A regulator of *Aspergillus fumigatus* extracellular proteolytic activity is dispensable for virulence.

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ABSTRACT

Virulence of the fungal pathogen *Aspergillus fumigatus* is in part based on the saprophytic lifestyle this mould has evolved. A crucial function for saprophytism resides in secreted proteases that allow assimilation of proteinaceous substrates. The impact of extracellular proteolytic activities on the pathogenesis of aspergillosis, however, remains controversial. In order to address this issue, characterisation of a conserved regulatory factor, PrtT, that acts on expression of secreted proteases was pursued. Expression of PrtT appears to be regulated post-transcriptionally, and the existence of an mRNA leader sequence implies translational control via eIF2α kinase signalling. Phenotypic classification of a *prtT*Δ deletion mutant revealed that expression of several major extracellular proteases is PrtT-dependent, resulting in the inability to utilise protein as nutritional source. Certain genes encoding secreted proteases are not regulated by PrtT. Most strikingly, the deletant strain is not attenuated in virulence when tested in a leukopenic mouse model, which makes a strong case for reconsidering any impact of secreted proteases in pulmonary aspergillosis.
INTRODUCTION

Diseases caused by the fungal pathogen of the genus *Aspergillus* represent a growing and clinically relevant threat in distinct medical settings (31). Aspergilloses have to be considered as a frequent complication during immunological therapies or disorders such as allogeneic stem cell transplantation and chronic granulomatous disease, respectively, and recent evaluations demonstrate the increasingly severe impact of this fungal infection on health economy (52). Among its various types, so-called invasive forms of aspergillosis are most severe and often life-threatening, especially for the immuno-compromised individual. Due to the commonly air-borne route of transmission, the pulmonary system represents the prime infection site in a susceptible host, and after successful germination of the infectious propagules, colonisation of the infected tissue and eventually haematogenous dissemination may occur. By far the most cases of aspergillosis are caused by the saprophyte *A. fumigatus*; however, other species of this genus are able to infect and harm individual patients (53).

Besides defined host factors (9), several fungal attributes are considered as virulence determinants in the host-pathogen system that results in aspergillosis (25). Based on recently gained insights into the cellular armamentarium that is expressed by members of this genus, the hypothesis that exclusive virulence factors contribute specifically to the virulome of *A. fumigatus* has been challenged. According to this, it is rather likely that the saprophytic life-style exhibited by aspergilli forms the basis for colonising a susceptible host, which is simply unable to clear the infection (51). Saprophytism is in part characterised by an absorptive nutritional mode, which is based on the extracellular degradation of the surrounding polymeric matrix and successive uptake of oligomeric break-down products. For the latter, specialised transport systems have evolved, whereas for the former practice a plethora of hydrolytic enzymes is expressed and secreted into the environmental vicinity.

Among those, proteases appear to play a prominent role, which is indicated by the huge array
of proteolytic activities that is presumably encoded by the *A. fumigatus* genome to comprise more than 100 proteases (33, 36). Three major proteases have been described to be secreted during infection by this fungus, an alkaline serine protease Alp, a metalloprotease Mep, and an aspartic protease Pep (32). Their production is triggered by the presence of proteinaceous substances in the growth medium, whereas free amino acids or oligopeptides repress their expression. Each sub-class of proteolytic activity appears not to be encoded by one particular locus but by a family of genes, which hampers a conclusive assessment of their role in virulence of *A. fumigatus*. Several points exist that would argue in favour of, as well as against, an involvement of extracellular proteolytic activities in the development of invasive aspergillosis: expression during infection could be demonstrated for several proteases (29, 35, 39, 40), and an elastinolytic activity expressed by *A. fumigatus* had been attributed ability to cause invasive aspergillosis (24). Also, the alkaline serine protease was able to induce structural changes in the actin cytoskeleton of cultured lung pneumocytes (22), and recently, a nutritional role for breakdown products of the proteinaceous matrix was postulated (14). Yet, in a singular case report the absence of elastinolysis of vessel walls during invasive aspergillosis was evident (10), and, more importantly, virulence attenuation could not be revealed for any *A. fumigatus* mutant deleted for single or multiple genes encoding extracellular proteolytic activities (15, 34, 42, 50).

