Genomics of *Aspergillus fumigatus*

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Summary

Aspergillus fumigatus is a filamentous fungal saprophyte that is ubiquitous in the environment. It is also a human pathogen and induces allergenic response, negatively impacting health care and associated costs significantly around the world. Much of the basic biology of this organism is only poorly understood, but the recent completion and publication of its genome sequence provides an excellent tool for researchers to gain insight into these processes. In this review we will summarize some of the more salient features revealed by analysis of the genome, including the search for candidate pathogenicity genes and the switch to a pathogenic lifestyle, allergen proteins, DNA repair, secondary metabolite gene clusters that produce compounds both useful and toxic, a theoretical capability of this asexual organism to reproduce sexually, signalling, and transcription. *A. fumigatus* was compared with the food biotechnology fungus *Aspergillus oryzae* and sexual fungus *Aspergillus nidulans,* as well as other fungi, in an attempt to discern key differences between these organisms.

Key words

Aspergillus, Genome sequence, Pathogen, Allergen, Sexuality

Genómica de *Aspergillus fumigatus*

Resumen

Aspergillus fumigatus es un hongo filamentoso saprobio cosmopolita con una amplia distribución en el ambiente. *A. fumigatus* es un patógeno humano muy importante que causa tanto enfermedades invasoras graves como enfermedades alérgicas de gran impacto en la salud pública mundial. La mayoría de los aspectos biológicos de este organismo son poco conocidos, pero, con la reciente publicación de la totalidad de su genoma, se abre un nuevo horizonte para ampliar las investigaciones científicas y conocer mejor su biología. En esta revisión haremos un resumen de algunas de las características mas sobresalientes obtenidas a través de estudios de su genoma incluyendo la búsqueda de genes causantes de su patogenicidad y del cambio de saprofito a patógeno, proteínas que inducen alergia, mecanismos de reparación del ADN, localización y agrupamiento de genes que producen metabolitos secundarios benéficos y tóxicos, la capacidad teórica de este organismo asexuado para reproducirse sexualmente, mecanismos de comunicacion y de transcription genetica. *A. fumigatus* ha sido comparado con *Aspergillus oryzae,* hongo filamentoso de gran importancia en biotecnologia de alimentos; *Aspergillus nidulans,* hongo con reproducción sexual, así como con una gran variedad de hongos en un intento de conocer mejor las diferencias claves entre estos organismos.

Palabras clave

Aspergillus, Secuencia genómica, Patógeno, Alérgeno, Sexualidad

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Aspergillus fumigatus is a ubiquitous filamentous fungal saprophyte that is also a primary and opportunistic pathogen and a major allergen. It is the prototypical airborne opportunistic pathogen, affecting a wide range of susceptible patient groups, particularly those with neutropenia, receiving corticosteroids, with T cell defects such as AIDS patients and the small number with inherited chronic granulomatous disease [10]. Invasive infection is usually fatal unless treated early and even then antifungal therapy is often unsuccessful. It is now the most common infectious cause of death among leukaemia and haematopoietic stem cell transplant patients [29]. Increasing numbers of patients in intensive care are recognised with this infection [30] and it is a common fungal postoperative infection [38]. In the environment, *A. fumigatus* is commonly found in the human habitat [19], including pillows [47] and in the epicentre of vegetable matter compost [42]. Its spore production is so prolific that human respiratory tract exposure is almost constant [25], making *A. fumigatus* exposure, along with taxes and death, one of the few certainties of life.

While the interaction of *A. fumigatus* spores and the human respiratory mucosa is understood to an extent [26], much of the basic biology of the organism, including its rapid growth rate, remarkable thermotolerance, apparent loss of sexuality, numerous secondary metabolic pathways producing both toxic and useful metabolites, and pathogenicity in general are poorly understood. The interaction of *A. fumigatus* and other airborne fungi with the immune system in atopic individuals, in particular the increasing evidence linking more severe asthma and sinusitis phenotypes with fungal allergy [11,48], is incompletely understood despite years of research. Recently direct hyphal exposure of the respiratory tract, with immediate allergen challenge, has been demonstrated [16].

