



Tools to study molecular mechanisms of *Aspergillus* pathogenicity

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The unique nature of *Aspergillus* fungi represents a challenge for scrutinizing the attributes that render these saprophytic microorganisms pathogenic or allergenic under certain environmental circumstances. Recent publication of the genomic sequence from an isolate of the major pathogen *Aspergillus fumigatus* denotes enormous progress in aiming at cellular features and gene products that contribute to its pathogenicity. Latest developments to study virulence-related characteristics comprise profiling techniques, conditional gene inactivation and precise manipulation of the genome by means of gene targeting. Advances in assessing the virulence potential of particular mutant strains in alternative test systems complement these approaches.

Aspergillus – saprophyte and opportunistic pathogen

The fungal kingdom comprises ~1.5 million species [1] but only a few of them have evolved to be pathogenic in mammals. Rapid progress in medicine accompanied by an increase in numbers of immunosuppressed patients has put fungal pathogens in the focus of interest for clinicians and medical microbiologists. Among the ~150 fungal species that are able to cause disease in a mammalian host, aspergilli are exceptional because they can elicit allergic responses or harm immunocompromised individuals, in most severe cases with a fatal outcome [2,3]. Their default way of life, however, is that of a saprophyte, which is characterized by the uptake of nutrients from a decaying organic substrate, and it is likely that the pathogenicity of this fungal species is based to a great extent on its saprophytic lifestyle.

The most frequent pathogen to cause aspergillosis (see Glossary) is *Aspergillus fumigatus* but species such as *A. terreus*, *A. flavus* or *A. niger* have also become increasingly evident in infections [4]. *Aspergillus nidulans* has evolved as the genetic model for the genus, and a comparative evaluation of the genome sequences from *A. nidulans* and *A. fumigatus* has not revealed specific virulence traits that are solely characteristic for *A. fumigatus* [5].

Several approaches can be followed to identify gene products that support *Aspergillus* pathogenicity. It is the objective of this review to provide an update on recent methods and advanced tools of *Aspergillus* molecular biology, along with alternative infection models that have been developed and established to study pathogenesis of *Aspergillus*.

Mimicking the disease – (alternative) test systems to study infection

Cell lines and amoebae

Pulmonary aspergillosis is characterized by the infection of an immunocompromised host through mitotic spores that are able to reach the lower respiratory tract. There, the fungus encounters two lines of defence by immune effector cells: alveolar macrophages that phagocytose and digest these conidia [6] and neutrophils that attack and lyse hyphae of germinated spores [7]. Accordingly, experiments in which *A. fumigatus* conidia are challenged with respective cell lines have commonly been used to assess resistance against attack by immune effector cells. Because the access to and handling of such cell cultures requires special expertise and infrastructural requirements (which might not be established in every research unit), an alternative test system to model phagocytosis by macrophages has been sought (Figure 1). Aside from early reports on the effect of spore diffusates from *Aspergillus* species on *Naegleria gruberi* and *Acanthamoeba castellanii* [8], no such model has been described for *Aspergillus fumigatus*. However, the social soil amoeba *Dictyostelium discoideum* could serve as a model: this haploid unicellular microorganism is a tractable host for studying uptake events or survival of a phagocytosed pathogen inside vacuolar compartments [9]. No data on the virulence potential of *Aspergillus* isolates against *D. discoideum* have been gathered so far, although it might be worth evaluating such an amoeba model that imitates the early steps of pathogenesis. This idea is strengthened by recent studies on the pathogenicity of the encapsulated fungus *Cryptococcus neoformans*, which used the *Dictyostelium* model as an alternative to macrophage cell lines [10]. Furthermore, this alternative phagocytosis system bears the potential for screening of *D. discoideum* mutants to assess cellular structures that support host immune cells in fungal eradication.

Vertebrates and insects

When studying the virulence potential of a particular *Aspergillus* strain, animal models for invasive pulmonary aspergillosis still represent an essential standard [11,12]. Especially rodents such as mice or rats but also guinea pigs, rabbits or birds are susceptible to *Aspergillus* infections, and both immunocompetent and immunosuppressed animals can be used in experimental studies [13]. Usually, an established regimen of immunosuppression is carried out before and after injection or inhalation of conidia to induce inflammation or to result in fungal growth inside

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Glossary

Aspergillosis: disease provoked by infection with *Aspergillus* species.

Differential fluorescence induction (DFI): a technique to monitor promoter activities by expression of a fluorescent protein that can be used as selectable marker in fluorescence-activated cell sorting.

Double-joint PCR (DJ-PCR): PCR-based fusion technique to combine three DNA fragments.

In vivo expression technology (IVET): promoter-trapping approach to select for promoters that are active in a specific ecological niche.

Molecular Koch's postulates: interpretation of Koch's postulates on a molecular basis; states that inactivation of a putative virulence gene should result in decreased pathogenicity and that its reversion or allelic replacement restores pathogenicity.

Nonhomologous end-joining (NHEJ): major cellular pathway to repair double-strand breaks, mediated by the DNA-end binding, heterodimeric Ku complex and the IV-XRCC4 ligase.

Parasexuality: processes that provide genetic variability and recombinant progeny in the absence of meiosis; characterized by diploidization through fusion of haploid nuclei in a heterokaryon to undergo mitotic recombination and spontaneous haploidization.