Knowledge on the regulatory means of *Aspergillus* to trigger expression and secretion of proteases is scarce. Early reports indicated that the presence of protein as sole source of nitrogen strongly induces expression of the major secreted *A. fumigatus* proteases, whereas free amino acids or small peptides repress their formation. Also the presence of a primary N-source such as ammonium results in very low levels of extracellular proteolytic activity, which implies that expression of secreted proteases is under control of the nitrogen catabolite repression system. A more detailed analysis of proteinase secretion by *A. fumigatus* in the presence of complex substrates such as gelatin, Matrigel, serum, BSA, or even pneumocytes
revealed a multifaceted pattern of expression for the major activities (11). Notably extracellular proteolysis, at least serine proteinase activity, is maintained in the presence of low (0.1%) concentrations of the protein hydrosylate peptone (4). Also in the model ascomycete *A. nidulans* an influence of the nitrogen source on proteinase secretion was documented as well as of the carbon source and the related catabolite repression system. There, the p53-like transcription factor XprG appears to play a general role in regulating the expression of extracellular proteases in response to starvation (20, 21).

In the biotechnological workhorse *A. niger*, however, a unique and specific regulator of extracellular proteolytic activity was recently identified in a forward genetics approach by random mutagenesis and screening on growth plates containing casein milk. The aim was to identify isolates that would exhibit reduced protein degradation during production of heterologous proteins (30). Among these *prt* strains, one particular, putatively regulatory mutant became of interest, and the corresponding *prtT* gene was identified to encode a Zn2Cys6 protein (37). Further analyses of disruptants in *A. niger* and *A. oryzae* validated a conserved function of PrtT in regulating the major protease genes in these two species of *Aspergillus*. Cross-species genome comparison revealed the interesting fact that *prtT* was present in several aspergilli but absent in the genome of *A. nidulans*, where most likely an evolutionary gene deletion event had occurred.

To address the role of secreted proteases in virulence of the pathogenic species *A. fumigatus* on a broad scale, we here describe our efforts to scrutinise the cellular function of its corresponding *prtT* gene. A comprehensive characterisation of a validated deletion mutant was carried out to reveal that the *A. fumigatus* *prtT* gene product is a transcriptional regulator of extracellular proteolytic activities that, however, is not essential for virulence of this opportunistic fungal pathogen.
MATERIALS AND METHODS

Strains, media, and growth conditions. For general cloning procedures the bacterial strain *Escherichia coli* DH5α was used (56), which was cultivated in LB (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) medium. Fungal strains of this study are listed in TABLE 1. Growth of *Aspergillus fumigatus* was carried out at 37°C on minimal medium prepared according to Käfer (18) supplemented with appropriate sources of carbon and nitrogen as specified. Concentrations of protein-containing supplements were 0.4% for casein, 0.1% for peptone, and 0.4% for BSA. Antibiotic concentrations were 100 µg/ml for ampicillin, 20 µg/ml for phleomycin, and 0.1 µg/ml for pyrithiamine.

Transformation procedures. *E. coli* cells were transformed after treatment with calcium/manganese (12). For *A. fumigatus*, a procedure of polyethylene glycol-mediated protoplast fusion was followed (38).

Manipulation of nucleic acids and plasmid constructions. Standard protocols of recombinant DNA technology were carried out (45). Phusion™ high-fidelity DNA polymerase was generally used in polymerase chain reactions (PCRs), and essential cloning steps were verified by sequencing at GATC BIOTECH. Sequence analyses were performed with the LASERGENE Biocomputing software package from DNASTAR. Fungal genomic DNA was prepared following the protocol of Kolar *et al.* (23), and Southern analyses were carried out as described (48, 49). Probes for non-radioactive hybridisations were generated and detected using the GENE IMAGES ALKPHOS DIRECT LABELLING AND DETECTION SYSTEM from GE HEALTHCARE. Autoradiographies were produced by exposing washed membranes to KODAK X-OMAT films. DNA extraction from homogenised lung tissue was performed using the QIAGEN DNEASY BLOOD AND TISSUE KIT. Samples of total RNA were isolated with the
TRIzol reagent of INVITROGEN and used in quantitative real-time PCRs (see below) after DNAse treatment and reverse transcription using the SUPERSCRIPT III FIRST-STRAND SYNTHESIS SUPERMIX from INVITROGEN.

