

The *zrfA* and *zrfB* Genes of *Aspergillus fumigatus* Encode the Zinc Transporter Proteins of a Zinc Uptake System Induced in an Acid, Zinc-Depleted Environment

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Zinc is an essential micronutrient that cells must obtain from the environment in order to develop their normal growth. Previous work performed at our laboratory showed that the synthesis of immunodominant antigens from *Aspergillus* spp., including *A. fumigatus*, was up-regulated by a low environmental concentration of zinc. These results suggested that a tightly regulated system for the fungus to grow under zinc-limiting conditions must underlie the ability of *A. fumigatus* to acquire zinc in such environments. In this work, we show that *zrfA* and *zrfB* are two of the genes that encode membrane zinc transporters from *A. fumigatus* in this system. Expression of these genes is differentially down-regulated by increasing concentrations of zinc in the medium. Thus, the transcription of *zrfB* is turned off at a concentration 50-fold higher than that for *zrfA* transcription. In addition, phenotypic analyses of single *zrfA*Δ and *zrfB*Δ mutants and a double *zrfA*Δ*zrfB*Δ mutant revealed that the deletion of *zrfB* causes a greater defect in growth than the single deletion of *zrfA*. Deletion of both genes has a dramatic effect on growth under acid, zinc-limiting conditions. Interestingly, in neutral or slightly alkaline zinc-depleted medium, the transcriptional expression of both genes is down-regulated to such an extent that even in the absence of a supplement of zinc, the expression of *zrfA* and *zrfB* is strongly reduced. This fact correlates with the growth observed in alkaline medium, in which even a *zrfA*Δ*zrfB*Δ double mutant was able to grow in a similar way to the wild-type under extremely zinc-limiting conditions. In sum, the zinc transport proteins encoded by *zrfA* and *zrfB* are members of a zinc uptake system of *A. fumigatus* that operates mainly under acid, zinc-limiting conditions.

Zinc is the second most abundant transition metal in cells after iron. Although it is found in cells in trace amounts, this element is an essential component of many enzymes. More than 300 different enzymes, including representatives of all six major functional enzyme classes, use zinc as a cofactor for their catalytic activity or/and structural stability by mediating the correct folding of specific domains (1). Thus, zinc is considered to be an essential micronutrient for all organisms, although excess amounts of zinc may prove poisonous for cells. As a consequence, a tight regulation of its availability is a critical issue in the normal growth of all organisms. However, as a highly charged metal ion, zinc cannot passively enter the cell through the hydrophobic lipid bilayer of the plasma membrane but instead requires specific membrane transporter proteins for its incorporation into cells (10).

Aspergillus fumigatus is a saprophytic fungus that inhabits soils, where it grows on organic debris and releases hundreds of conidia into the atmosphere. Conidia inhaled by immunocompetent individuals are efficiently eliminated by nonspecific immune cells, which constitute the first defense barrier (19). However, in the lung tissue of immunocompromised patients, extensive growth of *A. fumigatus* may result in invasive aspergillosis, the most fatal variety of aspergillosis (22). Where

A. fumigatus grows (in soil or in living tissue), its survival depends on its ability to obtain all the nutrients, including essential micronutrients from the environment such as iron, copper, or zinc, needed to support its growth. Since *A. fumigatus*, like most microorganisms, generally lives in environments that are nutrient limiting due to low initial availability or depletion caused by the growth of any other microorganism, this fungal pathogen has developed different mechanisms to obtain metal nutrients from its medium. In this context, much work has been done regarding the mechanism of iron uptake by pathogenic microorganisms (18). *Aspergillus* spp., including pathogenic strains of *A. fumigatus*, excrete different kinds of siderophores to mobilize extracellular iron with high affinity through siderophore transporters (23, 26). The acquisition of zinc has also been recognized as one of the key steps in the process of infection by any pathogen, since this metal is strongly sequestered by high-affinity zinc-binding proteins in mammalian hosts (29). However, not much work has been done to address this issue. In this line, we have previously shown that *A. nidulans* and *A. fumigatus* produce the immunodominant antigens Aspnd1 and Aspfd2, respectively, only when the fungi are grown in synthetic media without a zinc supplement (27). These antigens were specifically recognized by human sera obtained from patients with aspergilloma, indicating that these antigens are highly expressed when the fungus grows on living tissue (3). These results led us to investigate the mechanism of zinc uptake in this fungal pathogen. Furthermore, zinc uptake could represent a possible target for an antifungal chemotherapy (5), since *A. fumigatus* zinc uptake

