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Review

What makes *Aspergillus fumigatus* a successful pathogen? Genes and molecules involved in invasive aspergillosis

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ABSTRACT

Aspergillus fumigatus is an opportunistic pathogen that causes 90% of invasive aspergillosis (IA) due to *Aspergillus* genus, with a 50–95% mortality rate. It has been postulated that certain virulence factors are characteristic of *A. fumigatus*, but the "non-classical" virulence factors seem to be highly variable. Overall, published studies have demonstrated that the virulence of this fungus is multifactorial, associated with its structure, its capacity for growth and adaptation to stress conditions, its mechanisms for evading the immune system and its ability to cause damage to the host. In this review we intend to give a general overview of the genes and molecules involved in the development of IA. The thermotolerance section focuses on five genes related with the capacity of the fungus to grow at temperatures above 30 °C (*thtA*, *cgrA*, *afpmt1*, *kre2/afmnt1*, and *hsp1/asp f 12*). The following sections discuss molecules and genes related to interaction with the host and with the immune responses. These sections include β-glucan, α-glucan, chitin, galactomannan, galactomannoproteins (*afmp1/asp f 17* and *afmp2*), hydrophobins (*rodA/hyp1* and *rodB*), DHN-melanin, their respective synthases (*fks1*, *rho1–4*, *ags1–3*, *chsA-G*, *och1–4*, *mnn9*, *van1*, *anp1*, *glfA*, *pksP/alb1*, *arp1*, *arp2*, *abr1*, *abr2*, and *ayg1*), and modifying enzymes (*gel1–7*, *bgt1*, *eng1*, *ecm33*, *afpigA*, *afpmt1-2*, *afpmt4*, *kre2/afmnt1*, *afmnt2–3*, *afcwh41* and *pmi*); several enzymes related to oxidative stress protection such as catalases (*catA*, *cat1/catB*, *cat2/katG*, *catC*, and *catE*), superoxide dismutases (*sod1*, *sod2*, *sod3/asp f 6*, and *sod4*), fatty acid oxygenases (*ppoA–C*), glutathione tranferases (*gstA–E*), and others (*afyap1*, *skn7*, and *pes1*); and efflux transporters (*mdr1–4*, *atrF*, *abcA–E*, and *msfA–E*). In addition, this review considers toxins and related genes, such as a diffusible toxic substance from conidia, gliotoxin (*gliP* and *gliZ*), mitogillin (*res/mitF/asp f 1*), hemolysin (*aspHS*), festuclavine and fumigaclavine A–C, fumitremorgin A–C, verruculogen, fumagillin, helvolic acid, aflatoxin B1 and G1, and *laeA*. Two sections cover genes and molecules related with nutrient uptake, signaling and metabolic regulations involved in virulence, including enzymes, such as serine proteases (*alp/asp f 13*, *alp2*, and *asp f 18*), metalloproteases (*mep/asp f 5*, *mepB*, and *mep20*), aspartic proteases (*pep/asp f 10*, *pep2*, and *ctsD*), dipeptidylpeptidases (*dppIV* and *dppV*), and phospholipases (*plb1–3* and phospholipase C); siderophores and iron acquisition (*sidA–G*, *sreA*, *ftrA*, *fetC*, *mirB–C*, and *amcA*); zinc acquisition (*zrfA–H*, *zafA*, and *pacC*); amino acid biosynthesis, nitrogen uptake, and cross-pathways control (*areA*, *rhbA*, *mcsA*, *lysF*, *cpcA/gcn4p*, and *cpcC/gcn2p*); general biosynthetic pathway (*pyrG*, *hcsA*, and *pabaA*), trehalose biosynthesis (*tpsA* and *tpsB*), and other regulation pathways such as those of the MAP kinases (*sakA/hogA*, *mpkA–C*, *ste7*, *pbs2*, *mkk2*, *steC/ste11*, *bck1*, *ssk2*, and *sho1*), G-proteins (*gpaA*, *sfaD*, and *cpgA*), cAMP-PKA signaling (*acyA*, *gpaB*, *pkaC1*, and *pkaR*), His kinases (*fos1* and *tcsB*), Ca^{2+} signaling (*calA/cnaA*, *crzA*, *gprC* and *gprD*), and Ras family (*rasA*, *rasB*, and *rhbA*), and others (*ace2*, *medA*, and *srbA*). Finally, we also comment on the effect of *A. fumigatus* allergens (Asp f 1–Asp f 34) on IA. The data gathered generate a complex puzzle, the pieces representing virulence factors or the different activities of the fungus, and these need to be arranged to obtain a comprehensive vision of the virulence of *A. fumigatus*. The most recent gene expression studies using DNA-microarrays may be help us to understand this complex virulence, and to detect targets to develop rapid diagnostic methods and new antifungal agents.

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¿Qué hace que *Aspergillus fumigatus* sea un patógeno de éxito? Genes y moléculas involucrados en la aspergilosis invasora

Palabras clave:
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Aspergilosis invasora
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Termotolerancia
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Pared celular
Toxinas
Toma de nutrientes
Señalización y regulación
Alérgenos

R E S U M E N

Aspergillus fumigatus es un patógeno oportunista que causa el 90% de las aspergilosis invasoras (AI) con un 50–95% de mortalidad. Se ha postulado la existencia de factores de virulencia característicos, pero en *A. fumigatus* existe una gran variabilidad de factores de virulencia «no clásicos». Todos los estudios han demostrado que la virulencia de este hongo es multifactorial, asociada a su estructura, su capacidad de crecimiento y adaptación a condiciones de estrés, sus mecanismos de evasión del sistema inmune y su capacidad de causar daños en un huésped. En esta revisión se pretende dar una visión general de los genes y moléculas que intervienen en el desarrollo de la AI. La sección de termotolerancia incluye cinco genes relacionados con la capacidad de que el hongo crezca a más de 30 °C (*thtA*, *cgrA*, *afpmt1*, *kre2/afmnt1* y *hsp1/asp f 12*). En las siguientes secciones se discuten las moléculas y los genes relacionados con la interacción con el huésped y con la respuesta inmune. Estas secciones incluyen el β-glucano, el α-glucano, la quitina, el galactomanano, galactomanoproteinas (*afmp1/asp f 17* y *afmp2*), hidrofobinas (*rodA/hyp1* y *rodB*), la DHN-melanina, sus respectivas enzimas sintasas (*fks1*, *rho1-4*, *ags1-3*, *chsA-G*, *och1-4*, *mnn9*, *van1*, *anp1*, *glfA*, *pksP/alb1*, *arp1*, *arp2*, *abr1*, *abr2* y *ayg1*) y enzimas modificantes (*gel1-7*, *bgt1*, *eng1*, *ecm33*, *afpigA*, *afpmt1-2*, *afpmt4*, *kre2/afmnt1*, *afmnt2-3*, *afcwh41* y *pmi*), varias enzimas relacionadas con la protección del estrés oxidativo como catalasas (*catA, cat1/catB, cat2/katG, catC* y *catE*), superóxido dismutasas (*sod1-2*, *sod3/asp f 6* y *sod4*), oxigenasas de ácidos grasos (*ppoA-C*), glutatión transferasas (*gstA-E*) y otros (*afyap1*, *skn7* y *pes1*), y los transportadores de moléculas (*mdr1-4*, *atrF*, *abcA-E* y *msfA-E*). Esta revisión también incluye las toxinas y los genes relacionados, como la sustancia difusible de conidios, la gliotoxina (*gliP* y *gliZ*), la mitogilina (*asp f 1/mitF/res*), la hemolisina (*aspHS*), la festuclavina y la fumigaclavina A-C, la fumitremorgina, el verruculógeno, la fumagilina, el ácido helvólico, las aflatoxinas B1 y G1, y *laeA*. Dos secciones incluyen los genes y moléculas relacionadas con la absorción de nutrientes, la señalización y las regulaciones metabólicas implicadas en la virulencia, incluyendo enzimas, como las serin-proteasas (*alp/asp f 13*, *alp2* y *asp f 18*), metaloproteasas (*mep/asp f 5*, *mepB* y *mep20*), aspártico-proteasas (*pep/asp f 10*, *pep2* y *ctsD*), dipeptidilpeptidasas (*dppIV* y *dppV*) y fosfolipasas (*plb1-3* y fosfolipasa C); sideróforos y la adquisición de hierro (*sidA-G sreA*, *ftrA*, *fetC*, *mirB-C* y *amcA*); adquisición de zinc (*zrfA-H*, *zafA*, y *pacC*); biosíntesis de aminoácidos, absorción de nitrógeno, y regulación por *Cross-pathway Control* (*areA*, *rhbA*, *mcsA*, *lysF*, *cpcA/gcn4p* y *cpcC/gcn2p*); vías de biosíntesis generales (*pyrG*, *hcsA*, y *pabaA*) y biosíntesis de trehalosa (*tpsA* y *tpsB*); otras vías de regulación, como MAP quinasas (*sakA/hogA*, *mpkA-C*, *ste7*, *pbs2*, *mkk2*, *steC/ste11*, *bck1*, *ssk2* y *sho1*), proteínas G (*gpaA*, *sfaD* y *cpgA*), AMPc-PKA (*acyA*, *gpaB*, *pkaC1* y *pkaR*), histidin-quinasas (*fos1* y *tcsB*), señalización de Ca^{2+}(*calA/cnaA*, *crzA*, *gprC* y *gprD*), familia Ras (*rasA*, *rasB* y *rhbA*), y otros (*ace2*, *medA*, y *srbA*). Por último, también se comentan los efectos de los alérgenos de *A. fumigatus* (*Asp f 1* a *Asp f 34*) en la AI. Los datos obtenidos generan un complejo rompecabezas, cuyas piezas serían factores de virulencia o diferentes actividades del hongo, que se deben reunir para obtener una visión conjunta de la virulencia de *A. fumigatus*. Los estudios de expresión mediante *microarrays* de ADN podrían ser útiles para entender esta compleja virulencia, y para detectar dianas para desarrollar métodos rápidos de diagnóstico y nuevos agentes antifúngicos.

Aspergillus fumigatus is a well adapted saprophytic mold that produces large number of small airborne spores that can survive a wide range of environmental conditions, and accordingly are abundant in soil and decaying organic matter. Due to the 10,000–15,000l of air we inhale each day, humans are continuously in contact with these asexual spores[17] and it is estimated that an individual inhales several hundred conidia per day.[154] It is remarkable that, despite this constant exposure, most humans do not develop any illness attributable to these spores. In immunocompetent hosts, these spores do not normally cause harm because they are eliminated by pulmonary defense mechanisms.[17] However, in immunocompromised individuals, with altered or weakened immune responses, inhaled conidia are able to develop pulmonary mycoses known as aspergillosis. Aspergillosis can be regarded as a broad spectrum of diseases, each related to a spectrum of abnormal immune responses of the host.[217] Among them invasive aspergillosis (IA) stands out, with mortality rates greater than 50%, reaching 95% in certain situations[17,173] (Fig. 1). The higher mortality observed in the infections by *A. fumigatus* appears to be due to a weakened immune response, to the virulence of the microorganism itself and also, probably, to delays in establishing a diagnosis, which can prevent the success of treatments.[72] About 40 of the 250 species of *Aspergillus* have been reported to be human pathogens,[126] but although the spores of *A. fumigatus* are a small proportion of all the airborne spores within a hospital (0.3%), this fungus causes approximately 90% of the systemic infections due to *Aspergillus*.[40] Given this, it has been postulated that *A. fumigatus* has characteristic virulence factors.

The genome sequence of *A. fumigatus* has been made available recently.[201] About 9,630 predicted protein-coding genes have been described of which one-third have unknown functions.[85] During infection, fungi encounter dynamic changes in host conditions to which they must continually adapt to survive. This adaptation often requires substantial metabolic reprogramming,[91,263] with the simultaneous expression of virulence factors that mediate host cell damage.[51] A successful invasion strategy can involve large-scale alterations in protein expression and/or cellular differentiation[209]. The degree of host immunosuppression needed to develop IA could reduce the requirement for adaptive responses for infection in *A. fumigatus*. The importance of *A. fumigatus* infections is reflected by a number of reviews that have been published in the last few years concerning its biology and pathology, and the great effort being made to identify virulence factors.[13,19,21,40,41,69,72,112,133,153–155,183,193,208,232,272,285] All these studies have demonstrated that the virulence of this fungus is multifactorial and is due to a combination of biological characteristics of the fungus and the immune status of the patient. Some of these characteristics include the small size of its conidia (2–3 μm in diameter) which allows them to reach the human pulmonary alveoli, its thermotolerance and resistance to oxidative stress, and its high growth rate and nutritional versatility, among others. Other fungi share some of these features, but *A. fumigatus* possibly possesses a unique combination of different traits that make it the primary pathogenic mold in the world.[208]

With the use of sequenced genomes, we begin to be able to dissect some complex networks of fungal gene interactions such as metabolic regulation, autophagy, and sexuality. Although it is currently unclear, some authors have suggested that these networks have certain effects on the adaptive response of *A. fumigatus* to infections. Autophagy helps organisms to survive periods of nutrient stress by providing a source of recycled intracellular nutrients to fuel essential cellular functions.[209] In *Saccharomyces cerevisiae*,

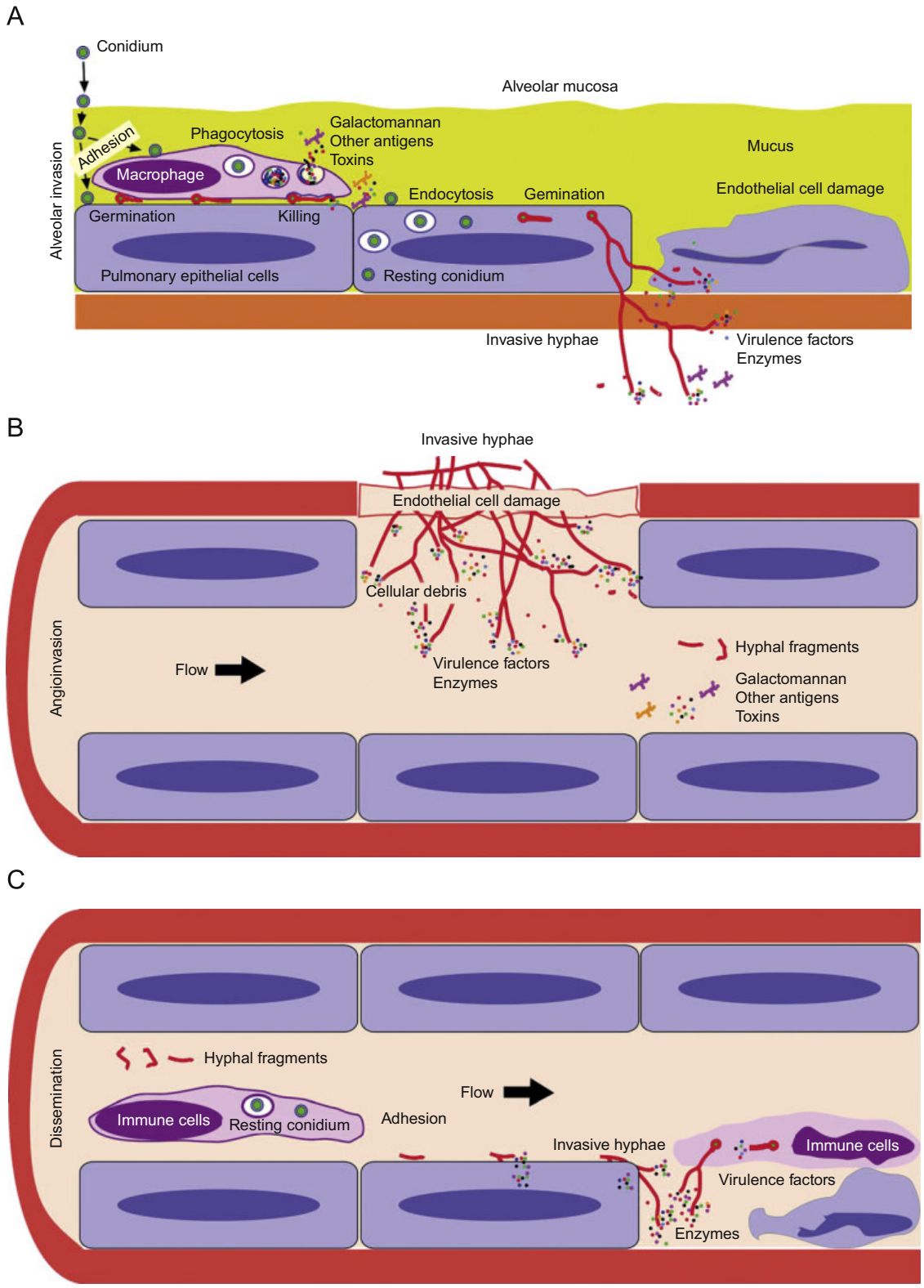

Fig. 1. Model of invasive aspergillosis development. (A) First step of colonization and invasion of pulmonary epithelium. (B) Invasion of blood capillaries and haematogenous dissemination of hyphal fragments, galactomannan and other molecules. (C) Dissemination and first step of invasion of deep organs.

