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Aspergillus fumigatus: saprophyte or pathogen?

Fredj Tekaiia¹ and Jean-Paul Latgé²

Large-scale genome comparisons have shown that no gene sets are shared exclusively by both *Aspergillus fumigatus* and any other human pathogen sequenced to date, such as *Candida* or *Cryptococcus* species. By contrast, and in agreement with the environmental occurrence of this fungus in decaying vegetation, the enzymatic machinery required by a fungus to colonize plant substrates has been found in the *A. fumigatus* genome. In addition, the proteome of this fungus contains numerous efflux pumps, including >100 major facilitators that help the fungus to resist either natural aggressive molecules present in the environment or antifungal drugs in humans. Environment sensing, counteracting reactive oxidants, and retrieving essential nutrients from the environment are general metabolic traits that are associated with the growth of the saprotrophic mold *A. fumigatus* in an unfriendly environment such as its human host.

Addresses

¹Unité de Génétique Moléculaire des Levures, URA 2171 CNRS and UFR 927, Université Pierre et Marie Curie, Institut Pasteur, 25, rue du Dr Roux, 75015 Paris, France

²Unité des Aspergillus, Institut Pasteur, 25, rue du Dr Roux, 75015 Paris, France

Corresponding author: Latgé, Jean-Paul (jplatge@pasteur.fr)

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Introduction

Aspergillus fumigatus is responsible for invasive aspergillosis (IA), a life-threatening disease that usually only occurs in the immunocompromised patient. The incidence of IA has increased tremendously during the past 10–20 years as medical practices that are now becoming more common, such as immunosuppression in the transplant patient and aggressive anti-cancer chemotherapy, predispose patients to IA. The incidence of IA varies among the patient population and can infect 15% of allogeneic transplant patients — the population at most risk. The mortality rates associated with proven IA infections caused by *Aspergillus* species range from 60–90%, again depending upon the type of patients infected [1,2,3••].

This review aims to investigate if comparative analysis of the increasing number of sequenced fungal genomes can answer one of the most frequently asked questions in this field of research to date: are there specific *A. fumigatus* genes that are responsible for human pathogenicity?

How unique is *A. fumigatus*?

Under the comparative conditions outlined in **Box 1**, it has been shown that the amount of ancestral duplication in the proteome of *A. fumigatus* is 40.6%. There are 1111 *A. fumigatus*-specific proteins that are only present in the *A. fumigatus* proteome (making up 11.2%). 42% of *A. fumigatus* proteins are exclusively conserved in Eukaryotes and 27% are ancient proteins (i.e. conserved in Archaea, Bacteria and Eukaryotes). Very few *A. fumigatus* proteins are exclusively conserved in the archaeal (<0.2%) and bacterial (<0.5%) domains. Domain-specific conservation profiles of each of the considered species can be found at <http://www.pasteur.fr/~tekaia/domspec.html>.

The number of *A. fumigatus* proteins that are exclusively conserved in *Aspergillus nidulans* and *Aspergillus oryzae* is 178 and 186, respectively, whereas 449 other proteins are exclusively and jointly conserved in the three *Aspergillus* species. Moreover, 549 proteins are exclusively conserved among *A. fumigatus* and the filamentous fungi *Fusarium graminearum*, *Magnaporthe grisea*, *Neurospora crassa*, *A. oryzae* and *A. nidulans*. No *A. fumigatus* proteins are shared uniquely with other human pathogens, which suggests that human pathogenic ascomycetous and basidiomycetous yeasts do not share a virulence pathway with *A. fumigatus*.

As a global approach cannot identify general pathways that are only present in *A. fumigatus* and other human pathogens, can a comparative analysis of gene sets that control specific pathways, such as those summarized in **Figure 1**, provide an insight into the basis of human infectivity by *A. fumigatus*?