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TABLE 1. Strains used in this study

Strain	Genotype or description	Reference or source
<i>A. fumigatus</i>		
CETC	Prototrophic wild type	CECT ^a
CEA17	Auxotrophic <i>pyrG</i> ⁻	7
AF14	Prototrophic wild type (isogenic to CEA17)	This work
AF01	<i>zrfAΔ::neo-pyrG-neo</i>	This work
AF02	<i>zrfAΔ::neo-pyrG-neo</i>	This work
AF03	<i>zrfAΔ::neo-pyrG-neo</i>	This work
AF04	<i>zrfBΔ::hisG-pyrG-hisG</i>	This work
AF05	<i>zrfBΔ::hisG-pyrG-hisG</i>	This work
AF13	<i>zrfBΔ::hisG-pyrG-hisG</i>	This work
AF09	<i>zrfAΔ::neo</i> (auxotrophic <i>pyrG</i> ⁻)	This work
AF06	<i>zrfAΔ::neo zrfBΔ::hisG-pyrG-hisG</i>	This work
AF10	<i>zrfAΔ::neo zrfBΔ::hisG-pyrG-hisG</i>	This work
AF11	<i>zrfAΔ::neo zrfBΔ::hisG-pyrG-hisG</i>	This work
AF15	<i>zrfAΔ::neo zrfBΔ::hisG</i> (auxotrophic <i>pyrG</i> ⁻)	This work
AF36	<i>zrfA-pyrG-neo zrfBΔ::hisG</i>	This work
AF37	<i>zrfA-pyrG-neo zrfBΔ::hisG</i>	This work
AF38	<i>zrfAΔ::neo zrfB-pyrG-hisG</i>	This work
AF39	<i>zrfAΔ::neo zrfB-pyrG-hisG</i>	This work
<i>S. cerevisiae</i>		
ZHY3	<i>MATα ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2 zrt2::HIS3</i>	34

^a Spanish Type Culture Collection.

proteins have no significant similarity with proteins exerting a similar function in mammals (20).

To date, microbial zinc uptake has been characterized functionally in detail only in the yeast *Saccharomyces cerevisiae*. Here, we report the functional characterization of an *A. fumigatus* zinc uptake system. Our results show that, unlike that observed in *S. cerevisiae*, the *A. fumigatus* zinc uptake system depends not only on a low environmental zinc concentration but also on environmental pH. In this work, we focus on the study of the mechanism that operates under acid, zinc-limiting conditions.