approximately 30 genes have been identified and collectively termed autophagy-related genes (ATGs)[127,128] and many of these are highly conserved across eukaryotes. In *A. fumigatus*, autophagy is required for conidiation, hyphal foraging, and maintenance of metal-ion homeostasis (all related to nutrient deficiency).[235,236] Nutrient deprivation is one of the antimicrobial mechanisms of the host, however, the number of secreted hydrolases encoded by the genome of *A. fumigatus*,[201,239] may allow this fungus to obtain nutrients from mammalian tissues without activating the autophagic network[209]. The fact that other pathogenic fungi use this mechanism to adapt to

the host makes autophagy a putative virulence factor that should be considered carefully in the future.

A. fumigatus, like many other clinically important fungal species, has traditionally been considered an asexual organism. However, the teleomorph phase of this fungus has been discovered and named *Neosartorya fumigata*.[204] The genome sequence of this fungus has made it clear that it occurs in two idiomorphs, MAT1-1 and MAT1-2, and strains of the two opposite mating types occur at the same frequency and are found in close proximity to each other.[204] Successful mating was obtained between unrelated, clinical isolates of *A. fumigatus*, and requires the presence of both mating-type idiomorphs.[270] Others authors showed the need for the expression of MAT1-1 and MAT1-2, as well as the expression of genes that encode factors involved in this process, such as genes encoding for sex pheromones and pheromone receptors.[80,212] For example, the *nsdD* gene, a conserved regulator of cleistothecium development, could be related to hyphal fusion and hence heterokaryon formation.[270] Heterothallism has now been discovered in four species of *Aspergillus* that affect human health or have an economic impact, namely *A. fumigatus*, *A. parasiticus*, *A. flavus*, and *N. udagawae*, but these fungi appear to have relatively low levels of fertility compared to other heterothallic or homothallic species of *Aspergillus* and require unusually precise environmental parameters to complete their sexual cycle.[142] There are different interpretations of this low fertility. Some authors favor the hypothesis that while fertility of these species is on the decline, this is compensated by their proficiency to reproduce asexually in a wider range of environmental conditions.[142] Other authors believe that the maintenance of all the machinery required for sex and the limitation of their access to sexual reproduction, has enabled the pathogenic fungi to proliferate rapidly in their environmental niche, but also to undergo genetic exchange, via sexual reproduction, in response to stressful conditions, for example, new environments, different host organisms, or changes in the human host, such as antimicrobial therapy.[200] Highly dynamic changes in *A. fumigatus* populations have been observed within a clinical setting, with new populations found in just a few months,[11] and coinfections with different related species of the *Aspergillus* genus have already been reported.[207] These data imply that there may be coinfections with different mating type strains, and surprisingly the possibility that mating could occur in hosts during fungal infection. The presence of a sexual cycle in *A. fumigatus* would have significant medical implications. Some data suggest a possible association between one idiomorph, the MAT1-1 mating type, and *A. fumigatus* invasiveness that might contribute to increased virulence and/or resistance to antifungal agents.[7] The study of sexual reproduction of this fungus and its possible relationship with virulence will remain a topic of interest in the coming years.[6]

The intention in this review is to give a general overview of the genes and molecules which have been associated with fungal virulence in the literature, the activities which they can perform and the importance that they could have in the development of IA.

Genes and molecules related to *A. fumigatus* virulence

Virulence factors are defined as pathogen determinants that cause damage to the infected host.[50] This definition includes genes the deletion of which reduces virulence of the reference strain without affecting normal growth, excluding therefore, genes encoding biosynthetic proteins.[208] Other genes related to *A. fumigatus* virulence, like catalases or secreted proteases, do not fit with this definition due to the redundancy of their gene families, and the difficulty of developing disruption of all the genes of a family in a single strain. Nevertheless, all of the genes that help and promote the growth of *A. fumigatus* in its environmental niche are also implicated in the pathogenesis of aspergillosis in the human host, and hence have to be considered as possible targets for new antifungal agents.[13]

The genes and molecules related to *A. fumigatus* virulence can be classified according to the process they are involved in, e.g., thermotolerance; cell wall composition and maintenance; resistance to the immune response; toxins; nutrient uptake during invasive growth; signaling, metabolism regulation and response to stress conditions; and allergens.

Thermotolerance

A. fumigatus is a thermophilic fungus able to grow at 55 °C and survive at more than 75 °C,[26,241] an essential ability to thrive in decaying organic matter and to infect mammalian hosts. Therefore, genes related to thermotolerance may also contribute to the virulence of this mold.[31] Until now, only four genes studied have been found to be necessary for thermotolerance (Table 1). The *thtA* gene is essential for *A. fumigatus* growth at 48 °C but does not contribute to the pathogenicity of the species.[54] Similarly, the *afpmt1* gene codes for an *o*-mannosyltransferase, necessary for growth over 37 °C, but is not involved in virulence.[311] A putative

Table 1
Aspergillus fumigatus thermotolerance genes and their relationship with virulence

Genes	Gene product	Function	Pathogenesis related activities	Effect of deletion[a]	Uses	References
thtA	Unknown	Function unknown; essential for growth at 48 °C		Normal virulence		54
afpmt1	Afpmt1 (*o*-mannosyl-transferase)	Necessary for growth > 37 °C	Cell-wall assembly and morphogenesis	Normal virulence		311
kre2 afmnt1	Kre2/Afmnt1 (α-1,2-mannosyltransferase)	Necessary to growth at 48 °C Cell wall integrity	Cell-wall assembly and morphogenesis	Hypovirulent	New antifungal target	289
cgrA	CgrA (nucleolar protein)	Ribosome biogenesis at 37 °C	Growth at 37 °C	Hypovirulent		32
hsp1/asp f 12	Hsp1/Asp f 12 (heat shock protein, Hsp90 family)	Chaperone	Chaperone activity and protein transport in growth at 37 °C Stress response during inflammation Autoimmunity Type I hypersensitivity			136

[a] Virulence assayed in animal model.

α-1,2-mannosyltransferase coded by *afmnt1* was also shown to be necessary for growth at 48 °C.[289] These authors showed that the Δ*afmnt1* mutant grows normally at 37 °C, and that the observed growth defect of the mutant at 48 °C can be attributed to cell wall instability resulting in leakage at the hyphal tips. This Δ*afmnt1* mutant was attenuated in a mouse model of infection, and showed an increased sensitivity to azoles.[289] Likewise the deletion of the *cgrA* gene, which is involved in ribosome biogenesis, produced a hypovirulent strain in a murine model of invasive aspergillosis but not in a fruit fly model, being so related to the growth at 37 °C.[32]

Cells exposed to non-lethal high temperatures become transiently resistant to subsequent heat shock, producing proteins named heat shock proteins (HSPs). Thermotolerance development is paralleled by expression of these HSPs.[203] HSPs have been identified as molecular chaperones conserved between organisms.[46] It has also been reported that a protein, Hsp1/Asp f 12,[136] classified as a member of the family of Hsp90 could be related to thermotolerance. In addition, the protein Hsp1/Asp f 12 may also play a role in protective immunity and autoimmunity, as it is one of the immunodominant antigens in allergic aspergillosis.[136]

Nierman et al.[201] studied the differences in gene expression between 30 and 37 °C and between 30 and 48 °C, and detected some upregulated genes at 37 °C, but to date none of the genes related to pathogenicity have been found to be more highly expressed at 37 °C than that at 48 °C. They concluded that host temperature alone is not sufficient to turn on many virulence-related genes. On the other hand, Do et al.[76] proposed that the thermal tolerance of *A. fumigatus* might be due to the efficient regulation of metabolic genes by HSPs. These authors used a state space model to examine transcriptional regulation and found a negative association between many HSPs and the metabolic genes they regulate. Little is known about *A. fumigatus* proteome changes at different temperatures, but a recent study has described 64 proteins to be up or downregulated from 30 to 48 °C.[4] Of them, Hsp 30/Hsp 42 and Hsp 90 showed the highest increase in expression during the heat shock response of *A. fumigatus.* More studies of changes in the proteome and their relationship with transcriptome changes could enhance our understanding of the thermoregulation of this fungus, and would help identify new possible targets for IA treatment.

Cell wall composition and maintenance

The cell wall is the main line of defense of the fungus against a hostile environment providing structural integrity and physical protection to the cell. The fungal cell wall is also the structure responsible for the interaction with the host and their components are often the targets of the host immune system during fungal infections. In *A. fumigatus*, the cell wall is mainly composed of polysaccharides (at least 90%) and proteins.[98] Among the polysaccharides there are linear β(1–3)-glucans (20–35%) branched with β(1–6) links (4%); linear β(1–3/1–4)-glucans (10%); α(1–3)-glucans (35–46%); chitins; and galactomannans (20–25%).[98,152,156] Fig. 2 shows a schematic drawing of the cell wall structure. The genes and molecules related to the cell wall and virulence included in this review are listed in Table 2. Additional layers in the outer part of the cell wall may be also present. A layer of hydrophobic components is detected on both hypha and conidia, and a melanin layer only on the conidia. The effect of melanin and hydrophobic components on the immune response is addressed in the next section. Further, the presence of sialic acids has been detected on the surface of conidia. These sialic acids are unsubstituted N-acetyl-neuraminic acids linked to galactose by α-2,6 bonds[29] and could play an important role in their adhesion to the extracellular matrix.[292]

The cell wall consists in a polysaccharide-based three-dimensional network and is now seen as a dynamic structure that is continuously changing as a result of the modification of culture conditions and environmental stress.[152] The maintenance of cell wall integrity and functionality as well as changes in cell wall composition to adapt to the environment of the host could be involved in pathogenicity. Genes participating in the biosynthesis of most of *A. fumigatus* cell wall components have been identified.[98] The study of these genes has revealed that mutant strains for enzymes required to synthesize cell wall polysaccharides were at least as virulent as the reference strain on almost all occasions.

The major polysaccharides in the *A. fumigatus* cell wall are the α(1–3)-glucans, and these have been shown to contribute to the virulence of diverse fungal pathogens. In particular, three α(1–3)-glucan synthase genes, *ags1*, *ags2*, and a*gs3*, have been identified and were found to be responsible for cell wall α(1–3)-glucan biosynthesis. The Δ*ags1* and Δ*ags2* strains were not defective in virulence,[22] while the Δ*ags3* mutant was hypervirulent in an experimental mouse model of aspergillosis.[174] Hypervirulence was correlated with an increased melanin content of the conidial cell wall, which could protect the cells from oxidative stress, and a quicker germination rate, that could evade macrophage killing. These authors did not observe significant changes in cell wall composition of the mutants, probably because of the redundancy between *ags1* and *ags3*.[13]

β(1–3)-glucan branched with β(1–6)-glucan form the skeleton of the wall, and these are covalently bound to chitin and β(1–3/1–4)-glucan. This component is an important fungal pathogen-associated molecular pattern (PAMP) being recognized by receptor dectin1 on immune cells,[29,43] and has different types of biological activity, triggering the activation of complement and inflammatory responses through mediators such as leukotrienes and TNFα[117]. β-glucan is a compound that is present in almost all fungi and has been used for the diagnosis of invasive mycosis,[117] its kinetics correlating very well with that of galactomannan in patients with IA.[218] Several authors have reviewed the synthesis of this component.[78,98] Briefly, β(1–3)-glucan synthase is a transmembrane complex formed by several different proteins.[78,98] The *fks1* gene encodes the catalytic subunit and some of the four *rho* genes (*rho1–4*) detected in *A. fumigatus* and may be the regulatory subunit of glucan synthase.[78,189] Although it is not a real virulence factor, Fks1 is essential for the fungus and its interest lies in being the target for the antifungal echinocandins. Research has also indicated that Rho1 and Rho3 are involved in controlling cell wall integrity and the cytoskeleton, and these are localized in the hyphal tip.[75] Therefore, in the future, Rho molecules could also be potential targets for developing new antifungal agents.

A. fumigatus has at least seven chitin synthase encoding genes, but just four of them have been assayed for virulence: *chsC*, *chsD*, *chsE*, and *chsG*.[14,177–179] Only *chsG* seems to have an influence on virulence, with a Δ*chsG* mutant strain having been shown to produce lower mortality rates than the reference strain in a mice infection model.[177] However, these results could also be explained by redundancy of this type of enzymes.

