B, Langfelder K, Schneider U, Schindel C, Brakhage AA. PKSP-dependent reduction of phagolysosome fusion and intracellular kill of *Aspergillus fumigatus* conidia by human monocyte-derived macrophages. Cell Microbiol 2002; 4: 793-803.
73. Jarque I, Andreu R, Salavert M, Gómez D, Pemán J, Gobernado M, Sanz MA. Valor de la detección del antígeno galactomanano de *Aspergillus* en el diagnóstico y seguimiento de la aspergilosis invasora en pacientes hematológicos. Rev Iberoam Micol 2003; 20: 116-118.
74. Kao R, Davies J. Fungal ribotoxins: a family of naturally engineered targeted toxins? Biochem Cell Biol 1995; 73: 1151-1159.
75. Kao R, Davies J. Molecular dissection of mitogillin reveals that the fungal ribotoxins are a family of natural genetically engineered ribonucleases. J Biol Chem 1999; 274: 12576-12582.
76. Kessler MM, Willins DA, Zeng Q, Del Mastro RG, Cook R, Doucette-Stamm L, Lee H, Caron A, McClanahan TK, Wang L, Greene J, Hare RS, Cottarel G, Shimer GH Jr. The use of direct cDNA selection to rapidly and effectively identify genes in the fungus *Aspergillus fumigatus*. Fungal Genet Biol 2002; 36: 59-70.
77. Khalaj V, Smith L, Brookman J, Tuckwell D. Identification of a novel class of annexin genes. FEBS Lett 2004; 562: 79-86.
78. Knutsen AP, Hutchinson PS, Albers GM, Consolino J, Smick J, Kurup VP. Increased sensitivity to IL-4 in cystic fibrosis patients with allergic bronchopulmonary aspergillosis. Allergy 2004; 59: 81-87.
79. Knutsen AP, Hutcheson PS, Slavin RG, Kurup VP. IgE antibody to *Aspergillus fumigatus* recombinant allergens in cystic fibrosis patients with allergic bronchopulmonary aspergillosis. Allergy 2004; 59: 198-203.
80. Kogan TV, Jadoun J, Mittelman L, Hirschberg K, Osherov N. Involvement of secreted *Aspergillus fumigatus* proteases in disruption of the actin fiber cytoskeleton and loss of focal adhesion sites in infected A549 lung pneumocytes. J Infect Dis 2004; 189: 1965-1973.
81. Kolattukudy PE, Lee JD, Rogers LM, Zimmerman P, Ceselski S, Fox B, Stein B, Copelan EA. Evidence for possible involvement of an elastolytic serine protease in aspergillosis. Infect Immun 1993; 61: 2357-2368.
82. Krappmann S, Bignell EM, Reichard U, Rogers T, Haynes K, Braus GH. The *Aspergillus fumigatus* transcriptional activator *CpcA* contributes significantly to the virulence of this fungal pathogen. Mol Microbiol 2004; 52: 785-799.
83. Kudo Y, Fukuchi Y, Kumagai T, Ebina K, Yokota K. Oxidized low-density lipoprotein-binding specificity of Asp-hemolysin from *Aspergillus fumigatus*. Biochim Biophys Acta 2001; 1568: 183-188.
84. Kudo Y, Ootani T, Kumagai T, Fukuchi Y, Ebina K, Yokota K. A novel oxidized low-density lipoprotein-binding protein, Asp-hemolysin, recognizes lysophosphatidylcholine. Biol Pharm Bull 2002; 25: 787-790.
85. Kumagai T, Nagata T, Kudo Y, Fukuchi Y, Ebina K, Yokota K. Cytotoxic activity and cytokine gene induction of Asp-hemolysin to murine macrophages. Nippon Ishinkin Gakkai Zasshi 1999; 40: 217-222.
86. Kumar A, Reddy LV, Sochanik A, Kurup VP. Isolation and characterization of a recombinant heat shock protein of *Aspergillus fumigatus*. J Allergy Clin Immunol 1993; 91: 1024-1030.
87. Kunert J. Effect of nitric oxide donors on survival of conidia, germination and growth of *Aspergillus fumigatus* in vitro. Folia Microbiol (Praha) 1995; 40: 238-244.
88. Kunert J. Effect of peroxynitrite on dormant spores and germlings of *Aspergillus fumigatus* in vitro. Folia Microbiol (Praha) 2000; 45: 325-329.
