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Disruption of the *Aspergillus fumigatus* Gene Encoding Nucleolar Protein CgrA Impairs Thermotolerant Growth and Reduces Virulence

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***Aspergillus fumigatus* CgrA is the ortholog of a yeast nucleolar protein that functions in ribosome synthesis. To determine how CgrA contributes to the virulence of *A. fumigatus*, a Δ*cgrA* mutant was constructed by targeted gene disruption, and the mutant was reconstituted to wild type by homologous introduction of a functional *cgrA* gene. The Δ*cgrA* mutant had the same growth rate as the wild type at room temperature. However, when the cultures were incubated at 37°C, a condition that increased the growth rate of the wild-type and reconstituted strains approximately threefold, the Δ*cgrA* mutant was unable to increase its growth rate. The absence of *cgrA* function caused a delay in both the onset and rate of germination at 37°C but had little effect on germination at room temperature. The Δ*cgrA* mutant was significantly less virulent than the wild-type or reconstituted strain in immunosuppressed mice and was associated with smaller fungal colonies in lung tissue. However, this difference was less pronounced in a *Drosophila* infection model at 25°C, which correlated with the comparable growth rates of the two strains at this temperature. To determine the intracellular localization of CgrA, the protein was tagged at the C terminus with green fluorescent protein, and costaining with propidium iodide revealed a predominantly nucleolar localization of the fusion protein in living hyphae. Together, these findings establish the intracellular localization of CgrA in *A. fumigatus* and demonstrate that *cgrA* is required for thermotolerant growth and wild-type virulence of the organism.**

Aspergillus fumigatus is a saprophytic filamentous fungus that inhabits soil, water, and organic debris, where it has an essential role in the recycling of carbon and nitrogen (37). The organism propagates itself by the release into the air of high concentrations of asexual spores (conidia), which are unavoidably inhaled on a daily basis (26, 46). Since the conidia are efficiently cleared by normal defenses, their inhalation is of minor consequence to healthy individuals. However, in the absence of adequate host immunity, the conidia germinate into highly invasive hyphae that cause severe lung damage and eventually disseminate to other organs. Patients with depressed immunity are at increased risk for infection with *A. fumigatus*, and the prognosis for invasive disease is very poor in these individuals (40). Of particular concern is the rising incidence of aspergillosis, a situation that has arisen as a consequence of aggressive cancer treatments and the widespread use of potent immunosuppressive regimens that support organ transplantation (33, 44, 54, 58).

Since *A. fumigatus* conidia are no more prevalent in the environment than the spores of some nonpathogenic molds (34), it is generally assumed that the organism has unique features that allow it to survive in humans, and thermotolerance has long been suspected to play a role (37). As a major component of the biomass in a self-heating compost pile, *A. fumigatus* has evolved mechanisms that allow it to grow well at 37°C and to tolerate temperatures approaching 60°C (6, 63). This is in striking contrast to most other common environmental molds with temperature optima in the range of 25 to 35°C (15). The ability to grow at 37°C is shared by all successful human pathogens and is a feature that has been shown to correlate with virulence potential (11, 27). Conversely, failure to tolerate 37°C is a strong indication that a fungus is unlikely to cause deep infections, and mutations that reduce thermotolerance in fungi are associated with attenuated virulence (2, 36, 51). However, the mechanisms by which these mutations affect thermotolerance are poorly understood, and since mutations that impair growth at high temperature have not been described in *A. fumigatus*, this has been a difficult question to address.

In this study, we demonstrate that the ability to grow rapidly at 37°C and the expression of wild-type (wt) virulence require the activity of CgrA, a protein that was previously shown to be the ortholog of a yeast nucleolar protein that has a role in pre-rRNA processing and 60S ribosomal subunit synthesis (8, 48).

MATERIALS AND METHODS

Culture conditions. wt strain H237 is a clinical isolate. Conidia were harvested from strains grown on *Aspergillus* minimal medium plates. Liquid cultures were grown in YG (0.5% yeast extract, 2% glucose) or *Aspergillus* minimal medium (16) as indicated. To measure growth rates, 5,000 conidia were spotted onto the center of a plate of minimal medium, and the change in the diameter of the colony with time was used as a relative indicator of the growth rate. In some experiments, hygromycin (Invivogen, San Diego, Calif.) was incorporated into

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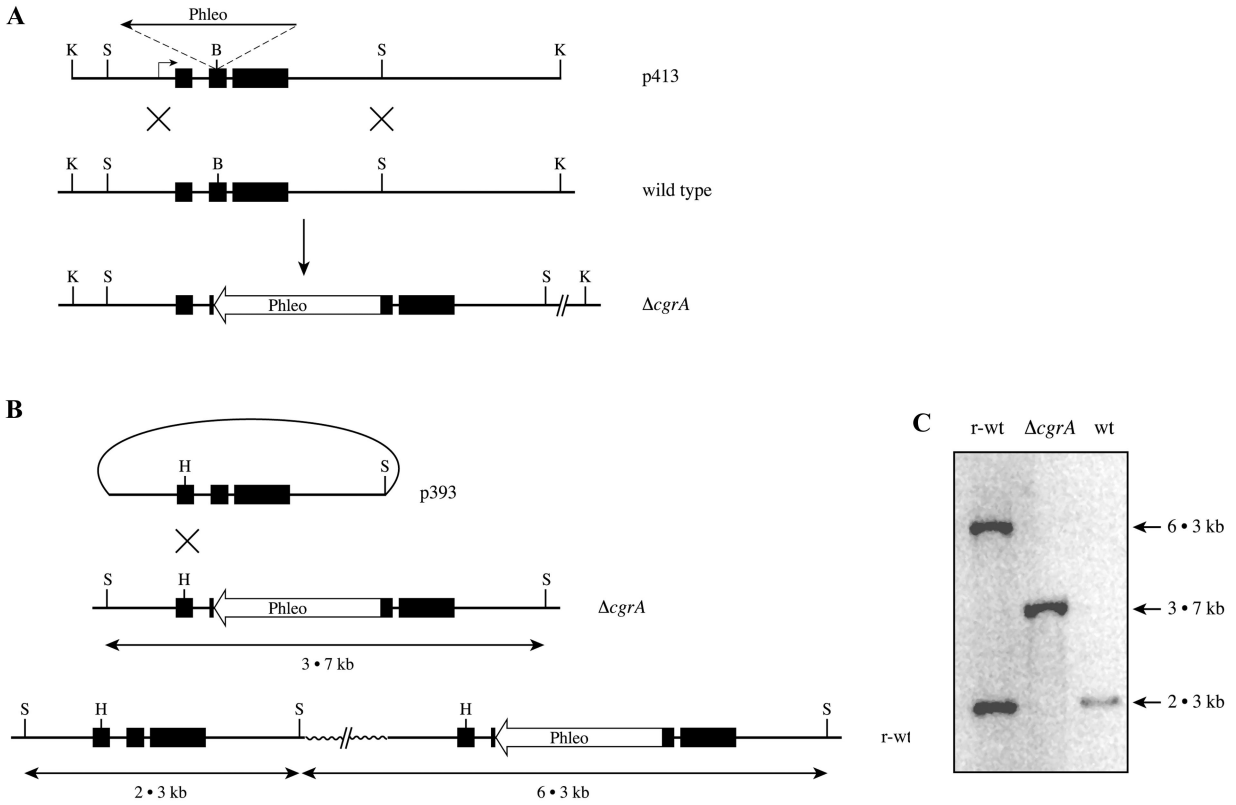

