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Nitrogen metabolism of *Aspergillus* and its role in pathogenicity

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Aspergilli represent unique pathogens. Based on their saprophytic life style they are able to colonize a variety of ecological niches, among them the immunocompromised individual. Distinct fungal attributes that play a role in pathogenicity of aspergilli have been described, and primary metabolism indisputably has to be taken into account for contributing to the virulence potential of this fungal genus. Here we present an overview of studies that focus on this aspect of nutritional versatility. In the predominant pathogenic representative *Aspergillus fumigatus* regulation of nitrogen utilization and sensing of nitrogen sources have been scrutinized with respect to pathogenicity. The impact of distinct metabolic pathways on virulence capacities could be evaluated by inspection of auxotrophic mutant strains. Among them, para-aminobenzoic acid-requiring mutants revealed that this biosynthetic route is strictly required for pathogenicity. For amino acid anabolism only lysine biosynthesis has been investigated in this regard. Fungal amino acid biosynthesis is generally subject to strict regulation mediated by the Cross-Pathway Control system, a conserved regulatory circuit evolved to counteract conditions of nutritional stress. A clear influence of the system on pathogenicity could be observed by targeting its transcriptional activator CpcA. However, additional metabolic characteristics as well as regulatory instruments that compensate environmental challenges need to be addressed in future research with the aim to assess the significance of fungal primary metabolism for pathogenicity of aspergillus species.

Keywords amino acid bioynthesis, aspergillosis, *Aspergillus fumigatus*, cross-pathway control, primary metabolism

Introduction

Filamentous fungi of the genus *Aspergillus* are ubiquitous and widespread in the environment. After their first description in 1792 by the Italian botanist Pier Antonio Micheli, Raper and Fennel were the first to categorize the genus into 18 groups containing 132 species, a number that nowadays has been extended to more than 180 [1,2]. The strongest morphological criterion defining the genus is the conidiophore, the asexual reproduction structure of the anamorph, which gave rise to the name of the genus. Common to all aspergilli is a mode of propagation via asexual conidiospores that are released into the environment. Following germination, these conidiospores produce a vegetative branched mycelium on suitable growth substrates [3–6]. Aspergilli are prime representatives of a saprobic lifestyle, feeding on dead matter and utilizing a broad range of growth substrates and organic material.

Over recent decades, aspergilli have attracted increasing attention as pathogens causing diseases collectively termed aspergilloses. First described in the 19th century, this formerly rare disease has become a major threat in the clinical environment for

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immunocompromised individuals [7,8]. Generally, every ailment caused by a fungus of the *Aspergillus* genus is defined as aspergillosis. Owing to the unique features of this fungal group, its associated syndromes are complex and diverse in nature [9,10]. Allergic diseases have been linked to aspergilli, but the more severe clinical forms of aspergillosis are characterised by saprophytic propagation of the fungus within the host [11]. Depending on the immune status of the infected individual, varying degrees of fungal infection can be monitored. Severe forms of mycoses are a major threat to immunocompromised patients, especially those with neutropenia or organ transplant patients.

The agents of aspergillus infection are airborne conidia, which, due to their small size, are able to reach the pulmonary alveoli, the primary site of infection. If these conidia are not cleared by the host, such as when an adequate host immune response is absent, they germinate and grow *in vivo* as pathogenic aspergilli. These generally have no specific nutritional requirements and can grow at elevated temperatures. Consequently, forms of so-called invasive aspergillosis (IA) may emanate, in which the fungus propagates and penetrates the surrounding lung tissue. IA is most severe and fatal in disseminated, systemic cases. According to the concept of the damage response framework [12], aspergilli represent unique pathogens due to their competence to harm individuals that display acute hyperactivity of their immune status or, alternatively, that are severely compromised in the immune system.

Factors supporting pathogenicity

As for opportunistic pathogens, the relevance of so-called virulence factors that are attributed to the virulence of aspergilli and support pathogenicity has to be evaluated with care [13]. Unlike 'true' pathogenic microorganisms, fungal pathogens often take advantage of a severely impaired innate immune response from the infected individual and propagate within the host analogous to any other growth substrate. Therefore, factors supporting the saprophytic lifestyle are likely to contribute to pathogenicity, and exclusive virulence factors that affect specifically *in vivo* growth remain rarely described [14].

