Aspergillus: A Multifaceted Genus

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Defining Statement

In this article, different aspects of ongoing work in the genus *Aspergillus* are discussed, ranging from toxin production, pathogenicity to humans and animals, traditional and modern biotechnological uses, genomics, and the use of *Aspergillus nidulans* as a model organism to study fundamental problems of cell and molecular biology.

Note

For *Aspergillus* genes and proteins, the standard nomenclature is followed, for example, the *benA* gene encodes the BenA protein, which is a *β*-tubulin. When genes or proteins of other species are mentioned, the standard nomenclature for each species is used.

What is *Aspergillus*?

An *Aspergillum* is an instrument used in the Roman Catholic mass to sprinkle holy water over the heads of the faithful. *Aspergillus* is a genus of the ascomycete fungi (see below). In 1729, Pietro Antonio Micheli, priest and botanist, described the asexual spore heads (conidiophores; see below) of a number of common molds. The heads of some of these molds showed rows of spores radiating from a globular central structure, which he thought resembled the *Aspergillum* he was familiar with. The morphology of the conidiophore is still an essential taxonomic marker. Fig. 1 compares the original Micheli's drawing with modern observations of conidiophores.
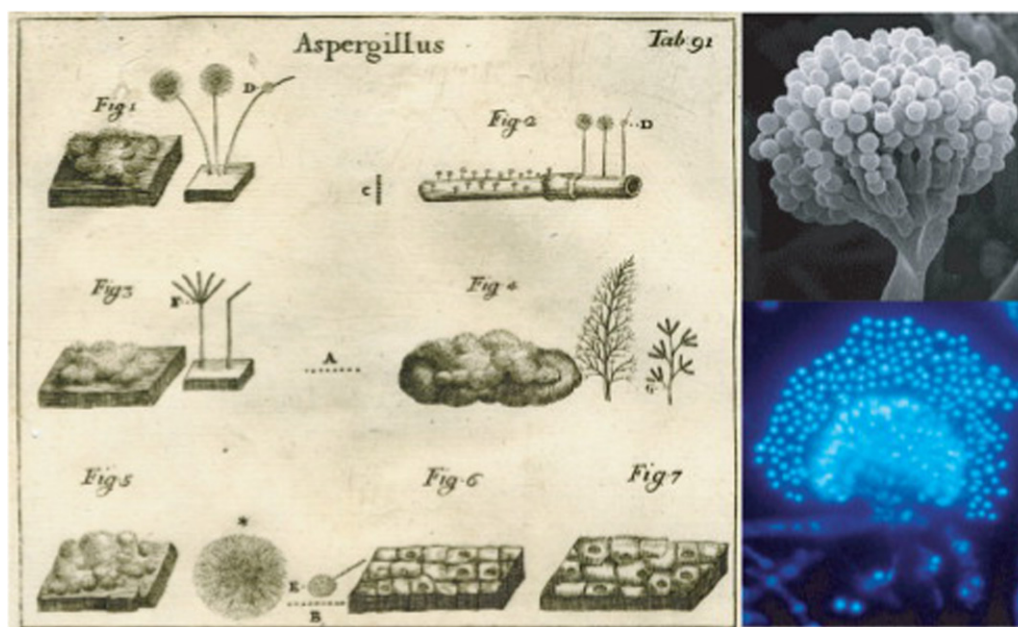

Fig. 1   The left panel shows a scan of copper-engraving 91 from Micheli's *Nova plantarum genera*, showing his drawings of *Aspergillus* conidiophores. The description in Micheli's text suggest that figure of the engraving, called *Aspergillus capitatus* (*muffa turchina*, blue mold) by Micheli, may correspond to *Aspergillus fumigatus* or a close relative. The right panel shows on the top a scanning electron micrograph of the conidiophore of *Aspergillus nidulans* and at the bottom an epifluorescence micrograph. The preparation is stained with DAPI which reveals the nuclei of the conidia and of the subjacent structures of the conidiophore. Both pictures on the right panel have kindly provided by Reinhard Fischer. Reproduced with permission from Kues, U., Fischer., R. (Eds.), 2006. The Micota I, Growth Differentiation and Sexuality. Berlin: Springer-Verlag. Micheli describes in his text the condiophore as been formed by a stalk, and a head, which he called 'placenta' carrying the conidia. See legend to Fig. 13 for the correspondence with the modern terminology.

The classification of the kingdom fungi into major groups (Phyla, such as Ascomycota and Basidiomycota) is based on the morphology of the sexual reproductive structures. The Aspergilli should be placed among the ascomycetes (see below), those fungi that have the products of meiosis placed in a sac or ascus. However, most of the fungi we call Aspergilli (see below) do not have sexual reproduction, and thus no asci. To solve this problem, mycologists created the group fungi imperfecti or otherwise called deuteromycetes in which they placed all fungi without known sexual reproduction. This is a mixed bag without any phylogenetic significance. This provokes ridiculous situations, by which a fungus would change genus, and in fact phylum, every time sexual reproduction is detected. Thus the 'imperfect' fungus *Aspergillus nidulans* (see below) becomes the 'perfect' fungus *Emericella nidulans*, and it is placed in a different phylum from *Aspergillus sydowii*, in spite of the fact that morphological and molecular data show the two organisms to be close relatives. Names like *Emericella*, *Eurotium*, and *Neosartorya* design Aspergilli with a sexual cycle (also called teleomorphs). Thus, *E. nidulans* is the teleomorph (perfect form) of *A. nidulans* (anamorph, imperfect form). No one but a mycologist would know that we are talking about one and same organism.

This situation exists for many other genera. The only solution to this conundrum is to completely abandon the division 'fungi imperfecti' and choose in each case one and only one name for a given genus. As early as in 1926, Thom and Church, Thom and Raper (1945), and Raper and Fenell (1973) proposed that "the generic name *Aspergillus* should be applied to all these fungi whether or not an ascosporic (sexual) stage was produced". The main morphological characteristics of the genus, drastically abbreviated from Raper and Fenell (1973), are "vegetative mycelium consisting of septate branching hyphae … Conidial apparatus developed as condiophores … conidiophores … broadening into turbinate elliptical, hemispherical, or globose fertile vesicles … bearing fertile cell or sterigmata … conidia (asexual spores) … produced successively from the sterigmata. Ascocarps (asci, containing sexual spores) found in certain groups only, unknown in most species". Recent work shows that the Aspergilli are as a whole a monophyletic group, where loss of sexual reproduction has occurred many times independently.

Not everyone agrees with the reasonable proposal of Raper and Fenell. A recent classification (2005) of the ascomycetes places the *Aspergillus*-related teleomorph genus names in the kingdom Fungi, subphylum Pezizomycotina, class Pezizomycetes, family Trichocomaceæ. The genera *Aspergillus* and the related *Penicillium* do not appear in this list as they are considered imperfect forms! Throughout this article, the generic name *Aspergillus* is used, as a genus comprising all the related 'teleomorphs' together with the forms where sexual reproduction is absent. The teleomorph name is also indicated when appropriate, as this is used in some important databases (as NCBI). The kiss of death to the concept of 'fungi imperfecti' was delivered by recent molecular data that show that the genomes of 'imperfect' Aspergilli, include the genes that determine mating types, these genes being clearly homologous to, and even placed in the same place in the chromosome as, the ones found in the 'perfect forms' ("The Genus Aspergillus in the Genomic Era").

Fungi of the genus *Aspergillus*, which includes about 200 species, are important in public health as toxin-producing food contaminants, as human and animal pathogens, as useful fungi in traditional and modern biotechnological processes, and finally one species has been used as a model to study a number of cellular processes. A recent development is the availability of eight complete genomes within the genus, a matter of obvious practical, taxonomical, and evolutionary importance.

Food Contamination by Aspergilli

Many organisms, including bacteria, fungi, and plants, produce secondary metabolites. These are molecules that can be very complex and are not obviously necessary for the viability of the organism. In fungi, they are produced during the stationary phase and their synthesis is usually coordinated with asexual sporulation (see below). Some secondary metabolites are extremely toxic, and when fungi grow on stored foods, they secrete them, provoking food spoilage and eventually intoxications that may be fatal. Among the Aspergilli, the two main culprits are strains of *Aspergillus flavus* and *Aspergillus parasiticus*, which secrete aflatoxins, a group of highly substituted coumarins. Strains that are closely related may vary drastically in their ability to produce the toxin. These saprophytic fungi can grow on a variety of foodstuffs, or even on plants before harvesting. In fact, *A. flavus* can be considered a weak opportunistic, nonspecific plant pathogen. The aflatoxins were discovered in 1960 when thousands of turkeys died in an English hatchery. The contaminated food was a ground peanut meal. The most serious contamination is that of maize. While this contamination results in loss of hundreds of million dollars every year to farmers in developed countries, the impact on human health is extremely serious in developing countries. Of the related compounds called aflatoxins, Aflatoxin B1 is one of the most toxic and carcinogenic compounds known, as judged by tests on laboratory animals. Maize stored under warm and humid conditions becomes contaminated with aflatoxigenic Aspergilli, and when consumed by humans or animals, this can lead to liver failure and death. Periodic outbreaks of acute aflatoxin poisoning occurred in East Africa, the latest in 2004, leading to 125 deaths. It is more difficult to assess the damage caused by chronic aflatoxin poisoning and the correlation of the toxin in food with the frequency of liver cancer. Controls on aflatoxin levels are tight in developed countries; they are, however, impossible to be enforced in developing countries, where people would store grains in their homes and the stored grain may be the only available food. Human aflatoxicosis is a disease of poverty.

The Aspergilli can contaminate food with other toxic molecules. Only the ochratoxins, produced by a number of Aspergilli and Penicillia will be discussed below. The ochratoxins comprise an isocumarin moiety and a phenylalanine ring joined by an amide bond. Ochratoxin contamination has been reported in many foodstuffs, including grapes, nuts, cacao, coffee beans, and spices. In poultry, and laboratory animals, ochratoxins provoke serious kidney lesions. It is difficult to assess damage to human health caused by chronic exposure to ochratoxins. They have been implicated as a cause of testicular cancer. The similarity of symptoms

of porcine mycotoxin nephropathy with that of Balkan endemic nephropathy, a disease localized to regions of Bulgaria, Romania, and former Yugoslavia, has implicated ochratoxins as causal agents of the disease. A similar case can be made for chronic interstitial nephropathy of northern Africa. A case of acute renal failure, almost certainly due to the exposure to an ochratoxin, has revived the hypothesis that exposure to this mycotoxin is the cause of the 'mummy curse', which is alleged to have killed archaeologists who have braved the prohibition to open royal tombs.

Aspergillus as Pathogens

The common fungus diseases are mild and superficial, while those that are deep-seated and endanger life are so rare that one man can seldom see enough cases to make any extensive study of them. (Henrici, Presidential Address to the Society of American Bacteriologists, 1939).

Aspergillus as Human Pathogens

The emergence of species of the genus *Aspergillus* as, in many cases, intractable human pathogens, has gone hand in hand with the progress of medicine. All Aspergilli encountered as causal agents of human or animal diseases are opportunistic pathogens. The Aspergilli are all saprophytes, usually growing in decomposing vegetal material. The main pathogen, *Aspergillus fumigatus*, thrives on compost.

Before the transplant era, *Aspergillus* infections were only encountered sporadically. Farmer's lung is a general name for an allergic disease that could be due to different causal agents, bacteria or fungi, of which the Aspergilli are the main culprits. It is an occupational disease associated with high exposure of spores, in environments such as grain silos. In the nineteenth century, two exotic occupational diseases associated with *A. fumigatus* were described: the *maladie de gaveurs de pigeons* and the *maladie de peigneurs de cheveux*. These pulmonary diseases were associated with people who force-fed pigeons and with people who sorted hair for wigs, respectively. A perusal of the Pathogenesis article by Austwick, included in Raper and Fennell's monograph of 1973, leaves the impression that a large number of *Aspergillus* species could be opportunistic pathogens, that pulmonary disease was basically an occupational hazard, that virtually every organ could be colonized by one or other *Aspergillus* species, and that once the fungus was established the prognosis was bleak. Henrici, compared invasive fungal diseases to autocatalytic processes, sluggish to start, but eventually becoming unstoppable. The comparison still holds today, except that immunodepression gives the fungus a head start. *A. fumigatus*, was then as now, the prevalent species, followed by *A. flavus*.

Three types of respiratory pathologies are associated with the Aspergilli. Exposure to the fungus can result in allergic diseases, such as farmer's lung and allergic bronchopulmonary aspergillosis, encountered mainly in asthmatic and cystic fibrosis patients. *Aspergillus* spores can germinate in preexisting cavities such as the sinuses or those present in the lung as a result of tuberculosis. This leads to localized Aspergillomas in immunocompetent subjects, which can be treated surgically and/or with appropriate drugs. Finally, the most threatening form is the invasive Aspergillosis, associated, in most but not in every case, with a depression of the immune system.

The ability to perform grafts of bone marrow cells in leukemic patients, of solid organs such as kidney, liver, and lung, has been accompanied by the emergence of invasive Aspergillosis. AIDS patients are also at risk, but *Aspergillus* spp. are encountered less frequently in these patients than *Pneumocystis carinii*, *Candida* spp., or *Cryptococcus neoformans*. Susceptible patients include those affected by neutropenia. Neutropenia can result from leukemia or from the chemotherapy used to control it, or be subsequent to treatment with immunosuppressant used in bone marrow, stem cell, or organ transplants. Patients of systemic diseases treated with immunodepressing drugs, mainly corticosteroids, are also at danger. In all these patients, germination of *Aspergillus* spores leads to invasive aspergillosis, usually of the lung, which breaking through the blood vessels can infect other organs. There seems to be no organ in which the fungus cannot grow in the absence of an appropriate immune response. In almost all cases, spores enter through the respiratory tract and germinate in the parenchyma of the lung, leading to invasion of the bronchiolar walls and the adjacent blood vessels. Invasive Aspergillosis has been classified into angioinvasive and bronchio-invasive forms, but this classification is somewhat artificial, as invasion of both bronchioles and arterioles can be seen in the same patient. This leads eventually to respiratory failure and death. Fig. 2 shows an *Aspergillus* mycelium grown in lung tissue.

A very recent review estimates an eight-fold increase in *Aspergillus*-disseminated infections, from the 1970s to the present day. Between 9% and 17% of all deaths in transplant recipients are due to *Aspergillus* infections according to recent data. The prognosis of invasive aspergillosis is grim; mortality in transplant patients infected with *Aspergillus* sp. is never lower than 60% for patients treated with antifungals and 100% in nontreated patients.

The most common encountered species in all *Aspergillus*-related pathologies is *A. fumigatus*, *A. flavus*, *Aspergillus niger*, *A. nidulans*, and *Aspergillus ustus* have also been recorded. One recent study of nosocomial infection reports that of 458 patients 154 were infected with *A. fumigatus* and 101 with *A. flavus*. The same and other studies establish a link between construction or renovation work in the vicinity of the hospital and frequency of invasive aspergillosis and conclude that even very low spore counts (1 spore per m^3) are dangerous to immunocompromised patients. Genotyping has shown that there are no specific pathogenic strains and suggests that every *A. fumigatus* strain present in the environment is a potential risk for immunodepressed patients. Recently, an upsurge of *Aspergillus terreus* infections has been observed. This is particularly worrying, as the organism is resistant to Amphotericin B, the drug most widely used to treat invasive Aspergillosis. The prevalence of *A. fumigatus* infection has not been explained. We are all continuously exposed to fungal spores and two obvious factors can be considered to explain the
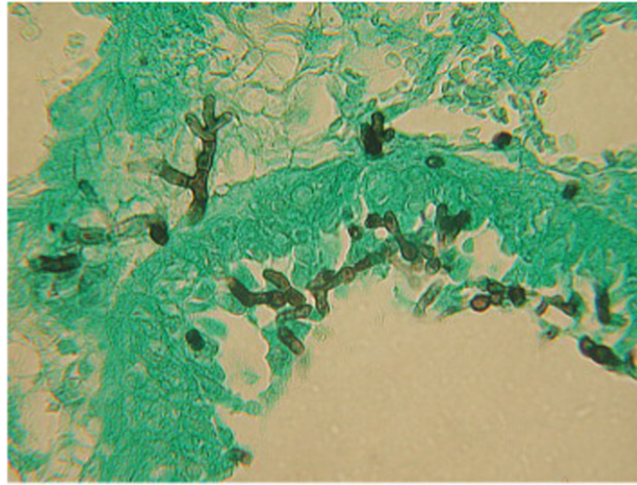
Fig. 2 A neutropenic mouse lung tissue, experimentally infected with *Aspergillus nidulans* is shown at 20 h postinfection. The fixed sections were stained with Grocotts Methenamine Silver. The pictures show hyphae (stained brown) actively growing in the lung tissue (stained green). Photograph kindly provided by Elaine Bignell.

prevalence of one or other species. The first is the spore density in specific environments. Unfortunately, many early studies simply report the density of 'Aspergillus' without any further species discrimination, let alone genotyping. It is generally accepted that the high frequency of A. fumigatus infections cannot be explained by a prevalence of the organism in the environment. The second parameter to be considered is spore size. The smaller the spores, the most likely they are to reach the alveolar tissue of the lung, as they will be less susceptible to removal by the mucociliary tissue of the respiratory tract. A. fumigatus spores are usually about 2–3 µm in diameter, at the lower end of the genus. Specific gravity of spores has, to my knowledge, never been measured. Another obvious parameter is thermotolerance, especially in relation to spore germination. However, it is unlikely that the combination of small spores and ability to germinate rapidly at 37°C be sufficient to explain the prevalence of A. fumigatus. Both characteristics are shared by A. fumigatus and A. nidulans, the latter being rarely encountered as an opportunistic pathogen. Another possibly interesting parameter is spore hydrophobicity. This is determined by a family of proteins called hydrophobins. Strains of A. fumigatus lacking a specific hydrophobin become more sensitive to macrophage killing. Sensitivity of different species to neutrophil and macrophage killing has been sporadically, but not systematically, assessed.

It is important to distinguish putative specific virulence determinants from essential metabolic processes, even if the latter can be potential drug targets. Only those processes, that when blocked, by mutation or otherwise result in reduced virulence but do not affect the growth of the fungus outside infected tissues, can be considered proper virulence determinants. This is of course conditional to the media in which the fungus is tested, my feeling is that the more we know about the metabolism of the fungus in the wild, the less we will be inclined to call a specific metabolic step a 'virulence determinant'. It is not surprising that engineered strains, deficient in essential biosynthetic pathways, or cell wall biosynthesis show reduced or no virulence. As an example, strains blocked in lysine biosynthesis show reduced virulence, but this tells nothing about virulence, it reveals that lysine is limiting in the alveolar environment. However, as some of these processes are fungal-specific, they are potential targets for antifungal drugs.

