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The *Aspergillus fumigatus* transcriptional activator CpcA contributes significantly to the virulence of this fungal pathogen

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Summary

We have cloned and characterized the *Aspergillus fumigatus cpcA* gene encoding the transcriptional activator of the cross-pathway control system of amino acid biosynthesis. *cpcA* encodes a functional orthologue of *Saccharomyces cerevisiae* Gcn4p. The coding sequence of the 2.2 kb transcript is preceded by two short upstream open reading frames, the larger one being well conserved among Aspergilli. Deletion strains in which either the coding sequence or the entire locus are replaced by a bifunctional dominant marker are impaired in their cross-pathway control response upon amino acid starvation, as demonstrated by analyses of selected reporter genes and specific enzymatic activities. In a murine model of pulmonary aspergillosis, *cpcAΔ* strains display attenuated virulence. Pathogenicity is restored to wild-type levels in strains with reconstitution of the genomic locus. Competitive mixed infection experiments additionally demonstrate that *cpcAΔ* strains are less able to survive *in vivo* than their wild-type progenitor. Our data suggest that specific stress conditions are encountered by *A. fumigatus* within the mammalian host and that the fungal cross-pathway control system plays a significant role in pulmonary aspergillosis.

Introduction

Saprophytic fungi use an array of mechanisms to achieve colonization of diverse substrates. Representatives of this ubiquitous ecological group are moulds of the genus *Aspergillus*, a family containing more than 180 species (Samson, 1999). Common to all Aspergilli is an anamorphic mode of propagation via asexual conidiospores that are released into the environment. These give rise to a vegetative, branched mycelium after germination on a suitable growth substrate.

The filamentous mould *Aspergillus fumigatus* is a ubiquitous component of the biosphere and has also been the subject of increasing interest in medical mycology over recent decades. This opportunistic pathogen is the main agent of several fungal infections collectively termed aspergilloses. Some allergic diseases, e.g. allergic bronchopulmonary aspergillosis (ABPA), have been linked to *A. fumigatus*, but the most severe clinical forms of aspergillosis are characterized by saprophytic propagation of the fungus within the host. Depending on the immune status of the infected individual, varying degrees of fungal infection by *A. fumigatus* can be monitored. Severe forms of *A. fumigatus* mycoses are a major threat to immunocompromised patients, especially neutropenic ones or organ transplant patients. The agents of aspergillus infection are airborne conidia, which, because of their small size, are able to reach the pulmonary alveoli, the primary site of infection. If these conidia are not cleared by the host, e.g. in the absence of an adequate host immune response, they germinate and grow *in vivo* as *A. fumigatus* has 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, and which display their most severe and fatal development in disseminated, systemic cases (Kontoyiannis and Bodey, 2002; Oren and Goldstein, 2002). The incidence of IA has been estimated in the US at 12.4 cases per million population per year and is associated with a very high mortality approaching 90% (Rees *et al.*, 1998; Lin *et al.*, 2001). In addition, the US economic burden associated with treatment of IA was estimated to be over $630 million per annum in 1996 (Dasbach *et al.*, 2000).

This increase in the incidence of IA has resulted in significant interest in the virulence mechanisms of *A. fumigatus*, although the concept of specific virulence factors remains to be evaluated for opportunistic pathogens

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(Latge and Calderone, 2002). Various aspects of *A. fumigatus* pathobiology have been implicated in host infection (Latge, 2001). Secreted proteolytic enzymes are likely to be required to promote host invasion, and several protease mutant strains have been generated (Monod *et al*., 1993; 1999; Reichard *et al*., 1997; 2000). As the conidiospore is the actual infectious propagule, cell wall components have been subject to scrutiny (Bernard and Latge, 2001). A putative role in virulence for conidial pigment, synthesized through the dihydroxynaphthalene–melanin pathway, has been proposed (Brakhage *et al*., 1999; Tsai *et al*., 1999). Mutant strains defective in the polyketide synthase locus *pksP* (*alb1*) have been shown to be more sensitive towards phagocytosis by monocyte-derived macrophages in comparison with wild-type conidia (Jahn *et al*., 1997; 2000; Langfelder *et al*., 1998; Tsai *et al*., 1998). Metabolic requirements within the host environment are known to influence propagation *in vivo*. A *pyrG*$^{-}$ strain, devoid of orotidine-5′-phosphate decarboxylase activity and therefore auxotrophic for uracil or uridine, is attenuated for virulence in an immunosuppressed murine intranasal challenge model (d'Enfert *et al*., 1996). Earlier indications towards nutritional needs during infection came from reports on *para*-aminobenzoic acid (PABA)-requiring mutants of *A. fumigatus*. Such mutants were unable to cause systemic infections upon intravenous (i.v.) injection in steroid-treated mice (Sandhu *et al*., 1976), a finding that has recently been further substantiated by a signature-tagged mutagenesis screen (Brown *et al*., 2000). *lysA2* mutants of *Aspergillus nidulans* have wild-type virulence characteristics, indicating that the host's lysine pool is not limiting for fungal colonization. To our knowledge, no further attempts to inspect *Aspergillus* amino acid auxotrophs for pathogenicity have been reported to date.

Fungal amino acid biosynthesis is vital to metabolism with feeding substrates entering from various metabolic routes. Generally, basal expression rates of the enzymatic activities that constitute the anabolic pathways are high, and pathway-specific regulatory systems have been characterized for every biosynthetic family. Moreover, a global regulatory system modulating fungal amino acid biosynthesis as a whole has been intensively studied, commonly referred to as the cross-pathway control (cpc) or general control of amino acid biosynthesis (Carsiotis and Jones, 1974; Carsiotis *et al*., 1974; Hinnebusch, 1986). Starvation for any of at least 11 of the proteinogenic amino acids is sufficient to derepress this system in *Saccharomyces cerevisiae*, resulting in elevated expression levels of its target genes such as genes encoding components of the amino acid and purine biosynthetic pathways or other cellular categories. The cross-pathway response read-out has been studied most thoroughly in the baker's yeast *S. cerevisiae*, in which whole-genome expression profiling has revealed the global nature of this regulatory circuit (Natarajan *et al*., 2001). The transcriptional activator protein Gcn4p, which shares similarity to the c-jun oncogene product (Struhl *et al*., 1988), is a central element of this network in baker's yeast. Upon induction, this DNA-binding protein is located to conserved *cis* elements within the promoter region of cpc targets where it enhances transcription initiation rates. Expression and activity of Gcn4p itself is subject to a complex pattern of regulation (Hinnebusch and Natarajan, 2002). Upstream open reading frames (ORFs) serve as translational barriers on the mature transcript but are omitted upon intracellular accumulation of uncharged tRNA molecules, a signal that is perceived by the sensor kinase Gcn2p (Wek *et al*., 1995).

We hypothesized that an active amino acid biosynthetic capability would be required for survival *in vivo* and, therefore, that the *A. fumigatus GCN4* orthologue would play an important role in the virulence of this opportunist fungal pathogen. Here, we present a detailed description of the cpc transcriptional effector from *A. fumigatus*, which is the gene product of the *cpcA* locus. We describe the generation of precise deletion mutants and characterize their response to amino acid starvation conditions. Pathogenicity of *cpcA* null mutants is determined in a murine model of invasive pulmonary aspergillosis. Our data clearly indicate a role for *A. fumigatus* CpcA upon host infection and support the function of the fungal cross-pathway control of amino acid biosynthesis as a general stress response system.

Results

cpcA *of* A. fumigatus *constitutes a highly conserved locus encoding a bZIP-type gene product*

To identify the effector of the *A. fumigatus cpc* system, we designed two degenerated oligonucleotides (FTD and KRL; see Table 1) according to a well-conserved region as identified by alignment of homologous nucleotide sequences from *cpcA* loci of *A. nidulans* and *Aspergillus niger* (Paluh *et al*., 1988; Wanke *et al*., 1997; Hoffmann *et al*., 2001). Polymerase chain reactions (PCRs) using these primers and genomic DNA from *A. fumigatus* clinical isolate D141 as template yielded a specific 450 bp amplicon that was cloned (pME2544) and sequenced. The sequence data confirmed that the amplicon contained an ORF that has high similarity to fungal cpc activators (not shown). Using this PCR product as probe, a genomic 1.5 kb *Eco*RI fragment comprising the complete coding sequence was isolated from a genomic sublibrary. Sequencing of both strands of this DNA fragment in pME2545 confirmed the presence of a putative ORF 810 bp in size encoding a putative polypeptide with high

Table 1. Fungal strains, plasmids and oligonucleotides constructed and used in this study.