Among the factors that may support pathogenicity, metabolism has to be taken into account as it is the very basis for growth and expansion of the fungal mycelium. Fungal metabolism makes this phylogenetic section unique and highly specialized to the environmental challenge. Aspergilli, in particular, share distinct metabolic features that assist their distribution in the environment and it is of logical consequence that their metabolic characteristics contribute to their pathogenic potential. In this short overview we will summarize the knowledge on nitrogen metabolism of aspergilli that has accumulated as far as it has been linked to pathogenicity attributes. Logically, we will concentrate on the predominant virulent representative of the species, *Aspergillus fumigatus*, but will also incorporate knowledge gained from the scientific model organism *Aspergillus nidulans*. Furthermore, we will essentially focus on selected studies of primary routes of metabolism, thereby excluding secondary metabolism which would exceed the scope of this review.

Utilization of nitrogen sources

Aspergilli can utilize a broad range of nitrogen sources, among them ammonium, nitrate, amino acids such as histidine or proline, or complex substrates such as collagen and elastin. Of particular interest is the pathway of nitrate assimilation, a key process in the global nitrogen cycle with enormous ecological and agricultural significance. Complete reduction of nitrate by the fungus yields ammonium, which is finally incorporated in the amino acids glutamate and glutamine that act as nitrogen donors. The latter three compounds are generally regarded as primary nitrogen sources for filamentous fungi, whereas nitrate, purines or amino acids are examples of secondary nitrogen sources [15]. The nitrate assimilation pathway is essentially made up of three components (Fig. 1A): a nitrate-specific transporter (CrnA), and two enzymes catalysing the step-wise reduction of nitrate via nitrite to ammonium (NiaD and NiiA) [16,17]. Furthermore, in aspergilli, a transcriptional activator encoded by the *areA* locus acts positively on genes required for the utilization of nitrogen sources, and the *nirA* gene product specifically induces the genes required for nitrate uptake and reduction [18,19]. Recent studies in *A. nidulans*, however, have challenged this simple model and indicate that NirA and AreA are able to form a transcriptional complex [20,21].

In *A. fumigatus*, the nitrate assimilation cluster has been mapped to the largest chromosome of the genome, and detailed inspection of the sequence of the locus has revealed a high degree of conservation and synteny [22]. A gene highly similar to the *niiA* gene of *A. nidulans* was found and the regions upstream and downstream were inspected for conserved gene loci. Downstream of the *niiA* coding sequence, the *crnA* regulatory gene of *A. fumigatus* was found, whereas upstream the *niaD* locus was mapped (Fig. 1B). Both genes encoding the nitrite and nitrate reductase activities are located adjacent to each other with an intergenic regulatory

