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In Vivo Analysis of *Aspergillus fumigatus* Developmental Gene Expression Determined by Real-Time Reverse Transcription-PCR▽

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Very little is known about the developmental stages of *Aspergillus fumigatus* during invasive aspergillosis. We performed real-time reverse transcription-PCR analysis on lung samples from mice with invasive pulmonary aspergillosis to determine the expression of *A. fumigatus* genes that are expressed at specific stages of development. In established infection, *A. fumigatus* exhibited mRNA expression of genes specific to developmentally competent hyphae, such as *stuA*. In contrast, mRNA of genes expressed by conidia and precompetent hyphae was not detected. Many genes required for mycotoxin synthesis, including *aspHS*, *gliP*, *mitF*, and *metAP*, are known to be expressed by developmentally competent hyphae in vitro. Interestingly, each of these genes was expressed at significantly higher levels during invasive infection than in vitro. The expression of *gliP* mRNA in vitro was found to be highly dependent on culture conditions. Furthermore, *gliP* expression was found to be dependent on the transcription factor StuA both in vitro and in vivo. Therefore, developmentally competent hyphae predominate during established invasive infection, and many mycotoxin genes are expressed at high levels in vivo. These results highlight the importance of the evaluation of putative virulence factors expressed by competent hyphae and analysis of gene expression levels during invasive infection rather than in vitro alone.

Aspergillus fumigatus is a ubiquitous saprophytic fungus that produces abundant airborne spores (conidia). Invasive infection with this organism can develop in patients who are immunocompromised from a variety of causes including chemotherapy, bone marrow or solid-organ transplantation, tumor necrosis factor inhibitor therapy, or underlying hematologic malignancy. In such patients, invasive aspergillosis is the most common filamentous fungal infection and is associated with a mortality rate of 30% to 90% (7, 10, 14, 17).

The typical portal of entry for *A. fumigatus* is the respiratory tract. Airborne conidia are inhaled and, because of their small size (2 to 3 μm), are deposited in the alveoli. In the absence of a robust immune response, these conidia germinate and invade the lung tissue before disseminating to other deep organs. Hyphae are the only form of the organism observed during invasive infection (12, 17). Surprisingly, little is known about the developmental cycle of *A. fumigatus* in vivo. In vitro, *A. fumigatus* conidia germinate to produce hyphae, which are initially unable to produce asexual reproductive structures in response to stimuli. After a fixed time period after germination, these hyphae become able to produce asexual reproduction structures (1). This shift from a state in which hyphae cannot undergo asexual reproduction to one in which they can is termed the acquisition of developmental competence. The time to acquisition of developmental competence is affected by temperature but is independent of nutrient status (25). Although mutagenesis studies in *Aspergillus nidulans* have identified mutants with a delayed onset of developmental competence, the specific genes controlling this developmental event have not yet been identified (3). While precompetent and competent hyphae are indistinguishable morphologically, the transition to developmental competence coincides with a coordinated alteration in the expression of over 400 genes in vitro (21). While some of the genes expressed by competent hyphae govern the production of conidiophores, many putative virulence factors are upregulated in response to the onset of developmental competence, including genes encoding mycotoxins, proteases, and allergens. In vitro, the transcription factor StuA plays a central role in governing the expression of these competence-associated genes. However, the deletion of *stuA* does not impair the acquisition of developmental competence (21). It was previously unknown whether hyphae become developmentally competent in vivo or if the *stuA* developmental pathway is active during invasive infection.

The determination of developmental competence is performed in vitro by exposing hyphae to conidiation signals and measuring the delay to the formation of conidiophores. Classically, this involves subculturing hyphal mats from liquid culture to an agar surface and visually observing the production of conidiophores (3). Given the few hyphae present during pulmonary infection, this method is not feasible for the determination of the timing of developmental competence in vivo. However, we recently performed whole-genome transcriptional analysis of *A. fumigatus* during the acquisition of developmental competence and have identified a subset of genes whose expression is specific to pre- or postcompetence (21).

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The expression of these genes can therefore provide a useful surrogate measure for determining the onset of developmental competence in vivo.

Here, we describe a method to analyze gene expression in vivo in the mouse model of invasive aspergillosis. Using this technique, we demonstrated that *A. fumigatus* hyphae express competence-associated but not precompetence-associated genes during invasive infection. We confirmed the mRNA expression of the developmental regulator *stuA* and of *stuA*-dependent genes in vivo (21). Finally, we analyzed the in vivo expression of a selection of genes essential for the biosynthesis of *A. fumigatus* mycotoxins and identified significant differences between their expression in vitro and their expression in vivo.

