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# *Aspergillus fumigatus* AcuM Regulates both Iron Acquisition and Gluconeogenesis

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## Summary

Relatively few transcription factors that govern the virulence of *Aspergillus fumigatus* are known. We constructed 11 *A. fumigatus* transcription factor mutants and screened them for altered virulence in *Galleria mellonella* larvae. We discovered that the zinc cluster transcription factor, AcuM, is essential for maximal virulence in this model, as well as in murine models of hematogenously disseminated and invasive pulmonary aspergillosis. Transcriptional profiling experiments suggested that AcuM suppresses *sreA* and induces *hapX* to stimulate expression of genes involved in both reductive iron assimilation and siderophore-mediated iron uptake. Consistent with these results, a Δ*acuM* mutant had reduced iron incorporation, decreased extracellular siderophore production, and impaired capacity to grow under iron-limited conditions. Interestingly, an *Aspergillus nidulans* Δ*acuM* mutant had normal extracellular siderophore production and growth under iron-limited conditions, indicating that AcuM does not govern iron acquisition in this organism. *A. fumigatus* AcuM also regulated genes involved in gluconeogenesis, and the Δ*acuM* mutant had impaired growth on gluconeogenic carbon sources. Deletion of *sreA* in the Δ*acuM* mutant restored iron uptake, extracellular siderophore production, and virulence, but not the defect in gluconeogenesis. Thus, AcuM represses SreA and thereby induces iron acquisition, a process that is essential for the maximal virulence of *A. fumigatus*.

## Introduction

*Aspergillus fumigatus* is a ubiquitous saprophytic mold that causes the majority of cases of invasive aspergillosis (Maschmeyer *et al.*, 2007; Patterson *et al.*, 2005). The incidence of invasive aspergillosis has risen substantially due to the increasing number of immunosuppressed patients (Marr *et al.*, 2002). Even with current antifungal therapy, the mortality associated with invasive aspergillosis is approximately 50%, and it approaches 100% when hematogenously disseminated disease is present (Patterson *et al.*, 2000; Patterson *et al.*, 2005; Pegues *et al.*, 2001). The mechanisms by which *A. fumigatus* causes invasive disease are incompletely understood. Identifying virulence factors of this fungus is important because this information holds promise for developing new approaches for diagnosing and treating invasive aspergillosis.

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One approach to identifying virulence factors is to determine the signaling pathways that regulate them. Transcription factors are highly useful in this regard, because they frequently govern the expression of multiple target genes. Thus, deletion of a single transcription factor gene has a higher probability of influencing virulence compared to deletion of a gene encoding a putative virulence factor. Among the 9900 genes annotated in *A. fumigatus* genome, approximately 322 specify transcription factors (Nierman *et al.*, 2005). To date, the list of transcription factors and transcriptional regulators that have been found to govern *A. fumigatus* virulence is relatively short. This list includes Ace2, SrbA, Hac1, CrzA, LaeA, ZafA, CpcA, MedA, and DvrA (Bok *et al.*, 2005; Cramer *et al.*, 2008; Ejzykowicz *et al.*, 2009; Ejzykowicz *et al.*, 2010; Gravelat *et al.*, 2009; Krappmann *et al.*, 2004; Moreno *et al.*, 2007a; Soriani *et al.*, 2008; Sugui *et al.*, 2007; Willger *et al.*, 2008).

To identify additional transcription factors that govern the virulence of *A. fumigatus*, we selected 12 genes that were predicted to specify *A. fumigatus* transcription factors that might influence pathogenicity, based on their homology to transcription factors in other organisms, expression in response to environmental stress, and uniqueness to *A. fumigatus* and other fungi. From this list, we constructed 11 transcription factor deletion strains and screened them for alterations in virulence. We discovered that the zinc cluster transcription factor, AcuM is required for maximal virulence during both hematogenously disseminated and invasive pulmonary aspergillosis in mice.

In other fungi such as *Saccharomyces cerevisiae* and *Aspergillus nidulans*, orthologs of *A. fumigatus* AcuM govern gluconeogenesis (Hynes *et al.*, 2007; Soontorngun *et al.*, 2007). We found that in *A. fumigatus*, AcuM not only regulates gluconeogenesis, but it also governs both reductive iron assimilation and siderophore-mediated iron uptake. In *Aspergillus* spp. iron acquisition under iron-depleted conditions is governed by SreA and HapX (Hortschansky *et al.*, 2007; Schrettl *et al.*, 2008). Under low iron conditions, expression of the GATA factor SreA is reduced, which results in derepression of the bZip transcription factor, HapX, as well as genes involved in both reductive iron assimilation and siderophore activity (Schrettl *et al.*, 2008). HapX binds to the CCAAT-binding complex and also induces the expression of iron assimilation and siderophore genes (Hortschansky *et al.*, 2007). We found that AcuM regulates iron acquisition under iron-depleted conditions mainly by repressing SreA and possibly by stimulating HapX.

## Results

### Screening of *A. fumigatus* transcription factor deletion mutants suggests that AcuM may be required for virulence

We selected 12 putative *A. fumigatus* transcription factors, based on their homology to transcription factors in other organisms, expression in response to environmental stress, and uniqueness to *A. fumigatus* and other fungi (Table 1). For 11 of these transcription factors, we were able to construct corresponding deletion mutants in the wild-type strain, Af293. However, despite multiple attempts, we were unable to delete gene Afu2g10770, which specifies an ortholog of Con7 in *Magnaporthe grisea*. Thus, this gene may either be essential in *A. fumigatus*, or required for growth under the selection conditions.

Next, we screened these deletion mutants for alterations in virulence using the *G. mellonella* larva model of invasive aspergillosis. Of the 11 mutants tested, only the Afu2g12330 deletion mutant had significantly reduced virulence (Table 1). Afu2g12330 was initially selected for deletion analysis because its gene product has 26% identity to *Candida albicans* Cwt1 (orf19.5849) and 25% identity to *Saccharomyces cerevisiae* Rds2 (YPL133C). Both of these zinc cluster transcription factors govern cell wall composition and integrity (Moreno *et al.*, 2003;Moreno *et al.*, 2008). Rds2 also regulates gluconeogenesis in *S. cerevisiae*

(Soontorngun *et al*., 2007); whether Cwt1 governs this process in *C. albicans* is currently unknown. A close ortholog of Afu2g12330 in *Aspergillus nidulans* is *acuM* (ANIA_06293; 54% amino acid identity), which also governs gluconeogenesis (Hynes *et al*., 2007). Based on the extensive homology between *A. nidulans* AcuM and the *A. fumigatus* Afu2g12330 gene product, we named Afu2g12330 *acuM*. Orthologs of *acuM* are present in many filamentous ascomycetes, including other species of *Aspergillus*, *Coccidioides* spp., *Paracoccidioides brasiliensis*, and *Penicillium* spp (Hynes *et al.*, 2007).

As annotated in the *A. fumigatus* genome sequencing project, the protein specified by *acuM* contains only 4 conserved cysteine residues, whereas most zinc cluster transcription factors have 6 (Naar and Thakur, 2009). Therefore, we amplified *acuM* cDNA by high-fidelity PCR and sequenced the resulting product to determine if there had been an error during genome sequencing. We found that there had been an error in intron prediction and that *acuM* actually specifies a 627 amino acid protein that contains the expected 6 cysteines. This corrected sequence has been deposited in Genbank (GU290314).

To confirm that the attenuated virulence of the Δ*acuM* mutant was due to the absence of AcuM, we constructed a Δ*acuM::acuM* complemented strain. The *acuM* mRNA expression in this strain was similar to that of the wild type strain, as determined by real-time PCR (data not shown). Next, we compared the virulence of the wild-type strain, Δ*acuM* mutant, and the Δ*acuM::acuM* complemented strain in *G. mellonella*. As predicted, complementation of the Δ*acuM* mutant with a wild-type copy of *acuM* restored virulence to wild-type levels (Fig. 1A), thus verifying that *acuM* is essential for maximal virulence in this model.

## The Δ*acuM* mutant has attenuated virulence in mouse models of hematogenously disseminated and invasive pulmonary aspergillosis

To determine whether AcuM governs *A. fumigatus* virulence in mammals, we tested the Δ*acuM* mutant in two different murine models of invasive aspergillosis. The first was a neutropenic model of hematogenously disseminated disease. In this model, the mice were immunosuppressed with cyclophosphamide and cortisone acetate, and then inoculated intravenously with *A. fumigatus* germlings. Mice infected with the Δ*acuM* mutant survived significantly longer than those infected with the wild-type and Δ*acuM::acuM* complemented strains ($p \leq 0.005$) (Fig. 1B). In the second model, the mice were immunosuppressed with high-dose cortisone acetate, and then invasive pulmonary disease was induced by placing them in an acrylic chamber filled with an aerosol of *A. fumigatus* conidia. Infection with the Δ*acuM* mutant also resulted in significantly delayed mortality in this model ($p < 0.005$ compared to the wild-type and Δ*acuM::acuM* complemented strains) (Fig. 1C). Collectively, these results indicate that *acuM* is necessary for the normal virulence of *A. fumigatus* during both disseminated and invasive pulmonary aspergillosis.

To verify the reduced virulence of the Δ*acuM* mutant, we investigated the pulmonary fungal burden of mice infected in the aerosol chamber by measuring the level of galactomannan in their lungs after 4 days infection (Sheppard *et al*., 2006). Preliminary in vitro studies demonstrated that the Δ*acuM* mutant released a similar amount of galactomannan into the medium as did the wild-type strain (data not shown), thus confirming that galactomannan can be used as a measure of organ fungal burden with this strain. As anticipated, the pulmonary galactomannan content of mice infected with the Δ*acuM* mutant was significantly lower than that of mice infected with either the wild-type or Δ*acuM::acuM* complemented strains ($p < 0.001$) (Fig. 2). Therefore, *acuM* is required for maximal growth of *A. fumigatus* in the lungs. Furthermore, the delayed mortality of mice with invasive pulmonary aspergillosis caused by the Δ*acuM* mutant was likely due in part to their lower pulmonary fungal burden.

### Analysis of the time course of acuM mRNA expression

To investigate the function of AcuM in *A. fumigatus*, we first used real-time PCR to analyze the time course of *acuM* mRNA expression in the wild-type strain when it was grown in *Aspergillus* minimal medium (AMM) with iron at 37°C. *acuM* appeared to be expressed constitutively as *acuM* mRNA levels were similar in swollen conidia, germlings and hyphae (data not shown).

