Deletion of the *Aspergillus fumigatus* Gene Encoding the Ras-Related Protein RhbA Reduces Virulence in a Model of Invasive Pulmonary Aspergillosis

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As *Aspergillus fumigatus* is the predominant mold pathogen in patients who lack functional innate immunity. The *A. fumigatus* rhbA gene was first identified as a transcript that was upregulated when the organism was grown in the presence of mammalian cells. To gain insight into the function of *rhbA* in the growth and pathogenesis of *A. fumigatus*, we constructed a strain that lacks a functional *rhbA* gene. The Δ*rhbA* mutant showed a significant reduction in virulence compared to the virulence of the wild type in a mouse model of invasive aspergillosis. Complementation of the deletion with the wild-type gene restored full virulence. Although the Δ*rhbA* mutant grew as well as the wild type on solid medium containing the rich nitrogen source ammonium, the growth of the mutant was impaired on medium containing poor nitrogen sources. Like the *Saccharomyces cerevisiae* *rhh1* mutant, the Δ*rhbA* mutant exhibited increased uptake of arginine. In addition, the Δ*rhbA* strain underwent asexual development in submerged cultures, even under ammonium-excess conditions. Growth of the mutant with poor nitrogen sources eliminated both the arginine uptake and submerged asexual development phenotypes. The mutant showed enhanced sensitivity to the TOR kinase inhibitor rapamycin. These findings establish the importance of *rhbA* for *A. fumigatus* virulence and suggest a role for *rhbA* in nutrient sensing.

The ability to adapt to changes in nutrient availability is an essential attribute of many successful pathogens. In fungi, nutrient-regulated signaling pathways have been clearly linked to pathogenesis in a number of species, but their contribution to the virulence of *Aspergillus fumigatus* is largely unexplored (14, 33). *A. fumigatus* is an opportunistic fungal pathogen that has emerged as a major cause of morbidity and mortality in patients who are neutropenic or lack intact innate immunity (21). As a saprophytic fungus, *A. fumigatus* can utilize a wide variety of carbon and nitrogen sources, and it has been hypothesized that such physiological versatility contributes to its status as the predominant mold pathogen (15).

The Rheb proteins comprise a family of Ras-related proteins that exhibit deviations from the consensus amino acid sequence in the first GTP-binding domain, as well as in the effector domain (24, 36, 41). Rheb proteins are conserved from lower eukaryotes to mammals, yet the mechanism by which they function remains elusive. In mammalian cells, Rheb interacts with Raf-1 in a cAMP-dependent manner and antagonizes Ras signaling (9, 43). In yeast, Rheb is required for the maintenance of wild-type sensitivity to the toxic arginine analog canavanine (36, 42). The canavanine hypersensitivity of *Saccharomyces cerevisiae* *rhh1* mutants is due to increased arginine uptake mediated by CAN1p, the basic amino acid transporter (36). Deletion of *rhh1* in *Schizosaccharomyces pombe* results in a phenotype that mimics nitrogen starvation, with cell cycle arrest and upregulation of *fnx1* and *mei2* (22).

The *rhbA* gene was first identified in a differential display reverse transcription-PCR screening analysis of transcripts upregulated when *A. fumigatus* was grown in cultures with human endothelial cells (26). This experimental design mimics the intimate contact between the pathogen and the endothelium during angioinvasion. The *rhbA* gene is the only Rheb homolog in the *A. fumigatus* genome, as confirmed by a search of The Institute for Genome Research genome database (http://www.tigr.org/tdb/e2k1/afu1/).

It has previously been demonstrated that nitrogen starvation in *A. fumigatus* is accompanied by increased expression of the Rheb homolog *rhhA*, suggesting that this gene plays a role in a nitrogen-regulated signaling pathway (24). Two main pathways regulating nitrogen metabolism have been identified in the fungi. Nitrogen catabolite repression (NCR) regulates the ability of an organism to discriminate between rich and poor nitrogen sources (1, 10). In the presence of a rich nitrogen source, such as ammonium, NCR prevents transcription of genes necessary for the utilization of poor nitrogen sources (34). In the presence of a poor nitrogen source, NCR is released. General control (GCN) or cross pathway control (CPC) is a means by which cells adapt to amino acid starvation, among other stresses (8, 16, 25). Many of the genes under the control of the GCN-CPC pathway encode amino acid and nucleotide biosynthetic enzymes (23).