Secondary metabolites and nonribosomal peptides vary considerably from one fungal species to the other, and thus they represent an interesting avenue of research bearing on virulence. These metabolites may have evolved in saprophytic organisms in response to the presence of competing organisms in a common environment. As such, they may be cytoxic and eventually involved in pathogenicity. One of these metabolites, gliotoxin, a substituted diketopiperazine, has received considerable attention. It been implicated in the suppression of the innate immune response, including apoptosis of neutrophils. However, specific inability to synthesize gliotoxin does not affect the virulence of A. fumigatus in the neutropenic mouse model. However, deletion of laeA, a gene necessary for the transcription of a large number of genes encoding enzymes of secondary metabolite synthesis (see 'Medically useful secondary metabolites' and 'Regulation of secondary metabolism'), including gliotoxin, does affect the virulence of A. fumigatus. Absence of LaeA leads to a pleiotropic phenotype, and the decreased virulence may result from a combination of factors. It seems that at present we simply do not know why A. fumigatus is the prevalent pathogen and why other Aspergilli are occasional pathogens. It is likely that a complex combination of characters is responsible for triggering the autocatalytic process proposed by Henrici. Opportunistic pathogens have not evolved as such, in a coevolutionary relationship with a host organism, it thus may be completely fortuitous that one or other of them be able to thrive in the tissues of immunocompromised patients.

It is important to determine which are the barriers that prevent fungal infections in immunocompetent subjects. Alveolar macrophage would get rid of ungerminated conidia, while polymorphonuclear neutrophils destroy hyphae mainly through the action of reactive oxygen species (ROS). One proposed mechanism involves the recognition of fungal cell wall constituents, such as β 1-3-glucans, by macrophage membrane receptors, leading to phagocytosis. Recent studies, however, imply a less clear-cut distribution of labor, with neutrophils also having an important role in preventing conidial germination, which correlates with the susceptibility of neutropenic

patients. Dendritic cells are able to ingest *Aspergillus* spores, thus being able to present specific antigens to T cells, a role shared with macrophage. Both $CD4^+$ T and $CD8^+$ T cells respond to fugal antigens, $CD4^+$ T cells produce cytokines, which further recruit neutrophils. The protective role of specific antibodies is subject to discussion, as they are found in infected patients, which they fail to protect. Recently, protective roles have been postulated for surfactants secreted by epithelial cells that interact with conidia and may facilitate phagocytosis. A crucial role in innate immunity to opportunistic fungi is carried out by PTX3, a protein belonging to the pentraxin family of secreted, soluble proteins. PTX3 is essential in conidial recognition by macrophage and dendritic cells, and homozygous knocked-out mice genes are highly susceptible to experimental infection. The study of the immune response to infection by *Aspergillus* spores has made considerable progress in recent years and may lead to treatments, which promote the recovery of the immune response of the patient as an alternative or in association with antifungal drugs.

Fungi are eukaryotes, more closely related to metazoans than to plants, that is why ascomycetes such as *Saccharomyces cerevisiae* and *A. nidulans* are useful models in molecular and cell biology. Many cell processes are common to the fungal and the animal cell, and that, in order to find effective antifungal agents, is necessary to identify those processes that will inhibit growth of the fungal cell without damaging the host. Flucytosine (5-fluorocytosine) has been used as an antimycotic since 1968. In clinical practice, it is used mainly in candidiasis. It affects nucleic acid synthesis and thus can hardly be considered a specific antifungal agent. Four other classes of compounds are currently used to treat fungal infections in clinical practice. The polyenes, such as Amphotericin B, interact directly with ergosterol in the fungal cell membranes leading to leakage of potassium ions and cell death. Amphotericin B, one of the most used antifungals, interacts with animal cell membranes also, which can lead to acute kidney failure. The azoles, such as fluconazole or the newly developed voriconazole, inhibit specifically lanosterol demethylase, blocking the synthesis of ergosterol. They are less toxic than Amphotericin B, and a case has been made to use voriconazole as a first line, rather than a second line, drug for the treatment of invasive Aspergillosis. However, they are not free of secondary effects. The allylamines such as Terbinafine also result in ergosterol depletion by inhibiting squalene epoxidase. Finally, the echinocandins are really specific antifungal drugs, as they affect the fungal-specific process, the synthesis of the glucans of the fungal cell wall by inhibiting noncompetitively β-1,3-glucan synthase. Better knowledge of fungal development and metabolism, the search for genes essential for the pathogen, but absent in, or not essential for the host should lead to the development of new specific antifungal drugs. A different and complementary approach is to reinforce the immunological response of the host. This includes the possible development of an antifungal vaccine. Besides the uncertainty as to whether protective antibodies can be produced, the large variety of fungi that can affect immunodepressed patients posits an additional difficulty. Recently, a whole roaster of new fungi appeared as opportunistic pathogens, such as *Fusarium*, black molds, and zygomycetes. Success against *Candida* has been followed by an increase of *Aspergillus* infections. Preventive treatment with voriconazole, effective against *Aspergillus*, has been followed by infections by a whole variety of zygomycetes. It has been proposed that a vaccine using β-1,3-glucan as antigen, which is a universal component of fungal cell walls, may be worth exploring. An early diagnosis is essential in the successful treatment of invasive fungal infections. Immunological detection of cell wall components such as galactomannan and 1-3-β-D-glucan and detection of fungal DNA by PCR are being developed and evaluated.

Aspergillus in Veterinary Medicine

Aspergilli are encountered, even if uncommonly, in veterinary practice. Here, as in the human disease, *A. fumigatus* is the most frequently encountered pathogen, followed by *A. flavus*. In mammals, canine sinonasal Aspergillosis, guttural pouch mycosis of horses, and bovine mycotic abortion are the most common diseases, but infection of other species and pulmonary and generalized aspergillosis has also been described. The horse disease is correlated with the presence of an extension of the Eustachian tube, the guttural pouch, an organ of uncertain physiological significance exclusive of horses, other Equidæ, and rhinos and tapirs. This organ could provide temperature and humidity conditions suitable for the growth of *Aspergillus*. Bovine mycotic abortion is correlated with confinement to sheds, which leads to exposure to high concentrations of spores. More surprising is the finding of pulmonary Aspergillosis in free-range dolphins. If the finding of *Aspergillus* infections in mammals is sporadic and infrequent, birds are at a much higher risk. The main pathogen is *A. fumigatus* and the route of entry is the respiratory tract. In a large postmortem study, 4% of more than 10,000 birds showed fungal infection of the respiratory tract, probably in most cases due to *A. fumigatus*. Aspergillosis affects both free-ranging and domestic birds. Turkeys, poultry, and waterfowl are commonly affected but fatal infections of penguins, ostriches, and rheas have also been reported. The susceptibility of birds to Aspergilli has been explained by both anatomical characteristics of the respiratory system and cellular differences related to innate immunity such as the absence of alveolar macrophage.

A. sydowii, a Specific Pathogen for Gorgonian Corals

An ecologically menacing new Aspergillosis affects Gorgonian (fan) corals, mainly but not exclusively *Gorgonia ventalina* (infections of *Gorgonia flabellum* and one outbreak affecting *Pseudopterogorgia americana* have been reported). Up to the present time, it has been recorded only in the Caribbean Sea, first identified in Saba in 1996 and studied intensively in the Florida Keys. In an epizootic starting in 1997, more than 50% of the sea fan corals were lost. A subsidence of the epizootic has been since reported. The organism responsible is exclusively *A. sydowii*. The restricted host–pathogen specificity contrasts with the situation described above for mammals and birds. This species is a common saprophyte, which can be isolated from a number of environments. Cultures isolated from diseased *G. ventalina* are infectious, while strains isolated from nonmarine environments are not. As only

Fig. 3   A specimen of the fan coral Gorgonia ventalina infected with *Aspergillus sydowii*. The infected areas are deep purple. The purple gall-like growths may be a result of the infection. A necrotic area surrounded by a deep-purpled ring can be seen at the bottom left of the colony. Bar 5 cm. The photograph has been kindly provided by Kiho Kim.

three and two strains respectively were analyzed, this experiment is not definitive. The pathogenic and nonpathogenic strains do not form separate clades when molecular markers are analyzed. As *A. sydowii* does not sporulate in seawater, aerial dissemination has been suspected. One hypothesis is that the spores are carried by dust storms, originating in the North Africa. While fungal spores are surely carried by dust storms, no genotyping work confirming this hypothesis has been reported. Warming and nutrient effluents, including nitrates, have also been blamed for the outbreak. Obviously, these possible causes are nonexclusive. It is possible that the decrease of the epizootic is due to selection for resistant strains of *G. ventalina*. Thus, sea fan infection by *A. sydowii*, besides being an ecological menace, provides an interesting opportunity to study a specific host–parasite interaction involving an *Aspergillus*, and the elucidation of the mechanism of resistance could lead to the discovery of new antifungal compounds, for which there is a crying need. An infected sea fan coral is shown in Fig. 3.

Useful Aspergilli

Aspergillus biotechnology ranges from the first steps of sake fermentation to the production of recombinant mammalian proteins. These processes are briefly summarized below.

Oriental Food Uses of *Aspergillus*

The use of Aspergilli in the food industry in the far East relies on the extracellular enzymes secreted by the fungus when grown on solid or semisolid substrates. These technologies originated in China more than 2000 years ago. An old review cites more than a hundred such different fungal fermentations. The main products are shoyu (soy sauce), miso (fermented soybean paste), and sake (rice wine). The production of soy sauce involves the fermentation of a mixture of cooked soybeans and wheat. The mixture is inoculated in traditional production by 'koji', which derives from a previous fermentation, or in more modern procedures by a spore suspension of specific strains of *Aspergillus oryzae* or *Aspergillus sojae*. A second fermentation is carried out by lactic acid bacteria and yeasts. In the production of sake, the Japanese wine derived from rice, the steamed rice is inoculated with spores of *A. oryzae*, and the hydrolyzed product is used as the substrate for alcoholic fermentation by *Saccharomyces sake*. The *Aspergillus* strains

used in the soy sauce production differ from those used for sake production, the former are selected for high protease, the latter for high amylase titers. Both *A. oryzae* and *A. sojae* belong to the *A. flavus* groups, and genomic analysis has confirmed the very close relationship between *A. oryzae* and *A. flavus*, while *A. sojae* is considered a domesticated strain of *A. parasiticus*. Through the centuries, the organisms have been in use, they have been selected for both high extracellular enzyme titers and nil toxin production, at least under fermentation conditions. *Aspergillus* fermented food products represent, according to a recent source, 2% of the gross national product of Japan.

Extracellular Enzymes Produced by Aspergilli: Aspergilli as Hosts for Recombinant Proteins

The Aspergilli are major producers of enzymes such as carbohydrate hydrolases, lipases, and proteases, used in a variety of industries such as food, beverages, detergent, and animal food additives industries. The first microbial enzyme to be marketed (1894) was an amylase, 'takadiastase', produced from *A. oryzae*. At least 27 different enzymes are produced industrially by the Aspergilli. Different species, mainly but not exclusively, of the *A. niger*, *A. oryzae*, and *A. sojae* groups have been optimized for the production of specific enzymes. In some cases, increased production has been achieved through proprietary recombinant procedures, which allows an increase in the copy number of homologous and in a few cases heterologous enzyme genes. Chymosin (rennin) is an enzyme essential for cheese production, which prior to its heterologous production by *A. niger var. awamori* (and other microorganisms), had to be extracted from calf's stomach. The stunning efficiency of some of the Aspergilli in the process of enzyme secretion (>20 g l^{-1}), the considerable experience of the fermentation industry, and the fact that many procedures involving Aspergilli are generally regarded as safe (GRAS) had suggested that the Aspergilli could be used as 'cell factories' for the production of heterologous proteins. This has been successful for some recombinant enzymes (chymosin, lipase, and phytase), but not for high valued, medically important mammalian proteins. Lactoferrin is produced in commercial quantities by recombinant strains of *A. awamori*. More research is needed to understand why some filamentous fungi are so efficient at secreting many fungal proteins but are inefficient as heterologous hosts. Tissue plasminogen activator and interleukin have been experimentally produced at a rate of 12–25 mg l^{-1} in a protease-less mutant of *A. niger*. A number of bottlenecks, such as specificity of glycosylation and the onset of the unfolded protein response by the translation of foreign proteins, are under active investigation.

Aspergillus and Production of Organic Acids

Depending on culture conditions, strains of *A. niger* are able to excrete a number of organic acids such as oxalic (used in metal leaching), citric, and itaconic acids and are thus used in their industrial production. Citric acid, a tricarboxylic acid, is an intermediate of the Krebs cycle. It is used in the food, beverage, and pharmaceutical industries. The annual production of citric acid, quoted for 2001, was 1 million tons. The main producing organisms are strains of *A. niger*. Since the ability of the organism to divert its metabolism to the production of citric acid was detected, industrial strains, that can convert over 90% of the carbon source in the culture media (carbohydrates) into citric acid were selected. Industrial carbon sources are low-grade molasses (typically sugar beet), but in principle many other residues of industrial process could be used. Specific culture conditions such as high concentrations of carbon source, low pH, and limitation of ions such as manganese are essential. It is not clear how the metabolism of the organism is diverted to citric acid overproduction. The production of citric acid implies that there is a bottleneck in the Krebs cycle, so that much more citric acid is produced than that is utilized in the cycle. Citrate itself inhibits phosphofructokinase I, the enzyme that catalyzes the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate, a crucial step in glycolysis. It has been proposed that under the culture conditions used, this inhibition is counteracted by other metabolites, thus removing this feedback inhibition of citrate production. Alternatively or additionally, tricarboxylic acid mitochondrial transporters leak out citrate from the mitochondrion, thus depleting the cycle.

Itaconic acid is a dicarboxylic acid, which is used in industry as a precursor of polymers used in plastics, adhesives, and coatings. New uses of itaconic acid-derived polymers are under active investigation. The production of itaconic acid for 2001 was quoted as 15,000 tons. There is a renewed interest in this chemical as industry searches for substitutes of petroleum-derived chemicals. Virtually all itaconic acid produced is by fermentation by specific strains of *A. terreus*. Itaconic acid production is a further perversion of the Krebs cycle, citrate is converted as normally into *cis*-aconitate, which for reasons unknown is, in some organisms, decarboxylated into itaconitate, which has no known metabolic role in the cell.

The fact that different strains of *Aspergillus* and more generally of fungi can divert metabolic pathways to the overproduction and secretion of useful chemicals, coupled with the fact that these organisms can grow on residues of processes such as sugar and ethanol production, open the possibility of engineering pathways to produce high value chemicals through 'green', low polluting, waste-eliminating procedures.

Medically Useful Secondary Metabolites

Of the useful fungal secondary metabolites, the most well-known are the β-lactam antibiotics, penicillin and cephalosporin, and their derivatives. Some Aspergilli, such as *A. nidulans*, produce low titers of isopenicillin N. This has been useful in the elucidation

of the genomic organization and regulation of the pathway. It has already been mentioned above (see Section "Aspergillus as Human Pathogens") that the echinocandins are specific antifungal drugs. Anidulafungin is a semisynthetic derivative of echinocandin B_0, produced by *A. nidulans* var. *echinulatus*. Anidulafungin has been recently introduced in clinical practice and it is specifically indicated to treat *Candida* infections of the digestive tract. The most widely used secondary metabolite produced by an *Aspergillus* is lovastatin, produced by *A. terreus*. This metabolite, as other statins, is used in medical practice to reduce cholesterol levels. The market for statins has been estimated at more than 12 billion US$ annually. Statins are specific inhibitors of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which reduces HMG-CoA to mevalonate. Statins are built around a common polyketide skeleton, have a structure similar to HMG, and act as competitive inhibitors of the HMG-CoA reductase. As other secondary metabolites, statins are synthesized by complex sequential steps, involving polyketide synthases, the genes coding for the cognate enzymes map in a 64 kb gene cluster. Gene clustering and its possible role in the synthesis of secondary metabolites is discussed in 'Regulation of secondary metabolism'.

A. (Emericella) nidulans as a Model Organism

The A. nidulans Genetic System

In 1953, Guido Pontecorvo published a 97-page review on the 'Genetics of *Aspergillus nidulans*'. Why did Pontecorvo and coworkers spend a considerable amount of time and energy to develop a genetic system for what was then an exotic organism? One of the key problems of biology was, at the time, that of the nature of the gene. The classical image of the gene was that of a discrete element, and mutations were considered to be alternative states of this element. The gene was an abstract concept whose molecular nature was elusive. The image of the gene as an indivisible, discrete unit was based on the experimental fact that mutations that did not complement were not separable in recombination experiments. That is, crosses of individuals carrying different alleles of the same gene, wild-type progeny were never obtained. 'Never' was a few dozen progeny in mice, a few thousands in *Drosophila melanogaster*. The modern reader may not grasp how fundamental the problem was at the time. In the 1940s, there were already exceptions to the 'nonrecombination' rule. In *D. melanogaster*, a few noncomplementing mutations could recombine in crosses to yield rare wild-type individuals. These mutations were called 'pseudo-alleles'. Pontecorvo was looking for a system where hundreds of thousands progeny could be scored. *A. nidulans* happened to be such an organism. By early 1950s, it became clear in Pontecorvo's laboratory, through the work of Alan Roper, followed by Bob Pritchard, that the gene was divisible. The paradigmatic work on the divisibility of the gene was carried out by Seymour Benzer using the bacteriophage T4.,"That is, that the gene as a working unit in physiological action is based on a chromosome segment larger than the unit of mutation or recombination" Roper (1953) and "The classical 'gene' which served at once as the unit of recombination, of mutation, and of function, is no longer adequate. These units require separate definition. A lucid discussion of this problem has been given by Pontecorvo" Benzer (1957). The phage system of Benzer was so powerful and elegant that *A. nidulans*, as a system to study the fine structure of the gene, seemed redundant. Nevertheless, a beautiful genetic system was there, ready for the taking. I dare say that in 1953 no system afforded the same degree of sophistication.