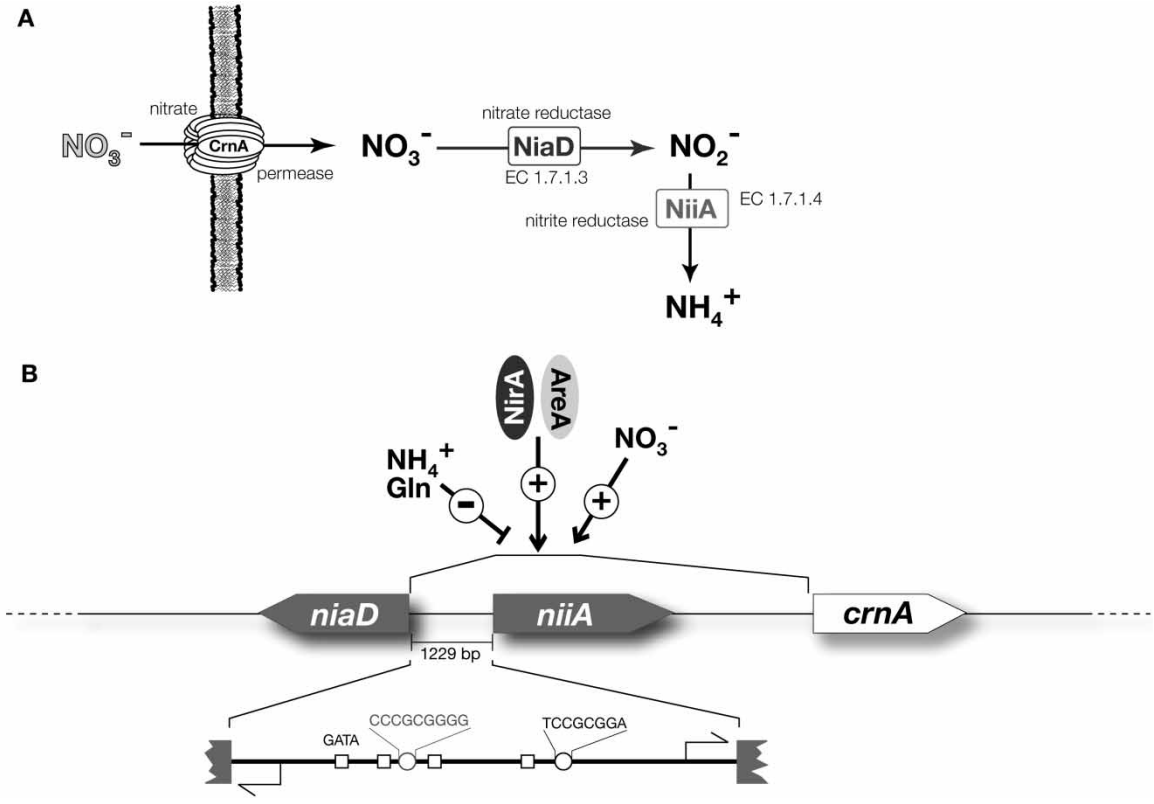

Fig. 1 Nitrate utilization in *Aspergillus*. (A) Major steps of nitrate assimilation in aspergilli are uptake of extracellular nitrate mediated by the CrnA permease followed by subsequent reduction via two reductase activities, NiaD and NiiA. The resulting ammonium is incorporated into the nitrogen pool after conversion to glutamate and glutamine. (B) Genetic organisation and regulation of the aspergillus nitrate assimilation cluster. The three encoding loci of the *niiA*, *niaD*, and *crnA* genes are located in the genome as a cluster, with the reductase-encoding genes transcribed divergently from one intergenic region. Transcriptional induction is driven by a variety of *cis* elements targeted predominantly by two DNA-binding proteins that influence transcription from the intergenic region as well as the *crnA* promoter and that may act as a transcription complex: the GATA factor AreA and the Zn binuclear cluster protein NirA. Conserved nucleotide sequences in the overlapping *niiA*/*niaD* promoters that are bound by the activators are shown, with the GATA motifs depicted as boxes and the NirA-binding elements as circles. As indicated, activation of the cluster requires the presence of nitrate and the absence of repressing metabolites like ammonium or glutamine.

region that mediates divergent transcription. On the nucleotide level, this bidirectional promoter region shares up to 43% identity with other *niiA*/*niaD* regulatory regions of aspergilli. Furthermore, it exhibits significant homology to its well-studied *A. nidulans* counterpart with respect to regulatory elements [17]. Conserved binding sites for the regulatory proteins NirA and AreA were found, which implies similar regulatory features of nitrate assimilation between these two species. As in *A. nidulans* [23], expression of the nitrate assimilation cluster in *A. fumigatus* is strictly repressed by ammonium and strongly induced by nitrate. No defined mutant strains deleted for one of the genes located in the nitrate assimilation cluster have yet been tested for their virulence capacities, but the role of the regulator gene *areA* in pulmonary aspergillosis has been scrutinized [24]. Based on its high degree of homology to the *A. nidulans* locus, the encoding gene in *A. fumigatus* was cloned and inactivated in a genetic wild-type background by disruption as well as replacement. Both strains were described to be impaired in utilization of certain nitrogen sources whereas growth rates of the mutants *per se* were not affected. The role of the *areA* gene in *A. fumigatus* virulence was examined by different approaches: the reversion rate of *areA::hyg*^{r} disruptants after causing pulmonary aspergillosis in neutropenic mice suggests that a functional *areA* gene is beneficial for propagation of the fungal pathogen within the lung of the host. Infection experiments with stable *areA*$^-$ mutant strains resulted in a delay in the development of pulmonary aspergillosis; nevertheless, mortality rates in these experiments were as high as for animals infected with the wild-type strain. Furthermore, extragenic suppressor mutants from the

areA null strain were tested in mixed infection experiments, and one of them displayed a strong growth advantage in competition to the *areA*$^{-}$ strain.