The galactomannans in the cell wall are composed of mannose chains (α-mannan), shorter than those of yeasts, with branches formed by small side chains of five molecules of β(1–5)-galactose linked to mannan.[98] Galactomannan synthesis requires mannosyl- and galactosyl-transferases. In the *A. fumigatus* genome there are orthologs of the *S. cerevisiae* genes related to mannan synthesis, four of the *OCH* genes, that initiate the synthesis of mannan chains, and orthologs of *MNN9*, *VAN1*, and *ANP1* genes,

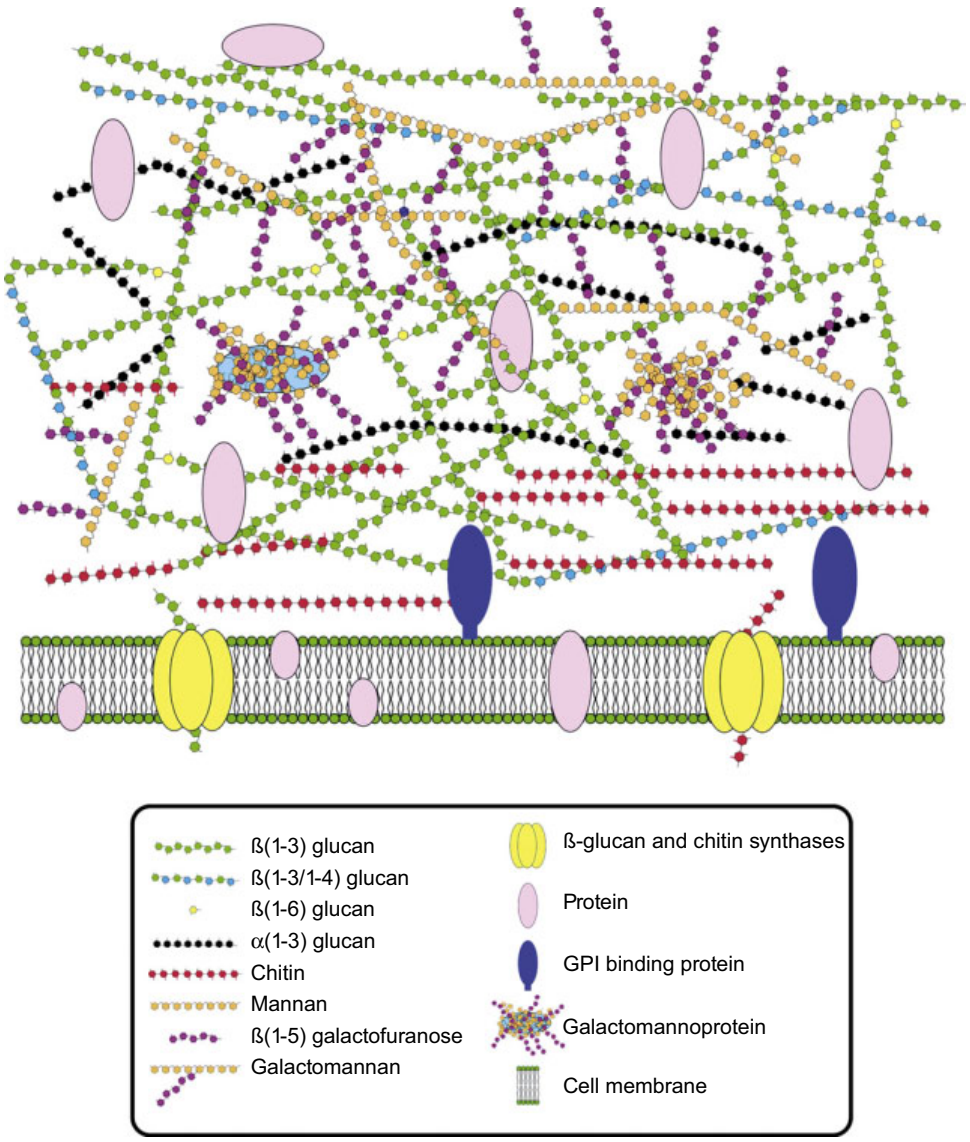

Fig. 2. Scheme of *Aspergillus fumigatus* cell wall.

which encode for mannosyltransferases.[98] The functional role of each gene remains unknown. Galactofuranose biosynthesis starts with the isomerization of UDP-galactopyranose to UDP galactofuranose by UDP galactomutase encoded by the *glfA* gene. Δ*glfA* strains displayed attenuated virulence in a low-dose mouse model of IA and showed an increased susceptibility to various antifungal agents.[244] UDP-galactomutase thus appears to be an appealing target for adjuvant therapy due to its absence from mammalian cells.[244] This galactomannan could be a PAMP of the fungus, and useful for adhesion to host components such as fibronectin and laminin, or to interact with pentraxin 3 and other surface receptors of macrophages, dendritic cells, and Langerhans cells.[29,112] Galactomannan is the principal exoantigen released during tissue invasion[154] and may activate the innate immune response away from the focus of the infection. At present, the galactomannan produced and released by *A. fumigatus* is used in a commercial test for the diagnosis of IA (Platelia® *Aspergillus*).[70,71]

Several proteins of the cell wall are also mannosylated. For example, *afmp1* and *afmp2* genes encode for a galactomannoprotein and a mannoprotein, respectively. Their role in virulence has not been investigated, but it is worth mentioning them as they are antigenic determinants and therefore possible candidates for serodiagnosis.[56,232,306] The addition of N-linked and/or O-linked oligosaccharides is a common modification of cell wall proteins. Mannosyltransferases play a crucial role in this process and most likely are also engaged in the generation of other glycoconjugates. Mannosyltransferases are localized in intracellular compartments of the secretory pathway, e.g., the Golgi apparatus or the endoplasmic reticulum (ER)[289] and initiate mannosylation of secretory proteins. In *A. fumigatus* three members of O-mannosyltransferases, orthologs of PMT family of *S. cerevisiae*, *afpmt1*, *afpmt2* and *afpmt4* have been detected. Two of these have been studied but were found not to be necessary for virulence. The Δ*afpmt1* mutant showed sensitivity to high temperatures, as mentioned above in the thermotolerance section, and also defects in growth and cell wall integrity, thereby affecting cell morphology, conidium formation, and germination in *A. fumigatus*.[311] Reduced expression of the *afpmt2* gene also led to delayed germination, retarded hyphal growth, reduced conidiation, and defects in cell wall integrity; but growth was not found to be temperature-sensitive.[83] The reduced production of Afpmt2 also caused actin rearrangement to fail.[83] The *afpmt4* gene has not yet been studied. The genome of *A. fumigatus* also harbors three putative α-1,2-mannosyltransferases genes with homology with

Table 2
Genes and proteins related with cell wall structure and virulence

Molecules/ genes	Gene product	Function	Pathogenesis related activities	Effect of deletion[a]	Uses	References
β-(1-3)-glucan		Cell-wall integrity	Immunomodulator Recognition and cellular adhesion		Diagnosis	29,43,117
fks1	Fks1 (catalytic subunit of β-(1–3)-glucan synthase complex)	Synthesis of β-(1–3)-glucan	Essential for fungal growth Cell-wall assembly and morphogenesis		Echinocandin target	189
rho1–4	Rho1-4 (Regulatory subunit of β-(1–3)-glucan synthase complex)	Synthesis of β-(1–3)-glucan	Cell-wall integrity Cytoskeleton control		New antifungal target?	75,114
rom2	Activator of rho	No essential				98,114
Glucanosyl-transferases						
gel1	Gel1 (Glucanosyl-transferase, GPI-anchored protein)	Elongation of β-(1–3)-glucan	Cell-wall assembly and morphogenesis		New antifungal target	187,190
gel2	Gel2 (Glucanosyl-transferase, GPI-anchored protein)	Elongation of β-(1–3)-glucan	Cell-wall assembly and morphogenesis	Hypovirulent		191
gel3–7	Gel3–7 (Glucanosyl-transferase, GPI-anchored protein)	Elongation of β-(1–3)-glucan	Cell-wall assembly and morphogenesis			98
bgt1	Bgt1 (Glucanosyl-transferase)	β-(1–6) branching of β-(1–3)-glucan	Cell-wall assembly and morphogenesis			188
eng1	Eng1 (β-(1–3)-endoglucanase)		Cell-wall assembly and morphogenesis			192
ecm33	Ecm33 (GPI-anchored protein)		Cell-wall assembly and morphogenesis	Hypervirulent		52,240
α-(1–3) glucan		Cell-wall integrity	Recognition and cellular adhesion			
ags1–2	Ags1–2(α-(1–3)-glucan synthases)	Sinthesis of α-(1–3) glucan	Cell-wall assembly and morphogenesis	Normal virulence		22
ags3	Ags3 (α-(1–3)-glucan synthases)	Sinthesis of α-(1–3) glucan	Cell-wall assembly and morphogenesis	Hypervirulent		174
Chitin **Chitin synthases**		Cell-wall integrity	Antigen Cell-wall assembly and morphogenesis Immunomodulator (generation chito-oligosaccharides)?		New antifungal target?	
chsA	ChsA (Chitin synthase class I)	Synthesis of chitin				195
chsB	ChsB (Chitin synthase class II)	Synthesis of chitin				195
chsC	ChsC (Chitin synthase class III)	Synthesis of chitin		Normal virulence		177
chsD	ChsD (Chitin synthase class VI)	Synthesis of chitin		Normal virulence		179
chsE	ChsE (Chitin synthase class V)	Synthesis of chitin		Normal virulence		178
chsF	ChsF (Chitin synthase class IV)	Synthesis of chitin				
chsG	ChsG (Chitin synthase class III)	Synthesis of chitin		Hypovirulent		177
afpigA	AfpigA (N-acetyl-glucosaminyl-transferase catalytic subunit, GPI-anchored protein)	Synthesis of chitin	Cell wall assembly and morphogenesis	Hypovirulent		160
Galactomannan		Cell-wall integrity	Extracellular antigen Immunomodulator Recognition and cellular adhesion		Diagnosis	70,71
och1–4	Och1-4 (mannosyl-transferases)	Synthesis of mannan	Cell wall assembly and morphogenesis			98
mnn9	Mnn9 (mannosyl-transferases)	Synthesis of mannan	Cell wall assembly and morphogenesis			98
van1	Van1 (mannosyl-transferases)	Synthesis of mannan	Cell wall assembly and morphogenesis			98
anp1	Anp1 (mannosyl-transferases)	Synthesis of mannan	Cell wall assembly and morphogenesis			98
glfA	GlfA (UDP-gal-mutase)	Synthesis of galactofuran	Cell wall assembly and morphogenesis	Hypovirulent in low dose aspergillosis model	New antifungal target	98
afmp1/asp f 17	Afmp1p/Asp f 17 (Galactomannoprotein)		Antigen, Type I hypersensitivity		Candidates for sero-diagnosis	306
afmp2	Afmp2 (Mannoprotein)		Antigen		Candidates for sero-diagnosis	56
pmi	Phosphomannose isomerase	Cell wall synthesis, morphology, conidiation, energy production	Cell wall assembly and morphogenesis			84

Table 2 (*continued*)

Molecules/ genes	Gene product	Function	Pathogenesis related activities	Effect of deletion[a]	Uses	References
Mannosyl-transferases					New antifungal target?	
afpmt1	Afpmt1 (*o*-mannosyl-transferase)	Glycosylation of protein Necessary for growth $>37\,^{\circ}C$	Cell-wall assembly and morphogenesis	Normal virulence		311
afpmt2	Afpmt2 (*o*-mannosyl-transferase)	Glycosylation of protein Actin re-arrangement	Cell-wall assembly and morphogenesis	Normal virulence		83
afpmt4	Afpmt4 (*o*-mannosyl-transferase)	Glycosylation of protein				
kre2/afmnt1	Kre2/Afmnt1 (α-1,2-mannosyltransferase)	Glycosylation of protein Necessary to growth at 48 °C Cell wall integrity	Cell-wall assembly and morphogenesis	Hypovirulent	New antifungal target?	289
afmnt2–3	Afmnt2-3 (α-1,2-mannosyltransferase)	Glycosylation of protein	Cell-wall assembly and morphogenesis			
afcwh41	Afcwh41 (α-glucosidase)	Cell wall integrity	Cell wall assembly and morphogenesis	Normal virulence		309

[a] Virulence assayed in animal model.

members of KTR family of *S. cerevisiae*. One of these, *kre2/afmnt1*, was studied by Wagener et al.[289] and given the findings this has been discussed above in the thermotolerance section. The function of the other two genes still remains unknown. The importance of α-1,2-mannosyltransferases for the synthesis of O- and N-linked carbohydrates and their possible role in the generation of other glycoconjugates, as well as the fact that humans do not possess any homologous enzymes, make α-1,2-mannosyltransferases promising targets for novel antifungal therapies.[289]

Other proteins present in the cell wall and related to virulence are linked to glycosyl-phosphatidyl-inositol (GPI) motifs. The glucanosyltransferases are enzymes linked to the cell membrane and the cell wall by GPI motifs. Some of these enzymes are thought to participate in the elongation of β(1–3)-glucan side chains.[13] For example, the Gel family which is composed of seven proteins coded for by *gel1–7*. One of these enzymes, encoded by the *gel2* gene, was observed to be related to virulence in a coinfection study. Specifically, the presence of DNA from a Δ*gel2* mutant strain was lower than the DNA of the reference strain in the lungs of coinfected mice.[191] Another gene, *afpigA*, encodes the catalytic subunit of a complex that catalyzes GPI anchor biosynthesis. The GPI anchor is not essential for viability, but does seem to be required for cell wall integrity, morphogenesis, and virulence in *A. fumigatus*, and accordingly disruption of this gene caused a hypovirulent strain in a model of infection.[160] However, the deletion of the *ecm33* gene, that codes for a GPI-linked protein, enhanced virulence and resulted in a higher rate of germination, with more resistant conidia but more susceptible hypha.[52,240]

The phosphomannose isomerase enzyme, Pmi1, is essential for viability and plays a central regulatory role in both cell wall synthesis and energy production in *A. fumigatus*. The deletion of this gene led to phenotypes showing defects in cell wall integrity, abnormal morphology, and reduced conidiation,[84] but their effect on virulence was not tested.

Zhang et al.[309] identified a gene in *A. fumigatus* encoding an α-glucosidase, *afcwh41*, involved in cell wall integrity, polarity, septation and conidiation, probably by affecting the proper function of the proteins required for cell wall synthesis. However, this gene was not essential for hyphal growth and virulence.

In addition, the polysaccharide matrix of the cell wall, mainly composed of α-glucans and galactomannans, can bind the hyphae of a colony to generate a biofilm. Such biofilms may have an impact on virulence increasing the resistance to antifungals, and concentrating the extracellular enzymes produced during growth, which are also necessary for tissue colonization and infection.[25] They might also help fungi to resist the immune response, although more studies are needed.