### Transcription profiling suggests that AcuM governs genes involved in reductive and siderophore mediated iron acquisition, and carbon metabolism

Next, we performed microarray analysis of the Δ*acuM* mutant to identify genes that require AcuM for normal expression. To simulate the relatively nutrient-poor conditions within the host, the organisms were grown in liquid RPMI 1640 medium at 37°C as described in Experimental Procedures. A total of 251 genes were down-regulated and 119 genes were up-regulated in the Δ*acuM* mutant compared to the wild-type strain and the Δ*acuM::acuM* complemented strain at the 18 h and/or 24 h time points. A complete list of these genes is contained in Supplemental Table S2. Gene ontology (GO) term analysis of the genes that were down-regulated in the Δ*acuM* mutant indicated that they were significantly ($p < 0.001$) enriched in terms related to iron acquisition and homeostasis, carbon metabolism, and synthesis of methionine and glutamate (Table 2). The genes contained in each of the categories are listed in Supplemental Table 3. The genes that were up-regulated in the Δ*acuM* mutant were not significantly enriched in any GO term. Although the AcuM orthologs, Rds2 and Cwt1 govern cell wall structure in *S. cerevisiae* and *C. albicans*, respectively (Moreno *et al.*, 2003; Moreno *et al.*, 2008), very few genes involved in cell wall synthesis showed AcuM-dependent expression, suggesting that AcuM does not play a significant role in governing cell wall structure in *A. fumigatus*.

*A. fumigatus* can obtain iron from the environment by two mechanisms, reductive iron assimilation and production of siderophores (Schrettl *et al.*, 2004). We found that two genes in the reductive iron assimilation pathway (*ftrA* and *fre2*) were significantly down-regulated in the Δ*acuM* mutant, as shown by the microarray data and verified by real-time PCR (Table 3). Furthermore, multiple genes involved in siderophore synthesis (*sidA*, *sidC*, *sidF*, *sidG*, and Afu3g03390) and siderophore transport (*mirB*, *sit1*, and Afu7g04730) were also down-regulated in this mutant (Table 3). Thus, the transcriptional profiling data suggested that AcuM controls both reductive iron assimilation and siderophore-mediated iron acquisition.

### Deletion of acuM results in increased expression of sreA and reduced expression of hapX

In *Aspergillus* spp. iron acquisition under iron-depleted conditions is governed by SreA and HapX (Hortschansky *et al.*, 2007; Schrettl *et al.*, 2008). Because AcuM also governs the expression of genes involved in these processes, we investigated the expression of *sreA* and *hapX* in the Δ*acuM* mutant. First, we verified that growth of wild-type *A. fumigatus* under iron-depleted conditions results in reduced *sreA* expression and increased *hapX* expression (Fig. 3A). Interestingly, *acuM* transcript levels were unaffected by the iron content of the medium.

When the Δ*acuM* mutant was grown under iron-limited conditions, *sreA* mRNA levels were 3-fold higher in this strain than in the wild-type strain (Fig. 3B). Also, *hapX* expression was reduced by 1.6-fold compared to the wild-type strain (Fig. 3C). These alterations in *sreA* and *hapX* transcript levels were due to the absence of *acuM* because they were restored to wild-type levels in the Δ*acuM::acuM* complemented strains (Figs. 3B and C). Collectively, these results suggest that *acuM* either directly or indirectly regulates the expression of *sreA* and *hapX*.

Deletion of SreA is known to result in the increased expression of 49 genes, most of which are involved in iron acquisition, siderophore production and siderophore transport (Schrettl *et al.*, 2008). The finding that *sreA* had increased expression in the Δ*acuM* mutant (Fig. 3B) predicted that many SreA-responsive genes would be down-regulated in this strain. Indeed, we found that 26 of 49 SreA-responsive genes had significantly reduced expression in the Δ*acuM* mutant (Fig. 4). It is known that many SreA-responsive genes are located in clusters along the chromosomes (Schrettl *et al.*, 2008). We determined that 5 of the 8 SreA-responsive gene clusters were down-regulated in the Δ*acuM* mutant (Fig. 4). These gene clusters were located on chromosomes 1, 3, 5 and 8, and contained genes involved in siderophore biosynthesis and transport. Collectively, these results suggest that AcuM governs siderophore-mediate iron acquisition at least in part by repressing SreA.

## AcuM is required for normal iron incorporation, extracellular siderophore production, and growth in iron deficient media

To test the predictions that AcuM governs both reductive iron assimilation and siderophore-mediated iron uptake, we first tested the capacity of the Δ*acuM* mutant to grow under iron limited conditions. These experiments were performed using organisms grown in Sabouraud broth containing the iron chelator, phenanthroline because the Δ*acuM* mutant grew more slowly than the wild-type strain in AMM, even with iron supplementation. Similar results have been reported with an *A. fumigatus* Δ*sidA* mutant (Hissen *et al.*, 2005). As predicted by the transcriptional profiling data, the Δ*acuM* mutant grew significantly more slowly than the wild-type or Δ*acuM::acuM* complemented strains under iron-limited conditions (Fig. 5A). This growth defect was rescued by supplementing the medium with iron. Next, we measured the capacity of washed germlings of the Δ*acuM* mutant to incorporate $^{55}FeCl_3$ from the medium. We found that the Δ*acuM* mutant incorporated approximately 50% less iron did than the wild-type and Δ*acuM::acuM* complemented strains ($p < 0.001$) (Fig. 5B). Because the germlings were washed prior to being tested in this assay, it is probable that the defective iron uptake of the Δ*acuM* mutant was due to impaired reductive iron assimilation rather than reduced extracellular siderophore production. Finally, we measured the accumulation of extracellular siderophore activity in the culture medium using the chrome Azurol S (CAS) assay (Schwyn and Neilands, 1987). When grown in AMM without iron, the extracellular siderophore activity of the Δ*acuM* mutant was reduced by at least 80% compared to the wild-type and Δ*acuM::acuM* complemented strains ($p < 0.001$) (Fig. 5C). Collectively, these results indicate that the impaired growth of the Δ*acuM* mutant under iron-limited conditions was due to defects in both reductive iron assimilation and extracellular siderophore production.

## AcuM is dispensable for siderophore production and growth under iron-limited conditions of *A. nidulans*

*A. fumigatus* AcuM shares significant homology with *A. nidulans* AcuM (Hynes *et al.*, 2007). Therefore, we investigated whether AcuM also governs iron homeostasis in *A. nidulans.* We found that an *A. nidulans* Δ*acuM* mutant did not have any significant defects in extracellular siderophore activity or growth under iron-limited conditions (Fig. 6). Therefore, AcuM does not appear to play a significant role in regulating siderophore-mediated iron assimilation or growth under iron-limited conditions in *A. nidulans*.

## The Δ*acuM* mutant is defective in gluconeogenesis

In *A. nidulans*, AcuM governs gluconeogenesis (Hynes *et al.*, 2007), and our transcriptional profiling results indicated that AcuM also controls gluconeogenesis in *A. fumigatus*. For example, *acuF* and *fbp1* were significantly down-regulated in the Δ*acuM* mutant (Table 3). These genes specify phosphoenolpyruvate carboxykinase and fructose-1,6,bisphosphatase, respectively, important enzymes that are specific for gluconeogenesis (Hynes *et al.*, 2007).

To verify that the Δ*acuM* mutant was defective in gluconeogenesis, we examined its growth in media containing carbon sources other than glucose. The Δ*acuM* mutant had a minor growth defect on AMM agar containing supplemental iron when glucose was the carbon source (Fig. 7). However, it was virtually unable to grow when the carbon source was ethanol, acetate, or proline. Taken together, these data indicate that AcuM is required for normal gluconeogenesis in *A. fumigatus*.

### AcuM is not required for resistance to environmental stress and antifungal agents

The AcuM orthologs, Cwt1 and Rds2, govern cell wall structure in *C. albicans* and *S. cerevisiae*, respectively (Moreno *et al.*, 2003; Moreno *et al.*, 2008). Also, *sreA*, whose expression is governed in part by AcuM, is required for normal resistance to oxidative stress and amphotericin B (Schrettl *et al.*, 2008). Therefore, we investigated the susceptibility of the Δ*acuM* mutant to various stressors and antifungal agents in iron replete conditions. This mutant was similar to the wild-type strain in its susceptibility to Congo red, calcofluor white, hydrogen peroxide, SDS, caspofungin, and amphotericin B (data not shown). Therefore, under the conditions tested, AcuM does not appear to influence cell wall integrity, or susceptibility to oxidative stress or antifungal agents.

### Absence of AcuM results in increased susceptibility to killing by neutrophil-like and macrophage-like HL-60 cells

Macrophages and neutrophils sequester iron and make it unavailable to microbial pathogens (Seifert *et al.*, 2008; Zarember *et al.*, 2007). In addition, *C. albicans* cells that have been phagocytosed by either macrophages or neutrophils have increased gluconeogenesis, suggesting that the phagocytic vacuole is a low-glucose environment (Barelle *et al.*, 2006). These data led us to test whether deletion of *acuM* influenced the susceptibility of *A. fumigatus* to leukocyte killing. We found that swollen conidia of the Δ*acuM* mutant were significantly more susceptible to killing by HL-60 cells that had been differentiated into either macrophage-like or neutrophil-like cells (Fig. 8). These results demonstrate that AcuM is required for *A. fumigatus* to resist killing by phagocytic leukocytes. They further suggest that increased susceptibility to phagocyte killing contributes to the attenuated virulence of the Δ*acuM* mutant.

### Deletion of *sreA* rescues the defects in iron acquisition and virulence of the Δ*acuM* mutant

SreA functions as a negative regulator repressor of iron acquisition (Schrettl *et al.*), and *sreA* transcript levels were increased by 3-fold in the Δ*acuM* mutant (Fig. 3B). This finding suggested that the reduced iron acquisition of the Δ*acuM* mutant was due in part to the elevated expression of *sreA*. To test this hypothesis, we constructed a Δ*acuM* Δ*sreA* double mutant. Deletion of *sreA* in the Δ*acuM* mutant resulted in higher transcript levels of the regulatory gene, *hapX*, as well as genes involved in siderophore biosynthesis and transport (*sidA*, *sigF*, and *mirB*), and high-affinity iron uptake (*ftrA* and *fre2*) (Fig. 9). However, mRNA levels of *sidG* were not significantly increased in the Δ*acuM* Δ*sreA* double mutant, suggesting that the expression of this gene may not be repressed by SreA in the absence of AcuM.