Recombineering: DNA engineering approach using homologous recombination in *Escherichia coli*.

RNA interference (RNAi): cellular phenomenon that results in gene-specific silencing, mediated by double-stranded RNA and degradation of its equivalent mRNA.

Signature-tagged mutagenesis (STM): insertional mutagenesis approach that uses pools of marker cassettes, which carry unique oligonucleotide signature tags that enable identification by hybridization.

the lung to model systemic or pulmonary aspergillosis, respectively (Figure 1). Mortality rates deduced from single inocula enable the approximate estimation of virulence for a given strain, but administering mixtures that contain equal amounts of spores from a wild-type and a mutant enables a more subtle assessment of the pathogenic potential. In such competitive infection experiments, the output ratio of mutant to wild-type spores is compared with the input proportion, and so-called competitive indices are calculated from these data [14].

A large body of infection studies has been carried out in murine models of aspergillosis to identify virulence-determining gene products. To avoid the high costs and logistical efforts associated with vertebrate models, alternative insect models were recently evaluated [15]. Despite their less sophisticated immune system (in comparison to mammals), insects respond to and counteract microbial pathogens using immune effector cells that reside in the hemolymph. Accordingly, their simple handling and availability makes them suitable for preliminary infection studies.

Caterpillars of *Galleria mellonella* – the greater wax moth – have been used to assess virulence differences between mutants of the fungal pathogen *Candida albicans*, and a certain degree of correlation to the murine infection model could be deduced [16]. More recently, the wax moth model was used to study the pathogenesis of *Cryptococcus neoformans* [17]. A limited number of studies have addressed the usefulness of these insects to study the pathogenesis of *A. fumigatus*. Practically, larvae are inoculated by injection of a small volume of a spore suspension through the cuticle of one of the pro-legs and fatal progression eventually results in the torpor of dark-brownish larvae (Figure 1). Virulence of specific *A. fumigatus* mutants has been studied by infecting *G. mellonella* larvae [18] but comprehensive data to estimate the validity of this alternative model are scarce. To date, the only example in

which virulence of an *A. fumigatus* mutant background was estimated in the murine and wax moth model is described for strains impaired in the nonhomologous end-joining (NHEJ) pathway [19,20].

A minihost model was established recently by Kontoyiannis and co-workers using the fruit fly *Drosophila melanogaster* [21]. In this insect, the Toll signalling pathway controls defence mechanisms against Gram-positive microbes and fungi [22]. Whereas wild-type *D. melanogaster* cannot be infected by *A. fumigatus* conidia, Toll-deficient mutants are highly susceptible. Fungal contagion is routinely triggered by pricking *Tl⁶³²/Tl^{I-RXA}* mutant flies at the dorsal thorax side with a sterile needle dipped into a concentrated spore suspension to result in infection of the hemolymph. Alternative modes of infection are ingestion to infect the fly gut or penetration through the exoskeleton after rolling (Figure 1). Virulence estimation can be completed a few days post-infection for a large group of infected flies, which makes this alternative infection model fast and statistically reliable. Furthermore, it enables testing of antifungal substances by feeding starved flies with drug-containing food [23]. Notably, two mutant backgrounds of *A. fumigatus* involved in thermotolerance and melanin synthesis were tested in the fruit fly model to give results that correlate with the murine system [23,24].

Some pros and cons of alternative test systems

Alternative test systems to identify virulence attributes of *Aspergillus* have evolved with valuable inputs from studies on other pathogenic microorganisms. Because none of these exhaustively mimic the process of *Aspergillus* infecting a human host, results from alternative test systems should always be considered as pre-screening guidelines to complement studies that use established animal models or primary and secondary cell lines. Because of the low optimal temperature for propagation of amoeba or insect cells (24.5 °C for *D. discoideum*, 30 °C for *G. mellonella*, 25 °C for *D. melanogaster*), virulence attributes of *Aspergillus* that are linked to its thermotolerance are not likely to be found in this kind of assay. However, the low costs and efforts in handling the test organisms, their simple tractability and the prospect of high-throughput screening make them attractive for future studies.

Gaining an overview on gene expression – transcriptomic and proteomic developments

Pathogenesis of *Aspergillus* depends on a plethora of genome-encoded factors [25], and detailed knowledge on such gene products aids in understanding the molecular basis for pathogenicity of a naturally saprophytic fungus. With the advance of molecular biology in *Aspergillus* research [26], screening procedures and straightforward approaches have become feasible by exact genetic manipulation. However, the firm interplay within the pathogen–host system represents a challenge, especially in profiling approaches, and analyses that scrutinize *in vivo* *Aspergillus* infection of an immunocompromised host are hampered by this setting.