The burden of non-allergic disease caused by *A. fumigatus* is substantial, particularly in immunocompromised individuals [10]. Antifungal prophylaxis has been of unproven value with current medication, and in any case the risk extends for months or years in post-transplant patients and those with AIDS or other intrinsic immunodeficiencies. No vaccine is available or in development. In diagnosed cases in the USA, the cost to the country of invasive aspergillosis was $600M in 1996 [9]. *Aspergillus* sinusitis, keratitis and chronic pulmonary aspergillosis including aspergilloma contribute to the international burden of disease. There remains a need for major prophylactic, diagnostic and therapeutic improvements, for which availability of genomic sequence is a key first step.

We summarize here some of the findings resulting from an international multi-institutional funding and sequencing effort, which started in 1998 and culminated in the complete genome sequence of *A. fumigatus* Af293 [33].

Genome overview

The genome of *A. fumigatus* Af293 was sequenced by the whole genome random sequencing method augmented by optical mapping [33]. Af293 contains eight chromosomes ranging in size from 1.8-4.9 Mb (Figure), for a total of 29.4 Mb of genomic sequence and 49.9% G+C. There are 9,926 predicted protein-coding genes with a mean gene length of 1,431 bp. About one-third of these predicted genes (3,288) are of unknown function. Additionally, there are at least 12 mitochondrial copies per nuclear genome. Comparisons to the genomes of *Aspergillus nidulans* [15] and *Aspergillus oryzae* [28] revealed ~500 genes unique to *A. fumigatus*, including genes encoding arsenate reductases and additional genes that may have been transferred from soil bacteria by means of a horizontal gene transfer process [33]. Other notable findings include a complete gene complement for heterothallic sex, a cell wall assembly process that is quite different in structural detail from any yeast, at least 28 gene clusters encoding proteins involved in secondary metabolism and mycotoxin production, evidence of numerous cell death pathway components [13], and at least 168 efflux pumps for drugs, toxins, and macromolecules. The genome sequence provides an unparalleled resource for the future understanding of this extremely prevalent fungus.

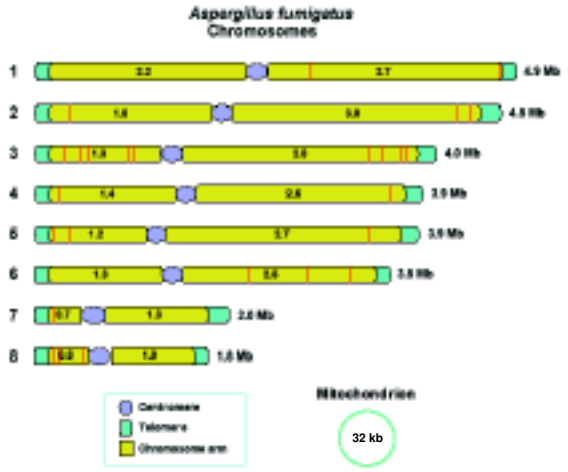

Figure. Illustration of the eight chromosomes of *A. fumigatus* Af293, showing lengths of arms as well as overall length, and locations of centromeres and telomeres. Red bars indicate locations of secondary metabolite biosynthetic gene clusters.

Pathogenicity genes and pathogenicity

A limited number of candidate pathogenicity genes and components of pathogenicity have been identified in *A. fumigatus* by assaying mutants in cultured macrophages or in animal models of invasive aspergillosis. These include the enzymes involved in pyrimidine biosynthesis, PyrG, and pigment biosynthesis, PksP [24]; a histidine kinase, fos-1 [6]; mycelial catalases Cat1 and Cat 2 [37]; the Ras-related protein RhbA [35]; cAMP signalling pathway components AcyA and GpaB [27]; the folate biosynthesis pathway component PabaA [4]; secreted proteases [20]; chitin synthase ChsG [31]; and the nutrient sensing system component CpcA [23]. Immunosuppressive substances in culture filtrates such as gliotoxin have also been suggested to be pathogenicity factors [1]. Differential display was used to compare gene expression in fungal cells grown on endothelial cells with that of cells grown without the endothelial cell contact [41]. Two up-regulated genes subsequently characterized encoded a regulatory subunit of a cAMP-dependent protein kinase and a *ras* gene family protein. Both proteins are involved in cAMP-mediated signalling [27], and this study therefore validates the potential of transcription profiling to reveal pathogenicity-related genes.