89. Kunert J. Further studies on the multiple forms of protease ALP of *Aspergillus fumigatus*. Mycoses 2001; 44: 307-310.
90. Kunert J, Kopecek P. Multiple forms of the serine protease Alp of *Aspergillus fumigatus*. Mycoses 2000; 43: 339-347.

91. Kurup VP, Kumar A, Kenealy WR, Greenberger PA. *Aspergillus* ribotoxins react with IgE and IgG antibodies of patients with allergic bronchopulmonary aspergillosis. J Lab Clin Med 1994; 123: 749-756.
92. Kurup VP, Shen HD, Banerjee B. Respiratory fungal allergy. Microbes Infect 2000; 2: 1101-1110.
93. Kurup VP, Xia JQ, Shen HD, Rickaby DA, Henderson JD Jr, Fink JN, Chou H, Kelly KJ, Dawson CA. Alkaline serine proteinase from *Aspergillus fumigatus* has synergistic effects on Asp f2-induced immune response in mice. Int Arch Allergy Immunol 2002; 129: 129-137.
94. Lai HY, Tam MF, Tang RB, Chou H, Chang CY, Tsai JJ, Shen HD. cDNA cloning and immunological characterization of a newly identified enolase allergen from *Penicillium citrinum* and *Aspergillus fumigatus*. Int Arch Allergy Immunol 2002; 127: 181-190.
95. Lamy B, Moutaouakil M, Latgé JP, Davies J. Secretion of a potential virulence factor, a fungal ribonucleotoxin, during human aspergillosis infections. Mol Microbiol 1991; 5: 1811-1815.
96. Langfelder K, Gattung S, Brakhage AA. A novel method used to delete a new *Aspergillus fumigatus* ABC transporter-encoding gene. Curr Genet 2002; 41: 268-274.
97. Langfelder K, Jahn B, Gehringer H, Schmidt A, Wanner G, Brakhage AA. Identification of a polyketide synthase gene (*pksP*) of *Aspergillus fumigatus* involved in conidial pigment biosynthesis and virulence. Med Microbiol Immunol 1998; 187: 79-89.
98. Langfelder K, Philippe B, Jahn B, Latgé JP, Brakhage AA. Differential expression of the *Aspergillus fumigatus pksP* gene detected in vitro and in vivo with green fluorescent protein. Infect Immun 2001; 69: 6411-6418.
99. Langfelder K, Streibel M, Jahn B, Haase G, Brakhage AA. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genet Biol 2003; 38: 143-158.
100. Latgé JP. *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev 1999; 12: 310-350.
101. Latgé JP. The pathobiology of *Aspergillus fumigatus*. Trends Microbiol 2001; 9: 382-389.
102. Latgé JP, Debeaupuis JP, Sarfati J, Diaquin M, Paris S. Cell wall antigens in *Aspergillus fumigatus*. Arch Med Res 1993; 24: 269-274.
103. Lee JD, Kolattukudy PE. Molecular cloning of the cDNA and gene for an elastinolytic aspartic proteinase from *Aspergillus fumigatus* and evidence of its secretion by the fungus during invasion of the host lung. Infect Immun 1995; 63: 3796-3803.
104. Liebmann B, Gattung S, Jahn B, Brakhage AA. cAMP signaling in *Aspergillus fumigatus* is involved in the regulation of the virulence gene *pksP* and in defense against killing by macrophages. Mol Genet Genomics 2003; 269: 420-435.
105. Liebmann B, Muhleisen TW, Muller M, Hecht M, Weidner G, Braun A, Brock M, Brakhage AA. Deletion of the *Aspergillus fumigatus* lysine biosynthesis gene *lysF* encoding homoaconitase leads to attenuated virulence in a low-dose mouse infection model of invasive aspergillosis. Arch Microbiol 2004; 181: 378-383.
106. Liebmann B, Muller M, Braun A, Brakhage AA. The cyclic AMP-dependent protein kinase a network regulates development and virulence in *Aspergillus fumigatus*. Infect Immun 2004; 72: 5193-5203.
107. Liu XQ, Yang J. Prp8 intein in fungal pathogens: target for potential antifungal drugs. FEBS Lett 2004; 572: 46-50.
108. Lopes Bezerra LM, Filler SG. Interactions of *Aspergillus fumigatus* with endothelial cells: internalization, injury, and stimulation of tissue factor activity. Blood 2004; 103: 2143-2149.