This system allows conventional meiotic genetics, carried out by analyzing the progeny contained in a fruiting body (cleistothecium). The cleistothecium may contain as many as 100,000 thousand asci, each containing eight ascospores, the product of a single meiosis and an additional mitosis. In the standard genetic analysis, the products are analyzed 'in bulk', without isolating single asci, as those are small and difficult to dissect. However, tetrad analysis is possible and was employed in early work. The power of resolution of the 'in bulk' genetic analysis has permitted fine structure mapping to the extent that mutations separated by 11 nucleotides have been resolved by recombination. *A. nidulans* strains carrying different markers can form heterokaryons. Nuclei in heterokaryons can rarely fuse, giving origin to stable diploids, which allow another layer of genetic analysis, developed by Pontecorvo and Etta Käffer, the parasexual cycle. Diploids can revert to haploids in which all markers in one chromosome segregate as a unit, allowing the rapid assignment to any new mutation to one of the eight chromosomes of the organism. Mitotic recombination also occurs and can be selected for in diploids, permitting the mapping of markers in relation to the centromere. The discovery of the parasexual cycle led to two developments. The first one was the possibility to carry out genetic analysis in the Aspergilli and the related Penicillia where a sexual cycle was not described. It is not known why stable diploids, different from the transient diploids that occur during the sexual cycle, can be obtained in these organisms and not in other filamentous ascomycetes. The second was the analogous development of systems, initiated by Pontecorvo, and based on cell fusion to carry out somatic genetics in mammalian cells. *A. nidulans* entered the molecular era when relatively efficient transformation techniques were worked out in 1983 followed by the development of replicating plasmids. We are witnessing a second methodological revolution, with the development of techniques and modified strains that allow to inactivate genes, introduce point mutations, change promoters, or add tags in a very simple and rapid way, opening the possibility of a high throughput reverse genetics. This, together with the availability of a complete genome and microarrays is changing again the prospects for this model organism. Usually a technique is first worked out in *A. nidulans*, and then that is applied to the other Aspergilli, such as the pathogenic or industrially important organisms mentioned in previous sections. The life cycle of *A. nidulans* is shown in Fig. 4.

Work carried out with *A. nidulans* has served as a model mainly in three aspects of cell and molecular biology (but a few others will be briefly discussed below). Historically, each of these three aspects can be related to a specific scientific school. David Cove and John Pateman initiated the investigation on control of gene expression in *A. nidulans*. The cell biology work derives from an
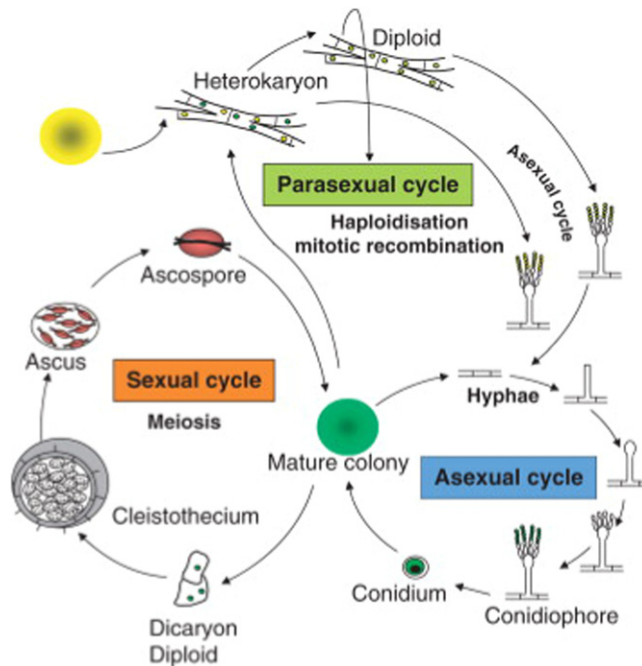
Fig. 4 Schematized life cycle of *Aspergillus nidulans.* Three cycles are shown, the asexual cycle, which has been described in the text (see A. *nidulans* as a model for cell biology and *A. nidulans* developmental pathways). In the sexual cycle, two nuclei divide synchronously as a dikaryon in a specialized structure. Eventually the nuclei fuse to give a diploid, which does not divide as such but undergo meiosis. No differentiated mating types exist; any given mycelium can generate fertile cleisthotecia. To cross two strains, nutritional and color markers are used. Classical genetics procedures are facilitated by the fact that one cleisthotecium derives from only one fertilisation event. In the parasexual cycle nuclei fuse in mycelia, outside the specialized developmental structure to yield a diploid, which does not undergo meiosis (at variance with diploids produced during the sexual cycle) but divides as such. Breaking up of the diploid (haploidization) and mitotic recombination are additional genetic tools. For clarity we have supposed that the heterokaryon and diploid formation are carried out between green (yA$^+$) yellow strains (yA$^-$) and nuclei are colored accordingly. Both diploids and heterokaryons can undergo the asexual cycle. The different structures are not shown to scale. Scheme kindly provided by Stéphane Demais and modified by the author.

early article by Ron Morris (1976) where he isolated mutations blocked in the cell cycle and in nuclear migration. Bill Timberlake initiated an analysis of the development of the conidiophore, work which profited from the early genetic work of John Clutterbuck, himself a product of the Glasgow school of genetics. Work on secondary metabolism stems from a confluence of this work with work carried out in other species of Aspergilli producing noxious chemicals.

The Mitochondrial DNA of *A. nidulans*

The possibility to construct heterokaryons allows the genetic study of mutations that occur in the mitochondrial genome, as these are cytoplasmically inherited. A few markers were characterized and a circular genetic map was established. In the late 1970s, two groups, led respectively by Hans Künzel and Wayne Davies, attempted to sequence the whole 33,000 bp mitochondrial DNA of *A. nidulans*. This was almost accomplished, except for a gap of around 200 bp. At the time, where the longest DNA sequenced was the 16,000 bp mitochondrial DNA of *Homo sapiens*, this was a less than trivial enterprise. A number of important results derived from this sequencing effort. It was possible to compare the whole DNA organization with that of the completely sequenced human mitochondrial DNA, and the ongoing sequence of the *S. cerevisiae* mDNA, an organism where sophisticated genetic and molecular studies were actively carried out. These comparisons were extended to other mitochondrial DNA sequencing projects such as those of *Neurospora crassa*, *Podospora anserina*, and *Schizosaccharomyces pombe*. A number of unidentified open reading frames (ORFs) were found in the mitochondrial DNA of *A. nidulans* and the human mitochondrial DNA, but not on that of *S. cerevisiae* or of *S. pombe*. These reading frames correspond to genes coding for complex I, the NADH dehydrogenase complex, which is absent in both model yeasts. The highlight of this work was the elucidation of the structure of class I introns. Metazoan mitochondrial genes have no introns, while introns of two different classes are present in the mitochondria of fungi and plants. Introns on the nuclear genome of the fungi are small, typically of >100 bp. Mitochondrial fungal introns are large, usually >1000 bp, and can contain ORFs. A comparison of the sequences of the mitochondrial introns of *A. nidulans* with those of wild-type and mutant strain of *S. cerevisiae* led to a model of the secondary structure of class I introns, including the proposal of a mechanism of intron splicing. Around the same time, it was published that the intron of the precursor of the ribosomal 26s gene of *Tetrahymena thermophila* was self-splicing. It was noticed that pairings that were postulated by us and by Bernard Dujon and François Michel for mitochondrial
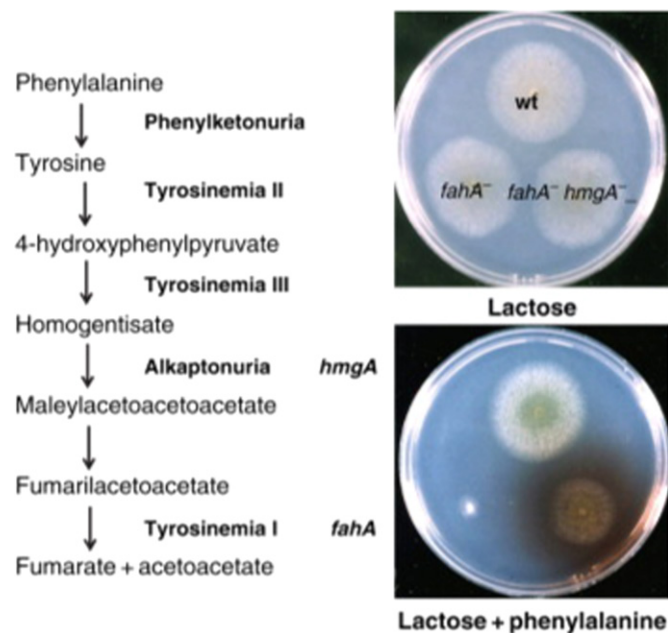
Fig. 5 *Aspergillus nidulans* as a model for human metabolic diseases. To the left the degradation of phenylalanine is shown, to the right of the pathway, the steps blocked in the cognate human metabolic diseases. In italics the relevant corresponding genes of *A. nidulans* are shown. To the right the toxicity of fumaryl-acetoacetate and the suppression of the toxicity by the upstream *hmgA* null is shown together with the secretion of the purple oxidation product of homogentisic acid. Lactose is used as a poor, nonrepressing carbon source, as phenylalanine catabolism is subject to carbon catabolite repression (see Section "Nitrogen and Carbon Utilization"). See text for details. Photographs of plates were kindly provided by Miguel Peñalva.

group I introns were conserved in this self-splicing intron; thus the model for splicing of mitochondrial class I introns became a model of self-splicing, which was confirmed experimentally. Self-splicing of introns was essential to the concept of ribozyme and eventually to that of a primeval RNA world. Thus, the sequence of the mitochondrial DNA of *A. nidulans* contributed, albeit somewhat indirectly, to present ideas on the origin of life.

A. nidulans as a Model for Genetic Metabolic Diseases

The metabolic versatility of the Aspergilli led a group of Spanish scientists to use mutants blocked in amino acid degradation to identify the enzymes and the genes of human metabolic diseases, including those of aromatic and branched amino acid catabolism. As stated in a review article "The metabolic capacity of *A. nidulans* for amino acid degradation largely resembles that of human liver". Fig. 5 shows the breakdown of phenylalanine and the cognate blocks in human diseases affecting this metabolism. The gene of *A. nidulans* coding for the fumaryl-acetoacetate hydrolase was cloned as a cDNA highly expressed in the presence of phenylacetic acid (*fahA*, Fig. 5). Mutations in the human homologue result in the serious disease tyrosinemia I, and the *A. nidulans* ORF shows 47% identity with the human gene. Satisfactorily, the growth of *A. nidulans* is strongly inhibited by the accumulation of this metabolite in *fahA*-deleted strains. In a second step, suppressor mutations of this inhibited phenotype were isolated. These pinpointed the gene (*hgmA*) coding for homogentisate dioxygenase. Not only these mutations suppressed the toxicity of phenylalanine seen in *fahA* nulls, they also resulted in the accumulation of a purple pigment (see Fig. 5). This is exactly the pigment that is accumulated in the urine of patients affected by a milder disease, alkaptonuria. The identification of the gene was straightforward and its sequence served to identify the hitherto unknown human gene and to identify the loss-of-function mutations present in a number of patients. Alkaptonuria was identified by Garrod in 1902 as an 'inborn error of metabolism' and shown to be inherited as single Mendelian gene. The work of Beadle and Tatum in *N. crassa*, and the one gene-one enzyme proposal arising from it, can be seen as a completion of Garrod early proposals. The identification of the human gene through the cloning of the *A. nidulans* gene is of more than historical importance and underlines the necessity of choosing the correct model system for a particular problem.

Control of Gene Expression

Nitrogen and Carbon Utilization

The Aspergilli can utilize a large number of metabolites as nitrogen and/or carbon sources. Early work with *A. nidulans* has established some fundamental concepts pertaining to the control of gene expression and metabolic regulation. Some of the first

eukaryotic pathway-specific regulatory genes (*nirA*, *uaY*; see below) were characterized in the 1960s. The genes encoding the key regulators for nitrogen (*areA*) and carbon catabolite repression (*creA*) were the first such genes to be described in any eukaryotic organism. Their mode of action was established by formal genetic analysis long before recombinant DNA technology came into existence.

In general, the genes coding for the enzymes involved in the utilization of a specific metabolite are only transcribed in the presence of a specific inducer. Inducers act by activating specific transcription factors, which in turn elicit the transcription of specific catabolic genes. Thus, nitrate activates the NirA protein, necessary for the transcription of the genes encoding nitrate and nitrite reductase and the nitrate transporters, acetaldehyde activates AlcR, regulating ethanol utilization, uric acid activates UaY, regulating at least eight scattered genes encoding enzymes and transporters involved in purine utilization, proline activates PrnA, regulating all the other genes of the proline utilization gene cluster, while *β*-alanine activates AmdR/IntA a protein that positively regulates the *amdS* gene (encoding acetamidase) and the *gabA* gene (encoding the *γ*-aminobutyrate transporter).

Almost all the pathway-specific transcription factors belong to a group of proteins that bind DNA through a specific fungal motif, the Zn binuclear cluster (Cys6Zn2). Most bind DNA as dimers, including the paradigmatic *S. cerevisiae* protein GAL4. AlcR is an exception, which uniquely binds as a monomer. NirA and UaY are localized in the nucleus as a result of induction. Nitrate induces by breaking the association of NirA with KapK (the orthologue of the mammalian and *S. cerevisiae* exportins Crm1P and CRM1). PrnA and AlcR are always nuclear, PrnA necessitating induction to bind its cognate sequences in the promoter.

The induction of genes involved in the utilization of nitrogen sources does not occur in the presence of preferred sources such as ammonium and glutamine, while the induction of genes involved in the utilization of carbon sources is strongly diminished in the presence of glucose. These processes, nitrogen metabolite repression and carbon catabolite repression, involve two additional regulators, AreA and CreA. AreA is a GATA factor, acting positively in synergy with the specific regulators (such as NirA or UaY). Ammonium and glutamine negate AreA function at a number of levels, including the stability of its cognate mRNA. The dependence on AreA is absolute for the *niiA-niaD* bidirectional promoter, driving the genes encoding nitrate and nitrite reductases, less marked for some of the genes of the purine utilization pathway.

CreA acts as a genuine repressor in the presence of favored carbon sources, negating the activation by or competing with the binding of the pathway-specific factors such as AlcR.

CreA is a Zn finger protein, with a Zn finger sequence extremely similar to Mig1p, the repressor mediating carbon catabolite repression in *S. cerevisiae* and related organisms. However, the similarity between CreA and Mig1p stops there. Little sequence conservation can be seen outside the DNA-binding domain. Neither the glucose signaling mechanism nor the downstream mechanism of transcriptional repression seems to be shared by Mig1p and CreA. Mig1 represses transcription by recruiting the Tup1/Ssn6p co-repressor complex, which is not the case in *A. nidulans* and most likely in the fungi where a CreA, rather than a Mig1p orthologue, is present.

The Aspergilli can use a number of metabolites as both carbon and nitrogen sources, the mechanism of regulation having been elucidated for the *prn* gene cluster (comprising five genes involved in the utilization of proline) and the *amdS* gene. Repression occurs only when both repressing carbon (glucose) and nitrogen sources (ammonium or glutamine) are present. This can be rationalized by thinking that if a repressing nitrogen source is present, it will be advantageous for the organism to use proline or acetamide as a carbon source, while if only a favored carbon source is present, it will still be advantageous to use proline or acetamide as a nitrogen source. Carbon metabolite repression requires the CreA repressor, while nitrogen metabolite repression operates through the inactivation of the AreA GATA factor. While, for example, in the nitrate assimilation pathway AreA is always essential for transcription to occur, for *prn* and *amdS*, it is only necessary when the CreA repressor is activated by a repressing carbon source. These regulatory patterns are conserved in the Aspergilli and more generally in the filamentous ascomycetes and are schematized in **Figs. 6–8**, while the nuclear-cytoplasmic shuffling of NirA is illustrated in **Fig. 9**. The *gabA* gene, encoding the *γ*-aminobutyrate transporter, is subject to an even more complex pattern of regulation. It is induced by *ω*-amino acids and subject to concomitant repression by nitrogen, carbon, and alkaline pH.

Regulation of Gene Expression by External pH

Soil organisms, such as the Aspergilli, respond to a variety of environments and it is not surprising that a system that regulates gene expression as a function of external pH has evolved. External pH regulates genes coding for extracellular enzymes or transporters or those encoding steps in the synthesis of exported metabolites. In neutropenic mice experimentally infected with *A. nidulans*, this process is necessary for virulence. Penicillin is synthesized by some Aspergilli but only at alkaline pH. The synthesis and uptake of siderophores is also regulated by pH.

The elucidation of the mechanism of pH regulation is a superb scientific achievement of the groups of Herb Arst and Miguel Angel Peñalva. The signal transduction pathway described below is conserved throughout the ascomycetes. The key actor is PacC, a transcription factor of the classical Zn finger type. In its active form, PacC acts as a positive transcription factor of alkaline-expressed genes (such as alkaline phosphatase or isopenicillin synthase) and as a repressor of acid-expressed genes (such as acid phosphatase or the *γ*-aminobutyrate transporter). At acidic pH (pH usually tested 4.0), there is no activation signal, and the protein is in an inactive form. In the full-length PacC (PacC[72]), intramolecular interactions hold the protein in a folded inactive form, which is largely excluded from the nucleus. At alkaline pH values (usually 8.0), the protein is activated by two proteolytic steps. The *palA*, *palB*, *palC*, *palF*, *palH*, and *palI* genes encode proteins involved in pH sensing and in signal transduction. Mutations in all these *pal* genes have an acidity-mimicking phenotype, while mutation in the transcription factor *pacC* can lead to acidity mimicking (loss-of-function mutations), alkalinity-mimicking or neutrality-mimicking phenotypes, where both 'alkaline' and 'acidic' genes are expressed. The pH sensor is probably PalH assisted by PalI, both of
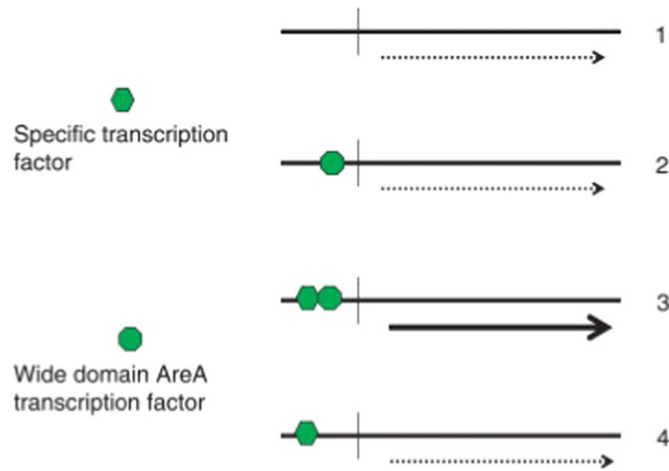
Fig. 6 General scheme of the transcriptional regulation of genes involved in the utilization of nitrogen sources. (1) In the absence of a specific inducer (such as nitrate) and in the presence of a preferred, repressing nitrogen source (ammonium, glutamine), neither the specific transcription factor nor the broad-domain GATA factor AreA is activated. No or only basal transcription is seen. (2) In the absence of a specific inducer in the presence of a nonrepressive nitrogen source, only AreA is active. No or only basal transcription is seen. (3) In the presence of a specific inducer and in the absence of a repressing nitrogen source, both transcription factors are active, full transcription is seen. (4) In the presence of both a specific inducer and a repressing nitrogen source, the specific transcription factor is active, but the AreA factor is inactive and no transcription is seen. In the nitrate utilization pathway a further mechanism is in act, as AreA is necessary both indirectly through its regulation of transporters for the uptake of the specific inducer (nitrate) and for the binding of the specific transcription factor (NirA) to DNA. Thus, the situation will be identical to that seen in Scheme 1, at the top of this figure.
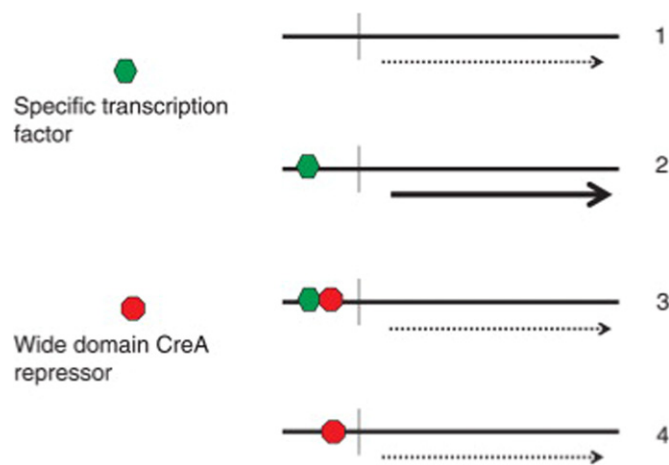