MATERIALS AND METHODS

Strains, media, and culture conditions. All fungal strains used in this work are listed in Table 1. All strains were routinely grown in either PDA complex medium (20 g/liter potato dextrose agar, 20 g/liter sucrose, 2.5 g/liter MgSO₄ · 7 H₂O) or AMMC medium [10 g/liter dextrose, 0.92 g/liter ammonium tartrate, 0.52 g/liter MgSO₄ · 7 H₂O, 0.52 g/liter KCl, 1.52 g/liter KH₂PO₄, 1.1 mg/liter H₃BO₃, 2.2 mg/liter ZnSO₄ · 7 H₂O, 0.5 mg/liter MnCl₂ · 4 H₂O, 0.5 mg/liter FeSO₄ · 7 H₂O, 0.16 mg/liter CoCl₂ · 2 H₂O, 0.16 mg/liter CuSO₄ · 5 H₂O, 0.11 mg/liter (NH₄)₂Mo₇O₂₄ · 4 H₂O, 5 mg/liter Na₂ EDTA, pH adjusted to 6.5 with 1.0 N NaOH] at 37°C for several days. For specific experiments, strains were also grown on SD base medium (5.7 g/liter yeast nitrogen base with ammonium sulfate [without amino acids, dextrose, phosphate, Cu, Zn, or Fe; purchased from Q-BIOgene], 20 g/liter dextrose, 1 g/liter KH₂PO₄, pH 4.5) which was made a complete SD medium when Fe³⁺, Cu²⁺, and Zn²⁺ were added to SD base medium at a final concentration of 0.74 μM, 0.16 μM, and 100 μM, respectively. Other media that were also used were CDDTE medium (20 g/liter dextrose, 3.0 g/liter NaNO₃, 0.5 g/liter MgSO₄ · 7 H₂O, 0.5 g/liter KCl, 1.0 g/liter KH₂PO₄, 0.01 g/liter FeSO₄ · 7 H₂O, 30 mM Trizma base, 250 μM EDTA, pH 8.7) and FBS medium (50% fetal bovine serum, 2% dextrose).

Metals were added at the concentrations specified for each experiment from sterile solutions of MnCl₂ · 4 H₂O (20 mM), FeCl₂ · 4 H₂O (100 mM), FeCl₃ · 6 H₂O (7.4 mM), CdCl₂ · 2 H₂O (100 mM), CoCl₂ · 6 H₂O (100 mM), NiCl₂ · 6 H₂O (100 mM), CuSO₄ · 5 H₂O (1.6 mM), or ZnSO₄ · 7 H₂O (14 mM) in water. All liquid media were sterilized by filtration. Solid media were prepared by adding sterile agar to get a 2% final concentration. In all cases, spores grown in

PDA medium were used as inoculum. Prewarmed liquid media were inoculated to a density of 5 × 10⁵ spores/ml and incubated at 37°C with shaking at 200 rpm.

To provide growth conditions at different pH values, a 200 mM PO₄²⁻, 400 mM K⁺ buffer was used supplemented with 200 μM TPEN [N,N,N',N'-tetrakis(2-pyridyl-methyl)ethylenediamine] (P4413; a specific zinc chelator obtained from Sigma). This buffer was added to complete SD medium, without a supplement of zinc, in which *A. fumigatus* was growing. SD medium was rendered slightly alkaline by including these ions as 57 mM K₂HPO₄ and 57 μM TPEN (resulting in an initial pH of 7.5); SD was made slightly acid by including 28.5 mM K₂HPO₄ together with 28.5 mM KH₂PO₄ plus 28.5 mM KCl and 57 μM TPEN (resulting in an initial pH of 6.5); SD medium was rendered acid by including 57 mM KH₂PO₄ plus 57 mM KCl and 57 μM TPEN (resulting in an initial pH of 3.5). These pH conditions were formally designated as alkaline, neutral, and acid, respectively. After 1 h of culture at 37°C, pH values did not change.

Yeast strain ZHY3 was routinely grown in yeast extract-peptone-dextrose complex medium at 28°C. For specific experiments, yeast strains were grown on SD medium (5.7 g/liter yeast nitrogen base [obtained from Q-BIOgene], 20 g/liter dextrose, 1 g/liter K₂HPO₄, 0.74 μM FeCl₃ · 6 H₂O, and 0.16 μM CuSO₄ · 5 H₂O) supplemented with 0.67 g/liter of a -LEU -URA dropout mixture (Q-BIOgene). Zinc was added at the final concentration indicated for each particular experiment from a stock solution of ZnSO₄ · 7 H₂O (14 mM).

DNA manipulations and analyses. Standard molecular biology procedures for DNA manipulation were used (25). Genomic DNA from *A. fumigatus* strains was obtained as previously described (3). For Southern blot analyses, 4 μg of DNA per lane was loaded onto a 0.8% agarose gel and transferred by capillarity to positively charged nylon membranes by standard protocols. Probes were digoxigenin labeled by random priming (DIG DNA labeling kit; Roche) and hybridized and detected according to the chemiluminescence method described in the manufacturer's recommendations (DIG nucleic acid detection kit; Roche).