FIG. 1. Disruption and reconstitution of the *cgrA* gene in *A. fumigatus*. (A) Schematic representation of the predicted Δ*cgrA* mutation (downward arrow) resulting from homologous recombination (×) between the plasmid containing a disrupted *cgrA* allele (p413) and the wt *cgrA* locus. The solid boxes represent exons of the *cgrA* gene, and the horizontal arrows indicate the transcriptional orientations of *phleo* and *cgrA*. The restriction sites are KpnI (K), SstI (S), Bsu36I (B), and HincII (H). (B) Schematic representation of the predicted reconstitution of *cgrA* (r-wt) in the Δ*cgrA* mutant, resulting from a single crossover between plasmid p393 (linearized at the HincII site) and the Δ*cgrA* mutant allele. (C) Southern blot analysis of SstI-digested genomic DNA from the wt, Δ*cgrA*, and r-wt strains using the 2.3-kb SstI fragment containing the *cgrA* gene as a probe.

radial-growth plates to compare strains for their sensitivity to this antibiotic. All radial-growth rates were calculated from the exponential part of the growth curves. For measurements of germination rates, conidia were inoculated into suspension cultures of YG medium, and the number of swollen conidia initiating a germ tube was scored microscopically with time.

Molecular cloning of the *A. fumigatus cgrA* gene. A cosmid library constructed from *A. fumigatus* isolate H237 in pWEB (52) was screened with a full-length *cgrA* cDNA probe from *A. fumigatus* (8), and one positive clone was isolated. The hybridization was mapped to a single 4-kb KpnI fragment in the cosmid, and this fragment was subcloned into pBluescript to make p383. Sequence analysis confirmed the presence of the *cgrA* gene. The hybridization and wash conditions used for library screening were as previously described (8). A comparison of the *A. fumigatus cgrA* cDNA sequence (8) with the genome sequence identified three exons with consensus fungal splice sites (5, 60): 5′(TG/GT). . .3′(CAG/G) (first intron) and 5′(AG/GT). . .3′(CAG/G) (second intron). The locations of the exons in the cDNA sequence are indicated in the GenBank sequence AY008837.

Genomic Southern blot analysis. Overnight cultures of *A. fumigatus* were frozen in liquid nitrogen, and genomic DNA was extracted from the crushed mycelial pellets by proteinase K digestion and phenol-chloroform extraction. Restriction digests of genomic DNA were fractionated on a 1% agarose gel and transferred to a charged nylon membrane (MSI, Inc., Westborough, Mass.). Filters were hybridized to a ^{32}P-labeled random-primed *cgrA* DNA probe and washed under stringent conditions, as previously described (4).

Disruption and reconstitution of *A. fumigatus cgrA*. To construct the *cgrA* disruption plasmid, a phleomycin resistance expression cassette containing the *Aspergillus nidulans gpdA* promoter, the *Streptoalloteichus hindustanus ble* gene encoding resistance to phleomycin, and the *Saccharomyces cerevisiae CYC1* terminator was amplified from pBCphleo (Fungal Genetics Stock Center) using the forward primer 5′-GAGATACTCCCTCAGGAATTAAAGCCTTCGAGCG TCCC and the reverse primer 5′-TATAAGAATCCTCAGGCGGAGAATATG GAGCTTCATCG (Bsu36I cloning sites in the oligonucleotides are underlined). The 1.4-kb PCR product was inserted into the 4-kb KpnI *cgrA* genomic subclone (p383) at the single Bsu36I site in exon 2 to create plasmid p413 (Fig. 1). The *cgrA* disruption cassette was excised from p413 by digestion with KpnI and introduced into *A. fumigatus* protoplasts using the following modification of the protocol from Oakley et al. (50). Conidia (10^9) were inoculated into 400 ml of YG medium and incubated for 14 h at 30°C at 200 rpm. After being washed in YG medium, 10 ml of the pellet was resuspended with an equal volume of 2× protoplasting solution prepared as follows: 200 mg of driselase (Interspex Products Inc., San Mateo, Calif.) was added to 10 ml of KCl-CA solution (1.1 M KCl–0.1 M citric acid adjusted to pH 5.8 with KOH) and incubated on ice for 30 min, and the starch carrier was removed by centrifuging it for 5 min at 3,600 × *g*; the clarified driselase solution was then added to an equal volume of KCl-CA solution containing 400 mg of bovine serum albumin (Sigma), 3,250 U of lyticase (Sigma), and 320 mg of β-D-glucanase (Interspex) and filter sterilized through a 0.45-μm-pore-size polyvinylidene difluoride membrane (Steri-Flip; Millipore). The hyphae were incubated in the protoplasting solution for 1 h at 30°C with gentle agitation, and the protoplasts were pelleted and resuspended in 20 ml of cold $MgSO_4$ solution (1.2 M $MgSO_4$–10 mM potassium phosphate buffer) before being overlaid in 5-ml aliquots onto an equal volume of protoplast-trapping solution (0.6 M sorbitol–100 mM Tris-HCl, pH 7.0). After being spun at 460 × *g* for 15 min at 4°C, the protoplasts were removed from the interface, washed once in MSC buffer (1 M sorbitol, 10 mM MOPS [morpholinepropanesulfonic acid], pH 6.5, 10 mM $CaCl_2$), and resuspended at 5×10^7/ml in MSC. For transformation, 200 μl of protoplasts was mixed with 10 μg of DNA and 50 μl of 60% polyethylene glycol 4000 (Fluka). After being incubated on ice for 30 min, an additional 500 μl of polyethylene glycol solution was added, mixed gently, and incubated at room temperature for 20 min. The transformed protoplasts were collected by microcentrifugation for 5 min, resuspended in 1,000 μl of MSC, and spread onto plates containing 20 ml of minimal medium supplemented with 1 M sorbitol. After being incubated at room temperature overnight, each plate was overlaid with 10 ml of minimal medium top agar containing 0.5% agar and 3.75

mg of phleomycin (Invivogen). Colonies arising on these primary plates were transferred onto secondary plates containing phleomycin, and conidia from the secondary plates were replated onto selective medium at low density to isolate colonies derived from single conidia. All subsequent experiments were performed on monoconidial isolates that were genotyped by Southern blot analysis.