While *A. fumigatus* clearly is well adapted as an environmental saprophyte, it also is capable of establishing invasive infection in immunocompromised human hosts. The ability to do this is not particularly prevalent among filamentous fungi, and so it is hoped that the availability of the genome sequence of this fungus will provide clues as to why it can act as a pathogen. A review of the genes that have been identified by mutant analysis to reduce virulence as discussed above include *pyrG*, *pksP*, *sidA*, *laeA*, and several others [33]. The range of functions performed by the products of these genes and their close association to fundamental components of the core metabolic infrastructure of the fungus suggest that the ability to survive in a human host is not the consequence of the presence of true virulence genes but of the metabolic capabilities it has evolved to succeed as a versatile saprophyte, including the ability to thrive at 37 °C. In support of this hypothesis is the observation that no genomic components are shared exclusively by *A. fumigatus* and other human pathogens such as the *Candida* or *Cryptococcus* species [43]. Indeed, the proteome infrastructure facilitating success in the competitive world of environmental life fuelled by decaying plant matter, environmental sensors, defence mechanisms against oxidative stress, the ability to enroll its numerous efflux pumps to export the chemical weapons of its neighbors as well as its own, and its high temperature versatility have likely provided the tools necessary to survive and thrive in a compromised human host. It also grows quickly, which may assist tissue invasion and destruction [34], and its growth rate is accelerated in the presence of corticosteroid [32].

Allergens

Allergy, with associated asthma, is an increasingly common condition. Fungi are one of the principal sources of allergens and likely to be a trigger for asthma in certain cases. More allergens have been characterised in *A. fumigatus* than in all other fungi combined. It is notable that *A. fumigatus* is a far more common source of allergy and asthma than either *A. nidulans* or *A. oryzae*. A comparative analysis of allergen genes in the three species [33] suggests that all *A. fumigatus* allergens have close homologs in the other two species with the exception of Asp f1, which encodes a ribonuclease toxin and the metalloprotease Asp f5, which has no homolog in *A. nidulans*. Comparison of *A. fumigatus* allergens with unknown function (Asp f2, f4, f7, f8, f9, f16 and f17) to *A. nidulans* and *A. oryzae* paralogs shows that these proteins have similarity to secreted glycosidases, specifically glucanases and cellulases, and may have association with the adhesion class of proteins from *Candida albicans.*

Analysis of newly described allergens [22] from *A. fumigatus* suggests that they can also be divided into two classes: highly conserved proteins and secreted glycosidases. All have close homologs in *A. nidulans*, *A. oryzae*, and other fungal species, and vice versa, a number of allergens present in other species have homologs in *A. fumigatus*, *A. nidulans* and *A. oryzae* [33]. The prevalence of *A. fumigatus* as an allergen cannot therefore be ascribed to possession of more allergen proteins, unless Aspf1 is a critical factor in provoking an allergic response. It is likely that *A. fumigatus* is either more common in the environment or better able to colonize tissue and withstand inflammatory responses; for example, *A. fumigatus* is the most thermophilic of the Aspergilli, capable of growing at temperatures above 50 °C.

DNA repair / DNA damage

The *A. fumigatus* genome encodes extensive information to protect its own integrity. Poly(ADP-ribose) polymerase-1 (PARP-1) plays a primary role in the process of poly(ADP-ribosyl)ation, activated post-translationally in response to DNA damage [18]. Poly(ADP-ribose) is short-lived in *vivo* since it is rapidly degraded by poly (ADP-ribose) glycohydrolase (PARG). We have observed the presence of PARP homologs in the three *Aspergillus* species analyzed and also in *Neurospora crassa*, but not in *Saccharomyces cerevisiae*. Enhanced PARP activity was reported in *A. nidulans* during sporulation induced programmed cell death [44]. Interestingly, no PARG genes are present either in the three sequenced *Aspergillus* species or in *N. crassa*. Clearly, these organisms must recycle their ADP-ribose by using other metabolic pathways.