A giant leap towards profiling analyses was aided by the *A. fumigatus* genome sequencing project [27,28] accompanied by release of the *A. nidulans* genome [29]. Analysis of

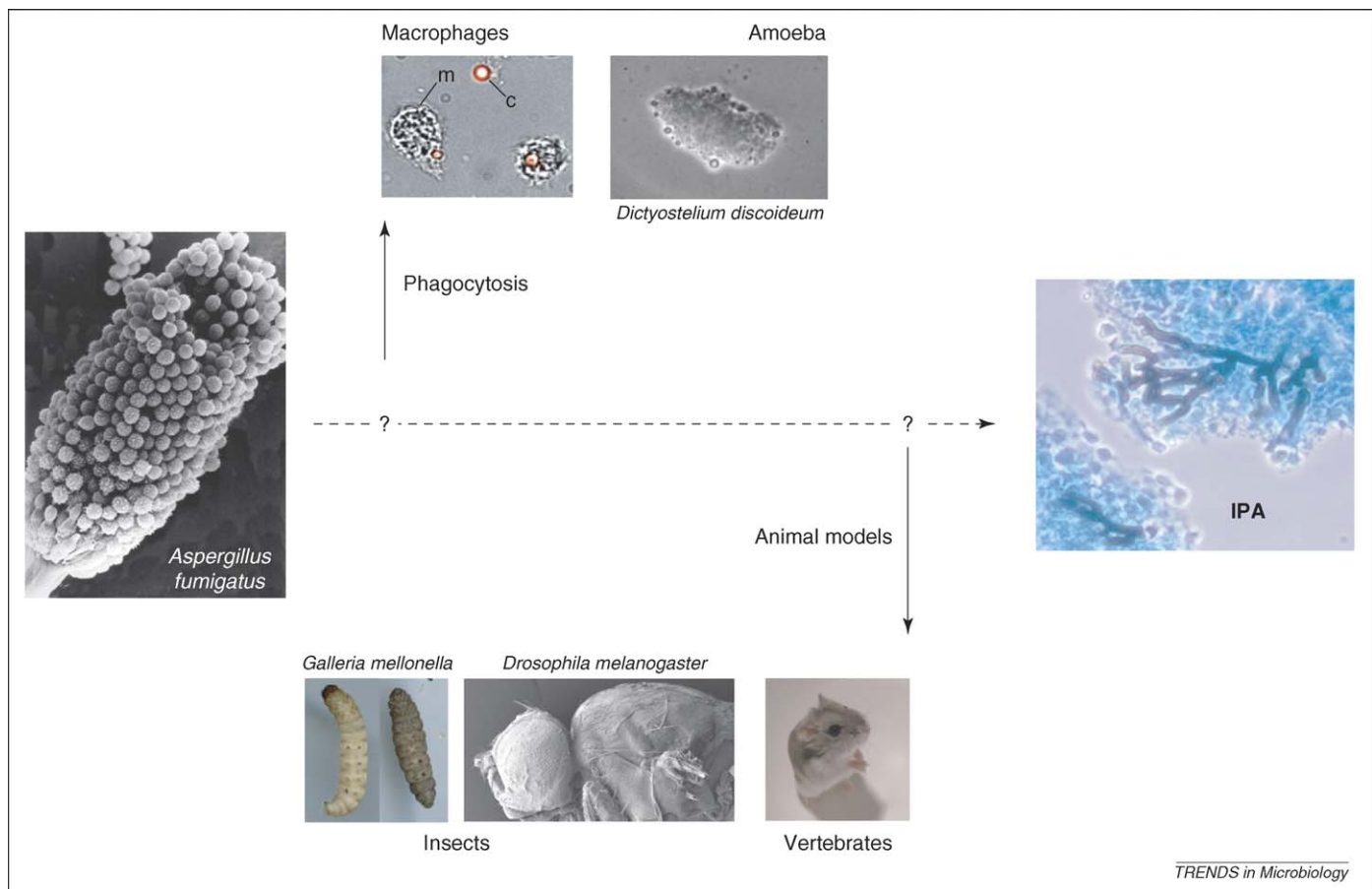


Figure 1. Established and alternative systems to model invasive aspergillosis. In the common route of infection, spores of aspergilli like *Aspergillus fumigatus* (left) are inhaled, germinate and propagate by hyphal growth inside the host lung to result in invasive pulmonary aspergillosis (IPA) as represented by the histological image (right). To study early events in *Aspergillus* infection, assays that use immune effector cells represent the standard, illustrated by macrophages (m) ingesting *A. fumigatus* conidia (c, red). Alternatively, microorganisms that are able to ingest small particles such as the social soil amoeba *Dictyostelium discoideum* might serve as genetically tractable models to examine phagocytosis events. To model invasive aspergillosis as a whole, animal infection studies are well established, primarily using vertebrates such as rodents. In recent times, alternatives were evaluated using insects such as larvae of the greater wax moth (*Galleria mellonella*) or fruit flies (*Drosophila melanogaster*). In the wax moth system, fatal infections result in brownish-pigmented, motionless larvae; in the fly minihost model, Toll-deficient flies are susceptible to fungal colonization. *A. fumigatus* conidiophore electron micrograph reproduced, with permission, © The Fungal Research Trust; image of conidia-ingesting alveolar macrophages kindly provided by M. Gunzer (GBF Braunschweig) and A.A. Brakhage (HKI Jena), Germany; scanning electron micrograph of *Drosophila* fly covered with *A. fumigatus* mycelium obtained liberally from D.P. Kontoyiannis (The University of Texas M.D. Anderson Cancer Center, USA).

the *A. fumigatus* sequence enabled the design of DNA arrays [30], which were used in first experiments to monitor the transcriptional read-out during temperature shifts [28]. Thermotolerance of *A. fumigatus* is regarded as a virulence-determining trait [31] and, therefore, these studies give hints on the contributing cellular features. Future studies will have to address the status of the *A. fumigatus* transcriptome in the course of infection. This task is impeded by contamination and low yields of RNA material but amplification steps to increase the input amounts in hybridizing experiments [32] can help to circumvent these difficulties.