Name	Description or sequence	Reference
Strains		
RH1408	*Saccharomyces cerevisiae gcn4-103*, *ura3-52* strain	Hinnebusch (1985)
D141	*Aspergillus fumigatus* wild-type strain, clinical isolate	Reichard *et al*. (1990)
AfS01	Strain D141 carrying deletion of *cpcA* coding sequence: Phleo^r, 5MT^s	This study
AfS02	Strain D141 carrying complete deletion of *cpcA* locus: Phleo^r, 5MT^s	This study
AfS03	Reconstituted *A. fumigatus* strain AfS01 by transformation with pME2561: Phleo^r, Hyg^r, 5MT^r	This study
AfS05	Reconstituted *A. fumigatus* strain AfS02 by transformation with pME2561: Phleo^r, Hyg^r, 5MT^r	This study
AfS07	*A. fumigatus* strain AfS01 reconstituted with *Bss*HII insert of pME2563: Phleo^s, 5MT^r	This study
AfS08	*A. fumigatus* strain AfS02 reconstituted with *Bss*HII insert of pME2563: Phleo^s, 5MT^r	This study
Plasmids		
pRS316	*URA3*-marked centromere vector for *S. cerevisiae*	Sikorski and Hieter 1989)
pRS316-GAL1	Yeast expression plasmid with *GAL1* promoter cloned in pRS316	Liu *et al*. (1992)
pAN7-1	Fungal ^{p}*gpdA::hph::trpC*^{t} expression plasmid conferring hygromycin resistance	Punt and van den Hondel (1992)
pAN8-1	Fungal ^{p}*gpdA::ble::trpC*^{t} expression plasmid conferring phleomycin resistance	Punt and van den Hondel (1992)
pCAD21	Conditional R6Kγ origin plasmid carrying bifunctional *zeo*^{R}*-A.f.pyrG* marker	Chaveroche *et al*. (2000)
pME1092	*GCN4* gene (2.8 kb *Sal*I–*Eco*RI) in pRS314	Albrecht *et al*. (1998)
pME2544	441 bp PCR amplicon obtained with primers FTD/RKL cloned in pBluescript II KS (pBS)	This study
pME2545	Genomic D141 *Eco*RI fragment (1.5 kb) encompassing *A. fumigatus cpcA* coding sequence in pBS	This study
pME2546	cDNA derived from *cpcA* transcript in pBK-CMV	This study
pME2547	$\approx$ 15 kb *Not*I fragment isolated from genomic D141 λ phage library ligated into pBS	This study
pME2548	5′ fragment (500 bp) derived from 5′ RACE mapping of *cpcA* transcript start cloned in pBS	This study
pME2549	pRS316-GAL1 with *GCN4* gene as 1.8 kb *Sca*I fragment from pME1092 in *Sma*I site	This study
pME2550	pRS316-GAL1 with *GCN4* coding sequence as flushed *Bst*EII–*Eco*RI 2.1 kb fragment in *Sma*I	This study
pME2551	pRS316-GAL1 with *cpcA* cDNA including uORFs as 2.2 kb *Bam*HI–*Not*I fragment from pME2546	This study
pME2552	pRS316-GAL1with *cpcA* coding sequence as 1.4 kb *Bam*HI–*Not*I amplicon by Sv10 and T7 on pME2546	This study
pME2553	pRS316 with complete *GCN4* locus as 2.8 kb *Sal*I–*Eco*RI fragment from pME1092	This study
pME2554	pRS316 with *cpcA* cDNA (*Bam*HI–*Not*I of pME2546) fused to *GCN4* promoter (*Sal*I–*Sca*I 475 bp from pME1092)	This study
pME2555	pRS316 with *cpcA* coding sequence (1.35 kb *Eco*RI of pME2546) behind *GCN4* promoter and 5′ leader (940 bp *Sal*I–*Bst*EII of pME1092)	This study
pME2556	Bifunctional marker module containing bacterial zeo^r (500 bp *Eco*RI–*Eco*RV of pCAD21) and fungal phleo^r (3.4 kb *Bgl*II–*Xba*I of pAN8-1) cassettes in pUC19 *Eco*RI–*Sma*I and *Bam*HI–*Xba*I	This study
pME2557	zeo^r/phleo^r module flanked by *cpcA* homology arms (amplicon Sv19/20 and Sv21/22 annealing products)	This study
pME2558	zeo^r/phleo^r module flanked by *cpcA* homology arms (Sv15/16 and Sv21/22 annealing products)	This study
pME2559	*cpcA*Δ(cds) deletion cassette obtained after recombination of pME2557 *Eco*RV–*Xba*I fragment on pME2547	This study
pME2560	*cpcA*Δ deletion cassette obtained after recombination of pME2558 *Eco*RV–*Xba*I fragment on pME2547	This study
pME2561	Genomic *cpcA Vsp*I fragment (3.3 kb) and Hyg^r cassette of pAN7-1 in pUC19	This study
pME2562	8 kb *Bss*HII fragment encompassing *A. fumigatus cpcA* locus ligated in pBS	This study
pME2563	pME2562 carrying point-mutated *Sal*I site (G $\rightarrow$ C at pos. +264) in *cpcA* coding sequence	This study
Oligonucleotides		
FTD	5′-TTC ACT GAT CTC AGC ACW CC-3′	
RKL	5′-CCT TGW CGC TCA AGC TTR CG-3′	
Sv02	5′-AGT CTC TCA AAC GAG G-3′	
Sv03	5′-ATA GAG CAC GAA GTC CTC C-3′	
Sv10	5′-CTG AGG ATC CAT GTC GAC CCC CAA CAT CGC-3′	
Sv15	5′-ATC TTC ACC ATA CAC CCA CAC AAT TAT CAG GAA TAT CAG TCT CAG TAT CCA TTG CAA-3′	
Sv16	5′-TTT GCA ATG GAT ACT GAG ACT GAT ATT CCT GAT AAT TGT GTG GGT GTA TGG TGA AGA-3′	
Sv19	5′-ATC CTC TGT CCT CAT CTC TTC G-3′	
Sv20	5′-GAT GCG GCG GTG ATA CAA C-3′	
Sv21	5′-CTA GCG ATG ATC GAT TGG TTA TCT TGA TGA CAT GCT GCG CTT GTG ATG TCT TGT TTG TTT AT-3′	
Sv22	5′-CTA GAT AAA CAA ACA AGA CAT CAC AAG CGC AGC ATG TCA TCA AGA TAA CCA ATC GAT CAT CG-3′	
Sv48	5′-TCT GGA GGA TAC AAT CAC TTT CC-3′	
Sv49	5′-AAT CCC ATT GAG TGC CTT TCA GC-3′	
Sv64	5′-TAA TCT ATT CAA AAG ATC TGA GG-3′	
Sv65	5′-TCT ACA CAC AGA TCT AGT TGG-3′	
Sv80	5′-AAC GAG CTC GAG GGG GGC GCC GAC GCA TCC-3′	