MATERIALS AND METHODS

Strains and media. *A. fumigatus* strain Af293, a clinical isolate, was a generous gift from P. T. Magee, University of Minnesota. The Δ*stuA* mutant and the complemented strain were described previously (21). Strains were grown on Sabouraud dextrose agar plates for 10 days at 37°C, and conidia were collected by flooding the plates with sterile phosphate-buffered saline containing 0.2% (vol/vol) Tween 80 (Sigma-Aldrich, St. Louis, MO). The conidia were concentrated by centrifugation and counted using a hemacytometer. For in vitro studies, 100-ml YPD (1% yeast extract, 2% peptone, 2% glucose) cultures were inoculated with 1×10^6 conidia/ml and grown in a shaking incubator for the indicated times at 37°C. Under these growth conditions, developmental competence occurs reproducibly between 10 and 12 h (21).

Animal experiments. Male BALB/c mice (National Cancer Institute, Bethesda, MD), 18 to 22 g, were used for these experiments. All experiments involving mice were approved by the institutional animal care and use committee according to the National Institutes of Health guidelines for animal housing and care. Mice were immunosuppressed at day −2 and day +3 (relative to infection) by intraperitoneal injection with cyclophosphamide (Western Medical Supply, Arcadia, CA) at 250 mg/kg and 200 mg/kg, respectively as well as with 250 mg/kg cortisone acetate (Sigma-Aldrich) subcutaneously on both days. Two methods of infection were used. For the study of later time points, mice were infected in an inhalational chamber by aerosolized conidia (1.2×10^{10} conidia), which resulted in an average inoculum of 2.4×10^3 conidia per mouse as described previously (22). For analysis of gene expression by the Δ*stuA* mutant and for testing gene expression at early time points, an intranasal model of infection was used because the Δ*stuA* mutant produced insufficient conidia for inoculation in the aerosol chamber (21). Therefore, for these experiments, mice were anesthetized with isofluorane (Western Medical Supply) and then infected by the intranasal instillation of a 25-μl volume containing 5×10^5 conidia of the various strains. All mice received ceftazidime (Western Medical Supply) at 5 mg per day subcutaneously to protect against bacterial infection. For all in vivo experiments, animals were sacrificed for gene expression studies only during the period where 90% of infected animals remained alive in order to avoid healthy survivor bias.

Isolation of total RNA. RNA was isolated from in vitro cultures as described previously (19). Briefly, *A. fumigatus* mycelia were harvested by filtration through Whatman filter paper, washed with sterile water, and then ground under liquid nitrogen using a mortar and pestle. Total RNA was isolated from ground mycelia using the RNeasy Plant Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. To extract total RNA from in vivo samples, lungs from infected mice were removed and immediately frozen in liquid nitrogen. Frozen lungs were then ground under liquid nitrogen, and the powder was resuspended in 600 μl TES buffer (1 mM Tris-HCl, 1 mM EDTA, 0.5% sodium dodecyl sulfate). Total RNA was then isolated using the hot phenol method. Briefly, after adding 600 μl acidic phenol, the solution was incubated for 30 min at 65°C and then cooled on ice for 5 min. The suspension was centrifuged at 16,000 × *g* for 3 min in a microcentrifuge, and the aqueous phase was transferred into a new tube. After phenol-chloroform extraction, the RNA was precipitated with LiCl, suspended in RNase-free treated water, ethanol precipitated, and finally resuspended in 60 μl RNase-free water.

Real-time RT-PCR. Contaminating genomic DNA was removed from RNA samples by treatment with 1 μl of Turbo-DNase (Ambion, Austin, TX) for 30 min at room temperature. DNase was then removed using an RNA Clean-Up kit (Zymo Research, Orange, CA). First-strand cDNA synthesis was performed using the Retroscript first-strand synthesis kit (Ambion). Gene-specific primers for expression analysis were designed with the assistance of online primer design software (Genscript, Piscataway, NJ). These primers are listed in Table 1. Whenever possible, the primers spanned an intron. For each primer pair, the amplification efficiency was determined by serial dilution experiments, and the resulting efficiency coefficient was used for the quantification of the products (20). Gene expression was analyzed with 500 nM primers by using the QuantiTect Sybr green PCR kit (Qiagen) and an ABI Prism 7000 thermocycler. Cycle conditions were 10 min at 90°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. Single PCR products were confirmed with the heat dissociation protocol at the end of the PCR cycles. Except where noted, the relative quantification of the mRNA levels of the target genes was determined using the ΔC_T method (18). Briefly, the amount of target was normalized to the endogenous reference gene *TEF1*: $\Delta C_T = C_T$ (target gene) $- C_T$ (*TEF1*), where C_T represents the cycle number required to reach a defined threshold target abundance. The relative mRNA levels were calculated as $x^{\Delta CT}$ (where x is primer efficiency) (20). All reactions were performed in duplicate, and the mixture included a negative no-reverse-transcription (RT) control in which reverse transcriptase was omitted.

