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Coding Tandem Repeats Generate Diversity in *Aspergillus fumigatus* Genes▽†

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Genes containing multiple coding mini- and microsatellite repeats are highly dynamic components of genomes. Frequent recombination events within these tandem repeats lead to changes in repeat numbers, which in turn alters the amino acid sequence of the corresponding protein. In bacteria and yeasts, the expansion of such coding repeats in cell wall proteins is associated with alterations in immunogenicity, adhesion, and pathogenesis. We hypothesized that identification of repeat-containing putative cell wall proteins in the human pathogen *Aspergillus fumigatus* may reveal novel pathogenesis-related elements. Here, we report that the genome of *A. fumigatus* contains as many as 292 genes with internal repeats. Fourteen of 30 selected genes showed size variation of their repeat-containing regions among 11 clinical *A. fumigatus* isolates. Four of these genes, Afu3g08990, Afu2g05150 (*MP-2*), Afu4g09600, and Afu6g14090, encode putative cell wall proteins containing a leader sequence and a glycosylphosphatidylinositol anchor motif. All four genes are expressed and produce variable-size mRNA encoding a discrete number of repeat amino acid units. Their expression was altered during development and in response to cell wall-disrupting agents. Deletion of one of these genes, Afu3g08990, resulted in a phenotype characterized by rapid conidial germination and reduced adherence to extracellular matrix suggestive of an alteration in cell wall characteristics. The Afu3g08990 protein was localized to the cell walls of dormant and germinating conidia. Our findings suggest that a subset of the *A. fumigatus* cell surface proteins may be hypervariable due to recombination events in their internal tandem repeats. This variation may provide the functional diversity in cell surface antigens which allows rapid adaptation to the environment and/or elusion of the host immune system.

Aspergillus fumigatus is a ubiquitous soil-dwelling fungus and the most common mold pathogen in humans (16, 32). It reproduces asexually by producing prodigious numbers of airborne conidia. In immunocompetent individuals, conidial inhalation can result in allergic reactions and limited colonization of the airways (5). In immunocompromised patients, inhaled conidia can germinate and invade the lung tissues, causing invasive aspergillosis, a severe and usually fatal infection (30 to 90% mortality when treated) (30). Because of the increase in the number of immunosuppressed patients following aggressive modern chemotherapy and immunosuppressive regimens, a fourfold increase in the number of cases of invasive aspergillosis has been seen in the last 15 years (30). Therefore, a better understanding of the molecular details of *A. fumigatus* virulence and host-pathogen dynamics is urgently needed.

Studies of both prokaryotes and eukaryotes suggest that variability of the cell surface proteins is a key determinant in pathogenicity. By varying their cell surface, pathogens can escape the immune system and/or colonize a broad range of tissues or substrates. Recent research shows that cell surface variability can be generated by hypervariable tandem repeats (TRs) located within exons of cell surface genes. These coding TRs are adjacent, in-frame coding DNA sequences of 2 to 200 nucleotides in length that are directly repeated. The repeated units may be completely identical or partially degenerate (31, 23). The number of these coding repeat copies often varies among different isolates, leading to expansion or contraction of amino acid blocks. In humans, the expansion of such coding repeats is associated with various diseases, including Huntington's disease and fragile X syndrome (19). In a number of prokaryotic genomes, repeats play an important role in generating variability in cell surface immunogenic antigens and adhesins, thereby evading the immune system or enhancing pathogenicity (9, 13).

Recently, it was shown that eukaryotic microbes, like their prokaryotic counterparts, may also employ coding TRs to generate functional variability at the cell surface. In the yeast *Saccharomyces cerevisiae*, for example, an increase in the number of coding repeats in the *FLO1* gene correlates with an increase in adhesion to plastics used in medical devices (34, 35). Similarly, repeat variation in the *Candida albicans ALS3* adhesin gene changes the cellular binding specificity (22). Moreover, clinical *C. albicans* isolates show variability in the number of repeats in various cell surface genes, indicating that this recombination process may play a role during infection, allowing cells to adapt rapidly to a fluctuating environment and/or evade the host immune system (39, 40).

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It is worth noting that besides the conidial hydrophobins (24), no *Aspergillus* adhesins involved in cell host or cell matrix attachment have been identified thus far. BLAST homology searches with sequences of known fungal adhesins (e.g., *S. cerevisiae FLO* genes, *C. albicans ALS* and *Hwp1* genes among others) have failed to identify significant homologs in *Aspergillus* spp.

We hypothesized that TR-containing genes in *A. fumigatus* may play an important role in host-pathogen interaction, possibly by generating diverse proteins important in pathogenic interaction. We performed a genome-wide analysis of all 9,926 *A. fumigatus* open reading frames (ORFs) to identify those containing internal coding repeats, using the ETANDEM software program, which assigns each intragenic repeat a numerical score based on its conservation and repeat number (35). Our screen yielded a range of genes with potentially functionally important diversity in repeat-encoded structures. Next, we identified those TR-rich ORFs containing both a signal peptide sequence and a glycosylphosphatidylinositol (GPI) anchor motif. These motifs target the proteins for secretion and attachment to the plasma membrane or cell wall (10, 17). The 10 highest-scoring TR-containing ORFs were analyzed for size variability among patient isolates of *A. fumigatus*. Four of the highest-scoring genes (Afu3g08990, Afu2g05150 [*MP-2*], Afu4g09600, and Afu6g14090) were found to have variable-size repeats. To investigate whether these ORFs indeed encode cell surface genes that could be important during infection, they were further analyzed for mRNA expression during development and in response to various compounds that induce stress. Three of the ORFs (Afu3g08990, Afu2g05150 [*MP-2*], and Afu6g14090) were deleted. The phenotypic characterization of the Afu3g08990-deleted mutant is described.

MATERIALS AND METHODS

Computational analysis. To find intragenic repeats, the EMBOSS ETANDEM software (http://emboss.sourceforge.net/apps/etandem.html) (26) was used to screen the sequences of all 9,926 *A. fumigatus* ORFs (http://www.cadre.man.ac.uk/Aspergillus_fumigatus/) (21). The ETANDEM threshold score was set at 35 (ETANDEM produces a score for every repeat; the score is calculated as a basic alignment score so that it combines repeat size, number, and purity in one number). This threshold was chosen because in a similar analysis in *S. cerevisiae*, it was experimentally shown that ORFs with an ETANDEM value greater than 35 have highly variable repeats (35). *A. fumigatus* ORFs were divided into three groups based on functional motifs: (i) group 1 ORFs are repeat-rich ORFs containing both a signal peptide sequence and a GPI anchor motif; (ii) group 2 ORFs contain a signal peptide sequence only; (iii) group 3 ORFs lack both motifs.

***A. fumigatus* strains and culture conditions.** Eleven *A. fumigatus* clinical isolates were used throughout this study (29). Feral strains were obtained from soil and air samples in the Tel-Aviv area (Israel).