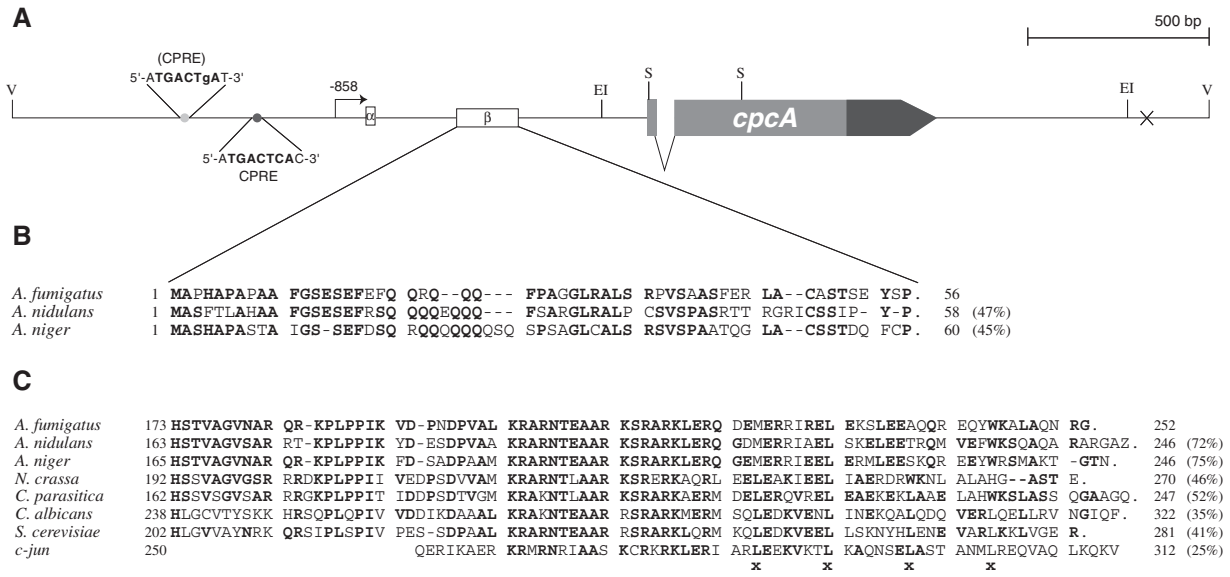

Fig. 1. The *cpcA* locus of *A. fumigatus* is highly conserved and encodes a bZIP-type gene product. A schematic overview of the *A. fumigatus cpcA* gene locus is shown (A). The two exons constituting the coding sequence are represented by the shaded arrow with the conserved leucine zipper region in dark grey. Boxes indicate the upstream open reading frames (uORFs) α and β in the 5′ leader region; the position of the transcriptional initiation site at position –858 is marked by a bent arrow. X indicates the polyadenylation site as determined in the cDNA insert of pME2546. Locations and sequences of the putative cross-pathway control elements (CPRE) are shown. EI (*Eco*RI), V (*Vsp*I) and S (*Sal*I) indicate positions of various restriction sites.

B. Deduced amino acid sequence of uORFβ aligned with its counterparts within *cpcA* leaders of *A. nidulans* (AF302935) and *A. niger* (CAA67604). Bold letters indicate conserved residues, and similarities are given in percentages on the right hand side.

C. Deduced peptide sequence of the *A. fumigatus* CpcA C-terminus encompassing the dimerization and DNA-binding domain in its alignment with corresponding regions of fungal orthologues from *A. nidulans* (CPCA, AAL09315), *A. niger* (CPCA, Q00096), *N. crassa* (CPC-1, P11115), *Cryphonectria parasitica* (CPC-1, P87090), *C. albicans* (CaGcn4p, AAF18140), *S. cerevisiae* (Gcn4p, NP010907) and the c-Jun bZIP-type leucine zipper (P05412). Positions and similarities are indicated, X marks conserved heptad positions of leucines that define the canonical leucine zipper dimerization domain.

identity to fungal cpc regulators (Fig. 1). The identified gene was termed *cpcA* (GenBank nucleotide sequence database accession number AF323678). To reveal possible intron sequences, a cDNA *cpcA* clone was isolated from a λ ZAP Express™ library (Reichard *et al*., 2000) derived from vegetative mycelium of *A. fumigatus* D141. The cDNA insert from a positive plaque was subcloned into the plasmid pBK-CMV to yield plasmid pME2546. Alignment of the pME2546 insert sequence with its genomic counterpart identified an intron of 51 bp located in close proximity to the most 5′ start codon identifiable in the coding region. The *cpcA* coding sequence therefore consists of two exons 25 and 734 bp in length and encodes a 252-amino-acid polypeptide with a calculated M_r of 27 113. In the cDNA insert of pME2546, the region preceding the CpcA translational start codon spans 830 bp, the polyadenylation site is located 560 nucleotides downstream of the translational stop codon. The encoded gene product has a high degree of similarity to fungal cpc activator proteins. In particular, the C-terminal half of the *A. fumigatus* CpcA closely matches those of its fungal counterparts as well as the DNA-binding domain of the proto-oncogene c-Jun (Fig. 1C). Similarity values for this region range from 75% to 25%, with a high proportion of residues important for dimerization and DNA binding being conserved.

During the course of our studies, an *A. fumigatus* genome sequencing project was initiated by an international group based at the Pasteur Institute, the Sanger Centre and The Institute of Genome Research (Denning *et al*., 2002). Upon alignment with the sequence determined from plasmids pME2545 and pME2546, a sequence contig harbouring the *A. fumigatus cpcA* locus could be identified at the TIGR *Aspergillus fumigatus* Genome Database (contig:4865:a_fumigatus). Close inspection revealed no mismatch between both sequences. By mining further sequence information from the genome database, the overall structure of the *cpcA* locus was determined (Fig. 1A). In the region upstream of the translational start codon, two small ORFs were identified, uORFα (–752 to –737) and uORFβ (–517 to –346), encoding putative polypeptides of 5 and 56 amino acids in length respectively. The deduced primary sequence of uORFβ shows high similarity to those mapped in the leader regions of *cpcA* from *A. nidulans* and *A. niger* (Fig. 1B) with calculated similarities of 47% and 46% respectively. In order to map the complete leader region preceding the *A. fumigatus cpcA* coding sequence, 5′

random amplification of cDNA ends (RACE) experiments were carried out. One distinct fragment was amplified from the 5′ end of reverse-transcribed cDNA (pME2548), and sequence determination indicated one transcriptional start site located at position –858 relative to the first AUG of the CpcA-encoding region. Both upstream ORFs are located within the leader sequence of the *cpcA* transcript. Further inspection of the genome sequence reveals conserved binding elements for the CpcA itself. *In vitro* studies have demonstrated that the *S. cerevisiae* orthologue Gcn4p binds to a palindromic sequence element constituted by a central C·G pair that is flanked by TGA half-sites (Oliphant *et al*., 1989). Furthermore, functional sequence variants of this so-called GCRE or CPRE (general control/cross-pathway control recognition element) have been described. In the region upstream of the *A. fumigatus cpcA* transcriptional initiation site, two motifs resembling a CPRE could be identified. The proximal one matches the consensus exactly (5′-A**TGACTCA**C-3′, –1062 to –1053), while the distal one contains one clear deviation from the canonical CPRE sequence (5′-A**TGACTgA**T-3′, –1273 to –1264).

Aspergillus fumigatus *CpcA is a functional orthologue of* S. cerevisiae *Gcn4p*

The most thoroughly investigated activator of a fungal cross-pathway control system is that of the baker's yeast *S. cerevisiae*. It is encoded by the *GCN4* locus, from which a transcript comprising an 846 bp coding sequence preceded by a 577 bp 5′ leader region is generated. Within this leader, four small upstream ORFs of two to three codons in size mediate stringent translational regulation of Gcn4p expression (Mueller and Hinnebusch, 1986). To test whether the *A. fumigatus cpcA* gene product can substitute its yeast orthologue, various expression cassettes were constructed for complementation of a haploid *gcn4* null strain (Fig. 2). In one set of expression plasmids, the complete *cpcA* cDNA insert from pME2546 or the encoding sequence only was expressed from the inducible *GAL1* promoter cloned in the low-copy-number shuttle vector pRS316, resulting in plasmids pME2551 and pME2552 respectively. Identical constructs were prepared for the *S. cerevisiae GCN4* coding sequence with (pME2549) or without the leader region (pME2550). The *S. cerevisiae* recipient RH1408 [*ura3-52*, *gcn4-103*] was transformed with the expression vectors, and abilities to grow in the presence of the antimetabolite 3-amino-triazole (3AT) were monitored by serial dilutions (Fig. 2A). On glucose-containing medium, only strains expressing the complete loci encompassing the leader regions were able to grow to a certain extent when starved for histidine by 3AT, because of the leakiness of the *GAL1* promoter. When the alternative carbon source raffinose was present, all strains expressing *GCN4* or *cpcA* grew in