Total RNA was isolated according to the protocol provided with the RNeasy Plant Mini kit (QIAGEN). Twenty micrograms of total RNA per lane was loaded onto 1% formaldehyde agarose gels and transferred by capillarity to neutral nylon membranes according to standard protocols (25). For Northern analysis, fragments of DNA containing most of the open reading frame (ORF) from each gene were labeled by random priming using the DNA Labeling Beads (dCTP) kit and [³²P]dCTP (3,000 Ci mmol⁻¹) (Amersham Pharmacia Biotech) and used as probes. Hybridization was carried out at 68°C in a solution containing 0.5 M NaH₂PO₄ (pH 7.2), 7% sodium dodecyl sulfate (SDS), and 1 mM EDTA. After hybridization, the blots were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.2% SDS at 68°C for 20 min. The 0.24- to 9.5-kb RNA ladder (Life Technologies) was used for sizing in formaldehyde-agarose gels. As internal RNA load and quality controls, a DNA fragment carrying a partial ORF of the actin gene (*actA*) from *A. fumigatus* was used as a probe.

Isolation of the *zrfA* and *zrfB* genes from *A. fumigatus*. A DNA fragment from each gene was isolated by a PCR amplification experiment using the degenerate primers JA01 and JA02 (Table 2). These primers were designed according to a pair of amino acid sequences conserved between Zrt1p and Zrt2p of *S. cerevisiae* (GenBank accession numbers CAA96975 and CAA97701, respectively). These sequences are separated in both cases by 153 and 151 amino acids, respectively. PCR amplification was performed with genomic DNA from the *A. fumigatus* CETC strain as template. Thirty-five cycles of PCR were performed with the following steps: 45 seconds at 94°C; 1 min at 54°C, and 1.5 min at 72°C. The reaction yielded a fragment with a predicted size of approximately 460 bp. The PCR product was subcloned into pGEM-T easy (Promega). Analysis of several transformants showed that two different plasmids had been obtained: pJAC1 and pJAC2 (Table 2). Sequencing of the PCR fragment carried by each plasmid revealed that two different PCR fragments were in fact amplified. The DNA fragment contained in pJAC1 was similar to a *ZRT1* DNA sequence while the one carried by pJAC2 was more similar to a *ZRT2* DNA fragment from *S. cerevisiae*. Next, both PCR products were excised from pJAC1 and JAC2 by EcoRI digestion, digoxigenin labeled by nonradioactive random priming, and used as probes to screen a λEMBL3 *A. fumigatus* genomic library as previously described (4). The detection of positive clones was done according to the colorimetric method described in the manufacturer's recommendations (DIG nucleic acid detection kit; Boehringer Mannheim). A 3.09-kb XbaI-XbaI DNA fragment containing the entire *zrfA* gene from *A. fumigatus* was subcloned into pBlueScript SK(-) to generate pZRF1g. Similarly, a 4.24-kb PvuII-SacI DNA fragment containing the entire *zrfB* gene was subcloned into pBlueScript SK(-) to generate pZRF24g.

Determination of growth rate of *A. fumigatus* strains. Dry weight (mg of mycelium per ml of culture) at each time was determined as previously described