Plasmids constructed during the course of this study are listed and briefly described in Table 1, Table 2 lists the sequences of relevant oligonucleotides. In detail, plasmids were constructed as follows: Generation of a gene replacement cassette for deletion of the prtT gene was executed according to the method of Kämper (19) by amplifying flanking regions of the target locus with the primer pairs AB17/20 and AB19/18 to yield a 5’ flank of 2.3 kb and a 3’ flank of 1.65 kb, respectively. After SfiI digestion, fragments were ligated to the SfiI-flanked phleomycin resistance cassette of pSK341, and the resulting replacement cassette was directly inserted in the cloning vector pJET1.2. From the resulting plasmid pSK462, a 6.8 kb replacement module was released by PmeI digestion prior to transformation of A. fumigatus. A reconstitution cassette was generated by insertion of the pyrithiamine resistance gene ptrA amplified from pSK275 with primers Sv551 and Sv556 into the blunted EcoRI site downstream of the prtT coding sequence, which had been inserted as 6.7 kb PCR amplicon with primers AB17/18 in pJET1.2. The complementing fragment was isolated from this plasmid pSK463 after PmeI digest.

Quantitative real-time PCR. qRT-PCRs were performed on a BIORAD MYIQ iCYCLER using the iQ SYBR GREEN SUPERMIX. Sequences of priming oligonucleotides are given in Table 3, gene-specific combinations are: AB34/35 (alp1), AB36/37 (pep1), AB38/39 (mep), AB30/31 (sedB), TubFw/TubRev (AFUA_1G10910, encoding β-tubulin), RTDpp4Fw/RTDpp4REv (dppIV), and AB28/29 (dppV). Per reaction, 0.3 µl of cDNA and 1 pmol of each primer were used to perform 40 temperature cycles as follows: 3’ of polymerase activation at 95°C, 15’’ at 95°C for initial denaturation, 15’’ at 60°C annealing, and 25’’ at 72°C elongation.
Fluorescence was determined at each elongation step. Melting curves were plotted to confirm specificity of each reaction, and results were quantified according to the comparative C\text{t} method (1).

5 **Proteolysis tests.** Two assays were executed for determination of proteolytic activities, one qualitative based on clearance of unprocessed X-ray film material (8) and another, quantitative one measuring hydrolysis of dye-coupled collagen (7, 17).

**Virulence model of pulmonary aspergillosis.** Outbred male mice (strain CD1, 20-28 g, Charles Rivers Breeders) were used for animal experiments. Immunosuppression was carried out by subcutaneous injection of 112 mg/kg hydrocortisone acetate and intraperitoneal injection of 150 mg/kg cyclophosphamide following a sequential protocol as described (47). Bacterial infections were prevented by adding 1 g/l tetracycline and 64 mg/l ciproxicin to the drinking water. Inocula of $1 \times 10^4$ conidiospores in 40 µl of saline were prepared by harvesting spores from 5-day-old slants of solid medium followed by filtration through miracloth (CALBIOCHEM) and washing with saline. Mice were anaesthetized by inhalation of isofluorane and infected by intranasal instillation. The weights of infected mice were monitored for 4 days twice daily before animals were culled to isolate their lungs. In order to assess fungal burdens as quantitative virulence criterion, qPCRs were performed on equivalent amounts (100 ng) of genomic DNA (gDNA) that had been extracted from equal amounts (250 mg) of homogenised lung tissue (5, 46) using several references for gDNA concentration and the oligonucleotides qPCRf and qPCRr to amplify the 18S rRNA locus from the *A. fumigatus* genome.
RESULTS

The *Aspergillus fumigatus* genome encodes an orthologue of PrtT.