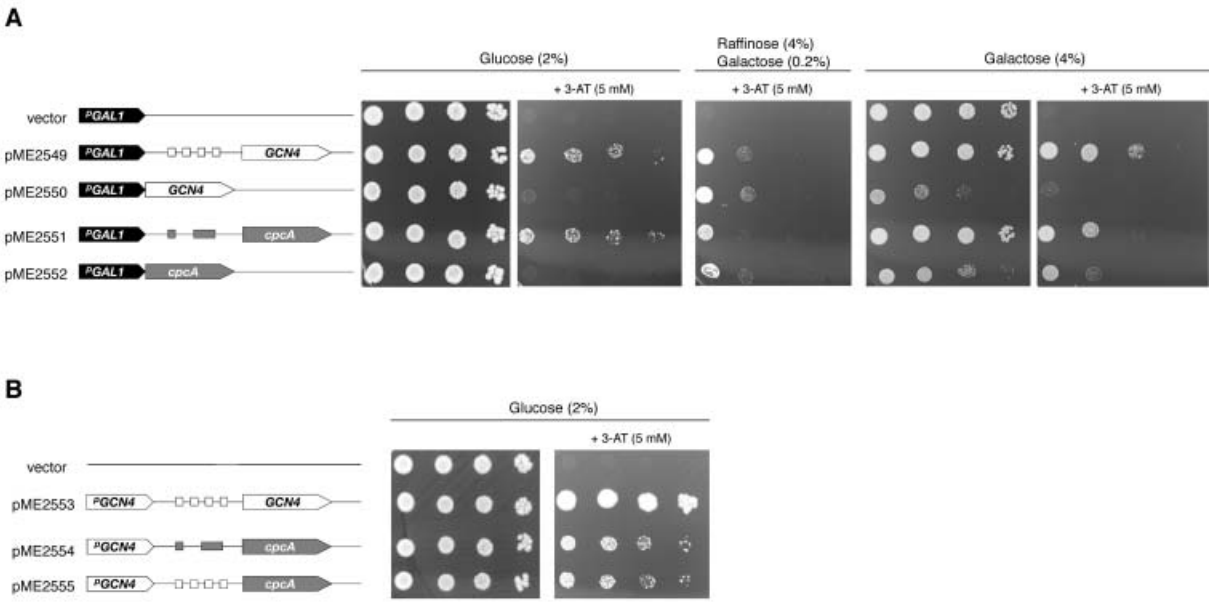

Fig. 2. *A. fumigatus* CpcA complements the histidine starvation phenotype of a *S. cerevisiae gcn4*Δ strain.
A. *GAL1* promoter-driven expression cassettes were transformed into *S. cerevisiae* recipient RH1408 [*ura3-52, gcn4-103*], and serial dilutions were spotted onto YNB minimal medium plates containing the indicated carbon sources and the false feedback inhibitor of histidine biosynthesis 3-amino-triazole (3AT). Upstream ORFs and coding sequences of *GCN4* and *cpcA* are presented in white and grey respectively.
B. Growth capacities of *S. cerevisiae* strains expressing the native *GCN4* gene or the *A. fumigatus cpcA* locus under the control of the endogenous *GCN4* promoter. Additionally, complementation capacities of the chimeric construct expressed from plasmid pME2555 are demonstrated.

the presence of 3AT, although only at high cell densities. In direct comparison, strains carrying *cpcA* fused to the *GAL1* promoter grew slightly less well under these conditions. Strong induction of gene expression using galactose as the sole C source revealed a stabilizing function of the leader regions harbouring short ORFs. Without starvation, growth of strains expressing the coding regions only was reduced. Additional exposure to 3AT abolished growth of RH1408 transformed with pME2550, and growth of RH1408 (pME2552) was markedly reduced. Expressing the complete *cpcA* cDNA fragment enhanced growth in comparison with the latter strain, and the best capacities to counteract histidine starvation were displayed by the transformant expressing the complete *GCN4* locus driven by *^{p}GAL1* (pME2549). Conclusively, unregulated overexpression of CpcA is toxic, as described for Gcn4p (Tavernarakis and Thireos, 1995). To test complementation abilities in a less artificial way, chimeric constructs were designed to express the *A. fumigatus* CpcA protein from the endogenous *S. cerevisiae GCN4* promoter (Fig. 2B). Plasmid pME2555 carries the complete *cpcA* cDNA insert fused to the *GCN4* promoter, whereas in pME2552, only the *cpcA* coding region is placed behind the *GCN4* promoter sequence and, additionally, the leader sequence. Both constructs were able to complement 3AT sensitivity of the *gcn4Δ* recipient to a certain extent, although not as well as a control construct carrying the *GCN4* locus (pME2553). In summary, the coding sequence of the *A. fumigatus cpcA* locus encodes a protein rendering a *S. cerevisiae* strain devoid of Gcn4p resistant to the drug 3AT. The *cpcA* 5′ leader sequence is functional in *S. cerevisiae*, as demonstrated by construct pME2554. Furthermore, both leader regions containing small uORFs are likely to repress translation of the downstream coding sequence, as indicated by pME2549 and pME2551 in the presence of galactose but without 3AT.

Deletion of the A. fumigatus cpcA *locus implies that both uORFs encode dispensable polypeptides*

In order to characterize the function of the *cpcA* gene product further, we constructed a series of *A. fumigatus* loss-of-function mutant strains. Deletion cassettes were generated exploiting a recombination system introduced by d'Enfert and coworkers (Chaveroche *et al.*, 2000). In a first step, a bifunctional dominant marker cassette was constructed that combines bacterial resistance to zeocin as well as fungal resistance towards phleomycin. Into this plasmid pME2556, appropriate *cpcA* 5′ and 3′ flanking homology arms were ligated, and the resulting cassette was used to replace the native *cpcA* sequence on a genomic template (pME2547) via recombinatorial cloning (Muyrers *et al.*, 2001). Using cassettes targeting different regions of the template, either the coding sequence or the complete locus including both upstream ORFs in the 5′ leader were replaced (Fig. 3A). The resulting deletion constructs of pME2559 (*cpcAΔcds*) and pME2560 (*cpcAΔ*) were introduced as linear fragments into the wild-type strain D141, and phleomycin-resistant transformants were selected. Primary transformants were colony purified, and two for each replacement made were chosen for inspection by Southern analysis (Fig. 3B) to confirm correct replacement of the endogenous *cpcA* region by single integration of the respective deletion cassette. One strain per mutant background, AfS01 and AfS02, was analysed in detail.

Growth of neither mutant strain on solid minimal medium was altered in comparison with their wild-type precursor D141, with respect to either colony appearance or sporulation capacities. To assess a putative influential role of the nitrogen source, the impact of a nitrogen-poor environment on radial growth rates was tested (Fig. 3C). Conidia were allowed to germinate on solid minimal media supplemented with various nitrogen sources, and hyphal extension kinetics were measured within 24 h time frames. Both mutant strains grew at constant rates comparable to that of the wild-type isolate D141, even when strains were propagated in the presence of a poor nitrogen source such as nitrate or proline. When AfS01 and AfS02 were starved for the amino acid tryptophan by addition of the structural analogue 5-methyl-DL-tryptophan (5MT), a clear growth phenotype was observable, with mutant strains displaying retardation of mycelial expansion and absence of conidiation (Fig. 3D). To confirm that the observed phenotypes were based on deletion of the *cpcA* gene, both mutants were reconstituted by ectopic integration of a fragment encompassing the complete *cpcA* locus (pME2561). The resulting strains, AfS03 and AfS05, were validated for single integration of the genomic fragment by Southern hybridization analysis (not shown). On 5MT minimal medium, both strains were able to counteract starvation induced by the inhibitor of tryptophan biosynthesis (Fig. 3D). Both strains grew well on 5MT plates and displayed proper conidiation like their wild-type predecessor D141.

Growth retardation upon amino acid starvation is characteristic for fungal mutant strains impaired in the cross-pathway control system. We therefore conclude from the phenotype of both deletion strains AfS01 and AfS02 that the *cpcA* gene encodes the presumed positive effector of the *A. fumigatus* cpc system. Furthermore, as we were unable to observe a clear difference between both deletion strains, which differ by the absence or presence of both upstream ORFs, we suggest that the polypeptides translated from each are dispensable for fungal growth, at least under the conditions tested.