Genes and molecules associated with resistance to immune response

As mentioned before, the small size of resting *Aspergillus* conidia means that some of the inhaled conidia are able to reach the respiratory zone of the lungs, beyond the ciliated epithelium. Various genes and molecules on surface structures of *A. fumigatus* form a set called PAMPs that interact with and activate the immune system. Host defense relies on soluble and cellular pattern recognition receptors; activation of the effector mechanisms of innate immunity, including the antimicrobial mechanisms of resident leukocytes in the lung, such as alveolar macrophages and dendritic cells; recruitment of other leukocytes; and activation of recruited leukocytes after their arrival at the site of infection. Several reviews focusing on immune response to *A. fumigatus* infections have been published in the recent years.[17,55,69,112,185] With these defenses weakened, conidia are able to germinate and form hypha within 12–15 h of arrival.[217]

In addition to the weakening of host immune response, *A. fumigatus* has a combination of characteristics that helps the fungus to evade or resist to immune response (Table 3). Pigmentation on *A. fumigatus* conidial surface has been shown to affect virulence by limiting C3 complement deposition and neutrophil activation.[275] Further, *A. fumigatus* has demonstrated an ability to bind Factor H, FHL-1, and C4BP on their surface to down-regulate the complement cascade,[27,180,288] and to produce a soluble complement-inhibitory factor, which may be lipid derived, that prevented the activation of the alternative pathways.[293,294] Moreover the thick fungal cell wall is largely resistant to direct lysis by the terminal membrane attack complex of the complement system.[132]

Various different types of behaviour have been detected on activation of immune cells through Toll-like receptors (TLR) by conidia and hyphae of this fungus. *A. fumigatus* conidia induce signal transduction after their recognition by TLR2 and TLR4; during tissue invasion, the conidia germinate into hyphae with loss of TLR4 stimulation, leading to a less pronounced stimulation of proinflammatory cytokines.[53,199] TLR4-mediated proinflammatory effects have been demonstrated to be important in the protection against

Table 3
Genes and molecules associated with resistance to host immune response

Molecules/genes	Gene product	Function	Pathogenesis related activities	Effect of deletion[a]	Uses	References
rodA/hyp1 and *rodB*	RodA/Hyp1 and RodB (Rodlets)	Dispersion and fixation to soil surfaces	Oxidative stress (ROS) protection Adhesion	Normal virulence		215,252,274
DHN-melanin[b]		Conidium protection Cell wall assembly Expression adhesins	ROS protection Reduction complement and neutrophil activation Adhesion			
Gen cluster	DHN-melanin synthesis					278
pksP/ alb1	PksP/Alb1 (Polyketide synthetase)	DHN-melanin biosynthesis cAMP signal transduction	ROS, phagocytosis and complement binding protection Immunosuppression	Hypovirulent		42,120,150
arp1	Arp1 (Scytalone dehydratase)	DHN-melanin biosynthesis	Reduction complement binding	Normal virulence		277,278
arp2	Arp2 (Hydroxynaphthalenes reductase)	DHN-melanin biosynthesis	Reduction complement binding	Normal virulence		277
abr1	Abr1 (Putative iron multicopper oxidase)	DHN-melanin biosynthesis		Normal virulence		277,278
abr2	Abr2 (Putative laccase)	DHN-melanin biosynthesis		Normal virulence		277
ayg1	Ayg1 (2-acetoacetil 1,3,6,8-tetra-hydroxynapthalene hydrolase)	DHN-melanin biosynthesis		Normal virulence		93,276
Catalases						
catA	CatA (Conidial-specific catalase)	H_2O_2 degradation	ROS protection	Normal virulence		216
cat1/catB	Cat1/CatB (Mycelial catalase)	H_2O_2 degradation	ROS protection	Δ*cat1*/Δ*cat2* hypovirulent		48,216
cat2/katG	Cat2/KatG (Mycelial catalase)	H_2O_2 degradation	ROS protection	Δ*cat1*/Δ*cat2* hypovirulent		
catC		H_2O_2 degradation	ROS protection?			208
catE		H_2O_2 degradation	ROS protection?			208
Superoxide dismutases (Sod)						
sod1	Sod1 (Cytoplasmic Cu,Zn-Sod)	O_2^- degradation	ROS protection	Δ*sod1*/Δ*sod2*/Δ*sod3* Normal virulence		113,147
sod2	Sod2 (mitochondrial Mn-Sod)	O_2^- degradation	ROS protection	Δ*sod1*/Δ*sod2*/Δ*sod3* Normal virulence		147
sod3/asp f 6	Sod3/Asp f 6 (cytoplasmic Mn-Sod, Cross-reactive pan-allergen)	O_2^- degradation	ROS protection Type I hypersensitivity Autoimmunity Antigen	Δ*sod1*/Δ*sod2*/Δ*sod3* Normal virulence	Diagnosis Confirm ABPA[c]	63,64,147
sod4	Sod4 (Mn-Sod)	O_2^- degradation	ROS protection			147
afyap1	Afyap1 (transcription factor)	Mediation ROS response	ROS protection	Normal virulence		158,225
skn7	Skn7 (transcription factor)	Mediation ROS response	ROS protection	Normal virulence		145
pes1	Pes1 (nonribosomal peptide synthase)	Peptide synthesis Stress resistance	ROS protection	Hypovirulent		227
Fatty acid oxygenases						
ppoA	PpoA (fatty acid oxygenase)	Prostaglandin synthesis related	Stress oxidative resistance	Δ*ppoA*/Δ*ppoB*/Δ*ppoC* hypervirulent		279
ppoB	PpoB (fatty acid oxygenase)	Prostaglandin synthesis related	Stress oxidative resistance	Δ*ppoA*/Δ*ppoB*/Δ*ppoC* hypervirulent		279
ppoC	PpoC (fatty acid oxygenase)	Prostaglandin synthesis related	Stress oxidative resistance	Δ*ppoA*/Δ*ppoB*/Δ*ppoC* hypervirulent		279
Glutathione transferases						
gstA-E	GstA–E		Stress oxidative resistance			47
Efflux transporters						
mdr1, mdr 2, and mdr-4	Mdr1, Mdr 2, and Mdr4 (ABC transporters)	Toxic molecule expulsion Antifungal resistance	Toxic molecules expulsion			196
mdr3	Mdr3 (major facilitator superfamily)	Toxic molecule expulsion Antifungal resistance	Toxic molecules expulsion			196
atrF	AtrF (ABC transporter)	Toxic molecule expulsion Antifungal resistance	Toxic molecules expulsion			256

Table 3 (*continued*)

Molecules/genes	Gene product	Function	Pathogenesis related activities	Effect of deletion[a]	Uses	References
abcA-E	AbcA-E (ABC transporters)	Toxic molecule expulsion Antifungal resistance	Toxic molecules expulsion			68
msfA-E	MsfA-E (major facilitator superfamily)	Toxic molecule expulsion Antifungal resistance	Toxic molecules expulsion			68

[a] Virulence assayed in animal model.
[b] DHN-melanin: melanin-1,8 dihydroxynaphthalene.
[c] Allergic bronchopulmonary aspergillosis.

IA.[28] Hence the tissue-invasive hypha of *A. fumigatus* is able to tilt the balance towards a non-protective Th2 response by a predominant TLR2 activation.[53] On the other hand, it has been demonstrated that *A. fumigatus* conidia can bind and become internalized by human epithelial cell lines,[214] which also may limit the induced levels of protective proinflammatory cytokines. These endocytosed conidia remained viable for relatively longer periods of time compared to conidia within macrophages,[296] and may eventually germinate and disseminate.[295]

Genes and molecules involved in resistance to immune response could be considered defensive virulence factors as proposed by Osherov.[208] It is well known that *A. fumigatus* has certain hydrophobic proteins on the surface of its conidial and aerial hyphae which help conidial dispersion, fixation to soil surfaces,[154,164] and conidial adherence to the respiratory epithelium,[274] and are related to the protection against the oxidative stress produced by alveolar macrophages.[215] These proteins are clustered in microfibrils called rodlets. *A. fumigatus* has at least six genes that code for hydrophobins, but only *rodA/hyp1* and *rodB* have been studied for virulence implication. The *rodA* gene encodes a small hydrophobic cysteine-rich polypeptide and the mutant strains for this gene showed high sensitivity to destruction by alveolar macrophages but were as virulent as the wild strain.[215,274] However, the Δ*rodA* strain produced smaller lung lesions and weaker inflammatory response than the reference strain.[252] On the other hand, the Δ*rodB* mutant did not show high sensitivity to killing by alveolar macrophages and did not lose their virulence.[215]

Another surface component of the fungi that has been associated with virulence is melanin, a pigment that protects the integrity of the genome in conidia from ultraviolet light, enzymatic lysis, and oxidation. The conidia of *A. fumigatus* possess a greyish-green melanin layer, absent in hyphae,[305] which contributes to their survival and longevity in the environment.[297] Some reviews have focused on the synthesis of melanin in pathogenic fungus and its importance.[15,151,221] This pigment appears adhered to the cell wall of the *A. fumigatus* conidia, coming into direct contact with the host immune system.[119,150] The presence of melanin on the surface of the conidium appears to protect the fungus in three ways. Firstly, as described above, the pigmentation on *A. fumigatus* conidial surface has been shown to affect virulence by limiting the activation of the complement cascade and neutrophils, and through interference with intracellular trafficking of phagocytised conidia.[29,275] Secondly, the wild pigmented strains have a 10- to 20-fold greater resistance against reactive oxygen species (ROS) than the white mutant strains, presumably due to their capacity to quench and detoxify these ROS.[154] Finally, the melanin could be masking β-glucan. 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*.[118,150,275] Melanin synthesis seems to be produced in the synthesis route of melanin-1,8 dihydroxynaphthalene (DHN-melanin) and is regulated by a cluster of six genes, *pksP/alb1*, *ayg1*, *arp1*, *arp2*, *abr1*, and *abr2*.[41,93,150,275,276,278] Of all these, 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 of this pathway. The deletion in other genes of this pathway produces conidia with different coloration, and in some cases with less deposition of complement (*arp1* and *arp2*), but they do not have any obvious effects on the virulence.[277,278] However, the Δ*pksP/alb1* mutant has been shown to produce a smooth white conidium, increased C3 deposition on the surface and increased phagocytosis and killing of conidia.[42,120] Resting conidia of this mutant strain express β-glucan abundantly on their surface encouraging its recognition through dectin1 receptors. Moreover, a product of the *pksP* gene could act as an immunosuppressant due to the presence of a functioning *pksP* gene, which is associated with inhibition of phagosome–lysosome fusion following conidial phagocytosis,[120] and may have a direct role in the virulence of the fungus in a murine infection model.[150] Melanin is also a structural component of the conidial wall that is required for correct assembly of the cell wall layers and the expression at the conidial surface of adhesins and other virulence factors.[221]

A. fumigatus also has specific enzymes for detoxification of ROS produced by macrophages and neutrophils, such as five catalases (*catA*, *cat1/catB*, *catC*, *catE*, and *cat2*)[48,208,215] and four superoxide dismutases (SODs): a cytoplasmic Cu/ZnSOD (Sod1), a mitochondrial MnSOD (Sod2), a cytoplasmic MnSOD (Sod3), and Sod4 displaying a MnSOD C-terminal domain.[87,113,147] Deletion of *catA*, a conidial catalase, resulted in increased susceptibility of conidia to H_2O_2 *in vitro*, but the virulence of the mutant strain did not change in a murine model.[215]. Disruptions of either *cat1* or *cat 2* genes, encoding the hyphal catalases, did not affect sensitivity to H_2O_2 *in vitro* or the virulence of mutants in animal infection models.[48,215] However, double mutant Δ*cat1*/Δ*cat2* exhibited reduced virulence in immunosuppressed rats.[48,215] In any case, as noted above, the redundancy of these genes for detoxification of ROS makes it difficult to verify their relationship with the virulence of the fungus. Fungal SODs that detoxify superoxide anions could be putative virulence factors for this opportunistic pathogen. During growth, Sod1 and Sod2 were highly expressed in conidia whereas Sod3 was only strongly expressed in mycelium and Sod4 was weakly expressed compared to other SODs.[147] The deletion of Sod4 was lethal. The Δ*sod1* and Δ*sod2* mutants showed an inhibition of growth at high temperatures and hypersensitivity to menadione, whereas the *sod3* mutant had only slightly delayed growth at high temperatures. The triple *sod1/sod2/sod3* mutant was characterized by a delay in conidial germination, lower rates of conidial survival over time during storage, the highest sensitivity to menadione and an increased sensitivity to killing by alveolar macrophages of immunocompetent mice. In spite of these phenotypes, no

significant virulence difference was observed between the triple mutant and the parental strain in experimental murine aspergillosis models with immunocompromised animals.[147] Recently, Lessing et al.[158] investigated the enzymatic ROS detoxifying system by proteome analysis of *A. fumigatus* challenged by H_2O_2. These researchers discovered that many of the identified proteins and genes were apparently regulated by a putative *S. cerevisiae YAP1* homologous gene. This gene codes for a bZip-type transcription factor that contributes to the response against oxidative stress. Deletion of this *afyap1* homologous gene in *A. fumigatus* led to drastically increased sensitivity to H_2O_2, but this mutant strain did not show attenuated virulence in a murine model of *Aspergillus* infection.[158] These data have been corroborated in another study by Qiao et al.[225] Other researchers have suggested that catalase activity in the Δ*afyap1* strain could be sufficient or more than sufficient to provide protection during incubation with neutrophils or *in vivo*, than after exposure to H_2O_2 *in vitro*.[69] These authors also argued that this similarity in virulence could be due to the use of a severe immunosuppression model, which made it difficult to detect small variations in virulence between mutants and their reference strains. Another transcription factor that contributes to the response against oxidative stress in yeast is *SKN7*. The homolog of this gene in *A. fumigatus* showed a similar role to the *YAP1* gene we have just discussed. The Δ*skn7* strain of *A. fumigatus* had an increased sensitivity to peroxides *in vitro* but this was not correlated with a modification of fungal virulence.[145] These results suggest that reactive oxygen intermediates have a relatively low importance in the destruction of the hyphae and conidia of *A. fumigatus*. Other mechanisms, such as the production of nitric oxide by macrophages or lactoferrin by neutrophils, a molecule with an ability to sequester iron, could be more relevant in the immune response against this fungus.[104,197,307] Therefore, the role of other genes and molecules of the fungus in combating stress should be studied.

Three glutathione transferases (GST) genes, termed *gstA–C* in *A. fumigatus*, have also been described.[47] The results from studying these genes suggested a role for these enzymes in the response of the organism to both oxidative stress and presence of xenobiotic compounds,[47] but they have not been tested for virulence. It has also been suggested that the nonribosomal peptide synthetase gene, *pes1*, contribute to the resistance of *A. fumigatus* to oxidative stress. Disruption of this gene led to decreased fungal virulence in a moth model system, as well as an increased susceptibility to oxidative stress and neutrophil-mediated killing, in addition to altered conidial morphology and hydrophobicity.[227]

Three fatty acid oxygenases encoding genes (*ppoA*, *ppoB*, and *ppoC*) have also been tested for their role in the virulence of *A. fumigatus*. The triple mutant strain was found to be hypervirulent in an invasive murine model and showed increased tolerance to H_2O_2 stress relative to that of the wild type.[279] These authors suggested that part of the increased virulence of the triple mutant strain might be due to the Ppo-generated prostaglandins, which could enhance host defense mechanisms, perhaps through initiation of inflammation responses involved in recruiting phagocytic cells.

Four genes that encode ATP-binding cassette (ABC)-type transporters (*mdr1*, *mdr2*, *atrF*, and *mdr4*), and one gene that codes for a protein of the major facilitator superfamily (MFS)(*mdr3*) related to azole resistance[149,196,256] have been described in *A. fumigatus*. Other genes (*abcA–E* and *mfsA–E*) that encode for these types of transporter could be related to voriconazole resistance.[68] These two classes of transporters or efflux pumps are associated with the membrane and could detoxify immune system components in a similar way to their involvement in resistance to antifungals.[208] Today, thanks to genome sequencing of *A. fumigatus*, at least 327 genes that encode putative multidrug resistance efflux pumps have been reported, including 49 ABC-type genes, and 278 genes that encode MFS proteins.[86,201]

However, despite the varied capabilities possessed by the fungal pathogen to evade host detection, it should be emphasized that the normal host defense is generally effective against most fungal infections and the host has first to be in an immune suppressed state before it becomes susceptible to opportunistic pathogens.[53]

Toxins

Mycotoxins can be described as a chemically diverse group of low molecular weight organic substances produced by fungi. These substances are formed in the hyphae during growth, and may be actively expelled into the environment, or released after the death of the hyphae. The presence of preformed mycotoxins in conidia means that the toxins must be incorporated during conidiogenesis. However, these substances might be also produced during germination. Toxins are apparently produced by the fungus to protect itself from predators and competitors in its ecological niche,[208] but they could also contribute to *A. fumigatus* pathogenesis, since they can directly attack the host (Table 4). Many of these toxins are secondary metabolites of these fungi. Depending on the mycotoxin, they can affect the synthesis of proteins, DNA and RNA, or alter the cell membrane, the consequences of which may be death or impairment of cellular functions.