In *S. cerevisiae*, gene regulation in response to nitrogen quality is mediated by a member of the target of rapamycin (TOR) family of PI-3 related kinases (29). In the presence of a rich nitrogen source, active TOR kinase mediates the cytoplasmic sequestration of Gln3p, a GATA-type transcription factor that is required for release of NCR (5). TOR kinases are specifically inhibited by the antibiotic rapamycin, and treatment of
growing S. cerevisiae yeasts with rapamycin results in a rapid release of NCR and cell cycle arrest (7, 35). Whereas vegetative cultures of S. pombe are not affected by treatment with rapamycin, nitrogen-limited cells become sensitive to the drug (39), suggesting that nitrogen metabolism in S. pombe is likewise regulated by TOR kinase. TOR has also been implicated in the control of GCN-CPC-regulated genes in S. cerevisiae (37). TOR-dependent regulation of either NCR or GCN-CPC has yet to be determined in any filamentous fungus.

Although nitrogen metabolism in A. fumigatus is not well characterized, A. fumigatus mutants lacking areA, the ortholog of S. cerevisiae GLN3, are unable to grow in the presence of poor nitrogen sources, suggesting that the pathways governing NCR in both A. fumigatus and S. cerevisiae are closely related (15). Moreover, areA mutants display reduced virulence in vivo, suggesting that variability in nitrogen utilization plays an important role in the pathogenesis of invasive pulmonary aspergillosis (IPA).

In order to increase our understanding of Rheb function in the fungi, we characterized an rhbA deletion mutant of A. fumigatus. In this paper, we describe construction of a strain of A. fumigatus that lacks the rhbA gene and demonstrate that there was reduced virulence of the mutant in a mouse model of IPA. Our results are consistent with a model in which the mutant is defective in nitrogen signaling and metabolic diversity is intimately linked to pathogenicity.

MATERIALS AND METHODS

Strains and media. The strain of A. fumigatus used in this study was H237, a clinical isolate with no known nutritional markers (15). Liquid cultures of H237 were grown in YG (0.5% yeast extract, 2% glucose) or Aspergillus minimal medium (11). H237 was propagated on Aspergillus minimal medium plates with 10 mM ammonium tartrate as the nitrogen source. Alternative nitrogen sources were added at a concentration of 10 mM unless otherwise specified.

Deletion of rhbA. An rhbA deletion construct was made by digesting a 2.5-kb rhbA genomic clone (GenBank accession number AF283573) in pPT7-Blue with BglII and removing a 1-kb fragment containing three of the four rhbA coding exons (5). A BamHI-XbaI fragment containing the hygromycin resistance cassette was removed from pMAD91 (40), blunted, and then ligated into the BglII-digested, filled-in-rhba genomic clone. Sequencing revealed that the hygromycin resistance cassette is in the inverse orientation to the rhbA gene. The rhbA deletion cassette was excised with BamHI and HindIII and was introduced into H237 by protoplast transformation as previously described (44). Hygromycin-resistant colonies were screened for homologous recombination by PCR by using primers that amplify the entire genomic clone. The 5′-GAGGCAAAAGGACGTCGCAAACGG primer sequence was 5′-GGTGCAAATCGCTTCCGCTGG-3′. Homologous recombination at 5′-GAGGCAAAAGGACGTCGCAAACGG was assessed by probing a Southern blot with exon 1 (data not shown). One putative homologous recombination was identified and confirmed by probing a Southern blot with exon 1, taking advantage of an EcoRI site present in the hygromycin resistance gene (Fig. 1B). The blot was also probed with a fragment of the hygromycin resistance gene and the pT7-Blue backbone to ensure that there was a single integration of the construct at the rhbA locus (data not shown).