Next, we investigated the phenotype of the Δ*acuM* Δ*sreA* double mutant. Consistent with transcriptional profiling results, deletion of *sreA* the Δ*acuM* background restored incorporation of $^{55}FeCl_3$ and extracellular siderophore production to wild-type levels (Figs. 10A and B). In addition, when tested in the neutropenic mouse model of disseminated aspergillosis, the Δ*acuM* Δ*sreA* double mutant was significantly more virulent than the Δ*acuM* mutant ($p = 0.003$). Mice infected with the Δ*acuM* Δ*sreA* double mutant survived slightly longer than those infected with the wild-type strain, but this difference was not significant ($p = 0.062$). Collectively, these results indicate that the reduced iron uptake,

decreased siderophore production, and attenuated virulence of the Δ*acuM* mutant are largely the result of increased expression of *sreA*.

## Discussion

AcuM was selected for inclusion in our screen for transcription factors that govern virulence because AcuM orthologs participate in governing cell wall integrity in *C. albicans* and *S. cerevisiae*, as well as gluconeogenesis in *S. cerevisiae* and *A. nidulans* (Hynes *et al.*, 2007; Moreno *et al.*, 2003; Moreno *et al.*, 2008; Soontorngun *et al.*, 2007). The extent of homology between *A. fumigatus* AcuM and *A. nidulans* AcuM predicted our findings that the Δ*acuM* mutant had reduced expression of genes involved in gluconeogenesis and defective ability to grow on gluconeogenic carbon sources. However, a novel discovery was that, in *A. fumigatus*, AcuM plays a central role in iron homeostasis, governing both high-affinity iron assimilation and siderophore-mediated iron uptake. This conclusion is supported by the transcriptional profiling results, as well as phenotypic data which showed that the Δ*acuM* mutant had reduced iron incorporation, decreased siderophore production, and impaired growth under iron-limited conditions.

In *Aspergillus* species, iron homeostasis is governed by the SreA and HapX transcription factors. We found that when the Δ*acuM* mutant was grown under iron-limited conditions, *sreA* mRNA levels were inappropriately elevated whereas *hapX* mRNA levels were abnormally low. Because deletion of *acuM* increased the transcript levels of *sreA* by 3-fold, but only reduced *hapX* transcript levels by 1.6-fold, it is likely that AcuM governs iron homeostasis predominantly via its effects on SreA. In support of this hypothesis, 26 of 49 of previously identified SreA-responsive genes (Schrettl *et al.*, 2008) had reduced expression in the Δ*acuM* mutant. Also, most of these AcuM-dependent genes were located in the same chromosomal clusters as the SreA-responsive genes. This overlap between AcuM and SreA is even more impressive when one considers that the transcriptional profiling experiments of the Δ*acuM* and Δ*sreA* mutants used organisms grown under significantly different conditions. In experiments with the Δ*acuM* mutant, the organisms were grown under iron-limited conditions, whereas the transcriptional profiling experiments of the Δ*sreA* mutants used organisms that had been initially iron starved and then switched to iron-replete conditions (Schrettl *et al.*, 2008). Our finding that deletion of *sreA* in the Δ*acuM* mutant restored iron uptake and extracellular siderophore production provides additional evidence that AcuM controls iron homeostasis mainly via SreA. At present, it is unknown whether AcuM directly binds to the promoter of *sreA* or whether it represses *sreA* transcript levels by an indirect mechanism.

Schrettl et al. (Schrettl *et al.*, 2008) also identified 9 iron repressed genes that were not under the control of SreA. Interestingly, 3 of these genes (Afu5g02330 [ribotoxin AspF1], Afu5g00720 [GCN5-related N-acetyltransferase], and Afu1g03150 [C-14 sterol reductase]) had reduced expression in the Δ*acuM* mutant. These results suggest that AcuM may also govern some responses to iron independently of SreA. In *A. nidulans*, HapX and SreA exhibit mutual transcriptional control, and each influences iron homeostasis (Hortschansky *et al.*, 2007; Schrettl *et al.*, 2008). The transcriptional profile of an *A. fumigatus* Δ*hapX* mutant has not been reported. However, some proteins whose levels are reduced in an *A. nidulans* Δ*hapX* mutant (Hortschansky *et al.*, 2007) correspond to genes that are down-regulated in the *A. fumigatus* Δ*acuM* mutant. This overlap includes 3-hydroxy-3-methylglutaryl CoA synthase (Afu3g10660), hydantoinase/oxoprolinase (Afu4g11460), and GMC oxidoreductase (Afu3g01580). We predict that the transcriptional profile of an *A. fumigatus* Δ*hapX* mutant would be even more similar to that of the Δ*acuM* mutant.

It is also notable that the transcriptional profiling studies of the *A. fumigatus* Δ*sreA* mutant indicated that SreA governs the expression of some genes involved in carbon metabolism (Schrettl *et al.*, 2008). At least one of these genes, Afu6g04920 (NAD-dependent formate dehydrogenase) (Kennedy *et al.*, 2009) was down-regulated in the Δ*acuM* mutant. This result provides an additional link between regulation of iron homeostasis and carbon metabolism in *A. fumigatus*. It further suggests that in *A. fumigatus*, AcuM may govern carbon metabolism partly by repressing SreA. However, deletion of *sreA* in the Δ*acuM* mutant did not restore its capacity to grow on gluconeogenic carbon sources (data not shown). Thus, gluconeogenesis is likely governed predominantly by AcuM, independently of SreA.

We found that the Δ*acuM* mutant had attenuated virulence in *G. mellonela* larvae. This virulence defect was subsequently verified in murine models of disseminated and invasive pulmonary aspergillosis. Our finding that deletion of *sreA* in the Δ*acuM* mutant restored both iron acquisition and virulence suggests that virulence defect of the Δ*acuM* mutant was largely due to impaired growth within the iron-limited environment of the host. Moreover, the near wild-type virulence of the Δ*acuM* Δ*sreA* double mutant, which remained defective in growth on gluconeogenic substrates, indicates that there are adequate glyoclytic substrates within the host and that gluoconeogenesis is dispensable for *A. fumigatus* virulence. Although the Δ*acuM* mutant had attenuated virulence in murine models of invasive aspergillosis, mice infected with this mutant still died. In contrast, the *A. fumigatus* siderophore mutants, Δ*sidA*, Δ*sidC*, Δ*sidD*, and Δ*sidF*, appear to have a much greater attenuation in virulence (Hissen *et al.*, 2005; Schrettl *et al.*, 2004; Schrettl *et al.*, 2007). The probable explanation for this result is that the expression of most of the genes involved in siderophore-mediated iron uptake was reduced, but not completely abrogated in the Δ*acuM* mutant. It is possible that these genes may be under the control of *hapX*, the expression of which was only partially governed by AcuM.

A surprising finding was that the *A. nidulans* Δ*acuM* mutant grew similarly to the wild-type strain under iron-limited conditions and had normal extracellular siderophore production. These results indicate that there has been significant transcriptional rewiring between *A. fumigatus* and *A. nidulans*. Although AcuM regulates gluconeogenesis in both species, it has evolved the additional function of controlling iron homeostasis in *A. fumigatus*. In *A. nidulans*, a second zinc cluster transcription factor, AcuK, functions with AcuM to control gluconeogenesis (Hynes *et al.*, 2007). An ortholog of AcuK is present in the *A. fumigatus* genome (Hynes *et al.*, 2007). Thus, it is possible that AcuK may also regulate iron homeostasis as well as gluconeogenesis in this organism. Transcriptional rewiring of metabolic regulatory pathways has been reported to occur between yeast such as *S. cerevisiae* and *C. albicans* (Martchenko *et al.*, 2007), which separated from each other 150 to 300 million years ago (Pesole *et al.*, 1995). *A. nidulans* and *A. fumigatus* diverged from each other over a similar time span, approximately 200 million years ago (Galagan *et al.*, 2005). During this time, AcuM and probably other genes that contribute to the enhanced pathogenicity of *A. fumigatus* developed a new function.

In summary, this work demonstrates that *A. fumigatus* AcuM governs both iron acquisition and gluconeogenesis. Its effects on iron acquisition are mediated mainly by repression of SreA. AcuM-regulated iron acquisition, but not gluconeogenesis is essential for maximal virulence during both hematogenously disseminated and invasive pulmonary aspergillosis. Although AcuM regulates iron homeostasis in *A. fumigatus* it does not do so in *A. nidulans*, indicating a significant divergence in transcriptional regulation between these two organisms has occurred.

## Experimental procedures

### Strains and growth conditions

*A. fumigatus* strain Af293 was used as the wild-type strain in all experiments with this organism. The *A. fumigatus* mutant strains used in the experiments are listed in Table 1. They were routinely grown on Sabouraud agar (Difco, Detroit, MI) at 37°C for 1 week prior to use. The *Aspergillus nidulans* control strain and Δ*acuM* mutant strain were a generous gift from Dr. Michael Hynes (University of Melbourne, Victoria, Australia) and grown as described (Hynes *et al.*, 2007). The conidia of all organisms were harvested by gently rinsing the agar plates with PBS containing 0.1% Tween 80 (Sigma-Aldrich, St. Louis, MO) and enumerated using a hemacytometer.

For iron-depleted conditions, the organisms were grown in either Sabouraud dextrose broth (Difco, Detroit, MI) containing 30 μM phenanthroline (Sigma-Aldrich) or liquid AMM containing 1 % glucose (wt/vol) and 10 mM ammonium tartrate, without supplemental iron (Hortschansky *et al.*, 2007). Iron–replete medium was prepared by supplementing Sabouraud dextrose broth plus phenanthroline with 1 mM $FeSO_4$, and the AMM with 50 μM $FeSO_4$. In each experiment, $2 \times 10^8$ conidia of the various strains were inoculated into 100 ml of culture medium and incubated at 37°C in a shaking incubator for 40 h.