To reconstitute the *cgrA* gene at its native locus, Δ*cgrA* protoplasts were transformed with a plasmid containing the entire *cgrA* gene in a 2.3-kb genomic fragment that was linearized at the HincII site in exon 1 (Fig. 1). Southern blot analysis was used to identify homologous reconstitution of the wt *cgrA* gene.

Intracellular localization of CgrA. Plasmid pgGFP contains the plant-adapted green fluorescent protein (GFP) driven by the *A. nidulans gpdA* promoter and has been used to follow GFP expression in *Cochliobolus heterostrophus* (42). A *cgrA-gfp* fusion gene was created in this vector by inserting the *cgrA* open reading frame (ORF) into the NcoI site located at the ATG codon for GFP. To do this, a second NcoI site in the *hph* gene was first disrupted by site-directed mutagenesis using the plus-strand oligonucleotide 5′-GCCGGTCGCGGAGGCTGATG GATGCGATCGCTG and the minus-strand oligonucleotide 5′-CAGCGATCG CATCCATAGCCTCCGCGACCGGC, creating the plasmid pgGFPΔNcoI. The *A. fumigatus cgrA* ORF was then PCR amplified from a full-length cDNA clone (8) using the forward primer 5′-CATGCCATGGTGTGATGGCA**ATG**TCGTC GGCAATCCC and the reverse primer 5′-CATGCCATGGCCGAGTGAAGT AACTTATTACGC (the ATG for the *cgrA* ORF is in boldface, and NcoI cloning sites are underlined). The PCR product was ligated into the remaining NcoI site in pgGFPΔNcoI, and the correct orientation was confirmed by sequence analysis. The resulting construct, p*cgrA*-gfp, was introduced into *A. fumigatus* protoplasts and plated on minimal medium that was stabilized by the addition of 1 M sorbitol. After overnight incubation at room temperature, each plate was overlaid with 10 ml of minimal medium containing 0.5% agar and 8 mg of hygromycin (Invivogen) to select for stable transformants.

The intracellular localization of the expressed fusion protein was visualized by live-cell imaging with a 63× oil objective on a Zeiss LSM-510 laser-scanning confocal microscope. The laser source was an argon laser set to 488 nm for GFP detection or a helium-neon laser set to 543 nm for propidium iodide (PI). For costaining with PI, coverslips containing germlings were fixed for 3 min at room temperature in a solution containing 3.7% formaldehyde, 0.2% Triton X-100, and 50 mM phosphate buffer, pH 7. The slides were then treated with a 10-mg/ml solution of RNase A (Sigma) for 1 h at 37°C and stained with a solution containing 12.5 μg of PI/ml.

Animal models. Mice were immunosuppressed with a single dose of the glucocorticoid triamcinolone acetonide (40 mg/kg of body weight injected subcutaneously in the nape of the neck on day −1) (14, 59) and a single dose of cyclophosphamide (150 mg/kg injected intraperitoneally on day −3) (12, 18, 19, 24, 30, 47). The mice were anesthetized with 3.5% isofluorane and inoculated intranasally with 10^5 conidia on day 0, and mortality was monitored for the next 14 days. Mice that appeared moribund were sacrificed by CO_2 inhalation. The lungs, kidneys, and brains from all mice that died, and from all mice remaining at the end of the experiment, were plated onto inhibitory mold agar, and genotypes were confirmed by genomic Southern blot analysis.

For experiments involving *Drosophila*, groups of 40 to 70 adult *spz* flies were infected with ~1,500 conidia by pricking them with a needle dipped in a conidial suspension. Confirmation of the inoculum size was obtained by plating dilutions of a homogenate of five flies. All dead flies were plated, and fungal outgrowth was used as a qualitative indicator of fungal infection. Oregon-R flies were used as the standard wt strain, and adult *spz* mutants were obtained by crossing flies with two null alleles of *spz* (spz^{rm7} and spz^{J97}; Bloomington Stock Center). The stocks and crosses were maintained on a standard cornmeal medium, and all experiments were performed at 25°C.

Statistics. Statistical significance for the mouse virulence experiment was assessed by Kruskall-Wallis analysis (analysis of variance on ranks), and pairwise analysis was performed post hoc by Dunn's procedure. *Drosophila* survival data were analyzed by the Mann-Whitney rank sum test. A test comparison with a P value of <0.05 was considered to be significant.

RESULTS

Disruption and reconstitution of the *A. fumigatus cgrA* gene. To create a strain of *A. fumigatus* that lacked *cgrA* function, a disruption plasmid was constructed by inserting the phleomycin resistance gene into exon 2 of the *cgrA* gene in a 4-kb KpnI genomic fragment (Fig. 1A, plasmid p413). This construction placed the phleomycin gene upstream of 70% of the coding region of *cgrA*. The entire disruption cassette was excised with KpnI and used to transform wt *A. fumigatus* protoplasts. Phleomycin-resistant colonies were genotyped by genomic Southern blot analysis, using the 2.3-kb SstI fragment that contains the entire *cgrA* gene as a probe (Fig. 1C). Homologous integrants were identified by the loss of the 2.3-kb wt SstI fragment and the appearance of the expected 3.7-kb SstI fragment (Fig. 1C). Single-copy integrants into the *cgrA* locus were confirmed by genomic Southern blot analysis using at least one other enzyme and probing with a genomic fragment located outside of the disruption cassette and a probe containing the phleomycin resistance gene (data not shown). The efficiencies of homologous recombination with this disruption cassette were 19, 14, and 34% in three separate transformations.

To demonstrate that any phenotype attributed to homologous targeting of *cgrA* was specifically due to loss of *cgrA*, a single-crossover strategy was used to reconstitute the wt *cgrA* gene at its native locus in the Δ*cgrA* mutant. A plasmid containing the entire *cgrA* gene but lacking the 5′ SstI site (Fig. 1B; p393) was linearized at the HincII site in the first exon of *cgrA* (Fig. 1B) and then used to transform Δ*cgrA* protoplasts. Since the phenotype of the Δ*cgrA* mutant was impaired growth at high temperature (see below), a temperature selection was performed to identify homologous reconstitution of *cgrA*. The transformed protoplasts were first allowed to recover for 40 h at room temperature before they were placed at 42°C. The next day, several hundred colonies were morphologically evident on plates containing protoplasts that were transformed with p393, but no colonies were evident on plates transformed with no DNA. Transformants were genotyped by genomic Southern blot analysis, and homologous reconstitution of the *cgrA* gene was identified by the regeneration of the wt 2.3-kb SstI fragment and the appearance of a 6.3-kb SstI fragment comprised of plasmid sequences linked to the original disrupted allele (Fig. 1B and C). Approximately 50% of the transformants tested had homologous reconstitution of the *cgrA* gene. The reconstituted strain was designated r-wt.