In order to identify the orthologous gene of the *A. niger* prtT locus, we interrogated the *A. fumigatus* genome sequence at the CADRE database (28). A BLAST search retrieved indeed an automatically annotated gene entry tagged with the locus identifier AFUA_4G10120. Its deduced gene product displays a high degree of conservation to the PrtT regulatory proteins of *A. niger*, *A. oryzae* and several others, with similarities ranging from 51% to 81%. The automatic annotation predicts a gene product of 696 residues with an unconserved N-terminal part based on the presence of an unusually long (386 nt) postulated intronic sequence. To map this region of the *prtT* transcript, diagnostic PCRs from reverse transcribed mRNA with several specific primer combinations covering the predicted 5’ region were conducted (Fig. 1A). Presence and size of the resulting amplicons indicate the absence of any splicing events for the first predicted intron, but hint at the existence of a long leader region of at least 690 nt preceding the translational start site on the *prtT* transcript.

Accordingly, we assume that one of the distal ATG triplets serves as actual translational start codon to result in a PrtT protein closely related to orthologous ones from other *Aspergillus* species (Fig. 1B). Based on similarity, the *A. fumigatus* PrtT is likely to comprise 613 amino acids. Efforts to map the exact transcriptional start sites by 5’ RACE failed, presumably due to insufficient expression of this putative transcription factor-encoding gene.

The deduced amino acid sequence from *prtT* contains a DNA binding domain of the C6 zinc finger domain class, which is highly conserved among its orthologous gene products (Fig. 1B). Besides this, no particular domains of specific function could be identified by various database searches.
PrtT is required for protein utilisation and degradation.

In order to gain insight into the cellular function of the *prtT* gene product, we constructed a full deletion strain by homologous gene replacement with a suitable selectable marker cassette (Fig. 2A). Southern analysis of several isolates confirmed the replacement event, and one representative was picked for further analysis (see below). Based on this deletant AfS61, a reconstituted strain, AfS62, was generated by introducing the *prtT* gene linked to a selectable marker at the resident genomic position, which could be confirmed by Southern analysis. The resulting set of strains was eventually subjected to phenotypic inspection on various types of growth media. The most strikingly phenotype was evident on plates supplemented with the protein BSA as sole source of nitrogen (Fig. 2B). On this type of medium, a clear growth retardation could be observed resulting in the near absence of mycelial expansion. Only after prolonged incubation of the growth plates a very sparse hyphal mesh was produced by strain AfS61, whereas proper growth was present for the wild-type progenitor and the reconstituted strain AfS62. Pre-digested protein, however, supported growth, as could be demonstrated by growing the *prtT*Δ strain on minimal medium in the presence of 0.1% peptone as nitrogen source. As a first indication on which cellular function this growth phenotype is based on, strains were propagated on minimal medium containing casein. In a wild-type situation, acidification results in protein precipitation around the growing colony to result in a turbid zone; upon prolonged growth, this precipitate zone clears up due to hydrolytic degradation by the abundantly secreted proteases. Mycelia of the *prtT* deletant strain did not display any clearance around or beneath the hyphal mat on casein plates, in contrast to the wild-type or the reconstituted strain (Fig. 2C). Accordingly, although casein is a different substrate, the growth defect of AfS61 in the presence of BSA might be due to the strain’s inability to break down this polymeric substrate.
A *prtT* deletant is strongly impaired in secreting proteolytic activities.