RESULTS

Hyphae express *stuA* mRNA and competence-associated genes in vivo. To determine the developmental status of hyphae during infection, we compared the in vitro and in vivo expression levels of *stuA* and three other genes that exhibited different regulation during the onset of developmental competence by microarray analyses in vitro (21). To ensure the detection of subpopulations of both types of hyphae, we examined two genes (*stuA* and *sspA*) that were upregulated in competent hyphae and two genes (*hsp70* and *ura7*) that were upregulated in precompetent hyphae in vitro (21). Details of these genes are presented in Table 1.

As predicted by our previous transcriptional profiling studies (21), developmentally competent hyphae grown in liquid culture displayed increased levels of expression of *stuA* and *sspA* after 24 h of culture compared to those after 4 and 8 h (Fig. 1). Conversely, the precompetence genes *hsp70* and *ura7* were strongly expressed after 4 and 8 h, but were markedly downregulated by 24 h, during the acquisition of developmental competence under these growth conditions (Fig. 1). When real-time RT-PCR was performed using samples from infected mouse lungs, the expression patterns of all four genes were similar to that of developmentally competent hyphae in vitro for all the time points examined between days 1 and 7 postinfection. Even using the higher-dose intranasal model of infection, we were unable to reliably detect mRNA from *A. fumigatus* from time points earlier than 24 h and thus cannot exclude a role for precompetent hyphae early in the establishment of disease. Collectively, these data suggest that after invasive aspergillosis is established, developmentally competent hyphae predominate, and there are few to no precompetent hyphae or conidia present.

Expression of *sspA* is StuA dependent in vitro and in vivo. The results of our previous transcriptional profiling experiments indicated that the expression of *sspA* is governed by StuA in vitro (21). Therefore, we determined if *sspA* mRNA expression was dependent on StuA in vivo using a Δ*stuA* mutant. We first verified that *sspA* expression required StuA in vitro. When the Δ*stuA* mutant was grown in liquid YPD medium, *sspA* expression was reduced after 1 day but not 3 days compared to that for the wild-type strain (Fig. 2). Complementation of the Δ*stuA* mutant with an intact copy of *stuA* restored *sspA* mRNA expression to wild-type levels. Next, we determined whether *sspA* expression was dependent on *stuA* in the

TABLE 1. Genes tested in this study and the primers used in real-time RT-PCR assays

Gene	Oligonucleotide sequence (5′–3′)[a]	Amplicon size (bp)	Gene accession no. or identifier (source or reference)
tef1	CCATGTGTGTCGAGTCCTTC (F) GAACGTACAGCAACAGTCTGG (R)	84	Afu1g06390
sspA	GACCGTCACTCTGACCTCAA (F) CTGTGGGAAGGGTAGTGCTT (R)	71	Afu8g03930 (this work)
stuA	GAGGACGAAGGGAGTCTCTG (F) ACCGTTGATCATGTGGTTGT (R)	83	Afu2g07900 (21)
hsp70	TGTCATCACCGTACCAGCTT (F) TGATGATGCGGAGAACATTT (R)	93	Afu8g03930 (this work)
ura7	CATCTTCGGCTCACAAGAGA (F) CCAAAGTGAACATCCGATTG (R)	113	Afu2g03930 (this work)
gliP	TCCAACAGTCAGAGGCATTC (F) CTTGAGGGATAATCGGTGGT (R)	103	AY838877 (13)
aspF1	TACCCGCACTGGTTCACTAA (F) GACGGTCACAGTCGGCTT (R)	94	M83781 (2)
18S rRNA	GGCCCTTAAATAGCCCGGT (F) TGAGCCGATAGTCCCCCTAA (R)	62	AB008401 (6)
mitF	AGCCGTGTCTGTTCTAGCTG (F) AGCTGTTGGTTGATGCATGT (R)	72	X58278 (16)
dmaW	GAATGGTTCTTACCGGGTTG (F) GTGTTGAATGGGTCTTGTGC (R)	118	XM_749235 (8)
metAP	GTTGTCCACGCTACGGAGTA (F) CGGTGATTCTCAGCTTCTCA (R)	132	Afu8g00410 (this work)
aspHS	AGTCCACTGGGACTGTCCAT (F) GCACCACCATACTTGTTCCA (R)	108	D16501 (11)

[a] F, forward; R, reverse.

lungs of mice with invasive aspergillosis. Surprisingly, we observed that in vivo, *stuA* deletion was associated with increased levels of expression of *sspA* after both 24 and 72 h of infection (Fig. 2B). Therefore, a factor other that StuA must also govern *sspA* expression during pulmonary infection.