To demonstrate that any phenotype seen in the ΔrhbA strain

RESULTS

rhbA gene is not essential in A. fumigatus. To determine if the rhbA gene is essential in A. fumigatus, a deletion construct that replaces three of the four rhbA coding exons with the hygromycin resistance gene was designed and introduced into A. fumigatus protoplasts (Fig. 1A). Hygromycin-resistant colonies were screened for homologous recombination by PCR (data not shown). One putative homologous recombination was identified and confirmed by probing a Southern blot with exon 1, taking advantage of an EcoRI site present in the hygromycin resistance gene (Fig. 1B). The blot was also probed with a fragment of the hygromycin resistance gene and the pT7-Blue backbone to ensure that there was a single integration of the construct at the rhbA locus (data not shown).
was due to deletion of \textit{rhbA} alone, a complemented strain was constructed by introducing the complementation construct into protoplasts of the \textit{H9004 rhbA} strain and selecting for phleomycin-resistant colonies. Single-locus integration of the complementation construct was confirmed by Southern blot probing with exon 1 (Fig. 1B).

\textit{A. fumigatus rhbA} is a virulence-related gene. To determine the effect of \textit{rhbA} deletion on the virulence of \textit{A. fumigatus}, \(5 \times 10^5\) conidia of the wild type, the \textit{rhbA} deletion strain, or the reconstituted strain were used to inoculate immunosuppressed mice, and mortality was monitored for 14 days. The mice inoculated with the \textit{H9004 rhbA} strain survived significantly longer than the mice infected with the wild type or the reconstituted strain \((P < 0.05)\) (Fig. 2). The same experiment repeated by using an inoculum containing 50-fold-fewer conidia again resulted in increased survival of mice inoculated with the \textit{ΔrhitA} strain compared to the survival of the mice inoculated with either the wild type or the reconstituted strain (data not shown). Introduction of the complementation construct into the \textit{ΔrhitA} strain restored wild-type virulence.

In a separate experiment, mice inoculated with each strain of the isogenic set were sacrificed on day 7 postinoculation, and the area of individual pulmonary lesions was measured in silver-stained sections by quantitative image analysis. The resulting measurements were used to calculate the average lesion area and the fungal burden for each strain of the isogenic set. The lesion area in mice inoculated with the \textit{H9004 rhbA} strain was \(496 \pm 82 \, \mu m^2 \) (\(n = 48\)), compared with values for mice inoculated with the wild type of \(1,921 \pm 457 \, \mu m^2 \) (\(n = 8\)) and values for mice inoculated with the reconstituted strain of \(1,564 \pm 311 \, \mu m^2 \) (\(n = 13\)) (mean ± standard error of the mean; \(n\) is the number of lesions in a section) \((P < 0.001)\). The lesions seen in mice inoculated with either the wild-type or the complemented strain were few and large, whereas those seen in mice inoculated with the \textit{ΔrhitA} strain were numerous and small. Since the number of lesions was greater in the \textit{ΔrhitA} strain-inoculated mouse sections than in either the wild-type or complemented strain-inoculated mouse sections, the percentage of total sectional area that contained lesions was calculated as a measure of fungal burden. The percentage of lesion area

![Diagram](http://iai.asm.org/)

**FIG. 1.** (A) Genomic organization of the \textit{rhitA} gene (top) and the \textit{rhitA} deletion construct (bottom). The \textit{rhitA} deletion construct was built by replacing the \textit{BglII} fragment of the \textit{rhitA} genomic clone with the hygromycin resistance gene. The restriction sites include \textit{EcoRI} (E), \textit{BglII} (B), \textit{SacI} (S), and \textit{KpnI} (K) sites. Numbers indicate the four coding exons. (B) Southern blot of genomic DNA from the wild type (\textit{rhitA}), the \textit{ΔrhitA} strain (\textit{ΔrhitA}), and the reconstituted strain (\textit{ΔrhitA} + \textit{rhitA}) cut with \textit{EcoRI} and probed with an \textit{rhitA} exon 1 probe. Homologous recombination of the deletion construct at the \textit{rhitA} locus results in the introduction of an \textit{EcoRI} site that reduces the size of the hybridizing band. Introduction of the complementation construct at a nonhomologous locus results in maintenance of the \textit{ΔrhitA} band and the appearance of a larger band corresponding to the integrated complementation construct.
in the ΔrhdA strain-inoculated mouse sections was less than that in sections from mice inoculated with the wild-type or complemented strain, but the difference was not significant due to high variation between mice (data not shown). Although there was a difference in average lesion area between strains, all strains had the ability to invade surrounding tissue (Fig. 3). Whereas rhdA is not required for invasive growth, the in vivo growth potential of the ΔrhdA strain is reduced. Taken together, these data support the hypothesis that rhdA plays a role in the pathogenesis of IPA.