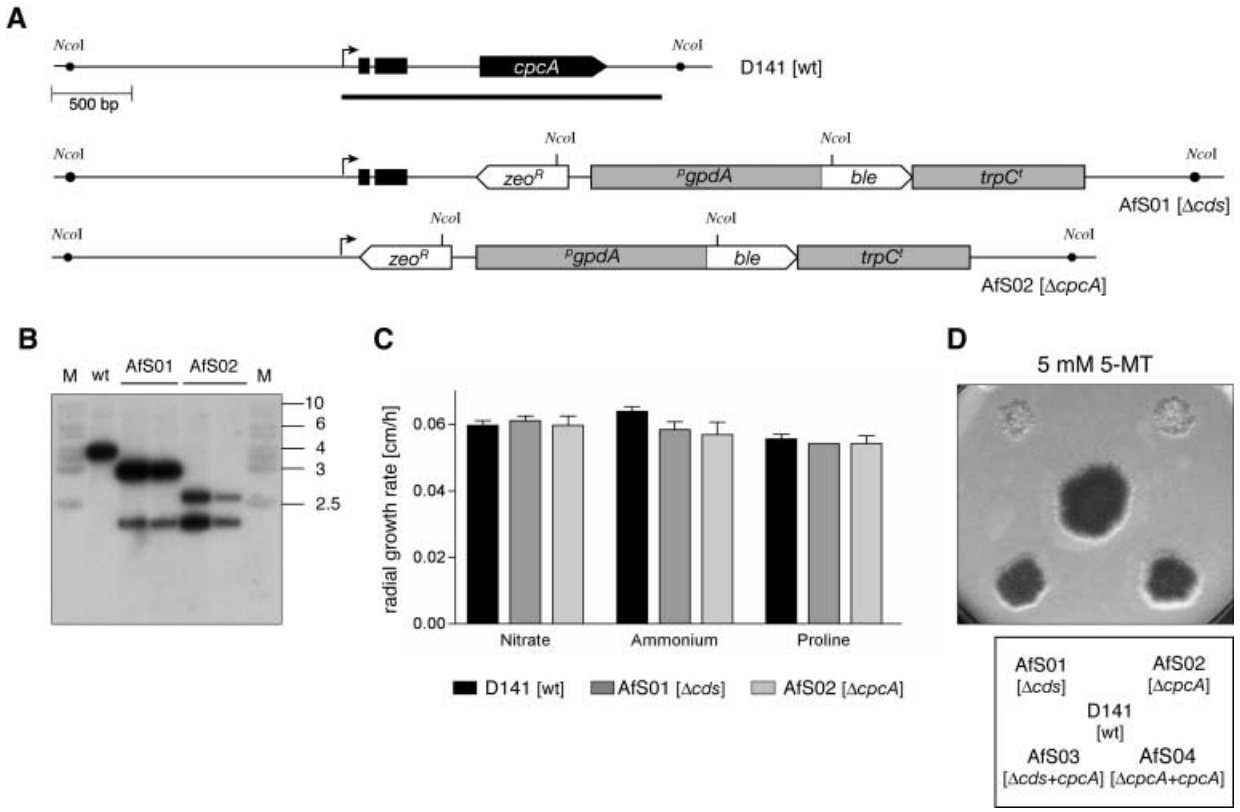

Fig. 3. *A. fumigatus* strains deleted for their *cpcA* locus are sensitive towards tryptophan starvation.
A. Schematic representation of the *cpcA* locus in the clinical isolate D141, the coding sequence deletion mutant AfS01 and the complete deletion strain AfS02. The native *cpcA* locus is shown and the position of the bifunctional zeo^R/phleo^R marker module. The black bar indicates the probe used for Southern analyses.
B. The autoradiography shows the hybridization pattern after separating *Nco*I-digested genomic DNA of the assigned *A. fumigatus* strains. M represents lanes with a DNA size marker with fragment sizes indicated.
C. Radial growth rates of wild-type (black bars) and *cpcAΔ* mutant strains (grey bars) on minimal medium containing various nitrogen sources. Nitrate and ammonium were supplemented at 10 mM concentration, L-proline at 5 mM.
D. Growth phenotypes of *cpcA* deletion strains in the presence of the tryptophan analogue 5-methyl-tryptophan (5MT). Strains were inoculated on minimal medium plates covered by membranes, allowed to germinate and transferred after 24 h to medium supplemented with 5 mM 5MT. Further growth was monitored for 2 days. Positions of the tested strains are given in the scheme underneath. AfS03 and AfS04 are derivatives of AfS01 and AfS02 that have been reconstituted by single-copy ectopic integration of a fragment encompassing the complete *cpcA* locus.

Aspergillus fumigatus cpcA*Δ* *mutant strains are impaired in their cross-pathway control response*

To gain further insight into the cross-pathway control system of *A. fumigatus*, we scrutinized the generated strains with respect to their metabolic read-out upon amino acid starvation conditions. Fungal strains were propagated vegetatively in the presence of 3AT at 10 mM concentration in order to induce histidine depletion, and samples were isolated at various time points after the onset of starvation. To one culture of each strain, histidine was added in combination with 3AT to assess the toxicity of the drug. Amino acid starvation results in elevated transcriptional initiation rates at the promoters of *cpc* target genes. Accordingly, steady-state transcript levels of the reporter genes *argB* and *aroC* were determined in Northern hybridization experiments (Fig. 4A). The chorismate mutase (CM)-encoding *aroC* transcript was monitored as an internal constitutive control, as transcription of the gene encoding this enzymatic activity has been described for several fungi to be independent of the cpc (Schmidheini *et al*., 1990; Krappmann *et al*., 1999; 2000). Transcript levels of *aroC* remained within a basal range in all strains tested during the entire time frame of 8 h of continuous 3AT challenge. In contrast, transcription of the ornithine transcarbamoyl transferase (OTCase)-encoding *argB* locus appeared to be elevated in the wild-type strain D141 upon exposure to 3AT. Transcript levels were strongly induced as little as 2 h after transfer to 3AT medium and remained at high levels throughout the time course. When propagated in the presence of 3AT and histidine, no increase in *argB* transcription was detected, which is in accordance with the model of 3AT inducing histidine starvation. To test any influence of His starvation on transcription of the *cpcA* locus itself, a probe derived from this gene was hybridized to the RNA samples. Basal

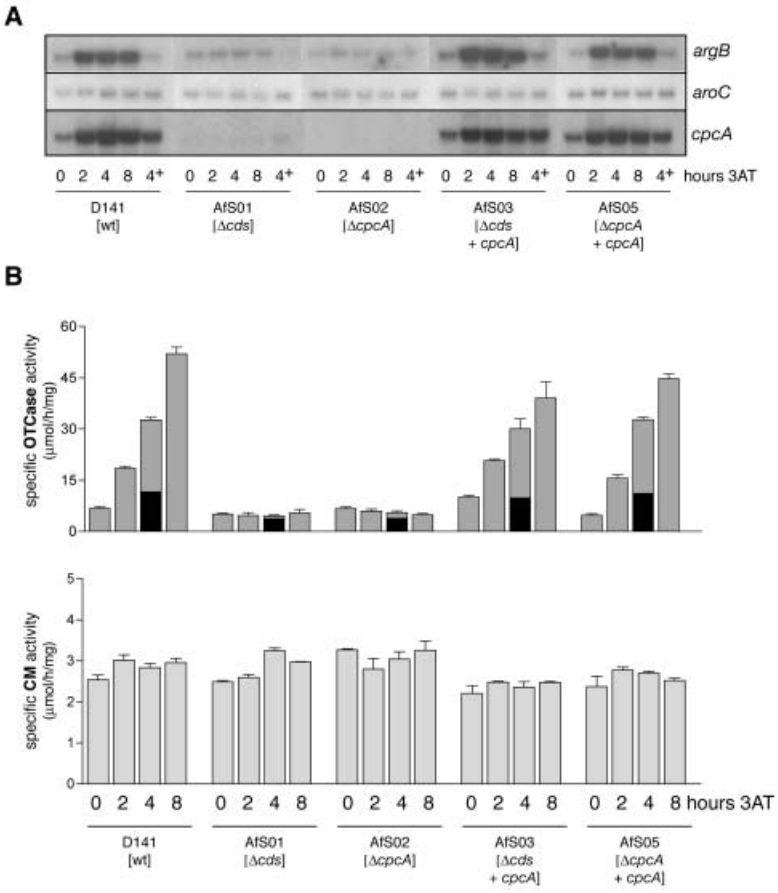

Fig. 4. The cross-pathway response is abolished in *A. fumigatus cpcA* deletion mutants. Strains were propagated in minimal medium and transferred to fresh cultures supplemented with 3AT. Samples were taken after the stated times for Northern hybridizations to monitor transcript steady-state levels (A) and for the determination of specific ornithine transcarbamoyl transferase (OTCase) and chorismate mutase (CM) activities from crude extracts (B). 4^+ and black bars, respectively, indicate growth in the presence of 3AT and histidine.

levels of the *cpcA* transcript were promptly elevated when D141 was challenged with 3AT and remained at high levels for up to 8 h. No transcript was detected in both mutant strains AfS01 and AfS02. Interestingly, *cpcA* transcript levels appeared to be derepressed in the presence of 3AT and histidine. For the reconstituted strains AfS03 and AfS05, wild-type expression was observed for all transcripts monitored, indicating that the observed transcriptional effects are caused solely by the deletion of *cpcA*.