Instead of excising damaged bases, some DNA repair enzymes act by reversing DNA damage. Photoreactivation (PHR) is a general term used to refer to the ability of cells to make use of visible light to reverse the toxic effects of UV irradiation [12]. *A. fumigatus* has a single PHR gene that encodes a protein with similarity to Phr, a deoxyribopyrimidine photolyase from *Fusarium oxysporum.*

The nucleotide excision repair (NER) pathway mainly removes bulky adducts caused by environmental agents [14]. NER is highly conserved among eukaryotes. One major difference between the bacterial and eukaryotic NER systems is that many more proteins are needed to carry out each step in eukaryotic NER [12]. In *S. cerevisiae* and humans, NER is coupled to transcription and requires the assembly of a large multiprotein complex known as the "repairosome" [14]. In *Aspergillus* species almost all human NER homologs are present, except for *RPA3*, *DDB2*, and *RAD23A*.

There are at least two independent pathways that have evolved to repair double-strand breaks (DSBs) [5]: homologous recombination (HR) and non-homologous DNA end joining (NHEJ). HR is an important means of DNA repair in filamentous fungi and yeasts. In the *Aspergillus* species we have identified two genes (*uvsC* and possibly *uvsE*) encoding proteins related to the single Rad51 of *S. cerevisiae* and the single RecA of *Escherichia coli*. In mammals, most of the repair of DSBs is carried out by NHEJ rather than by homologous recombination [12]. At least three proteins specifically required for NHEJ in humans, XRCC5-7, cooperate to make up the DNA-dependent protein kinase complex composed of Ku80/86 (XRCC5), Ku70 (XRCC6) and DNA-PKCs (XRCC7). This complex is thought to function by binding to DNA ends and stimulating DNA ligase activity [46]. As found in yeast, putative homologs of Ku70 and Ku86 were identified in *Aspergillus* species. Although an XRCC7 homolog was not found in the *A. nidulans* genome, LIG4, which is responsible for the ligation of the DNA fragments, was observed. It appears, therefore, that these three *Aspergillus* species have most of the DNA repair genes that are found in *Homo sapiens*, including PARP, and that these genes are quite conserved in the three species.

Secondary metabolism genes and clusters

Filamentous fungi display many unique characteristics that render them of great interest to the research community. Among these characteristics is the production of natural products, or secondary metabolites [21]. These

compounds often have obscure or unknown functions in the producing organism but have tremendous importance to humankind. Secondary metabolites display a broad range of useful antibiotic and immunosuppressant activities as well as less desirable phyto- and mycotoxic activities. The distribution of natural products is characteristically restricted to certain fungal taxa, particularly the Ascomycetes.

Because of the great interest in these compounds, efforts have been expended in the last decade to clone and characterize the genes involved in their biosynthesis. Accumulating data from these studies support a model of fungal secondary metabolite gene clusters containing most if not all of the genes required for product biosynthesis. The locations of these clusters in *A. fumigatus* are biased towards the telomeres (Figure), in contrast to those of *A. nidulans*, which tend to be subtelomeric, i.e., located within 500 kb of the chromosome ends. A compilation of available data indicates that many clusters contain regulatory genes and/or genes associated with resistance to the metabolite. In some cases there are also several genes with no apparent role in production of the metabolite in question. Of course, there are exceptions for all of these generalities.