Based on the hypothesis that the *prtT* deletion mutant is impaired in expressing extracellular proteases, a degradation assay was performed with this strain when grown in liquid culture in the presence of BSA as well as low amounts of peptone (4). The latter substrate supports growth of the mutant but does not suppress secretion of extracellular proteases in the wild-type (data not shown). Accordingly, these culture conditions allow proper data evaluation due to comparable growth of the analysed strains. Supernatants from the BSA/peptone cultures and from cultures supplemented with alternative sources of nitrogen were subjected to a crude assay for proteolytic activity by spotting aliquots on sheets of unprocessed X-ray films. A clearing zone could only be observed for strains expressing PrtT, which is based on gelatin hydrolysis in the light-sensitive layer (Fig. 3A). From these assays it was evident also that presence of the primary nitrogen source ammonium suppresses expression of extracellular proteolytic activities. To assess degradation of the protein substrate in the course of fungal growth, supernatants from the BSA/peptone cultures were subjected to SDS-PAGE and proteins visualised by Coomassie staining (Fig. 3B). After 24 hours, degradation of BSA was evident for the wild-type strain as well as the reconstituted isolate, whereas the *prtTΔ* strain was apparently unable to degrade this substrate. Upon prolonged cultivation, complete hydrolysis of the growth substrate could be observed for the strains carrying the *prtT* gene; the *prtT* deletant, however, was able to break down BSA to a limited extent, resulting in a distinct degradation product of approximately 50 kDa in the culture’s supernatant. Shift experiments with pre-grown mycelia of Afs61 transferred to BSA-containing but peptone-lacking medium did not result in this degradation pattern (data not shown), indicating that the expression of the accountable proteolytic activity is linked to fungal growth. To quantify the actual proteolytic capacities from the culture supernatants, colorimetric assays with the unspecific substrate azocoll were carried out. In this assay, almost no detectable activity was evident in supernatants from the *prtTΔ* strain Afs61, in sharp contrast to the wild-type.
progenitor ATCC 46645 or the reconstituted strain AfS62 (Fig. 3C). In summary, these data indicate that the presence of prtT is vital for the expression of extracellular proteases by A. fumigatus, which enables this saprophyte to assimilate proteinaceous growth substrates.

5 **PrtT regulates transcription of major secreted proteases.**

As it was evident that the prtT gene product would regulate the expression of extracellular proteolytic activities, we monitored steady-state transcript levels of several selected genes encoding major secreted proteases of A. fumigatus, in particular alp1 (AFUA_84G11800), mep (AFUA_8G07080), pep1 (AFUA_5G13300), sedB/tppA (AFUA_4G03490), dppIV (AFUA_4G09320) or dppV (AFUA_2G09030), which encode a serine alkaline protease (16), metalloprotease (15), aspergillopepsin (43), a sedolisin (41), and two didpetidyl-peptidases (2, 3), respectively (Fig. 4). Upon a shift from minimal medium supplemented with primary sources of carbon and nitrogen to medium containing BSA as sole N-source, expression of any of the major extracellular protease-encoding transcripts alp1, mep, and pep1 increased in the wild-type isolate, and this transcriptional induction was absent in strain AfS61 carrying the prtT deletion. Transcript levels of the sedolisin-encoding gene sedB did not increase significantly upon a shift to BSA-containing medium, whereas transcription of the two genes encoding extracellular dipetidyl-peptidases, dppIV and dppV, was strongly induced when BSA was present. This regulatory pattern was unaltered in the prtTA genetic background for the dppV gene. For dppIV, however, a much lesser range of induction was evident resulting in basal levels of transcription. This clearly demonstrates that induced expression of some of the major secreted proteases is regulated on the transcriptional level by the PrtT regulator; however, this regulation is not entirely comprehensive and the expression pattern for some extracellular proteases might not exclusively depend on PrtT, as demonstrated for the didpetidyl-peptidase-encoding genes dppIV and dppV.
The \textit{prtT} gene product is not a virulence determinant in leukopenic mice.

In order to assess any contribution of the PrtT regulator to \textit{A. fumigatus} virulence, the corresponding deletion mutant AfS61 and its wild-type progenitor ATCC 46645 were tested in a murine model of pulmonary aspergillosis. From small groups (n=5) of leukopenic animals that had been infected with low doses of fungal spores, similar weight loss profiles were determined for the \textit{prtT}\textsuperscript{Δ} deletion strain and the wild-type isolate (Fig. 5A). These data correlate with disease progression and were essentially very similar for either \textit{A. fumigatus} strain. Moreover, lungs of infected animals were isolated four days post infection to extract genomic DNA from the homogenised tissue. From these samples the fractions of fungal DNA, corresponding to the fungal burden in the respective organ, were determined by quantitative PCR amplifying the 18S ribosomal DNA locus (Fig. 5B). No significant differences were evident between the cohorts of infected animals, which indicates that fungal growth is unaltered for the \textit{prtT}\textsuperscript{Δ} strain. Although fungal burden is not directly indicative of virulence, fungal growth is a recognised pathogenicity trait (44). Accordingly, it is likely that regulated expression of the major extracellular proteases is not a virulence determinant of \textit{A. fumigatus} in the course of pulmonary aspergillosis, at least not in the employed disease model.
DISCUSSION