Expression of mycotoxins differs in vitro and in vivo. *A. fumigatus* secretes a number of toxic molecules in vitro. However, it is unknown whether the genes encoding most of these molecules are expressed in vivo. We therefore compared the in vitro and in vivo expression levels of genes involved in the biosynthesis of six putative mycotoxins (*aspF1*, *dmaW*, *metAP*, *gliP*, *aspHS*, and *mitF*) (Table 2). The expression levels of the first three of these genes are predicted to be StuA dependent and those of the latter three genes are predicted to be StuA independent based on our previous microarray studies (21). All of the mycotoxin synthetic genes were significantly upregulated during experimental aerosol infection with strain Af293 compared with their level of expression during growth for the same period of time in liquid YPD medium (Fig. 3).

In vivo expression of *gliP* (gliotoxin) is dependent on *stuA*. The high level of expression of *gliP* by competent hyphae in vivo suggests that gliotoxin production may be linked to developmental competence. These data, however, contrast with data from our previous microarray study in which the gliotoxin biosynthetic cluster was not found to be developmentally regulated. An important point is that this study was performed using organisms grown in liquid YPD medium, in which *gliP* expression is very low (21). We therefore hypothesized that *gliP* expression may be developmentally regulated only under the appropriate permissive growth conditions. To test this hypothesis, we examined the expression of *gliP* in wild-type strain Af293, the Δ*stuA* mutant, and the *stuA*-complemented strain both in vivo and in different liquid culture media. For in vitro experiments, we tested *gliP* expression at 24 h and at 72 h in both YPD and RPMI 1640 media, the latter of which was previously shown to induce high levels of gliotoxin production and is commonly used to mimic in vivo growth (4, 15).

During in vitro growth in YPD medium, very low levels of *gliP* mRNA expression were detectable in all strains. In contrast, growth in RPMI medium induced high levels of both *stuA* and *gliP* mRNA in strain Af293 but not in the Δ*stuA* mutant, while complementation of the Δ*stuA* mutant with an intact copy of *stuA* restored both *stuA* expression and *gliP* expression (Fig. 4). Similarly, during intranasal infection, the expression levels of *stuA* and *gliP* were markedly reduced in samples from mice infected with the Δ*stuA* mutant (Fig. 4).