Comprehensive data on expression of *Aspergillus* proteins under varying conditions are still limited. A first evaluation on the proteome of *A. fumigatus* was recently published by Brakhage and co-workers, who described the protein patterns after growth on different carbon sources [33]. Moreover, the report presents optimized protocols for sample preparation and electrophoretic separation, which will prove valuable in future proteomic approaches. In upcoming experiments, the implementation of multiplex detection techniques [34] that enable simultaneous

evaluation of sample mixtures will be of worth; these are yet to be established in *Aspergillus* proteomic research.

Given these recent developments, studies on aspergilli have entered the post-genomic era, and data on the cellular characteristics that render members of this genus pathogenic can now be collected.

Genomic screens and modulation of gene expression

Genetic screening protocols have a long standing tradition in fungal molecular biology and are valuable in identifying genes that fulfil specific biological tasks [35]. An excellent example of an *in vivo* screen was described by Holden and co-workers, who carried out signature-tagged mutagenesis to identify *A. fumigatus* genes that are essential for pathogenesis in a murine model of pulmonary aspergillosis [36]. From an array of 4648 random insertion mutants that were used to infect mice in mixed infection experiments, 35 were cleared from the pool of mutants. Among them, the *pabaA* gene was validated to be essential for virulence, which was in accordance with previous findings in *A. nidulans* [37]. Other methods to identify genes induced in a niche-specific way during infection, such as *in vivo* expression technology

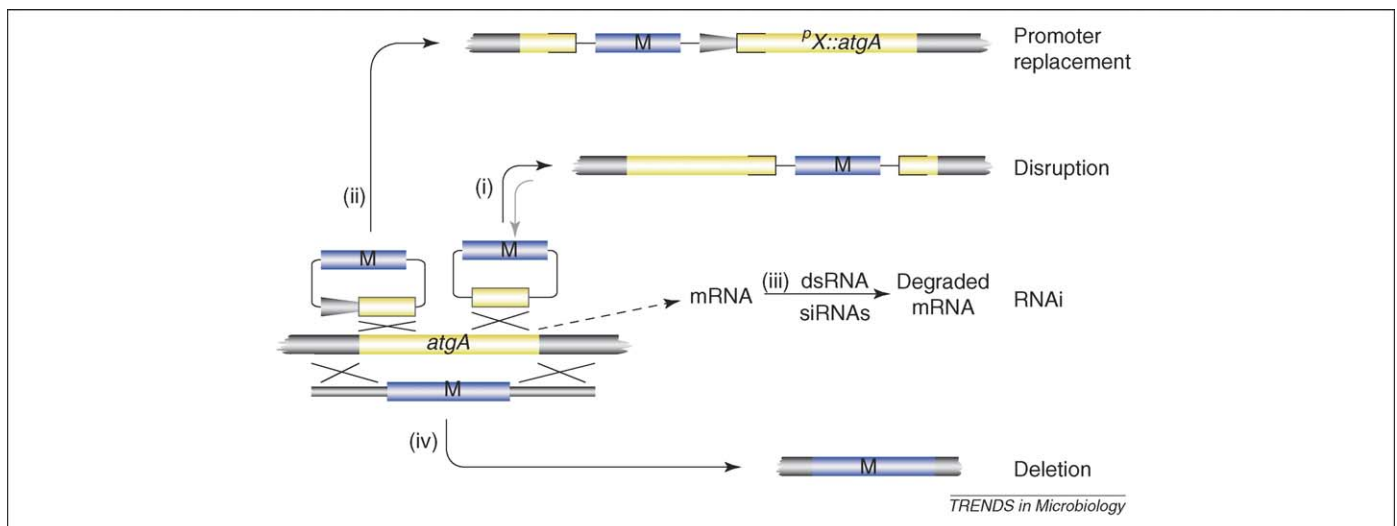


Figure 2. Strategies to modulate gene expression in *Aspergillus*. To modulate expression of an *Aspergillus* target gene (*atgA*, yellow), several molecular approaches can be followed. (i) Integration of a partial fragment (yellow box) linked to a selectable marker gene (M, blue) results in a disruption, from which a truncated version of AtgA can still be expressed. Reversal of the integration event in the absence of selection pressure (light-grey arrow) accounts for genetic instability. (ii) Integration of a regulable promoter-fused construct leads to promoter replacement, by which expression of *atgA* can be modulated through environmental conditions. (iii) Degradation of the *atgA* mRNA can be achieved to a certain extent by RNA interference after introduction of double-stranded RNA (dsRNA) or small interfering RNA molecules (siRNAs). (iv) A complete null phenotype is accomplished by abolition of the coding sequence using a replacement cassette. In this approach, the selectable marker gene is flanked by sequence stretches homologous to the target locus, and homologous recombination results in the desired *atgAΔ* deletion.

or differential fluorescence induction [38], still await exploitation in *Aspergillus* virulence studies.