109. Lumbreras C, Gavaldà J. Aspergilosis invasora: manifestaciones clínicas y tratamiento. Rev Iberoam Micol 2003; 20: 79-89.
110. Madan T, Priyadarsiny P, Vaid M, Kamal N, Shah A, Haq W, Katti SB, Sarma PU. Use of a synthetic peptide epitope of Asp f 1, a major allergen or antigen of *Aspergillus fumigatus*, for improved immunodiagnosis of allergic bronchopulmonary aspergillosis. Clin Diagn Lab Immunol 2004; 11: 552-558.
111. Mambula SS, Sau K, Henneke P, Golenbock DT, Levitz SM. Toll-like receptor (TLR) signaling in response to *Aspergillus fumigatus*. J Biol Chem 2002; 277: 39320-39326.
112. Markaryan A, Morozova I, Yu H, Kolattukudy PE. Purification and characterization of an elastinolytic metalloprotease from *Aspergillus fumigatus* and immunoelectron microscopic evidence of secretion of this enzyme by the fungus invading the murine lung. Infect Immun 1994; 62: 2149-2157.
113. Martínez-Ruíz A, Kao R, Davies J, Martínez del Pozo A. Ribotoxins are a more widespread group of proteins within the filamentous fungi than previously believed. Toxicon 1999; 37: 1549-1563.
114. Meier A, Kirschning CJ, Nikolaus T, Wagner H, Heesemann J, Ebel F. Toll-like receptor (TLR) 2 and TLR4 are essential for *Aspergillus*-induced activation of murine macrophages. Cell Microbiol 2003; 5: 561-570.
115. Mellado E, Aufauvre-Brown A, Gow NA, Holden DW. The *Aspergillus fumigatus chsC* and *chsG* genes encode class III chitin synthases with different functions. Mol Microbiol 1996; 20: 667-679.
116. Mellado E, Dubreucq G, Mol P, Sarfati J, Paris S, Diaquin M, Holden DW, Rodríguez-Tudela JL, Latgé JP. Cell wall biogenesis in a double chitin synthase mutant ($chsG^-/chsE^-$) of *Aspergillus fumigatus*. Fungal Genet Biol 2003; 38: 98-109.
117. Mennink-Kersten MASH, Donnelly JP, Verweij PE. Detection of circulating galactomannan for the diagnosis and management of invasive aspergillosis. Lancet Infect Dis 2004; 4: 349-357.
118. Mitchell CG, Slight J, Donaldson K. Diffusible component from the spore surface of the fungus *Aspergillus fumigatus* which inhibits the macrophage oxidative burst is distinct from gliotoxin and other hyphal toxins. Thorax 1997; 52: 796-801.
119. Miyoshi S, Shinoda S. Microbial metalloproteases and pathogenesis. Microbes Infect 2000; 2: 91-98.
120. Monod M, Capoccia S, Lechenne B, Zaugg C, Holdom M, Jousson O. Secreted proteases from pathogenic fungi. Int J Med Microbiol 2002; 292: 405-419.
121. Monod M, Jaton-Ogay K, Reichard U. *Aspergillus fumigatus*-secreted proteases as antigenic molecules and virulence factors. Contrib Microbiol 1999; 2: 182-192.
122. Monod M, Paris S, Sanglard D, Jaton-Ogay K, Bille J, Latgé JP. Isolation and characterization of a secreted metalloprotease of *Aspergillus fumigatus*. Infect Immun 1993; 61: 4099-4104.
123. Monod M, Paris S, Sarfati J, Jaton-Ogay K, Ave P, Latgé JP. Virulence of alkaline protease-deficient mutants of *Aspergillus fumigatus*. FEMS Microbiol Lett 1993; 106: 39-46.
124. Monod M, Togni G, Rahalison L, Frenk E. Isolation and characterisation of an extracellular alkaline protease of *Aspergillus fumigatus*. J Med Microbiol 1991; 35: 23-28.
125. Moragues MD, Amutio E, García-Ruiz JC, Pontón J. Utilidad de la detección de galactomanano en el diagnóstico y seguimiento de la aspergilosis invasora en pacientes hematológicos. Rev Iberoam Micol 2003; 20: 103-110.
126. Morrison BE, Park SJ, Mooney JM, Mehrad B. Chemokine-mediated recruitment of NK cells is a critical host defense mechanism in invasive aspergillosis. J Clin Invest 2003; 112: 1862-1870.