### Construction of *A. fumigatus* mutant strains

Each of the *A. fumigatus* mutant strains in Table 1 was generated using split-marker approach with a minor modification of our previously described method (Sheppard *et al.*, 2005). To delete *acuM* (Afu2g12330), a DNA fragment encompassing 1416 bp upstream of the *acuM* protein coding region was amplified from *A.fumigatus* Af293 genomic DNA by high-fidelity PCR using primers AcuM-F4 and AcuM-F3 (Supplemental Table S1). This fragment was cloned into plasmid pNLC106 (Catlett *et al.*, 2002), yielding (pAcuM-HY), which contained the 5' portion of the *hph* hygromycin resistance cassette. Next, a DNA fragment containing the upstream *acuM* flanking region and the 5' portion of the hygromycin resistance cassette was amplified by high-fidelity PCR from pAcuM-HY using primers AcuM-F4 and HY. To generate the second half of the split marker, 1516 bp of the *acuM* downstream flanking sequence was PCR amplified using primers AcuM-F2 and AcuM-F1. Next, the 3' region of the hygromycin resistance cassette was amplified from plasmid pAN7-1 (Punt *et al.*, 1987) using primers HYG-F and YG. The downstream sequence was combined with the 3' resistance cassette by fusion PCR using primers AcuM-F1 and YG. *A. fumigatus* Af293 was then transformed by protoplasting with the two fragments containing the upstream and downstream flanking sequences of *acuM* (Weidner *et al.*, 1998). Hygromycin-resistant clones were screened for disruption of *acuM* by colony PCR using the primers AcuM-VER-UP and AcuM-VER-R (Table S1). The other mutant strains of *A. fumigatus* were generated in a similar manner using the primers listed in Supplemental Table S1.

To complement the Δ*acuM* mutant, the *acuM* open reading frame along with 2968 bp of upstream sequence and 918 bp downstream sequence was amplified from genomic DNA of strain Af293 by high-fidelity PCR using primes AcuM-REV-F and AcuM-REV-R (Supplemental Table S1). The resulting 6180 bp DNA fragment was ligated into pGEM-T Easy (Promega Corp., Madison, WI) and sequence verified. This fragment was excised from pGEM-T Easy by NotI digestion and cloned into plasmid p402, which contains the phleomycin resistance gene (Richie *et al.*, 2007). The resulting plasmid was linearized with AfeI and transformed into the Δ*acuM* mutant. Phleomycin-resistant clones were screened by whole cell PCR using primers AcuM-REV-VER-F and AcuM-REV-VER-R (Supplemental Table S1) to select clones with the proper integration.

Disruption of *acuM* and correct integration of a single copy of the *acuM* complementation plasmid were verified by Southern blotting with a 569 bp fragment encompassing the hygromycin resistance cassette and the 5' *acuM* flanking sequence.

To delete *sreA* (Afu5g11260) in the Δ*acuM* background, the Gateway® cloning system (Invitrogen, Carlsbad, CA) was used to fuse *sreA* flanking sequences with the *ble* phleomycin resistance cassette as follows. A 1402 bp sequence of the *sreA* upstream flanking sequence was amplified from *A. fumigatus* Af293 genomic DNA by high-fidelity PCR using the primers SreA-gate1 and SreA-gate2 (Table S1). This fragment was first cloned into the pENTR-D-TOPO plasmid, and then transferred to the pBL plasmid so that the 3' end of the fragment was positioned next to the 5' end of the *ble* cassette. Using PCR with the SreA-gate1 and BL primers, a 2285 bp product containing the upstream *sreA* flanking region and the 5' portion of the phleomycin resistance cassette was amplified from this plasmid. Similarly, a 1242 bp fragment of the *sreA* downstream flanking sequence was generated by PCR, cloned in pENTR-D-TOPO, and then transferred to pLE immediately downstream of the *ble* cassettee. A 2015 bp product containing the 3' portion of the phleomycin resistance cassette followed by the downstream *sreA* flanking region was amplified from this plasmid by PCR with the primers LE and SreA-gate4. Finally, the *A. fumigatus* Δ*acuM* mutant was transformed with the two fragments containing the upstream and downstream flanking sequences of *sreA*. Phleomycin-resistant clones were screened for disruption of *sreA* by colony PCR using the primers SreA-ext1 and SreA-ext4 (Table S1). Positive clones were then screened for the absence of *sreA* mRNA by real-time PCR.

## Galleria mellonella model of invasive aspergillosis

The various transcription factor mutants were screened for virulence in the *Galleria mellonella* larva model of disseminated aspergillosis (Fan *et al.*, 2007; Gravelat *et al.*, 2009; Jackson *et al.*, 2009; Mylonakis, 2008; Reeves *et al.*, 2004; Renwick *et al.*, 2006). *G. mellonella* larvae in the final instar stage were obtained from Vanderhorst Wholesale (St. Mary's, OH). Larvae were stored at room temperature in wood shavings in the dark prior to use and were used within 1 week of delivery. The larvae were injected with $5 \times 10^6$ conidia in 5 μl of phosphate buffered saline (PBS) in the last pro-leg with a Hamilton syringe. Each strain of *A. fumigatus* was used to infect 30 to 35 larvae, and the experiments were repeated 2 or 3 times. The infected larvae were maintained in Petri dishes in a dark humidified incubator at 37°C. Mortality was assessed based on lack of movement in response to tactile stimulation.

## Murine model of hematogenously disseminated aspergillosis

Male BALB/c mice (Taconic Farms Inc, Hudson, NY) were immunosuppressed with cortisone acetate (Sigma-Aldrich) administered subcutaneously at 250 mg/kg on days −2 and +3 relative to infection, and with cyclophosphamide (Western Medical Supply, Arcadia, CA) administered intraperitoneally at 250 mg/kg on days −2 and +3. The mice received daily intraperitoneal injections of 5 mg of ceftazidime while they were immunosuppressed to prevent bacterial infections. Germlings of the three strains were prepared by incubating conidia in Petri dishes containing Sabouraud dextrose broth ($5 \times 10^5$ conidia/ml) at 37°C for 3 h, then storing them at 4°C overnight, and incubating them at 37°C for an additional 4 h the next day. After gently rinsing the resulting germlings twice with PBS, the organisms were harvested with a cell scraper, suspended in PBS, and enumerated with a hemacytometer. The immunosuppressed mice were inoculated with $3 \times 10^3$ germlings via the lateral tail vein, and the inocula were verified by quantitative culture. Control mice were immunosuppressed, but not infected. In each experiment, 5 to 10 mice were infected with each strain.

### Murine model of invasive pulmonary aspergillosis

To induce invasive pulmonary aspergillosis, male BALB/c mice were immunosuppressed with 5 doses of 10 mg cortisone acetate administered subcutaneously every other day, starting on day −4 relative to infection and finishing on day +4 (Ejzykowicz *et al.*, 2009; Spikes *et al.*, 2008). The mice received daily intraperitoneal injections of 5 mg of ceftazidime while they were immunosuppressed. They were infected by placing them for 1 h in an acrylic chamber into which $10^9$ conidia/ML were aerosolized (Sheppard *et al.*, 2004). Control mice were immunosuppressed, but not infected. In the survival studies, 11 mice were infected with each strain of *A. fumigatus*. Shortly after inoculation, 3 mice from each group were sacrificed, after which their lungs were homogenized and quantitatively cultured to verify conidial delivery to the lungs. The remaining mice were monitored for survival. The survival experiments were repeated twice and the results were combined.

The pulmonary fungal burden was assessed by measuring the pulmonary galactomannan content using the Platelia Aspergillus kit (Bio-Rad, Hercules, CA) as previously described (Ejzykowicz *et al.*, 2009; Gravelat *et al.*, 2009). Briefly, 10 to 12 mice per strain were infected as above. Three mice were sacrificed after inoculation with each strain to verify pulmonary conidial delivery by quantitative culture. The remaining mice were sacrificed after 4 days of infection. Their lungs were harvested, weighed, and then homogenized in ice cold PBS containing protease inhibitor cocktail (Sigma-Aldrich). The homogenates were clarified by centrifugation and aliquots of the resulting supernatants were stored at −80°C for later analysis. To determine the galactomannan content, each pulmonary homogenate was diluted 1:10 in ultra-pure water and processed according to the manufacturer's instructions. The resulting optical densities were compared with a standard curve, which was made using serial dilutions of a pool of lung homogenates from 5 heavily infected immunosuppressed mice (7 days after intranasal infection with strain Af293). All animal studies were approved by the Institutional Animal Use and Care Committee, and performed according to the National Institutes of Health guidelines for animal housing and care.

### Microarray analysis

To obtain RNA for microarray analysis, conidia of the various strains were used to inoculate RPMI 1640 medium (Cat. No. R6504; Sigma-Aldrich) buffered with 4-morpholinepropanesulfonicacid to pH 7.0 (34.5 g/l; Sigma-Aldrich). This medium contains 0.2% glucose as the carbon source and 0.03% glutamine as the nitrogen source. For the 8 h time point, a one liter flask containing 500 ml of medium was used. For the 18 and 24 h time point, 250 ml flasks containing 100 ml of medium were used. The concentration of conidia in these flasks was $5 \times 10^5$ cells per ml. After incubating the organisms in a shaking incubator at 37°C for 8, 18, and 24h, the resulting hyphae were harvested by filtration and snap frozen in liquid nitrogen. Next the RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Germantown, MD) following the manufacturer instructions.

An *A. fumigatus* Af293 DNA amplicon microarray containing triplicate probes for 9516 genes was used in this study (Nierman *et al.*, 2005). The labeling reactions with RNA and hybridizations were performed as described in the J. Craig Venter Institute standard operating procedure (Http://pfgrc.jcvi.org/index.php/microarray/protocols.html). cDNA from the Δ*acuM* mutant was hybridized against cDNA from the wild-type strain in two biological replicates and against cDNA from the Δ*acuM*::*acuM* complemented strain in two biological replicates. Dye swaps were performed. Because hybridization against the wild-type and Δ*acuM*::*acuM* complemented strains gave similar results, the data were combined for the final analysis. The gene expression ratios were $\log_2$-transformed and imported into JCVI MultiExperiment Viewer software (http://www.tm4.org/mev.html) (Saeed *et al.*, 2006). The Significance Analysis for Microarrays (Tusher *et al.*, 2001) method was used

(false discovery rate of 0.1%) to identify genes subject to differential transcriptional regulation between the control strains and the Δ*acuM* mutant after 18h and/or 24h of growth. Genes down-regulated in the Δ*acuM* mutant were further analyzed by the Expression Analysis Systematic Explorer (Hosack *et al.*, 2003) within JCVI MultiExperiment Viewer to identify overrepresented Gene Ontology (GO) terms and Pfam domains. Using Fisher's exact test and stepdown Bonferroni correction, a cutoff value of adjusted $P < 0.05$ was set to assess statistical significance.

Selected microarray results were verified by real-time PCR using the primers listed in Supplemental Table S1. Relative gene expression levels were quantified by the $2^{-\Delta\Delta CT}$method using *TEF1* as the reference gene (Ejzykowicz *et al.*, 2009; Gravelat *et al.*, 2008; Sheppard *et al.*, 2005). The real-time PCR experiments were performed using 3 biological replicates, each tested in triplicate.