A diffusible, heat-stable substance, with a mass of less than 14 kDa, can be rapidly extracted from the surface of the conidium. This diffusible substance has been shown to affect competent macrophages, inhibiting the respiratory burst, phagocytosis and the release of cytokines by macrophages,[30,181] and its effect is reversible. This component has still not been identified, but may allow the fungus to remain in the lungs and express its pathogenic effects. In particular, it has been associated with the pathogenicity level of *A. fumigatus* strains, but not all strains produce it.[30]

Ergot alkaloids are a complex family of indole-derived mycotoxins that affect the nervous and reproductive systems of exposed individuals through interactions with monoamine receptors.[59] The ergot alkaloids festuclavine and fumigaclavines A–C are present in or on conidia of *A. fumigatus*.[59] An ergot alkaloid gene cluster in *A. fumigatus* genome has been described,[60] of which the *dmaW* gene has been studied. This gene encodes a dimethylallyl tryptophan synthase that appears to control a determinant step in ergot alkaloid biosynthesis, as when *dmaW* was knocked out all known ergot alkaloids were eliminated from *A. fumigatus*.[60] Another recently studied gene, *easA* encodes an enzyme which catalyzes the reduction of the chanoclavine-I aldehyde alkene to dihydrochanoclavine aldehyde, and facilitates an intramolecular reaction to generate the immediate precursor to festuclavine.[58] Some other genes, like the 4-dimethylallyltryptophan N-methyltransferase encoded gene, *fgaMT*[237] and a dehydrogenase gene, *fgaDH/fgaOx2*, that catalyzed the oxidation of chanoclavine-I to chanoclavine-I aldehyde,[291] have also been reported. However, none of these genes have yet been tested for virulence.

Gliotoxin is the major and the most potent toxin produced by *A. fumigatus*.[143] It belongs to the family of epipolythiodioxopiperazines, which are characterized by a disulfide bridge across a piperazine ring which is essential for their toxicity.[97] Gliotoxin has several immunosuppressive roles including inhibition of macrophage phagocytosis, mitogen-activated T cell proliferation,

Table 4
Toxins related to the direct attack to the host organism

Molecules/ genes	Gene product	Function	Pathogenesis related activities	Effect of deletion[a]	Uses	References
Conidium toxin diffusible substance			Reversible macrophage inhibition			30,181
Gliotoxin			Inhibition of macrophages, neutrophils, and T lymphocytes Ciliostasis Epithelial cells damage Apoptosis			10,81,206,213,262
Putative cluster gliotoxin synthesis (12 genes)						
gliP	GliP (nonribosomal peptide synthetase)	Gliotoxin biosynthesis		Hypovirulent in cortisone treated non-neutropenic mice Normal virulence in neutropenic mice		61,137,261,269
gliZ	GliZ (transcriptional regulator)	Gliotoxin biosynthesis regulation		Normal virulence		37
asp f 1/mitF/ res	Asp f 1/MitF/Res (mitogillin, restrictocin)	Ribotoxin	Protein biosynthesis inhibition Cytotoxin Apoptosis Type I hypersensitivity	Normal virulence	Diagnosis	12,224,257
aspHS	AspHS (hemolysin)	Hemolytic activity	Hemolytic and cytotoxic activity (erythrocytes, macrophages and endothelial cells)			135
Festuclavine		Ergot alkaloid	Nervous and reproductive systems disfunction			59
Fumigaclavine A–C		Ergot alkaloid	Nervous and reproductive systems disfunction			59
Cluster ergot alkaloid genes						60
dmaW/ fgaPT2	DmaW/FgaPT2 (Dimethil-allyl-tryptophan synthase)	Ergot alkaloid biosynthesis				60
fgaDH / fgaOx2	FgaDH /FgaOx2 (Dehydrogenase)	Ergot alkaloid biosynthesis				291
fgaMT	FgaMT (4-dimethyl-allyl-tryptophan N-methyltransferase)	Ergot alkaloid biosynthesis				237
aesA	AesA (Chanoclavine-I aldehyde alkene reductase)	Ergot alkaloid biosynthesis				58
Fumitremorgin A–C		Neurotropic toxins	Nervous system disfunctions			
Cluster fumitremorgin synthesis (nine genes)						171
ftmA-I	FtmA-I	Fumitremorgin biosynthesis				106,107,125,171
Verruculogen			Production on infections?			
ftmOx1	FtmOx1 (α-ketoglutarate-dependent dioxygenase)	Formation of verruculogen				264
Fumagillin		Antitumor antibiotic (inhibition angiogenesis)	Ciliostasis Inhibition of endothelial cell proliferation			45
Helvolic acid		Fusidanes (steroidal antibiotics)	Inhibition macrophage (respiratory burst) Ciliostasis and respiratory epithelium damage			181
Cluster helvolic acid synthesis (nine genes)						166,182
AfuOSC3	AfuOSC3 (oxidosqualene cyclase)	Helvolic acid biosynthesis				166
Aflatoxin B1 and G1			Production on infections?			219
laeA	LaeA (nuclear protein, Arg and His methyl-transferases homology)	Regulation of secondary metabolism and 10% genome expression		Hypovirulent		36,38,220

[a] Virulence assayed in animal model.

mast cell activation, cytotoxic T-cell response, and monocyte apoptosis.[81,194,262,301] It also inhibits the NADPH of neutrophils,[280] suppresses ROS production and impairs neutrophil phagocytic capacity,[206] reduces the ciliary movement of epithelial cells and leads to epithelial cells damage.[10] It has also been reported that gliotoxin induces ROS-facilitated apoptotic cell death by activating the *Bak* gene of mice, a member of proapoptotic Bcl-2 family.[213] It has been proven that this toxin is produced in experimental animal aspergillosis[159,234] as well as in human IA, with serum concentrations of 166–785 ng/ml in 80% of patients with IA.[159] Although some studies have reported that a low proportion of strains produce this toxin,[77,92] a recent study reported that gliotoxin is produced by more than 95% of *A. fumigatus* isolates from both clinical and environmental origins,

while it is less often produced by other *Aspergillus* species.[138] A putative cluster of 12 genes involved in gliotoxin biosynthesis was discovered.[96] The *gliZ* gene controls expression of the remaining 11 genes in this cluster,[37] while *gliP* encodes a multimodular nonribosomal peptide synthase that catalyzes the condensation of serine and phenylalanine, the first step of the pathway.[16] In neutropenic models of IA, the mutant strains for these two genes were as virulent as the reference strain.[37,61,137] Nevertheless, in non-neutropenic mice treated with cortisone, the virulence of *gliP* mutant strains was lower than the reference strains.[261,269] These results suggest that gliotoxin induces neutrophil apoptosis[261] and a direct role of gliotoxin in aspergillosis virulence in non-neutropenic immunocompromised individuals.

A. fumigatus is able to produce ribotoxins, proteins that have a highly specific activity against the sarcin/ricin domain universally preserved in 28S ribosomal RNA, inhibiting protein biosynthesis.[123,124] One of these proteins is restrictocin, also known as mitogillin, encoded by the *asp f 1/mitF/res* gene. This toxin is related to the allergic process, since it is one of the immunodominant antigens of allergic aspergillosis.[12] Mitogillin is secreted *in vivo* by *A. fumigatus*[148] and has strong toxic effects that can cause cell death at low concentrations.[224] Ok et al [205] showed *in vitro* that Asp f 1 is also able to induce cytokine release and apoptosis in human immature dendritic cells. This immunomodulator effect could be helping the immune evasion of *A. fumigatus*. However, the deletion of *asp f 1/mitF/res* did not affect fungal virulence in a neutropenic model of IA.[257] The fungus also produces a hemolysin encoded by the *aspHS* gene. This molecule has hemolytic activity on rabbit and sheep erythrocytes, cytotoxic effects on macrophages and endothelial cells *in vitro*,[135] and can be detected during infection *in vivo*[304].

It is worth mentioning that in a recent study the levels of expression of certain of the genes discussed above (*gliP*, *aspHS*, *asp f 1*, and *dmaW*) were determined by real-time RT-PCR analysis, and higher expression was observed *in vivo* than *in vitro*.[102] These results suggest an overexpression of these toxins during infection.

Other toxins produced by *A. fumigatus* are helvolic acid and fumagillin. Helvolic acid is part of a small family of steroidal antibiotics known as fusidanes. At high concentrations it can affect the oxidative burst of macrophages,[181] the metabolism of low density lipoproteins[254] and *in vivo* it induces ciliostasis and rupture of epithelial cells.[10] On the other hand, fumagillin is an antitumor antibiotic that inhibits angiogenesis and *in vitro* directly inhibits endothelial cell proliferation and cilial movement in respiratory epithelium.[45] The active concentrations of these toxins are considerably higher than those of gliotoxin, but it is still unknown in what concentrations are produced *in vivo*[232]. It has also been reported that fumitremorgin A[302] fumitremorgin B[165], and fumitremorgin C[77], neurotropic toxins that cause tremors, seizures, and abnormal behavior in mice, are produced in a dose-dependent manner. Another toxin described to be produced by *A. fumigatus* that causes tremors is tryptoquivaline A.[303] Further toxins, such as aflatoxin B1 and G1, and verruculogen, have been detected in culture filtrates of *A. fumigatus*, but their presence during infection has not yet been demonstrated.[219] Other genes involved in the biosynthesis of these toxins have also been identified, such as *ftmOx1*, that encodes a non-heme Fe(II) α-ketoglutarate-dependent dioxygenase, which catalyses the endoperoxide formation of verruculogen in *A. fumigatus*.[264] Fumitremorgin biosynthesis seems to be encoded by a cluster of nine genes, *ftmA–I*,[171] and most of which have been described recently.[106,107,125,171] A cluster of nine genes involved in helvolic acid biosynthesis has also been described.[166,182] However, none of these genes have yet been tested for virulence.

The transcription factor *leaA* is a global regulator of secondary metabolite biosynthesis[38] that modulates the expression of approximately a 10% of the genome of this fungus.[220] The deletion of this gene in *A. fumigatus* blocked the production of almost all secondary metabolites, including gliotoxin,[268] and a *leaA* mutant strain was hypovirulent after intranasal inoculation of neutropenic mice.[36] These authors also showed that Δ*laeA* mutants lost pigment production and their conidia were more susceptible than wild type *A. fumigatus* conidia to phagocytosis by macrophages.

Nutrient uptake in invasive growth

Mammalian organisms present a broad variety of microenvironments in which *A. fumigatus* must survive to cause disease, and these environmental conditions can rapidly change depending on the current stage of infection.[298] Normal nutrient uptake systems, used in their ecological niche, might serve the fungus during infection, but it is possible that other systems could be activated by environmental conditions. Table 5 shows the major molecules and genes related to virulence covered in this section.

A. fumigatus can obtain important nutrients from destruction of host tissue. *A. fumigatus* secretes extracellular enzymes, most of them proteases, that degrade and recycle organic matter in the environment, but during infection they could serve to break down the structural barriers of the host and to obtain nutrients. As indicated above, one of the host antimicrobial mechanisms is nutrient deprivation, and the amount of secreted hydrolases encoded on the genome[201,239] may allow *A. fumigatus* to obtain nutrients from mammalian tissues without the need to activate the autophagic network.[209] Several articles have reviewed these proteases and their relationship with pathogenicity.[112,133,208,232] Some of these proteases can degrade collagen and elastin, which are the main components of the lung matrix. Various researchers have demonstrated a clear link between elastase activity of *A. fumigatus* strains and their invasiveness,[35,130] so the fungus seems to be able to adapt to the host environment increasing elastase activity.[95] However, other authors found no statistical correlation between the existence of elastase or acid proteinase activity and the development of invasive disease.[5] These enzymes include serine alkaline protease (Alp) from the family of subtilisins, which can degrade elastin, collagen, fibrinogen, and casein,[130,228] and corresponds to the allergen Asp f 13; Alp2, a serine protease that is associated with the cell wall;[229] and a vacuolar serine protease, the allergen Asp f 18.[251] The extracellular metalloprotease Mep can degrade collagen and elastin,[172,255] and is also known as Asp f 5 allergen. Other metalloproteases have been identified in *A. fumigatus* such as that encoded by the *mep20* gene[226] or the intracellular metalloproteinase encoded by the *mepB* gene, which appears to be associated with the cytoplasmic degradation of small peptides.[115] Another group of extracellular enzymes produced by *A. fumigatus* are aspartic proteases, also called aspergillopepsins. Two aspergillopepsins have been identified, a secreted aspergillopepsin (Pep)[157] which matches the known Asp f 10 allergen, and another one associated to the cell wall (Pep2).[230,231] A novel aspartic protease, CtsD, has been described in culture supernants.[287] The expression of the *ctsD* gene was absent under nutrient-rich conditions, but it was detected, *in vivo*, in a *Galleria mellonella* infection model.[287] In culture supernatants of *A. fumigatus* two members of dipeptidylpeptidases family (Dpp) have also been detected, DppIV and DppV, which cut at the amino-terminal end of peptides and proteins. These enzymes can bind to collagen, and even to hormones and cytokines, and degrade them. Their role in T cell activation has also been described.[23,24]

Finally *A. fumigatus* also secretes phospholipases, which break the ester bond of phosphoglycerides and thus may destabilize the host cell membranes causing cell lysis.[232] Activity of phospholipases A–D has also been detected in culture filtrates of

Table 5
Genes and molecules related with nutrient uptake in invasive growth

Molecules/genes	Gene product	Function	Pathogenesis related activities	Effect of deletion[a]	Uses	References
Enzymes		Nutrient obtention	Tissue destruction/ invasion			
alp/asp f 13	Alp/Asp f 13, orycine (alkaline serine protease, Elastase)	Protein degradation (elastin, collagen, fibrinogen and casein)	Tissue destruction/ invasion Type I hypersensitivity	Hypovirulent		121,130,228,271
alp2	Alp2 (cell wall alkaline serine protease)	Protein degradation (elastin)	Tissue destruction/ invasion	Normal virulence		229
asp f 18	Asp F 18 (vacuolar serine protease, related with Alp2?)	Protein degradation	Tissue destruction/ invasion Type I hypersensitivity	Normal virulence		249,251
mep/asp f 5	Mep/Asp f 5 (extracellular metalloprotease glycosylated)	Protein degradation (collagen and elastin)	Tissue destruction/ invasion Type I hypersensitivity	Normal virulence		63,121,172,255
mepB	MepB (Intracellular metalloprotease)	Protein degradation		Normal virulence		115
mep20	Mep20 (metalloprotease)	Protein degradation	Tissue destruction/ invasion	Normal virulence		226
pep/asp f 10	Pep/Asp f 10, aspergillopepsine F (aspatic endopeptidase)	Protein degradation (collagen)	Tissue destruction/ invasion Type I hypersensitivity	Normal virulence	Diagnosis	63,157,231
pep2	Pep2 (aspatic protease cell wall-associated)	Protein degradation	Tissue destruction/ invasion	Normal virulence		230
ctsD	CtsD (extracellular aspartic protease)	Protein degradation (starvation condition produced)	Tissue destruction/ invasion			287
dppIV	DppIV (glycoprotein)	Dipeptidyl-peptidase activity (Protein degradation)	Protein degradation (collagen, hormones and cytokine) T lymphocyte activation			24
dppV	DppV (glycoprotein)	Dipeptidyl-peptidase activity (Protein degradation)	Protein degradation (collagen, hormones and cytokine) T lymphocyte activation		Diagnosis	23
plb1-3	Plb1, Plb2 and Plb3 (phospholipases B)	Phospolipid degradation Membrane destruction	Tissue destruction/ invasion			250
Phospholipase C		Phospolipid degradation Membrane destruction	Tissue destruction/ invasion			33
Iron acquisition		Fe uptake				
sidA	SidA, L-ornithine hydroxilase	First common step of synthesis of siderophores	Iron acquisition Essential to virulence	No virulent	New antifungal target	111,246
sidC	SidC	Synthesis of ferrocrocin and hydroxyferricrocin	Intracellular iron storage	Hypovirulent	New antifungal target	247
sidD	SidD	Synthesis of fusarinine C and triacetylfusarinine C	Extracelular Iron acquisition	Hypovirulent	New antifungal target?	247
sidF	SidF	Synthesis of fusarinine C and triacetylfusarinine C	Extracelular Iron acquisition	Hypovirulent	New antifungal target?	247
sidG	SidG	Synthesis of triacetylfusarinine C	Extracelular Iron acquisition	Hypovirulent	New antifungal target?	247
sreA	SreA (transcription factor, GATA family protein)	Iron acquisition regulation		Normal virulence		248
ftrA	FtrA (iron permease)	High affinity permease		Normal virulence		246
fetC	FetC (Putative ferroxidase)					246
mirB	MirB (Siderophore transport protein)	Siderophore transport gene				223
mirC	MirC (Siderophore transport protein)	Siderophore transport gene				223
amcA	AmcA (putative mitochondrial carrier for ornithine)	Putative mitochondrial carrier for siderophore precursor ornithine				223
Zn acquisition						
zrfA	ZrfA (putative zinc transporter)	Zn acquisition in acidic pH	Zn acquisition			8
zrfB	ZrfB (putative zinc transporter)	Zn acquisition in acidic pH	Zn acquisition			8
zrfC	ZrfC (putative zinc transporter)	Zn acquisition in neutral or alkaline pH	Zn acquisition			9