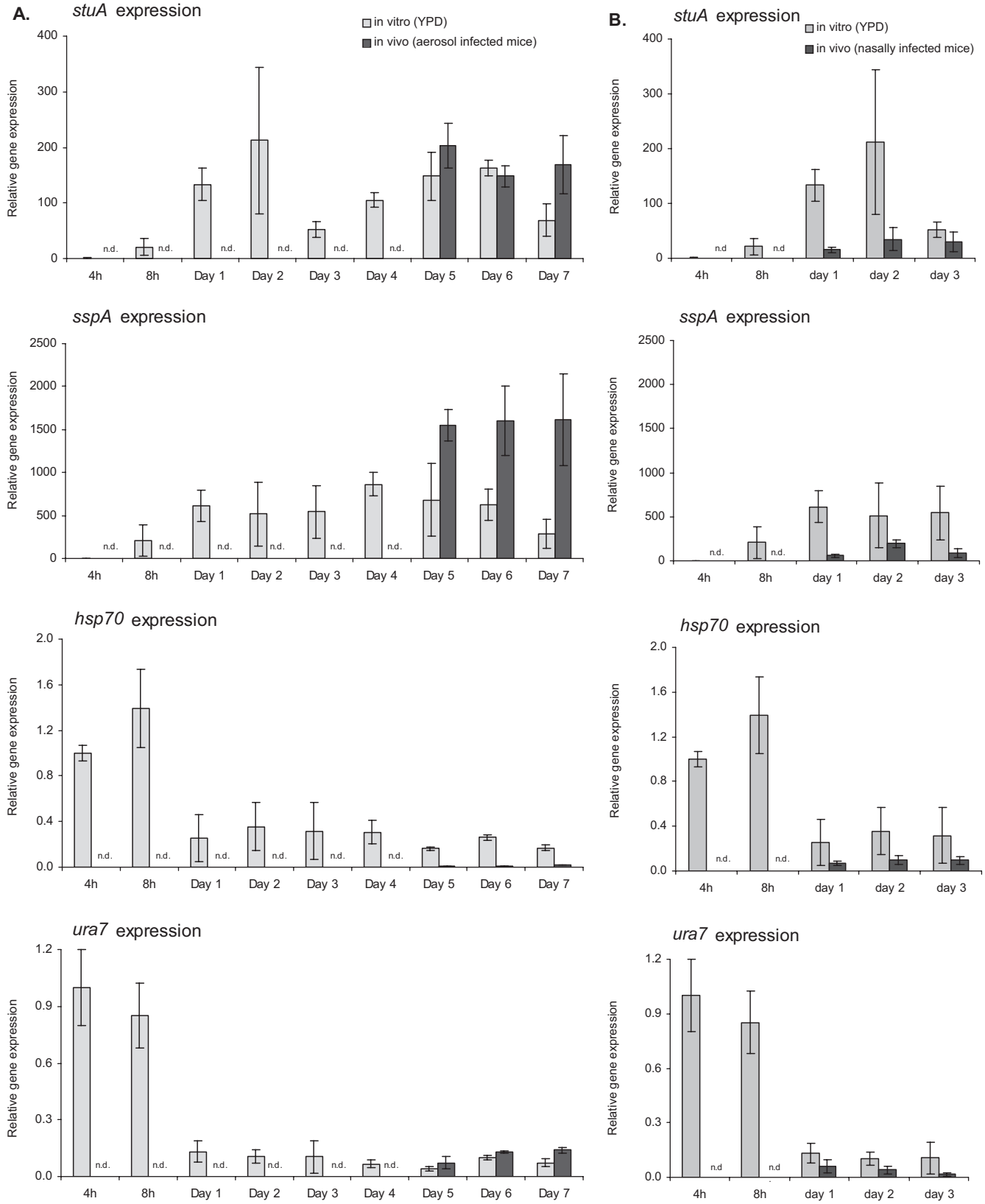

FIG. 1. Comparative in vitro and in vivo expression levels of *stuA*, *sspA*, *hsp70*, and *ura7* determined by real-time RT-PCR. RNA in vitro was derived from cultures that were grown for 4 h, 8 h, and 1 to 7 days at 37°C in YPD medium. For in vivo data, mice were infected with wild-type strain Af293 in an inhalational chamber (A) or intranasally (B). At the indicated time points, infected lungs were removed, and total RNA was then used for real-time RT-PCR analysis ($n = 3$ per time point). The expression levels of all genes were normalized to the expression level of the endogenous control gene *TEF1*. The expression value at 4 h for each gene was used as the baseline. Arrows indicate the onset of developmental competence in vitro. n.d. indicates that fungal RNA was not detected at this time point.