Fig. 7 General scheme of the transcriptional regulation of genes involved in the utilization of carbon sources. (1) In the presence of 'neutral' carbon source (such as glycerol) and the absence of an inducing carbon source, neither the specific positive-acting transcription factor nor the CreA repressor are bound to the promoter. No or only basal transcription is seen. (2) In the presence of an inducer carbon source, the specific transcription factor (such as AlcR in the ethanol utilization pathway) is bound to DNA and active, full transcription is seen. (3) In the presence of both inducing and repressing carbon sources, the specific transcription factor is active but the CreA repression partially or totally negates its effect. No or only basal transcription is seen. (4) In the presence of only a repressing carbon source, only the CreA repressor is bound to DNA, no or only basal transcription is seen.

which are plasma membrane proteins. The C-terminus cytoplasmic tail of PalH interacts directly with PalF, a member of the arrestin family, which is, similarly to the mammalian arrestins, phosphorylated and ubiquinated. These modifications occur at alkaline pH and are dependent on the PalH and PalI proteins. Under alkaline pH conditions, PalA binds to motifs flanking a specific protease-sensitive sequence in the C-terminus of the full-length PacC (PacC[72]). PalA interacts directly with the A. nidulans orthologue of Vps32, a protein involved in the formation of multivesicular endosomes. PacC/PalA interaction renders PacC sensitive to a specific cleavage, catalyzed by PalB, a protease of the calpain family. PacC[72] is cleaved to PacC[53]. The cleaved form of PacC becomes susceptible to further processing by the proteasome, yielding PacC[27]. This is the active form of PacC, strictly localized in the nucleus, where it activates genes expressed at alkaline pH and represses genes expressed at acid pH. This account leaves open the mechanism of pH sensing and the connection between the arrestin-like PalF and PalA-PalB. The interactions of PalA with components of the mature endosome, and recent work on the related signal transduction pathway in *S. cerevisiae*, strongly suggests that endocytosis provides this connection. PalC, the unplaced actor of the process, has a functionally important Bro1 domain (also present in PalA), a domain of possible interaction with Vps32, strengthening the
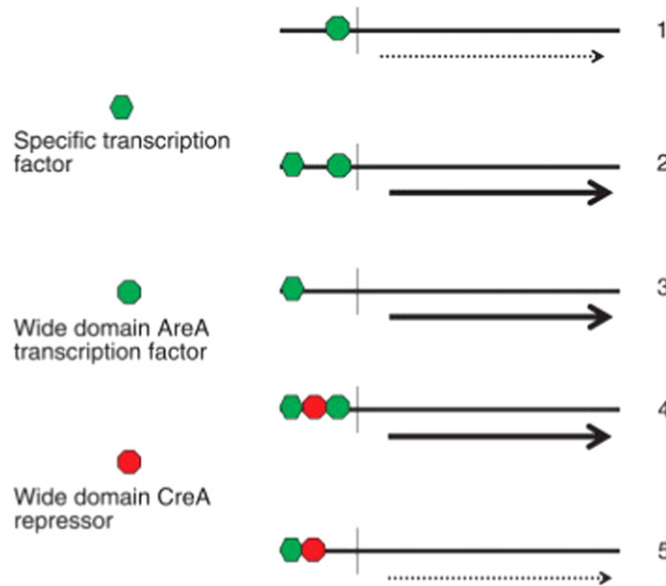
Fig. 8 General scheme of the transcriptional regulation of genes involved in the utilization of metabolites that can serve as both nitrogen and carbon sources. (1) In conditions where the inducer is not present the specific transcription factor (PrnA in the proline utilization gene cluster) is not bound to the promoter. In the scheme shown, under neutral conditions (e.g., urea as nitrogen source, lactose as carbon source) AreA would be bound, and CreA would not be bound. No or only basal transcription is seen. This applies to every other combination (not shown) where the specific inducer is absent. (2) Same conditions but in the presence of the inducer (proline in the example given in the text), both the specific transcription factor (such as PrnA) and AreA are bound, full transcription is seen. (3) In the presence of the inducer and a repressing nitrogen source (ammonium or glutamine) but no repressing carbon source. Only the specific transcription factor is bound. Full or almost full transcription. (4) In the presence of the inducer and a repressing carbon source (glucose), but no repressing nitrogen source. The three regulatory proteins are bound; AreA negates the repressing action of CreA. Full or almost full transcription. (5) In the presence of inducer (such as proline) and both carbon and nitrogen repressing metabolites (ammonium or glutamine and glucose). The specific transcription factor (such as PrnA) is bound and CreA negates its action. Efficient repression. No or only basal transcription is seen.
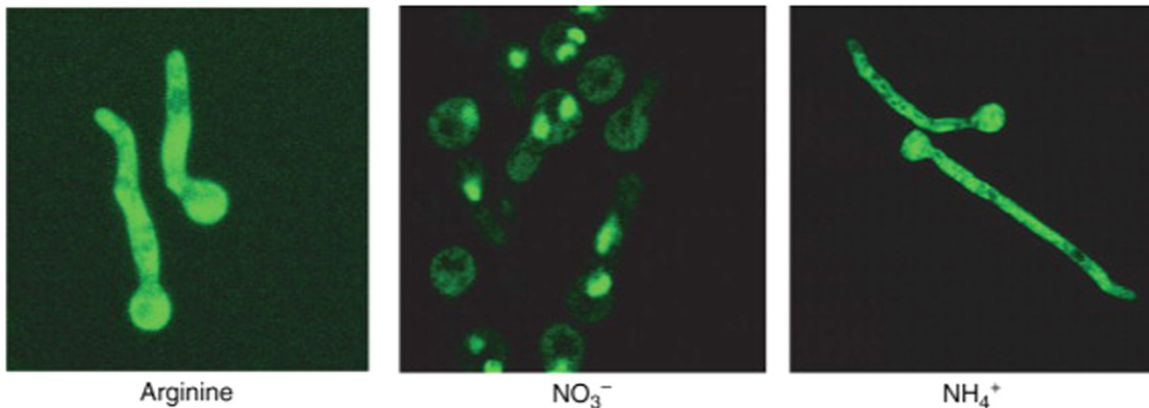

Fig. 9 Cytoplasmic and nuclear localization of the NirA transcription factor. A construction where the whole NirA transcription factor is fused to green fluorescent protein (GFP) substitutes the NirA wild-type gene. This construction is competent to mediate induction by nitrate. NirA is localized in the cytoplasm in the presence of a noninducing, nonrepressing nitrogen source (arginine), of a repressing nitrogen source (ammonium) and localizes in the nucleus only when an inducing nitrogen source is present. See text for details. This figure illustrates also the technology of gene fusions and epifluorescence microscopy, which is been extended to all other Aspergilli and many filamentous fungi, including animal and plant pathogens. The original pictures were kindly provided by Joseph Strauss.

endosomal connection of the pH signaling pathway. The YPXL/I motif recognized by PalA is also recognized by its putative mammalian orthologue, AIP1/Alix, a protein involved in a variety of functions, including the budding of the human HIV virus from infected cells. The whole process has tantalizing similarities with the Hedgehog signaling pathway in metazoans, leading to the proteolytic activation of the Zn finger transcription factor *cubitus interruptus*/Gli, posing the question of whether these pathways are evolutionarily related. A simplified version of the pH signaling process is shown in Fig. 10.
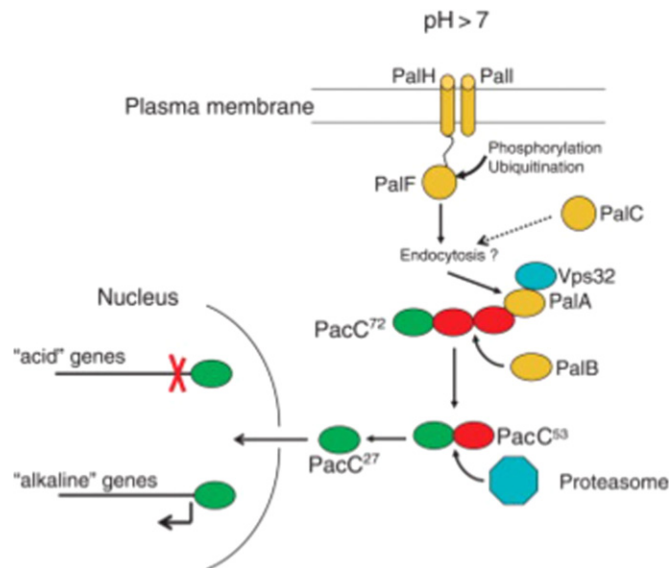
Fig. 10 Simplified scheme of the regulation of gene expression by external pH, redrawn from a number of articles of the groups of Arst and Peñalva. Alkaline pH is sensed by the PalH and PalI proteins, the signal is transduced to the PalF arrestin via the C-terminus of PalH. PalF is phosphorylated and ubiquinated and signals PalA, which has been shown to interact with the endosomal protein Vps32. The role of PalC is hypothetical. PalA leads to the opening of PacC72, which is cleaved by PalB at a specific site. PacC53 is further processed by the proteasome, leading to the active form PacC27. In this simplified scheme both proteolytic processing steps are shown in the cytoplasm, in fact the second step may occur in either or both the cytoplasm and the nucleus. In yellow, proteins of the pH signal transducing pathway, in blue cellular proteins or complexes nonspecific for the pathway. The 'active' portion of PacC is shown in green, the inhibitory, cleaved portions are shown in red.

Specific Regulatory Mechanisms Acting at the Level of Transporters

The control of transporter synthesis and activity is a key step in metabolic regulation, as the activity of specific transporters modulates the entry of metabolites that serve as inducers or repressors of specific pathways. Work with *A. nidulans* has led to the identification of two new control processes affecting transporters, besides their tight specific transcriptional regulation. The transcription of a number of transporters is activated during the isotropic phase of conidial germination (see below). This is a developmental control, which bypasses other specific control systems. Recent transcriptomic work suggests that this mechanism occurs for many transporters and is general for the filamentous ascomycetes. It can be proposed that germinating fungal spores explore an unknown environment by expressing a whole range of transporters, to progress to specific induction once the spore has germinated. The second mechanism is posttranslational. In the presence of a favored nitrogen source such as ammonium, both purine and amino acid transporters are internalized to the vacuole, where they are possibly destroyed. This posttranslational mechanism is synergistic with but independent from the nitrogen metabolite repression mechanism (described above). Fig. 11 illustrates this process.

Regulation of Secondary Metabolism

Fungi produce an astonishing variety of secondary metabolites. Fungal toxins, the β-lactam antibiotics and lovastatin, have already been mentioned. In the pregenomic days, conventional genetic analysis led to the identification, cloning, and sequencing of a number of genes encoding biosynthetic steps for a number of secondary metabolites, while many more metabolites were identified as secreted by a variety of Aspergilli. As many secondary metabolites involve nonribosomal peptide or polyketide synthases, putative fungal metabolite gene clusters have been identified in a number of fungal genomes. In *A. fumigatus*, the estimate is that of 22 secondary metabolite gene clusters. The best studied pathways are those leading to the biosynthesis of isopenicillin in *A. nidulans*, aflatoxin in *A. flavus*, and the aflatoxin precursor sterigmatocystin in *A. nidulans*. Secondary metabolism synthesis occurs late during mycelial growth and is generally correlated with conidiation and shares with this process some of its signaling pathway. Pathway-specific transcription factors have been characterized for the aflatoxin, sterigmatocystin, and gliotoxin pathways. The clusters of aflatoxin and sterigmatocystin biosynthesis include the regulatory gene *aflR*, necessary for the expression of the rest of the genes of the cluster. AflR belongs to the Cys_6Zn_2 family of specific fungal activators. It is not known whether the activation of AflR involves a specific metabolite or if it is only activated by the 'fluffy' signaling pathway to be described below (*A. nidulans* developmental pathways). No pathway-specific activator has been described for isopenicillin biosynthesis, which is regulated by a number of environmental parameters, including extracellular pH.

Clustering of genes is variable for genes involved in primary metabolism. In contrast, the genes of secondary metabolism biosynthesis are as a rule organised in large clusters. The 70 kb aflatoxin cluster comprises 25 coregulated genes. The gliotoxin gene cluster comprises 12 genes. Does the clustering of secondary metabolism genes have an evolutionary and/or functional
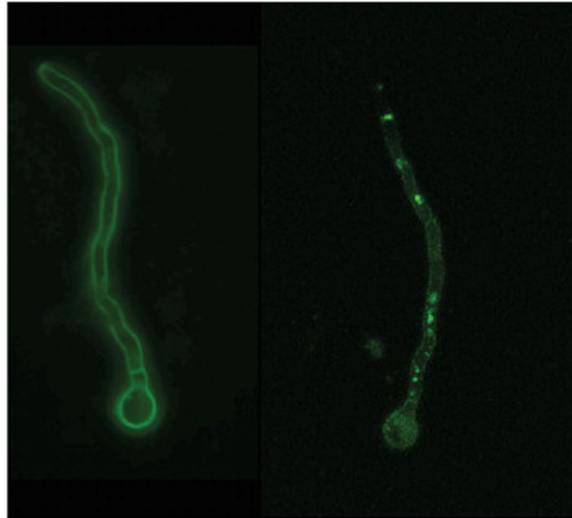
Fig. 11 Posttranslational regulation of transporters. The left panel shows the membrane localization of a fusion with the green fluorescent protein of the proline transporter (PrnB-GFP). The cognate gene *prnB* maps in the proline gene cluster, its transcriptional regulation is schematized in Fig. 8 (see Section "Nitrogen and Carbon Utilization"). Conidiospores were grown for 16 h at 25°C in the presence of urea as nitrogen source, and induced with L-proline after 10 h. In the right-hand panel ammonium was added for the last 2 h. In the left panel, the PrnB-GFP fusion strongly stains also the basal septum. Localization in septa is a characteristic of all membrane proteins studied up to now. Confocal microscope images were kindly provided by Vicky Sophianopoulou.

significance? Possibly the two divergently transcribed genes responsible for isopenicillin-N synthesis have been horizontally transferred from a *Streptomyces* to an ancestor of the Aspergilli and Penicillia. There is no evidence for horizontal transfer for any other secondary metabolite gene cluster. Comparative genomics is providing some clues, even if not yet an answer, to the significance of secondary metabolite gene clustering. There is a significant bias toward the location of secondary metabolite clusters in subtelomeric regions. The fact that species of Aspergilli differ widely in the secondary metabolites they produce correlates with the mapping of the cognate genes in genomic regions where synteny between species is broken.

A fundamental advance in the understanding of the regulation of secondary metabolism arises from the discovery of the global regulator LaeA in the laboratory of Nancy Keller. LaeA is conserved in filamentous fungi, but not in yeasts. LaeA shows a domain typical of histone methyltransferases, the SAM domain, while lacking a second domain found in these enzymes, the SET domain. LaeA regulates positively the synthesis of isopenicillin, sterigmatocystin, gliotoxin, and lovastatin. The global role of LaeA has recently been investigated by transcriptomic studies with *A. fumigatus*. Of the 22 gene clusters, a deletion of *laeA* diminishes clearly the transcription of 13. Thus LaeA is a broad, but not a universal regulator of secondary metabolism. Recent work points to a role of LaeA in remodeling chromatin structure. In *A. nidulans*, deletion of a number of genes universally involved in gene silencing in heterochromatin result in premature secondary metabolite production. More strikingly, these deletions act as partial suppressors of a *laeA* deletion. Thus the exciting possibility arises that LaeA acts by reversing a heterochromatic state of the secondary metabolite gene clusters. Thus the study of the regulation of secondary metabolism may lead to an understanding of the role and genomic distribution of heterochromatin in filamentous ascomycetes.

A. nidulans as a Model for Cell Biology

The life cycle of the Aspergilli includes a number of tightly regulated developmental pathways, from the germination of conidia or ascospores to the formation of complex structures involved in sexual (cleistothecia) or asexual (conidiophore) spore formation. The germination of conidiospores, but not that of ascospores, has been well studied. Conidiospores can stay dormant and viable for many years and contain (in *A. nidulans*) one nucleus arrested in the G1 phase. When plated on suitable media, they go through a phase of isotropic growth, where the conidium swells. The first mitosis may occur in this phase or after the emergence of the germ tube (Fig. 12). Mitosis occurs synchronically, up to the eight nuclei stage when a perforated septum appears basally (Fig. 11). Other septa are laid during hyphal growth out every – three to four nuclei. Only the nuclei comprised between the septum and hyphal tip are competent to divide and they do so synchronously. A second germ tube can arise from the conidiospore at 180° from the first one. Nuclei in nonapical compartments became again competent to divide when the conidiophore is developed (see below) and when branches arise from subapical compartments. Thus a highly coordinated process occurs, involving the regulation of mitosis, the establishment of a primary polar axis, the establishment of secondary polar axes in branches, nuclear migration, the laying down of septa and finally the appearance of another highly polarized structure, the conidiophore. Some processes, such as hyphal polar growth and the
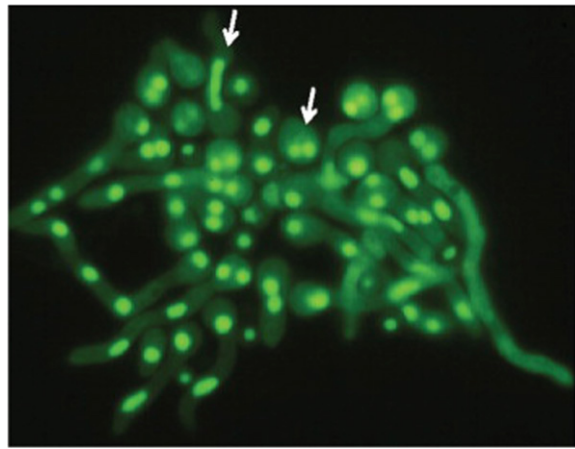
Fig. 12 Conidial germination. A group of germinating conidia from A. nidulans are shown. They are stained with the green fluorescent protein (GFP) fused to a strong nuclear localization signal, driven by a strong constitutive promoter. One white arrow indicates a conidia where the first mitosis has occurred before the production of the germinal tube, another mitosis occurring concomitantly with germination. Note that the signal is not lost during mitosis, which as in other fungi is closed. Photograph by Ana Pokorska in the laboratory of the author.

deposition of septa, are specific of fungi, while others are common to all eukaryotes, and the A. nidulans work matches and has added considerable information to the work carried out in S. pombe and S. cerevisiae. In both yeasts and most cells in higher eukaryotes, mitosis is followed by cytokinesis, where the two daughter cells separate. This is not exactly the case in filamentous fungi, where the whole mycelium is one syncytium, subdivided by perforated septa. It must be stressed that during conidiogenesis the situation resembles budding, with proper cytokinesis, as metullae, phialides, and conidia are uninucleate cells, while ascospores are binucleate (see 'A. nidulans developmental pathways'). Thus an understanding of Aspergillus cytokinesis involves understanding the generation of these different patterns. The determination of hyphal polarity and the related problem of the relationship of mitosis with septum formation are active fields of research at present, and recent work has shown that while some of the determinants of polarity and cytokinesis are common with the yeasts, some are entirely novel. In particular, a specific ceramide synthase is essential for polarity, probably by generating specific lipid rafts at the growing tip, which in turn would be involved in the localization of other polarity determinants such as formin, an actin nucleating protein. Particular to filamentous fungal growth is the Spitzenkörper, a subapical organelle that acts as a vesicle supply center. The challenge for future research is to understand the coordination of signaling pathways, the polarization of the actin cytoskeleton, the formation of lipid rafts, and the activity of the Spitzenkörper to reach a complete understanding of polarity determination.