A. fumigatus liquid cultures used for genomic DNA and total RNA preparation were grown on YAG (0.5% [wt/vol] yeast extract, 1% [wt/vol] glucose, and 10 mM $MgCl_2$), supplemented with trace elements and vitamins (1) at 37°C for 16 to 20 h. To evaluate gene expression during conidial germination and growth, conidia (5×10^6 conidia/ml) were grown in stationary culture at 37°C in YAG and harvested at different time points (0, 6, 12, and 20 h). The response to cell wall-disrupting reagents was examined by growing conidia for 12 h on stationary liquid minimal medium (MM) containing 70 mM $NaNO_3$, 1% glucose, 12 mM KPO_4, pH 6.8, 4 mM $MgSO_4$, 7 mM KCl, and trace elements and then supplementing for an additional 4 h with 7.8 μg/μl calcofluor (Sigma Chemical Co., St. Louis, MO), 1.25 μg/ml Congo red (Sigma), 0.2 μg/ml caspofungin (Merck & Co, Inc., NJ), 0.00375% H_2O_2 (Sigma), 2.5 mg/ml β-D-glucanase (InterSpex Products Inc., Foster City, CA), 31.25 μg/ml sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories Inc., Hercules, CA), 0.6 μg/ml amphotericin B (Sigma), or 0.16 μg/ml itraconazole (Jansen, Piscataway, NJ).

DNA and RNA analysis. A. *fumigatus* genomic DNA was extracted by the MasterPure yeast DNA purification kit (Epicenter Biotechnologies, Madison, WI) with modifications for *A. fumigatus* as described by Jin et al. (12). Total RNA was prepared by the "hot SDS/phenol" protocol (20).

For reverse transcription-PCR (RT-PCR), total RNA was treated with DNase (Ambion Inc., Austin, TX) according to the manufacturer's instructions. RNA concentration was assessed, and 3-μg RNA samples were treated with DNase (DNA-free; Ambion, Austin, TX) and taken for the RT reaction with PowerScript reverse transcriptase (Clontech Laboratories Inc., Mountain View, CA). PCR was performed with the Red Load *Taq* master mix (Larova GmbH, Teltow, Germany) with the following designed primer pairs (see Table S1 in the supplemental material). The primers were designed 30 to 50 bp upstream and downstream of the repeat domain based on the *A. fumigatus* strain Af293 genomic DNA sequence (21). Primer length ranged between 22 and 32 bp, and the melting temperature ranged from 55 to 70°C. Aliquots were taken at cycles 18, 20, 25, and 30, and the PCR products were analyzed by gel electrophoresis. Analysis was performed on samples taken during the logarithmic phase of the PCR. Each PCR was independently performed three times on the same RNA sample. Control DNase-treated RNA samples that lacked reverse transcriptase were PCR amplified in parallel and showed no amplified fragments. Densitometric analysis was carried out using the TINA 2.0 software package (Molecular Dynamics Inc., Sunnyvale, CA). Densitometric values were normalized by calculating the ratio between the experimental point and *gpdA* expression. Final values were derived by calculating the ratio between the normalized experimental and reference points. Statistical analysis was performed using the Student two-tailed *t* test.

Gene deletion. A 5,215-bp DNA fragment flanking the *A. fumigatus* Afu3g08990 gene was generated by PCR, using the Expand high-fidelity PCR system (Roche Diagnostic, Penzberg, Germany) and primers *Asc*I-*08990* 5′ and *Asc*I-*08990* 3′ (see Table S1 in the supplemental material). These primers were designed to contain an AscI restriction site at their 5′ end. The Afu3g08990 gene, including 354 bp upstream and 342 bp downstream of the ORF was then removed by digestion with Acc65I and BmgBI and replaced with a hygromycin-selectable marker to produce the p*Afu3g08990*-D plasmid (see Fig. 6A). The hygromycin cassette, containing 5′ and 3′ Acc65I and BmgBI restriction sites, respectively, was generated by PCR amplification using primers *Hyg* 5′ and *Hyg* 3′ (see Table S1 in the supplemental material). For transformation, 10 μg of spin-purified AscI-digested p*Afu3g08990*-D plasmid was used. Transformation was performed as described by Romano et al. (27). For Southern blot analysis, *A. fumigatus* genomic DNA was extracted as described above. Southern hybridization analysis was performed as described previously (11). Briefly, 10-mg fungal genomic DNA samples were digested with BamHI and run on a 1% (wt/vol) agarose gel. The cleaved DNA was transferred to a Nytran N nylon membrane (Schleicher & Schuell) and hybridized with an [α-^{32}P]dCTP-radiolabeled Afu3g08990 5′ probe at 65°C. The probe was generated by PCR with primers *08990-S5′* and *08990-S3′* (see Table S1 in the supplemental material).

The 8990-RI (RI means random integration) control strain was isolated as a transformant in which the p*Afu3g08990*-D plasmid had integrated randomly outside the Afu3g08990 locus, resulting in a hygromycin-resistant strain containing the intact Afu3g08990 gene. The Afu3g08990 KI-1 (KI means knock-in) control strain was prepared by complementing the Afu3g08990 D1 strain with plasmid pAN-8.1 containing the p*gpdA-phleomycin* cassette (25) and the 5,215-bp DNA fragment flanking the *A. fumigatus* Afu3g08990 gene described above. Three Afu3g08990 KI strains were obtained and verified for Afu3g08990 mRNA expression by RT-PCR. All three strains were phenotypically identical to the control wild-type strain Af293 as assessed by germination and adhesion experiments (data not shown).

Construction of the p*Afu3g08990-myc* plasmid. The p*Afu3g08990-myc* plasmid containing Afu3g08990 with two N-terminal c-*myc* tags was created as follows: two PCR fragments of Afu3g08990 containing (i) the Afu3g08990 promoter region, start codon, and leader sequence and (ii) the Afu3g08990 coding sequence, terminator region, and 5′ c-*myc* tag were obtained by amplification of *A. fumigatus* genomic DNA with the oligonucleotides *08990 prom 5′* and *3′* and *08990-myc 5′* and *3′* primers (see Table S1 in the supplemental material). The two fragments were gel purified, subcloned into the pGEM T/A cloning vector (Promega Corp., Madison, WI), verified by sequencing, digested with FseI/NotI and NotI/AscI, respectively, and inserted into the pGEM T/A–hyg plasmid. This plasmid contains the hygromycin resistance cassette and 5′ FseI/AscI cloning sites. The resulting plasmid, p*Afu3g08990-myc*, was subsequently transformed into *A. fumigatus* strain Af293 to generate the Afu3g08990-myc1 strain expressing *myc*-tagged Afu3g08990 protein.

Phenotypic characterization of the Afu3g08990-deleted mutant. Hyphal growth rate and germination studies were performed by plating 10^3 to 10^5 freshly

TABLE 1. Classification of repeat-containing *A. fumigatus* ORFs

Gene	Function[a]	ETANDEM score	Repeat size (bp)	No. of repeats	% Conservation[b]	Allelic variability[c]
ORFs with a leader and GPI anchor consensus sequence						
Afu3g08990	Hypothetical	432	18	32	89.1	+
Afu2g05150	MP-2 antigenic galactomannoprotein	301	39	10	93.6	+
Afu4g09600	Conserved hypothetical	292	198	3	91.2	+
Afu6g14090	CFEM[d] domain protein	134	6	40	79.2	+
Afu4g03240	MP-1 antigenic galactomannoprotein	72	12	38	59.2	−
Afu7g00970	Hypothetical	49	3	82	60.6	−
Afu3g00270	Glucanase (Bg12p-like)	46	45	7	64.4	−
Afu1g16190	Glucanase (Crfl-like)	45	3	94	58.5	−
Afu3g00880	Conserved hypothetical	42	18	8	70.8	−
Afu2g07800	GPI-anchored protein	35	12	14	63.7	−
ORFs with leader sequence only						
Afu1g01020	NACHT[e] domain protein	929	99	14	87.1	−
Afu2g18090	Conserved hypothetical	402	48	12	89.1	−
Afu7g08500	Tenascin-like glycoprotein[f]	250	126	4	97.3	ND
Afu3g07400	Hypothetical	146	105	3	89.8	−
Afu6g06670	Conserved hypothetical	113	15	12	85.6	+
Afu2g03540	Mucin-like[f]	88	57	3	92.4	+
Afu8g00630	Conserved hypothetical	76	24	6	84.7	−
Afu5g06880	Hypothetical	75	15	12	75.0	+
Afu8g07170	Conserved hypothetical	72	153	3	74.5	−
Afu5g11300	OTU-like[g] cysteine protease	67	255	2	81.6	−
ORFs without leader and GPI anchor consensus sequence						
Afu7g07100	NACHT domain protein	1,032	126	11	91.8	ND
Afu7g08290	WD[h] repeat protein	670	126	8	89.5	+
Afu7g08310	Hypothetical	400	132	5	90.3	+
Afu6g09340	Hypothetical	388	18	26	93.4	+
Afu4g10350	Polyubiquitin (UbiD)	302	228	3	88.7	−
Afu7g08240	Hypothetical	276	171	3	93.6	−
Afu8g06150	Sensor histidine kinase[f]	232	12	24	92.4	+
Afu6g09360	Conserved hypothetical	226	72	6	84.5	+
Afu5g15090	Hypothetical	218	18	17	88.6	+
Afu7g07060	Hypothetical	190	132	3	90.7	+