A. fumigatus is a grass eater

A. fumigatus is one of the most common inhabitants of the air-borne fungal flora [13]. Its ubiquity in nature suggests that this fungus has a saprophytic lifestyle in decaying organic and plant materials. Growth on plants requires an enzymatic armamentarium that is able to degrade plant cell wall polysaccharides [14]. Indeed, a survey of the *A. fumigatus* genome has shown that it encodes a wide range of glycosylhydrolases that have the capacity to degrade the major plant cell wall polymers (**Table 1**).

Box 1 Comparison of the *A. fumigatus* genome with prokaryotic and eukaryote genomes.

Analysis of *A. fumigatus* protein sequences (9925 available from the Institute of Genome Research [TIGR] on January 2005) was performed following the methodology used for large-scale proteome comparisons [4,5]. The proteome of *A. fumigatus* was compared to that of each of the 102 species that were surveyed (including 29 eukaryotic, 20 archaeal and 53 bacterial species), using the blastp program with the pam250 substitution matrix and the seg filter. *A. fumigatus* was specifically compared to two other *Aspergillus* species *A. oryzae* and *A. nidulans*, three filamentous fungi *Neurospora crassa*, *F. graminearum* and *M. grisea* (the last two of which are phytopathogens), the human pathogenic yeasts *Candida albicans*, *Candida glabrata* and *Cryptococcus neoformans*, and the yeasts models *S. cerevisiae* and *S. pombe* [6,7–11]. When each eukaryotic species was used as target, the blastp significance threshold e-value was set at 10^{-9} . The results of all bidirectional pairwise-predicted proteome comparisons allowed estimation of: the level of ancestral duplication in each species, the ancestral conservation, the number of shared orthologs between pairs of species, and the conservation profile of each protein across all considered species.

Duplicated proteins were included in partitions and clusters. Partitions are disjoint subsets, each of which includes members that are significantly similar to at least another member of the same subset and that have no similarity to members that are not included in the subset. Each non-unique protein is then assigned a partition denoted Pn.m, where n is the number of proteins in the partition and m is an arbitrary order. In parallel, the same set of non-unique proteins is clustered using the MCL algorithm (Markov Cluster algorithm by Stijn van Dongen; a cluster algorithm for graphs <http://micans.org/mcl/>). The clustering was performed using $-\log(\text{blastp e-values})$ and an inflation index $I=3.0$ [12]. Each non-unique protein is assigned a cluster of the form Cp.q, where p is the number of proteins in the cluster and q an arbitrary order. Each protein belongs to both a partition (Pn.m) and an MCL cluster (Cp.q), which we concatenate to form the final 'family' assignment Pn.m.Cp.q to the protein. The term 'singleton' is assigned to proteins that do not have significant matches. Partitioning and clustering have been performed for all non-unique proteins in each of the 102 surveyed species and the results are organized in a table format (<http://www.pasteur.fr/~tekaia/AFUM/AFUMagc>) where each line corresponds to a protein described by its partition-MCL family if non-unique or by the term singleton if unique. Also present is a list of the best homologs of *A. fumigatus* in each of the considered species, as well as their respective partition-MCL families. These comparative results have been used in this study particularly for determination of the number of proteins shared uniquely with one or some species (viz protein sets are exclusively conserved in one or in some species but are not present in the 102 other genomes analysed).

This genomic survey showed that a similar number of enzyme families are found in these, so called, saprotrophic or phytopathogenic species and in *A. fumigatus*, which suggests that the primary ecological niche of *A. fumigatus* is the plant. Interestingly, no global differences have been demonstrated between the enzymes produced by true phytopathogens (such as *M. grisea* or *F. graminearum*) and saprotrophic *Aspergilli*. Non-phytopathogenic fungi such as the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* do not possess these enzymes, which demonstrates that there is a direct correlation