Although the Aspergilli are rich in secondary metabolic capacity, there are significant differences between them. Despite its reputation as a toxin-free species used safely in the food industry, *A. oryzae* has a remarkably large secondary metabolic potential [28] compared to *A. fumigatus*, known for its toxin production. *A. fumigatus* produces at least the following secondary metabolites, some of which are clearly mycotoxins: fumitremorgin A, verruculogen, gliotoxin, fumagillin, fumigaclavine, helvolic acid, sphingofungins, brevianamide F, and phthioic acid (for full details see the mycotoxin section of The *Aspergillus* Website, http://www.aspergillus.man.ac.uk/indexhome.htm?secure/mycotoxin/index.php~main). Genome analysis of *A. fumigatus* has identified gene clusters for gliotoxin biosynthesis [33] and ergot alkaloid (fumigaclavine) biosynthesis [8]. In contrast, wild-type *A. oryzae* produces only cyclopiazonic acid, maltoryzine, and 3-nitropropionic acid. We already know that the aflatoxin pathway present in *A. oryzae* is not expressed, and this may be true of many of the other clusters. The novel nuclear protein LaeA, likely a methyltransferase, has recently been described as a global regulator of secondary metabolite production in *A. nidulans* and *A. fumigatus* [3]. Loss of this gene eliminates sterigmatocystin, aflatoxin, lovastatin, gliotoxin and penicillin production, and results in an avirulent *A. fumigatus* mutant [2]. Homologs exist in *A. oryzae* and *Aspergillus flavus*.

A second puzzle is the diversity of nonribosomal peptide synthase (NRPS) and polyketide synthase (PKS) genes within this genus. While there are a few genuine orthologs (Table), including genes responsible for the biosynthesis of penicillin, two siderophores, an asexual spore pigment precursor, and aflatoxin/sterigmatocystin, most appear to be paralogs. This suggests that there is a relatively rapid evolution of the "non-essential" secondary metabolic repertoire, including deletion (or gain?) of clusters (cf. penicillin, aflatoxin and carotenoid pathways). Some clusters, including the gliotoxin cluster, appear to be recently duplicated, suggesting that duplication and rapid diversification also contributes to the variability of non-essential secondary metabolites produced by the Aspergilli.

Table. PKS, NRPS and other secondary metabolic gene functions where known function is proven or highly likely.

Pathway/Species	*A. oryzae*	*A. fumigatus*	*A. nidulans*
Penicillin	+	-	+
Siderophore Sid2*	+	+	+
Siderophore SidC*	+	+	+
Conidial pigment WA	+	+	+
Aflatoxin/sterigmatocystin	+	-	+
Carotenoid	+	-	-

*NRPS required, but not strictly secondary metabolism since essential for growth.

Sex

One of the unexpected findings from genome sequence analysis is that *A. fumigatus* appears to contain all the genes known to be required for sexual reproduction, although the presence of sexual cycles in the fungus has never been reported. Sexual reproduction in ascomycete filamentous fungi is governed in part by two different mating type genes that establish sexual compatibility. One gene, *MAT-1*, encodes a high mobility group (HMG) box domain protein, while the other, *MAT-2*, encodes an alpha domain [39]. Homothallic fungi contain both genes and are capable of mating with identical mating types (self-crossing). Heterothallic fungi possess only one of the two genes and require a partner with a different mating type gene (obligate outcrossing). The two mating type genes in heterothallic species typically occupy the same chromosomal location in different haploid strains but lack sequence similarity, and are thus termed idiomorphs rather than alleles.

The non-pathogenic fungus *A. nidulans* is homothallic, while *A. fumigatus* is known to reproduce only through asexual mitotic spores (conidia). However, analysis with 61 genes that are implicated in the mating process or pheromone response in fungi reveals that every gene (except for one of the two mating type genes, *MAT-1*) that can be identified in *A. nidulans* is also present in *A. fumigatus* [15]. A subset of these genes has been previously reported for *A. fumigatus*, including an α-factor like pheromone precursor and the receptors for both the **a**-factor and α-factor like pheromones [40]. Surprisingly, strain Af293 contains one mating type gene (*MAT-2*), while another strain sequenced by Celera Genomics (CEA10) coincidently contains the opposite gene (*MAT-1*), suggesting that they may be a heterothallic pair of the species. Population surveys have shown that both mating types are almost equally present in nature, resembling those found in typical heterothallic species [36]. These findings raise a series of questions as to if *A. fumigatus* is capable of sexual reproduction, and, if so, under what conditions.