127. Mousavi SA, Robson GD. Entry into the stationary phase is associated with a rapid loss of viability and an apoptotic-like phenotype in the opportunistic pathogen *Aspergillus fumigatus*. Fungal Genet Biol 2003; 39: 221-229.
128. Mousavi SA, Robson GD. Oxidative and amphotericin B-mediated cell death in the opportunistic pathogen *Aspergillus fumigatus* is associated with an apoptotic-like phenotype. Microbiology 2004; 150: 1937-1945.
129. Moutaouakil M, Monod M, Prevost MC, Bouchara JP, Paris S, Latgé JP. Identification of the 33-kDa alkaline protease of *Aspergillus fumigatus* in vitro and in vivo. J Med Microbiol 1993; 39: 393-399.
130. Mouyna I, Henry C, Doering TL, Latgé JP. Gene silencing with RNA interference in the human pathogenic fungus *Aspergillus fumigatus*. FEMS Microbiol Lett 2004; 237: 317-324.
131. Mouyna I, Sarfati J, Recco P, Fontaine T, Henrissatz B, Latgé JP. Molecular characterization of a cell wall-associated beta(1-3)endoglucanase of *Aspergillus fumigatus*. Med Mycol 2002; 40: 455-464.
132. Mouyna I, Monod M, Fontaine T, Henrissat B, Lechenne B, Latgé JP. Identification of the catalytic residues of the first family of beta(1-3)glucanosyltransferases identified in fungi. Biochem J 2000; 347: 741-747.
133. Muhlschlegal F, Fonzi W, Hoyer L, Payne T, Poulet FM, Clevenger J, Latgé JP, Calera J, Beauvais A, Paris S, Monod M, Sturtevant J, Ghannoum M, Nozawa Y, Calderone R. Molecular mechanisms of virulence in fungus-host interactions for *Aspergillus fumigatus* and *Candida albicans*. Med Mycol 1998; 36: 238-248.
134. Nascimento AM, Goldman GH, Park S, Marras SA, Delmas G, Oza U, Lolans K, Dudley MN, Mann PA, Perlin DS. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. Antimicrob Agents Chemother 2003; 47: 1719-1726.
135. Netea MG, Van der Graaf C, Van der Meer JW, Kullberg BJ. Recognition of fungal pathogens by Toll-like receptors. Eur J Clin Microbiol Infect Dis 2004; 23: 672-676.
136. Netea MG, Van der Meer JW, Kullberg BJ. Toll-like receptors as an escape mechanism from the host defense. Trends Microbiol 2004; 12: 484-488.
137. Nigam S, Ghosh PC, Sarma PU. A new glycoprotein allergen/antigen with the protease activity from *Aspergillus fumigatus*. Int Arch Allergy Immunol 2003; 132: 124-131.

138. O'Brian GR, Fakhoury AM, Payne GA. Identification of genes differentially expressed during aflatoxin biosynthesis in *Aspergillus flavus* and *Aspergillus parasiticus*. Fungal Genet Biol 2003; 39: 118-127.
139. Okumura Y, Ogawa K, Nikai T. Elastase and elastase inhibitor from *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger*. J Med Microbiol 2004; 53: 351-354.
140. Oliver BG, Panepinto JC, Fortwendel JR, Smith DL, Askew DS, Rhodes JC. Cloning and expression of *pkaC* and *pkaR*, the genes encoding the cAMP-dependent protein kinase of *Aspergillus fumigatus*. Mycopathologia 2002; 154: 85-91.
141. Pain A, Woodward J, Quail MA, Anderson MJ, Clark R, Collins M, Fosker N, Fraser A, Harris D, Larke N, Murphy L, Humphray S, O'Neil S, Pertea M, Price C, Rabbinowitsch E, Rajandream MA, Salzberg S, Saunders D, Seeger K, Sharp S, Warren T, Denning DW, Barrell B, Hall N. Insight into the genome of *Aspergillus fumigatus*: analysis of a 922 kb region encompassing the nitrate assimilation gene cluster. Fungal Genet Biol 2004; 41: 443-453.
142. Panepinto JC, Oliver BG, Amlung TW, Askew DS, Rhodes JC. Expression of the *Aspergillus fumigatus rheb* homologue, *rhbA*, is induced by nitrogen starvation. Fungal Genet Biol 2002; 36: 207-214.