TABLE 2. Oligonucleotides and plasmids used in this study

Primer or plasmid	Sequence ^a or description	Source or reference
Primers		
JA01	CTBGARTTYGGHATCATCTTYCA	
JA02	GCCAYTTDCCGATVARRGCCAT	
JA09	GCACATGAGAGTCGTTTGTGC	
JA10	CCTCGAGTCACCGCTACAACC	
JA25	AACTGAGCGGAATACTCCTCG	
JA36	AAGCTAGCGGCTACCCCTACGACGTCCCCGACTACGCCCTTCGACCCCTCCAACGTTGACC	
JA37	CCTGGTTAACGATCATTACGCCCACTTGCC	
JA45	AAGCTAGCGGCTACCCCTACGACGTCCCCGACTACGCCCAAGGCCTTCACACTCTTCTGG	
JA46	GTCAGTTAACGAGCGAGATTAGGCC	
RTZ22	GGGAGTGCAATTATAAGAGTCGCG	
Plasmids		
pJAC1	PCR product (partial sequence of <i>zrfA</i>)	This work
pJAC2	PCR product (partial sequence of <i>zrfB</i>)	This work
pMC5-HSET	Carries the <i>ZRT1</i> gene from <i>S. cerevisiae</i>	13
pNPN1	Smaller version of plasmid pCDA14 (7)	This work
pNPN2	Derivative from pNPN1 which carries the <i>pyrG-hisG</i> cassette flanked by sites NsiI-BstZ17I upstream of gene <i>pyrG</i>	This work
pPYRG1r	pUC19 derivative carrying the <i>pyrG-hisG</i> cassette flanked by sites EcoRI/BstZ17I upstream of the <i>pyrG</i> gene	This work
pPYRG2	pUC19 derivative carrying the <i>hisG-pyrG-hisG</i> cassette flanked by EcoRI/SmaI/XbaI and HpaI/SpeI sites	This work
pRS416	A centromeric plasmid for expression of genes in <i>S. cerevisiae</i>	28
pZRF1g	pBluescript carrying a 3.09-kb XbaI fragment containing the <i>zrfA</i> gene	This work
pZRF24g	pBluescript carrying a 4.24-kb PvuII-SacI fragment containing the <i>zrfB</i> gene	This work
pZHA1	pMC5-HSET in which the NheI-HpaI fragment had been replaced by the ORF of <i>zrfA</i>	This work
pZHA2	pMC5-HSET in which the NheI-HpaI fragment had been replaced by the ORF of <i>zrfB</i>	This work
pZRF1-D4	pZRF1g in which the BstZ17I-StuI fragment had been replaced by a 6.84-kb HpaI fragment containing the <i>neo-pyrG-neo</i> cassette	This work
pZRF2-D6	pZRF24g in which the XmnI-StuI fragment had been replaced by a 3.88-kb HpaI-SmaI fragment containing the <i>hisG-pyrG-hisG</i> cassette	This work
pZRF18-D10	pNPN2 in which a 2.74-kb NsiI-EcoRV fragment from pZRF1g was ligated into NsiI/BstZ17I	This work
pZRF25-D7	pPYRG1r in which a 3.32-kb EcoRI-PvuII fragment from pZRF24g was ligated into EcoRI/BstZ17I	This work

^a Restriction sites are underlined.

(2). The generation time (μ) during the log phase (exponential growth) was determined by the formula $\mu = (t_f - t_0)/n$, where n is the number of generations calculated from the formula $n = (\log W_f - \log W_0)/\log 2$, in which W_f is the dry weight at the end of the time period (t_f) and W_0 is the dry weight at the beginning of the time period (t_0). The generation times calculated for each strain are the averages from three independent experiments. Using data on generation times, the growth rate (α , h^{-1}) was calculated from the formula $\alpha = \ln 2/\mu$.

Construction of plasmids used for *S. cerevisiae* transformation. The ZHY3 yeast strain was transformed with the centromeric plasmids pZHA1 and pZHA2 (Table 2), which carry the *zrfA* and *zrfB* genes, respectively, from *A. fumigatus*. In these plasmids, the expression of either the *zrfA* or *zrfB* ORFs from *A. fumigatus* is driven by the *ZRT1* promoter of *S. cerevisiae*. To construct these plasmids, only the encoding region of the *zrfA* and *zrfB* genes of *A. fumigatus* was obtained by PCR using plasmid pZRF1g or pZRF24g as a template and the oligonucleotides JA36-JA37 and JA45-JA46, respectively, as primer pairs (Table 2). The quality of PCR products was confirmed by sequencing. The NheI restriction site was included in oligonucleotides JA36 and JA45. The HpaI restriction site was included in oligonucleotides JA37 and JA46. PCR products were digested with NheI and HpaI and used to replace the NheI-HpaI fragment of plasmid pMC5-HSET, which contains nearly the entire ORF of *ZRT1* (13). Strain ZHY3 was independently transformed to Ura3⁺ strains with plasmids pRS416 (28) and pMC5-HSET, which were used as a negative and positive control, respectively. Transformation was carried out according to the LiAc procedure (11). All strains were selected on the appropriate SD dropout medium supplemented with 500 μ M ZnSO₄ · 7 H₂O.