Signalling

Regulation of metabolic functions in response to environmental change is essential for survival of free-living microbes. Two-component regulatory systems are phosphorelay pathways that are utilized by both prokaryotes and eukaryotes to regulate cellular responses to environmental changes. The *A. fumigatus* genome has 13 putative histidine kinase genes, compared to 15 in the genomes of *A. nidulans* and *A. oryzae*. It also possesses two response regulators and a single histidine-containing

phosphor-transmitter protein. Relative to *S. cerevisiae, Schizosaccharomyces pombe,* and *N. crassa* this is a significantly larger number of histidine kinases, suggesting a greater importance and diversity for these regulatory systems in the genus *Aspergillus*.

Mitogen-activated protein (MAP) kinase pathways are found in all eukaryotes and regulate cellular responses to extracellular signals, such as growth factors in metazoans, the mating pheromone alpha factor in *S. cerevisiae,* and responses to stressors such as changes in osmolarity or the presence of reactive oxygen species. Thus MAP kinase pathways are implicated in regulating cellular physiology in developmental programs and environmental stress. The genomes of *A. fumigatus*, *A. nidulans* and *A. oryzae* each contain four genes that encode MAP kinases that contain the consensus sequence TGY or TEY in the activation loop. They also contain a fifth potential MAP kinase that is also found in *N. crassa* but has the sequence TTY in the activation loop. Other components of the MAP kinase signaling pathways are also found in the genomes of these three species and include MAP kinase kinases, MAP kinase kinase kinases as well as a homolog of the negatively regulating tyrosine phosphatase. These observations suggest the existence of at least three MAPK-mediated signal transduction pathways based on *S. cerevisiae* homologs for osmoadaption, mating/hyphal growth, and regulation of cell integrity with interaction between them to regulate different aspects of responses to environmental stimuli and infection in *A. fumigatus* [17].

Transcription associated proteins

The availability of sequences from genomes of many organisms, including many fungi, has made it possible to perform comparative analysis of Transcription-Associated Proteins (TAPs) across several fungi and other unrelated organisms using the TRANSFAC database of eukaryotic transcription factors [45]. Sequences comprising the three *Aspergillus* genomes, four additional fungal genomes, one apicomplexan and one plant genome were included. Just under half of the 85 profile HMMs present in the TRANSFAC database matched Aspergilli proteins, indicating that *Aspergillus* TAPs exhibit a high degree of taxon-specificity, as has been previously observed for *S. cerevisiae* and *S. pombe* TAP families [7]. Thirty-nine of these *Aspergillus*-matching HMMs also match budding yeast, and 38 match fission yeast sequences, indicating a high degree of similarity in the types of domains associated with transcriptional control in these groups of fungi. When the abundances of the genes that match these HMMs are normalized against genome size the majority of the sequences are present at equivalent levels in all seven fungal genomes. However, the numbers of proteins containing the most common DNA-binding motif observed in fungi (PF00172, the Zn(2)-Cys(6) zinc finger) show considerable variation across the seven fungal species, with an average of 67.9 per 10,000 genes for budding and fission yeast, and an average of 137.9 per 10,000 genes for the filamentous fungi. Furthermore, the Aspergilli genomes contain on average 166 Zn(2)-Cys(6) zinc finger-containing proteins per 10,000 genes contrasting with an average of 95 per 10,000 for the *Magnaporthe grisea* and *N. crassa* genomes. This 1.7 fold increase in the abundance of this type of DNA-binding domain may be a reflection that the Aspergilli reside in highly diverse environments, and so require more sophisticated transcriptional networks and contain dispensable metabolic pathways.

Conclusion

This brief review illustrates the power of bringing a genome sequence to the study of the biology and pathogenicity of an organism. *A. fumigatus* biology is booming with interest, partially sparked by the public availability of the *A. fumigatus* and *A. nidulans* genomes, and partly by a greater awareness of the significance of the burden of invasive disease on human societies. The number of publications on *A. fumigatus* has steadily increased. Serious projects are being initiated to use this newfound wealth of genome information and the availability of genome sequence derived *A. fumigatus* glass slide microarrays will enable researchers to explore the particular attributes and capabilities of the fungus that allow it to pose such a consequential health risk to humans.

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