Loss of CgrA impairs hyphal growth of *A. fumigatus* at elevated temperature. *A. fumigatus* is a typical filamentous fungus that expands radially at a linear rate, a feature that allows invasion into unexploited substrate (55). To determine how loss of *cgrA* would impact hyphal growth, the colony morphology of the Δ*cgrA* mutant was compared to that of the wt and r-wt. After 7 days of growth at room temperature (22°C), all three strains achieved similar sizes, although the Δ*cgrA* colony was slightly smaller and had fewer conidia (Fig. 2A). At 37°C, both the wt and r-wt strains reached the edge of the plate within 3 days and were covered with conidia. At the same time point, the Δ*cgrA* colony was much smaller and lacked conidia (Fig. 2A), but it was able to continue to grow at a reduced rate.

The radial-growth rates of the three strains were compared by plotting the changes in colony diameter with time. The wt and r-wt strains had indistinguishable growth rates at room temperature, expanding radially by 6.6 mm/day (Fig. 2B). The Δ*cgrA* mutant showed a slightly extended lag period relative to the wt at this temperature, but once radial growth was established, the extension rate of the Δ*cgrA* mutant was similar to that of the wt (Fig. 2B). The slight delay in conidiation observed in the Δ*cgrA* mutant at room temperature (Fig. 2A) may be a consequence of this extended lag period.

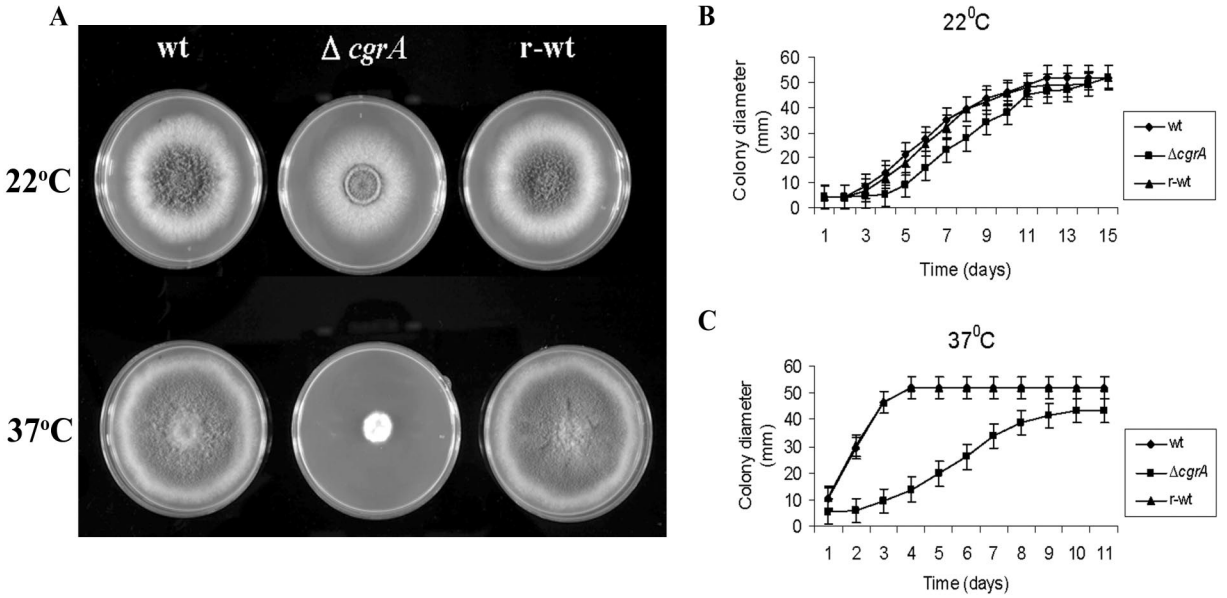

FIG. 2. Impaired radial-growth rate of the Δ*cgrA* mutant. (A) Colony morphologies of the wt, Δ*cgrA*, and r-wt strains after 7 days of growth at room temperature (22°C) or 3 days of growth at 37°C. (B and C) Conidia from the wt, Δ*cgrA*, and r-wt strains were spotted into the center of a plate of *Aspergillus* minimal medium, and the change in the diameter of the colony with time was measured at 22 (B) and 37°C (C). The first measurement was taken on day 1, 24 h after inoculation. The experiment was performed in triplicate, and the values shown are averages ± standard errors of the mean.

At 37°C, both wt and r-wt strains increased their growth rates almost threefold relative to those at room temperature, achieving a colony expansion rate of 18 mm/day and reaching the edge of the plate within 4 days of inoculation (Fig. 2C). By contrast, the Δ*cgrA* mutant was unable to increase its growth rate beyond what it could achieve at room temperature, growing at 6.3 mm/day until it reached a plateau 10 days later. The Δ*cgrA* colony failed to extend completely to the edge of the plate, possibly due to the depletion of nutrients resulting from the extended incubation time. This temperature-sensitive phenotype was not complemented by growth on osmotically stabilized medium, which argues against a loss of wall integrity as the reason for the temperature sensitivity (data not shown).

A comparison of the radial-growth rates of the wt, r-wt, and Δ*cgrA* strains at various temperatures is shown in Fig. 3A. The mutant grew progressively more slowly as the temperature increased above 37°C, and a temperature of 48°C was not permissive for growth. By contrast, the wt strain grew optimally between 37 and 42°C and retained the ability to grow at 70% of its optimal growth rate at 48°C.

The Δ*cgrA* mutant is hypersensitive to translation inhibition. Yeast mutants that are deficient in ribosome biogenesis often display increased sensitivity to aminoglycoside antibiotics that decrease translational fidelity (7, 32, 65, 70). To determine whether the Δ*cgrA* mutant was hypersensitive to impaired ribosome function, the radial-growth rates of the wt, r-wt, and Δ*cgrA* strains were compared in the presence and absence of the aminoglycoside hygromycin. As shown in Fig. 3B, the Δ*cgrA* mutant was unable to grow in the presence of a concentration of hygromycin that had little effect on the wt or r-wt strain. The hygromycin hypersensitivity of the Δ*cgrA* mutant was also evident at 22°C (data not shown).