[a] Based on annotation provided by CADRE (http://www.cadre.man.ac.uk/) unless specified otherwise.
[b] Percent conservation between repeats.
[c] Presence (+) or absence (−) of variability of a gene in isolates. Two genes were not detected (ND) in the majority of isolates by PCR.
[d] CFEM is an eight cysteine-containing domain. CFEM-containing proteins are proposed to have important roles in fungal pathogenesis (15).
[e] NACHT, predicted nucleoside triphosphatase domain (14).
[f] Based on BlastP results with an E value of $<10^{-10}$.
[g] OTU, ovarian tumor.
[h] WD-40 repeats coordinate the binding of multiprotein complex assemblies (18).

harvested spores ml^{-1} onto 96-well plates in 200 μl liquid MM at 37°C. At various time points, growth was observed under a grid-mounted Olympus CK inverted microscope at a magnification of ×200. The lengths of the germlings ($n = 50$) were measured in microns.

Adhesion of *A. fumigatus* conidia to extracellular matrix (ECM) derived from A549 lung cells was performed as described by Wasylnka and Moore (37). Adherent conidia were counted under a grid-mounted Olympus CK inverted microscope at a magnification of ×400. Statistical analysis was performed using the Student two-tailed *t* test.

Microscopy. For Afu3g08990-*myc* immunostaining, conidia were grown on coverslips in liquid MM for 8 h, incubated in fixation solution (37% formaldehyde, 50 mM HEPES, pH 6.7, 5 mM $MgSO_4$, 25 mM EGTA, pH 7.0) for 45 min and washed in phosphate-buffered saline containing 1% bovine serum albumin (PBS-BSA) for 5 min. Nongerminated, freshly harvested conidia were used for the 0-h time point. Mouse-derived primary antibody c-*myc* (9E10) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was diluted 1:100 in PBS-BSA and added to the coverslips for 1 h. Coverslips were washed twice with PBS containing 0.1% (wt/vol) Nonidet P-40 for 5 min. Alexa Fluor 488 anti-mouse secondary antibodies were used according to the manufacturer's instructions (Signal-Amplification Kit for Mouse Antibodies; Invitrogen Molecular Probes, Carlsbad, CA). Images were obtained by fluorescence microscopy on an Olympus BX40 microscope equipped for fluorescence, at a total magnification of ×1,000. Images were recorded with a digital Olympus DP70 camera.

Murine model for systemic aspergillosis. Six-week-old female ICR mice were injected intraperitoneally with 200 mg cyclophosphamide kg of body $weight^{-1}$ 3 days prior to conidial infection. Mice were inoculated intravenously via the tail vein with an inoculum of 2.5×10^5 conidia/mouse of freshly harvested Af293 wild-type and Afu3g08990 D1-deleted conidia. To prolong neutropenia, additional cyclophosphamide (70 mg kg^{-1}) was administered 2 and 5 days after infection. Systemic aspergillosis was followed up for 21 days. Statistical analysis of mouse survival was performed by the GraphPad Prism 4 software (GraphPad Software). *P* values of <0.05 were considered significant in this analysis. Animal studies were performed in accordance with Tel-Aviv University institutional policies.

RESULTS

Genome-wide search for *A. fumigatus* ORFs containing TRs. The *A. fumigatus* genomic sequence was analyzed to identify coding TRs with the ETANDEM software program, which

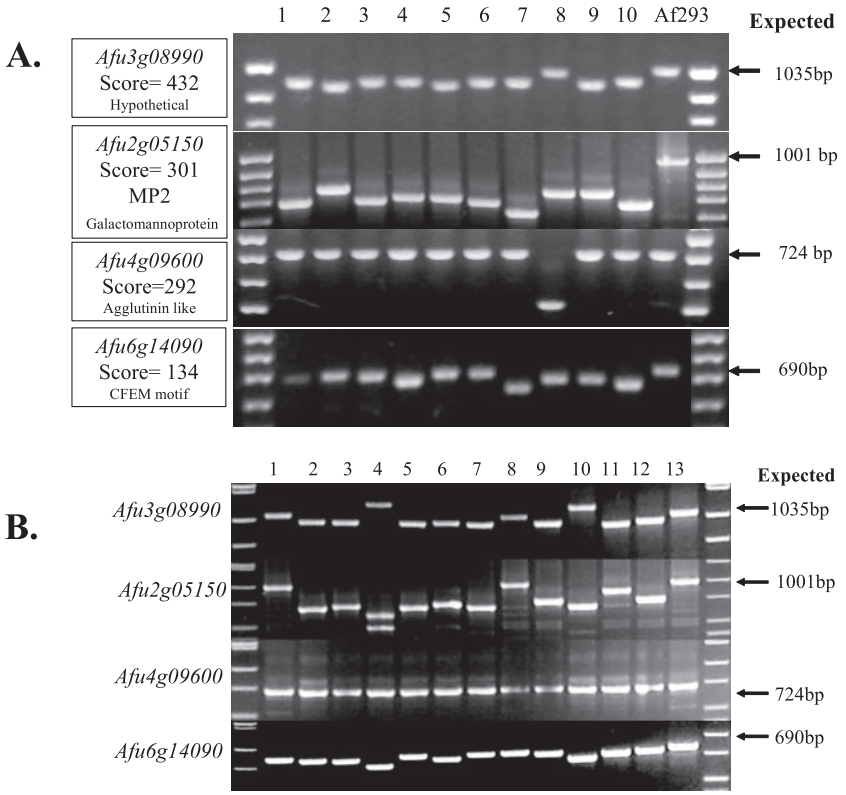


FIG. 1. TR regions in *A. fumigatus* ORFs containing a leader sequence and GPI anchor show size variability. The TR region of the 10 top-scoring ORFs containing a leader sequence, GPI anchor, and tandem repeat region were amplified by PCR in 11 clinical *A. fumigatus* isolates (A) and 13 environmental *A. fumigatus* isolates (B). The results for 4 of the 10 genes in this group, Afu3g08990, Afu2g05150, Afu4g09600, and Afu6g14090 (ETANDEM scores of 432, 301, 292, and 134, respectively), which showed size variation between the strains are shown. The expected sizes of the repeat fragments in the sequenced *A. fumigatus* strain Af293 are shown to the right of the gels. The other six genes showed no strain-dependent size variation (data not shown). Note that the occurrence of multiple bands in some of the PCR amplifications may reflect mispriming because of sequence differences between strain Af293 and the other strains.

provides a numerical score for repeats in a nucleotide sequence. From a total of 9,926 *A. fumigatus* putative ORFs, 292 ORFs (2.94%) were identified as containing TRs with an ETANDEM score of ≥35 (see Table S2 in the supplemental material). Many repeats were trinucleotide repeats, but some ORFs contain repeat units as long as 285 nucleotides (Afu5g09030 and Afu4g13630). The highest copy number found was 223 (Afu7g01800) (see Table S2 in the supplemental material). We found that all repeat units contain a multiple of 3 nucleotides, so that repeat variation would not cause a frameshift mutation. Of the 292 ORFs with TRs with an ETANDEM score of ≥35, 75 (0.75%) had a putative leader sequence motif in their N-terminal region and 10 (0.10%) also had a putative GPI anchor consensus sequence (see Table S2 in the supplemental material).