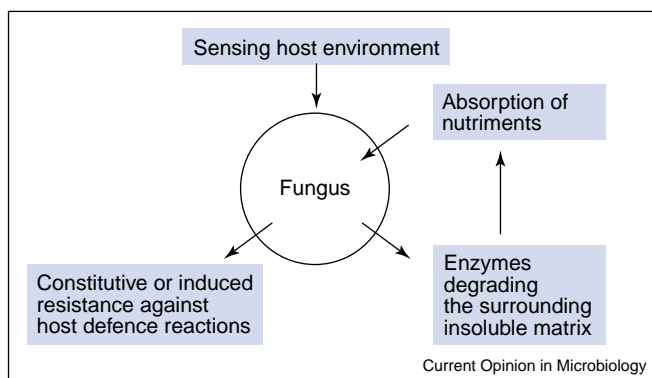
between genome characteristics and the ecological niche. However, even though *A. fumigatus* has genes that encode laccase activity, it does not have genes homologous to lignin peroxidase or to manganese peroxidase that are present in other lignin-degrading fungi such as *Phanerochaete chrysosporium* [15]. This survey suggests that *A. fumigatus* plays a major role in leaf but not in hard wood degradation, a result in agreement with its ubiquitous colonization of compost.

Sensing the environment

Two-component phosphorelay systems are a major mechanism by which some organisms sense and adapt to their environment [16,17**]. Fungal histidine kinases (HKs) are hybrids, which means that they function in multistep phosphorelays. In these phosphorelays, the phosphate is transferred from the response regulator (RR) domain of the hybrid HK to a second histidine residue in a histidine phosphotransfer domain (HPt), and then to a second RR domain. These systems have been implicated in the regulation of virulence in both plant and animal pathogens. In *A. fumigatus*, 13 HK genes have been identified with orthologs that are either limited to other *Aspergillus* and filamentous species or common to all Eukaryotic species. Despite this cross-species conservation, little is known about the signals perceived by and the functions of the orthologous HKs that can vary greatly from species to species. *SLN1* is an essential HK in *S. cerevisiae* and acts as a sensor of the osmotic environment of the cell. *SLN1* of *Candida albicans*, unlike its *S. cerevisiae* ortholog, is not essential but is involved in hyphal formation and virulence [18]. Currently, the role of the *SLN1* homolog in *A. fumigatus* has not been clearly defined. By contrast, another member of the same cluster, *FOS1*, is known to be essential for the virulence of *A. fumigatus* [19,20]. Despite the upstream presence of numerous HKs, *A. fumigatus*, like other filamentous ascomycetes examined and in contrast to plants and bacteria, contain only one HPt domain protein that is homologous to the yeast *YPD1*. This organization of HKs should allow the integration of multiple environmental inputs into a single signaling pathway. Future functional characterization should elucidate whether the expansion of the number of HKs can be translated in terms of specific sensing of the host environment by *A. fumigatus*.

Another mechanism that is involved in *A. fumigatus* virulence involves the cyclic AMP signaling pathway, which has been shown to be associated with the pathobiology of several human and plant pathogenic fungi [21]. It has been shown that the deletion of *GPAB*, a G protein α subunit, which is an upstream stimulator of adenylate cyclase, led to drastically attenuated virulence of *A. fumigatus* in a mouse infection model of IA in the absence of any growth retardation [22*]. Conidia of *A. fumigatus* *GPAB* mutant were killed more efficiently by human

Figure 1



Key biological traits and steps during infection. A pathogenic mold senses the environment and if it is favourable it will react by germinating. Germination is associated with the secretion of enzymes that degrade external polymers into nutriments that are then directly assimilated by the fungus. During growth, the fungus is able to counteract aggressive external environments.

monocyte-derived macrophages compared to conidia of the wild-type strains.

Responses to a hostile environment

Following inhalation of conidia by the immunocompetent host, the innate cellular immune system (comprised of alveolar macrophages and neutrophils) is responsible for the killing of the conidia. The anti-*fumigatus* activity of

phagocytes primarily requires oxidative mechanisms to function [23].