## Quantification of iron incorporation

The capacity of the different strains to incorporate ferric iron from the medium was determined by a minor modification of our previously described method (Fu *et al.*, 2004). Briefly, $5\times10^6$ conidia/ml of the various *A. fumigatus* strains were incubated in AMM without iron containing 1 mM ferrozine (Sigma-Aldrich) and 1 mM ascorbic acid (Sigma) in a shaking incubator at 37 °C for 8 hours. Next, the swollen conidia were harvested, washed twice with assay buffer (AMM without iron, 10 mM MOPS pH 6.1, and 2% glucose), and enumerated. After adjusting the concentration of conidia to $10^8$ cells per ml, 200 μl of this suspension was added to 400 μl of assay buffer containing 1.5 μM $^{55}FeCl_3$ and the organisms were incubated at 37°C for 60 minutes. The suspension was then chilled on ice, after which the organisms were washed three times with washing buffer (1 mM EDTA, 20 mM $Na_3$ citrate pH 4.2, 1 mM $KH_2PO_4$, 1 mM $CaCl_2$, 5 mM $MgSO_4$, 1 mM NaCl, 0.05% Tween 80). The fungal associated $^{55}Fe$ was measured by liquid scintillation counting. Each experiment was performed in triplicate on 3 different occasions.

## Measurement of extracellular siderophore production

The CAS assay was used to measure the total extracellular siderophore activity of the different strains (Schwyn and Neilands, 1987). This assay is based on competition for iron by the indicator dye (CAS) and the siderophore produced by the microorganism. Removal of iron from CAS by the siderophore causes it to change color (from blue to orange), which is measured by absorbance at 630Å. Siderophores were generated by adding $3 \times 10^8$ conidia to AMM without iron, and incubating them at 37°C in a shaking incubator. For the *A. fumigatus* studies, the incubation time was 30 h. Because *A. nidulans* had greater extracellular siderophore activity than *A. fumigatus*, a 24 h incubation time was used. Next, the conditioned medium was collected by filtration, and an aliquot was added to the CAS assay solution. After adding shuttle solution to the mixture, the $OD_{630}$ was determined using a spectrophotometer. A standard curve was constructed using different dilutions of ferrichrome. The extracellular siderophore activity of each strain was measured in 3 independent experiments.

## Effects of different carbon sources on growth

To test the effects of different carbon sources on growth, AMM plus iron without glucose was supplemented with 0.5% ethanol, 50 mM acetate, 50 mM L-proline, or 1% glucose (Hynes *et al.*, 2007). Serial 10-fold dilutions of conidia ranging from $10^5$ to $10^2$ cells in a volume of 5 μl were spotted onto AMM agar plates. The plates were incubated at 37 °C for 40 h and then imaged to determine the relative sizes of the colonies. Each experiment was repeated 3 times.

### Susceptibility to environmental stress and antifungal agents

To determine susceptibility to environmental stress, $10^3$ conidia or germlings of the various *A. fumigatus* strains were added to individual wells of 96-well plates containing serial 2-fold dilutions of the various stressors in Sabouraud broth. These stressors included Congo red (5 to 250 μg/ml), calcofluor white (5 to 250 μg/ml), hydrogen peroxide (0.1 to 5 mM), SDS (0.0002 to 0.01%), caspofungin (0.001 to 0.2 μg/ml), amphotericin B (0.25 to 2.2 μg/ml). The plates were incubated at 37°C for 48 h, after which the growth in the wells was scored visually. The minimal inhibitory concentration was defined as the concentration of that stressor that inhibited growth by at least 80% compared to organisms grown in the absence of that stressor.

### *A. fumigatus* killing by macrophage-like and neutrophil-like HL-60 cells

HL-60 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium, supplemented with glutamine (Irvine Scientific, Santa Ana, CA), 10% fetal bovine serum (Gemini BioProducts, Woodland, CA), 1% penicillin, streptomycin, and 50 μM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) at 37°C in 5% $CO_2$. To induce differentiation into macrophage-like cells, the HL-60 cells were stimulated with 50 nM 12-o-tetradecanoyl-phorbol-13-acetate for 2 days (Collins, 1987). To induce differentiation into neutrophil-like cells, the HL-60 cells incubated in 1.3% (v/v) DMSO and 2.5 μM retinoic acid for 3 days (Spellberg *et al.*, 2005). Conidia from the 3 strains *A. fumigatus* were incubated in RPMI 1640 medium at 37°C for 4 h to obtain swollen conidia. Next, $10^3$ swollen conidia were added to polypropylene tubes containing differentiated HL-60 cells in 1 ml RPMI 1640 medium supplemented with 5% pooled human serum (Sigma-Aldrich) to achieve a target to effector cell ratio of 1:50. After incubation at 37°C in 5% $CO_2$ for 3 h, the tubes were vortexed vigorously, and the number of surviving organisms was determined by quantitative culture. Swollen conidia incubated in the absence of HL-60 cells were processed in parallel as a negative control. Each experiment was performed in triplicate and repeated 3 times.

### Statistical analyses

The survival data were analyzed using the Log-Rank test, and pulmonary fungal burden results were analyzed with the Wilcoxon Rank Sum test. The data from the in vitro experiments were analyzed by Analysis of Variance. A $p$ value of $\leq 0.05$ was considered to be significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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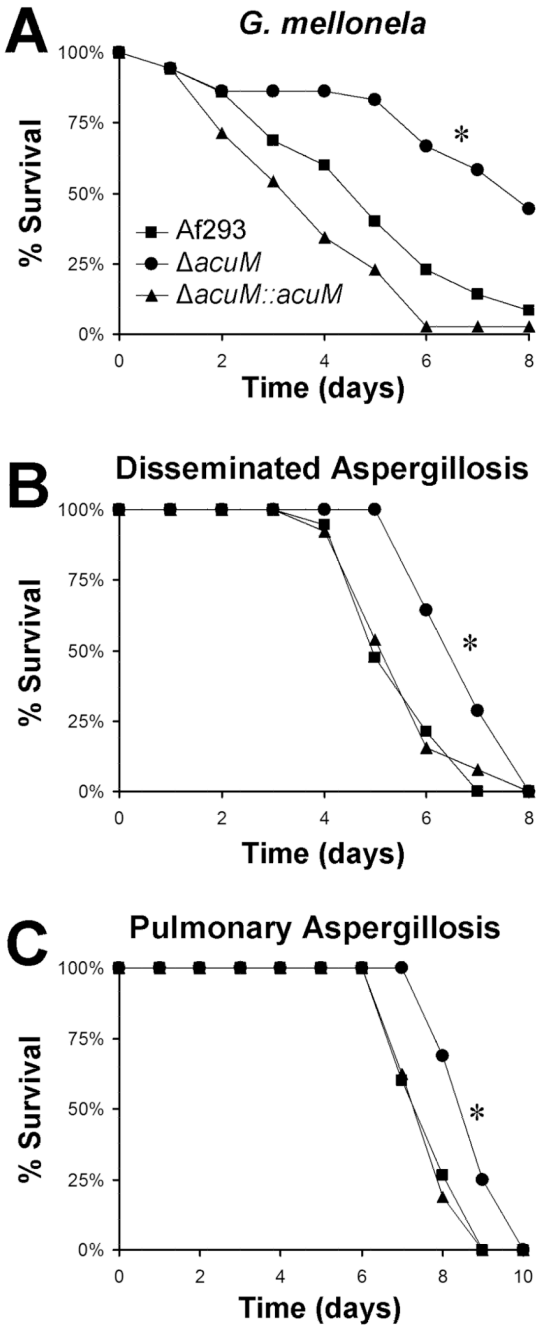


**Fig. 1.**

Deletion of *acuM* results in reduced virulence in *G. mellonella* and murine models of invasive aspergillosis. (A) Survival of *G. mellonella* larvae after injection with conidia of the indicated strains. Representative results of one of three experiments, each with 35 larvae per strain. (B) Survival of neutropenic mice with hematogenously disseminated aspergillosis. Mice were immunosuppressed with cyclophosphamide and cortisone acetate, inoculated intravenously with germlings of the indicated strain, and followed for survival. Results are the combined data from two experiments for a total of 13–19 mice per strain. (C) Survival of non-neutropenic mice with invasive pulmonary aspergillosis. Mice were immunosuppressed with cortisone acetate and then inoculated by placing them in an acrylic chamber filled with

an aerosol of conidia from the indicated strains of *A. fumigatus*. Results are the combined data from two experiments for a total of 15–16 mice per strain. **P* ≤ 0.005 compared to Af293 and the Δ*acuM*::*acuM* complemented strains.