Construction of plasmids used for *A. fumigatus* transformation. To obtain a *zrfA*Δ-null mutant, plasmid pZRF1-D4 was constructed. This plasmid carries a transforming DNA designed to delete most of the ORF of *zrfA*. To construct this plasmid, a 6.84-kb HpaI-HpaI fragment obtained from plasmid pNPN1, which contains the *neo-pyrG-neo* cassette, was used to replace a 381-bp BstZ17I-StuI

fragment of the ORF from *zrfA* in plasmid pZRF1g. Similarly, in order to obtain a *zrfB*Δ-null mutant, a 0.75-kb XmnI-StuI fragment of the ORF of *zrfB* in plasmid pZRF24g was replaced by a 3.88-kb SmaI-HpaI fragment, which contains the *hisG-pyrG-hisG* cassette, to generate plasmid pZRF2-D6. The *hisG-pyrG-hisG* cassette is carried by plasmid pPYRG2, which was constructed at our lab. To obtain revertant strains for either gene *zrfA* or *zrfB*, plasmids pZRF18-D10 and pZRF25-D7, respectively, were constructed. A 2.74-kb NsiI-EcoRV fragment from pZRF1g was ligated into NsiI-BstZ17I sites in plasmid pNPN2, which carries the cassette *pyrG-neo*, to generate plasmid pZRF18-D10. To construct plasmid pZRF25-D7, a 3.32-kb EcoRI-PvuII fragment from pZRF24g was ligated into EcoRI-BstZ17I sites in plasmid pPYRG1r, which carries the cassette *pyrG-hisG*. Plasmid pZRF2-D4 was linearized by digestion with NheI and NotI, plasmid pZRF2-D6 was linearized by PvuI and NotI digestion, plasmid pZRF18-D10 was linearized by XmnI and NsiI digestion, and plasmid pZRF25-D7 was linearized by digestion with PvuII. In all cases, linearized DNA was extracted with phenol-chloroform-isoamylalcohol, precipitated with isopropanol, resuspended in STC buffer (1 M sorbitol, 10 mM CaCl₂, 10 mM Tris-HCl, pH 7.5), and used for transformation as described below.

Protoplast generation and transformation of *A. fumigatus*. The protocols used by other laboratories for the preparation of *A. fumigatus* protoplasts did not work in our hands. However, using such protocols as references, we designed a new procedure which did work in a reproducible fashion. Briefly, conidia were collected in 0.1% Tween 80 from a PDA plate. About 50 ml of liquid AMMC medium supplemented with 0.05% uracil and 0.12% (wt/vol) uridine was inoculated with 8×10^8 spores. The culture was incubated at 28°C for 15 h to induce germination. Mycelia were collected by centrifugation at 3,000 rpm for 10 min at room temperature and washed twice with 0.6 M MgSO₄ · 7 H₂O. Conidia were resuspended in 10 ml of 1.2 M MgSO₄ · 7 H₂O. A fresh protoplasting solution was prepared as follows: 1 ml bovine serum albumin (10 mg/ml), 100 mg glucanex (lot no. L1412; Sigma), 0.1 ml zymolyase 20T (50 mg/ml), and 0.2 ml β-gluc-