In human pathogenic fungi, melanins have been shown to play a protective role in pathogenicity. In *A. fumigatus*, conidial dihydroxynaphthalene-melanin has been also recognized as a virulence factor [24,25,26]. This hydrophobic pigment, which is present on the conidial surface, quenches reactive oxygen species and protects the pathogen against damage by macrophage and neutrophils [27]. In *A. fumigatus*, a cluster of seven genes has been shown to be involved in the synthesis of this pigment. Comparative genomics has shown that homologs of this cluster were found in most filamentous fungi, including plant pathogens such as *M. grisea*. This result suggested the lack of specificity for the protective role of conidial pigments during infection, but point out its necessary role to withstand ultraviolet light radiations in nature and accessorially reactive oxidants in humans. In addition to their association with pigment biosynthesis, secondary metabolite pathways might play a direct role in fungal virulence, as was shown recently for the global regulator *LAEA* [28].

Pathogenic microorganisms have also developed a network of oxidoreductases and metabolites to neutralise phagocyte reactive oxygen intermediates (ROIs). The major anti-oxidant molecules and interconnecting pathways that are thought to be active in *A. fumigatus* are shown in Figure 2 [29]. *A. fumigatus* has the entire armamentarium to combat oxidative stress, but no difference can be seen between this and that of non-pathogenic fungi.

Another way in which to withstand the toxic molecules that passively enter the fungal cell is by use of an efficient efflux system. *A. fumigatus* encode >40 ATP-binding cassette transporters. This is more than twice the number of ABC transporter homologs that are present in yeasts