Taken together, these results suggest that the nitrogen sources that are accessible to the fungus during growth in the lung of the host are unlikely to be ammonium or glutamine and that the *areA* gene product contributes to fungal growth in the murine lung. The nature of the extragenic *areA*$^{-}$ suppressor strain implicates that a metabolic pathway that is under control of AreA is important for fungal growth in the lung tissue.

Besides nitrogen utilization, sensing of convenient nitrogen sources and uptake of nitrogen-containing components is of metabolic significance. Recently, a mitogen-activated protein (MAP) kinase of *A. fumigatus* was shown to be involved in nitrogen sensing [25]. The SakA kinase is well conserved among fungi and its orthologues like Hog1p of *Saccharomyces cerevisiae* or HogA of *A. nidulans* play a prominent role in osmotic stress responses [26–28]. Deletion mutants of *sakA* were generated in *A. fumigatus*, and besides an impaired transcriptional response upon hyperosmotic conditions these mutants displayed altered germination characteristics in dependency of the nitrogen source. In the presence of reduced nitrogen, *sakAΔ* strains germinated as well as the wild-type, but in the presence of nitrate or nitrite a much higher rate of germination was observed after 12 hours. Conclusively, the SakA MAP kinase pathway regulates germination negatively in response to the nitrogen source. Nevertheless, the *sakA* deletion strain displayed no reduction in virulence in a murine model of invasive pulmonary aspergillosis, demonstrating that a functional osmotic stress response pathway even with its impact on nitrogen sensing is not required for pathogenicity of this fungus.

A more convincing aspect of nutritional versatility and its connection to pathogenicity of *A. fumigatus* was demonstrated by studies on the Ras-related RhbA protein [29]. *rhbA* encodes the only Rheb homologue in the *A. fumigatus* genome and was initially characterized to be transcriptionally upregulated during *A. fumigatus* propagation in the presence of human endothelial cells. Deletion studies revealed that the gene is not required in general, but that proper growth of *A. fumigatus* on poor nitrogen sources like proline, histidine or nitrate is reduced in a *rhbAΔ* background. Moreover, deletion of *rhbA* resulted in increased arginine uptake in the presence of a rich nitrogen source and hypersensitivity to the TOR kinase inhibitor rapamycin. Most strikingly, *rhbA* is required for full virulence of *A. fumigatus*. When infected with the deletion mutant, a group of immunosuppressed mice displayed significantly longer survival periods than mice infected with the wild-type progenitor or a reconstituted *rhbAΔ; rhbA*$^{+}$ strain. By inspection of individual pulmonary lesions, the *in vivo* growth potential of the *rhbA* deletion mutant was found to be reduced. Conclusively, RhbA influences nitrogen signalling in *A. fumigatus* and, possibly as a result, contributes to its virulence; this emphasizes the impact of nutritional flexibility on fungal pathogenicity.

Metabolic routes affecting pathogenicity

Fungal biosynthetic routes have always been of interest as the anabolic features of these organisms are often distinct from the ones displayed by animals or plants, therefore making them attractive target sites for counteracting fungal pathogens. Early studies on the virulence capacities of specific auxotrophic *Aspergillus* mutants came from Purnell [46], who used a defined set of auxotrophic strains of the genetically tractable species *A. nidulans* to evaluate their relative virulence in mice after intravenous inoculation. Based on cumulative mortality data, four groups could be classified: auxotrophies for biotin, nicotinic acid, thiamine/4-methyl-5-hydroxyethyl-thiazole, methionine, and proline/arginine did not result in any decrease in virulence; a moderate level of virulence could be detected for strains requiring purine (*ad3*), riboflavin, or thiamine. A more pronounced reduction in virulence was observed for *ad20*, *paba6*, *pyro4* or *orn4* mutants auxotroph for purines, p-aminobenzoic acid, pyridoxine or ornithine/arginine, respectively. Avirulent mutants constituted the most interesting group. Here, four loci were associated with a complete loss of virulence, *ad14* and *ad23* resulting in purine auxotrophy, *paba1* leading to p-aminobenzoic acid requirement, and *pyro*, causing pyridoxin auxotrophy. Taken together, these results support the impact of metabolic and nutritional versatility on fungal pathogenicity. Nevertheless, the data have to be evaluated with care owing to the virulence testing model, which mimics a systemic infection and not a pulmonary aspergillosis. The primary site of infection is the lung of the host, and nutritional requirements at this spot determine the capacities of the fungal pathogen to expand and spread in the infected host.