To investigate the cpc response of *A. fumigatus* in more detail, enzymatic activities from crude extracts prepared during 3AT-induced histidine starvation were determined (Fig. 4B). Again, chorismate mutase activity was chosen as the internal standard, and specific activities were not significantly altered for strains D141, AfS01 or AfS02. In contrast, specific OTCase activities served as a proper indicator for any derepression of the cpc status. In D141, a basal OTCase activity level of 6.8 μmol h^{-1} mg^{-1} was elevated to 18.5 during the first 2 h of 3AT treatment. This value increased to 52.0 μmol h^{-1} mg^{-1} over the next 6 h. Cultivation of the wild-type strain in minimal medium supplemented with 3AT as well as histidine did not increase OTCase activities significantly above basal levels. When the *cpcAΔ* mutant strains AfS01 and AfS02 were treated accordingly, no derepression of the cpc system as mirrored by OTCase activities could be detected. When the reconstituted strains AfS03 and AfS05 were monitored, again a strong derepression of specific OTCase activities was detected upon depletion of histidine pools by 3AT, which was absent in the additional presence of the amino acid itself. Again, basal levels of CM activities remained unchanged in these strains throughout the experiment. We conclude that onset of amino acid starvation conditions induces a strong and prompt derepression of the *A. fumigatus* cross-pathway control system, a response that is abolished upon deletion of the *cpcA* locus.

Aspergillus fumigatus *strains defective in their cpc display attenuated virulence in a murine model of pulmonary aspergillosis*

Virulence capacities of the *cpcA* deletion strains were assessed and compared with respect to their wild-type progenitor. We chose to do so using a neutropenic murine model of pulmonary aspergillosis. Neutropenic mice were infected by intranasal instillation of 2×10^5 conidia of each strain, and the health status of the animals was monitored over 10 days after infection with emphasis on weight loss

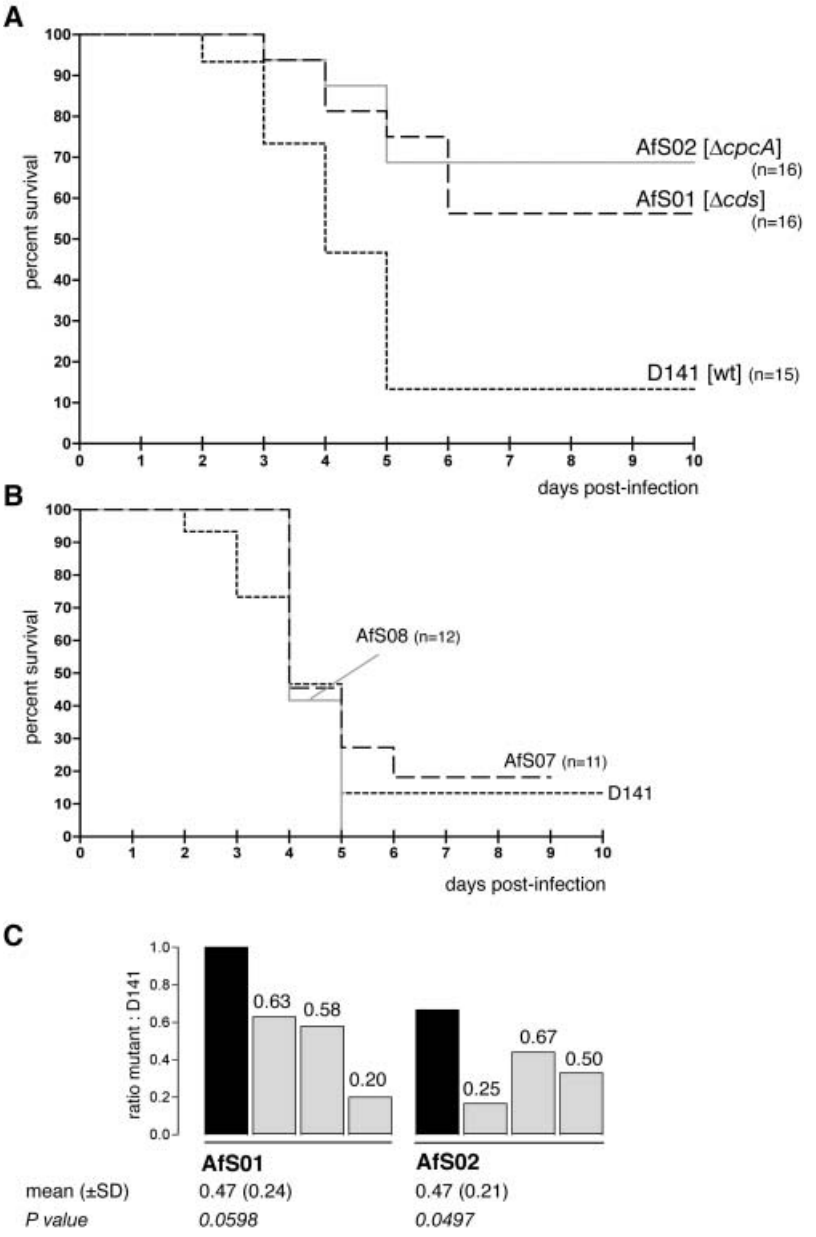

Fig. 5. *cpcA* mutant strains of *A. fumigatus* are attenuated in a murine model of pulmonary aspergillosis.
A. Survival plots for groups of neutropenic CD1 mice intranasally infected with 2×10^5 conidia of *A. fumigatus* wild-type (dotted line), AfS01 (*Δcds*, broken line) or AfS02 (*ΔcpcA*, grey line). Animals received an intraperitoneal dose of 150 mg kg^{-1} cyclophosphamide on days −3, −1, 2 and every third day plus a subcutaneous dose of 112.5 mg kg^{-1} hydrocortisone acetate on day −1. Statistical significance was determined using the log rank method. Both *A. fumigatus* AfS01 ($P = 0.0034$) and AfS02 ($P = 0.0013$) are attenuated for virulence compared with the wild-type progenitor strain D141. No difference was seen in the virulence of AfS01 and AfS02 ($P = 0.5543$).
B. *A. fumigatus cpcA* reconstituted strains have the same virulence as wild-type cells. Groups of immunocompromised mice were inoculated with 1×10^5 conidia of AfS07 (broken line) or AfS08 (grey line), and survival rates were recorded for 9 days. For comparison, survival of mice infected by the wild-type progenitor D141 is included on the graph. No differences in virulence between the wild-type strain D141 and strain AfS07 ($P = 0.4922$) or AfS08 ($P = 0.8757$) were detected. Similarly, AfS07 and AfS08 had the same virulence characteristics ($P = 0.2312$).
C. Outcome of competitive infection experiments. Three neutropenic mice each were infected with mixed inocula of mutant strain AfS01 or AfS02 in combination with conidia of D141 (input ratio, black bars). Animals were culled 4 days after infection, and the ratio of colony-forming units from either mutant strain relative to the wild type was assessed from homogenized lungs (output ratio, grey bars). Competitive indices (CI) deduced from these ratios are given on top of each output bar. From these, mean competitive indices and standard deviations (± SD) were calculated, statistical significance (*P*-value) was assessed using a two-tailed one-sample *t*-test comparing with 1.0, which is the expected CI for a strain with the same virulence as the wild-type strain.