**rhdA deletion mutant exhibits a reduced growth rate on poor nitrogen sources.** To determine if the reduced growth potential seen in mouse lungs was limited to in vivo growth, the in vitro growth rates of the isogenic set were measured on various solid media. The rhdA deletion mutant exhibited a significantly reduced growth rate on minimal medium containing the poor nitrogen sources proline, histidine, and nitrate compared to the growth rate of either the wild type or the reconstituted deletion strain (Fig. 4). On minimal medium containing the rich nitrogen source ammonium, however, the ΔrhdA strain grew at a rate similar to the growth rates of the wild type and the reconstituted mutant. Similarly, the ΔrhdA strain exhibited the wild-type growth rate, 0.082 ± 0.001 cm h⁻¹, compared with a growth rate of 0.086 ± 0.001 cm h⁻¹ for the mutant, on Sabouraud dextrose agar, a medium containing a pancreatic digest of casein and neopeptone as sources of nitrogen.

**Deletion of rhdA results in increased uptake of arginine that is nitrogen source dependent.** In *S. cerevisiae*, deletion of RHB1 results in hypersensitivity to the toxic arginine analog canavanine that is due to increased arginine uptake (36). It has previously been demonstrated that expression of *A. fumigatus* rhdA is able to complement the canavanine hypersensitivity of an *S. cerevisiae* rhd1 mutant, suggesting that these genes have overlapping functions (24). To determine if the ΔrhdA strain might exhibit a phenotype similar to that of the *S. cerevisiae* rhd1 mutant, we assessed the pattern of uptake of arginine in the isogenic set. In minimal medium containing ammonium, the slope of the line generated by plotting the amount of arginine taken up per milligram (dry weight) versus time was greatly increased in the ΔrhdA strain (Fig. 5A). Calculation of the initial velocities from the plots revealed that the ΔrhdA strain exhibited a nearly threefold-higher initial velocity of arginine uptake than either the wild type or the complemented strain (data not shown). The magnitude of this result is similar to that seen in the *S. cerevisiae* rhd1 mutant. In the presence of a poor nitrogen source (10 mM sodium nitrate or 10 mM proline), however, the rates of arginine uptake were similar for all members of the isogenic set (Fig. 5B).

![FIG. 3. Similar-size lesions from lung sections of mice inoculated with the isogenic set. (A) Wild type; (B) ΔrhdA mutant; (C) reconstituted strain. The sections were stained with silver stain.](http://iai.asm.org/)

![FIG. 2. Cumulative mortality of immunosuppressed mice inoculated intranasally at zero time with 5 × 10⁶ conidia of the wild type (rhdA), the ΔrhdA strain (ΔrhdA), or the reconstituted strain (ΔrhdA + rhdA). The asterisk indicates that the P value is <0.05.](http://iai.asm.org/)
Rhba strain undergoes asexual development in submerged culture that is nitrogen source dependent. Asexual development in Aspergillus species is dependent on several factors, including the acquisition of competence and the presence of an air-water interface (2). Under nutrient deprivation conditions, asexual development can be induced in a submerged culture (31). After 18 to 24 h of growth in minimal medium containing 10 to 40 mM ammonium tartrate, gross observation of cultures of the Δrhba mutant revealed the production of a vibrant green pigment. Microscopic observation of the cultures revealed the presence of conidiophores and masses of dark green conidia (Fig. 6A). After a similar amount of time in rich medium (YG) or minimal medium with a poor-quality nitrogen source (10 mM sodium nitrate or 10 mM proline), the hyphae appeared normal with no pigment or signs of development (swollen vesicles or conidiophores) (Fig. 6C).