The 10 ORFs with the highest ETANDEM scores from each of the following groups were selected for analysis of strain-specific size variation: repeat-rich ORFs containing both a leader and GPI anchor consensus sequence, a putative leader sequence, and neither of these motifs. Table 1 summarizes the main characteristics of the repeats in these 30 ORFs. As a starting point, we analyzed all 30 ORFs for size variability of the repeat region among 11 *A. fumigatus* clinical isolates. Subsequent analysis focused on the group 1 leader and GPI sequence-containing genes, as they are most likely to encode potential cell surface proteins.

***A. fumigatus* ORFs with high ETANDEM scores have variable-size repeat regions.** We performed a PCR analysis on genomic DNA obtained from 11 clinical *A. fumigatus* strains with primers designed to amplify the region of repeats from the 30 ORFs described above (Table 1; see Table S1 in the supplemental material). All three gene categories contained ORFs with variable-size repeat regions. Group 1 ORFs, having a leader sequence and GPI anchor motif, contained four ORFs with variable-size repeat regions (Afu3g08990, Afu2g05150 [*MP-2*], Afu4g09600, and Afu6g14090; ETANDEM scores of 432, 301, 292, and 134, respectively) (Fig. 1A), whereas six group 1 ORFs with lower ETANDEM scores (Afu4g03240, Afu7g00970, Afu3g00270, Afu1g16190, Afu3g00880, and Afu2g07800; ETANDEM scores of 72, 49, 46, 45, 42, and 35, respectively) showed no size variability (Table 1 and data not shown). Group 2 ORFs, having only a leader sequence, contained three ORFs with variable-size repeat regions (Afu6g06670, Afu2g03540, and Afu5g06880; ETANDEM scores of 113, 88, and 75, respectively) (Table 1; see Fig. S1A in the supplemental material). Group 3 ORFs, lacking both motifs, contained seven ORFs with variable-size repeat regions (Afu7g08290, Afu7g08310, Afu6g09340, Afu8g06150, Afu6g09360, Afu5g15090,

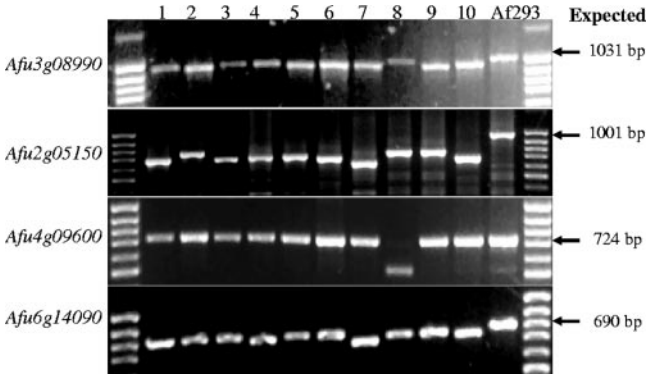


FIG. 2. The variable-size ORFs containing a leader sequence and GPI anchor are expressed genes and are transcribed into variable-size mRNA. RT-PCR amplification of the TR region in Afu3g08990, Afu2g05150, Afu4g09600, and Afu6g14090 ORFs was performed with mRNA prepared from the 11 clinical *A. fumigatus* isolates after 12 h of growth. The results reveal the same pattern of size variability in mRNA molecules as at the genomic DNA level of the respective genes.

and Afu7g07060; ETANDEM scores of 670, 400, 388, 232, 226, 218, and 190, respectively) (Table 1; see Fig. S1B in the supplemental material). Group 1 ORFs, containing both a leader sequence and GPI anchor motif, showed a significant correlation between a high ETANDEM score and size variability (correlation coefficient $r = 0.869$, $P < 0.01$), whereas group 2 and 3 ORFs did not ($P > 0.1$).

We next focused on the analysis of group 1 ORFs with variable-size repeat regions, Afu3g08990, Afu2g05150 (*MP-2*), Afu4g09600, and Afu6g14090 containing a leader sequence and GPI anchor motif in greater detail, because they might potentially encode cell surface proteins.

Afu3g08990, Afu2g05150, and Afu6g14090 ORFs show repeat size variability in environmental isolates of *A. fumigatus*. To assess whether the variability in size of the TR region in group 1 ORFs is unique to the patient isolates of *A. fumigatus* as opposed to environmental isolates, we performed a PCR analysis of genomic DNA obtained from 13 environmental *A. fumigatus* isolates, using the same repeat-spanning primer sets described above. We observed size variability in three of the four ORFs (Afu3g08990, Afu2g05150, and Afu6g14090) but not in Afu4g09600 (Fig. 1B). This suggests that (i) the size variation of TRs in these genes is not a specific attribute of clinical *A. fumigatus* isolates and that (ii) the size variation seen in Afu4g09600 in the single patient isolate (isolate 8) is probably a relatively rare occurrence (one size variant in 24 isolates tested in total). Notably, microsatellite typing of clinical and environmental *A. fumigatus* isolates has also established that there are no geographical or temporal associations of clinical or environmental genotypes (2, 28).

Afu3g08990, Afu2g05150, Afu4g09600, and Afu6g14090 are expressed genes and produce variable-size mRNA. To verify that the variability pattern is conserved at the mRNA level, an RT-PCR assay was performed on the four ORFS in group 1 exhibiting variability at the genomic DNA level. Figure 2 shows that the same pattern of variability seen at the genomic DNA level in the repeat regions of Afu3g08990, Afu2g05150, Afu4g09600, and Afu6g14090 is also found at the mRNA transcript level. This strongly suggests that the protein products of these genes also show variability among the clinical isolates.

Afu3g08990, Afu2g05150, Afu4g09600, and Afu6g14090 undergo expansion or contraction of discrete repeat units. To assess whether the size variability results from expansion or contraction of discrete repeats, we sequenced the reverse-transcribed mRNA from the most variable clinical isolates and the Af293 control strain with primers flanking the repeat regions of the four genes (Fig. 3; see Fig. S2 in the supplemental material). The results indicate that size variability is due to expansion or contraction of discrete in-frame coding repeats, so that deletion or addition of repeat units does not alter the reading frame.