and signs of respiratory distress. Whereas infection with D141 resulted in 87% mortality by day +5 after infection within a group of 15 animals, AfS01 and AfS02 resulted in mortality of 44% and 31% by days +6 and +5 respectively (Fig. 5A). Statistical analyses using a log rank method supported the significance of the survival curves. Saline-inoculated mice remained healthy throughout the experiment, as did immunocompetent mice infected with the wild-type strain. From all mice culled, fungal colonies were regrown from the lungs, indicating that the nature of pulmonary distress was due to aspergillosis. The number of colony-forming units (cfu) extracted from the lungs of animals that were judged as survivors was reduced in comparison with those suffering from aspergillosis. Further inspection of organs from the infected mice revealed a preference in systemic spreading of the fungal pathogen: occasionally, colonies could be grown from the kidneys (nine out of 35 animals); in only two animals was dissemination of the wild-type strain into the liver observed. To confirm that the reduced virulence of our deletion strains was solely attributed to the genetic lesion at the *cpcA* locus, we aimed at reconstitution of both mutants. Strains AfS07 and AfS08 were isolated upon transformation of AfS01 and AfS02, respectively, with a genomic DNA fragment encompassing the complete *cpcA* locus of *A. fumigatus* (pME2563). To rule out any cross-contamination by the original isolate D141, a silent mutation had been introduced in the coding sequence of this

complementing fragment. Furthermore, this particular mutation destroys a native *Sal*I recognition site in the *cpcA* coding sequence. Primary transformants were screened for enhanced growth in the presence of 5MT, colony purified and confirmed for their *cpcA* genotype by Southern analyses as well as their cpc$^+$ phenotype by determination of OTCase activities upon 3AT challenge (not shown). Diagnostic PCR covering the silent mutation followed by *Sal*I restriction of the amplicon confirmed the reconstituted nature of both strains AfS07 and AfS08. These strains therefore genetically resemble their wild-type progenitor as closely as possible but are distinguishable from it, which excludes any contamination during the reconstitution procedure. Intranasal infection of neutropenic mice with 1×10^5 conidia of either reconstituted strain resulted in severe signs of pulmonary distress and mortality rates of 82% for AfS07 after 9 days and 100% at day +5 for AfS08 (Fig. 5B). Calculated *P*-values based on log rank tests indicate that statistically significant differences between the reconstituted strains and the wild-type isolate D141 do not exist. Therefore, we presume that the observed attenuation in virulence of strains AfS01 and AfS02 is solely attributed to their *cpcAΔ* genotype.

To scrutinize the reduced virulence of *A. fumigatus* strains impaired in their cpc response further and to address any kind of growth retardation *in vivo* at early stages of infection, competitive infection experiments were carried out with either mutant strain in challenge with the wild-type progenitor. Mixed inocula containing a total of 1×10^4 cfu were applied to three mice for each strain pair (AfS01/D141 or AfS02/D141). Animals were sacrificed 4 days after infection, and the ratio between both input strains was determined for competitive index (CI) calculation (Chiang and Mekalanos, 1998; Brown *et al*., 2000). Mean CI values of 0.47 were obtained in both instances (Fig. 5C), indicating a competitive advantage for the wild-type isolate over either *cpcAΔ* strain *in vivo*. We therefore conclude that *cpcA* deletion strains of *A. fumigatus* are less capable of survival *in vivo*, resulting in severely attenuated virulence observed for these strains in our animal model of pulmonary aspergillosis.

Discussion

Aspergillus fumigatus is a unique human pathogen as it represents a severe menace to individuals showing a hyperactive or an impaired immune system, resulting in serious allergic reactions or life-threatening invasive mycoses respectively. It is widely accepted that the pathogenicity of *A. fumigatus* is multifactorial, and we have identified a conserved eukaryotic regulatory network that contributes to the virulence of this filamentous fungus.

Transcriptional derepression of numerous genes upon amino acid starvation conditions is a widespread phenomenon displayed by lower eukaryotes. Previous studies have revealed that the system underlying this response is highly conserved among fungi, as demonstrated by extensive data on the general control of *S. cerevisiae* and other yeasts (Hinnebusch, 1986; Bode *et al*., 1990), including the human commensal *Candida albicans* (Tripathi *et al*., 2002), or the cross-pathway control systems of filamentous fungi such as *A. nidulans*, *A. niger* or *Neurospora crassa* (Paluh *et al*., 1988; Wanke *et al*., 1997; Hoffmann *et al*., 2001). Furthermore, orthologues of effectors essential for the signal transduction cascade of this system are found in mammalian genomes (Zhang *et al*., 2002). Cellular functions of cpc systems have been elucidated in great detail. First studies on the general control response of *S. cerevisiae* were focused on compensation of amino acid starvation conditions but, in recent reports, a much broader scope of the cellular response directed by the *GCN4* gene product has been revealed (Natarajan *et al*., 2001). Further studies on *C. albicans* have shown that histidine depletion additionally triggers a morphogenetic response of this human pathogen that is dependent on CaGcn4p (Tripathi *et al*., 2002). In the ascomycete *A. nidulans*, increased activity of the regulator protein CpcA is associated with a reversible block of fruit body formation, supporting an interconnection of the cross-pathway control with cellular differentiation (Hoffmann *et al*., 2000). Our data provide evidence that the cross-pathway control system of amino acid biosynthesis is present and functional in *A. fumigatus* and that it is required for full virulence capacities of this saprophyte. The *cpcA* locus encodes the transcriptional effector of this system and displays high similarity to its relatives within the genus *Aspergillus*. Furthermore, complementation studies in *S. cerevisiae* show that CpcA is a functional orthologue of the Gcn4p regulator of gene expression, at least with respect to amino acid starvation conditions. The presence of upstream ORFs within a long 5′ leader sequence is characteristic of fungal cpc activator genes. In *S. cerevisiae*, four short uORFs mediate the translational regulation of Gcn4p expression; in filamentous fungi, two such uORFs are generally found to precede the actual coding sequence. Based on the assumption of the reinitiation model (Gaba *et al*., 2001), spacing between the distal and proximal translational barrier is crucial for translational control. On the *GCN4* mRNA, uORF1 and uORF4 are separated by 198 nucleotides (nt), whereas uORFα and uORFβ of *A. fumigatus cpcA* are separated by 220 nt. Therefore, it is tempting to speculate on conserved regulatory mechanisms of translational control between the two species. Our complementation studies reveal that the *cpcA*-encoded gene product alone is able to rescue a yeast *gcn4Δ* mutant from amino acid starvation conditions, so the presence of upstream ORFs regulating expression of the transcriptional activator is not strictly

necessary for heterologous complementation. The major role for fungal cpc systems lies in sensing and responding to environmental stress conditions, with the focus on depletion of intracellular amino acid pools. Additional stress conditions such as purine starvation, glucose limitation or high salinity have been identified in *S. cerevisiae* to trigger increased synthesis of the cpc transcriptional activator protein (Hinnebusch and Natarajan, 2002). For Aspergilli, no stress condition besides amino acid deprivation has been described to induce the cpc response, and we were not able to detect any *in vitro* phenotype of *A. fumigatus cpcA* deletion strains on high-salinity media (not shown). Considering the reduced virulence capacities of *A. fumigatus areA* mutants (Hensel *et al*., 1998), it is likely that the host's lung represents a nitrogen-poor environment. We were not able to detect any *in vitro* phenotype of *cpcA* deletion mutants with respect to nitrogen source quality, so we conclude that the utilization capacities of different nitrogen sources are not affected by the cross-pathway control system. Fungal purine–pyrimidine biosynthesis has been described as being influenced by the cpc system (Natarajan *et al*., 2001), and adenine auxotrophs of *A. nidulans* have been characterized as being avirulent (Purnell, 1973), although different *ad*$^-$ mutations differed in their effects on virulence. Both *cpcA* deletion mutants display no specific nutritional requirements and are therefore prototrophic for amino acids, purines or pyrimidines. Nevertheless, we cannot exclude an *in vivo* starvation situation because of specific metabolic imbalances or stress situations, and it is likely that the scope of the cpc response in *Aspergillus* exceeds amino acid starvation, so future studies will have to address this issue.