Some of the highlights of the work relating to the cell cycle are indicated below, where A. nidulans has served as an eukaryotic model, while some specific aspects of Aspergillus development are summarized in 'A. nidulans developmental pathways'.

The judicious use of mutants resistant to the tubulin inhibitor benomyl led to the identification of the first α- and β-tubulin-encoding genes in any organism. It was then shown that the tubulins are involved in nuclear and chromosomal movement. The crowning of this work was the discovery of γ-tubulin by Berl and Liz Oakley. A benA (encoding one of the isoforms of β-tubulin) temperature-sensitive mutant, benA33, results in microtubules that are hyperstable (rather than nonfunctional) at the non-permissive temperature. Three suppressors of benA33 mapped in a gene that when cloned and sequenced was shown to code for a new tubulin. This tubulin is critical for the nucleation of microtubules in all eukaryotes where it has been studied. The establishment of the function of γ-tubulin illustrates the use of A. nidulans as a model organism. While the inactivation of the cognate gene is lethal, the mutation could be maintained in a heterokaryon (see Section "The A. nidulans Genetic System"). As conidia are uninucleate, heterokaryons will produce two types of conidia, one of which carries the disrupted allele, where the phenotype caused by the mutation during conidial germination can be assessed microscopically. The disruption does not affect germination, but blocks nuclear division and to some extent nuclear migration. DNA is replicated, chromosomes condense, but spindles are not assembled. Thus, work that started with the isolation of tubulin inhibitor-resistant mutants led to the discovery of a new tubulin, which in all organisms is crucial for microtubule nucleation in centrosomes and in fungi (which have a closed mitosis) in spindle polar bodies.

In the seminal Morris article of 1976, a large number of conditional mutants were characterized. These were temperature-sensitive mutants, which either failed to enter mitosis at the nonpermissive temperature (nim, never in mitosis), were blocked at different stages (bim, blocked in mitosis), or where the nuclei failed to migrate (nud, nuclear distribution), while sep mutants are defective in septum formation. Eventually, the cognate genes were cloned and sequenced, suppressors were isolated and identified, to give a growing picture of the genes involved in basic processes of cell biology.

Cellular motors of the myosin class associate with actin filaments, while kinesis and dyneins move cargo (vesicles and organelles) along microtubules. nudA encodes the dynein heavy chain, nudG the dynein light chain, while other nud mutants

defined hitherto undescribed regulatory proteins of the dynein complex. In particular, *nudF* encodes a close homologue of the human protein LIS1, which is mutated in Miller–Dicker lysencephaly, a human hereditary disease of the nervous system where neurons fail to migrate in the hemizygote. NudC, a protein that interacts with NudF is also conserved from fungi to mammals. It is likely that the primary effects of NudC/NudF in organisms with an open mitosis are in cytokinesis, a role obviously that can only be partially conserved in a syncytial organism with a closed mitosis such as *A. nidulans*. This pioneering work, which exploited both the *A. nidulans* genetic system and its specific morphology, has guided the work leading to the understanding of the function of the dynein complex in the nervous system.

At variance with dyneins, kinesin genes are highly redundant and only one was identified through mutant screens. This is *bimC*, which defines a specific class of plus-end conserved kinesins. Mutants in this gene are defective in spindle pole separation and are thus blocked in nuclear division and provided the first direct evidence the kinesins are involved in mitosis.

In the genetic screen, no mutants blocked in G1 were found, mutants blocked in the S-phase map at five loci, others blocked in the transition of G2 to mitosis map at six loci. Among the genes so defined, some are orthologues of genes previously known from *S. pombe*. *nimX* (not identified in the screen) encodes the orthologue of the cyclin-dependent *S. pombe* cdc2 kinase. The homologue of the cdc13 cyclin B is encoded by *nimE*, while the phosphatase activity necessary for the activation of NimX^{cdc2} is encoded by *nimT*. NimA, on the contrary, is a newly discovered serine/threonine kinase, which defines a whole class of proteins conserved throughout the eukaryotes. NimA functions downstream of NimX^{cdc2}/cyclin B, which would then have two independent functions, one to promote spindle formation, through the activation of other kinases, the second to activate NimA, which in turn is necessary for chromosome condensation. NimA is necessary for entry into mitosis, mutants showing duplicated spindle polar bodies, while its destruction by proteolysis is necessary for exit from mitosis. There is a considerable evidence for similar roles in mitosis for NimA homologues in higher eukaryotes. A human protein, Pin1, interacting with NimA was identified in a two-hybrid screen. Pin1 mutants have a phenotype reciprocal to that of NimA mutants, suggesting that Pin1 (PinA in *A. nidulans*) is involved in the inactivation of NimA. Pin1 is a universally (in eukaryotes) conserved peptidyl-prolyl isomerase that catalyzes specifically the isomerization of prolyl bonds in a P-Ser/Thr-Pro dipeptide, increasing its rate by about 1000 times, thus allowing a drastic change in the peptide backbone conformation, NimA is only one of its substrates, another one being cdc2/cyclin B. It has recently been shown in HeLa cells that Pin1 is necessary for entry into mitosis, associates with mitotic chromosomes, and it strongly stimulates cdc2 phosphorylation. The discovery of Pin1 and its involvement in mitosis has led to flurry of activity, concerning its possible role in cancer, but more cogently in the onset of Alzheimer's disease. Mice homozygously deleted for the *Pin1* gene develop a neuronal degeneration with many of the histological characteristics of Alzheimer's. Both *tau*, a microtubule-associated protein, and APP (amyloid precursor protein) are phosphorylated at Ser/Thr-Pro motifs. These proteins are hyperphosphorylated and insoluble in Alzheimer's. It had been proposed that the key regulator of the state of these proteins is actually Pin1, which would displace the equilibrium toward the nonphosphorylated, soluble forms.

The anaphase-promoting complex (APC) is an ubiquitin ligase that targets key mitotic proteins such as cyclins and directs them to the proteasome. Mutants in its components will be expected to be blocked in metaphase and to show a *bim* phenotype. Two such components were first identified among the *bim* mutants. BimE was identified first as a negative regulator of mitosis. Biochemical work in *Xenopus* oocytes showed that a protein that copurified with APC (APC1) is the orthologue of BimE. *bimA* encodes the APC3 component. Once all chromosomes are attached to microtubules, APC activation results in degradation of securin. This releases and activates separase, a protease that cleaves cohesin. As cohesin keeps sister chromosomes together, this cleavage is the prerequisite for anaphase. *bimB* encodes separase. A component of cohesin, *sudA*, was identified as a suppressor of a *bimD* allele, which itself results in an anaphase block characterized for defective chromosome separation. Finally, mutations in *bimG* result in large, polyploid nuclei that fail to complete anaphase. Nuclei are clumped and conidia fail to germinate highlighting the link between the regulation of mitosis and the establishment of polarity. BimG is a phosphatase, showing striking identity with mammalian phosphatases of the PP1 class. BimG is localized to the spindle polar bodies, to the nucleolus, to the tip of the hypha, and transiently in the septum. There is no hint as to what are the substrates of BimG in the mitosis, septum formation, and polarity establishment.

A. nidulans Developmental Pathways

In the sexually reproducing Aspergilli, the mycelial mat can follow two different developmental pathways. Meiosis and the formation of asci that contain ascospores, occurs in specialized structures, the cleistothecia. In *A. nidulans*, mature cleistothecia are globose, darkly pigmented structures of 100–200 μm in diameter. Ascospores have a characteristic bivalve morphology (about 4 μm × 3.5 μm) showing two equatorial crests. Ascospore ornamentation is a valuable taxonomical character in the sexually reproducing Aspergilli. The protocleistothecium is generated from vegetative hyphae, which coil in a spherical structure developing into a cleistothecium, surrounded by specialized, modified hyphal cells called hülle cells. *A. nidulans* is homothallic; two genetically identical nuclei can fuse to give diploids which, as in all other filamentous ascomycetes, are immediately committed to meiosis. In heterothallic Aspergilli, the sexual cycle occurs only when nuclei of opposite mating types meet in heterokaryons. Some nonsexual Aspergilli (*A. flavus* and *A. parasiticus*) form structures, sclerotia, which may be developmentally related to the cleistothecium. Conceptually, we can distinguish two processes in the development of the mature cleistothecium. One is the morphological process that leads to cleisthotecia, surrounded by hülle
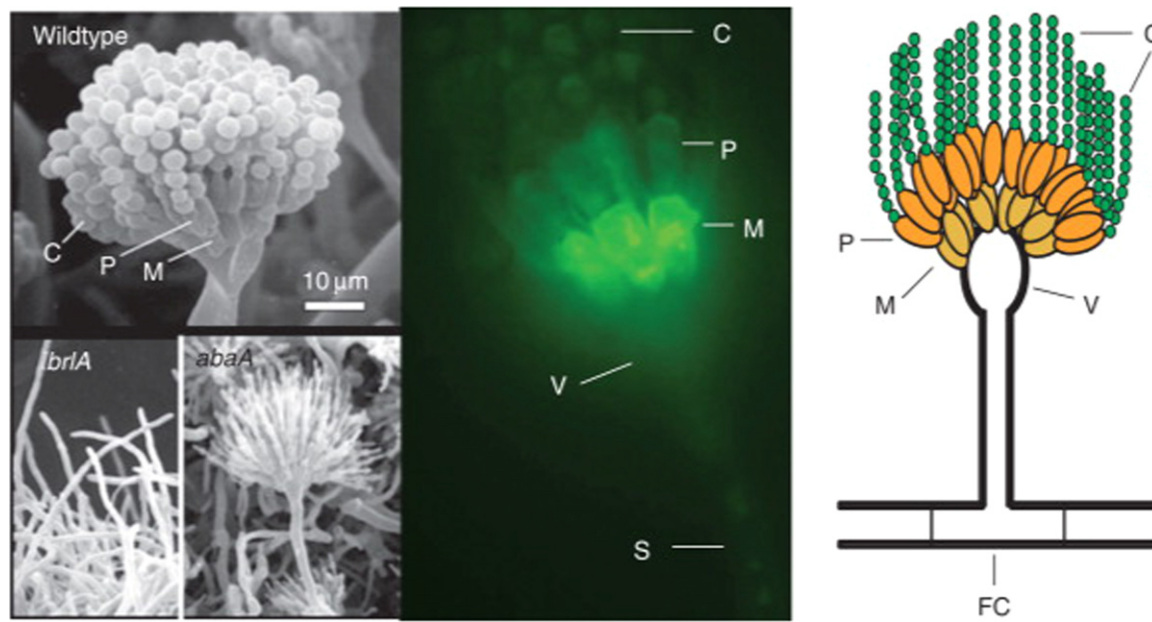
Fig. 13 The condiophore of *Aspergillus* nidulans. In the left panel scanning electron microscopy images of condiophore of the wild-type and two mutant strains are shown, these carry loss-of-function mutations in the *brlA* (bristle) and *abaA* (abacus) gene respectively. The center panel illustrates the developmental process by showing the expression of a membrane protein (the UapA transporter fused to the green fluorescent protein (GFP)), which is specifically expressed in the metula stage, which is then diluted on in the phialides and conidia, the same transporter is then expressed again during conidial germination (see text). The right panel show a schematic representation of the conidiophore of *A. nidulans*, metulae and phialides are arbitrarily colored to facilitate identification. FC, foot compartment; S, conidiophore stalk; V, Vesicle; M, metula; P, phialide; C, conidia. Notice that for some metulae in the left and center panels the two cognate phialides can be clearly seen. The pictures in the left hand panel has been kindly provided by Reinhrad Fischer. Reproduced with permission from Kues, U., Fischer., R. (Eds), 2006 The Micota I, Growth Differentiation and Sexuality. Berlin: Springer-Verlag. One of the center panel by George Diallinas and Areti Pantazopoulou.

cells. The second is the behavior of nuclei, which in the primordium of the cleisthotecium form dikaryons, in which two nuclei divide synchronously. Dicaryotic nuclei fuse into transient diploids, which undergo immediate meiosis, followed by two mitoses leading to eight binucleate haploid ascospores per ascus. These processes can be experimentally separated, as it is possible to obtain morphologically perfect cleisthothecia that do not contain asci. Many genes, including transcription factors and G-coupled receptors, have been implicated in either or both processes. The availability of the genome and possibility of following tagged proteins through the developmental processes should lead to an understanding of the sexual maturation process, including the roles of mating types in homothallic and also heterothallic species. Very recent work has established that both α and HMR mating type genes (see Section "The Genus *Aspergillus* in the Genomic Era") are necessary for fertility but not for cleistothecial formation. At present, we cannot yet draw a scheme of the developmental pathway leading to the formation of sexually mature, fertile cleistothecia.

The second developmental pathway is the formation of asexual conidia, which is present in all Aspergilli. These are formed from a specific structure, the conidiophore, which is the taxonomic marker of the genus. Conidiophores sizes range from 50 to 70 μm long in *A. nidulans* as much as 5 cm in *Aspergillus giganteus*. The structure of the conidiophore is shown in **Fig. 13**. From the flat mycelial mat, a stalk grows from a foot compartment at a right angle from the mat. The stalk then swells into a multinucleate vesicle. From the vesicle a first series of cells arise, the metulae or primary sterigmata. About 60 metulae are formed in each vesicle. Each metula buds at its tip to give two or three uninuclear phialides, also called secondary sterigmata. From the phialide, uninuclear conidia bud, only one nucleus enters each conidium. The process is repeated, in such a way that clonal rows of conidia are formed, the last conidium to be formed is adjacent to the metula, the first and oldest being the most distal one. This process is not identical in all Aspergilli; some species, called uniseriate (such as *A. fumigatus*), have only one series of sterigmata from where conidia arise directly, while in some Aspergilli, conidia contain more than one nucleus (such as *A. oryzae*).

Two approaches were used to study this process. In the first one, mutants were isolated and blocked in different steps of conidiophore development; in the second, mycelia were synchronized, and by the technique of 'cascade hybridization', an early methodology to define a transcriptome, it was determined which genes were expressed at different stages of conidiophore development. John Clutterbuck published in 1969 the seminal article of the study of the conidiophore developmental pathway, while the first cascade experiment in any organism was published by the Timberlake Laboratory in 1980. Clutterbuck described a number of mutants blocked in different steps of conidiophore development. In *bristle* mutants (*brlA*),
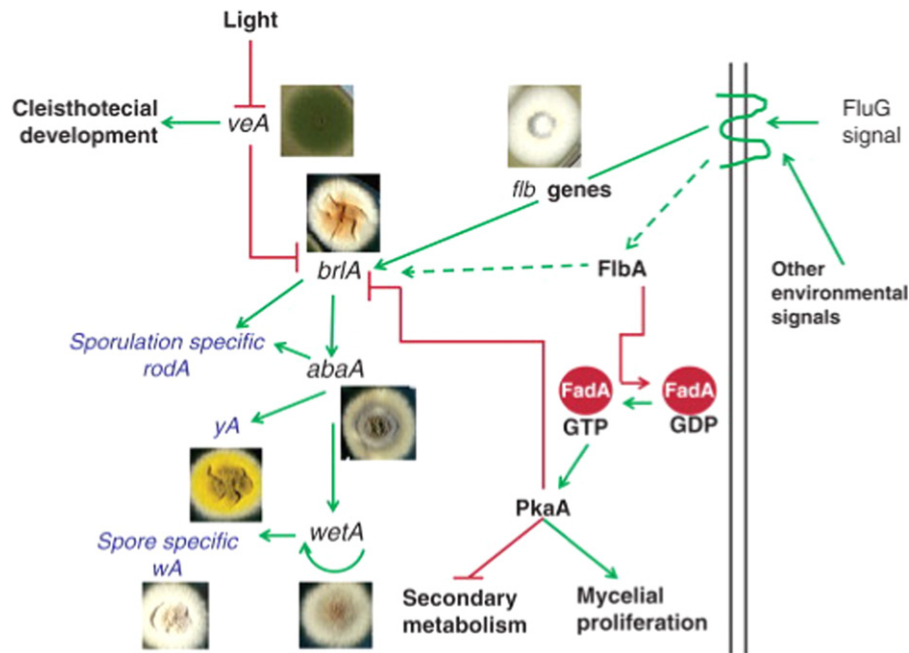
Fig. 14 Simplified scheme of the development of the conidiophore. The central developmental pathway, involving the products of the BrlA, AbaA, and WetA transcription factors is shown. In blue, target genes. Three are specifically shown, rodA encoding a conidial specific hydrophobin (see Section "Aspergillus as Human Pathogens"), yA encoding the last step of the synthesis of the wild-type green pigment and wA, encoding a polyketide synthase which is the first step in the synthesis of the conidial pigment. The insets show the morphological phenotypes of veA1, brlA, abaA, wetA, yA, and wA mutations. To the right a simplified version of the signalization pathway defined by dominant and recessive fluffy mutations. The inset shows the morphological phenotype of a fluffy mutant. The transmembrane receptor of the FluG signal has not been identified. The insets correspond approximately to the size of colonies grown on complete medium at 37°C for 48 h. Pictures from the laboratory of the author or kindly provided by Reinhard Fischer. All signals are shown as affecting the expression of brlA, but this is for simplicity's sake, as we do not know if these are direct or indirect. PkaA may affect the BrlA protein posttranslationally. To simplify the scheme the β and γ subunits interacting with FadA are not shown.

conidiophore stalks that fail to complete the developmental pathway originate from the mycelial mat. In *abacus* mutants (*abaA*), sterigmata continue to give row after row of additional sterigmata, without ever terminally differentiate phialidae or conidia (Fig. 14). *Stunted* mutants (*stuA*) result in short conidiophores with conidia being made directly from the vesicle, while in *medusa* (*medA*), metulae do not immediately differentiate and produce series on metulae before giving origin to phialides. Finally, *wet* mutants (*wetA*) do not affect the development of the conidiophore, but result in defective conidia that autolyze. Once these genes were cloned, their function was analyzed by inactivating them, following their expression pattern and overexpressing them conditionally using the tightly regulated *alcA* promoter (see above). BrlA is a Zn finger transcription factor, which directly regulates the expression of *abaA*. AbaA is also a transcription factor, which regulates *brlA* in a feedback loop and *wetA*. WetA regulates late-expressed and conidial-specific genes such as cell wall genes, and it is supposed to be a transcription factor. StuA has the characteristics of a transcription factor and limits in some unknown way the spatial distribution of the BrlA and AbaA proteins as well as being involved in conidiophore elongation and wall thickening, while MedA regulates the temporal expression of *brlA* along the developing conidiophore. Downstream of the three core regulators, BrlA, AbaA, and WetA, there are target genes, which are activated at different stages of the conidial developmental process. Some of these, as genes involved in spore pigmentation, were known from the earlier days of *A. nidulans* genetics, others were identified by the cascade hybridization methodology. These target genes show a variety of regulation patterns, some like *wA* (see Fig. 14) being under the control of WetA, some like *yA* under the control of AbaA, some others requiring, in order to be expressed, various combinations of the three transcription factors. The number of downstream genes has been estimated <100 by genetical procedures and at about 1200 by cascade hybridization. This huge difference could be partly explained by the fact that the inactivation of at least some sporulation-specific genes does not lead to any visible phenotype and that overexpression of many metabolic genes occurs during sporulation.