Loss of *cgrA* impairs germination at elevated temperature. Since new ribosome production is a major synthetic process during the early minutes of germination in filamentous fungi (9, 45), we determined how loss of CgrA would affect the rate of germ tube formation. At 37°C, wt and r-wt conidia started forming germ tubes within 6 h, and by 9 to 10 h of incubation, 100% of the conidia had germinated (Fig. 4B). This was in contrast to Δ*cgrA* conidia, which required 16 h of incubation at 37°C before germ tubes became evident and an additional 6 h of incubation before 100% of the conidia had germinated (Fig. 4B). This difference was much less evident at room temperature; the Δ*cgrA* mutant had a short delay in the start of germination relative to wt and r-wt conidia, but all three strains had completely germinated within 4 h of each other (Fig. 4A).

A CgrA-GFP fusion protein is nucleolar and complements the Δ*cgrA* mutation. It has been demonstrated that CgrA localizes to the nucleolus when heterologously expressed in *S. cerevisiae* (8). To determine the intracellular localization of CgrA in its native species, the *cgrA* gene was fused in frame to the plant-adapted GFP and expressed in wt *A. fumigatus* as an ectopically integrated transgene under the control of the *A. nidulans gpdA* promoter. The intracellular localization of the fusion protein was determined by fluorescence confocal microscopy. A strain expressing GFP alone showed diffuse hyphal fluorescence (Fig. 5B). Conidia from the CgrA-GFP-expressing strains initially showed very low levels of GFP fluorescence, but after 3 h of growth in rich medium, intense nuclear fluorescence became evident and persisted throughout hyphal growth (Fig. 5D). Costaining with the DNA-specific dye PI showed that the GFP fluorescence was concentrated in the nucleolar region, defined as the region of the nucleus that stained weakly with PI (Fig. 5E and F). The nucleolus occupies a substantial proportion of the nuclear volume due to the high

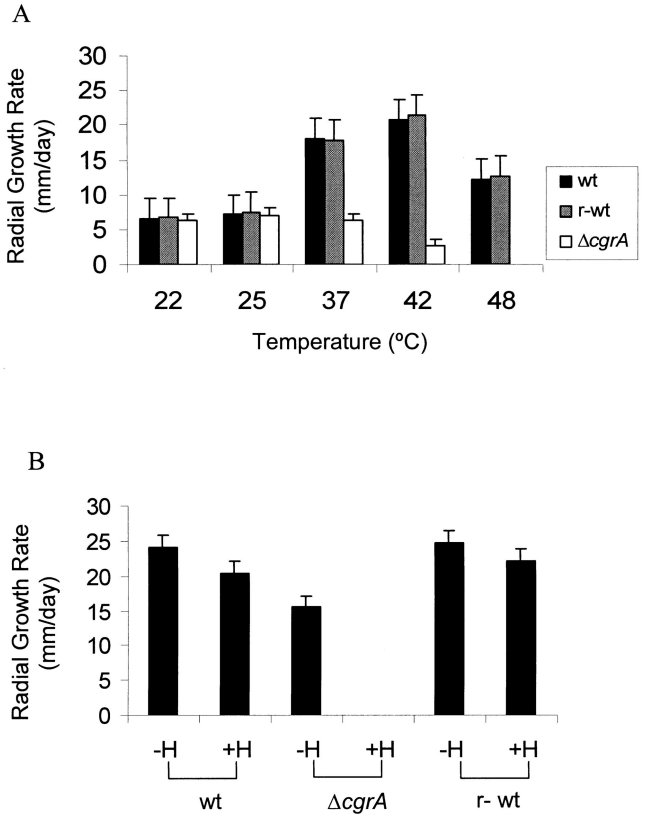

FIG. 3. (A) Comparison of radial growth rates at 22 to 48°C. The radial growth rates of the wt, r-wt, and Δ*cgrA* strains on minimal medium are shown for the indicated temperatures. The experiment was performed in triplicate, and the values shown are averages ± standard errors of the mean. (B) Hygromycin sensitivity of the Δ*cgrA* mutant. Conidia from the wt, r-wt, and Δ*cgrA* mutant were spotted onto a yeast-peptone-dextrose plate in the absence (−H) or presence (+H) of 62.5 μg of hygromycin/ml and incubated at 37°C for 8 days.

demand for ribosomes in an organism that has a relatively small genome (23, 68). The strain shown in Fig. 5 harbors two ectopically integrated copies of the *cgrA-gfp* transgene, and Northern blot analysis showed that *cgrA-gfp* was overexpressed fivefold relative to *cgrA* levels in the wt (data not shown). However, strains with single ectopic integrations with less than twofold overexpression showed the same nucleolar localization of the CgrA-GFP fusion protein (data not shown), suggesting that nucleolar localization is not an artifact of overexpression. Moreover, since expression of the *cgrA-gfp* fusion in the Δ*cgrA* mutant was able to increase the growth rate at 37°C (Fig. 6), the observed nucleolar localization of CgrA is likely to be appropriate for the biological activity of this protein. However, the inability of the *cgrA-gfp* fusion to fully complement the Δ*cgrA* mutant suggests that CgrA function was somewhat impaired by the presence of the GFP tag. Expression of *cgrA-gfp* in the wt had no effect on the growth rate (data not shown).

Loss of CgrA impairs virulence in *A. fumigatus*. The ability to grow readily at 37°C distinguishes *A. fumigatus* from most other common environmental molds (15, 63) and has been speculated to contribute to virulence (37). To determine how loss of thermotolerance in the Δ*cgrA* mutant would affect growth in vivo, we compared the virulence of the Δ*cgrA* mutant in two infection models. The first model used mice that were immunosuppressed with a single dose of triamcinolone acetonide and cyclophosphamide. The mice were inoculated intranasally with 10^5 conidia on day 0, and mortality was monitored for 2 weeks. As shown in Fig. 7A, most of the animals inoculated with the wt or r-wt strain died within the first week, with a median survival time of 5 to 6 days. By contrast, all of the mice inoculated with the Δ*cgrA* mutant survived the first week, and 60% of them survived to day 14. Histological examination of lung tissue from mice infected with the wt strain showed extensive fungal growth surrounding bronchioles 2 days after inoculation (Fig. 8). At the same time point, only sporadic germlings could be identified in lungs from mice infected with the Δ*cgrA* mutant (Fig. 8). The Δ*cgrA* mutant had formed distinct fungal colonies in the lung 4 days after inoculation, but colonies of the wt strain were much larger (Fig. 8).