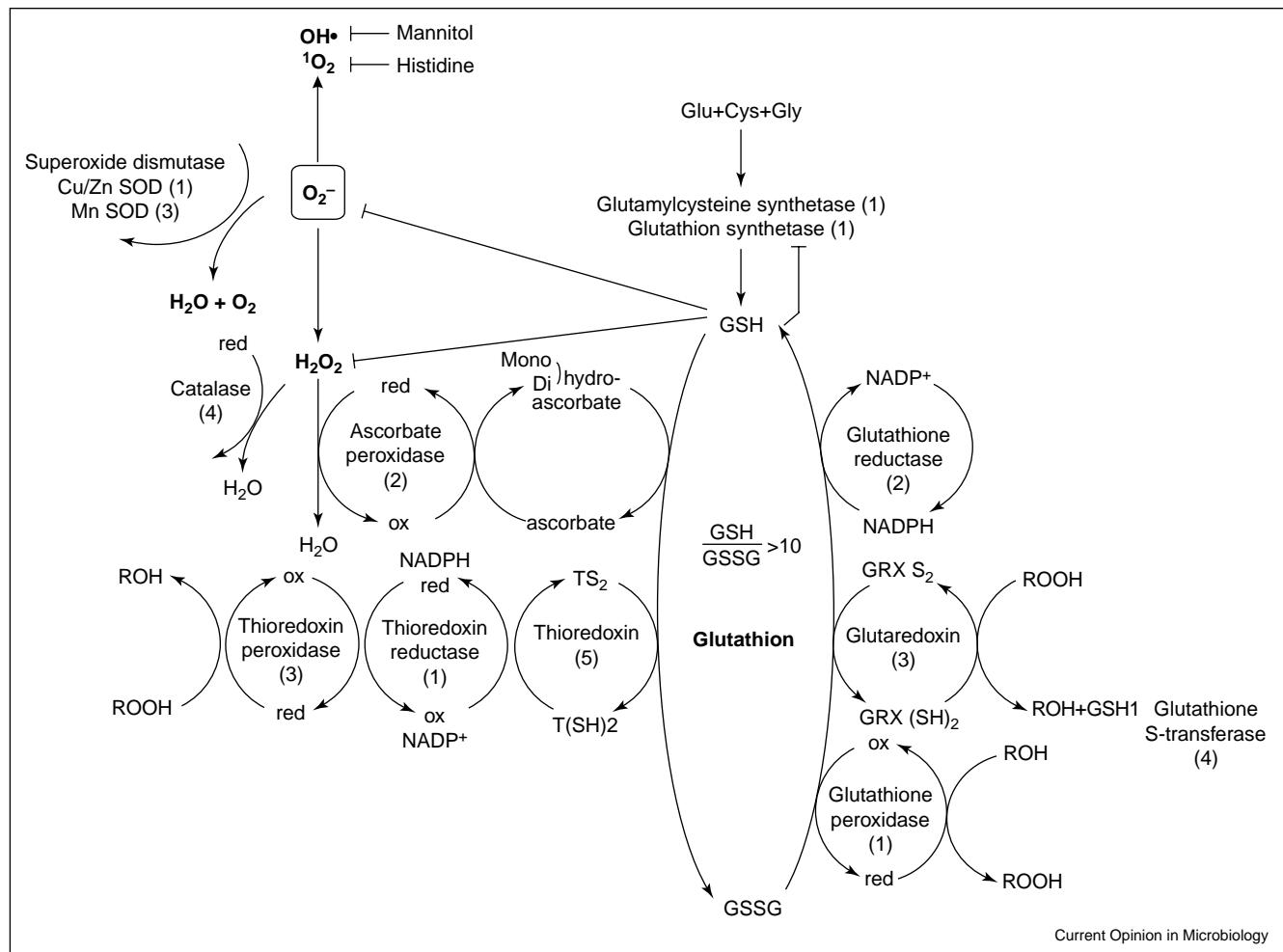
Table 1

Predicted glycosylhydrolase families found in the genome sequence showing the ability of *A. fumigatus* to degrade the plant cell wall.

Cell wall components	Enzymes	Genes ^a
Cellulose	Endo β glucanase	+
	Cellobiohydrolase	+
	β glucosidase	+
Hemicellulose	Exo β glucanase	+
	EndoXylanase	+
	β xylosidase	+
	AcetylxyLANesterase	+
	β mannanase	+
	Arabinoxylanase	+
	Glucuronoxylanase	+
	Glucuronidase	+
	Feruloyl esterase	+
Pectin	Polygalacturonase	+
	Rhamnogalacturonase	+
	Pectin lyase	+
	Pectate lyase	+
	Pectin methylsterase	+
	Arabinase	+
	Arabinofuranosidase	+
Lignin	Mn-peroxidase	–
	Lignin peroxidase	–
	Laccase	+
Others	Tannase	+
	Cutinase	+

^a + indicates the presence of at least one homolog in the *A. fumigatus* genome sequence; – indicates the absence of homologs.

Figure 2



Mechanisms and genes used by *A. fumigatus* to combat reactive oxidants of the phagocyte defence reactions (adapted from [29]). Numerous proteins are able to counteract reactive oxidants such as singlet oxygen ($^1\text{O}_2$), hydroxyl radical (OH^\bullet), superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) mentioned in this figure. Two main pathways (which are based on oxidases or glutathion) have been identified in the genome of *A. fumigatus*. The number of genes of each protein family found in the genome of *A. fumigatus* are indicated in parenthesis.

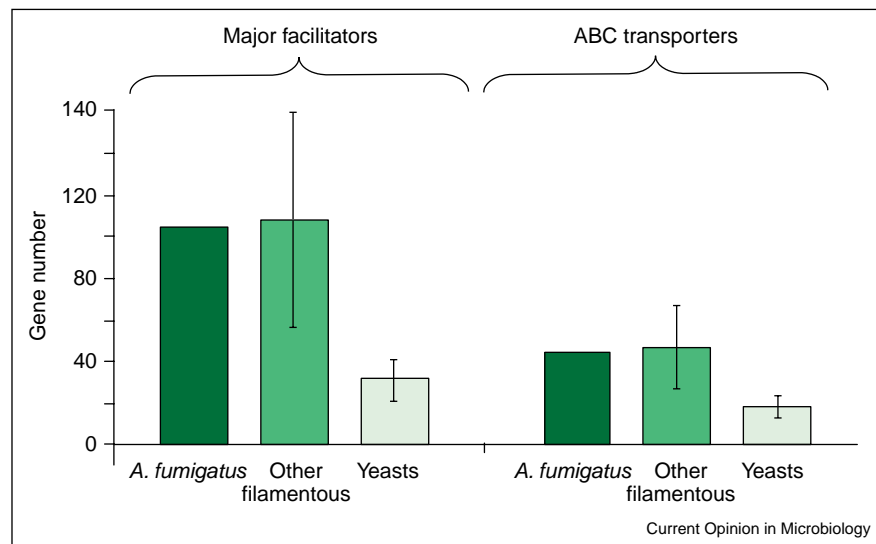
(Figure 3) [30,31]. The genome of *A. fumigatus* also contains >100 genes that encode major facilitator transporters, a family of proteins that is more than five times larger than in yeast and that is absent from higher Eukaryotes. Accordingly, *A. fumigatus* should possess an enhanced capacity for the efflux of toxic metabolites. However, this species does not possess a larger number of transporters than non-pathogenic filamentous fungi. The high number of efflux pumps in *A. fumigatus* could originate from the ecological niche of *A. fumigatus* where it is known that plants and soil (micro)organisms are able to secrete a broad range of toxic compounds. A similar observation was found in the saprophytic *Pseudomonas aeruginosa*, which contains a much higher number of efflux pumps than non-saprophytic bacteria [32]. This high number of efflux pumps and their limited specificity could be the reason for the lack of serious azole-resistance