Changes in mRNA levels of Afu3g08990, Afu2g05150, Afu4g09600, and Afu6g14090 during germination and growth of Af293. We performed RT-PCR on the four variable-size GPI-anchored repeat-containing genes with primers flanking the repeat region (Fig. 4). Afu6g14090 mRNA is present throughout the life cycle of the mold, including the dormant

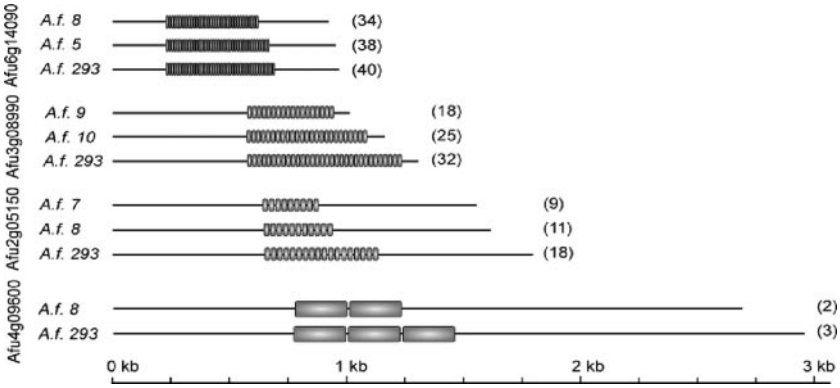


FIG. 3. Afu3g08990, Afu2g05150, Afu4g09600, and Afu6g14090 ORFs undergo expansion or contraction of discrete repeat units. The cDNA sequences of the variable-size TR regions in Afu6g14090, Afu3g08990, Afu2g05150, and Afu4g09600 show discrete changes in the number of repeated sequence units. The Afu6g14090 ORFs of *A. fumigatus* (*A.f.*) isolates 8 and 5 and *A. fumigatus* strain Af293 (293) contain 34, 38, and 40 repeats, respectively (repeat numbers are in parentheses to the right of the maps). The Afu3g08990 ORFs of isolates 9 and 10 and Af293 contain 18, 25, and 32 repeats, respectively. The Afu2g05150 ORFs of isolates 7 and 8 and Af293 contain 9, 11, and 18 repeats, respectively. The Afu4g09600 ORF of isolate 8 contains two repeats, whereas that of strain Af293 contains three repeats.

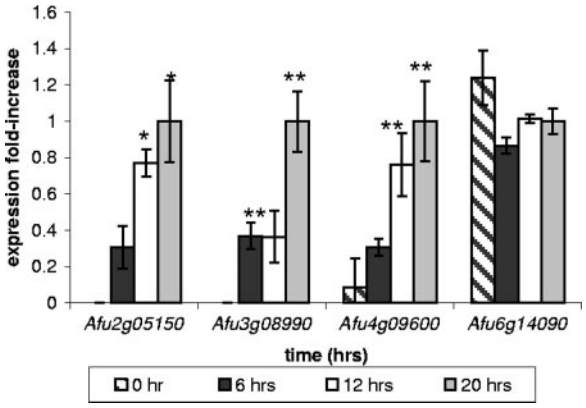


FIG. 4. mRNA levels of Afu3g08990, Afu2g05150, Afu4g09600, and Afu6g14090 ORFs during conidial germination and hyphal growth. Gene expression was examined at different time points (0, 6, 12, and 20 h) in *A. fumigatus* strain Af293 by RT-PCR. Afu6g14090 was consistently expressed (0 to 20 h). Afu2g05150, Afu3g08990, and Afu4g09600 were expressed at low or undetectable levels in dormant conidia (0 h) and were upregulated during conidial germination and hyphal growth (6 to 20 h). Expression was normalized to *gpdA*, a housekeeping gene that was constitutively expressed throughout growth. Final values were derived by calculating the ratio between the normalized experimental and 20-h time points. The values are the means ± standard deviations (error bars) for three wells. Values that are significantly different from the value for the 0-h time point are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$).

conidium stage (0 h). Afu3g08990, Afu4g09600, and Afu2g05150 mRNAs are present at undetectable or low levels in dormant conidia (0 h) and at increasing levels during germination (6 h) and hyphal growth (12 to 24 h).

Changes in mRNA levels of Afu3g08990, Afu2g05150, Afu4g09600, and Afu6g14090 in response to agents that disrupt the cell wall and plasma membrane. We reasoned that genes involved in cell wall architecture or maintenance would be differentially expressed during cell wall stress. We therefore assessed the expression of Afu3g08990, Afu2g05150, Afu4g09600, and Afu6g14090 in the Af293 control strain in response to growth in the presence of reagents that disrupt the fungal cell wall (caspofungin, Congo red, calcofluor, or β-glucanase) or plasma membrane (amphotericin B, itraconazole, or SDS) or induce oxidative stress (H_2O_2). For a positive control, we measured *MDR1* expression, which has been previously shown to increase in the presence of azoles (7) (Fig. 5). The results indicate a reduction in mRNA levels for the following ORFs and conditions: Afu2g05150 with caspofungin, β-glucanase, amphotericin B, and H_2O_2 and Afu3g08990 and Afu4g09600 with Congo red, amphotericin B, and H_2O_2. Increased mRNA levels were observed for the following ORFs and conditions: Afu2g05150 and Afu3g08990 with itraconazole and SDS, Afu4g09600 with itraconazole, and Afu6g14090 with amphotericin B (Fig. 5). As expected, *MDR1* mRNA levels were strongly elevated in the presence of the azole itraconazole. Our results indicate that Afu2g05150, Afu3g08990, Afu4g09600, and Afu6g14090 mRNA levels increase in response to several of the membrane-destabilizing agents. In contrast, Afu2g05150, Afu3g08990, and Afu4g09600 mRNA levels decrease in response to several of the cell wall-destabilizing agents and in response to amphotericin B and H_2O_2.