143. Panepinto JC, Oliver BG, Fortwendel JR, Smith DL, Askew DS, Rhodes JC. Deletion of the *Aspergillus fumigatus* gene encoding the *Ras*-related protein *RhbA* reduces virulence in a model of invasive pulmonary aspergillosis. Infect Immun 2003; 71: 2819-2826.
144. Paris S, Boisvieux-Ulrich E, Crestani B, Houcine O, Taramelli D, Lombardi L, Latgé JP. Internalization of *Aspergillus fumigatus* conidia by epithelial and endothelial cells. Infect Immun 1997; 65:1510-1514.
145. Paris S, Debeaupuis JP, Crameri R, Carey M, Charles F, Prevost MC, Schmitt C, Philippe B, Latgé JP. Conidial hydrophobins of *Aspergillus fumigatus*. Appl Environ Microbiol 2003; 69: 1581-1588.
146. Paris S, Wysong D, Debeaupuis JP, Shibuya K, Philippe B, Diamond RD, Latgé JP. Catalases of *Aspergillus fumigatus*. Infect Immun 2003; 71: 3551-3562.
147. Pazos C, del Palacio A. Diagnóstico precoz de la aspergilosis invasora en enfermos neutropénicos mediante la detección bisemanal de galactomanano en suero con Platelia® Aspergillus. Rev Iberoam Micol 2003; 20: 99-102.
148. Pazos C, Pontón J, del Palacio A. Contribution of (1→3)-ß-D-glucan chromogenic assay to diagnosis and therapeutic monitoring of invasive aspergillosis in neutropenic adult patients: a comparison with serial screening for circulating galactomannan. J Clin Microbiol 2005; 43: 299-305.
149. Pepeljnjak S, Slobodnjak Z, Segvic M, Peraica M, Pavlovic M. The ability of fungal isolates from human lung aspergilloma to produce mycotoxins. Hum Exp Toxicol 2004; 23: 15-19.
150. Philippe B, Ibrahim-Granet O, Prevost MC, Gougerot-Pocidalo MA, Sánchez-Pérez M, Van der Meeren A, Latgé JP. Killing of *Aspergillus fumigatus* by alveolar macrophages is mediated by reactive oxidant intermediates. Infect Immun 2003; 71: 3034-3042.
151. Pott GB, Miller TK, Bartlett JA, Palas JS, Selitrennikoff CP. The isolation of *FOS-1*, a gene encoding a putative two-component histidine kinase from *Aspergillus fumigatus*. Fungal Genet Biol 2000; 31: 55-67.
152. Pratt WB, Galigniana MD, Harrell JM, DeFranco DB. Role of hsp90 and the hsp90-binding immunophilins in signalling protein movement. Cell Signal 2004; 16: 857-872.
153. Priyadarsiny P, Swain PK, Sarma PU. Expression and characterization of Asp fI, an immunodominant allergen/antigen of *A. fumigatus* in insect cell. Mol Cell Biochem 2003; 252: 157-163.
154. Pylkkanen L, Gullsten H, Majuri ML, Andersson U, Vanhala E, Maatta J, Meklin T, Hirvonen MR, Alenius H, Savolainen K. Exposure to *Aspergillus fumigatus* spores induces chemokine expression in mouse macrophages. Toxicology 2004; 200: 255-263.
155. Ramachandran H, Banerjee B, Greenberger PA, Kelly KJ, Fink JN, Kurup VP. Role of C-terminal cysteine residues of *Aspergillus fumigatus* allergen Asp f 4 in immunoglobulin E binding. Clin Diagn Lab Immunol 2004; 11: 261-265.
156. Ramachandran H, Jayaraman V, Banerjee B, Greenberger PA, Kelly KJ, Fink JN, Kurup VP. IgE binding conformational epitopes of Asp f 3, a major allergen of *Aspergillus fumigatus*. Clin Immunol 2002; 103: 324-333.
157. Ramesh MV, Sirakova TD, Kolattukudy PE. Cloning and characterization of the cDNAs and genes (*mep20*) encoding homologous metalloproteinases from *Aspergillus flavus* and *A. fumigatus*. Gene 1995; 165: 121-125.
158. Reeves EP, Murphy T, Daly P, Kavanagh K. Amphotericin B enhances the synthesis and release of the immunosuppressive agent gliotoxin from the pulmonary pathogen *Aspergillus fumigatus*. J Med Microbiol 2004; 53: 719-725.