More specific inspections were performed by testing strains that carry established auxotrophic mutant alleles or targeted deletions of biosynthetic gene loci. The pyrimidine biosynthesis pathway has been explored by testing *A. fumigauts pyrG*$^{-}$ strains [30]. The *pyrG* gene product catalyses the final step in the *de novo* UMP biosynthesis and corresponding mutants are auxotroph for uracil/uridine [31]. When inoculated

in immunosuppressed mice, the mutant strains turned out to be almost completely avirulent, a trait that was diminished by the addition of uridine to the animal's drinking water or by re-constitution of the mutant by the *A. niger pyrG* gene. Detailed *ex vivo* inspection of germination and growth characteristics of the *A. fumigatus pyrG*$^{-}$ mutant strains revealed that only high uracil/uridine supplementation restored wild-type germination kinetics, and even then growth rates remained at 85% of wild-type levels. In summary, the uracil/uridine pool seems limiting in the murine lung, which accounts for drastically reduced virulence capacities of *A. fumigatus pyrG*$^{-}$ strains due to impaired germination.

Holden *et al*. [49] were able to establish another supplement strictly required for *Aspergillus* pathogenicity. Early reports on mutants of *A. fumigatus* that require the vitamin precursor para-aminobenzoic acid (PABA) had indicated that these mutants are incapable of causing systemic infections after intravenous injection. By testing *A. nidulans pabaA1* auxotrophs, the avirulence of such strains could be supported in pulmonary infection models [32]. The *pabaA*-encoded PABA synthetase converts chorismate to PABA, which is a late step in folate biosynthesis. Mice infected with conidia of *A. nidulans pabaA1* strains stayed healthy throughout the experiment and did not show any signs of pulmonary distress. Histologic inspections revealed that no fungal infection had been established, and no fungal colonies could be re-grown from homogenized lung tissues. Furthermore, supplementation of the drinking water with PABA restored virulence and also demonstrated that the requirement for that metabolite in lung tissue persists during germination as well as growth in the lung parenchyma. Further evidence came from an *in vivo* screening report employing the signature-tagged mutagenesis (STM) approach [33]. Using pools of 96, a total of 4648 mutant strains carrying distinct signature tags were inoculated in neutropenic mice. After recovery of the fungi from the lungs following development of pulmonary aspergillosis, the input pool was screened with the recovered pool for the absence of specific mutants. Among them, a severely attenuated mutant could be identified in which an insertion event at the *pabaA* locus had occurred. The *A. fumigatus pabaA* gene was cloned and a targeted deletion of this gene confirmed the complete avirulence of *A. fumigatus* strains that are impaired in folate biosynthesis.