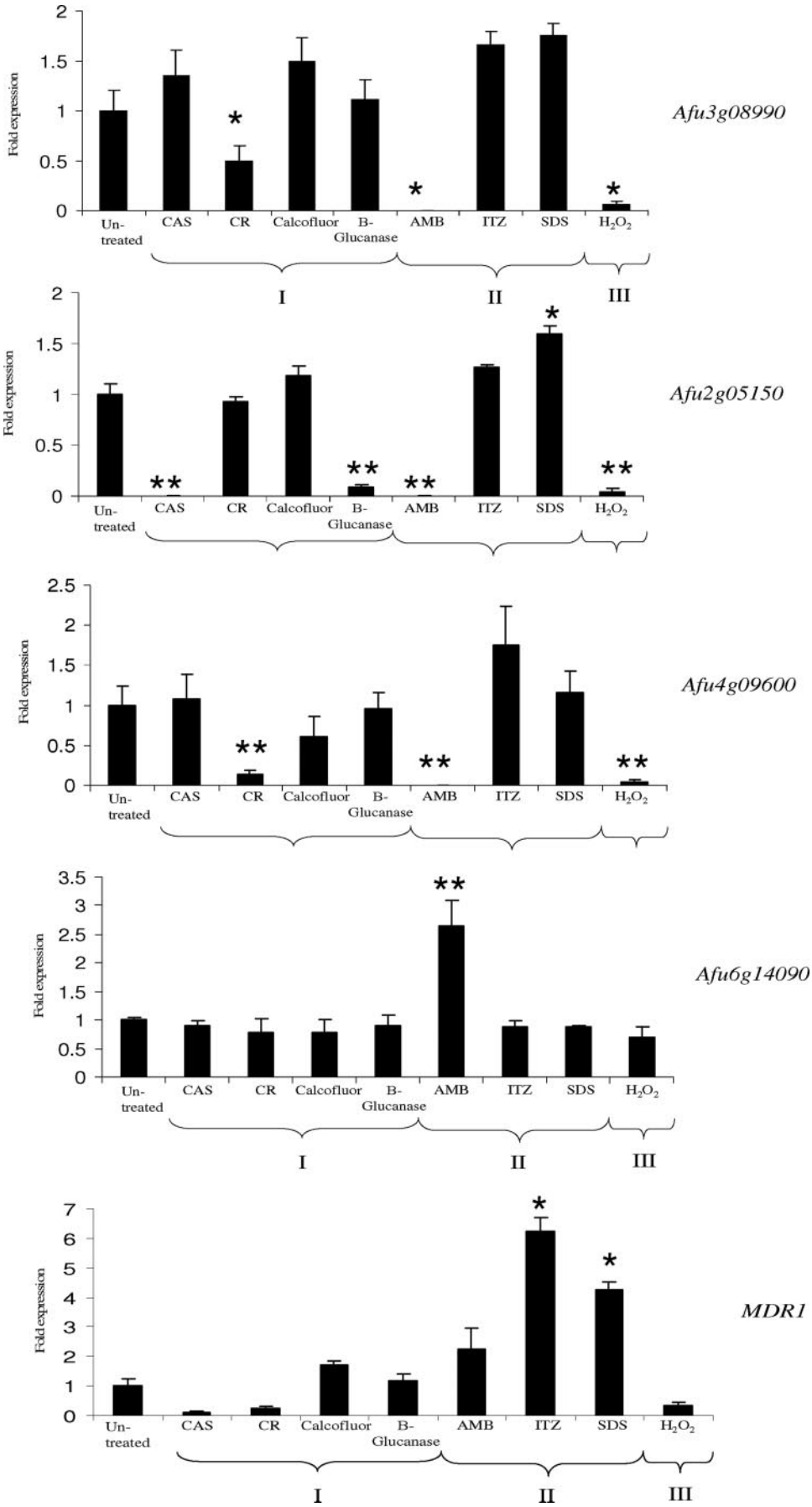
Deletion of Afu3g08990 results in rapid germination and decreased adherence of conidia. We deleted the Afu2g05150, Afu3g08990, and Afu6g14090 genes to determine their function. A clear mutant phenotype was observed following deletion of the Afu3g08990 gene, and it will be described in this report. The Afu3g08990 gene was deleted as described in Materials and Methods (Fig. 6A). After transformation of p*Afu3g08990*-D into strain Af293, 20 hygromycin-resistant transformants were purified and screened with PCR for putative insertion mutants. Three putative mutants were identified and further characterized by Southern blot analysis (Fig. 6B). Based on these analyses, all three transformants were disrupted in the Afu3g08990 gene alone (Afu3g08990 D1 to D3). An Afu3g08990-reconstituted strain (Afu3g08990 KI-1) was prepared as described in Materials and Methods and used as a control throughout this study.

The Afu3g08990 D1 to D3 mutant strains grew normally on rich YAG medium or defined MM agar plates, indicating that the Afu3g08990 gene is not essential for growth (data not shown). Microscopic analysis in liquid MM demonstrated that the Afu3g08990 D1 to D3 strains germinate earlier than the wild-type, 8990-RI (random insertion) and Afu3g08990 KI-1 reconstituted strains (Fig. 7A).

To determine whether *A. fumigatus* Afu3g08990 D1 to D3 conidia show altered adherence to extracellular matrix (ECM) proteins produced by host organisms, the relative levels of conidial binding to polystyrene (uncoated plastic control), laminin (a constituent of the ECM), and intact A549 lung ECM were compared to wild-type and random insertion cells. Conidia from the three Afu3g08990-deleted strains (Afu3g08990 D1 to D3) showed an approximately twofold reduction in adhesion to ECM compared to those from the wild-type Af293, Afu3g08990 KI-1 reconstituted, and 8990-RI strains (Fig. 7B). No differences were seen in the ability of the various strains to adhere to polystyrene or laminin (data not shown).

The Afu3g08990 protein is localized to the cell wall. To determine the subcellular localization of the Afu3g08990 protein, we generated the *A. fumigatus* strain Afu3g08990-myc1 expressing a *myc*-tagged version of the gene under its endogenous promoter (see Materials and Methods for details). Immunofluorescence analysis was performed on Afu3g08990-myc1 dormant conidia (0 h) and conidia germinated for 8 h on coverslips and analyzed by indirect fluorescence using c-*myc*-specific monoclonal antibodies. Afu3g08990 protein was labeled in the cell walls of dormant and germinating conidia and in the hyphal tips of strain Afu3g08990-myc1, whereas no labeling was seen in the control strain Af293 (Fig. 8).

Deletion of Afu3g08990 does not affect virulence in a murine model for disseminated aspergillosis. To determine whether Afu3g08990 is involved in pathogenicity, we tested the Afu3g08990 D1 strain for virulence in an immunocompromised murine model for disseminated aspergillosis. For a control, we included Af293, the parental *A. fumigatus* strain. Freshly harvested conidia were carefully counted and adjusted to the same density (2.5×10^5 conidia/mouse) before injection into the lateral tail vein. The number of mice alive in each group ($n = 12$) was reported every day over a 21-day study period. Figure S3 in the supplemental material shows survival curves obtained during the course of the experiment. The results indicate that there were no significant



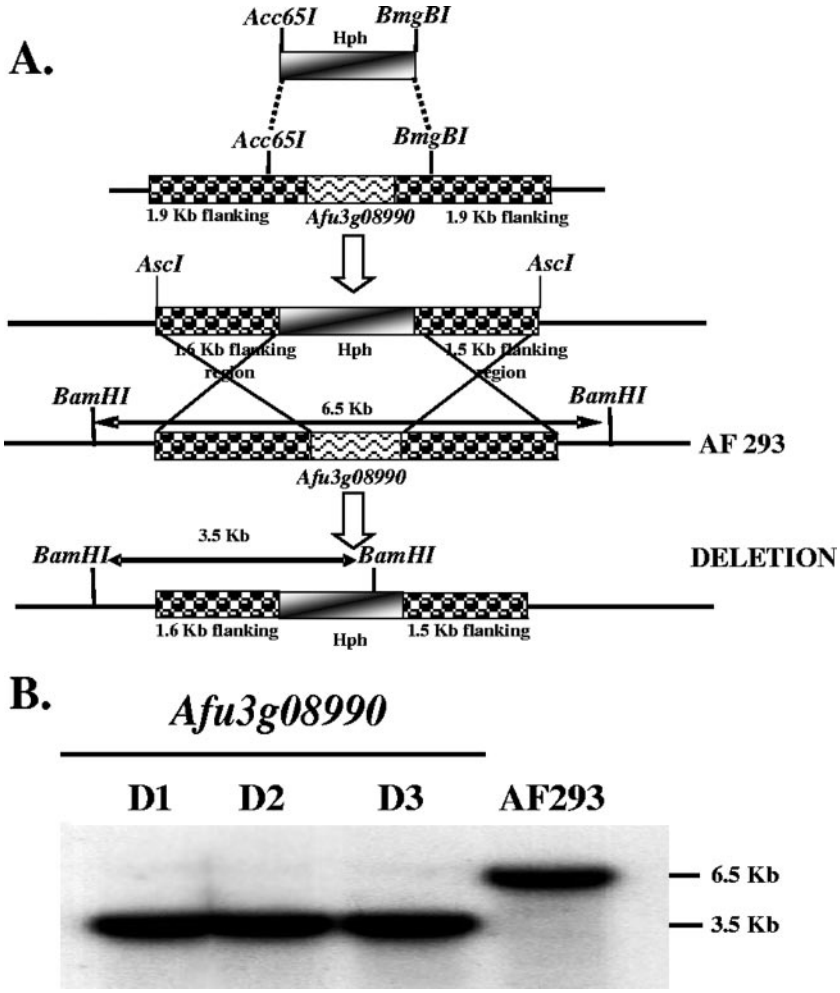


FIG. 6. Disruption of the Afu3g08990 gene in *A. fumigatus*. (A) Schematic representation of the Afu3g08990 wild-type locus and the plasmid p*Afu3g08990*-D AscI-cut insert used for disruption. Hph, hygromycin cassette. (B) Southern blot verification of the *A. fumigatus* Afu3g08990 D1 to D3 isolates and control Af293 wild-type strain. For the Southern analysis, genomic DNA (10 μg/well) was digested with BamHI and hybridized with ^{32}P-labeled Afu3g08990-5′ DNA probe, resulting in ~3.5-kb and 6.5-kb fragments for the *A. fumigatus* Afu3g08990-disrupted and Af293 control strain, respectively.