Pathogenicity of the ubiquitous saprophyte A. fumigatus has been subjected to extensive scrutiny for the last years, since this filamentous fungus appears to lack bona fide virulence factors. In order to address the open question about the contribution of secreted proteolytic activities to the fungal virulome, we directed our efforts towards a conserved regulator of extracellular protease expression, the prtT gene product. Characterisation of a defined deletion mutant revealed that PrtT is indeed required for transcription of several genes encoding secreted proteases, which in turn enable A. fumigatus to assimilate proteinaceous substrates. However, the absence of regulation on expressing extracellular proteases appears not to influence virulence. It is evident that by our in vitro characterisation of the prtTΔ mutant we address the saprophytic capacity of A. fumigatus only in part. Yet, the data on PrtT-dependent expression of extracellular proteolytic activities give insights into fungal characteristics that contribute to saprophytism.

In our analysis of the automatically annotated prtT gene we became aware of an unusually long leader sequence preceding the coding region that is translated into the conserved regulatory protein. Diagnostic PCRs indicate that this leader is at least 690 nucleotides in length with no splicing events being evident. For the A. niger orthologue of prtT, a comparable situation was described with the widest-reaching stretch upstream of the translational start codon being 800 nt long (37). Leader stretches are common among genes that are modulated in their expression by post-transcriptional mechanisms, for instance translational regulation via small upstream open reading frames (uORFs). Sequence analysis of the postulated leader region indeed indicates the presence of various AUG triplets followed by in-frame by stop codons. Accordingly, a regulatory mechanism acting on PrtT expression via eIF2α kinase signalling seems likely (54). In this scenario, nutritional stress such as the presence of protein as secondary source of nitrogen would trigger phosphorylation of the
translation initiation factor subunit eIF2α and relaxation of the inhibitory functions of uORFs in the \textit{prtT} mRNA leader region to result in proper translation of the actual coding sequence. In line with this is the fact that steady-state levels of the \textit{prtT} transcript do not change upon a shift from the primary N-source ammonium to BSA (data not shown), a situation in which extracellular proteases become abundantly expressed. Yet, additional regulatory mechanisms, such as phosphorylation or regulated translocation, may modulate PrtT activity as well.

The observation that expression of extracellular proteolytic activity is abolished by the presence of ammonium accounts for an influence of the wide-domain regulatory system of nitrogen catabolite repression (NCR), which is essentially mediated by the GATA-type transcription factor AreA \cite{6,55}. Regulation of expression by PrtT appears to be more specific as it acts on transcription of a subset of secreted proteases, among them the major activities Alp, Mep, and Pep. The fact that these genes are targets of PrtT-mediated regulation is properly illustrated by the phenotypic behaviour of the corresponding deletant: growth on protein as sole source of nitrogen and carbon became markedly reduced as degradation of this substrate is considerably impaired. Several other protease-encoding genes might not be affected by PrtT, as it is supported by the unaltered expression of \textit{dppV} or \textit{sedB}, and also basal levels of protease expression might still be present in the \textit{prtTΔ} deletion background. This is corroborated by low transcript levels for the dipeptidyl-peptidase-encoding gene \textit{dppIV} in the \textit{prtTΔ} deletant and the observation that prolonged propagation of this strain in BSA-containing medium results in limited but distinct degradation of this protein (Fig. 3B). This specific pattern may result from the action of a specific endoprotease expressed in low amounts or bound to the fungal cell wall. Future studies might explore the PrtT-directed transcriptome and its associated proteome or secretome, respectively, to reveal the depth of the PrtT regulon. Furthermore, the mechanism of regulating PrtT activity remains to be
clarified as well as the signal transduction cascade that mediates the degradative response of *A. fumigatus* when challenged with proteinaceous substrates.