Fungal biosynthesis of amino acids has always been of particular interest as fungi are generally able to synthesize all 20 proteinogenic amino acids *de novo,* in contrast to animals. The biochemical syntheses of amino acids are based on very diverse pathways, which generally start from intermediates of glycolysis, the pentose phosphate pathway, or the citric acid cycle. Depending on common precursor molecules, biosynthetic routes can be classified into six major groups, which constitute the biosynthetic families of amino acids. Aspergillus strains impaired in any of these biosynthetic routes have been generated and several mutants have been characterized on the molecular level [34–38]. Lysine biosynthesis has attracted special attention as higher fungi synthesize this essential amino acid by eight steps from α-ketoglutarate via the α-aminoadipate pathway, whereas most other microorganisms as well as plants follow the diaminopimelinic acid route [39,40]. Furthermore, an intermediate of lysine biosynthesis (α-aminoadipate) is a precursor for penicillin G, and regulation of either branch of this anabolic route is of special significance in investigating the interplay of primary and secondary metabolism [41]. Early studies on lysine biosynthesis in pathogenic fungi have monitored five enzymatic activities that contribute to the anabolic pathway [42]. On the molecular level, the *lysF* genes of *A. nidulans* and *A. fumigatus*, which code for the homoaconitase enzyme that converts homoaconitate to homoisocitrate, were cloned and characterized; the *lysA* gene of *A. nidulans* catalysing the final step in lysine biosynthesis has been subjected to intense studies recently [43–45]. Virulence studies have been carried out with lysine-requiring mutants of both *Aspergillus* species, *A. nidulans* and *A. fumigatus*, and the results drawn from these experiments are differing. Using *A. nidulans lysA2* mutants, virulence persisted when neutropenic mice were infected with $2{\cdot}10^6$ conidia, although a trend towards lower mortality rates could be detected. In mixed infection experiments, however, a clear advantage of a prototrophic *A. nidulans* strain over the *lysA2* auxotrophic strain was evident, indicating that lysine is necessary but not essential for infection and that the requirement for lysine might attenuate virulence [32]. Optimizing a low-dose infection model for mice and applying a deletion mutant of *A. fumigatus* in which the complete *lysF* coding sequence had been replaced, Brakhage *et al*. [44] were able to show that lysine auxotrophy results in severely attenuated virulence. When infected with $5{\cdot}10^3$ conidia by intranasal inhalation, all animals receiving the *lysFΔ* mutant remained healthy, in contrast to mice infected with the wild-type progenitor strain in which survival rates as low as 10% were monitored over the course of the experiment. Application of 10^4 conidia resulted in a slight decrease of survival, so the *A. fumigatus lysFΔ* mutant strain was considered almost avirulent. Consequently, the

latter results imply a lysine requirement of *A. fumigatus* during invasive growth within the lung, which may be more pronounced than that of *A. nidulans*. Owing to the differences in the animal models used for invasive pulmonary aspergillosis, care must be taken in deciding whether this reflects a substantial difference between *A. nidulans* and *A. fumigatus*.

The Cross-Pathway Control system as virulence determinant

Regulation of the flux through any metabolic pathway is of immense importance for every organism. Anabolism of amino acids has to be regulated tightly as biosynthesis of these translational precursors is an essential and also energy-consuming cellular process. Besides pathway-specific regulation, global regulation of the biosynthetic routes has evolved in fungi [46–48]. The so-called Cross-Pathway Control (CPC) system (also known as General Control [GC] of amino acid biosynthesis) acts in response to environmental stress conditions, amino acid deprivation being the most prominent (Fig. 2). In its very components, a sensor kinase and a transcriptional activator protein make up this conserved regulatory system. Depletion of amino acids is reflected intracellularly by the accumulation of uncharged