    This scheme does not account for the signaling pathway that leads to the onset of conidiation, that is, the formation from the mycelial mat of the conidiophore stalk. All the early work was carried out in a standard Glasgow strain, which is really a hyperconidiating constitutive mutant carrying the *veA1* mutation. This partial loss-of-function mutation leads to profuse conidiation in the dark. In strains carrying this mutation, when the mycelia are transferred from submerged culture to an air interphase, conidiation occurs synchronously in the dark, which allows the monitoring of the expression of relevant genes. The *veA*$^{+}$ (wild-type) strains behave quite differently. In these strains, conidiation is light inducible, and *veA*$^{+}$ strains produce

profusely cleisthothecia in the dark. *veA* null mutants do not produce cleisthothecia at all. Blue light irradiation result in exclusion of the VeA protein from the nucleus, whereas the VeA1 mutant protein lacks precisely the nuclear localization signal and is always present in the cytoplasm. VeA mediates also, directly or indirectly, the response to polyunsaturated fatty acids, which have been shown to provide a sporogenic signal that is alternative or additive to light, the ratio of different unsaturated fatty acids driving development toward the sexual or the asexual cycle. The *veA* gene product behaves formally in the absence of light as a repressor of conidiation and an activator of cleistothecial development. Thus, VeA must directly or indirectly repress the expression of *brlA*. Fig. 13 and Fig. 14 illustrate and summarize the process of conidiophore development.

In order to study the signalization of *brlA*, another set of mutants was isolated. These are strains where a conidiophore does not develop, 'fluffy' mutants, which make a fast growing, colorless, undifferentiated mycelium. The study of fluffy mutations has revealed a complex signaling pathway, which determines both the conidiation pathway and the production of secondary metabolites. Recessive mutations of the *fluG* gene lead to a fluffy phenotype. FluG codes for an enzyme that is responsible for the synthesis of a diffusible product, which activates, through an unknown receptor, a signaling cascade specified by several *flb* genes. Dominant mutations in the *fadA* gene also lead to a fluffy phenotype. FadA is a subunit of a trimeric G-protein, which in its GTP-bound form activates a protein kinase (PkaA) that through phosphorylation of target proteins represses secondary metabolism, promotes growth, and represses conidiation. The FlbA protein, also characterized by fluffy loss-of-function mutations, acts downstream of the FluG signal to shift FadA to the GDP-bound inactive form. Fig. 14 summarizes the 'fluffy' signaling pathway and its possible relationship with the developmental pathway leading to conidiation. Recent work has shown that the 'fluffy' class of mutations has not been saturated and that there are at least three concurrent signaling pathways. This complex signaling pathway (which has been drastically simplified here and in Fig. 14) conceals, however, a conceptual problem. Fluffy mutants are not only aconidial but they are also foremost, fast proliferating mutants. By studying fluffy mutants, we are studying the signals that control and limit mycelial proliferation, and the complete or partial loss of conidiation could be a necessary result of the enhanced proliferative activity. In fact, if we slow down the growth of fluffy mutants by using very poor carbon sources, quite good conidiation can be seen. Mutants that are specifically altered in the signals upstream of bristle should not be fluffy, but 'bald', where condiophores do not arise from a normally proliferating mycelial flat mat. However, 'bald' mutants were not reported in the early mutant screens, and it will be most interesting to know if they could be isolated at all.

The Genus *Aspergillus* in the Genomic Era

In the last few years, complete genomic sequences have been established for *A. nidulans*, *A. oryzae*, *A. fumigatus*, *A. terreus*, two different strains of *A. niger*, *A. flavus*, *Aspergillus clavatus*, and *Neosartorya fischeri* ('perfect' name for *Aspergillus fischerianus*). Articles have been published describing the genomes of *A. nidulans*, *A. fumigatus*, *A. oryzae*, and *A. niger*. Dedicated Web sites exist for all the species except for the ongoing projects of *A. clavatus* and *N. fisheri*; the predicted genes and proteins of the latter can be found in the NCBI database. For *A. nidulans*, where a detailed genetic map exists, a correlation of the genetic and physical maps is available, even if the *in silico* identification of many of the classically mapped genes with autocalled genes is far from complete. Genome annotation is in different stages of completion in different species and so is the availability of microarrays. The availability of complete genomes has stimulated technological developments that allow high throughput gene inactivation and substitution. The genomes reflect the high metabolic versatility of the genus and have highlighted the evolutionary divergence of the different species. Telomeres have been assigned to each *A. nidulans* chromosome and this can be carried out for all the other member of the genus. Putatively active transposons of different families have been identified, including the interesting finding that helitrons, a newly described family of rolling-circle replicating eukaryotic transposons, are present and active in *A. nidulans* but not in the other species. The last release of the *A. nidulans* genome predicts 10,701 proteins as coded by the genome of this species, while for *A. niger*, the predicted number is 14,165. Interestingly, all the genomes sequenced contain mating type genes and other genes known or presumed to be involved in the sexual cycle. The homothallic *A. nidulans* and *N. fischeri* contains unlinked genes for both the α and HGM mating types, but their location in the genome suggest that homothallism has evolved independently in these species. Interestingly, for *A. fumigatus*, *A. oryzae*, and *A. flavus*, strains of opposite mating types have been found in nature, opening the possibility that in fact all (or at least many) Aspergilli can undergo the sexual cycle. Another interesting finding is the variability and phylogenetic scatter of the genes putatively involved in the production of secondary metabolites. As an example, the genome of *A. niger* contains a gene cluster similar to that involved in the synthesis of fumonisin in *Giberella* (*Fusarium*) *moniliformis*, while these genes are absent in other Aspergilli, thus positing the question of whether convergent evolution or horizontal transfer is involved. The considerable knowledge accumulated in *A. nidulans* may permit to extrapolate to other Aspergilli, for example, by asking which are the genes regulated by transcription factors such as NirA, AreA, or PacC (see Section "Control of Gene Expression"), investigate promoter structure *in silico*, or more subtly to investigate the factors that make the conidiophore of *A. nidulans* biseriate, that of *A. fumigatus* uniseriate and that of *A. clavatus* characteristically elongated and uniseriate. At present, genome data have grown faster than the capacity of the research community to make use of them, a general problem of the genomic era. It is hoped that the comparison of genomes will lead to a better understanding of problems such as the evolution of metabolism, the evolution of the sexual and parasexual cycles, the evolution of silencing mechanisms, the isolation of useful new secondary metabolites, the possible engineering of pathways to produce new metabolites, and the identification of specific fungal essential genes that could lead the development of highly specific antifungal agents.

See also: Antifungal Agents; Fungal Infections, Systemic

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Relevant Websites

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   Fungal Genetics Stock Center
http://www.aspergillus.org
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http://www.fieldmuseum.org
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Appendix A: *Aspergillus*: A Multifaceted Genus. 2018 Update

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Abstract

Aspergillus is one of the most ubiquitous and best studied genera of filamentous fungi. It comprises model experimental species, species of industrial importance and noxious ones which are either opportunistic pathogens or secrete potent toxins. Reverse genetics methodologies, genomics, transcriptomics and new imaging methods have both led to a refinement of basic research with the model organism *Aspergillus nidulans* and to its extension to experimentally fastidious species of both medical and industrial importance. The phylogeny of the genus and its relations with other fungi has benefited from whole genome approaches. This update follows closely the sections of the 2009 edition of the Encyclopædia, with the addition of two sections, one dealing specifically with the sexual cycle, the second with viruses infecting different species of *Aspergillus*.

Keywords: Animal pathogens, *Aspergillus*, Genomics, Human pathogens, Industrial strains, Model organisms, Secondary metabolites, Sex, Sporulation, Transcriptomics.

Introduction

In 2007 (3rd edition of the Encyclopaedia of Microbiology, published 2009), there were 26,641 Pub Med entries for "*Aspergillus*", today (October 2018), there are 46,233. In 2007 the genomes of *A. nidulans*, *A. oryzæ*, *A. fumigatus*, *A. flavus*, *A. fischerianus* and *A. niger* were available. A recent article reports the genome analysis of 19 species. The Joint Genetic Institute includes 85 genomes of *Aspergillus* species, together with genomes of many other related organisms such as *Penicillium* and *Monascus spp*. Species Fungorum, a taxonomical site maintained by Kew Gardens, has 823 records for different *Aspergillus* species, from *A. acanthosporus* to *A. zutongii*. In 2017, 37 new species of *Aspergillus* were described. Thus, to update our knowledge of the genus *Aspergillus* is a daunting task. I thus apologise if some of my choices of the work reported appear somewhat subjective.

Contrary to what I have stated, Pier Antonio Micheli was not ordained as a catholic priest. Poor, and with no formal education, several priests and friars stimulated his vocation as a naturalist. Besides being considered "the father of Mycology" Micheli devised (1729), following Redi on flies (1668) but preceding Spallanzani (1765–1767) and Pasteur (1864) ingenious experiments leading to the disproving of spontaneous generation. His contribution went far beyond descriptive mycology and has a place in the history of the experimental method. In his experiments, he used a *Botrytis* sp. and a *Mucor* sp., together with "*Aspergilli capitati, capitulo glauco*", which could have been *A. fumigatus* or *A. glaucus*. This work was the very first use of *Aspergillus* (and other fungi) as model organisms to investigate a general biological problem. The most accessible account of the experimental work of Micheli is G.C. Ainsworth, *Introduction to the History of Mycology* (1976), reissued by Cambridge University Press. Even for us who don't read Latin, the *Nova plantarum genera*… of Micheli, 1729 (an example on **Fig. 1** left panel, chapter printed above, third edition), can now be downloaded from the library of the Royal Botanical Gardens in Madrid, I will follow up some of the sections of the 2009 publication, and try to point out major advances for each of these.

What are the Aspergilli, taxonomy

The dual nomenclature of "perfect" and "imperfect" forms has almost died out. On the whole, the term *Aspergillus* is used for all species. In 2012, the international commission on *Aspergillus* and *Penicillium* decided: "Option 5: Compromise: Keep the name *Aspergillus* (as option 2), and treat other names as optional when it has a meaning (but always together with the *Aspergillus* name)." In practice, almost everyone uses *Aspergillus*. *Aspergillus* is, within the fungi, a genus in the phylum Ascomycota, sub-phylum Pezizomycotina, Class Eurotiomycetes, Order Eurotiales, Family Trichomomaceæ. All evidence points out to the genus being monophyletic.

Food contamination by Aspergillus

Aflatoxin B1 is metabolised in the liver to an 8,9-exo-epoxide derivative. It is this derivative which is mutagenic and carcinogenic. Hepatitis viruses (mainly Hepatitis B) and chronic exposure to aflatoxin are strongly synergic in the development of hepato-carcinoma. In 2013 a major scare followed the finding of levels of aflatoxins above the permitted threshold in milk in Holland and Germany. Apparently, this originated from animal feed imported from Serbia. No further negative consequences for human health were reported. Aflatoxin poisoning may not only be a disease of poverty, but also a result of globalisation.

Aspergillus (mainly fumigatus) as human pathogens

While concentrating below on invasive pulmonary aspergillosis, the actual number of cases of the non-life-threatening Allergic Pulmonary Aspergillosis and Chronic Pulmonary Aspergillosis conditions (the latter affecting mainly patients with a previous, underlying lung disease) is actually far higher than those with invasive pulmonary aspergillosis. Invasive Pulmonary Aspergillosis continues to be a major pathology for immunocompromised patients, the mortality rate being >50%. It is still not clear why *A. fumigatus* is the major species involved. However, patients suffering from chronic granulomatous disease (a genetic condition resulting from mutations affecting any of 5 sub-units of NADPH-oxidase) are uniquely sensitive to invasive infection by *A. nidulans*. Ability of *A. fumigatus* to form biofilms contributes to pathogenicity and drug resistance. Melanin

presence in the conidiospores, hydrophobicity of the latter and early production of the toxic gliotoxin are still considered contributing factors. The exopolysaccharide galactosaminogalactan (GAG, a linear polysaccharide composed of galactose bound to N-acetyl galactosamine in no specific arrangement) has shown to be essential for adherence of *A. fumigatus* hyphae to both cells and artificial substrates, while also masking cell wall antigens, thus suppressing host inflammatory responses. Mutants deleted for the epimerase gene necessary for GAG synthesis (*uge3*) are critically impaired in virulence in mice. Orthologues of *uge3* are present throughout the Aspergilli (84% identity with *A. nidulans*) and GAG is present in many fungal species, including *A. nidulans*. However, the amounts of mycelial cell-wall bound GAG are significantly higher in *A. fumigatus* and the relatives amounts of N-acetyl-galactosamine in the polymer are also higher. An early expression of *uge3* during conidial germination, could be actually a long-searched for specific pathogenicity factor. A consensus seems to have been reached that there are no specific pathogenic factors, but rather a serendipitous constellation of characteristics in this species has pre-disposed it to become an opportunistic pathogen.

I correct an inaccuracy in the 2009 version of this article. The specificity of 5-flourocytosine to inhibit fungal growth is based on its conversion to 5-fluorouracil by cytosine deaminase, an enzyme absent in human cells. However, toxicity affecting both liver and bone marrow has been reported. It is not widely used in the treatment of Aspergillosis, as *A. fumigatus* is not very sensitive to it, due to the repression of the cognate transporter at physiological pHs.

It is essential to develop new antimycotic agents, given the appearance of azole resistant strains. *A. fumigatus* resistant strains are present in the environment, in all probability due to the widespread use of azoles in agricultural practice. Nevertheless, *in patient* resistance can also arise. The most common genotype of pan-azole resistance strains includes a tandem duplication of the promoter region of *cyp51*(encoding lanosterol 4-α-demethylase) leading to eight-fold over-expression of the gene, together with a mutation in the ORF, L98H being the most frequent. Advances in treatment have relied mostly in developing new azoles and echinocandins and/or new ways to deliver already currently employed drugs. Nevertheless, new agents targeting specific aspects of fungal metabolism are being developed, among these specific inhibitors of cell wall. Some newly developed Nikkomycins, inhibitors of chitin synthases are in Phase 2 clinical trials. The most promising new antifungal drug, VL2397, produced by *Acremonium perscinum* is effective against *A. fumigatus*, and is undergoing Phase 2 clinical trials. It was discovered by a brute force screen, which used infected silk-worms (larvae of *Bombix mori*) as the test-organism. The mechanism of action of VL2397 is not known, but it is structurally similar to siderophores (Fe^{3+} chelators) and is incorporated into the mycelium by siderophores transporters. Siderophore synthesis is well known and essential for *A. fumigatus* pathogenicity. The latter has suggested a possible "trojan horse" approach where an antimycotic drug is conjugated with a siderophore, which then delivers it to the fungal cell.

Aspergillus sydowii *as a gorgonian coral pathogen*

Recent work has disproved the existence of specific pathogenic strains of *A. sydowii*. A study including both morphological and molecular markers did not find any evidence favouring the "African dust hypothesis". The snail *Cyphoma gibbosum*, a specialist predator of gorgonian corals, may be responsible from spreading the disease. While the prevalence of the disease was quite alarming shortly after its discovery in 1995, the epizootic outbreak has now subsided to relatively low endemic levels. Selection of resistant gorgonian corals, is supposed to have been the main mechanism for this outcome.

Extracellular enzymes produced by Aspergilli

An exciting development is the very recent isolation and characterisation of a strain of *A. tubingensis* (a member of the *A. nigri* -black Aspergilli- group) able to degrade polyester polyurethane (PU). It was estimated (2006) an annual global production of 8 million tons of PU increasing by 4%–5% year. PU is usually disposed in land-fills (forbidden in several European countries) or burned with the production of both carbon monoxide and hydrogen cyanide. To determine the detailed enzymology and the regulation of the process, together with the possible use of *A. tubingensis* in PU bio-degradation, alone or within a microbial community, would be of obvious importance.