survival differences between the mouse groups infected with the wild-type Af293 and the Afu3g08990-disrupted *A. fumigatus* strains ($P = 0.69$). It is important to note that virulence genes are often redundant, so that deletion of a single gene often does not result in significant changes in pathogenicity.

DISCUSSION

In this study we analyzed the entire *A. fumigatus* genome for ORFs containing coding TR regions and assessed the variability in the lengths of these regions in different isolates. Coding repeat regions have been previously implicated in generating

FIG. 5. Changes in the mRNA levels of Afu3g08990, Afu2g05150, Afu4g09600, and Afu6g14090 ORFs in response to cell wall-disrupting reagents. *A. fumigatus* strain Af293 was grown in the presence of reagents that (I) disrupt the fungal cell wall (caspofungin [CAS], Congo red [CR], calcofluor, or β-glucanase [B-glucanase]) or (II) the plasma membrane (amphotericin B [AMB], itraconazole [ITZ], or SDS) or (III) induce oxidative stress (H_2O_2). mRNA levels of Afu3g08990, Afu2g05150, Afu4g09600, and Afu6g14090 were examined. *MDR1* expression, which is activated in the presence of azoles, was used as a positive control (7). Afu2g05150, Afu3g08990, and Afu4g09600 expression was strongly reduced in the presence of the antifungal drug AMB and in response to oxidative stress (H_2O_2). Afu2g05150, Afu3g08990, and Afu4g09600 expression increased in response to membrane damage by either SDS or ITZ. Expression was normalized to the constitutively expressed housekeeping gene *gpdA*. The values are the means ± standard deviations (error bars) for three wells. Values that are significantly different from the value for the untreated cells are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$).

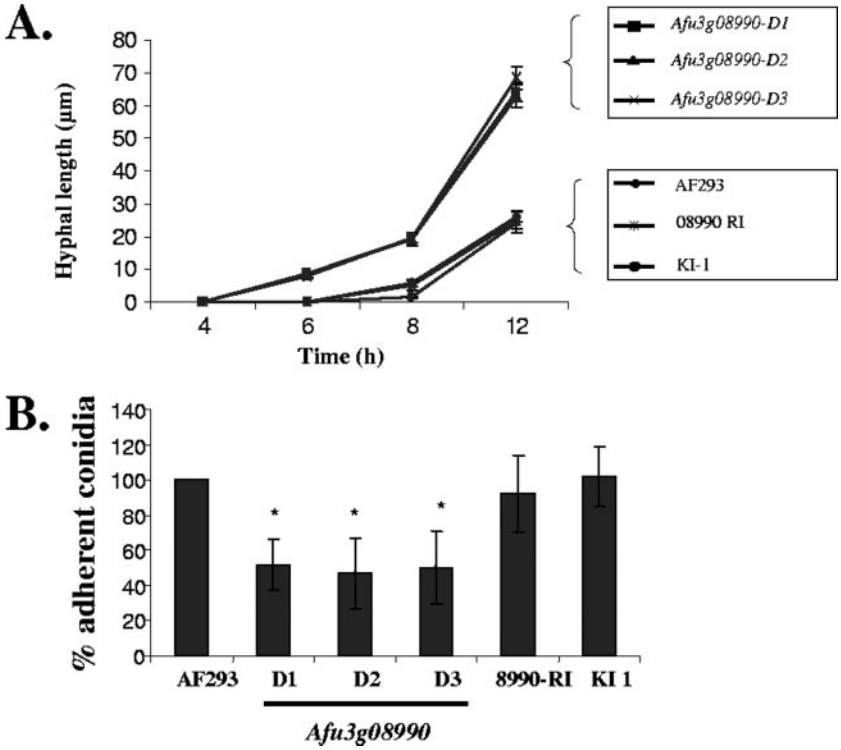


FIG. 7. Rapid germination and reduced adherence of the Afu3g08990-deleted *A. fumigatus* strain. (A) Quantitative analysis of mutant hyphal growth. Each time point is calculated as the mean ± standard error (error bar) of 50 hyphae. (B) Reduced adherence of the Afu3g08990-deleted strains D1 to D3 compared to the wild-type Af293 strain, random integrant 8990-RI, and reconstituted KI-1 strains. The results shown are the means plus standard deviations (error bars) for four independent experiments. Values that are significantly different from the value for the wild-type Af293 strain are indicated by asterisks (*, $P < 0.05$).

functional variation and diversity in a wide range of organisms, including humans, yeasts, and bacteria (35). Emphasis was placed on the analysis of repeat-containing ORFs having a leader sequence and GPI anchor because of their possible localization to the plasma membrane and cell wall and their potential involvement in pathogenesis (22, 35, 36).

***A. fumigatus* genes undergo expansion and contraction of coding repeat regions.** Our main findings were that expansion and contraction of coding TR regions occur in *A. fumigatus* genes and that a significant proportion of those genes encode putative cell surface proteins. Out of 30 selected ORFs with high ETANDEM scores, 14 (47%) showed variability in repeat

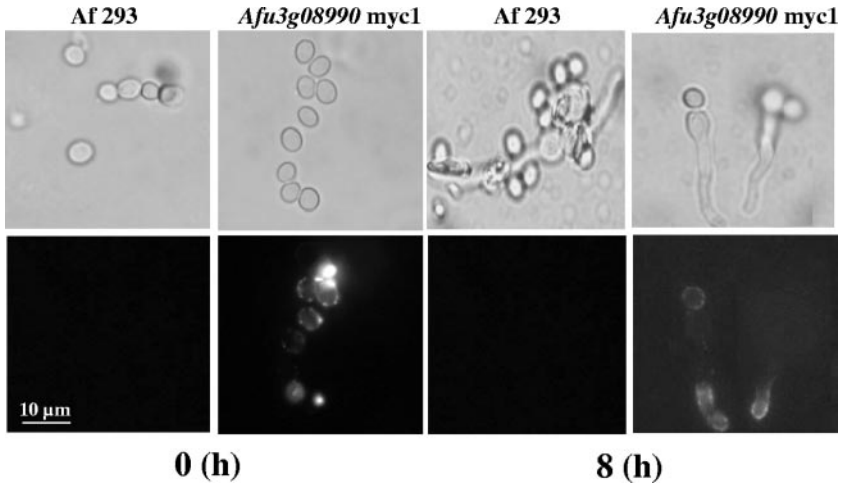


FIG. 8. The Afu3g08990 protein is localized to the cell wall. Immunofluorescence analysis of Afu3g08990 protein localization in freshly harvested, ungerminated (0 h) and germinated (8 h) conidia expressing Afu3g08990-myc. No immunofluorescence is detectable in untransformed control wild-type (Af293) cells. (Top) Cells viewed by light microscopy; (bottom) Afu3g08990-myc-tagged fluorescent images.

number among independent *A. fumigatus* patient isolates and four (28%) encoded putative cell surface proteins.