Table 5 (*continued*)

Molecules/genes	Gene product	Function	Pathogenesis related activities	Effect of deletion[a]	Uses	References
zafA	ZafA (zinc-responsive transcriptional activator)	Induction of *zrfC* and *asp f 2* expression in zinc-limiting media	Zn acquisition Essential to virulence	No virulent	New antifungal target	9,186
pacC	PacC (transcriptional regulator)	Repression of *zrfC* and *asp f 2* at acidic pH				8,9
N acquisition						
areA	AreA (transcriptional regulator)	Nitrate transport and processing regulator	N acquisition	Normal virulence		110
rhbA	RhbA (GTPase, Ras-related protein)		N acquisition	Hypovirulent		211
cpcA	CpcA (transcriptional activator)	Putative amino acid biosynthetic pathways regulator	N acquisition	Hypovirulent		134
mcsA	McsA (methylcitrate synthase)			Hypovirulent	New antifungal target	116
lysF	LysF (homoaconitase)	Lysine biosynthesis	Aminoacids biosynthesis and growth	Hypovirulent	New antifungal target?	162
hcsA	HcsA (homocitrate synthase)	First enzyme of α-aminoadipate pathway (lysine biosynthesis)	Amino-acids biosynthesis and growth	Hypovirulent in pulmonary aspergillosis Normal virulence in inoculation by intravenous via	New antifungal target?	245
pyrG	PyrG (orotidine-5'-phosphate decarboxilase)	Pyrimidine biosynthesis	DNA biosynthesis and growth	Hypovirulent		74
pabaA	PabaA (*p*-aminobenzoic acid synthase)	Folate biosynthesis	Folate biosynthesis and growth	Hypovirulent		44
tpsA and *tpsB*	TpsA and TpsB	Trehalose biosynthesis	Response to heat shock	Δ*tpsA*/Δ*tpsB* hypervirulent		3

[a] Virulence assayed in animal model.

A. fumigatus.[34] The genes *plb1*, *plb2*, and *plb3* of *A. fumigatus* encode for B phospholipases, which are characterized by their phospholipase, lysophospholipase, and lysophospholipase transacylase activity.[250] Two of them, Plb1 and Plb3, are known to be secreted.[250] The genome of *A. fumigatus* codes for at least another three putative secreted phospholipases.[208] Although these enzymes have been considered virulence factors for other species such as *C. albicans* or *C. neoformans*, in clinical isolates of *A. fumigatus* the production of B phospholipases is lower than in environmental isolates, making unlikely, if not excluding, their involvement in the virulence of the fungus. This could be explained by the secretion of other phospholipases by *A. fumigatus*, such as phospholipase C which has not been detected in other species and is produced in a higher proportion in clinical than environmental isolates.[33] It should however be noted that while high phospholipase production was found to be associated with development of invasive aspergillosis, not all isolates that caused invasive diseases have displayed high phospholipase activity.[5]

Different proteases may play unique or overlapping roles during pathogenesis, and is difficult to obtain evidence of them as individual virulence factors.[298] Only one mutant strain of *A. fumigatus* in a 33-kDa protein, coded for by the *alp/asp f 13* gene and which has elastase activity, has produces lower rates of mortality when neutropenic mice were infected by intranasal inoculation.[130] However none of these extracellular enzymes, metalloproteases,[115,121] alkaline proteases[121,184,271] or aspartic proteases[231] have demonstrated a direct role in virulence, probably due to their redundancy. It is worth noting that there are at least 99 putative secreted proteases for the *A. fumigatus* genome.[168,201]

Recently, the biosynthesis of trehalose has been linked to virulence in pathogenic fungi. Trehalose is a non-reducing disaccharide the expression of which increases during the life cycle of *A. fumigatus*. Its concentration also increases after heat shock but not in response to other types of stress and in this fungus it is related with reduction in pathogenicity.[3] In *A. fumigatus* the proteins involved in threhalose biosynthesis are encoded by two genes, *tpsA* and *tpsB*. The deletion of both genes showed conidia with delayed germination at 37 °C and susceptibility to oxidative stress. The double mutation was required to block the trehalose accumulation, and this double mutant was hypervirulent in murine model of IA and was also associated with alterations in the cell wall and resistance to macrophage phagocytosis.[3]

The uptake of certain components is essential for most organisms and the ability to acquire these components in limiting environments, such as in the human host, is a necessary requirement for virulence of human pathogens. One of these limiting components in the human host is iron. *A. fumigatus* can acquire iron in two different ways, by reductive iron assimilation and by siderophore-assisted iron uptake, both of which are induced upon iron starvation[246]. The reductive mechanism for iron assimilation consists in the reduction of ferric to ferrous iron and the subsequent uptake of ferrous iron by the FtrA/FetC complex.[246] Inactivation of the high affinity iron permease FtrA did not produce a reduction in virulence in a murine infection model, suggesting that virulence of *A. fumigatus* does not depend on reductive iron assimilation.[246] By contrast, the inactivation of the *sidA* gene, which catalyses the first step of the biosynthesis of all known siderophores, namely the hydroxylation of L-ornithine,[111] was found to be absolutely essential for virulence.[111,246] Siderophores are low-molecular weight proteins ($Mr < 1500$), that act as ferric iron-specific, high-affinity chelators.[198] *A. fumigatus* possesses at least four siderophores: fusaricine C and triacetylfusaricine C are excreted for iron

acquisition; and ferricrocin and hydroxyferricrocin are used for intracellular iron storage.[247] The study and deletion of the four genes needed for the biosynthesis of these two types of siderophores, *sidC*, *sidD*, *sidF*, and *sidG* revealed that the nonribosomal peptide synthetase, *sidC*, is involved in intracellular siderophore biosynthesis and that this type of siderophore is required for germ tube formation, asexual sporulation, resistance to oxidative stress, catalase A activity, and virulence.[247] The strains with deletion of *sidD* and *sidF* genes, which are involved in biosynthesis of extracellular siderophores, were found to have attenuated virulence in animal infections and partial sensitivity to oxidative stress.[247] The acquisition of iron is also regulated by the protein SreA, of the GATA family, but as this gene acts as a repressor under high iron conditions, its genetic inactivation results in over-accumulation of iron. Although the Δ*sreA* strain showed increased sensitivity to iron and oxidative stress, it did not demonstrate a role in virulence in a murine infection model.[248] Certain other genes, including *amcA*, a putative mitochondrial carrier for the siderophore precursor ornithine, and the siderophore transport gene *mirB*, have shown to be upregulated during iron starvation conditions,[223] but have not yet been studied for their role in virulence. As humans do not produce siderophores, most of these genes, and particulary *sidA* and *sidC*, could be good targets for new antifungal therapies.

Zinc is another essential element for fungal growth. The genome of *A. fumigatus* contains three putative zinc transporter-encoding genes (*zrfA–C*) whose expression is regulated by both pH and the environmental concentration of zinc.[8,9] Two of these transporters, coded by genes *zrfA* and *zrfB*, are transcribed at higher levels and are required for fungal growth under acidic zinc-limiting conditions, while they are not required for growth in neutral or alkaline zinc-limiting media,[286] the conditions found in lung tissues. It has recently been described that the *zrfC* gene encodes a transporter devoted to obtaining zinc from alkaline zinc-limiting media.[9] This gene is adjacent to the *asp f 2* gene, which encodes an allergen secreted by *A. fumigatus*. In alkaline and extreme zinc-limiting conditions, the transcriptional regulators ZafA and PacC induce the simultaneous transcription of *zrfC* and *asp f 2* genes. Specifically, ZafA upregulates the expression of zrfC and Asp f 2 under zinc-limiting conditions regardless of the environmental pH, whereas PacC represses the expression of these genes under acidic growth conditions.[9] The role in virulence of these transporters has not yet been studied. However, the deletion of the transcriptional regulator *zafA* gene impairs the germination and growth capacity of *A. fumigatus* in zinc-limiting media and the Δ*zafA* strain abrogated *A. fumigatus* virulence in a murine model of IA.[186] The *zafA* gene may constitute a new target for the development of chemotherapeutic agents against *Aspergillus*, especially since no *zafA* orthologues have been found in mammals.[186]

Nitrogen metabolism has also been related to *A. fumigatus* virulence. Several sources of nitrogen may be used by *A. fumigatus*, such as nitrate or amino acids released during host tissue destruction or biosynthesized in their metabolism. The proteins that are involved in nitrate transport and processing are transcriptionally regulated by the *areA* gene.[69] The study of an Δ*areA* mutant strain in a neutropenic model of IA showed similar virulence to the reference strain. However, this mutant strain presented a delayed-growth phenotype in the lung tissue.[110] The expression of another gene, *rhbA*, was induced under nitrogen starvation conditions.[210] This gene codes for a Ras-related protein and has been considered a virulence factor because Δ*rhbA* mutant strains displayed a significantly lower virulence in a murine infection model.[211] Amino acids can be another source of nitrogen for microorganisms but not all amino acids are readily available in mammalian hosts during infections.[298] The *cpcA* gene of the Cross-Pathway Control (CPC) system (also known as General Control of amino acid biosynthesis) is activated in amino acid-limiting conditions. It has been proposed that this system regulates the *A. fumigatus* amino acid biosynthetic pathways, and the deletion of this gene produced mutants with decreased virulence.[134] The deletion of essential functional genes, such as *lysF*, which encodes a homoaconitase of lysine biosynthesis, produces mutants with decreased virulence in murine models of IA.[162] The fungal α-aminoadipate pathway is also essential for lysine biosynthesis, and the first pathway specific enzyme, homocitrate synthase (HcsA), has recently been described.[245] The *hcsA* deletion mutant was lysine auxotrophic, but although virulence of the mutant was strongly attenuated in murine models of bronchopulmonary aspergillosis, the mutant retained full virulence when injected intravenously.[245] Therefore, inhibition of fungal lysine biosynthesis does not appear to provide a suitable target for new antifungals, at least not for disseminating invasive aspergillosis. The degradation of amino acids could be important in *A. fumigatus* pathogenesis, and during invasive growth the amino acid metabolism can produce propionyl-CoA accumulation, which is a toxic metabolite. The fungus metabolizes propionyl-CoA via the methylcitrate cycle.[169] Recently the deletion of *mcsA* gene, which codes for the first enzyme of the methylcitrate cycle, a methylcitrate synthase, has been studied. This mutant strain displayed attenuated virulence in a murine model of IA, so that this activity does provide a suitable target for new antifungals.[116]

The genome of *A. fumigatus* contains some putative genes for the uptake of other essential elements such as magnesium or phosphate, but none of them have yet been studied for their role in virulence. That is the case, for example, of four putative inorganic phosphate transporters and six secreted acid phosphatases.[208]

Like other essential genes, strains with deletion of the *pyrG* gene that encodes an orotidine-5′-phosphate decarboxylase and catalyzes the last step of pyrimidine biosynthesis, has a reduced virulence and produced a low germination rate in murine models of IA.[74] In the same way, mutant strains lacking the *pabaA* gene, that encodes for *p*-aminobenzoic acid synthase and is involved in folate biosynthesis, showed a severe reduction of virulence.[44] In the case of these two latter genes, their involvement in virulence is attributed to a supposed low concentration of pyrimidine and *p*-amino benzoic acid *in vivo*.

Signaling, metabolic regulation and response to stress conditions

The environmental conditions found by pathogenic fungi in the colonization and infection of the host are different to those found in their normal environmental niche. The signals must be detected and transmitted through mechanisms of gene regulation and metabolism, enabling the fungus to adapt to them. Several regulatory mechanisms have been studied in *A. fumigatus* including mitogen-activated protein kinase (MAPK) pathways, signal transduction pathways activated by G-proteins, Ras proteins, histidine kinases, calcium signaling, and a CPC system, among others (Table 6).