159. Reichard U, Buttner S, Eiffert H, Staib F, Ruchel R. Purification and characterisation of an extracellular serine proteinase from *Aspergillus fumigatus* and its detection in tissue. J Med Microbiol 1990; 33: 243-251.
160. Reichard U, Cole GT, Hill TW, Ruchel R, Monod M. Molecular characterization and influence on fungal development of ALP2, a novel serine proteinase from *Aspergillus fumigatus*. Int J Med Microbiol 2000; 290: 549-558.
161. Reichard U, Cole GT, Ruchel R, Monod M. Molecular cloning and targeted deletion of *PEP2* which encodes a novel aspartic proteinase from *Aspergillus fumigatus*. Int J Med Microbiol 2000; 290: 85-96.
162. Reichard U, Monod M, Odds F, Ruchel R. Virulence of an aspergillopepsin-deficient mutant of *Aspergillus fumigatus* and evidence for another aspartic proteinase linked to the fungal cell wall. J Med Vet Mycol 1997; 35: 189-196.
163. Reichard U, Monod M, Ruchel R. Molecular cloning and sequencing of the gene encoding an extracellular aspartic proteinase from *Aspergillus fumigatus*. FEMS Microbiol Lett 1995; 130: 69-74.
164. Rhodes JC, Oliver BG, Askew DS, Amlung TW. Identification of genes of *Aspergillus fumigatus* up-regulated during growth on endothelial cells. Med Mycol 2001; 39: 253-260.
165. Roeder A, Kirschning CJ, Rupec RA, Schaller M, Korting HC. Toll-like receptors and innate antifungal responses. Trends Microbiol 2004; 12: 44-49.
166. Romani L, Bistoni F, Gaziano R, Bozza S, Montagnoli C, Perruccio K, Pitzurra L, Bellocchio S, Velardi A, Rasi G, Di Francesco P, Garaci E. Thymosin alpha 1 activates dendritic cells for antifungal Th1 resistance through toll-like receptor signaling. Blood 2004; 103: 4232-4239.
167. Romani L, Bistoni F, Puccetti P. Fungi, dendritic cells and receptors: a host perspective of fungal virulence. Trends Microbiol 2002; 10: 508-514.
168. Rovira M, Puig de la Bellacasa J. Detección de antígeno galactomanano de *Aspergillus* en el trasplante alogénico de progenitores hematopoyéticos. Rev Iberoam Micol 2003; 20: 111-115.
169. Sarma PV, Purkayastha S, Madan T, Sarma PU. Expression of an epitopic region of AspfI, an allergen/antigen/cytotoxin of *Aspergillus fumigatus*. Immunol Lett 1999; 70: 151-155.
170. Saxena S, Madan T, Muralidhar K, Sarma PU. cDNA cloning, expression and characterization of an allergenic L3 ribosomal protein of *Aspergillus fumigatus*. Clin Exp Immunol 2003; 134: 86-91.
171. Schaffner A, Douglas H, Braude A. Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to *Aspergillus*. Observations on these two lines of defense in vivo and in vitro with human and mouse phagocytes. J Clin Invest 1982; 69: 617-631.
172. Schaffner A, Douglas H, Braude AI, Davis CE. Killing of *Aspergillus* spores depends on the anatomical source of the macrophage. Infect Immun 1983; 42: 1109-1115.
173. Schrettl M, Bignell E, Kragl C, Joechl C, Rogers T, Arst HN Jr, Haynes K, Haas H. Siderophore biosynthesis but not reductive iron assimilation is essential for *Aspergillus fumigatus* virulence. J Exp Med 2004; 200: 1213-1219.
174. Schulz T, Senkpiel K, Ohgke H. Comparison of the toxicity of reference mycotoxins and spore extracts of common indoor moulds. Int J Hyg Environ Health 2004; 207: 267-277.
175. Segurado M, López-Aragón R, Calera JA, Fernandez-Abalos JM, Leal F. Zinc-regulated biosynthesis of immunodominant antigens from *Aspergillus* spp. Infect Immun 1999; 67: 2377-2382.
176. Shankar J, Nigam S, Saxena S, Madan T, Sarma PU. Identification and assignment of function to the genes of *Aspergillus fumigatus* expressed at 37 degrees C. J Eukaryot Microbiol 2004; 51: 428-432.
177. Shen HD, Lin WL, Tam MF, Chou H, Wang CW, Tsai JJ, Wang SR, Han SH. Identification of vacuolar serine proteinase as a major allergen of *Aspergillus fumigatus* by immunoblotting and N-terminal amino acid sequence analysis. Clin Exp Allergy 2001; 31: 295-302.