In the murine model system of pulmonary aspergillosis, statistically significant reductions in virulence were detected for *A. fumigatus cpcA*Δ strains. Although these mutants remained virulent to a certain extent, survival rates were above 50%, which accounts for the strongly reduced pathogenic capacities of strains impaired in the cross-pathway control system. Monitoring the weight loss profiles of the animals infected showed that mice inoculated with either *cpcA* mutant strain had lost weight in a less progressive way than their wild type-infected counterparts (not shown). Histological inspection of mice culled as a result of respiratory distress or at the end of the experiment revealed no differences with respect to the extent of growth, growth pattern or invasiveness of fungal foci. Both mutant strains are therefore not drastically impaired in their ability to grow *in vivo*. To yield first hints on the reasons for virulence reduction, competitive infection experiments were carried out. Although statistical parameters for the results are not satisfying, a trend can be drawn from these data, which implies that an *A. fumigatus cpcA*Δ strain has reduced capacities in establishing aspergillosis. As the pathogenicity of *A. fumigatus* depends on the cpc system to a certain extent, we hypothesize that environmental stress is faced by the fungal pathogen upon host infection and that a signal transduction cascade triggering the cpc response read-out, which is generated by transcriptional activity of CpcA, is required to counteract this stress signal. The absence of amino acids is unlikely to represent the metabolic stress condition, supported by the prototrophic nature of *cpcA*Δ strains, and the exact nature of the stress signal remains to be identified. Imbalances in amino acid pools might trigger the cpc response *in vivo* to adjust expression levels of amino acid biosynthetic activities. Alternatively, CpcA might be required for basal transcription of a virulence-determining *A. fumigatus* gene, making the influence of CpcA on pathogenicity indirect.

The impact of metabolism on *A. fumigatus* pathogenicity has been addressed before by the identification of various mutants impaired in biosynthetic pathways. Reduced or abolished virulence of such auxotrophs gave insight into limiting nutritional pools in the mammalian host. Exact data on the physiological situation within a mammalian lung, the primary site of infection, are not available, so defining environmental conditions at this ecological niche remains speculative. Using *A. fumigatus* mutants impaired in specific physiological pathways such as metabolism, pH regulation, osmotic balance, etc., is an approach well-suited to gaining clues to such issues. We describe here the first example of a metabolic mutant of *A. fumigatus* that is prototrophic but displays reduced virulence. Although applying a single-gene deletion strategy, we were able to identify the cross-pathway control system as required for virulence, based on the fact that one of the major regulatory genes of this system was targeted. As noted by Latge (2001), regulatory genes are promising candidates for elucidating the pathogenicity mechanisms of *A. fumigatus*, and detailed studies on them are likely to complement broad-range strategies such as signature-tagged mutagenesis (STM) screening or restriction enzyme-mediated integration (REMI) mutagenesis (Brown *et al*., 1998; 2000).

Experimental procedures

Strains, media and culture conditions

Bacterial strains were *Escherichia coli* DH5α [F$^-$, ϕ80dΔ(*lacZ*)M15^{-1}, *Δ*(*lacZYA-argF*)U169, *recA1*, *endA1*, *hsdR17* (r_K^-, m_K^+), *supE44*, λ$^-$, *thi1*, *gyrA96*, *relA1*] (Woodcock *et al*., 1989) for general cloning procedures and KS272 [F$^-$, *ΔlacX74*, *galE*, *galK*, *thi1*, *rpsL*, *ΔphoA*(*Pvu*II)] carrying the pKOBEG plasmid for recombination (Chaveroche *et al*., 2000). *E. coli* strains were propagated in LB or LBLS (1% bacto-tryptone, 0.5% yeast extract, 1% or 0.5% NaCl, pH 7.5) medium. Fungal strains used in this study are listed in Table 1. Growth of *A. fumigatus* strains was carried out at 37°C on minimal medium prepared and supplemented

according to Käfer (1977). For radial growth rate determination, the procedure described by Panepinto *et al*. (2003) was applied by measuring colony diameters arising from 1×10^4 freshly harvested conidia spotted in triplicate on minimal media containing various nitrogen sources. *S. cerevisiae* strain RH1408 was cultivated on minimal medium YNB (0.14% yeast nitrogen base, 0.5% ammonium sulphate). Antibiotic concentrations were 100 μg ml^{-1} for ampicillin, 25 μg ml^{-1} for chloramphenicol, 50 μg ml^{-1} for zeocin, 20 μg ml^{-1} for phleomycin and 200 μg ml^{-1} for hygromycin B.

DNA manipulations and plasmid constructions

Standard protocols of recombinant DNA technology were carried out (Sambrook *et al*., 1989). *Pfu* proofreading polymerase was generally used in PCRs (Saiki *et al*., 1986), and essential cloning steps were verified by sequencing on an ABI Prism 310 capillary sequencer. Fungal genomic DNA was prepared according to the method of Kolar *et al*. (1988), and Southern analyses were carried out as described previously (Southern, 1975). Total RNA samples were isolated using the TRIzol reagent from Invitrogen followed by Northern hybridization according to the protocols cited by Brown and Mackey (1997). Random primed labelling was performed with the Stratagene Prime-It II kit in the presence of [α-^{32}P]-dATP (Feinberg and Vogelstein, 1983). Autoradiographies were produced by exposing the washed membranes to Kodak X-Omat films.

Transformation protocols for *E. coli* were either for calcium/manganese-treated cells (Hanahan *et al*., 1991) or for electroporation (Dower *et al*., 1988) with a Bio-Rad GenePulser at 2.5 kV in 0.2 cm cuvettes. *A. fumigatus* was transformed by polyethylene glycol-mediated fusion of protoplasts as described previously (Punt and van den Hondel, 1992). *S. cerevisiae* strain RH1408 was transformed according to Elble (1992).

Plasmids used and constructed during the course of this study are listed and described briefly in Table 1, together with essential oligonucleotides used to construct them. Sv19 and Sv80 were used to PCR amplify a 468 bp fragment from the genomic *cpcA* locus. The *Sal*I–*Xho*I-digested amplicon was used to replace the native *Sal*I fragment in pME2562 to yield complementation plasmid pME2563. Furthermore, primers to amplify gene-specific hybridization probes by PCR are specified as follows: a fragment targeting the *A. fumigatus aroC* transcript has been generated using primer combination Sv48/Sv49; the amplicon derived from Sv64 combined with Sv65 represents an *argB*-specific template. Sv02 and Sv03 served as gene-specific priming oligonucleotides in 5′ RACE experiments, which were carried out with an Invitrogen system according to the manufacturer's instructions.

Biochemical methods

Crude extracts were prepared by grinding washed and shock-frozen mycelia to a fine powder and extracting soluble proteins with buffer (100 mM Tris-HCl, 200 mM NaCl, 20% glycerol, 5 mM EDTA, pH 8) at 4°C in the presence of the protease inhibitor phenylmethylsulphonyl fluoride. Protein contents were determined by the procedure of Bradford (1976). Enzymatic activities of chorismate mutase (CM, EC 5.4.99.5) were assayed essentially as described previously (Schmidheini *et al*., 1989), and ornithine carbamoyltransferase (OTCase, EC 2.1.33) activities were determined according to Tian *et al*. (1994).

Animal model studies and histology

Outbred male mice (strain CD1, 20–28 g; Charles River Breeders) were used for animal experiments. Immunosuppression was executed with hydrocortisone acetate (112 mg kg^{-1} subcutaneous) and cyclophosphamide (150 mg kg^{-1} intraperitoneal) according to the protocol of Smith *et al*. (1994). Bacterial infections were prevented by adding tetracycline (1 g l^{-1}) and ciproxicin (64 mg l^{-1}) to the drinking water. Inocula of up to 2×10^5 conidiospores in 40 μl of saline were prepared by harvesting spores from 5-day-old slants of solid medium followed by filtration through miracloth and washing with saline (Aufauvre-Brown *et al*., 1998; Tang *et al*., 1993). Mice were anaesthetized by inhalation of halothane and infected by intranasal instillation. The weights of infected and control animals were monitored for up to 10 days twice daily, and mice developing severe pulmonary illness, characterized by respiratory distress, hunched posture and poor mobility, or 20% weight loss were culled. Organs were prepared and homogenized in saline for colony counts, and lungs from various animals were preserved for histology in 4% formaldehyde (Tang *et al*., 1993). For the determination of competitive indices (CI; Brown *et al*., 2000), mixed infection experiments were carried out using inocula of 1×10^4 spores in 40 μl of saline, and animals were culled on day +4 after infection. Wild-type and mutant colonies were distinguished by plating in duplicate on minimal media with or without phleomycin.

Statistical analyses

The log rank method was used to evaluate mortality rates in single-strain infection experiments using the GraphPad PRISM® software. CIs are defined as the output ratio of mutant to wild-type fungal colonies divided by the input ratio of mutant to wild-type fungal colonies (Chiang and Mekalanos, 1998). Statistical significance was assessed by a two-tailed one-sample *t*-test compared with 1.0, which is the expected CI for a strain with wild-type virulence.

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