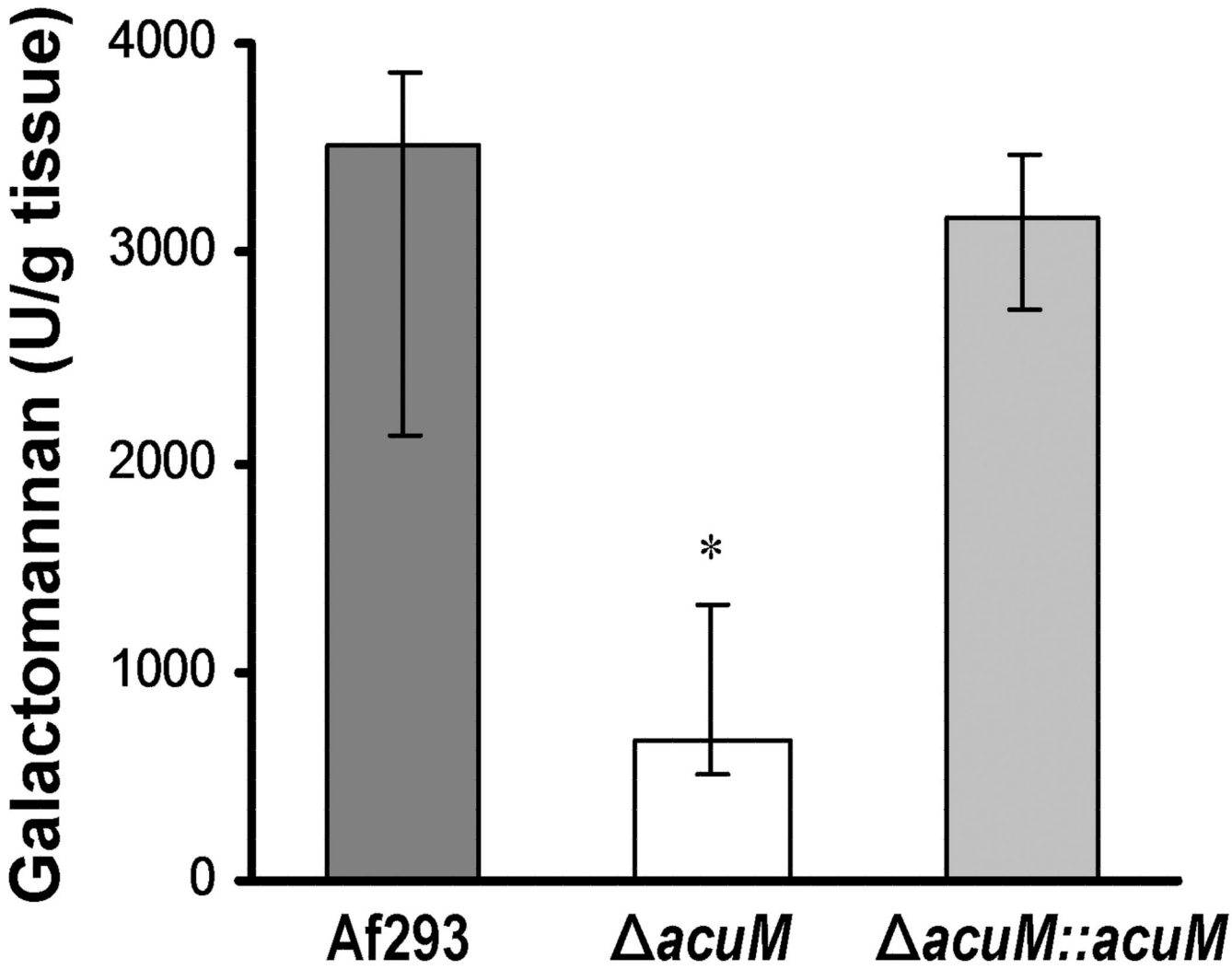


**Fig. 2.**

Mice infected with the Δ*acuM* mutant had a lower pulmonary fungal burden. Non-neutropenic mice were infected with the indicated strains in the aerosol chamber and then sacrificed after 4 days for determination of pulmonary galactomannan content. Results are median ± interquartile range of 7 to 9 mice per strain. **P* < 0.001 compared to Af293 and the Δ*acuM*::*acuM* complemented strain.

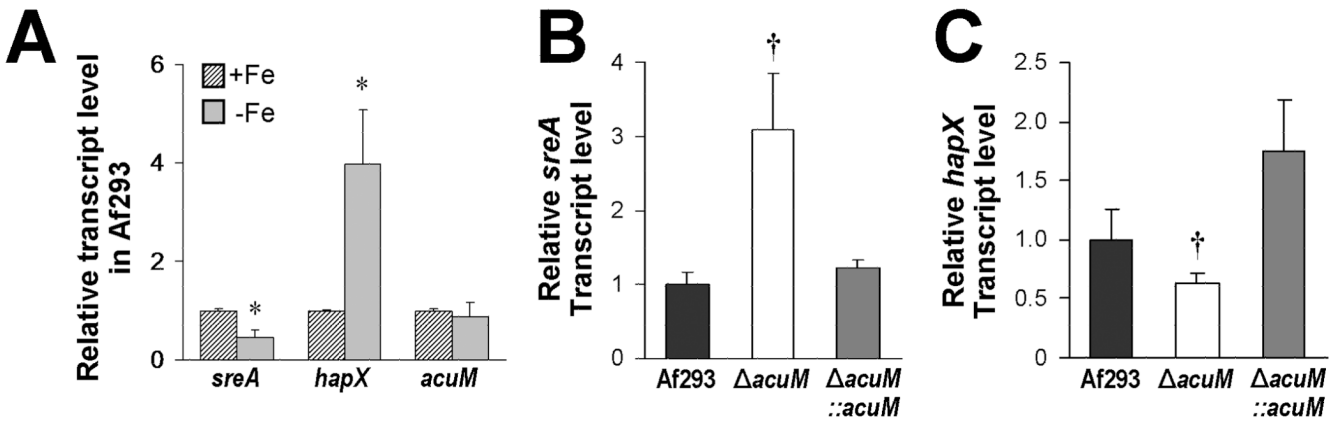


**Fig. 3.**

Effects of extracellular iron and *acuM* deletion on *sreA* and *hapX* mRNA levels. (A) *A. fumigatus* Af293 was grown in *Aspergillus* minimal medium (AMM) containing ferrozine and $FeSO_4$ (+Fe) or AMM containing ferrozine without $FeSO_4$ (−Fe) for 24 h, after which the relative transcript levels of the indicated genes were determined by real-time PCR. (B and C) The indicated strains of *A. fumigatus* were grown for 24 h in AMM containing ferrozine without $FeSO_4$, and then the relative transcript levels of *sreA* (B) and *hapX* (C) were determined by real-time PCR. Results are the mean ± SD of three biological replicates, each tested in triplicate. *$P < 0.05$ compared to organisms grown in the presence of $FeSO_4$; †$p < 0.05$ compared to Af293 or the Δ*acuM*::*acuM* complemented strain.

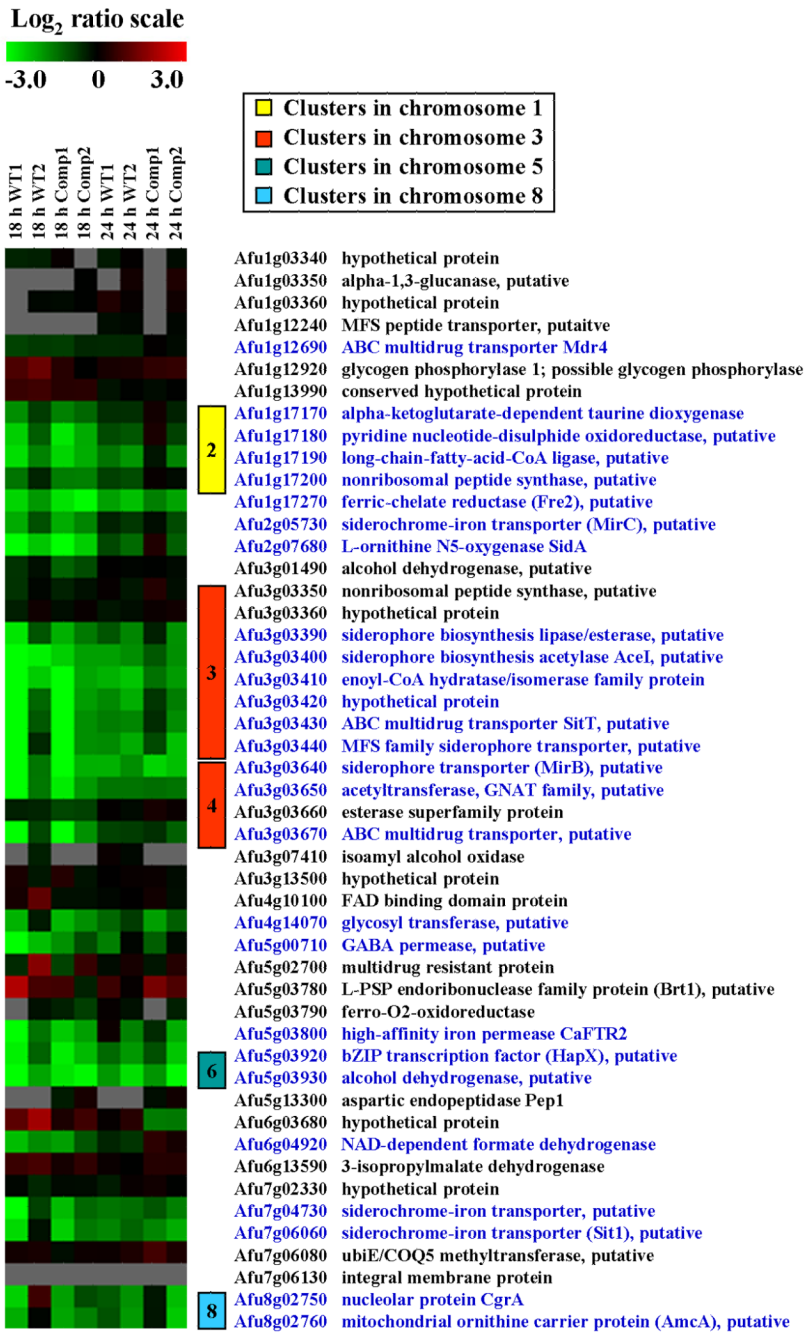


**Fig. 4.**

Cluster analysis of SreA-responsive gene expression in the Δ*acuM* mutant. Microarray data comparing the response of the Δ*acuM* mutant with Af293 (WT) and the Δ*acuM*::*acuM* complemented strain (Comp) after 18 and 24 h incubation in RPMI 1640 medium at 37°C are shown. The bar at the top indicates the colors that correspond to the observed expression ratios. The genes are displayed in the order of their chromosomal location and the vertical colored boxes indicate gene clusters. The numbers in these boxes correspond to the SreA-responsive gene clusters in (Schrettl *et al.*, 2008). The gene names in blue font denote genes that had significantly reduced transcript levels in the Δ*acuM* mutant compared to the control strains.

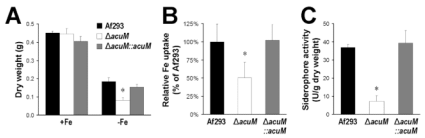


**Fig. 5.**

Deletion of *acuM* resulted in decreased growth in a low iron medium, impaired iron incorporation, and reduced extracellular siderophore production. (A) Dry weight of the indicated strains after 40 h of incubation in Sabouraud broth containing phenanthroline with (+Fe) or without $FeSO_4$ (−Fe). (B) Relative incorporation of $^{55}FeCl_3$ after 1 h by washed germlings of the indicated strains. (C) Extracellular siderophore activity of the indicated strains after 30 h of growth. Results are mean ± SD of three (A and B) or four (C) experiments, each performed in triplicate. **P* < 0.001 compared to Af293 and the Δ*acuM*::*acuM* complemented strain.