The main finding of this study lies in the observation that an *A. fumigatus* strain deleted for the *prtT* gene is as virulent as its progenitor in a murine model of pulmonary aspergillosis testing leukopenic animals. From the observed phenotypes displayed by the deletant it is obvious that PrtT contributes to protein degradation and therefore most likely to the saprophytic lifestyle of *A. fumigatus*, though it appears not to be essential for pathogenicity in the aforementioned animal model. This kind of aspergillosis model, which is devoid of phagocytic cells of the innate immune system, is appropriate when testing for growth or fitness of an *A. fumigatus* mutant at the site of infection, two qualities strongly related to fungal virulence (44). We chose not to employ a standard model following progression of deaths among cohorts of infected animals but instead quantifying fungal burden as a read-out for virulence, based on the consideration that this would reveal even subtle differences more consistently. Data from the corresponding qRT-PCRs strongly indicate that the *prtTΔ* strain is not attenuated, a conclusion that is supported by the weight loss profiles of the infected animals in the course of the infection experiments. Yet, as regulation of extracellular protease expression by PrtT is possibly not comprehensive, we cannot rule out that other than the PrtT-affected secreted proteases contribute to substrate tapping or invasion during aspergillosis. Moreover, it might well be that extracellular proteases are involved in interacting with the primary lines of immune defence, an aspect that is not covered by our study. Virulence tests in the according model using cortisone-treated animals did however not reveal any attenuation in virulence for a *prtTΔ* mutant (Nir Osherov, pers. comm.). In summary, these findings resemble the insights gained from various protease deletion mutants (15, 34, 42) and do not support a role of secreted proteases in pulmonary aspergillosis. The impact of cell wall-linked proteolytic activities, however, remains to be addressed.
One noteworthy aspect of the prtT gene lays in the fact that it is absent in the less pathogenic species A. nidulans. Quantification of extracellular proteolytic activity from a wild-type strain of A. nidulans revealed levels similar to the ones expressed by the A. fumigatus prtTΔ deletant (our unpublished observation). This indicates that A. nidulans has not evolved compensatory means, and it may be reasoned that the prtT gene loss event is evolutionary recent. It is tempting to speculate that the associated deletion event in A. nidulans might have affected its virulence potential. Our data do not support such a hypothesis, and based on the multifactorial nature of Aspergillus virulence it has to be assumed that other additional variations make the difference between the saprophyte A. nidulans and the pathogen A. fumigatus.

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REFERENCES


FIGURE LEGENDS

FIG. 1. A. fumigatus prtT encodes a conserved putative transcription factor of the Zn2Cys6-type. (A) The existence of the first annotated intron was examined by diagnostic PCRs covering the 5’ region to indicate that the prtT coding sequence is preceded by an unusually long leader sequence. The putative N-terminus based on the putative intron is depicted in gray, the actual coding sequence in black. Priming oligonucleotides used are Sv589 (1), Sv576 (2), and AB51 (3), calculated fragment sizes amplified from genomic DNA (g) or cDNA (c) are indicated on the left margin, a 100 bp DNA marker (M) was used as size standard. Results were consistent among strains ATCC 46645 and an unrelated A. fumigatus isolate (D141, data not shown). The larger amplicon with primers Sv589 and AB51 (1/3) from cDNA is most likely due to contamination with genomic DNA. (B) Global alignment of the deduced A. fumigatus PrtT sequence with fungal orthologues from A. niger (A.n.; accession number XP_001402055), A. oryzae (A.o.; BAF74781), A. clavatus (A.c.; XP_001271888), and Penicillium crysogenum (P.c.; CAP98521). Consensus residues are boxed in black, the grey bar indicates the position of the highly conserved Zn2Cys6 DNA binding domain.

FIG. 2. The prtT gene supports protein assimilation by A. fumigatus. (A) Deletion and reconstitution of the prtT locus. The genomic situation at the target locus is shown schematically for the wild-type progenitor ATCC 46645, the deletant AfS61, and the reconstituted strain AfS62. On the right hand side confirmative Southern analyses after digestion of genomic DNA with BspHI and EcoRV, respectively, and hybridisation with a 3’-specific probe (black bar) are displayed. (B) Growth tests on minimal medium supplemented with BSA ± peptone as nitrogen source. Media were point-inoculated with equal amounts of spores and incubated at 37°C to reveal a severe growth retardation of the
$prtT\Delta$ strain when undigested protein serves as nitrogen source, a phenotype that is completely reversed when hydrolysed protein such as peptone is added. (C) Growth on casein-containing medium shows the absence of a clearance halo around the growing edge of the AfS61 [$prtT\Delta$] colony and therefore deficient degradation of precipitated protein.