isolates in *A. fumigatus* despite the large quantity of azole fungicides that are sprayed in nature to combat phytopathogenic fungi.

Thermophily

Thermophily is a requirement for *Aspergillus* pathogenicity. *A. fumigatus* is the most frequently found thermophilic fungus. It is able to grow at 55°C and can survive temperatures of up to 75°C [33,34]. Because of its thermophily it is an essential component of the compost microflora. Until now only two genes have been directly associated with thermophily in *Aspergillus*: first, the *THTA* gene that allows the fungus to grow at 48°C but is not important for virulence [35], and second, the *CGRA* gene that is a nucleolar protein involved in ribosome biogenesis [36]. These two proteins are ubiquitous in the fungal world, even among non-thermophilic species. Thermo-

Figure 3



The number of genes coding for ABC transporters and major facilitators in *A. fumigatus*, yeasts and other filamentous fungi. 44 ATP binding cassette transporters are clustered in two families of 15 and 29 members in *A. fumigatus*. In contrast, analysis of *A. fumigatus* genome has shown that the 105 major facilitators are organised in a single cluster. Note that the number of efflux pumps is extremely high in *Aspergillus* and other molds. Mean \pm standard deviation is calculated on five molds and nine yeast species.

phyly could result from amino acid changes as sequence comparisons have shown that amino acid substitutions result in a thermostabilization of the *A. fumigatus* phytase [37]. However, the direct association between a protein and thermophily as a whole remains unknown. Similarly, in yeast, growth at high temperatures is a complex polygenic trait [36]. However, mutations in homologous genes do not lead to a similar growth temperature phenotype. For example, in *Cryptococcus neoformans*, deletion of calcineurin, α -1,3-glucan synthase, *RAS1*, or MAP kinase (*MPK1*) results in thermosensitive mutants [38–40]. In *Wangiella dermatitidis*, some chitin synthase mutants are unable to grow at 37 °C [41]. By contrast, in *A. fumigatus*, none of these mutations lead to a thermosensitive phenotype, which is an indication that thermophily in fungi is controlled by different genes and that these genes might be differently regulated in *A. fumigatus* than in other fungi.

Chasing for salts

Pathogens have developed mechanisms to acquire iron from the host. Blood serum is generally fungistatic because of the presence of transferrin. *A. fumigatus* possess siderophores of the hydroxamate family that are able to remove iron from transferrin *in vitro* and also have a system for reductive iron assimilation. Mutations in the *SIDA* gene that encodes ornithine oxygenase — an essential step in the biosynthesis of the *A. fumigatus* siderophores ferriochelin and triacetylfusarinine — resulted in the inability of the deleted strain to grow in low-iron medium as well as in mouse, whereas deletion of the

FETC and *FTRA* genes, which are responsible for the ferrous assimilation system, does not influence virulence [42,43].