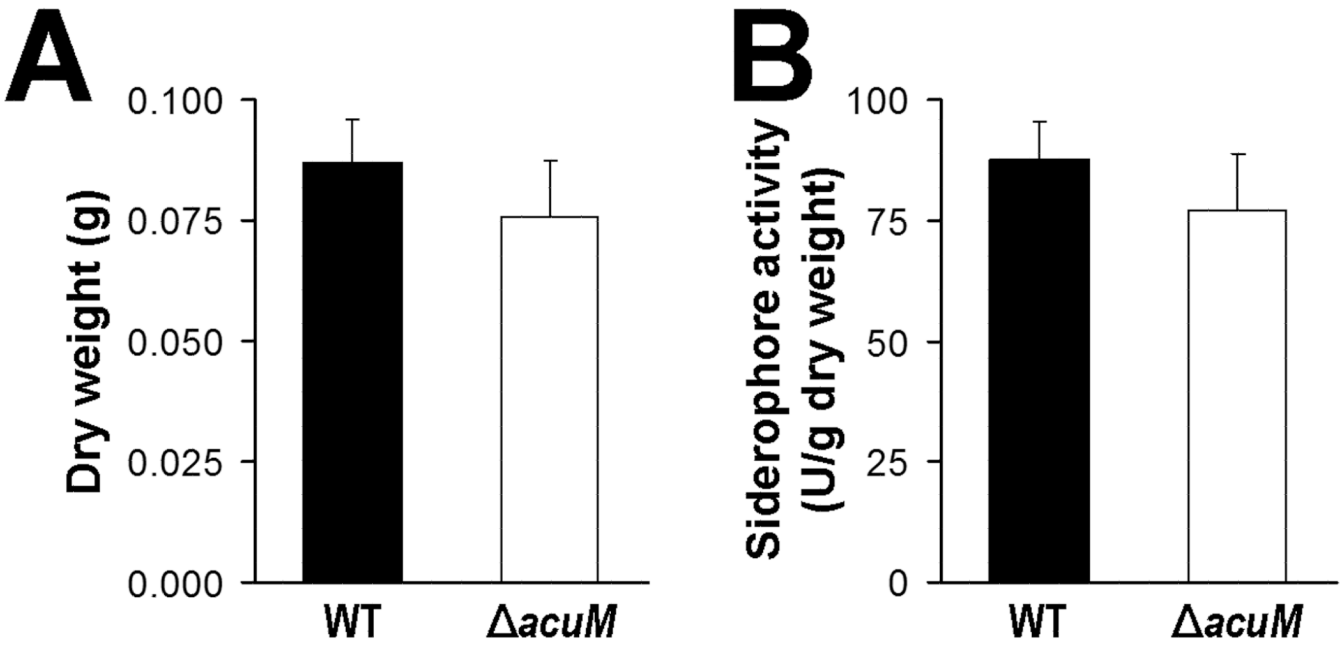


**Fig. 6.**

Role of *A. nidulans* AcuM in extracellular siderophore production and growth under iron limited conditions. (A) Dry weight of the indicated strains after 24 h growth in AMM without iron. (B) The indicated strains of *A. nidulans* were grown in AMM without iron for 24 h, after which the total extracellular siderophore activity was determined. Results are mean ± SD of three independent experiments.

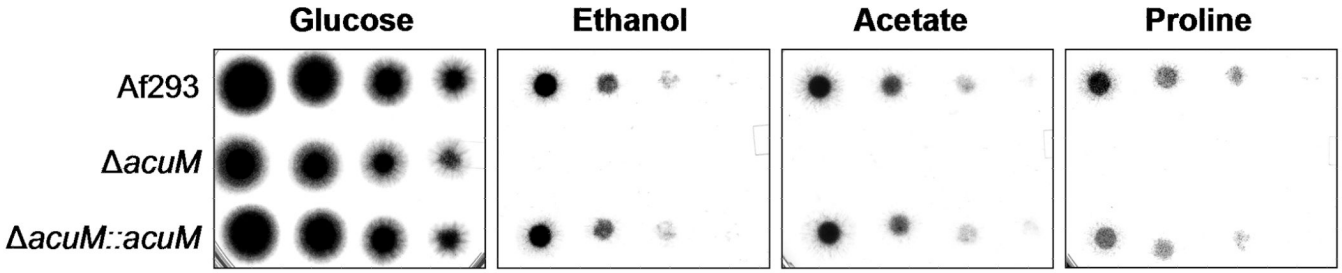


**Fig. 7.**

AcuM is required for normal growth on gluconeogenic carbon sources. Serial 10-fold dilutions of the indicated strains were grown on *Aspergillus* minimal medium supplemented with $FeCl_3$ and containing the indicated carbon sources. The plates were imaged after incubation at 37°C for 40 h.

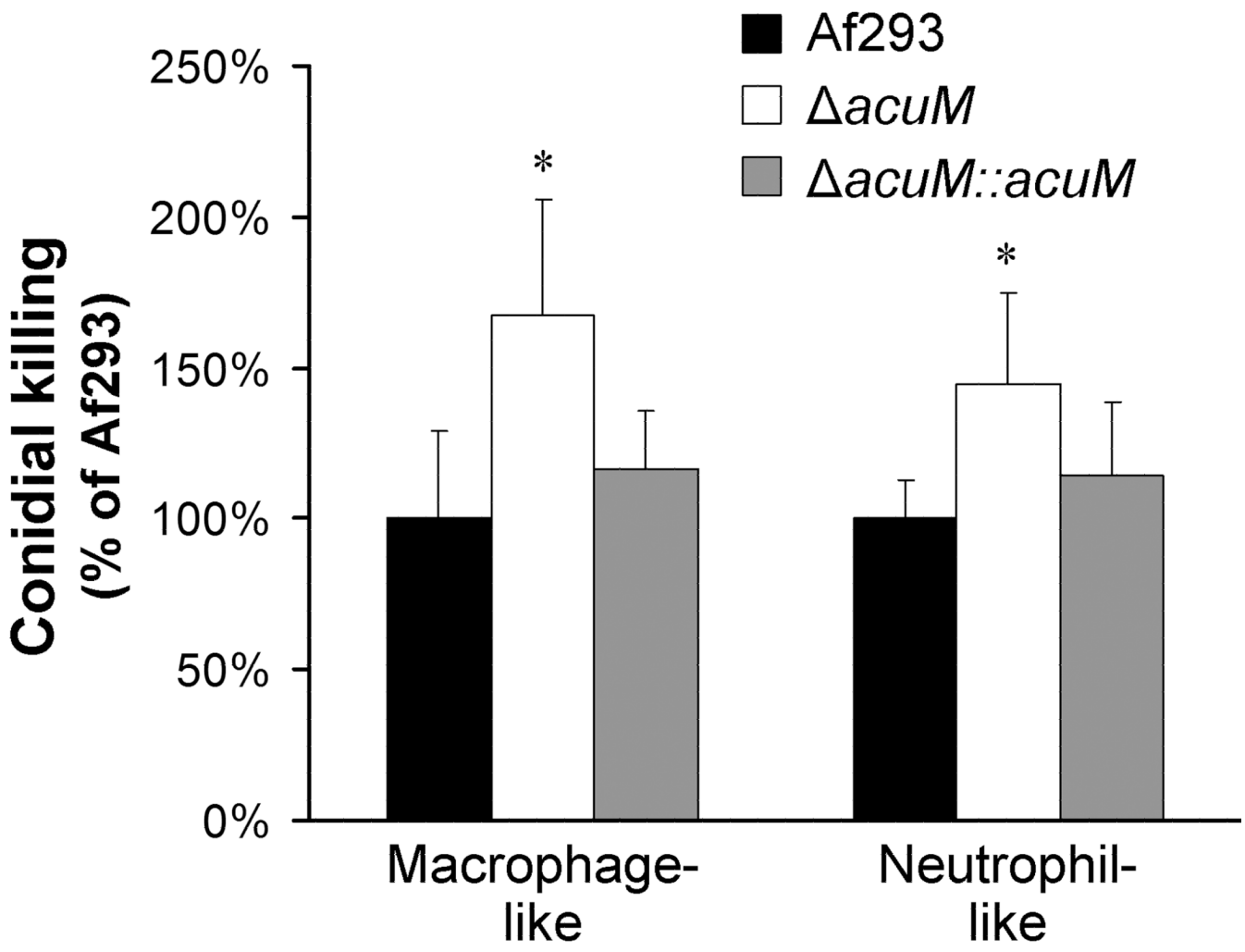


**Fig. 8.**

Increased susceptibility of the Δ*acuM* mutant to phagocyte killing. Swollen conidia of the indicated strains were incubated for 3 h with HL-60 cells that had been differentiated into macrophage-like or neutrophil-like cells. The number of organisms killed was determined by quantitative culture. Results are mean ± SD of three independent experiments each performed in triplicate. **P* < 0.01 compared to Af293 or the Δ*acuM::acuM* complemented strain.