Fungi, like other eukaryotes, can regulate their cellular physiology in response to environmental changes via MAPK pathways. These environmental changes include conditions of stress (increased osmolarity, heat shock, high concentrations of heavy metals, and reactive oxygen species), nutrient limitation, disruption of cell wall integrity, and mating pheromones.[176] For a better understanding of MAPK pathways in *Aspergillus* see the review of May.[175] The MAPK pathways consist in three protein kinases that act subsequently by phosphorylation. The genome of *A. fumigatus* has four MAPK described genes, *sakA/hogA*, *mpkA*,

Table 6
Molecules and genes involved in signaling, metabolic regulation and response to stress conditions

Molecules/ genes	Gene product	Function	Pathogenesis related activities	Effect of deletion[a]	Uses	References
MAP kinase[b] pathways						
sakA/hogA	SakA/HogA (MAP kinases)	Stress regulation (osmotic, C and N starvation) Regulation of conidium germination	Response to stress			300
mpkA	MpkA (MAP kinases)	Regulation of cell wall integrity signaling Regulation of pyomelanin formation	Response to stress	Normal virulence		283,284
mpkB	MpkB (MAP kinases)	Mating (putative pheromone)	Response to stress			175
mpkC	MpkC (MAP kinases)	Regulation of conidium germination	Response to stress			233
ste7	Ste7 (MAPK kinases)	Mating				175
pbs2	Pbs2 (MAPK kinases)	Osmotic regulation				175
mkk2	Mkk2 (MAPK kinases)	Cell wall integrity				175
steC/ste11	SteC/Ste11 (MAPKK kinases)	Mating				175
bck1	Bck1 (MAPKK kinases)	Cell-wall integrity				175
ssk2	Ssk2 (MAPKK kinases)	Osmotic regulation				175
sho1	Sho1(adaptor protein)	HOG–MAPK pathway[c]	Response to stress	Normal virulence		167
G-proteins						
gpaA	GpaA (G protein α subunit)	Regulation vegetative growth and conidium germination				170
sfaD	SfaD (G protein β subunit)	Regulation vegetative growth and conidium germination Regulation metabolite production (gliotoxin, etc.)				253
gpgA	GpgA (G protein γ subunit)	Regulation vegetative growth and conidium germination Regulation metabolite production (gliotoxin, etc.)				253
cAMP-PKA signaling						
acyA	AcyA (adenylate cyclase)	cAMP signal transduction				161
gpaB	GpaB (G protein α subunit)	cAMP signal transduction, Stimulator of adenylate cyclase		Almost avirulent		163
pkaC1	PkaC1 (cAMP-dependent PKA catalytic subunit)	cAMP signal transduction		Almost avirulent		163
pkaR	PkaR (PKA regulatory subunit)	cAMP signal transduction		Hypovirulent		310
His kinases		Osmolarity stress response Dicarboximide fungicides resistance Cell-wall assembly				222
fos1	Fos1 (histidine kinase)		Stress response	Hypovirulent		57,222
tcsB	TcsB (histidine kinase)					79
Other kinases (Cross-Pathways Control)						
cpcA/ gcn4p	CpcA/Gcn4p (Transcriptional activator)	Putative amino acid biosynthetic pathways regulator	N acquisition	Hypovirulent		134
cpcC/ gcn2pC	CpcC/Gcn2p (eIF2a kinase)	Sensor kinase, in amino acid starvation, down-regulation of general translations Derepress *cpcA* in nutritional stress conditions	N adquisition Adaptation amino acid starvation	Normal virulence		243

Table 6 (*continued*)

Molecules/ genes	Gene product	Function	Pathogenesis related activities	Effect of deletion[a]	Uses	References
Ca^{2+} signaling						
calA/cnaA	CalA/CnaA (calcineurine catalytic subunit A)	Septum formation Conidiophore development	Stress response	Hypovirulent	Adjunct therapeutic target	265,266
crzA	CrzA (zinc finger transcription factor)	Ca^{2+}–Mn^{2+}-tolerance	Stress response	Hypovirulent	New antifungal target	62,258
gprC and *gprD*	GprC and GprD (putative G protein-coupled receptors)	Stress signals via modulation of the calcineurin pathway	Adaptation stress signaling	Hypovirulent		99
Ras family						
rasA	RasA (GTPase)	Hyphal growth and asexual development Cell wall integrity				88
rasB	RasB (GTPase)	Germination and growth rates		Hypovirulent		90
rhbA	RhbA (GTPase, Ras-related protein)		N acquisition	Hypovirulent		211
ace2	Ace2 (transcription factor)	Pigment production and conidiation		Hypervirulent		82
medA	MedA	Adherence Biofilm formation	Host interactions (adherence to pulmonary epithelial cells, endothelial cells and fibronectin)	Hypovirulent	New antifungal target	103
srbA	SrbA (related with SREBP[d], homolog to Sre1)	Ergosterol biosynthesis Maintenance of cell polarity	Hypoxia adaptation Azole resistance	Hypovirulent		299

[a] Virulence assayed in animal model.
[b] Mitogen activated protein kinase (MAP kinase). MAP kinase kinase (MAPK kinase). MAPK kinase kinase (MAPKK kinase).
[c] High osmolarity glycerol (HOG) MAPK signaling pathway.
[d] Sterol regulatory element-binding proteins (SRBEP).

mpkB, and *mpkC*, three putative MAPK kinases (MAPKK) and three MAPKK kinases (MAPKKK). The three MAPKKs are Ste7 like, Pbs2 like, and Mkk2 like, suggesting their possible roles in mating, osmotic regulation, and cell wall integrity, respectively. Similarly, the MAPKKKs are SteC/Ste11, Bck1, and Ssk2, with possible relations in mating, cell-wall integrity, and osmotic regulation, respectively.[175] Of all of these genes, the *sakA* is the most intensively studied. This gene is necessary for the osmotic stress response, it negatively regulates conidial germination in response to less-preferred nitrogen sources; and is activated upon either carbon or nitrogen starvation during vegetative growth.[300] On the other hand, the *mpkA* regulates cell wall integrity signaling and pyomelanin formation,[284] and *mpkC* regulates conidial germination in response to the carbon source in the medium.[233] The *mpkA* deletion has been carried out but no influence was observed on virulence of the mutant strain in a murine infection model,[283] while the other genes have not been yet tested for virulence.

The high osmolarity–glycerol (HOG) MAPK (HOG–MAPK) signaling pathway plays an important role in regulating morphology, growth, and adaptation to stress and virulence in a number of fungal pathogens. The Sho1 adaptor protein is an important element of the two upstream branches of the HOG–MAPK pathway in *S. cerevisiae*. However, although the deletion of this gene in *A. fumigatus* produces a mutant sensitive to oxidative stress, it was still as virulent as the wild-type strain in an immunosuppressed mouse infection model.[167]

Many signal transduction pathways are activated by heterotrimeric G-proteins whose activation is frequently coupled to cell surface receptors. In fungi, G-proteins play integral roles in germination, vegetative growth, cell cycle control, mating, cell–cell fusion, morphogenesis, chemotaxis, pathogenicity, and secondary metabolism.[253] The system consists of a membrane bound G-protein coupled receptor (GPCR), heterotrimeric G-protein α, β, γ subunits, and a diverse group of effectors. The G protein α subunit, GpaA, mediates signaling for vegetative growth and negative-regulation of conidiation in *A. fumigatus*,[170] while the β subunit, SfaD, and γ subunit, GpgA, play crucial roles in proper control of vegetative growth, spore germination, asexual development and production of certain metabolites.[253] The deletion of the *sfaD* and *gpgA* genes resulted in no or very low gliotoxin detection,[253] suggesting a possible role of these proteins in gliotoxin biosynthesis.

The *gpaB* gene encodes a G protein α subunit involved in cAMP signal transduction that was found to be an upstream stimulator of adenylate cyclase, *acyA*. Deletion of these genes was studied and the mutant strains showed reduced conidiation, and also a slower growth rate in the Δ*acyA* mutant strain.[161] The same effect was observed with the deletion of *pkaC1* gene, which encodes the cAMP-dependent protein kinase A (PKA) catalytic subunit.[163] The Δ*gpaB* and Δ*pkaC1* strains were almost avirulent in an animal infection model of IA.[163] The regulatory subunit of PKA is encoded by the *pkaR* gene. A Δ*pkaR* mutant had reduced growth and germination rates, increased susceptibility to oxidative stress, and reduced virulence in an immunosuppressed mouse model of IA.[310] However, the reduced virulence of Δ*pkaC1* and Δ*pkaR* observed in mice could be a general outcome of impaired growth.[208] Recent studies have also related the cAMP–PKA signal transduction pathway with pigment formation[105] and the nuclear duplication cycle.[94] In fact, the sporulation and expression of the *pksP/alb1* gene, which codes for the first enzyme of melanin production, is controlled by the cAMP signal transduction pathway, which includes a G protein α subunit, acenylate cyclase,

and protein kinase A.[42,161] Recently, two putative G protein-coupled receptors, GprC and GprD, have been characterized.[99] Deletion of the corresponding genes resulted in drastic growth defects, including reduced hyphal extension, retarded germination and elevated levels of hyphae branching. Furthermore, compared with the wild type, the sensitivity of the mutant strains towards reactive oxygen intermediates was greater, and the mutants displayed attenuated virulence in a murine infection model. These authors concluded that the receptors are involved in integrating and processing stress signals via modulation of the calcineurin pathway.

Ras proteins are monomeric GTPases which act as molecular switches that transduce signals from the outside of the cell to signaling cascades inside the cell. In *A. fumigatus*, three of these proteins have been studied: RasA, RasB, and RhbA. The first, RasA, appears to have a crucial role in hyphal growth and asexual development, and its function is linked to cell wall integrity,[88] while deletion of the *A. fumigatus rasB* gene caused decreased germination and growth rates as well as a diminished virulence in a mice infection model.[90] The role of *rhbA* gene was discussed above in the nutrient uptake section.

In fungi, two-component histidine kinases are involved in response mechanisms to extracellular changes in osmolarity, resistance to dicarboximide fungicides, and cell-wall assembly.[222] The *A. fumigatus* genome has at least 15 putative histidine kinase genes, of which only two have been studied, *fos1* and *tcsB*.[208] The Δ*fos1* mutant strain did not exhibit any detectable defects in either hyphal growth or morphology when grown on solid or liquid media[222] but it had significantly lower virulence than the wild-type strain.[57] The Δ*tcsB* mutant was similar to the wild type strain with regard to growth and morphology[79] but its role in virulence has not been established.

Calcium signalling through the Ca^{2+}-binding protein, calmodulin, and the Ca^{2+}–calmodulin-dependent phosphatase, calcineurin, has been associated with a multitude of processes, including stress response, mating, budding, and actin-based processes[66] as well astolerance to antifungal drugs.[65,73,131,242,266,290] Notably, this pathway is highly conserved throughout the fungal kingdom.[238] Calcineurin is a heterodimeric protein formed by a catalytic subunit A, and a calcium-dependent regulatory subunit B. Steinbach et al.[265] demonstrated that the *calA*/*cnaA* gene, which codes for the calcineurin subunit A, is implicated in virulence. A Δ*cnaA* mutant strain exhibited decreased filamentation, morphological conidial defects and attenuation of pathogenicity compared to infection with the wild-type in several different animal models. In agreement with these results, Da Silva Ferreira et al.[67] showed that the *calA* gene is not essential in *A. fumigatus*, but its deletion results in severe defects in branching and conidial architecture and limited growth. A recent study has also suggested that calcineurin is involved in septum formation and conidiophore development.[122] Indeed, calcineurin may be an excellent target for adjuvant in combination with other cell wall inhibitors against *A. fumigatus*.[266] A key target of calcineurin is the zinc finger transcription factor CrzA, a homologue of the *S. cerevisiae* transcription factor Crz1.[258] The Δ*crzA* mutant of *A. fumigatus* resulted in a strain with significant defects in conidial germination, polarized hyphal growth, cell wall structure, and asexual development[62] and produced a significantly lower mortality rate in a neutropenic murine model of invasive pulmonary aspergillosis.[62,258] Fortwendel et al.[89] have obtained data suggesting that the Ras and calcineurin pathways act in parallel to regulate cell wall formation and hyphal growth, and additionally, that the calcineurin pathway elements *cnaA* and *crzA* play a major role in proper chitin and glucan incorporation into the *A. fumigatus* cell wall. Soriani et al.[258] also demonstrated a role of *crzA* in the mediation of cellular tolerance to increased concentrations of calcium and manganese. Thus, *crzA* is an attractive fungus-specific antifungal target for the treatment of IA.[62]

A conserved signal transduction cascade linking environmental stress to amino acid homeostasis is the CPC system that acts via phosphorylation of the translation initiation factor eIF2 by a sensor kinase. As noted before, the *cpcA* gene encodes the transcriptional activator of the CPC-system of amino acid biosynthesis and Δ*cpcA* strains displayed attenuated virulence in a murine model of IA.[134] On the other hand, the *cpcC* gene encodes the CPC eIF2a kinase. The Δ*cpcC* deletion mutant showed increased sensitivity towards amino acid starvation but it was not impaired in virulence in a murine model of pulmonary aspergillosis.[243]

The transcription factor Ace2 influences virulence in other fungi. *A. fumigatus* contains an ortholog of this gene, *ace2*, which governs pigment production, conidiation, and virulence[82]. Mice immunosuppressed with cortisone acetate and infected with the Δ*ace2* mutant showed accelerated mortality, greater pulmonary fungal burden, and increased pulmonary inflammatory responses than mice infected with wild type strain. This hypervirulence of the Δ*ace2* strain was related to reduced expression of *ppoC*,

Table 7
Allergens of *A. fumigatus* related with activation of Type I hypersensitivity

Genes	Gene product	Function	Pathogenesis related activities	Effect of deletion[a]	Uses	References
asp f 1/mitF/res	Restrictocin, mitogillin	Ribotoxin	Protein biosynthesis inhibition Cytotoxin Apoptosis Type I hypersensitivity	Normal virulence	Diagnosis	12,224,257
asp f 2	Asp f 2	Fibrinogen binding protein Zn metabolisms?	Adhesion Type I hypersensitivity		Diagnosis	9,18
asp f 3	Asp f 3, peroxisomal protein (PMP, redoxin)	Peroxisomal membrane Protein	Type I hypersensitivity		Diagnosis	109
asp f 4			Type I hypersensitivity		Diagnosis Confirm ABPA	64,140
mep/asp f 5	Mep/Asp f 5 (extracellular metalloprotease glycosylated)	Protein degradation (collagen and elastin)	Tissue destruction/ invasion Type I hypersensitivity			63,121,172,255
sod3/asp f 6	Sod3/Asp f 6 (cytoplasmic Mn superoxide dismutase, Cross-reactive pan-allergen)	O_2^- degradation	ROS protection Type I hypersensitivity Autoimmunity Antigen		Diagnosis Confirm ABPA[b]	63,64,147

Table 7 (*continued*)

Genes	Gene product	Function	Pathogenesis related activities	Effect of deletion[a]	Uses	References
asp f 7	Asp f 7		Type I hypersensitivity		Diagnosis	63
asp f 8	Asp f 8, 60S acidic ribosomal protein P2 (cross-reactive pan-allergen)	Protein synthesis (elongation step)	Type I hypersensitivity Autoimmunity		Diagnosis	141
asp f 9/crf1	Asp f 9/Crf1 (cell wall glucanase)	Cell wall assembly	Type I hypersensitivity		Diagnosis	63
pep/asp f 10	Pep/Asp f 10, aspergillopepsine F (aspatic endopeptidase)	Protein degradation (collagen)	Tissue destruction/ invasion Type I hypersensitivity		Diagnosis	63,157,231
asp f 11	Asp f 11 (peptidyl-prolyl cis-trans isomerase, cyclophilin, cross-reactive pan-allergen)	Peptide synthesis Chaperone and cell signaling function	Type I hypersensitivity Autoimmunity		Diagnosis	87
hsp1/asp f 12	Hsp1/Asp f 2 (heat shock protein, Hsp90 family)	Chaperone	Chaperone activity and protein transport in growth at 37 °C Stress response during inflammation Autoimmunity Type I hypersensitivity			136
alp/asp f 13	Alp/Asp f 13, orycine (alkaline serine protease, Elastase)	Protein degradation (elastin, collagen, fibrinogen and casein)	Tissue destruction/ invasion Type I hypersensitivity	Hypovirulent		121,130,228,271
asp f15	Asp f 15, (serine protease, homolog Asp f 13)		Tissue destruction/ invasion Type I hypersensitivity		Diagnosis	64
asp f 16	Asp f 16	Putative glycosylhidrolase	Type I hypersensitivity			20
mp1/asp f 17	Mp1/Asp f 17 (relation with Afmp1?)	Cell wall galactomannoprotein	Adhesion Type I hypersensitivity		Diagnosis	64
asp f 18	Asp f 18 (vacuolar serine protease, related with Alp2?)	Protein degradation	Tissue destruction/ invasion Type I hypersensitivity	Normal virulence		249,251
asp f 22	Asp f 22, Enolase	General metabolism	Type I hypersensitivity			144
asp f 23	Asp f 23, 60S ribosomal protein L3	Protein synthesis	Type I hypersensitivity			
asp f 27	Asp f 27 (peptidyl-prolyl cis-trans isomerase, cyclophilin, cross-reactive pan-allergen)	Peptide synthesis Chaperone and cell signaling function	Type I hypersensitivity Autoimmunity		Diagnosis	101
asp f 28	Asp f 28 (thioredoxin, cross-reactive pan-allergen)	Protein disulfide oxidoreductases	Type I hypersensitivity Autoimmunity		Diagnosis	
asp f 29	Asp f 29 (thioredoxin, cross-reactive pan-allergen)	Protein disulfide oxidoreductases	Type I hypersensitivity Autoimmunity		Diagnosis	
asp f 34	Asp f 34, PhiA	Cell wall protein	Type I hypersensitivity		Diagnosis	100
	Asp f 56kD	Protease	Type I hypersensitivity			202
	Asp f AfCalAp		Type I hypersensitivity			282
	Asp f GST (glutathione S-transferases)	Detoxification with glutathione	Type I hypersensitivity			

[a] Virulence assayed in animal model.
[b] Allergic bronchopulmonary aspergillosis.

ecm33, and *ags3* detected in this mutant. It is known that *A. fumigatus* mutants with null or reduced expression of these genes have increased virulence in mice.