An approach to study factors required for viability lies in the identification of the encoding genes. Because *A. fumigatus* does not display a sexual cycle under standard laboratory conditions, its parasexual genetics must be used to identify mutants deleted for such genes [39]. A more direct approach to assess essentiality of a candidate gene lies in conditional silencing of its expression (Figure 2). A regulable promoter that drives transcription of the gene in question and replaces the original 5' region serves this task, as demonstrated by fusing the ethanol-inducible *alcA* promoter sequence to the nuclear migration factor-encoding gene *nudC* of *A. fumigatus* [40]. Other established promoter sequences often lack tight repression characteristics, therefore novel, non-leaky promoters are needed. One such example is the isocitrate lyase gene *acuD* from *A. fumigatus*, which is almost completely repressed in the presence of glucose as the sole source of carbon, whereas expression is triggered by acetate feeding [41].

An alternative way to achieve downregulation of gene expression is provided by RNA interference (RNAi). Recently, this method was successfully applied to silence gene expression in *A. fumigatus* [42] and other *Aspergillus* species [43,44]. Thus, RNAi could serve as an alternative to gene targeting when studying functions of genes and gene families. However, results from other fungi imply that RNAi-induced reduction of gene expression can be incomplete and, therefore, might not necessarily result in a null phenotype [45].

In summary, genetic screens and modulation of gene expression are powerful approaches to study factors involved in *Aspergillus* pathogenicity. Although few studies have been carried out in this field, recent progress in manipulating the genomes of *Aspergillus* species has made smart screens and comprehensive studies feasible.

Gene targeting strategies to identify virulence-determining factors

To assess the cellular function of an uncharacterized protein, complete inactivation of its encoding gene has become a standard procedure. This candidate approach is a prerequisite to test defined strains in experimental models of aspergillosis, especially for the study of *Aspergillus* genes that encode putative virulence-determining factors. Moreover, in line with the molecular concept of Koch's postulates [46], generation of a mutant followed by reconstitution is necessary to define a gene product as a virulence-determining factor in microbial pathogenesis.

Genetic markers

A versatile marker system has been developed in *Aspergillus* molecular biology (Table 1), which was first based on complementation of nutritional requirements to select for transformants [47–49]. This approach uses a homologous or heterologous gene that replaces the gene of interest to complement an underlying auxotrophy but is not always practicable as position effects might interfere with expression to result in additional phenotypes [50]. Especially when assessing the virulence of *A. fumigatus* mutants in animal models, such phenotypes based on insufficient expression of the marker gene must be excluded because the influence of nutritional versatility on the pathogenicity of this fungus has been demonstrated in several studies [37,51,52]. A more suitable strategy lies in the use of resistance markers that can be selected for in a wild-type background. Commonly used marker cassettes for *Aspergillus* are the *Escherichia coli* phosphotransferase-encoding *hph* gene or the *Streptoalloteichus hindustanus* *ble* gene, which confer resistance towards hygromycin or phleomycin, respectively [53]. In addition, the *bar* gene of *Streptomyces hygroscopicus* has been used to select glufosinate-resistant transformants of *A. nidulans* [47]. Moreover, two additional

Table 1. Characteristics of selected genetic markers in *Aspergillus* molecular biology^a

Marker gene or module	Phenotype	Marker type	Counter-selection	Recyclable	Recombineering	Refs
<i>argB</i>	Arginine ⁺	M				[49]
<i>lysB</i>	Lysine ⁺	M				[49]
<i>pyroA</i>	Pyridoxin ⁺	M				[47]
<i>riboB</i>	Riboflavin ⁺	M				[47]
<i>pyrG</i>	Uracil/uridine ⁺ , 5FOA ^S	M	Yes			[48]
<i>pyrGI/zeo^R</i>	Uracil/uridine ⁺ , 5FOA ^S	M	Yes		Yes	[61]
<i>neo^R-pyrG-neo^R</i>	Uracil/uridine ⁺ , 5FOA ^S	M	Yes	Yes		[56]
<i>zeo^R-pyrG-zeo^R</i>	Uracil/uridine ⁺ , 5FOA ^S	M	Yes	Yes	Yes	[57]
<i>niaA</i>	Nitrate utilization ⁺ , chlorate ^S	M	Yes			[58]
<i>sC</i>	Sulfate utilization ⁺ , selenate ^S	M	Yes			[59]
<i>aurA^R</i>	Aureobasidin A ^R	Res				[55]
<i>ptrA</i>	Pyrithiamine ^R	Res				[54]
<i>PgpdA::ble::trpC^t</i>	Phleomycin ^R	Res				[53]
<i>PgpdA::hph::trpC^t</i>	Hygromycin ^R	Res				[53]
<i>PamdS::bar::glaA^t</i>	Glufosinate ^R	Res				[47]
<i>PgpdA::ble::trpC^t/zeo^R</i>	Phleomycin ^R	Res			Yes	[62]
<i>loxP-PgpdA::ble/tk::trpC^t-loxP</i>	Phleomycin ^R , FUDR ^S	Res	Yes	Yes		[60]

^aAbbreviations: 5FOA, 5-fluoro-orotic acid; FUDR, 5-fluoro-2'-deoxyuridine; M, metabolic marker; P, promoter sequence; ^R, resistance; Res, resistance marker; ^S, sensitivity; ^t, terminator sequence; plus symbol (+) denotes prototrophy.

drug-resistance genes are described for transformation of *Aspergillus* species: the aureobasidin A resistance gene *aurA^R* and a *ptrA* allele, which enables growth in the presence of pyrithiamine [54,55]. The *ptrA* selection system can be applied for a range of *Aspergillus* species, whereas aureobasidin A is effective against *A. nidulans* and *A. niger* only.