A significant number of *A. fumigatus* genes undergoing expansion and contraction of coding repeat regions encode putative cell wall proteins. Four of the 14 variable-size TR genes (28%) (Afu3g08990, Afu4g09600, Afu2g05150, and Afu6g14090) contain a leader sequence and putative GPI anchor motif, suggesting that they are localized at the plasma membrane or cell wall. This is a significantly high proportion, considering that this category makes up only 0.8% of the ORFs identified in the *A. fumigatus* genome (21). We demonstrate that all four are expressed genes and are transcribed into variable-size mRNAs and presumably proteins. Three of the four genes (Afu3g08990, Afu6g14090, and Afu4g09600) are of unknown function and have not been previously described. Afu3g08990 encodes a hypothetical protein conserved specifically in the aspergilli. It contains a variable 6-amino-acid repeat (P-G/S/E-QPS-V/A) showing significant homology to repeats found in the immunoglobulin A-binding beta antigen of *Streptococcus agalactiae* and to the extended rod domain of mammalian type XXI collagen. This variable repeat-rich region was recently successfully used to subtype 55 outbreak isolates of *A. fumigatus*. The method was able to identify "clonal" and genotypically distinct *A. fumigatus* isolates, and results were concordant with another discriminatory genotyping technique, the *Afut1* restriction fragment length polymorphism typing method (companion paper [1a]). Afu2g05150 (*AFMP2*) encodes an immunogenic protein (Afmp2p) of unknown function belonging to the antigenic mannoprotein superfamily (4). It contains a variable-size Ser/Thr/Pro-rich repeat region (repeat region rich in serine, threonine, and proline [Ser/Thr/Pro]) (amino acid residues 239 to 368) composed of a 13-amino-acid repeat. Afmp2p is found in the cell wall and culture medium of *A. fumigatus*. Patients with aspergilloma and invasive aspergillosis develop a specific antibody response against this protein (4). The fourth leader- or GPI anchor-containing ORF, Afu6g14090, has an N-terminal CFEM domain (amino acid residues 18 to 85) adjacent to the variable-size Ser/Thr/Pro-rich repeat region (amino acid residues 140 to 219). CFEM is a fungus-specific eight-cysteine-containing domain. Some CFEM-containing proteins, such as the Pth11p receptor from *Magnaporthe grisea* and the Rbt5p plasma membrane-anchored heme-binding protein in *Candida albicans*, are proposed to play important roles in fungal pathogenesis (15, 38).

The repeat regions of the putative *A. fumigatus* cell wall proteins are rich in serine, threonine, and proline amino acid residues. All four of the leader or GPI anchor repeat variable-size genes have repeat units that are unusually rich in Ser, Thr, and Pro amino acid residues (51% of the residues in the protein encoded by Afu3g08990 are Ser/Thr/Pro; Afu4g09600 protein, 26%; Afu2g05150, 82%; and Afu6g14090, 54%). Ser/Thr-rich repeat regions are characteristic of fungal cell surface proteins and in particular adhesins, such as *C. albicans ALS*, *Candida glabrata EPA1*, and *S. cerevisiae FLO* genes (34, 36). They are proposed to adopt an extended rod-like structure by virtue of Ser/Thr-linked O glycosylation, acting as a stalk to present the N-terminal ligand-binding domain at the cell wall surface (8). Several mammalian proteins, including the mucins and human glycoprotein Ib alpha, also contain Ser/Thr/Pro-rich repeat regions. Like the fungal adhesins, the mucin repeat domains are extensively O glycosylated, making these proteins protease resistant, highly hydrated, and antigenic (3).

Deletion of the leader or GPI anchor repeat-containing variable-size gene Afu3g08990 results in a mutant phenotype characterized by rapid conidial germination and reduced adherence to ECM. Deletion of Afu3g08990 resulted in a clear mutant phenotype characterized by rapid conidial germination and reduced adherence to ECM. The early germination of the Afu3g08990-deleted mutant compared to the wild-type Af293 strain is intriguing. Early germination has been previously shown in a mutant lacking *A. fumigatus* Ecm33, a GPI-anchored cell wall protein involved in cell wall integrity and virulence (27). A possible explanation for this phenotype is that deletion of *A. fumigatus* Ecm33 and Afu3g08990 leads to the formation of a softer, more pliable cell wall, enabling germination to proceed more rapidly. Strikingly, we observed reduced adhesion of Afu3g08990-deleted conidia to lung cell ECM compared to the wild-type Af293 strain. In contrast, the mutant adhered normally to polystyrene and laminin, suggesting some specificity in the alteration of the conidial surface. Alveolar ECM is a specialized ECM composed of laminin, type IV and V collagen, entactin, chondroitin sulfate proteoglycan, heparan sulfate proteoglycan, and fibronectin (6). Our results suggest that Afu3g08990-deleted conidia are impaired in their ability to adhere to one of these proteins or to a combination of these proteins. Decreased adhesion of *A. fumigatus* conidia following targeted gene deletion has been reported for only one gene. Mutant conidia lacking the outer conidial cell wall protein RodAp show decreased adhesion to laminin and BSA (33). The reduced conidial adhesion we have observed following Afu3g08990 gene deletion may be an indirect result of changes to the cell wall architecture or it may suggest a more direct role for the Afu3g08990 protein in adhesion.

The protein encoded by Afu3g08990 is localized at the cell wall. To enable us to visualize the protein encoded by the Afu3g08990 gene, we labeled it with a *myc* epitope tag downstream of the leader sequence. Using *myc* tag-specific antibodies and immunofluorescence microscopy, we show that the Afu3g08990 protein is localized to the cell walls of dormant conidia. During germination, the protein is found in the spore cell wall and at the emerging hyphal tip. These results suggest that it may play a role in both conidial adhesion and germination as well as in the later stages of polarized hyphal growth.

Deletion of Afu3g08990 does not alter fungal virulence. We demonstrate that Afu3g08990 gene deletion does not result in altered virulence in an immunocompromised murine model for disseminated aspergillosis. Assuming that the mutant phenotype observed in vitro is maintained in vivo, this suggests that the ability to germinate more rapidly or the reduction in adherence does not directly affect virulence or that the changes cancel out each other. Moreover, analysis of model organisms, such as *S. cerevisiae*, shows that adhesion genes are often members of large gene families with partially redundant function (34). Hence, deletion of a single gene often does not result in severe changes in virulence. Additional animal models and in particular a murine model for pulmonary aspergillosis will be tested to further assess the virulence of the Afu3g08990-deleted mutant.

In summary, this work describes the identification of all ORFs in the *A. fumigatus* genome that contain internal tandem

repeats. Analysis of 30 repeat-containing ORFs in clinical isolates of *A. fumigatus* showed that about 50% of the repeats are variable, with some variations leading to large changes in the size of the mRNA and thus, the corresponding protein. Hence, as is the case in some prokaryotic pathogens, intragenic repeats may serve as hypervariable elements that confer cell surface variability on the pathogenic fungus *A. fumigatus*, allowing fast adaptation during infection and/or immune evasion. We show that the small group of putative cell surface genes (as identified by the presence of leader and GPI sequences) is significantly enriched in intragenic repeats. Deletion of one of these genes, Afu3g08990, which encodes a protein localized at the cell wall, results in rapid germination and decreased adherence to ECM, suggesting it plays a role in defining cell surface properties.

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