MedA is a development regulated protein that governs adherence, host interactions, and virulence in *A. fumigatus*.[103] These authors studied a Δ*medA* strain and demonstrated a dramatic reduced conidiation, and impaired biofilm production and adherence to plastic, as well as adherence to pulmonary epithelial cells, endothelial cells, and fibronectin *in vitro*. This mutant also exhibited reduced virulence in both invertebrate and mammalian models of IA. These results suggest that MedA downstream targets mediate virulence and might provide novel therapeutic targets for IA.

The presence of *A. fumigatus* causes significant inflammation in the sites of infection. It is known that levels of oxygen are significantly lower at sites of inflammation.[298] Accordingly, during infection, *A. fumigatus* may be exposed to rapid changes in oxygen concentration, even reaching extremely low levels, depending upon the tissue infected and current immune response. The mechanisms of hypoxic adaptation of the aerobic *A. fumigatus* are currently unknown. Willger et al.[298,299] have hypothesized that a putative Sre1 homolog in *A. fumigatus* (SrbA), related to the sterol regulatory element-binding proteins (SREBPs), could also act as an indirect sensor of oxygen levels and could regulate the transcription of genes required for adaptation to hypoxic environments. These authors have

demonstrated that the *srbA* gene plays a critical role in ergosterol biosynthesis, azole resistance, and the maintenance of cell polarity in *A. fumigatus*.[299] The Δ*srbA* strain was almost avirulent in mouse models of IA, and loss of this gene, affects the expression of 87 genes related sterol biosynthesis and hyphal morphology, as demonstrated by expression analysis using DNA microarrays.[299] Hypoxia adaptation is likely an important virulence attribute of pathogenic molds.

Allergens

A. fumigatus produces a significant number of allergenic molecules which show reactions with IgE in asthmatic patients and patients with allergic bronchopulmonary aspergillosis (ABPA). Data concerning all known *A. fumigatus* allergens are collected by "Allergome, a platform for allergen knowledge"[1] and the Allergen nomenclature website,[2] and are summarized in Table 7 of this review. Only 23 molecules currently hold an official name of allergen, and have names in the range Asp f 1–Asp f 34. One of these, Asp f 15, has been proposed to be removed from the list due to it having been demonstrated that it is identical to Asp f 13, and the Asp f 6 allergen has shown a high degree of homology with Asp f 9.[64] On the other hand, there are three candidates to be considered as allergens, Asp f 56 kDa (a protease), Asp f AfCalAp, and Asp f GST (related to glutathione-S-transferase). However, the sequence of Asp f 56 kDa is not predicted to be encoded in any of the sequenced *Aspergillus* genomes.[64] Some of these allergens have known structural, toxic or enzymatic functions, and their relationship with virulence has been discussed in previous sections of this review. However, other allergenic components do not have virulence activities except as allergens. All *Aspergillus* allergens reacted with IgE in asthmatic patients and with ABPA.[129] Some *A. fumigatus* allergens showed cross-reactivity with various conserved proteins including some human proteins. Among these, Asp f 6 (Mn-Sod), Asp f 8 (P2 acidic ribosomal protein), Asp f 11 and Asp f 27 (cyclophilins), and Asp f 28 and Asp f 29 (thioredoxins) have been shown to belong to families of cross-reactive pan-allergens.[64] This fact could imply autoimmunity problems in human patients.

Allergenic behaviour of the aforementioned molecules, due to their presence on conidia, their release by the destruction of the conidia by pulmonary phagocytes, or their production during the growth of fungus is unclear in IA. We were able to identify two different situations, namely, the infections caused by Aspergillus in immunocompetent or immunocompromised patients. In immunocompetent patients *Aspergillus* can produce several hypersensitivity diseases due to these allergens, such as ABPA, allergic rhinosinusitis, asthma, and aspergilloma. Inhalation of fungal spores, often considered the traditional route of exposure, has been associated with the induction or exacerbation of these respiratory diseases. Large numbers of inhaled fungal spores are removed from the lungs prior to germination,[139] but a few conidia could escape phagocytosis and may begin to germinate. Dormant or nonviable *A. fumigatus* conidia uptake is associated with IFN-γ production and Th1 responses, while hyphae or swollen (germinating) conidia induce IL-4 production and eosinophil recruitment, a hallmark of allergic inflammation and Th2 responses.[39,273] Therefore, successful germination is likely to contribute to the development of fungal allergy. Specific structures, factors secreted by fungi or released by killed conidia, can play an important role in allergic sensitization, but the environmental and patient-specific factors (such as the personal history of previous contact in early life immune development) are also critical to acquire tolerance or allergic sensitization in immunocompetent individuals. All *Aspergillus* allergens appear to activate a Type I hypersensitivity response in sensitized patients with production of high affinity IgG and IgE antibodies.[232] In immunocompromised patients with debilitated innate immune responses, these allergenic compounds can increase the risk associated with aspergillosis because they may redirect the immune response to the fungus by the activation of Th2 lymphocytes, a response that does not seem to be efficient in eliminating this fungus.[108] Some of these allergens have been studied for their usefulness for diagnosis (see Allergome[1] and Table 7).

Gene expression assays

During the last years only a few studies have investigated *A. fumigatus* gene expression during infection. Zhang et al.[308] analysed the expression of certain virulence factors *in vivo* and *in vitro* concluding that *in vitro* measurements of transcription compared to transcription in infected lung tissue demonstrated low levels of *fos-1* and *rhbA* genes, and 20–40-fold increases in *cpcA*, *lysF*, and *pabA* genes, while the *pksP* gene was only detected *in vivo*. Gravelat et al.[102] performed real-time reverse transcription-PCR analysis on lung samples from mice with invasive pulmonary aspergillosis to determine the expression of *A. fumigatus* genes that are expressed at specific stages of development. This study revealed that in established infections, *A. fumigatus* exhibited mRNA expression of specific genes to develop competent hyphae, such as *stuA*. The acquisition of competence is referred to the shift of hyphae from a state in which they cannot undergo asexual reproduction to one in which they can. In contrast, mRNA of genes expressed specifically by conidia and precompetent hyphae was not detected. Many genes required for mycotoxin synthesis, including *aspHS*, *gliP*, *mitF*, and *metAP*, were expressed at significantly higher levels during invasive infection than *in vitro*. On the other hand, the expression of *gliP* mRNA *in vitro* was found to be highly dependent on culture conditions. Furthermore, this expression was found to be dependent on the transcription factor *StuA* both *in vitro* and *in vivo*. These results highlight the importance of the evaluation of putative virulence factors expressed by competent hyphae and the analysis of gene expression levels during invasive infection rather than *in vitro* alone.

Gene expression assays have also been developed to analyse the function of various proteins, comparing the gene expression profiles of the mutant against those of the reference strain. For example, Soriani et al.[259] searched the metabolic pathways influenced by *A. fumigatus* transcription factor AfCrzA after a short pulse of calcium, by determining the transcriptional profile of *A. fumigatus* wild type in comparison to Δ*afcrzA* mutant strains. Similarly, Twumasi-Boateng et al.[281] described, on the basis of transcriptional profile studies, the role for BrlA in the response to nitrogen depletion and for StuA in the regulation of secondary metabolite clusters in *A. fumigatus*. Using transcriptomic analysis, other authors have investigated the exit from dormancy of *A. fumigatus* conidia[146] and the genes differentially expressed in conidia and hyphae of this fungus upon exposure to human neutrophils.[267] Gene expression assays with DNA microarrays are also being used to study the adaptation of *A. fumigatus* to different stress conditions such as hypoxia,[299] heat shock,[201] and antifungals activities such as voriconazole[68]. Finally, DNA microarray-based studies have also been used for the detection and identification of fungal pathogens, including *A. fumigatus*.[49,260]

Conclusions

A. fumigatus is an opportunistic pathogen whose ability to produce disease is inextricably linked to the host immune response. The most recent progress in research has revealed how components of the immune system are able to eliminate the fungus and that the

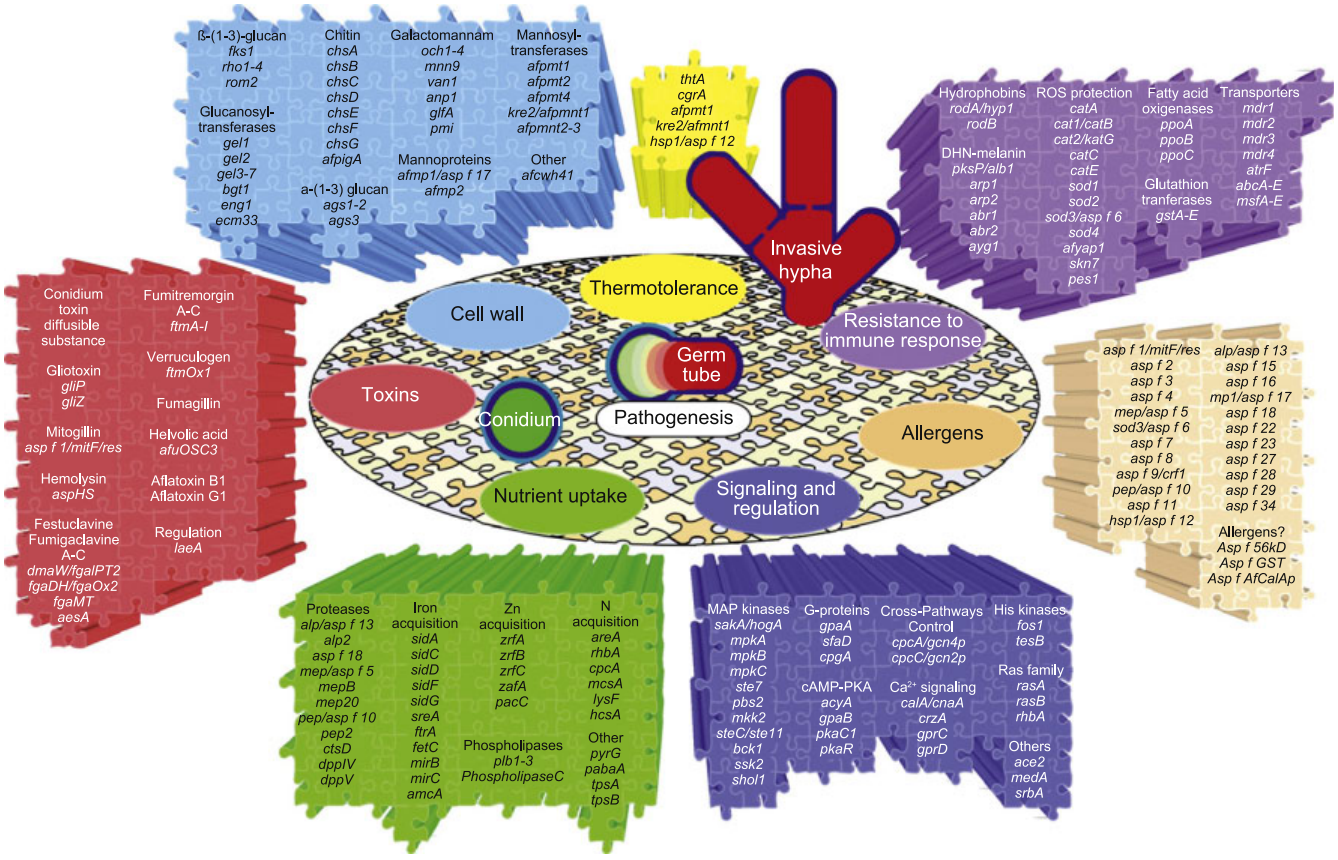

Fig. 3. Summary of genes and molecules associated with the virulence of *Aspergillus fumigatus* contained in this review.

weakness of immune system has a role in the development of aspergillosis. Likewise, some of the mechanisms that the fungus uses to evade immune responses, to obtain nutrients and to cause damage to the host and thus generate an IA, have been identified. If we consider only the classical definitions of virulence factors, i.e., a component of a pathogen that allows it to cause disease, we would probably have difficulties in deciding what is or is not a virulence factor in human fungal pathogenesis. In fact, that would exclude, for example, normal or adaptive mechanisms of the fungi to grow in different environmental niches, which are extensively used during the colonization of a human host. In *A. fumigatus* a great variability of "non-classical" virulence factors have been described, associated with its structure, its capacity to grow and adapt to stress conditions, its mechanisms for evading the immune system and its ability to cause host damage. As detailed in this review, a large number of genes and molecules have been identified and investigated in some depth as potential virulence factors. However, none of them have proven to be sufficiently important to fully explain the virulence of *A. fumigatus*. In most cases, the experiments based on the loss of gene/function by mutation have shown only small declines in virulence, unless the genes involved regulation of multiple activities of fungal adaptation and growth were eliminated. The pleiotropic effect of certain genes, the function of various genes associated with the virulence in the normal growth of *A. fumigatus*, and the redundancy due to the existance of several genes with the same activity, complicate the process of studying virulence factors of *A. fumigatus* with mutant strains. On the other hand, virulence studies use animal models with high levels of immunosuppression, which can also lead to failure to detect the effect on the virulence of the mutant strains. Likewise, the animal immunosuppression used, focusing mainly on causing neutropenia, only simulates the situation in neutropenic patients without providing any data for the other types of patients with aspergillosis. From all this data, the idea has emerged that the pathogenesis of diseases caused by this fungus in immunocompromised patients is very complex. As shown in Fig. 3, we could imagine a complex puzzle, the pieces of which would be virulence factors or the different activities of the fungus, and our task would then be to complete this puzzle to obtain a comprehensive vision of the virulence of *A. fumigatus*. We begin to understand the intricacies of its metabolism but much remains to be learned concerning the activity of this fungus *in vivo*. Furthermore, understanding changes in the host microenvironment, including hypoxia, pH, available nutrients, and immune responses, and how these signals are processed by the fungus, could be useful to determine the efficacy and effectiveness of particular antimicrobial strategies. The data so far have helped to improve diagnosis and identify new targets for antifungal development, which in combination with currently available therapies can improve the prognosis for IA patients. Expression studies using DNA microarrays of *A. fumigatus* during invasion or interaction with immune responses may help to provide a more rapid and profound understanding of the virulence capabilities of this fungus, as well as their adaptation mechanisms based on networks of complex metabolic and genetic regulation systems, in order to find new possible targets for detection and treatment of the disease. In particular, these expression studies using DNA microarrays are being applied to different stress conditions such as heat shock and antifungal activity.

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