Similarly, *A. fumigatus* requires high amount of magnesium to grow *in vitro*. Acquisition of magnesium *in vivo* in the phagolysosome has indeed been shown to be an essential requirement for bacterial pathogens such as *Mycobacterium tuberculosis* or *Salmonella typhimurium* [44]. Magnesium acquisition is under the control of MgtC, a membrane protein of unknown function that is known to be essential for pathogenicity as *mgtC* bacterial mutants are unable to grow in the phagocyte. *mgtC* is one of the few bacterial genes found in *A. fumigatus* and its role is currently being investigated.

Phosphate is another ion that is essential for fungal growth. The amount of phosphate present in the serum, 1 mM, is insufficient for growth of *A. fumigatus*, which require ten times more Pi than this. Many extracellular phosphatases and phospholipases (at least 5 times less genes for both families in yeast than in *A. fumigatus*) that are repressed by the presence of phosphate in the growth medium, have been identified in the genome of *A. fumigatus*. In addition to their use for recovering phosphate, phospholipase can have a direct role in pathogenicity by the perturbation of host membrane integrity [45,46].

Zinc is another macroelement that *A. fumigatus* must acquire from the environment, and this has been shown

to be important for microbial infection. A family of five zinc transporters, of which two have been recently analysed, are shown to be involved in zinc transport [47]. Knowledge of their role in pathogenicity awaits the results of further animal studies.

Conclusions

Genomic data gathered to date and the biological items pinpointed in Box 2 suggest that *A. fumigatus* virulence results from the immunosuppression or genetic deficiency of the host rather than from specific and unique fungal determinants. Encountering an immunocompetent host is indeed a dead end for the fungus.

Rather than trying to identify specific fungal virulence factors, perhaps we should consider that the life-threatening *A. fumigatus* is a saprotrophic fungus that only becomes pathogenic for very simple biological reasons:

Box 2 A few biological points of consideration.

1. Resistance of *A. fumigatus* to host defence reactions is associated with the presence of melanin. *Aspergillus niger* conidia, however, are more resistant to phagocytosis than those of *A. fumigatus*, but are not responsible for IA in patients. How significant is resistance to phagocytosis in determination of fungal virulence?

2. *Aspergillus flavus* is another widely distributed *Aspergillus* species that is isolated from soil, plant products or insects. This is also a human pathogen. A close phylogenetic association has been found between *A. flavus* and the 'generally regarded as safe' species *A. oryzae*, which is used in industry. Although recent studies have shown genetic distinctiveness between *A. oryzae* and most aflatoxin-producing strains of *A. flavus*, some *A. flavus* strains are indistinguishable from *A. oryzae* strains [48]. *A. oryzae* strains used in the biotechnology industry are able to infect and kill mouse when the mice are immunocompromised with cortisone acetate (JP Latgé, unpublished). These data indicate that, in contrast to the generally admitted idea, many molds including the biotech fungus *A. oryzae* have the enzyme armamentarium to invade lung tissues.

3. *Neosartorya fischeri* is a food contaminant that is taxonomically closely related to *A. fumigatus*. It is regarded as a useful comparative genomic tool to identify virulence factors of *A. fumigatus* as it was not considered to be a human pathogen. However, the finding that *Neosartorya* is able to infect immunocompromised animals (Latgé, unpublished) and humans [49,50] makes such genomic comparison a questionable method for the identification of virulence genes.

4. Growth rate has been directly correlated to virulence. Genome analyses have not, however, been able to identify the network of regulatory genes that control growth. Differences in growth rate might explain why *A. nidulans* is less pathogenic than *A. fumigatus*, as at 40°C *A. nidulans* grow less than *A. fumigatus*. 1 log difference is seen in the amount of conidia needed for the two species to reach the same mortality in an experimental IA model. In spite of its lower pathogenicity, *A. nidulans* has been considered and sometimes proven to be a useful model to understand *Aspergillus* virulence [51]. The debate regarding the accuracy of the use of *A. nidulans* is still on.

it is present in high concentrations in the atmosphere, it grows faster than any other airborne fungi at 40 °C and it can overcome the defence of the host not because it has developed specific systems but because the host colonized has a very weak defence immunity. This should direct future studies towards the host rather than the fungus to understand the pathobiology of *A. fumigatus*.

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