Δrhba strain is hypersensitive to the TOR kinase inhibitor rapamycin. TOR kinase regulates the response to nitrogen availability in both S. cerevisiae and S. pombe (7, 19). To determine if the rhba deletion phenotype might be due to aberrations in TOR kinase signaling, the sensitivity of the isogenic set to rapamycin was assessed (Fig. 7). The growth rate of the rhba deletion strain was less than one-half that of the wild type or the reconstituted strain when the organisms were grown in

![Fig. 4](image-url)  
FIG. 4. Radial growth rates of the wild type (rhba) (closed bar), the Δrhba strain (Δrhba) (open bar), and the reconstituted strain (Δrhba + rhba) (gray bar) on Aspergillus minimal medium with various nitrogen sources at a concentration of 10 mM. NT, ammonium tartrate; NO3, sodium nitrate; HIS, histidine; PRO, proline. One asterisk indicates that the P value is <0.05; two asterisks indicate that the P value is <0.001.

![Fig. 5](image-url)  
FIG. 5. (A) L-[U-14C]arginine uptake in germlings of the wild type (rhba), the Δrhba strain (Δrhba), and the complemented strain (Δrhba + rhba) after incubation for 1 h in fresh minimal medium with 10 mM ammonium tartrate as the nitrogen source. (B) L-[U-14C]arginine uptake in germlings of the wild type, the Δrhba strain, and the complemented strain after incubation for 1 h in fresh minimal medium with 10 mM proline as the nitrogen source. Each graph is representative of several experiments.
the presence of rapamycin on ammonium-containing medium. The magnitude of growth inhibition seen on medium containing poor nitrogen sources was similar to that on ammonium, suggesting that rapamycin sensitivity cannot be ablated by the release of NCR (data not shown). This hypersensitivity to rapamycin is compatible with the explanation that loss of \( \text{rhhA} \) function may alter signaling through a TOR kinase pathway.

**DISCUSSION**

Rheb family members have been identified in multiple eukaryotic species, but their precise function is unclear. In the mammalian system, Rheb has been reported to antagonize Ras signaling and to be affected by the activation state of the cAMP-protein kinase A signaling pathway (41, 43). In the two yeast systems in which Rheb has been studied, \( \text{S. cerevisiae} \) and \( \text{S. pombe} \), functional Rheb is required for maintenance of wild-type sensitivity to the arginine analog canavanine (36, 42).

Immunosuppressed mice inoculated with the \( \Delta \text{rhhA} \) strain survived significantly longer than immunosuppressed mice inoculated with the wild type, implicating \( \text{rhhA} \) in aspects of the pathogenic process. The reduced average lesion area seen in mouse lungs inoculated with the \( \Delta \text{rhhA} \) strain suggests that the mutant has a reduced ability to grow in vivo. On solid medium containing poor-quality nitrogen, the mutant also exhibited a

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**FIG. 6.** Photomicrographs of the wild type (B and D) and the \( \Delta \text{rhhA} \) strain (A and C) taken from submerged cultures after 24 h of incubation in minimal medium containing 40 mM ammonium tartrate (A and B) or 40 mM sodium nitrate (C and D) as the nitrogen source.

**FIG. 7.** (A) Representative plates showing the wild type (\( \text{rhhA} \)), the \( \Delta \text{rhhA} \) strain (\( \Delta \text{rhhA} \)), and the reconstituted strain (\( \Delta \text{rhhA} + \text{rhhA} \)) after 48 h of growth on \( \text{Aspergillus} \) minimal medium with (NT-R) and without (NT) 5 ng of rapamycin ml\(^{-1}\). (B) Radial growth rate of the wild type (black bar), the \( \Delta \text{rhhA} \) strain (open bar), and the reconstituted strain (gray bar) on \( \text{Aspergillus} \) minimal medium with (NT-R) and without (NT) 5 ng of rapamycin ml\(^{-1}\). Ammonium tartrate was the nitrogen source. The asterisk indicates that the \( P \) value is <0.005.
lower growth rate. The in vivo reduction in lesion area may result from a decreased ability of the organism to utilize available nutrients. The reduced virulence of the *A. fumigatus* *arcA* mutant, which is severely impaired in utilization of poor nitrogen sources, also supports the hypothesis that nutritional versatility plays a role in pathogenesis (15).

The *rhhA* transcript was first identified in a differential display reverse transcription-PCR screening analysis for transcripts upregulated during coculture with human endothelial cells (26). It was proposed that transcripts obtained from this analysis were candidates for virulence-related genes, as intimate contact with endothelial cells is a prerequisite for disseminated disease. By demonstrating that *rhhA* contributes to the virulence of the organism, we validated the model in which the transcript was identified, prompting further study of the genes encoding other upregulated transcripts.