Counter-selection systems that enable screening for the absence of a gene have been of value in fungal molecular biology for marker rescue. Three counter-selectable loci have been described for aspergilli: the *pyrG* gene (and orthologues thereof), the presence of which is toxic when the drug 5FOA is supplemented [56,57]; the *niaA* gene, which confers sensitivity towards chlorate [58]; and the *sC* system, which mediates sensitivity towards selenate [59]. Usually, these markers are flanked by direct repeats to enable recombination and, therefore, excision from the genome. Such systems require genetic lesions in the background in which they are introduced, causing auxotrophies once the cassette has been excised. Because this might interfere with successive experiments, a negative selection system that could be applied in wild-type strains would be beneficial. Such a recyclable marker system that enables positive and negative selection in a wild-type background of *A. fumigatus* was developed recently. The system uses a fusion of the phleomycin-resistance gene *ble* to a viral thymidine kinase-encoding gene that confers toxicity for nucleoside analogues (Figure 3). Moreover, excision of this module by transient expression of the Cre recombinase can be achieved because of flanking *loxP* sites. Accordingly, this recyclable marker enables multiple gene deletions and reconstitution of genetic lesions by homologous replacement, as validated in *A. fumigatus* by promoter replacement of the *veA* locus after deletion of the *pabaA* gene [60].

Gene targeting

Two strategies to target a gene and to impair its functionality are established in *Aspergillus* molecular biology: disruption or deletion (Figure 2). By transforming a construct that contains a fragment of the target locus together with a selectable marker, interruption of the coding sequence by homologous integration is achieved. Such a disruption event can result in expression of a truncated but still functional gene product, and its probable genetic instability is a further drawback. A more reliable approach lies in complete deletion of the target locus by replacement, in which a marker is flanked by sequence stretches homologous to the gene to be replaced, and two crossover events (in contrast to one crossover event in case of a disruption) result in the desired genomic situation. Gene targeting in filamentous fungi is often hampered by low frequencies of homologous recombination (HR) and results in integrations of a transformed DNA fragment out of the target region. In aspergilli, this experimental drawback is also evident, making the incorporation of long stretches of at least 1kb of homologous flanking sequence into a replacement cassette necessary.

Precise manipulation of large DNA molecules can be achieved using *E. coli* strains that express recombination systems, an approach that was successfully applied to generate suitable replacement cassettes for deletions in *Aspergillus* [61–63]. However, this recombineering approach requires the isolation of a DNA fragment that comprises the target locus. PCR-based methods are of use to circumvent this step but are limited in accuracy and the size of the amplified fragment. However, recent approaches to construct replacement modules are predominantly PCR-based because of their straightforwardness. Commonly, stretches of flanking sequences are amplified and then fused to a convenient marker cassette. This can be