178. Shen DK, Noodeh AD, Kazemi A, Grillot R, Robson G, Brugere JF. Characterisation and expression of phospholipases B from the opportunistic fungus *Aspergillus fumigatus*. FEMS Microbiol Lett 2004; 239: 87-93.
179. Shen HD, Tam MF, Chou H, Han SH. The importance of serine proteinases as aeroallergens associated with asthma. Int Arch Allergy Immunol 1999; 119: 259-264.
180. Shibuya K, Takaoka M, Uchida K, Wakayama M, Yamaguchi H, Takahashi K, Paris S, Latge JP, Naoe S. Histopathology of experimental invasive pulmonary aspergillosis in rats: pathological comparison of pulmonary lesions induced by specific virulent factor deficient mutants. Microb Pathog 1999; 27: 123-131.

181. Shinohara C, Hasumi K, Endo A. Inhibition of oxidized low-density lipoprotein metabolism in macrophage J774 by helvolic acid. Biochim Biophys Acta 1993; 1167: 303-306.
182. Sirakova TD, Markaryan A, Kolattukudy PE. Molecular cloning and sequencing of the cDNA and gene for a novel elastinolytic metalloproteinase from *Aspergillus fumigatus* and its expression in *Escherichia coli*. Infect Immun 1994; 62: 4208-4218.
183. Slaven JW, Anderson MJ, Sanglard D, Dixon GK, Bille J, Roberts IS, Denning DW. Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, *atrF*, in the presence of itraconazole in an itraconazole resistant clinical isolate. Fungal Genet Biol 2002; 36: 199-206.
184. Smith JM, Tang CM, Van Noorden S, Holden DW. Virulence of *Aspergillus fumigatus* double mutants lacking restriction and an alkaline protease in a low-dose model of invasive pulmonary aspergillosis. Infect Immun 1994; 62: 5247-5254.
185. Starcher BC. Elastin and the lung. Thorax 1986; 41:577-585.
186. Tang CM, Cohen J, Krausz T, Van Noorden S, Holden DW. The alkaline protease of *Aspergillus fumigatus* is not a virulence determinant in two murine models of invasive pulmonary aspergillosis. Infect Immun 1993; 61: 1650-1656.
187. Thau N, Monod M, Crestani B, Rolland C, Tronchin G, Latgé JP, Paris S. Rodletless mutants of *Aspergillus fumigatus*. Infect Immun 1994; 62: 4380-4388.
188. Tomee JF, Kauffman HF. Putative virulence factors of *Aspergillus fumigatus*. Clin Exp Allergy 2000; 30: 476-484.
189. Tsai HF, Chang YC, Washburn RG, Wheeler MH, Kwon-Chung KJ. The developmentally regulated *alb1* gene of *Aspergillus fumigatus*: its role in modulation of conidial morphology and virulence. J Bacteriol 1998; 180: 3031-3038.
190. Tsai HF, Fujii I, Watanabe A, Wheeler MH, Chang YC, Yasuoka Y, Ebizuka Y, Kwon-Chung KJ. Pentaketide melanin biosynthesis in *Aspergillus fumigatus* requires chain-length shortening of a heptaketide precursor. J Biol Chem 2001; 276: 29292-29298.
191. Tsai HF, Washburn RG, Chang YC, Kwon-Chung KJ. *Aspergillus fumigatus arp1* modulates conidial pigmentation and complement deposition. Mol Microbiol 1997; 26: 175-183.
192. Tsai HF, Wheeler MH, Chang YC, Kwon-Chung KJ. A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. J Bacteriol 1999; 181: 6469-6477.
193. Tsunawaki S, Yoshida LS, Nishida S, Kobayashi T, Shimoyama T. Fungal metabolite gliotoxin inhibits assembly of the human respiratory burst NADPH oxidase. Infect Immun 2004; 72: 3373-3382.
194. Varga J, Toth B. Genetic variability and reproductive mode of *Aspergillus fumigatus*. Infect Genet Evol 2003; 3: 3-17.
195. Warris A, Voss A, Verweij PE. Hospital sources of *Aspergillus* species: New routes of transmission? Rev Iberoam Micol 2001; 18: 156-162.
196. Wasylnka JA, Moore MM. Adhesion of *Aspergillus* species to extracellular matrix proteins: evidence for involvement of negatively charged carbohydrates on the conidial surface. Infect Immun 2000; 68: 3377-3384.