Since the mouse model challenges the organism to grow at 37°C, a second model was used to examine the virulence of the Δ*cgrA* mutant in *Drosophila*, a well-studied invertebrate host that is usually maintained at 25°C. Since the radial-growth rate of the Δ*cgrA* mutant was indistinguishable from that of the wt at this temperature (Fig. 3A), we predicted that the wt and Δ*cgrA* strains would express similar levels of virulence in the fly. To test this hypothesis, groups of 40 to 70 adult *spz* mutant

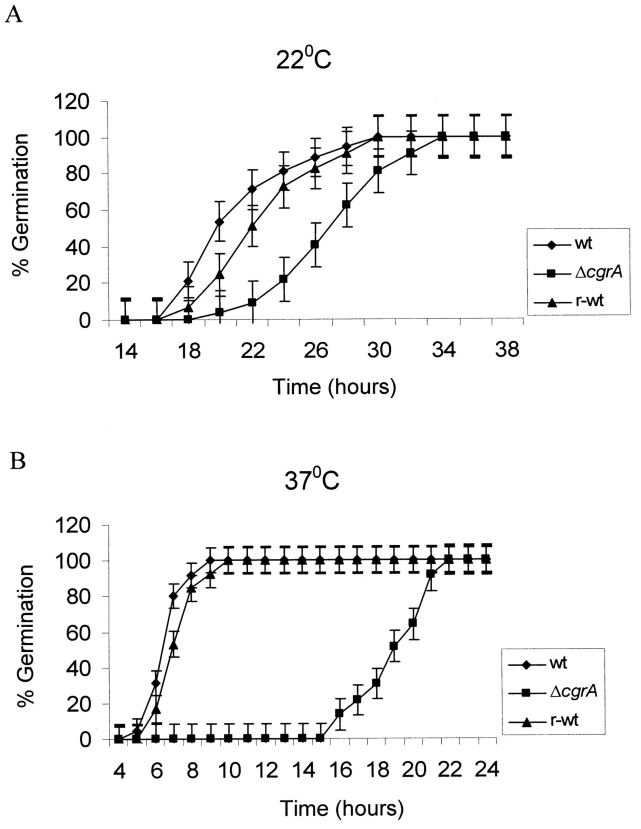

FIG. 4. Impaired germination of the Δ*cgrA* mutant. The percentages of swollen conidia that had initiated a germ tube after the indicated periods of growth in liquid cultures in YG medium are shown for the wt, r-wt, and Δ*cgrA* strains at 22 and 37°C. The experiment was performed in triplicate, and the values shown are averages ± standard errors of the mean.

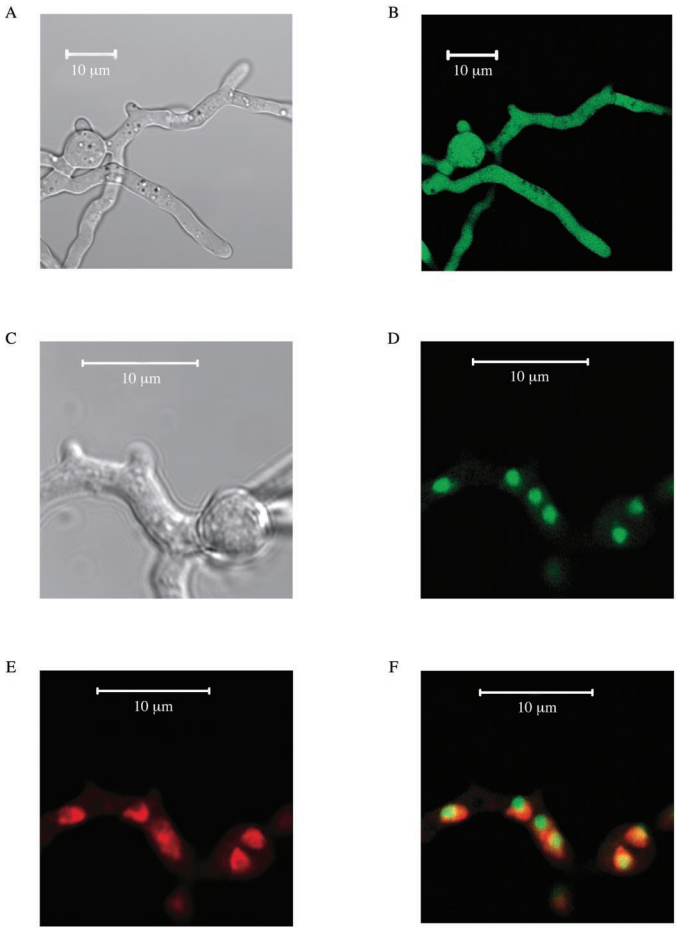

FIG. 5. Subcellular localization of CgrA. (A and B) wt *A. fumigatus* expressing GFP alone is shown in a differential interference contrast image (A) and a corresponding fluorescent image (B). (C to F) Hypha from a wt strain expressing the CgrA-GFP fusion protein and counterstained with the DNA-specific dye PI. (C) Differential interference contrast image. (D) GFP fluorescence. (E) PI fluorescence. (F) Overlay of the PI and GFP fluorescence showing GFP localization to the PI-excluded nucleolar region.

flies were infected with ~1,500 conidia and monitored for 120 h for mortality (Fig. 7B). The majority of the flies inoculated with wt conidia died within 5 days, and death was associated with fungal outgrowth from each fly. In comparison to the mouse model, the virulence of the Δ*cgrA* mutant in the fly was much closer to that of the wt, which is consistent with the ability of the mutant to grow with wt kinetics at 25°C in vitro. The small difference in survival between the wt and Δ*cgrA* strains in the fly model was statistically significant however, suggesting that the growth of the mutant may be somewhat impaired compared to that of the wt in this model.

DISCUSSION

Pathogenic fungi must deal with a variety of adverse environmental conditions during the course of an infection. The ability to meet these challenges requires the expression of many gene products, which in turn depends upon an adequate supply of ribosomes from the nucleolus. For example, serial analysis of gene expression analysis of the transcriptional response of *Cryptococcus neoformans* to a temperature shift from 25 to 37°C in vitro showed upregulation of multiple components of the translational machinery (61). A high abundance of translation-related tags was also identified in *C. neoformans* cells isolated from the central nervous systems of infected rabbits (62). Interestingly, a comparison of the abundance of translation-related serial analysis of gene expression tags in these two studies found that 73% of them were most abundant in vivo relative to the in vitro situation (62). In *Candida albicans*, different clinical isolates are well known to exhibit great variability in the number of ribosomal DNA (rDNA) repeats, suggesting that rRNA synthesis is regulated in nature by expansion and contraction of the rDNA units (35, 56, 57), allowing growth adaptation in response to the environment. This is supported by the fact that strains of *C. albicans* with higher numbers of rDNA units can be obtained by selecting for rapidly growing variants (56). Differences in the expression of rRNA have also been reported between pathogenic and nonpathogenic species of *Mycobacteria*, where slow growth is associated with reduced rDNA gene dosage and the presence of promoters that drive lower levels of rRNA transcription (25).