Aspergillus nidulans *as a model organism*

The reverse genetics of the Aspergilli has seen a number of methodological advances. Transformation can be carried out now directly with composite DNA molecules assembled from PCR products. Arguably the most useful advance has been the availability of strains (first obtained for *Neurospora crassa*) inactivated for the *nkuA* gene, necessary for non-homologous end-joining recombination, thus virtually eliminating the problem of non-homologous integration in transformation experiments. Transformation frequencies were improved for a number of Aspergilli by direct transfer of the Ti plasmid of *Agrobacterium tumefaciens*, which can also compete with or complement transposon-based methods for random inactivation of genes. Recently CRISPR-Cas9 methods have been adapted to a variety of Aspergilli. The advances on reverse genetics had the effect of displacing research -at least in quantitative terms- from the model organism *A. nidulans* to species of industrial or medical importance, as these methods partially by-pass the need for sophisticated classical genetics. For 2018 there are in PubMed (up to October), 112 entries for *A. nidulans* compared with 486 for *A. fumigatus* and 369 for *A. niger*.
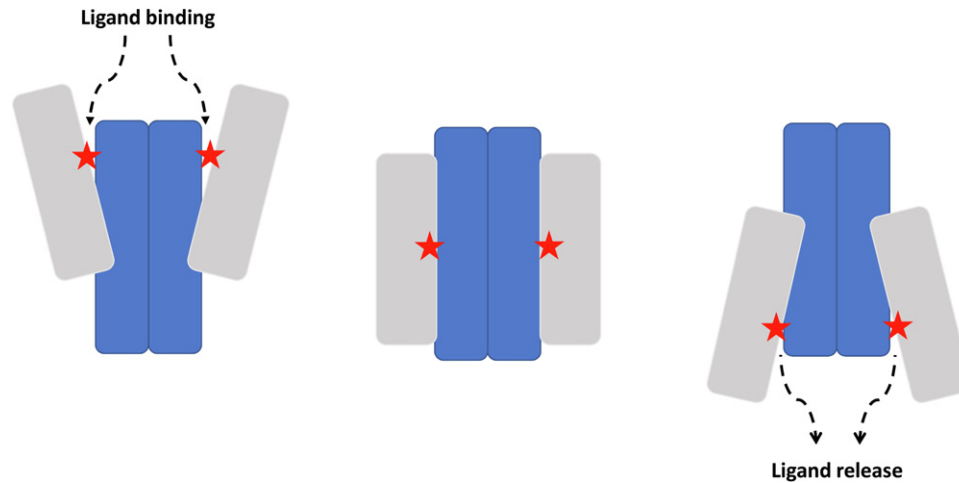
Fig. A1 A very schematic cartoon of the elevator model which accounts for all the structural and mutational data obtained for the UapA transporter. Grey, core domain which includes substrate binding residues. Blue, gate/dimerisation domain which includes both gate residues, which limit substrate specificity, and the dimerisation surface. Red star, substrate of the transporter. The core domain slides and twists around the dimerisation domain, transporting the ligand from the extracellular space (up, left scheme) to the cytosol (down, right scheme). A similar model accounts for the properties of the bacterial uracil transporter UraA, a member of the same transporter family (NAT) as UapA. I thank Sotiris Amillis and George Diallinas for help with this figure.

A number of fluorescent proteins are available to generate fusions to investigate cell trafficking and intracellular protein-protein interactions. This, coupled with striking advances in cell imagining has allowed extremely sophisticated studies of the cell biology of filamentous fungi with *A. nidulans* an *N. crassa* as the key model organisms. The articles of many of the groups working on *Aspergillus* cell biology are routinely accompanied by high-quality movies showing intracellular traffic.

I will detail below some recent contributions to fundamental cell and molecular biology based on work with *A. nidulans*. Progress has been achieved in understanding the signaling of some of the regulatory proteins mentioned in the 2009 version of this article (see **Figs. 6**, **7** and **8** of the 2009 edition printed above, and related text pages). The activation of the CreA repressor, mediating carbon catabolite repression is complex and surely different form that of that of the isofunctional Mig1p *of Saccharomyces cerevisiæ*. A ubiquitination/de-ubiquitination cycle is involved in the activation of CreA in *A. nidulans*. The positive-acting GATA factor AreA responds to the presence of favored nitrogen sources through several mechanisms. An important one is RNA stability, intracellular glutamine destabilising the *areA* mRNA. The protein mediating this process, and also the degradation of other mRNAs involved in the utilization of nitrogen sources, RrmA has been identified. RrmA is quite conserved even in very basal fungal species such as *Rozella allomyces* (Cryptomycota) and even in Oomycetes (Stramenopiles, related to brown algae), organisms with a "fungal lifestyle", some of which are important plant pathogens.

"**Fig. 9** of the 2009 edition printed above" shows the nitrate-induced localization to the nucleus of NirA, the specific transcription factor necessary for the expression of the three genes of the nitrate utilization gene cluster. Strauss and co-workers have shown that in the absence of nitrate, a conserved methionine in the Nuclear Export Sequence is oxidised to methionine sulfoxide by a specific enzyme. Nitrate elicits the reduction of the oxidised methionine, nuclear localization and activation of NirA. While the intramolecular structural changes resulting from this oxidation/reduction cycle are not yet elucidated, sulphoxidation of methionine is a novel signaling mechanism, probably conserved throughout the nitrate induction mechanism in the Pezizomycotina.

The continuing work of the laboratories of Miguel Peñalva and Herb Arst have led to a more profound understanding of the signaling pathway leading to the activation by proteolysis of the transcription factor PacC, illustrated in **Fig. 10** of the 2009 edition. Endocytosis, as it was then proposed, is not involved, but nevertheless, the ESCRT complexes (Endosomal Sorting Complexes Required for Transport) participate in PacC activation. The ESCRT multi-protein complexes, conserved throughout eukaryotes, mediate multi-vesicular body biogenesis, a process which internalise membrane proteins into endosomes leading to their eventual degradation in vacuoles. They are involved in other processes such as membrane excision during cell division and viral budding. In alkaline conditions, the ubiquitinylated PalF binds to PalH and recruits proteins of the ESCRTI and ESCRTII complexes. These, in turn recruit the ESCRTIII protein Snf7/Vps32 (*S. cerevisiæ* nomenclature, shown in "**Fig. 9** of the 2009 edition, printed above" to bind PalA in the cytoplasm, see below). The polymer of Snf7 interacts with a PalA/PalC complex that in turn recruits PalB, the protease which catalyzes the first proteolytic step leading to the activation of PacC. The whole process occurs in a plasma membrane-bound rather than a cytoplasmic location as proposed previously and shown in "**Fig. 9** of the 2009 edition, printed above". Data from related work in *S. cerevisiæ* and the basal yeast species *Yarrovia lypolitica* point to an overall conservation of this signaling pathway in the ascomycetes. An unresolved problem is how PalH senses extracellular pH, leading to its binding of PalF. The signaling of pH regulation in fungi, provides a further example of the multiple cellular functions of the ESCRT complexes. This work led to the discovery of a novel signaling system involved in cation homeostasis. Mutations in genes encoding proteins of the
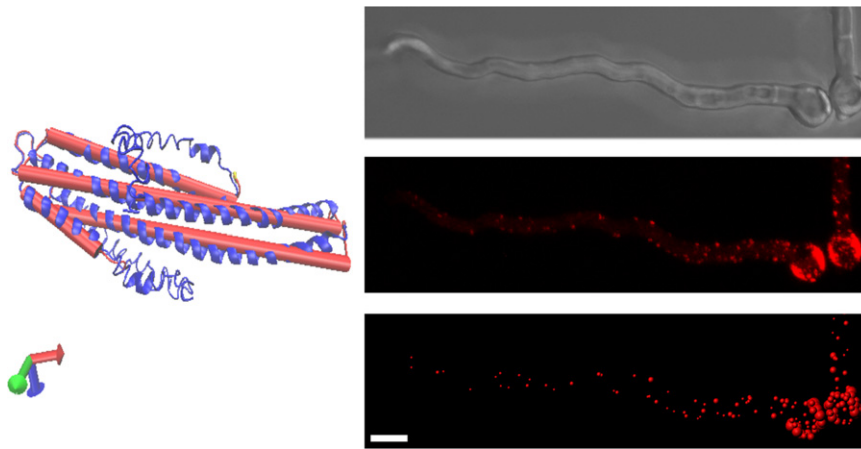
Fig. A2   Left panel: A model of the PilA eisosomal protein of *A. nidulans* (blue) superposed to the solved cartoon structure of the LspA protein of *S. cerevisiæ* (red) showing the typical BAR domain structure. Model obtained with I-Tasser (Reproduced from Yang, J., Yan, R., Roy, A., *et al.*, 2015. The I-TASSER Suite: Protein structure and function prediction. Nature Methods 12, 7–8) and visualised with VMD 1.9.4 (Reproduced from Humphrey, W., Dalke, A., Schulten, K.,1996. VMD - Visual Molecular Dynamics. Journal of Molecular Graphics 14, 33–38). Right panel: Eisosomes in a 14 h germling of *A. nidulans* grown at 25°C. Top, DIC, Middle, PilA-mRFP fusión protein fluorescence, Bottom PilA-mRFP three dimensional reconstruction. Scale bar 5 μm. Picture courtesy of Alexandros Atanasopoulou and Vicky Sophianopoulou. Detailed methods to be found in the recent publication of these authors.

ESCRT complexes lead to severely impaired growth. Suppressors of this phenotype map in two genes, *sltA* and *sltB*. These encode respectively a Zn-finger transcription factor and protease/pseudokinase self-activating protein, required for SltA activation. SltA and SltB are required for cation tolerance; mutations in either *slt* gene also result in oversized vacuoles. The range of targets of SltA includes both vacuolar and plasma membrane cation transporters. Another transcription factor CrzA, regulated by the calcineurin/calmodulin system also participates in cation homeostasis. CrzA is widely conserved among eukaryotes, while the SltA system is specific to the Pezizomycotina, a sub-phylum of the Ascomycota (see above).

Post-translational regulation of transporter fate is now well established (**Fig. 11** of the 2009 edition, printed above). A new phenomenon, endocytosis as a direct result of transport activity has been shown for the uric acid-xanthine transporter of *A. nidulans*. Several *A. nidulans* transporters have been dissected by mutagenesis coupled with molecular modelling of docked substrates. The highpoint of this work has been the determination of the actual structure of the UapA xanthine/uric acid transporter complexed with xanthine. Substrate specificity is determined by the interaction of the substrate binding domain with gate domains along the substrate translocation pathway. The structure confirms predictions based on mutational evidence and strongly suggests an "elevator model" where in a UapA dimer, a core domain which binds the substrate sliding down a dimerisation/gate domain, thus leading to the internalisation of the substrate (cartoon in **Fig. A1**).

Work with *S. cerevisiæ* has defined specific compartments in the cell membrane. One of these (MCC), containing the arginine transporter Can1p is organised thanks to a number of other proteins, some tetraspan membrane proteins such as Sur7 and Nce102, others localized immediately below the membrane. These are two BAR domain proteins which generate furrows in the membrane called eisosomes, which typically appear as punctate structures. Eisosomes were described as sites of endocytosis in *S. cerevisiæ*, but recent work points out rather to a protective role in relation to endocytosis. Eisosome organization has been studied sparingly in a few other species, but in detail in *Aspergillus nidulans*. There are two BAR domain eisosomal paralogues in the Aspergilli, PilA and PilB. Eisosomes in conidia and ascospores contain both PilA and PilB, while mycelial eisosomes include PilA but not PilB. Eisosomes are quite an enigma, as the cognate proteins are almost universally conserved in ascomycetes, but their deletion has virtually no phenotype (**Fig. A2**).

The laboratory of Steve Osmani has established the fate of all nuclear pore components during mitosis in *A. nidulans*, his results challenging the traditional drastic opposition of closed and open mitosis processes. Further collaborative work with the laboratory of Eduardo Espeso has aimed to establish the role and fate during mitosis of the 14 putative nuclear transport (importins and exportins) proteins found in the genome of *A. nidulans*. This work identified six essential nuclear genes involved respectively in a general protein import pathway, a general nuclear export pathway, an mRNA/nucleoprotein export pathway and a possible specific carrier of the RanA GTPase. Hydrolysis of GTP, catalyzed by RanA, provides the energy for all nuclear entry/exit transactions. The essential proteins studied remain partially attached to the nucleus or nuclear envelope during mitosis. The KapK/CrmA export pathway had been previously shown to mediate NirA and AreA localization while the importin $\alpha 1/\beta 1$ is responsible for the import of VeA. The detailed knowledge of nuclear import/export pathways coupled with that of the role transcription factors involved in metabolism, cell cycle and morphogenesis would allow a unique thorough description of nuclear entry/exit processes coupled with gene expression in this organism.

The Golgi apparatus of *A. nidulans*, studied in detail in the laboratory of Miguel Peñalva, is a dynamic network, which is polarized early during apical extension and remains intact during mitosis. They have also established that the biogenesis of secretory vesicles fuelling apical extension is governed by the recruitment to the trans-Golgi network of the RAB11 GTPase, which

Fig. A3   A complex secondary metabolism gene cluster of *A. fumigatus*. Partial view of a sub-telomeric region of chromosome VIII of *A. fumigatus*. All the genes in this cluster and the near-by cluster involved in the biosynthesis of fumitremorgin are regulated by LaeA, and those shown in the figure also by the specific transcription factor encoded by *fapR* (in blue in the scheme). In red, genes encoding enzymes involved in fumagillin biosynthesis; in green genes, involved in pseurotin biosynthesis, interspersed yellow genes involved in neither, but nevertheless FapR-regulated. I have indicated the names of only the two "signature" genes, *fmaB* encodes a polyketide synthase, *psoA* a hybrid polyketide synthase non-ribosomal peptide synthase. Redrawn and simplified from Wiemann, P., Guo, C.-J., Palmer, J.M. *et al.*, 2013. Prototype of an intertwined secondary-metabolite supercluster. Proceedings of the National Academy of Sciences of the United States of America 110 (42), 17065–17070.

is mediated by the TRAPPII oligomeric GTPase exchange factor (GEF). This latter finding is of general importance as RAB11 function in the establishment and maintenance of polarity during development has been established in several animal systems, including human cells, *Xenopus* and *Drosophila*.

The filamentous nature of the Aspergilli (and in general all Pezizomycotina as opposed to the single celled yeast models) and their growth by apical extension implies an active directional intracellular traffic. *A. nidulans* has been, together with *N. crassa* the model to study these processes. Molecular motors (dynein, kinesins, myosin-5) are involved in the long-distance movement of organelles, such as secretory vesicles, early endosomes and peroxisomes. Classical and molecular genetic analysis together with newly developed imaging techniques is leading to a complete analysis of the growing hyphae, defining the role of exocytosis and endocytosis at the hyphal tip. One substantial advance is the molecular and functional definition of the Spitzënkorper. This German term, coined in 1924, referred to dark staining organelle at the tip of hyphae. The Spitzënkorper results from an accumulation of secretory vesicles involved in exocytosis and hyphal expansion at the apex before fusing with the plasma membrane, and thus can be considered as the organelle determining hyphal apical extension.

Aspergillus *mitochondrial genomes*

Total genome sequencing necessarily includes mitochondrial DNA and thus it is possible to assemble and compare these genomes within and outside the genus. A systematic approach for all available *Aspergillus* mitochondrial genomes has not been carried out. The comparison of mitochondrial genomes of six species of *Aspergillus* and three of *Penicillium* showed a variation in size mostly due to class I introns, proteins encoded in their open reading frames and "accessory genes", that is mitochondrial encoded genes of unknown function. Curiously, *A. nidulans* where very early work on the mitochondrial genome, intron positions and structure was carried out, was not included in this survey.

Secondary metabolites and their synthesis

The availability of whole genome sequences led to the realisation that only a small minority of fungal secondary metabolites (SM) had been detected by standard chemical methods. The genes encoding SM synthetic enzymes are usually clustered and they include as a key enzyme a polyketide synthase and/or a non-ribosomal peptide synthase (such as that catalysing the condensation of L-α-aminoadipic acid, L-cysteine and L-valine into a tripeptide in the synthesis of the penicillins). The presence of SM gene clusters is extremely variable even between different strain of the same species. This had led to the development of informatic programmes to detect secondary metabolism gene clusters. Genome analysis has led to the identification of 56 SM gene clusters in *A. flavus*, 26 in *A. fumigatus* and 56 in *A. nidulans*. This is probably the tip of the iceberg. The potentiality of Aspergilli to produce novel compounds is illustrated by the identification in 2017 of *A. hancockii*, (a member of the *flavus* group) which when cultured on rice, produces, besides a number of hydrolytic enzymes, 69 secondary metabolites, of which 11 were novel. Gene clusters involved in SM metabolite synthesis can be organised in different patterns. In *A. fumigatus* trichothecene synthesis genes are split in two clusters, while a single super- cluster, regulated by LaeA encodes the enzymes involved in the synthesis of the unrelated, fumitremorgin, fumagillin and pseurotin, the latter two in an interwoven (Fig. A3).

Under standard laboratory conditions most of SM gene clusters are not expressed. We are faced here with two problems, one conceptual and one practical. The first is to identify and dissect the signaling which leads to the expression of each secondary metabolite gene cluster. The second, is to elicit the expression of each cluster under controlled conditions, such as to be able to assay the biological activity of each of the many yet undescribed secondary metabolites. These are problems not specific to the Aspergilli, as secondary metabolites gene clusters are present in fungi, bacteria and plants, and perhaps in other poorly studied microbial eukaryotes. The most obvious method is to try as many culture conditions as feasible and monitor the metabolites produced by standard chemical methods. Transcriptome analysis could then be used to identify the cognate genes or gene clusters. Many secondary metabolite gene clusters include a specific transcription factor gene. Its overexpression being a straightforward way to activate the gene cluster. The overexpression of *laeA* led to expression of several secondary metabolite gene clusters. Hetero-logous expression of SM genes, coupled with the substitution of native promoters by inducible, well characterized ones, has been used to investigate the products of several polyketide synthases and has led to the elucidation of the biosynthetic pathway of the *A. fumigatus* meroterpenoid pyripyropene, a potentially useful inhibitor of cholesterol synthesis. A promising avenue is the construction of gene libraries of $\approx$ 100 Kb in a multi-copy, self-replicating plasmid, comprising potentially each of the SCM gene clusters in a given genome, followed by transformation and expression in *A. nidulans*. Using this methodology (FAC-MS, Fungal Artificial Chromosomes-Metabolic Scoring) seventeen SC metabolites from different Aspergilli were identified and assigned to their cognate gene clusters. Finally, the expression of some SM clusters was achieved serendipitously. In a forward mutation screen,
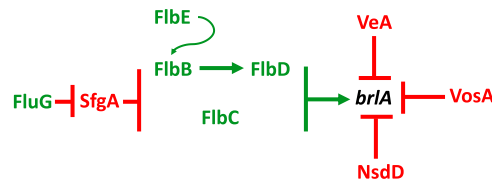
Fig. A4 The complex regulation of the expression of the *brlA* gene. This figure complements Fig. 14 of the 2009 edition, printed above. In red negative regulators and in green positive regulators of expression. Red connectors, repression, green arrows induction of expression. The squiggle arrow indicates the role FlbE in FlbB apical localization.

laeB (not a paralogue of *laeA* see above) was identified, where a deletion results in loss of stergimatocystin synthesis but also unexpectedly in eight new secondary metabolites being produced. The deletion of a protein kinase (*mpkA*, see below, genomic section) resulted in the activation of the Asperindin A gene cluster in *A. nidulans*. Life-saving (penicillin) or deadly (aflatoxin B) fungal secondary metabolites were at first discovered serendipitously. Statins were discovered after a systematic search of HMG-CoA reductase inhibitors in the culture broths of 3800 strains of fungi. This illustrates the problem of trying to find a function and/or application of the plethora of secondary metabolites produced by fungi and revealed by genome screens. A combination of methods, both bioinformatic and experimental high-throughput screening will be necessary to address this challenge.