Inoculation legend presented on the right hand side is valid for (B) and (C).

FIG. 3. PrtT is required for expression of extracellular proteolytic activities. (A) Supernatants from cultures supplemented with various sources of nitrogen were spot on an unprocessed X-ray film to test for the presence of proteolytic activities. In the presence of the primary N-source ammonium, expression of secreted proteases that would hydrolyse the gelatin-containing light-sensitive layer is absent, but not in the presence of protein or the proteolysis product peptone. In contrast to the wild-type isolate ($wt$) and the reconstituted strain ($rec.$) the $prtT\Delta$ mutant apparently is impaired in producing extracellular proteases. Drop zones are indicated by dashed circles, untreated medium (m) was included in the spot tests. (B) Visualisation of protein degradation by SDS-PAGE after 24 and 48 hours of cultivation supports the near-complete absence of extracellular proteases in the culture supernatants containing BSA and peptone when inoculated with the $prtT\Delta$ deletion strain AfS61. (C) Quantitative assessment of extracellular proteolytic activities secreted by the wild-type isolate, the $prtT\Delta$ deletion strain and the reconstituted strain when grown in the presence of BSA and peptone reveals the almost-complete absence of extracellular proteases in the $prtT\Delta$ culture supernatant.

FIG. 4. PrtT regulates transcriptional induction of several secreted proteases. Steady state levels of transcripts from several protease-encoding genes were monitored by qRT-PCR after a shift from minimal medium containing $\text{NH}_4^+$ as nitrogen source to BSA-containing cultures.
for 2 hours. Expression rates were normalised to mRNA levels of the β-tubulin-encoding gene AFUA_1G10910 and set arbitrarily to 1 for the wild-type strain grown in minimal medium, except for expression of dppIV, which could not be detected in either strain grown in NH₄⁺-containing medium; in this particular case, the transcript level of the BSA-grown wild-type was set arbitrarily to 100.

FIG. 5. The prtTA deletant strain grows at rates comparable to the wild-type reference in the lungs of leukopenic mice. Shown are weight loss data of infected animals (A) and Cₜ values from qPCRs performed on DNA samples extracted from corresponding lung tissues (B). Similar disease progressions and quantities of fungal burden can be deduced from these data, which indicates that the deletion mutant AfS61 is not impaired in virulence in this kind of disease model.
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<th>Name</th>
<th>Description</th>
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<tr>
<td>ATCC 46645</td>
<td><em>Aspergillus fumigatus</em> wild-type strain</td>
<td>(13)</td>
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<tr>
<td>5 AIS61</td>
<td><em>prtT</em>Δ deletion mutant [<em>prtT::loxP-phleo&lt;loxP]</em></td>
<td>this study</td>
</tr>
<tr>
<td>AIS62</td>
<td>reconstituted strain [<em>prtT&lt;ptrA&gt;</em>]</td>
<td>this study</td>
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<tr>
<td>pJET1.2</td>
<td>positive selection vector, pUC19 derivative [<em>bla</em>, MCS in <em>eco47IR</em>]</td>
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<tr>
<td>pSK275</td>
<td>plasmid containing <em>ptrA</em> cassette conferring resistance towards pyrithiamine</td>
<td>(27)</td>
</tr>
<tr>
<td>pSK341</td>
<td><em>Aspergillus</em> marker cassette conferring resistance towards phleomycin [<em>gpdA::ble/tk::trpC</em>]</td>
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<td>10 pSK462</td>
<td>replacement cassette for deletion of <em>prtT</em> in <em>A. fumigatus</em></td>
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<td>pSK463</td>
<td>reconstitution cassette containing <em>prtT</em> gene coupled to <em>ptrA</em> resistance cassette</td>
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TABLE 2. Oligonucleotides used in this study

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Figure 3

A.

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B.

m | wt | rec. | prtTΔ |
---|----|------|-------|
24 h |     |      |       |
48 h |     |      |       |

C.

ext. 550 nm / g dry weight

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Figure 4
Figure 5

A. ATCC 46645 [wt]

B. C₁ values

days post infection

weight %

wt   prtTΔ