As an aid to understanding the function of *rhhA* in *A. fumigatus*, we are able to draw upon the data generated by Rheb deletion in the model yeasts *S. cerevisiae* and *S. pombe*. The *S. cerevisiae* *rhh1* mutant exhibits increased uptake of arginine that is complemented by expression of both the *S. pombe rhh1* gene and the *A. fumigatus rhhA* cDNA (24, 36). The *S. pombe rhh1* mutant displays a phenotype similar to that seen when the organism is nitrogen starved (22).

Like the *S. cerevisiae rhh1* mutant, the Δ*rhhA* strain exhibited increased uptake of arginine when it was grown in the presence of ammonium, corroborating the hypothesis that these two genes have overlapping functions. The uptake phenotype, however, was reversed by growth on poor-quality nitrogen. In the *S. cerevisiae* *rhh1* mutant, transcription of arginine permease was reported to be unaltered compared to transcription in the wild type, suggesting that translation, transporter activity, or localization, rather than transcription, is the cause of the increased arginine uptake (36). It is possible that release of NCR by the presence of a poor nitrogen source exerts overriding regulation on the function of the arginine permease in *A. fumigatus*.

Deletion of *rhh1* from *S. pombe* results in cell cycle arrest and accumulation of *fnx1* and *mei2* mRNA (22). The response of *A. fumigatus* to nitrogen starvation has not been explored; however, it has been reported that wild-type *A. nidulans* can be induced to undergo asexual sporulation in submerged culture in response to either carbon or nitrogen starvation (31). The asexual development in submerged culture seen in the Δ*rhhA* strain may indicate that the mutant is inappropriately initiating a starvation response similar to that seen in *S. pombe rhh1* mutants. Growth in medium containing poor-quality nitrogen ablated the development aspect of the Δ*rhhA* phenotype. These data are consistent with a model in which release of NCR could override the starvation response pathway. Furthermore, the fungal lesions in the lungs of mice inoculated with the Δ*rhhA* strain showed no indication of asexual development, which is consistent with the hypothesis that in vivo growth involves a response to poor-quality nitrogen.

In both *S. cerevisiae* and *S. pombe*, the response to nitrogen limitation is regulated by the activity of TOR kinase in *S. cerevisiae*. TOR kinase activity is decreased in response to the absence of rich nitrogen, allowing the release of NCR (7, 35). TOR also affects translation initiation, the stability of nutrient transporters, and the autophagic response (4, 18, 27). In contrast, TOR activity is required for cell cycle arrest in response to nitrogen starvation in *S. pombe* (39).

Altered sensitivity to rapamycin is a hallmark phenotype of defective TOR kinase signaling in *S. cerevisiae*, as evidenced by deletion or mutation of numerous TOR effectors (5, 12, 17, 38). Treatment of *S. cerevisiae* yeasts with rapamycin leads to rapid release of NCR, whereas the phenotypic consequences of treating the *S. pombe* yeasts with rapamycin are evident only under nitrogen-limited conditions (39). As with deletion of *rhhA* in *A. fumigatus*, deletion of *S. cerevisiae* *RHH1* also results in rapamycin hypersensitivity (Panepinto, unpublished data), supporting the assertion that the Δ*rhhA* phenotype may result from an aberrance in TOR-dependent nutrient signaling.

The ability to adapt to changes in nutrient availability is an important attribute of pathogenic microorganisms. Preventing *Histoplasma capsulatum* from acquiring calcium by deletion of CBP1 results in an avirulent phenotype (28). Mutants of *A. fumigatus* that are auxotrophic for para-aminobenzoic acid are also avirulent (6), as are uracil auxotrophs of *Candida albicans* and *A. fumigatus* (13, 20). The decreased virulence of both the Δ*rhhA* and Δ*arcA* mutants suggests that versatility in nitrogen utilization contributes to the growth of *A. fumigatus* in vivo. Understanding the signaling pathways regulating the metabolic versatility of *A. fumigatus* may uncover additional genes that are important for the pathogenesis of invasive aspergillosis.

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