197. Wasylnka JA, Moore MM. Uptake of *Aspergillus fumigatus* conidia by phagocytic and nonphagocytic cells in vitro: quantitation using strains expressing green fluorescent protein. Infect Immun 2002; 70: 3156-3163.
198. Wasylnka JA, Moore MM. *Aspergillus fumigatus* conidia survive and germinate in acidic organelles of A549 epithelial cells. J Cell Sci 2003; 116: 1579-1587.
199. Wasylnka JA, Simmer MI, Moore MM. Differences in sialic acid density in pathogenic and non-pathogenic *Aspergillus* species. Microbiology 2001; 147: 869-877.
200. Watanabe A, Kamei K, Sekine T, Higurashi H, Ochiai E, Hashimoto Y, Nishimura K. Cytotoxic substances from *Aspergillus fumigatus* in oxygenated or poorly oxygenated environment. Mycopathologia 2004; 158: 1-7.
201. Watanabe A, Kamei K, Sekine T, Waku M, Nishimura K, Miyaji M, Kuriyama T. Immunosuppressive substances in *Aspergillus fumigatus* culture filtrate. J Infect Chemother 2003; 9: 114-121.
202. Watanabe A, Kamei K, Sekine T, Waku M, Nishimura K, Miyaji M, Tatsumi K, Kuriyama T. Effect of aeration on gliotoxin production by *Aspergillus fumigatus* in its culture filtrate. Mycopathologia 2004; 157: 19-27.
203. Weidner G, d'Enfert C, Koch A, Mol PC, Brakhage AA. Development of a homologous transformation system for the human pathogenic fungus *Aspergillus fumigatus* based on the *pyrG* gene encoding orotidine 5'-monophosphate decarboxylase. Curr Genet 1998; 33: 378-385.
204. Weig M, Frosch M, Tintelnot K, Haas A, Gross U, Linsmeier B, Heesemann J. Use of recombinant mitogillin for improved serodiagnosis of *Aspergillus fumigatus*-associated diseases. J Clin Microbiol 2001; 39: 1721-1730.
205. Wheeler MH, Bell AA. Melanins and their importance in pathogenic fungi. In McGinnis MR (Ed.) Current topics in medical mycology. Vol. 2. New York, Springer-Verlag, 1988: 338-387.
206. Woo PC, Chan CM, Leung AS, Lau SK, Che XY, Wong SS, Cao L, Yuen KY. Detection of cell wall galactomannoprotein Afmp1p in culture supernatants of *Aspergillus fumigatus* and in sera of aspergillosis patients. J Clin Microbiol 2002; 40: 4382-4387.
207. Woo PC, Leung AS, Lau SK, Chong KT, Yuen KY. Use of recombinant mitogillin for serodiagnosis of *Aspergillus fumigatus*-associated diseases. J Clin Microbiol 2001; 39: 4598-4600.
208. Wright MS, Clausen HK, Abrahamsen TG. Liver cells respond to *Aspergillus fumigatus* with an increase in C3 secretion and C3 gene expression as well as an expression increase in TLR2 and TLR4. Immunol Lett 2004; 95: 25-30.
209. Xue T, Nguyen CK, Romans A, May GS. A mitogen-activated protein kinase that senses nitrogen regulates conidial germination and growth in *Aspergillus fumigatus*. Eukaryot Cell 2004; 3: 557-560.
210. Yuen KY, Chan CM, Chan KM, Woo PCY, Che XY, Leung ASP, Cao L. Characterization of *AFMP1*: a novel target for serodiagnosis of aspergillosis. J Clin Microbiol 2001; 39: 3830-3837.
211. Youngchim S, Morris-Jones R, Hay RJ, Hamilton AJ. Production of melanin by *Aspergillus fumigatus*. J Med Microbiol 2004; 53: 175-181.
212. Zhang MX, Brandhorst TT, Kozel TR, Klein BS. Role of glucan and surface protein BAD1 in complement activation by *Blastomyces dermatitidis* yeast. Infect Immun 2001; 69: 7559-7564.
213. Zhao Y, Liu J, Wang J, Wang L, Yin H, Tan R, Xu Q. Fumigaclavine C improves concanavalin A-induced liver injury in mice mainly via inhibiting TNF-alpha production and lymphocyte adhesion to extracellular matrices. J Pharm Pharmacol 2004; 56: 775-782.