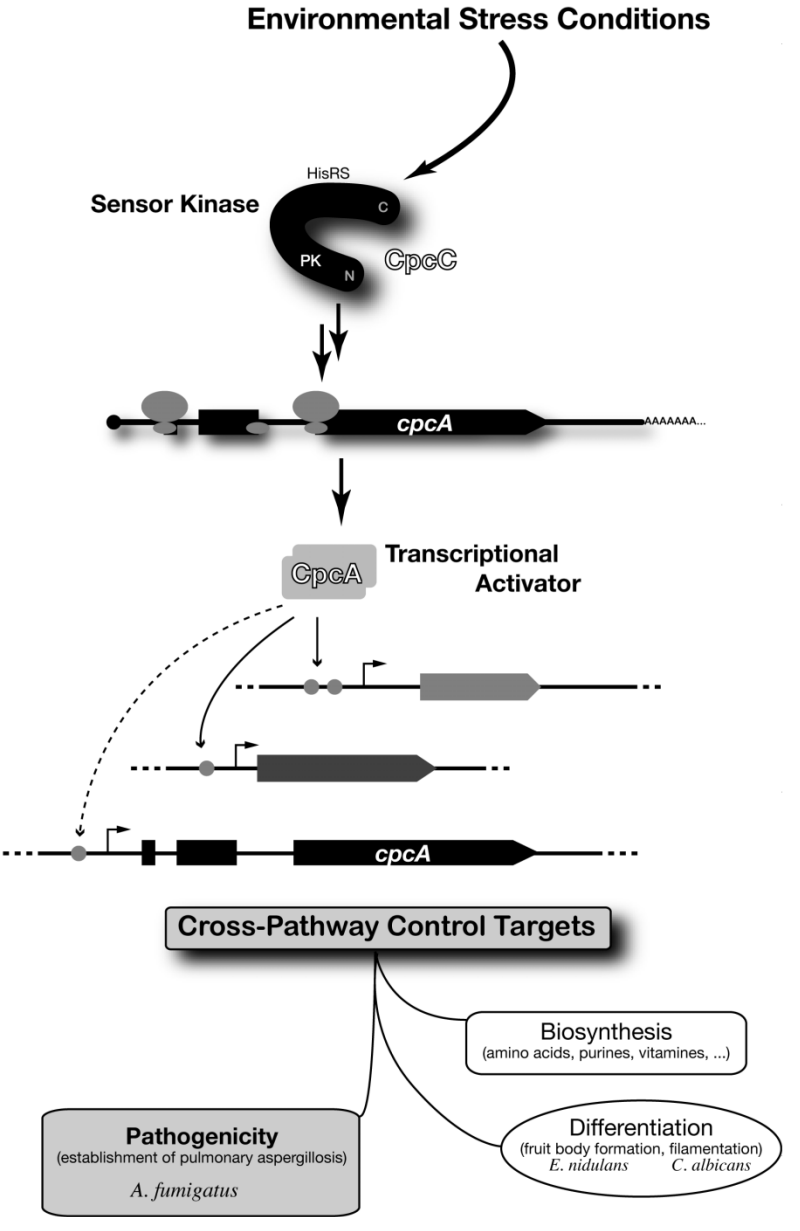

Fig. 2 The fungal Cross-Pathway Control system. A schematic overview on the regulatory circuit is given. One central component is a bi-partite sensor kinase, in aspergilli encoded by the *cpcC* gene, which carries a protein kinase (PK) domain and a tRNA-binding domain homologous to histidyl-tRNA synthetase enzymes (HisRS). Conditions of environmental stress are perceived by this sensor to influence translation initiation. Accordingly, one particular mRNA coding for a transcriptional activator, the aspergillus *cpcA* gene product, is expressed at elevated levels. Binding of the positive effector to conserved elements located in promoter regions of downstream target genes creates a transcriptional read-out, the presumed transcriptional auto-regulation of *cpcA* expression is indicated by the dotted arrow. As a global regulatory system, the Cross-Pathway Control influences a variety of cellular attributes in fungi, key examples are indicated.

tRNA molecules, which are bound by the HisRS domain of an eIF2α kinase. Phosphorylation of this subunit of translation initiation factor leads to down-regulation of general translation, accompanied by increased expression of a specific transcript encoding the activator to generate a transcriptional read-out with the purpose to counteract the starvation condition.