To determine the extent to which a nonlethal defect in ribosome biogenesis would affect the virulence of *A. fumigatus*, we disrupted the function of a gene that was previously shown to have a role in 60S ribosomal subunit synthesis in *S. cerevisiae*

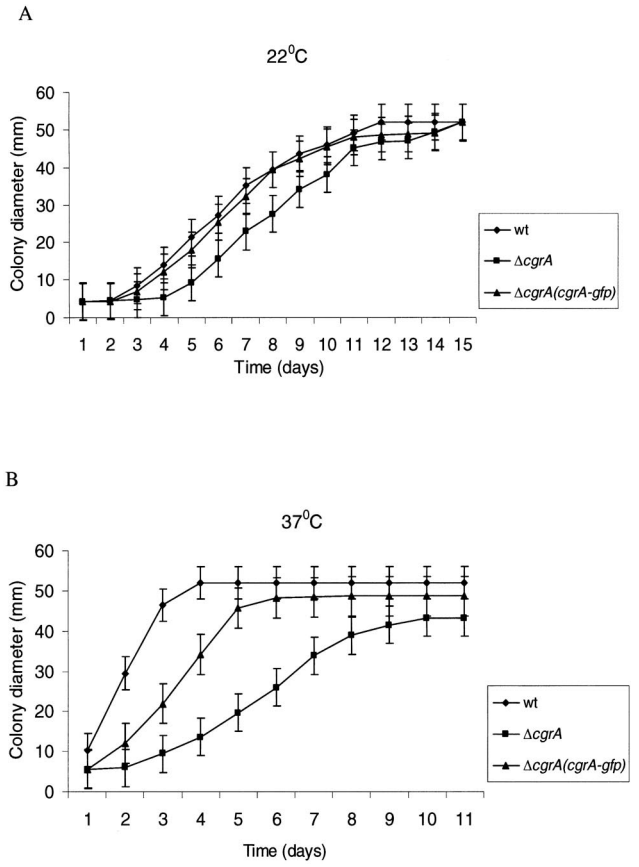

FIG. 6. Complementation of the thermosensitive Δ*cgrA* phenotype by a *cgrA-gfp* fusion gene. The radial growth of the wt, Δ*cgrA* and the Δ*cgrA* strain expressing *cgrA-gfp* from an ectopically integrated transgene are compared at 22 (A) and 37°C (B). The growth rate of the wt strain expressing the *cgrA-gfp* fusion gene was indistinguishable from that of the wt (data not shown).

(8, 48). This particular nucleolar protein was selected because it has no clear human homolog, suggesting a level of evolutionary divergence that could be exploited in future therapies. The protein is highly conserved among fungi, and the *A. fumigatus* ortholog, CgrA, is able to complement yeast *cgr1* mutants and to localize to the yeast nucleolus when heterologously expressed (8, 48). We found that CgrA function in *A. fumigatus* was dispensable for normal growth rates in vitro at room temperature. The Δ*cgrA* mutant showed a slight delay in the start of radial outgrowth at this temperature, suggesting that it may take longer for it to become competent for hyphal growth (Fig. 2A). This may reflect the small germination delay that was also observed at 22°C (Fig. 4A). Although loss of CgrA function had little effect on germination and hyphal growth at room temperature, it was critical to establish the rapid growth rates that are characteristic of *A. fumigatus* at 37°C and above. The Δ*cgrA* mutant was also hypersensitive to hygromycin, suggesting that the combined effects of translation inhibition and a loss of CgrA activity create a defect in the translational machinery that is incompatible with hyphal growth. These findings are consistent with our data from yeast showing that *cgr1* mutants are both thermosensitive (unpublished) and hypersensitive to aminoglycosides (65).

Not all mutations in ribosome biogenesis cause growth defects at elevated temperature. In yeast, some mutations that affect ribosome biogenesis are lethal (64), some have little or no growth phenotype (22, 43), and others display comparable growth rates at 30 and 37°C but are cold sensitive (69). Our data for *A. fumigatus* indicate that CgrA is required for rapid growth at elevated temperature, which is striking for an organism that grows well at a broad range of temperatures. This may reflect a direct role for CgrA in thermotolerant ribosome biogenesis or may represent an indirect effect of the loss of CgrA on the overall fitness of the organism, leading to a decline in thermal tolerance. Experiments to distinguish between these possibilities are under way.

Thermotolerance is often cited as a factor that contributes to the virulence of *A. fumigatus*, since many nonpathogenic environmental molds, and several avirulent mutant fungal strains, grow poorly at mammalian body temperature (2, 15, 36, 51). Since the Δ*cgrA* mutant was growth impaired at 37°C, its capacity to cause disease was assessed in a mouse model of invasive aspergillosis. Both the wt and r-wt strains caused a rapidly fatal infection in immunosuppressed mice, with a me-

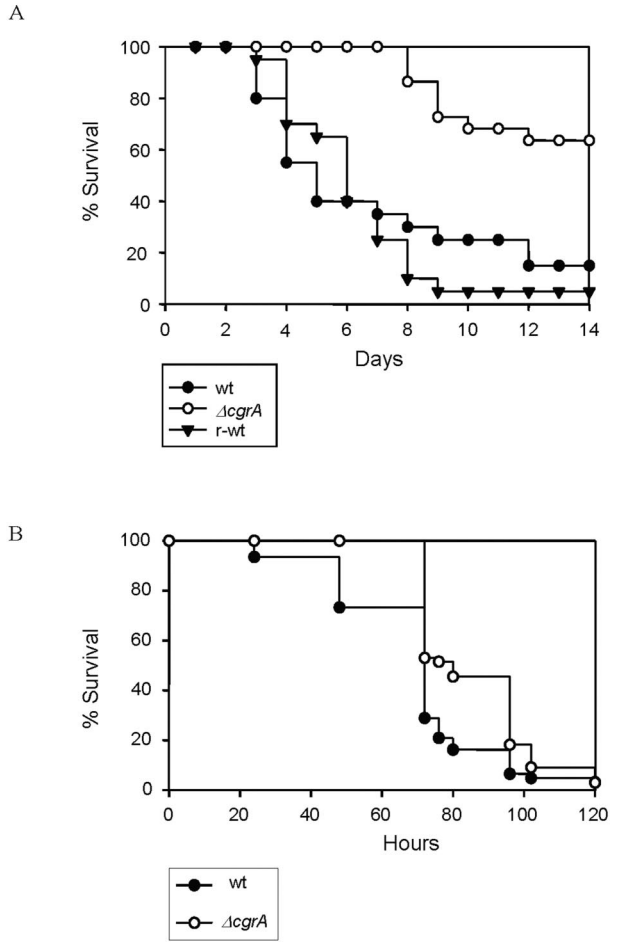

FIG. 7. Virulence of wt and Δ*cgrA* conidia in two infection models. (A) Groups of 20 to 22 immunosuppressed mice were infected with 10^5 conidia and monitored for 2 weeks. (B) Groups of 40 to 70 adult *spz* flies were inoculated with 1,500 conidia and monitored for 120 h at 25°C. *P* is <0.05 for both experiments.