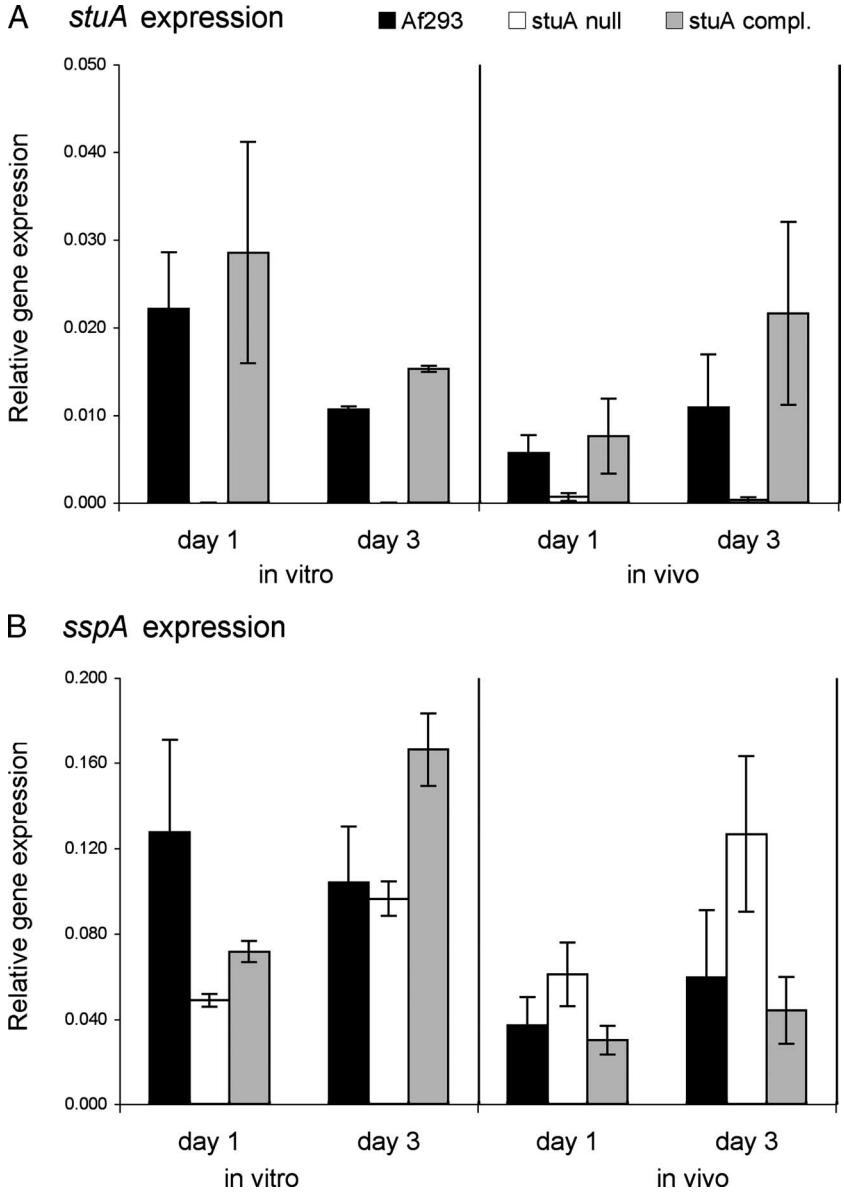

FIG. 2. mRNA expression of *sspA* is differentially dependent on StuA in vitro and in vivo. (A) In vitro and in vivo expression of *stuA* mRNA as determined by real-time RT-PCR. (B) Relative mRNA expression of *sspA* determined by real-time RT-PCR in vitro and in vivo. In vitro cultures of each strain were grown for 24 h at 37°C in YPD. Mice were infected intranasally with the wild-type strain Af293, the Δ*stuA* mutant, and the *stuA*-complemented strain ($n = 3$ mice per time point), and lungs were removed for expression analysis at 24 and 72 h after infection. The expression levels of all genes were normalized to the expression level of the endogenous control gene *TEF1*.

TABLE 2. Genes encoding or necessary for synthesis of toxins that were analyzed in this study

Gene	Toxin	Cellular activity
gliP	Gliotoxin	Cytotoxic; NF-κB inhibitor
aspF1	Mitogillin	RNase; inhibition of protein synthesis
mitF	Alpha-sarcin	RNase; inhibition of protein synthesis
aspHS	Asp-hemolysin	Red blood cell lysis
metAP1	Unknown polyketide	Unknown
dmaW	Fumitremorgin?	Neurotoxic

Complementation of the Δ*stuA* mutant with an intact copy of *stuA* also restored both *stuA* and *gliP* expression in vivo. Collectively, these results suggest that *gliP* expression and, by extension, gliotoxin production are likely dependent on StuA during murine infection.

DISCUSSION

The developmental transcription factor StuA is required for the normal production of conidia. However, the mRNA expression of *stuA* coincides with the onset of developmental competence and is maintained at high levels in competent hyphae even in the absence of conidiation (21). Our finding