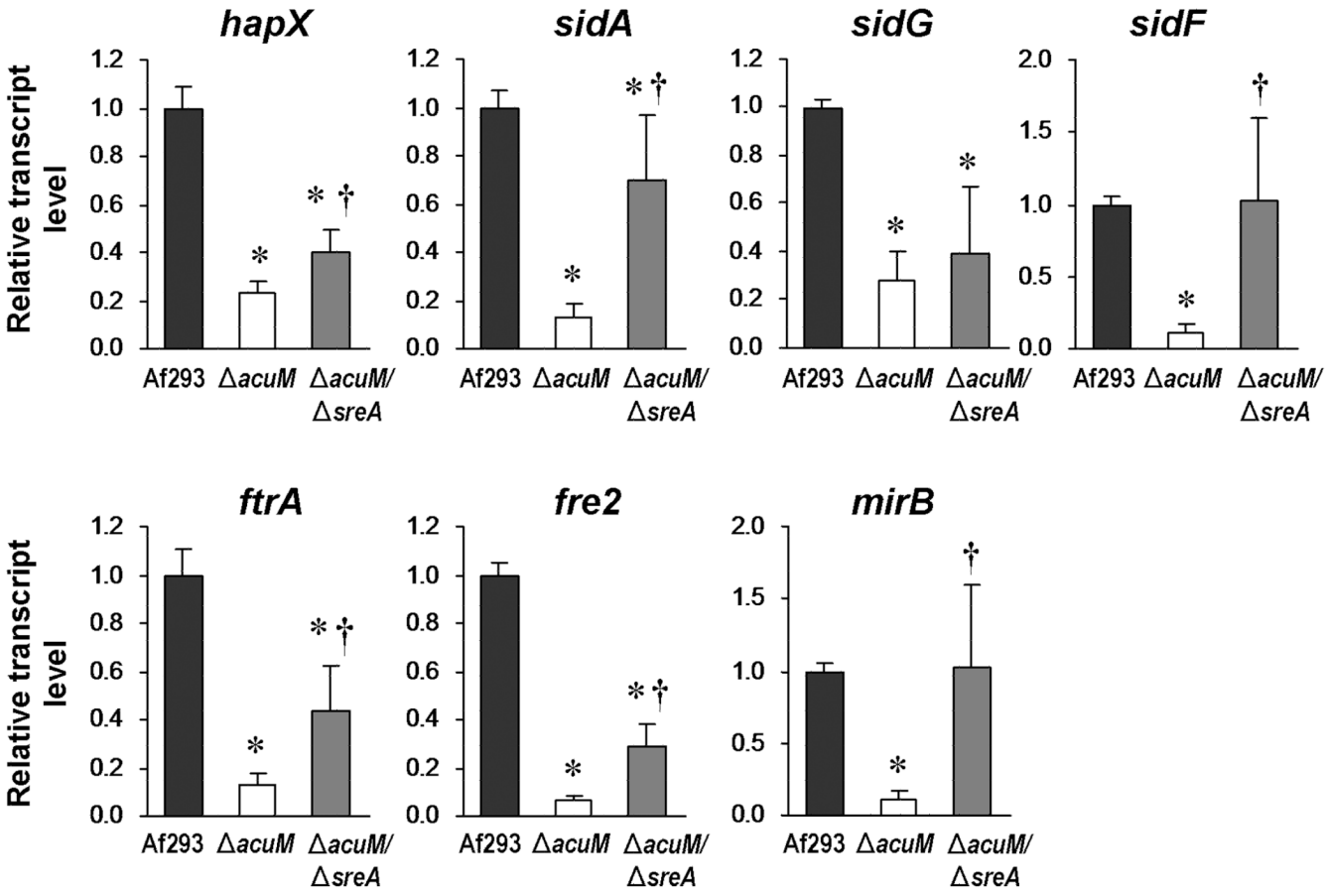


**Fig. 9.**

Deletion of *sreA* in the Δ*acuM* mutant results in enhanced expression of genes involved in iron acquisition. The indicated strains of *A. fumigatus* were incubated for 24 h at 37°C in AMM without iron containing 300 μM ferrozine, after which the relative transcript levels of the indicated genes were determined by real-time PCR. Results are the mean ± SD of 3 biological replicates, each tested in triplicate. **P* < 0.05 compared to Af293, †*p* < 0.05 compared to the Δ*acuM* single mutant.

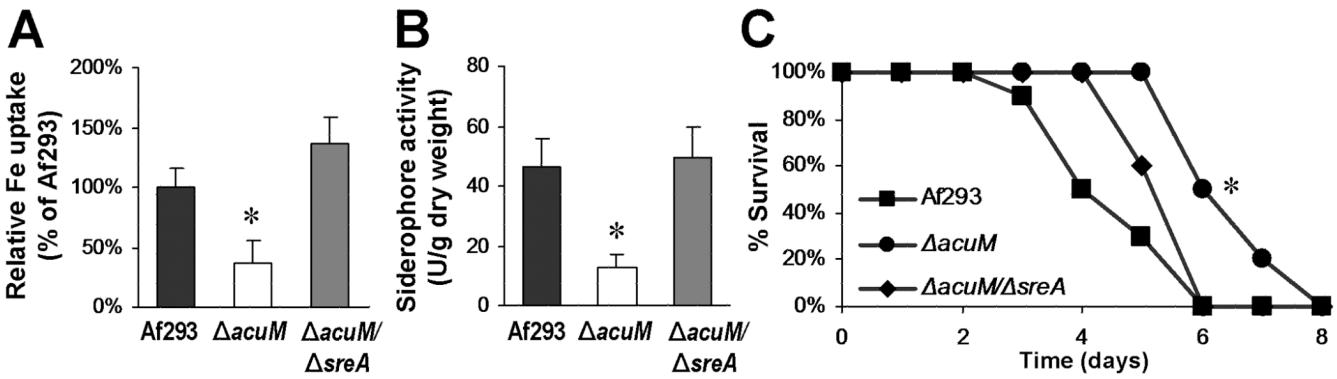


**Fig. 10.**

The Δ*acuM* Δ*sreA* double mutant has wild-type iron uptake, extracellular siderophore production, and virulence. (A) Relative incorporation of $^{55}FeCl_3$ after 1 h by washed germlings of the indicated strains. Results are mean ± SD of three experiments, each performed in triplicate. (B) Extracellular siderophore activity of the indicated strains after 30 h of growth. Results are mean ± SD of three experiments, each performed in triplicate. (C) Survival of neutropenic mice after intravenous inoculation with conidia of the indicated strains. Each strain was inoculated into 10 mice. *$P < 0.005$ compared to Af293 and the Δ*acuM* Δ*sreA* double mutant.

## Table 1

List of *A. fumigatus* genes that are predicted to specify transcription factors, and for which null mutants were constructed.

| Strain | *A. fumigatus* Gene | Ortholog | Function of ortholog | Virulence of null mutant (Median survival time in days)[a] |
|---|---|---|---|---|
| Af293 (wild-type) | | | | 8.5 |
| ΔAfu1g02860 | Afu1g02860 | *Fusarium graminearum* TRI6 | Regulator of mycotoxin production (Pinson-Gadais *et al.*, 2008; Proctor *et al.*, 1995; Seong *et al.*, 2009) | 7.5 |
| ΔAfu1g04140 | Afu1g04140 | *A. fumigatus* specific transcription factor | | 7.5 |
| ΔAfu2g04600 | Afu2g04600 | *Aspergillus. parasticus* SugR | Regulator of sugar utilization (Yu *et al.*, 2000) | 7.5 |
| Δ*acuM* | Afu2g12330 (*acuM*) | *Saccharomyces cerevisiae* Rds2; *Candida albicans* Cwt1; *Aspergillus nidulans* AcuM | Regulator of cell wall integrity and gluconeogenesis (Moreno *et al.*, 2003; Moreno *et al.*, 2007a; Moreno *et al.*, 2008; Soontorngun *et al.*, 2007) | 12.0[b] |
| Δ*acuM* Δ*sreA* | Afu2g12330 (*acuM*) Afu5g11260 (*sreA*) | *A. nidulans* SreA | Negative regulator of iron homeostasis | ND[c] |
| ΔAfu2g16310 | Afu2g16310 | Fungal specific transcription factor | | 8.0 |
| ΔAfu4g09710 | Afu4g09710 | *A. nidulans* NosA | Regulator of sexual development (Vienken and Fischer, 2006) | 8.0 |
| Δ*steA* | Afu5g06190 (*steA*) | *S. cerevisiae* Ste12; *C. albicans* Cph1 | Regulator of filamentation and mating (Braun and Johnson, 2000; Lewis *et al.*, 2002; Liu *et al.*, 1994; Ramer and Davis, 1993; Vallim *et al.*, 2000) | 9.0 |
| ΔAfu5g10130 | Afu5g10130 | *A. fumigatus* specific transcription factor | | 7.0 |
| ΔAfu6g07170 | Afu6g07170 | *A. nidulans* RosA | Regulator of sexual development, repressor of NosA (Vienken *et al.*, 2005) | 10.0 |
| Δ*yap1* | Afu6g09930 (*yap1*) | *S. cerevisiae* Yap1 | Regulator of oxidative stress tolerance (Delaunay *et al.*, 2000) | 9.0 |
| ΔAfu8g05460 | Afu8g05460 | | Up-regulated in heat shock (Do *et al.*, 2009) | 7.0 |
| | Afu2g10770 | *Magnaporthe. grisea* Con7 | Essential for appressorium formation and disease-related morphogenesis (Odenbach *et al.*, 2007) | ---[d] |

[a] Survival of *G. mellonella* larvae after infection with $10^6$ conidia of the indicated null mutant Data are the combined results two experiments, each using 20–30 larvae per strain

[b] $P < 0.001$ compared to larvae infected with Af293

[c] Not determined

[d] Deletion mutant for this gene was unable to be constructed

## Table 2

Overrepresented functional categories of *A. fumigatus* genes that were down-regulated in the Δ*acuM* mutant compared to the wild-type and Δ*acuM*::*acuM* complemented strain.

| GO Term | *P* value | |
|---|---|---|
| | 18 h | 24 h |
| Siderophore-iron transport | 0.17 | 0.0000015 |
| Iron ion homeostasis | 0.45 | 0.00028 |
| Gluconeogenesis | 0.00035 | 0.00095 |
| Glyoxylate cycle | 1.0 | 0.00092 |
| One-carbon compound metabolism | 0.00036 | 1.0 |
| Methionine metabolism | 0.0000088 | 1.0 |
| Glutamate biosynthesis | 0.0012 | 0.0000045 |

## Table 3

List of genes involved in siderophore synthesis and transport, and iron uptake that were down-regulated in the Δ*acuM* mutant compared to Af293.

| *A. fumigatus* gene | Fold-change in mRNA level in the Δ*acuM* mutant | | | Putative function |
|---|---|---|---|---|
| | Microarray (18 h)[a] | Microarray (24 h)[a] | Real-time PCR (24 h)[b] | |
| Afu1g17270 (*fre2*) | −3.2 | −2.4 | −2.3 | Ferric-chelate reductase |
| Afu5g03800 (*ftrA*) | −2.8 | −1.6 | −1.6 | High-affinity iron permease |
| Afu2g07680 (*sidA*) | −4.0 | −1.4 | −4.2 | L-ornithine $N^5$-oxygenase |
| Afu1g17200 (*sidC*) | −1.7 | −1.2 | −1.7 | Nonribosomal peptide synthase |
| Afu3g03420 (*sidD*) | −3.3 | −2.0 | −3.1 | Fusarinine C nonribosomal peptide synthetase |
| Afu3g03650 (*sidG*) | −3.0 | −1.9 | −11.4 | Fusarinine C acetyltransferase |
| Afu3g03400 (*sidF*) | −3.4 | −2.1 | −7.2 | Siderophore biosynthesis acetylase |
| Afu3g03390 | −2.3 | −1.7 | −5.0 | Siderophore biosynthesis lipase/esterase |
| Afu3g03640 (*mirB*) | −4.0 | −2.7 | −4.1 | Siderophore transporter |
| Afu7g06060 (*sit1*) | −2.2 | −2.1 | −2.1 | Siderophore transporter |
| Afu7g04730 | −2.4 | −1.8 | −2.1 | Siderophore transporter |
| Afu6g07720 (*acuF*) | −9.4 | −6.4 | −16.2 | Phosphoenolpyruvate carboxykinase |
| Afu4g11310 (*fbp1*) | −2.9 | −1.7 | −7.2 | Fructose-1,6-bisphosphatase |

[a] Mean of 4 biological replicates in RPMI 1640 medium

[b] Mean of 3 biological replicates in AMM without iron and with 300 μM ferrozine