Thorough studies in baker's yeast *Saccharomyces cerevisiae* have revealed that the scope of the GC-directed transcriptome far exceeds that of amino acid biosynthetic genes. In this fungus, starvation for amino acids, purines, or glucose limitation elicits the transcriptional reprogramming mediated by the sensor kinase Gcn2p and the DNA-binding factor Gcn4p. Whole-genome expression profiling revealed that a large fraction of the genes encoded by the yeast genome is affected by histidine starvation as induced by the false feed-back inhibitor 3-amino-triazole (3AT) [49]. Among them, about 500 genes were dependent on Gcn4p for induction, therefore assigning them as targets of this positive effector. Besides amino acid biosynthetic genes of every family, the regulatory circuit influenced genes contributing to purine-pyrimidine biosynthesis or vitamin-cofactor anabolic pathways. Diverse genes involved in peroxisomes, autophagy, and amino acid transport or mitochondrial carriers were found to be affected in their expression profile. Furthermore, loci coding for regulatory gene products such as transcription factors, protein kinases or regulatory subunits of protein phosphatases could be identified as Gcn4p targets. Consistently, the yeast transcriptional activator Gcn4p was designated as a master regulator of gene expression. Expression of this regulator itself is mediated on different levels as translational induction, alteration of stability or regulation of its function, and conserved binding sites located in the 5′ regulatory region of target genes generally mediate transcriptional activation by this factor [50–52]. In aspergilli, previous work has focused on the transcriptional activator of the CPC system, which is encoded by the *cpcA* locus [53–55]. CpcA is conserved among aspergilli with distinct features and domains of the gene product being well conserved. It belongs to the bZIP-type of transcriptional activators based on the C-terminal leucine zipper region necessary for dimerization and DNA binding. Common to all *cpcA* loci of aspergilli that have been characterized to date is an unusual long leader region that precedes the coding sequence on the mRNA transcript. Within this 5′ leader, two conserved small upstream open reading frames can be found that are likely to mediate translational regulation of CpcA expression comparable to the mechanism in *S. cerevisiae* [56]. In contrast to the baker's yeast [57], *cpcA* transcription is promptly induced upon amino acid starvation conditions. Consensus sequences for CpcA binding can be found in the 5′ untranslated region of *cpcA*, therefore transcriptional auto-regulation is likely to contribute to CpcA expression. The influence of the system on fungal virulence has been addressed in studies on the CPC of *A. fumigatus* [55]. In this organism, the functional orthologue of yeast Gcn4p has been cloned and characterized, accompanied by targeted deletion of the encoding locus. The deletion mutants displayed no special nutritional requirements and they were indistinguishable from their wild-type ancestor, except for reduced growth capacities in the presence of the anti-metabolite 5-methyl-tryptophan. Virulence capacities of the deletion mutants were assessed in a murine model of pulmonary aspergillosis by infecting cohorts of immunosuppressed animals as well as by competitive infection experiments. Noticeably, *A. fumigatus* mutants void of the transcriptional activator were reduced in pathogenicity and were outgrown by their wild-type progenitor in the murine lung. Attenuation of virulence was completely restored by homologous reconstitution of the *cpcAΔ* strains. Conclusively, the presence of the CPC transcriptional regulator is required for full virulence of this opportunistic fungal pathogen and, therefore, the CPC system contributes to pathogenicity of *A. fumigatus*. Nevertheless, whether this is a specific trait of *A. fumigatus* or a general attribute of the pathogenic potential of aspergilli remains to be demonstrated. Furthermore, the actual level of CpcA necessary to support pulmonary aspergillosis has not been determined to date. Owing to the complex regulation of CpcA expression, the transcriptional activator is present at low levels in unstarved cells. Therefore, it is tempting to test strains that have an impaired CPC signal transduction cascade and are unable to increase CpcA expression. A prime target for these studies is the sensor kinase that perceives the actual stress condition to mediate de-repression of *cpcA* translation. Preliminary inspection of the *A. fumigatus* genome sequence has revealed such an eIF2α kinase encoded by a locus similar to *Neurospora crassa cpc-3* or yeast *GCN2* [58,59]. This *cpcC* locus has the capacity to encode a gene product that exhibits a strong degree of conservation within distinct domains characteristic for the CPC sensor kinase module, like a degenerated kinase part, an eIF2α kinase domain, a HisRS element required for binding of uncharged tRNAs, or a C-terminal end necessary for ribosome association and dimerization. Generation of corresponding *cpcCΔ* deletion mutant strains and testing them for their virulence capacities

will shed further light on the influence of the conserved CPC regulatory circuit in aspergillosis.

Conclusion

Knowledge on primary metabolism of aspergilli is incomplete, and especially for the opportunistic pathogen *A. fumigatus* the majority of biosynthetic routes have not been studied in great detail. Addressing specific pathways with respect to pathogenicity is a promising approach to evaluate prerequisites of fungal virulence as well as targets for antifungal therapies. Fungal metabolism is generally typical for that of a eukaryotic organism, with specific features making it distinct and unique within the eukaryotic kingdom. Metabolism is crucial for fitness and therefore for germination, growth and expansion of the vegetative mycelium, either in the environment or at the primary site of infection within a host. Furthermore, the capacity to counteract metabolic stress contributes to the capability to escape from immune effector cells, as conditions of stress and starvation are encountered by the fungal pathogen within specific cellular compartments [60]. Testing of mutant strains that are blocked at a certain step within a metabolic pathway yields insights on specific nutritional requirements during host infection. Single-gene deletion strategies appear to be invalid as a means to comprehensively uncover metabolic features that contribute to pathogenicity, owing to the fact that virulence of *A. fumigatus* is likely to be multifactorial. A more promising approach is the targeted inactivation of metabolic regulatory circuits of metabolism or factors that affect a cluster of genes, in order to identify general metabolic features that contribute to fungal virulence. Additionally, creative *in vivo* screening approaches, as demonstrated by the STM screening methodology [33], are necessary to complement the strategies aiming on identification of fungal-specific metabolic issues that contribute to virulence of aspergilli.

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