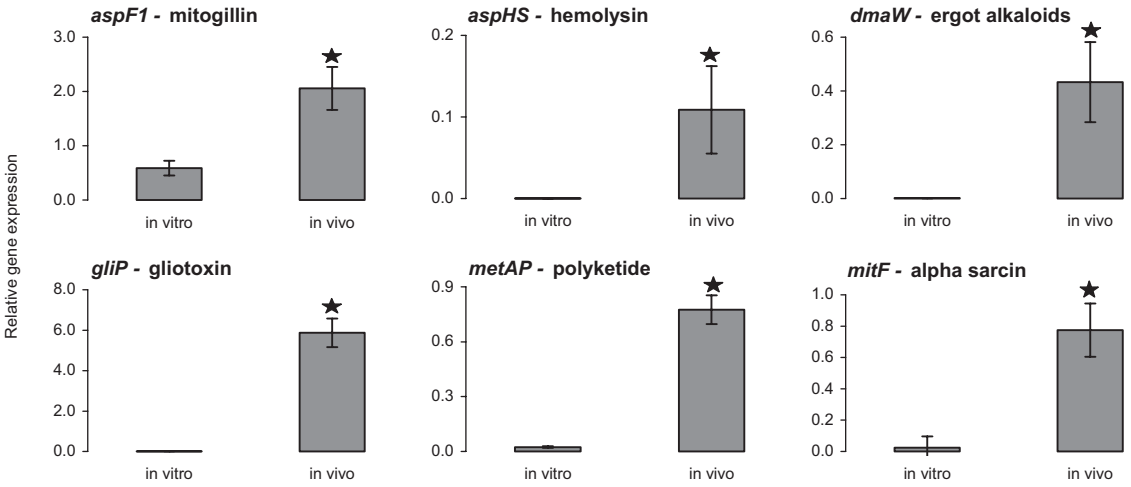

FIG. 3. Mycotoxin expression is induced in vivo. Comparative in vitro and in vivo expression levels of *gliP*, *aspHS*, *aspF1*, *metAP1*, *dmaW*, and *mitF* were determined by real-time RT-PCR. RNA in vitro was derived from cultures that were grown for 5 to 8 days at 37°C in YPD medium. For in vivo data, mice were infected with wild-type strain Af293 in an inhalational chamber. On days 5 to 8 after infection, the infected lungs were removed, and total RNA was then used for real-time RT-PCR analysis ($n = 3$ mice per time point, with 12 mice total). Results from all four time points in vivo were very similar and were combined. Average expression values for each gene were normalized to values for *TEF1* expression. ⋆ indicates statistically different results from in vitro expression by log-rank test ($P < 0.05$).

that *stuA* is expressed in vivo therefore suggests that hyphae are developmentally competent during pulmonary infection and that they at least have the potential to produce conidia, even though it is still doubtful that conidia are produced within the host. The *sspA*, *hsp70*, and *ura7* genes are also highly differentially regulated during the onset of developmental competence. The expression pattern of all three genes during the infection is consistent with that of developmentally competent hyphae grown in vitro. These data support the hypothesis that hyphae are competent during established invasive pulmonary infection. These results have important implications given that the gene expression profile of competent hyphae is very different from that of precompetent hyphae (21). For example, when investigating the interactions of *A. fumigatus* with host cells in vitro, it may be relevant to test competent hyphae in addition to the more commonly used germlings. One caveat to our study is that we were unable to reliably detect mRNA from *A. fumigatus* at very early time points. Thus, it remains likely that precompetent hyphae may play a role in initiating pulmonary infection.

Transcriptional profiling of *A. fumigatus* genes in vivo is a valuable tool for investigating the pathogenesis of invasive disease. As demonstrated by the results of this study, direct extrapolation of in vitro transcriptional analyses must be performed with caution. While expression levels of some genes (such as *stuA* and the precompetence-associated genes) were similar in vitro and in vivo, important discrepancies in the expression levels of several mycotoxins were observed. Although most mycotoxin genes were expressed at higher levels in mice than in vitro, the most striking difference between in vivo and in vitro gene expression was found with *gliP*. We detected only extremely low *gliP* mRNA levels during the growth of the wild-type strain in YPD medium, while high-level expression was easily detected from mouse lungs infected with the same strain using both intranasal and inhalational models of invasive aspergillosis. Indeed, growth in RPMI 1640 medium was required to induce levels of *gliP* mRNA expression comparable to those seen during pulmonary infection. The importance of this observation is exemplified by our finding that *gliP* expression, and likely gliotoxin production, is dependent on StuA in vivo. In our previous microarray studies (21), this StuA dependence was not observed because the organisms were grown in YPD medium, which resulted in very low levels of *gliP* expression in the wild-type strain as well as in the Δ*stuA* mutant. Thus, although StuA is necessary but not sufficient for *gliP* expression, other signal transduction pathways must also govern the expression of this gene. Interestingly, similar differences between in vivo and in vitro toxin expression levels were also found when the global regulator *laeA* was studied. The disruption of *laeA* resulted in impaired gliotoxin production in vitro; however, gliotoxin was still detected in mice infected with the Δ*laeA* strain (5).

Collectively, these studies of genes that exhibit StuA dependence in vivo suggest that the *stuA* pathway is active during invasive infection and governs the expression of key candidate virulence genes such as *gliP*. Gliotoxin production has proven to be an important virulence factor in the nonneutropenic murine model of invasive aspergillosis through the induction of leukocyte apoptosis (23, 24). In previous studies of the role of *stuA* in virulence, the deletion of *stuA* was not associated with a reduction in virulence despite the impaired gliotoxin production (21). These studies, however, were performed in neutropenic mice, in which the absence of gliotoxin has no effect on virulence (5, 9, 23, 24). These findings may also reflect the precocious germination of the Δ*stuA* null mutant or the contribution of other StuA-dependent genes to overall virulence.