The importance of chromatin organization in secondary metabolism gene expression is under active investigation and has revealed a role for the Compass complex (involved in methylation of H3K4), histone deacetylation, H3K4 and K9 methylation and heterochromatin protein I in secondary metabolite gene cluster silencing. The link between secondary metabolism and developmental regulation has been brought to the forefront with the discovery that LaeA, a global regulator of secondary metabolism, forms a complex with VeA and a VeA-like protein, VelB. As established previously, VeA is enriched in the nucleus in the light, but the distribution of LaeA and VelB does not seem to be affected by light/dark conditions. This physical interaction was unexpected and opens a new avenue of research. In a thorough screen several new positive regulators of secondary metabolism cluster were discovered. A global negative-acting putative DNA binding protein, McrA was detected in *A. nidulans* and is conserved in many other ascomycetes. McrA seems to be a global regulator, involved in the repression of other genes beside secondary metabolite gene clusters. A cogent model linking the function of LaeA in secondary metabolism production with the light-regulated roles of VeA in the shift from the asexual to the sexual cycle is not yet available, neither is a comprehensive scheme of how the different positive and negative regulators interact.

While several environmental signals may be involved in triggering secondary metabolite production, a quite striking finding is that at least some secondary metabolite biosynthesis can be triggered by a close direct interaction of *A. nidulans* with *Streptomyces hygroscopicus*. Conversely, a non-identified *Aspergillus* species secretes a specific bacteriostatic metabolite (structure known, but biosynthetic pathway and genes involved not determined), which turns on the synthesis of nitrous oxide by a non-identified, co-cultured *Streptomyces* species, which activates the biosynthesis by the bacterium of a fungistatic agent, heronapyrrole B. These two studies underline the importance both conceptual and practical of studying secondary metabolism production in relevant eco-logical settings.

Aspergillus *asexual sporulation*

The pathway leading to the differentiation of the conidia in *A. nidulans* as shown in Fig. 14 of the main text still holds with some additional complications and refinements.

Research has proceeded in two directions. In the first, the details of the pathway signaling the key transcription factor BrlA were ironed out. To somewhat simplify, the central role of BrlA is confirmed, different paths leading of conidiogenesis are different ways of turning on *brlA* transcription. The Velvet complex responds to environmental stimuli, such as blue and red light, carbon levels, exposure of the hyphae to the atmosphere, osmotic and/or oxidative stresses, which lead to VeA cytoplasmic as opposed to nuclear localization, with resulting de-repression of the asexual sporulation pathway.

The "FluG factor" (Fig. 14) can be bypassed by an adduct of dehydroaustinol (a meroterpenoid) and diorcinol (a derivative of the secondary metabolite orsellinic acid). As the adduct is lypophilic, it may go through the membrane without the need of a specific receptor or transporter, which would leave open the identity of the intracellular interactor of the adduct.

In Fig. 14 of the main text it is indicated that a number of genes called *flb* (for fluffy, low *brlA* expression) are involved in the signaling of *brlA*. FlbB is a basic-leucine zipper transcription factor which is targeted to the hyphal apex, where it complexes with FlbE. The latter is essential for the apical localization of FlbB. In response to an unknown signal (perhaps mediated by the hypothetical receptor of the FluG signal) FlbB detaches itself from FlbE, travels in a retrograde fashion and eventually into the nuclei of aerial cells. FlbB transit through the apex is a prerequisite for its transcriptional competence, which was unexpected. Once inside the nucleus, FlbB elicits the synthesis of FlbD, a transcription factor of the cMyb-type. A dimer of both cooperate with a third transcription factor, of the Cys2His2 type, FlbC, to turn on *brlA* transcription. Uniquely among the Flb and Flu proteins, FldD is also required for the formation of the peridium, the wall of the sexual fruiting bodies. In addition to the central regulators showed in Fig. 14 a number of negative regulators of asexual sporulation have been identified. SfgA, is Zn^{++} 2 Cys6 factor (motif called $Cys_6 Zn_2$ in page 411, shared by a large number of fungal transcription factors). SfgA represses the FlbB/D/C pathway, epistasis relations indicate that FluG acts relieving SfgA repression. VosA belongs to the same family as VeA and acts in vegetative growth by
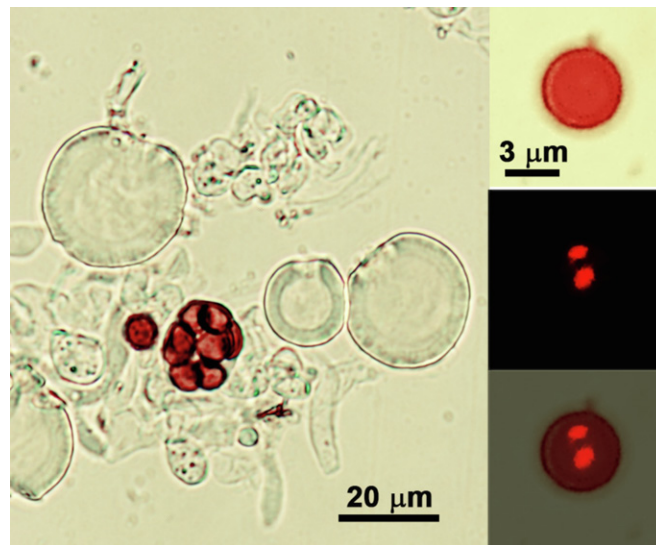
Fig. A5 Left panel, shows partial contents of a crushed cleistothecium. In the center an entire ascus with eight brown-red pigmented ascospores. To the left of it, an ascospore from a broken ascus. The large pale round cells are hülle cells. Right panel: Isolated ascus, top light microscopy, center epifluorescence of nuclei labelled with histone H1 fused to a red-fluorescent protein, bottom merge of top and central panels. Photographs courtesy of Eszter Bokor and Zszsanna Hamari.

repressing *brlA*, finally NsdD is a repressive GATA factor, which on the other hand is necessary for sexual development. The latter two factors act by repressing *brlA*. A simplified scheme is shown in **Fig. A4**. A number of viruses (see below) affect conidiation of different species of *Aspergillus*. The interaction of these viruses with the asexual sporulation pathway is an unexplored area of research.

The second direction is to investigate how general is the conidiation induction pathway worked out for *Aspergillus nidulans*; within the Aspergilli and beyond. The first approach is obviously genomic, that is, to investigate which components of the conidiation cascade are present in which species. Some of these genes are not limited to the Aspergilli, or indeed to the Pezizomycotina. Twentyfive genomes species of *Aspergillus* show a complete conservation of the genes involved in asexual sporulation. The central regulatory network composed by *brlA*, *abA* and *wetA* is conserved almost without exception throughout the searched *Aspergillaceæ*. Once relevant genes are identified in a given species, standard methods could be used to check if their function is conserved. An example of this work is the investigation of the role of transcriptional activator WetA in *A. nidulans*, *A. flavus* and *A. fumigatus*. This work included RNAsec and Chip-sec (see below). The repression feed-back loop of *brlA* by WetA (not shown in **Fig. 14** of the main text) is common to the three species, as it is the conidial color-less and autolysed phenotype. However, a number of downstream target genes are species specific. Previous work had established a pervasive role for WetA in *A. flavus*, including in vegetative growth. Travelling far away from the Aspergilli, in the wheat pathogen *Zymosteria tritici* (Pezizomycotina, Dothideomycetes) genome we find homologues of many of the genes involved in the sporulation pathway in the Aspergilli, including the central regulator *brlA*. Systematic deletion of these genes revealed that the orthologues of *abaA*, *brlA* and *flbB* do not have a crucial role in asexual sporulation, while *stuA* deletions failed to produce spores. This illustrates the limits of model systems and that apparently orthologous genes can be used in different ways in developmental pathways.

Aspergillus Sex

For an old-fashioned mycologist, this title would be an oxymoron, as Aspergilli by definition don't have sex, and when they have, they also have to change name. Following the cogent phylogenetic evidence that the apparently non-sexed Aspergilli and their sexual relatives form a monophyletic clade, I will discuss them together. In the original article I mentioned sex in two contexts, first while describing the sexual cycle of *A. nidulans*, which led to its development as a model organism, secondly in the context of developmental pathways. The mutational approach, which was a resounding success in the dissection of asexual sporulation has not delivered such a cogent picture for the process of fruiting body (cleisthotecial) formation, in spite of some interesting mutations having been characterized. It seems clear, however, that the formation of fruiting bodies, differentiation of hülle cells and fertilisation/nuclear fusion and meiosis are separable processes. It may be necessary to summarize again the process of sexual reproduction in the Pezizomycotina, the sub-phylum of the ascomycetes to which the Aspergilli belong. This has clear differences with other ascomycetes, the "yeasts" (properly Saccharomycotina and Taphrinomycotina). In specialised structures, two nuclei, which could be of the same or of different strains, divide synchronically in a common cytoplasm. This stage is called the dikaryon. Fertilisation occurs when the two nuclei fuse, giving origin to a diploid nucleus. This nucleus never divides mitotically, but goes immediately into meiosis resulting in four haploid cells. These may divide once more mitotically, typically resulting in eight ascospores, contained within an ascus (sack). Asci of *A. nidulans* comprise eight bi-nucleate

ascospores (Fig. A5). *A. nidulans* is a homothallic species, no strains of different mating types are extant. Heterothallic ascomycetes came in two mating types and only strains of opposite types can mate. This is the case for *S. cerevisiæ* and *Schizosaccharomyces ponbe*, yeasts, which follow a different path to meiosis without the dicaryon stage (see above). Two heterothallic Aspergilli *A. (Neosartoria) fennelliæ* and *A. (Emericella) heterothallicus* were described about 50 years ago. In heterothallic ascomycetes mating type is determined by alternative genes (called idiomorphs), which are non-homologous but occupy, however the same chromosomal location (I am ignoring here the silent mating type loci, present in some yeasts, and which underlie mating type switching as they are not relevant to this discussion). These two genes encode DNA binding proteins comprising two different domains. One mating type gene encodes an α-box protein (MAT1-1 abbreviated MAT1). The alternative mating type gene (MAT1-2, abbreviated MAT2) encodes a protein including a HMG-box (high mobility group-box). There are usually several paralogues of HMG proteins in the genome (e.g., 7 in *A. nidulans*) of which one is concerned with determining the mating type. In the genome of the homothallic *A. nidulans*, both HMH-box and α-box encoding genes are present in different chromosomes and necessary for the sexual cycle, while in the "asexual" *A. fumigatus* and *A. oryzae* only the HMG-box encoding gene and only the α-box encoding gene were detected respectively. This was coherent with *A. nidulans* being homothallic, but suggested that the other two species were heterothallic rather than asexual, and it was a serendipitous occurrence which mating type was found in the single sequenced strain. Indeed, targeted sequence of many isolates of *A. fumigatus* yielded strains with either one or other mating types. In *N. fischeri* (*A. fisherianus*) a homothallic species of the *Fumigati* group, both mating type genes are present. In a more recent survey comprising 18 *Aspergillus* species, all species were found to have either one or the other mating type (thus possibly being heterothallic), while in addition to *A. nidulans* and *A. fischerianum A. glaucum* also has both. All species genomes included the pheromone and pheromone receptor encoding genes first described in *A. nidulans*. Thus, probably none of the species involved in this survey is asexual. Population genetics studies had suggested recombination in *A. fumigatus*, *A. flavus* and *A. parasiticus*. The discovery of strains of the same species with complementary mating types led directly to attempts to obtain sexual crosses in the laboratory. This was strikingly successful for *A. fumigatus* (which lead to a suggested name change to *Neosartoria fumigata*, unfortunate in my opinion) and successively for a few other species, including members of the biotechnologically important black *Aspergilli*, such as *A. tubingensis*. We should start doubting whether asexual Aspergilli exist at all. Similar work has been carried out with Penicillia, and some other members of the Pezizomycotina. There has been an unresolved debate, as to which of the modes of sexual reproduction (homothallic or heterothallic) is primitive and which derivative. The two mating types should not be confused with the fact, that when crossing two strains of *A. nidulans*, distinguished by genetic markers, in any one cleisthotecium either nucleus can behave as a "female" or "male" nucleus. Both mitochondrial markers and nuclear markers with a maternal effect have been used to establish this fact, which was known from classical genetical work, but has not been investigated further.

Viruses of *Aspergillus*

I did not address this subject in the original article, but recent work prompts me to include it here. Following work with *Penicillium*, it was published in 1970 that both *A. niger* and *A. fœtidus* (a close relative and probably a strain of *A. niger*) contain viral particles and that double stranded RNA extracted from these was able to elicit interferon production in mice. Double stranded RNA viruses were reported in a number of *Aspergillus* species. No extracellular transmission seems to occur. Transmission was achieved through heterokaryons, and also between species through protoplast fusion. Most viral infections are asymptomatic. This contrasts with the well-studied killer phenotype of *S. cerevisiæ*, which is caused by a dsRNA. Interspecies transfer to *A. nidulans* showed that one specific RNA virus supressed the dicer-argonaut silencing pathway, while a second one actually was degraded to siRNAs, presumably though the action of dicer. Recently there has been a revival in the search and study of double stranded viral RNAs, using multiplex RNAseq. Most of the recent work concerns *A. fumigatus*. Many strains of *A. fumigatus* were screened for dsRNAs which revealed viruses belonging to several different families. Some alterations of morphology were reported, but no glaring correlation with pathogenicity or fungicide resistance was found. The virology of the Aspergilli is an underexplored area, mainly in respect of the phenotypic effects of viral infection. Old work (some of it of the 50s) showed cytoplasmic transmissible morphological phenotypes in a number of Aspergilli. The correlation of these with viral infections deserve a re-investigation.

Aspergillus *in the post-genomic era*

In the original article I referred to the "genomic" era of *Aspergillus* research. It has now become customary to refer to "post genomics" to highlight the challenges posited by an accumulation of data that goes beyond the capabilities of a research community. I have referred throughout this update to the contribution that genome availability has made to different research areas (SM, Asexual and sexual sporulation, fate of nuclear envelope proteins). *Aspergillus* is arguable the genus with most genomes sequenced and annotated. The availability of a genome does not automatically lead to the knowledge of the proteome. Gene models, derived from genome sequencing are based on algorithms that detect the start and end of open reading frames and the intron/exon structure of each gene in the genome. The latter is a particularly vexed problem. In spite of the existence of algorithms trained on different species, it is not uncommon to find erroneous gene models in data bases. I have personally encountered spuriously fused genes, promoters interpreted as introns, dubious start codons and incorrect intron/exon organization. While alternative splicing is less frequent in fungi than in other eukaryotes; it does occur, including in the Aspergilli, and to detect it is essential to establishing a complete correct proteome. Correct gene models are essential to interpret proteomes obtained under different conditions analyzed by mass spectrometry. The corollary of genome availability is transcriptomics, and "next generation" sequences techniques (RNAsec) have displaced other methods to investigate global gene expression. The application of RNAsec to the complete transcriptome has two objectives, firstly, to obtain correct gene models for all genes in a given

genome. Secondly, to establish the transcriptome under different growth conditions or at different developmental stages. Whole transcriptomes are available for *A. nidulans A. flavus, A. oryzae, A. niger, A. fumigatus* among others. These were used to investigate general metabolic competence, secondary metabolism gene expression, developmental switches, resting conidia, response to drugs and alteration of expression in response to specific mutations and the transcriptional competence of specific transcription factors. As an example, FlbB mentioned in the context of asexual sporulation (see above), has other functions including the positive regulation of four secondary metabolism gene clusters. It is also possible to draw the complete chromatin landscape of a specific genome, combining chromatin immunoprecipitation (ChIP) microarray hybridization (Chip-Chip) or by parallel sequencing of precipitated DNA (Chip-Seq). Chip-Seq has been used to study the histone H3 acetylation profile of *A. nidulans* co-cultivated with *S. rapamycinicus* (see above Section "Regulation of Secondary Metabolism"), which led to the discovery of BasR a Myb-like transcription factor, which mediates the response to co-cultivation.

In both *S. certevisiæ* and *N. crassa*, knock-out strains are available for every gene. In *A. nidulans* knock-out cassettes can be obtained from the Fungal Genetics Stock Center. Using this methodology, all kinases of *A. nidulans* were deleted, and the phenotypes of their deletions studied. In principle such throughput methods can be used for any single gene family or groups of genes.

The availability of whole genomes has contributed to establish phylogenetic relations within the Aspergilli and of the Aspergilli with other fungi. A very recent article reports whole genomes for 23 species of *Aspergillus* section *nigri*. The availability of genomes opens new areas of *in silico* research. The evolution of gene clustering goes beyond secondary metabolism. Pre-genomic work has shown clustering in a number of primary metabolism pathways in *A. nidulans*, usually absent in *S. cerevisiæ* and *N. crassa*. Comparative genomics allows the study of how clustering arises, changes and vanishes within and beyond the genus. An example of this is provided by recent work on the nicotinate utilisation gene cluster of *A. nidulans*. Horizontal gene transfer (HGT) within eukaryotes is subject to controversy. There is no question that, notwithstanding our ignorance of its mechanism, transfer within the fungi, to the fungi and beyond has occurred. Already mentioned was the transfer of genes involved *β*-lactam synthesis from a *Streptomyces* to an ancestor of Aspergilli and Penicillia. Examples derived from genome analysis are the acquisition by *A. niger* fumonisin gene cluster from a *Fusarium* sp. and the acquisition by *Podospora anserina* (Sordariomycetes) of the sterigmatocystin gene cluster from an *Aspergillus*. Algorithms have been developed to detect HGT and applied specifically to *Aspergillus* species (HGT-finder). This predicts, as an example, 715 possible HTG events for *A. nidulans*. SM genes are over represented in this sample which confirms previous proposals. However, specific, individual phylogenies are necessary to validate the findings of HGT-finder. As for other taxa, the availability of whole genomes reveals a surprising role for HGT in genome evolution and metabolic capabilities. An untapped area is the evolution of transposable elements, limited recently to an investigation of LINE (long interspersed nuclear elements) retroposons in strains of *A. fumigatus*.

Acknowledgments

Further Reading

I have mostly indicated very recent reviews. Due to space limitations some very important work is not cited below. The reader can easily access the original work with either the name of the senior author or of the specific gene/protein indicated in the text. When a review was not available I have indicated only the most recent article for each subject, which usually leads to a complete set of references.

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