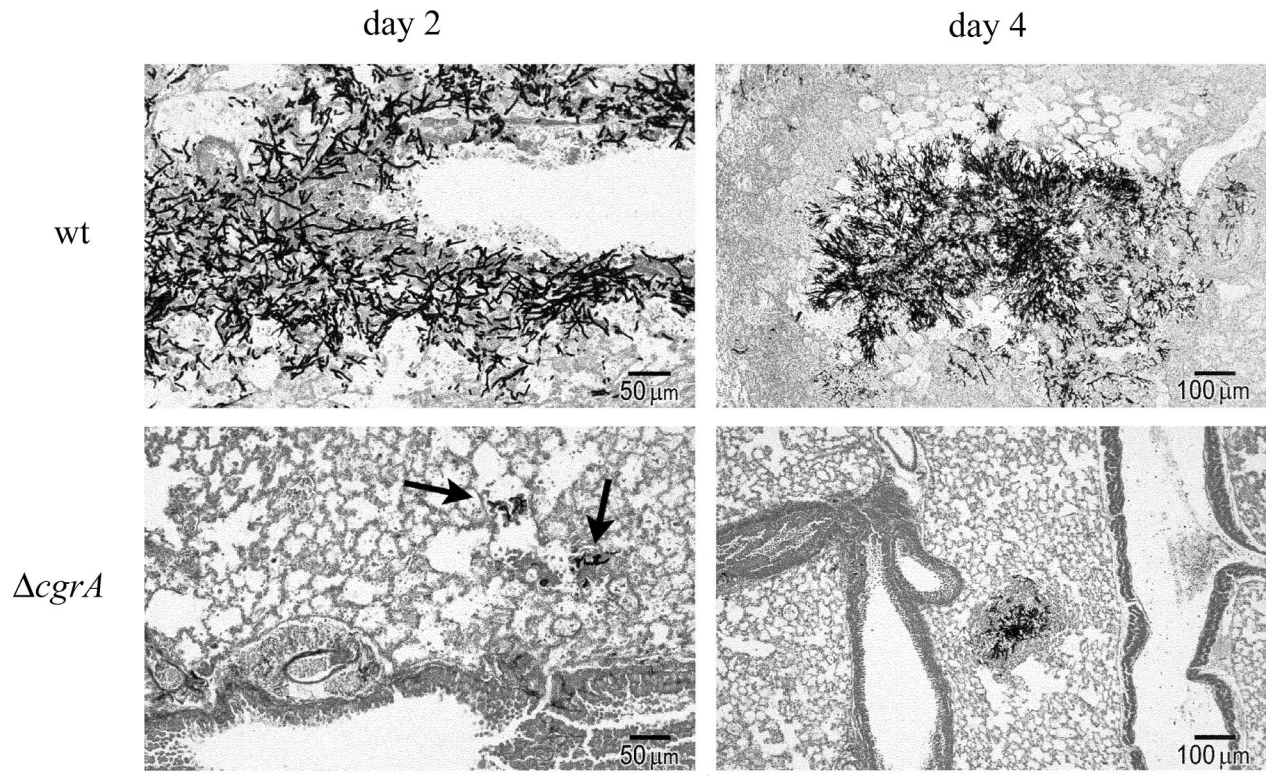

FIG. 8. Histologic analysis of lung tissue from infected mice. At 2 and 4 days following inoculation, lung sections from mice infected with wt *A. fumigatus* or the Δ*cgrA* mutant were stained with methenamine silver nitrate to reveal the morphologies of fungal colonies. Only sporadic germlings could be identified in lungs from mice infected with the Δ*cgrA* mutant on day 2 (arrows).

dian mortality of 5 to 6 days. The Δ*cgrA* mutant was clearly attenuated in virulence, showing a delay in both the onset and progression of mortality that likely reflects the impaired growth of the mutant at mammalian body temperature. Since the radial growth of the Δ*cgrA* mutant in vitro was indistinguishable from that of the wt at 25°C, we tested the prediction that loss of *cgrA* would have little or no effect on virulence in *Drosophila*, an invertebrate species that is maintained at 25°C and has been used to study the pathogenesis of a number of important pathogens (17, 20, 38). During a fungal infection, the *Drosophila* Toll receptor on the surfaces of fat-body cells is activated by a cleaved form of a cytokine-like protein called Spaetzle (Spz), which is present in the *Drosophila* hemolymph. The interaction between Spz and Toll initiates an intracellular signaling cascade through the threonine-serine kinase Pelle, which culminates in the synthesis of antifungal peptides (31, 66). Although *A. fumigatus* is normally a weak pathogen for insects (28), mutants in the *Drosophila* dorsoventral regulatory cassette Spatzle/Toll/Cactus are highly susceptible to fungal infections (3, 39). We found a clear difference in virulence between wt and Δ*cgrA* strains in the mouse, but the difference was much less dramatic in the *Drosophila* infection model. This suggests that the Δ*cgrA* mutant grows almost as well as the wt in the fly, and this is consistent with the comparable growth rates of the two strains at 25°C in vitro. Although it is difficult to extrapolate from growth on plates to growth in the host, the small difference in virulence observed between the wt and Δ*cgrA* strains in the *Drosophila* model achieved statistical significance, raising the possibility that the Δ*cgrA* mutant has a minor growth detriment in vivo that was not evident in vitro.

A. fumigatus grows readily at 37°C and is one of the few fungal species with the ability to thrive at temperatures between 45 and 55°C (15). This classifies *A. fumigatus* as thermotolerant rather than thermophilic, since true thermophilic fungi have growth maxima at or above 50°C (41). Several lines of evidence suggest that high temperature is a significant stress for the process of ribosome biogenesis. For example, bacterial heat shock proteins or chaperones have been shown to be required for ribosome biogenesis at high temperature (1, 10, 21, 29). In eukaryotes, heat stress adversely affects nucleolar morphology, and protection is conferred by the rapid nucleolar translocation of chaperones (13, 49, 53, 67). This implies that *A. fumigatus* has evolved specific mechanisms to circumvent such problems during growth in the host. Understanding more about the relationship between CgrA and thermotolerant growth may provide insight into how fungal pathogens meet the challenge of ribosome biogenesis at elevated temperature and may offer new avenues for the development of antifungal technology.

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