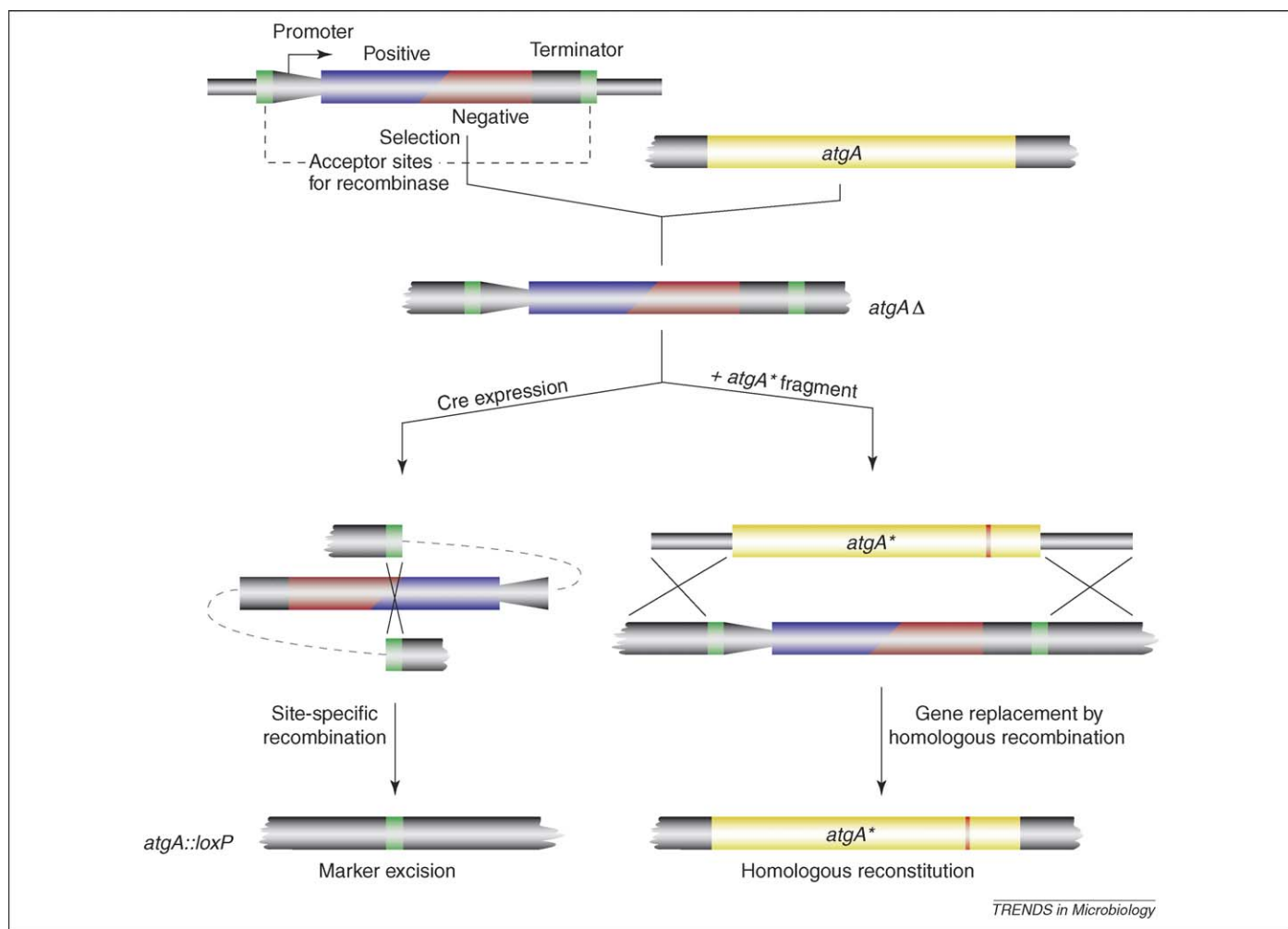


Figure 3. Advanced gene manipulation in *Aspergillus*. A system of gene replacement and reconstitution or marker rescue was designed and could be validated in *Aspergillus fumigatus*. The marker module consists of regulatory elements that drive expression of a resistance marker gene (positive selection) fused to a counter-selectable gene (negative selection); acceptor sites for site-specific recombination flank the construct. After replacement of the *Aspergillus* target gene (*atgA*) to result in a deletion (*atgAΔ*), and as a result of negative selection, homologous reconstitution of the genetic lesion is possible (*atgA**). By contrast, transient recombinase expression from an autonomously replicating plasmid results in excision of the marker (*atgA::loxP*) to enable another round of gene replacement.

achieved again by PCR, using the double-joint method (DJ-PCR) [64] or, alternatively, by ligation. With ligation, incorporation of specific restriction endonuclease recognition sequences forces generation of the desired cassette and avoids by-products [65].

Other approaches have aimed to improve the ratio of homologous versus ectopic integration. The bipartite marker concept is based on the assumption that directing the cellular HR machinery to the replacement cassette aids in its integration at the target site. Accordingly, increased gene targeting rates were observed for *A. nidulans* in transformation experiments with two overlapping fragments spanning a marker module [66]. An alternative approach was recently applied to *Aspergillus* molecular biology, based on findings in *Neurospora crassa* [67]. In this method, deletions in genes encoding subunits of the Ku heterodimer, which is required for the nonhomologous end-joining process, resulted in an increase in the relative rate of homologous integration. Accordingly, *A. fumigatus* strains with deletions of the corresponding *akuA* or *akuB* gene display high gene-targeting efficiencies (Figure 4), which was also validated for *A. nidulans*, *A. oryzae* and *A. sojae* [19,20,47,68]. Moreover, the low rates of ectopic

integration enable shortening of the flanking homology arms to a size of 100–500 bp. Tests on NHEJ-deficient *A. fumigatus* mutants in two different model systems did not reveal any reduction in virulence, which supports the feasibility of this genetic background in gene deletion and virulence testing projects. However, further investigations must strengthen this claim, considering the data from the model eukaryote *Saccharomyces cerevisiae* on the involvement of NHEJ and the Ku complex in fundamental processes such as DNA repair or telomere maintenance [69].

Manipulating *Aspergillus* genes depends on several components, such as a marker system, strategies to construct replacement modules and reliable targeting efficiencies. Recent developments have addressed each of these issues, and by the improvements that have been achieved, a more straightforward and precise way of generating mutant strains has been established.

Concluding remarks and future perspectives

The molecular toolbox to study genes that contribute to the pathogenicity of *Aspergillus* species – in particular *A. fumigatus* – has expanded enormously in recent times,