In addition, we found that the levels of expression of a number of other StuA-independent genes that encode toxin biosynthetic genes are upregulated during infection. Although the role of most of these metabolites in virulence remains undefined, their expression during infection suggests that they

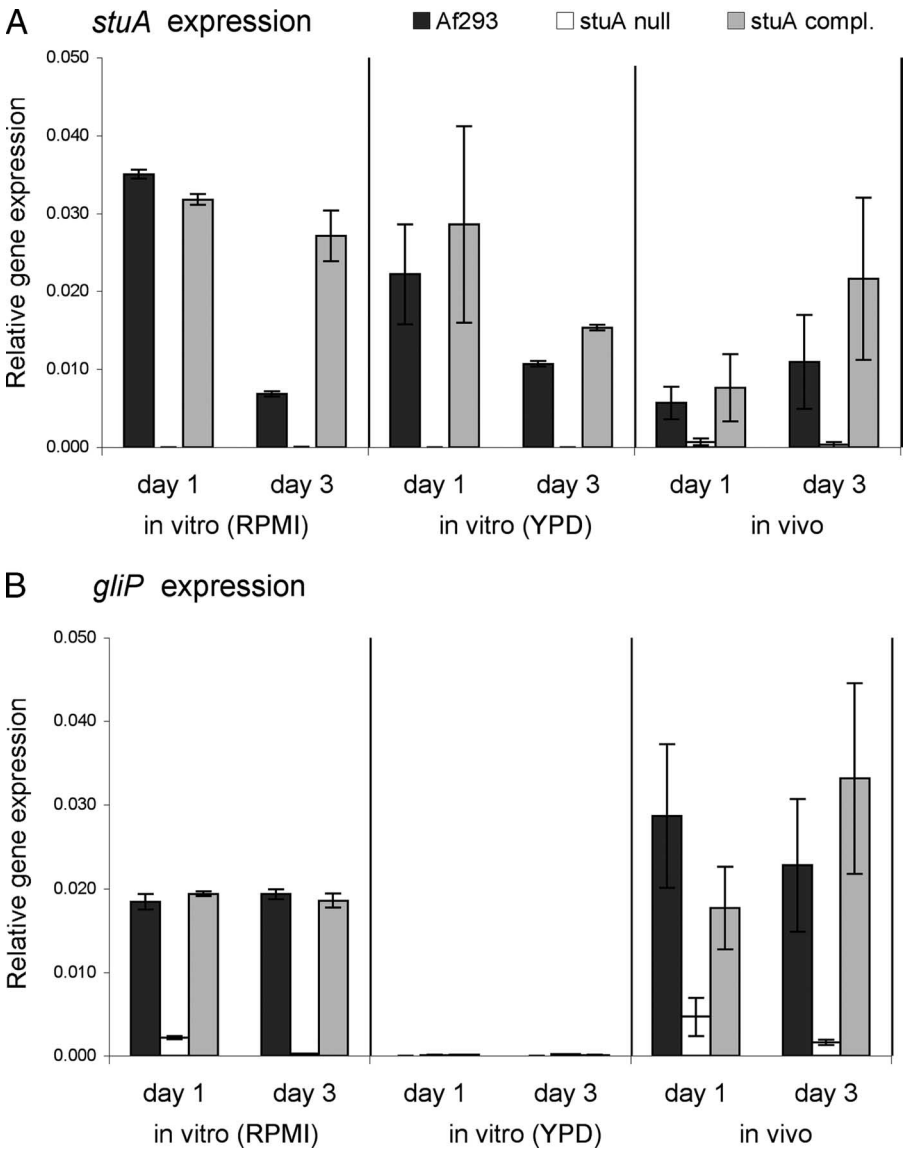

FIG. 4. Comparative in vitro and in vivo expression levels of *gliP* determined by real-time RT-PCR. Expression values are normalized to *TEF1* expression values. (A) RNA in vitro was derived from cultures of wild-type strain Af293, the Δ*stuA* mutant, or the *stuA*-complemented strain, which were grown for 24 h and 72 h at 37°C in YPD or RPMI medium. Note that data for *stuA* expression in YPD and in vivo from Fig. 2 are used for comparative purposes. (B) Mice were infected by intranasal instillation of wild-type strain Af293, the Δ*stuA* mutant, or the *stuA*-complemented strain. Twenty-four hours and 72 h after infection, infected lungs were removed for total RNA extraction, and the mRNA levels of *gliP* were determined by real-time RT-PCR. All experiments were repeated in triplicate on at least two separate occasions.

may represent interesting candidates for further detailed study by gene disruption and other methods.

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