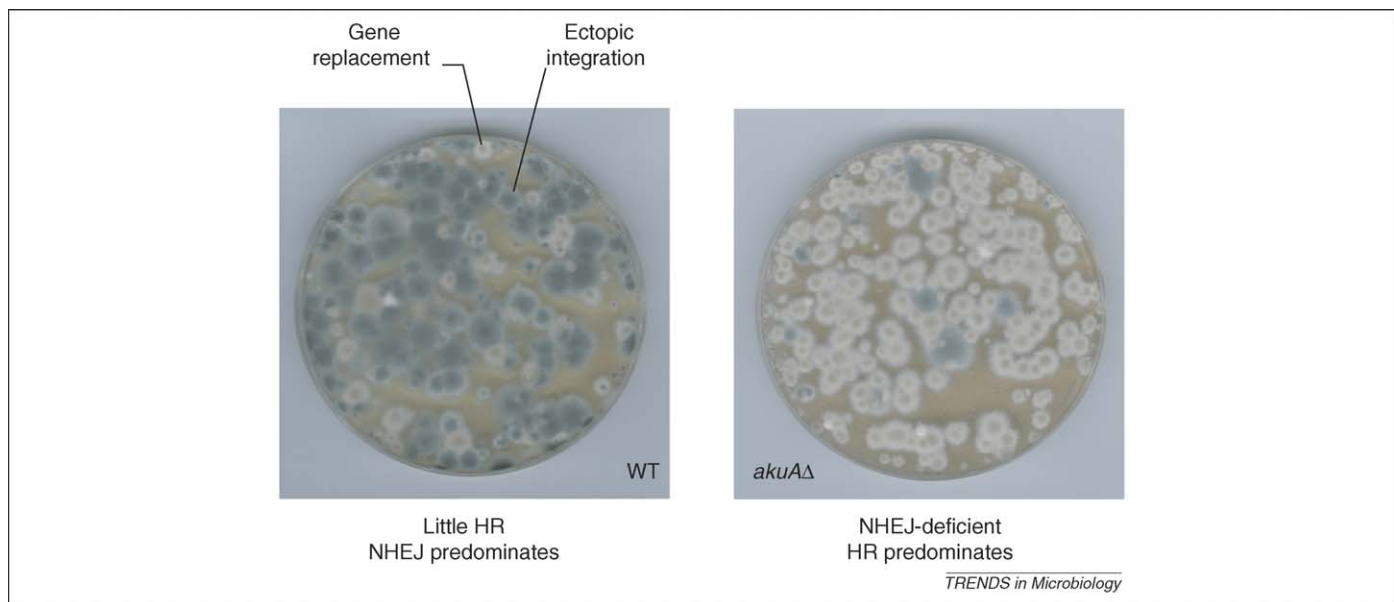


Figure 4. Gene targeting in *Aspergillus* mutants deficient in nonhomologous end-joining. The homologous recombination (HR) and nonhomologous end-joining (NHEJ) pathways determine the modes of DNA integration in *Aspergillus*. NHEJ supports ectopic integration of a transformed DNA fragment, HR results in the desired gene replacement event. Inactivation of the Ku heterodimer, which is essential for the NHEJ process, increases the relative rate of HR. This is evident when comparing primary transformation plates using a wild-type (WT) or Ku^- (*akuAΔ*) genetic background as a recipient to generate a spore colour mutant strain of *Aspergillus fumigatus* that produces brown colonies instead of green ones: in the wild-type strain, ectopic integration is the main mode for a corresponding gene replacement cassette to yield a bulk of green-coloured transformants (left), whereas in the NHEJ-deficient mutant strain, HR and brownish colonies predominate (right).

Box 1. What makes a 'grass-eater' become a man-eater? Future objectives in studying *Aspergillus* pathogenicity

Can the cryptic sexuality of *Aspergillus fumigatus* be exploited?

The isolation of recombinant progeny from a meiotic cycle is a powerful classical genetic technique. Given the chance that *A. fumigatus* has the capacity for mating and sexual development [70], activation of its cryptic sexual cycle would greatly improve manipulation tasks for this organism.

Is there a standard in testing mutant strains?

Testing *A. fumigatus* mutants in murine models of aspergillosis is the most common way to identify virulence-determining factors. However, differences are evident for almost every component of such test systems, ranging from genetic backgrounds and genetic markers to infection routes and doses. Accordingly, common protocols for mutant generation, characterization and reconstitution or even a central platform for virulence testing are considered necessary.

Which genes determine pathogenicity?

Publication of the *A. fumigatus* genome sequence has meant that the generation of a deletion mutant collection has become feasible, supported by advances in genetic manipulation of this fungus. To exploit the recombination capacities of the NHEJ-deficient genetic background, transient inactivation of the Ku complex would be advantageous over the use of *aku⁻* mutants because this background might interfere with individual mutations or fitness in a long-term perspective. Moreover, expression systems that enable *in vivo* manipulation need to be evaluated to study the influence of particular *A. fumigatus* genes during infection.

complemented by the development of feasible test systems to assess the virulence potential of defined mutant strains. A clear trend can be deduced from these latest advances that points to a comprehensive collection of deletion mutants to enable large-scale virulence tests. Such an array of deletion mutants will surely contribute to our understanding of the cellular attributes of *Aspergillus* that render this saprophytic fungal species pathogenic under

certain conditions. A currently unaddressed problem, however, lies in the quantitative nature of *Aspergillus* pathogenicity; scrutinizing single genes might not be of value in studying this multifactorial trait. Choosing candidates that regulate clusters of genes, like signal transduction components or transcription factors, could assist in addressing this matter. By using recyclable markers, the combined impact of several genes or gene families can be assessed. Moreover, a variety of additional tasks must be addressed in the future (Box 1) to strengthen research on the molecular mechanisms of *Aspergillus* pathogenicity.

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