ronidase (lot no. G8885; Sigma) were dissolved in 7 ml of 1.2 M $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, buffered with 80 ml of 1.0 M K_2HPO_4 and 0.92 ml of 1.0 M KH_2PO_4 , and incubated at room temperature for 5 min. This protoplasting solution was added to the spore suspension and incubated at 32°C for 4 h with gentle shaking. After incubation, the suspension was brought up to 20 ml with 1.2 M $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, overlaid with 20 ml of TB medium (0.6 M sorbitol, 0.1 M Tris-HCl, pH 7.5), and centrifuged at 3,000 rpm for 15 min at room temperature in a bucket rotor. Protoplasts were recovered from the interphase and washed once with ST medium (1 M sorbitol, Tris-HCl, pH 7.5) and twice with STC buffer. Protoplasts were resuspended in 0.8 ml of STC buffer. Aliquots of 0.3 ml were used to transform protoplasts of the *pyrG*⁻ *A. fumigatus* strain CEA17 using 40 µg of DNA. Next, 75 µl of a 60% polyethylene glycol solution was mixed with the DNA-protoplast mixture and incubated for 20 min on ice. Next, 0.75 ml of additional polyethylene glycol solution was added, mixed, and incubated at room temperature for 20 min. Protoplasts were pelleted and resuspended in 0.2 ml of STC buffer, mixed with 3 ml of soft agar medium, and laid out on an AMMC plate. Plates were incubated at 37°C until *pyrG*⁺ fungal transformants had developed. Up to 100 independent transformants for each mutant were reisolated on AMMC medium, and suspensions of conidia from each were prepared in 1.0 ml of water. The correct integration at the expected locus can take place only by means of two recombination events. Those transformants which still bore the gene to be deleted at its original locus were discarded by PCR analysis of genomic DNA obtained from spores as described below. The correct recombination events at the expected loci were verified by Southern blot analyses using the appropriate probes.

Spontaneous *pyrG*⁻ fungal strains from a *pyrG*⁺ independent clone were selected on AMMC medium containing uracil (0.05%), uridine (0.12%), and 5-fluoroorotic acid (1 mg/ml). These *pyrG*⁻ fungal strains were then used in a second round of transformation to generate a double mutant strain.

Screening of transformants of *A. fumigatus* by PCR. Genomic DNA was obtained from spores resuspended in 0.1 ml of 10 mM EDTA and 50 mM Tris-HCl (pH 8.0). Next, 0.1 ml of a denaturing solution (0.2 M NaOH, 1% SDS) was mixed with the spore solution and incubated at 65°C for 30 min. After cooling at room temperature for 10 min, pH was neutralized and SDS was precipitated with 0.1 ml of 3.0 M potassium acetate (pH 5.5). The suspension was cleared by centrifugation for 8 min at 4°C, and the DNA was precipitated with isopropanol, air dried, and resuspended in 10 µl water. The PCR was carried out in a final volume of 25 µl, using 1 µl of DNA suspension as a template. The oligonucleotides used for this purpose must have a GC content up to 60%, preferably either CG or GC at their 3' end. Oligonucleotides JA09 and JA10 were used in a rapid PCR-based screening in order to discard transformants carrying the *neo-pyrG-neo* cassette integrated at a locus other than the *zrfA* locus. The same oligonucleotides were used in a rapid PCR-based screening for positive identification of *zrfAΔ pyrG*⁻ pop-out strains. Following the same approach, oligonucleotides RTZ22 and JA25 were used to discard transformants carrying the *hisG-pyrG-hisG* cassette integrated at a locus other than the *zrfB* locus.

Construction of an isogenic strain of *A. fumigatus* for use as a control. As described above, the *pyrG*⁻ CEA17 strain was used as a recipient for transforming DNA for the construction of *A. fumigatus* mutants. This strain was generated by chemical mutagenesis, and the mutation at the *pyrG* locus of this strain has been well characterized at the molecular level (7). However, although in most aspects of its biology this strain has been reported to be similar to the wild-type strain from which it was generated (7), it cannot be precluded that some functions might have been reduced or lost due to additional mutations in loci other than *pyrG*. Thus, in order to avoid any misinterpretation of the phenotype of the mutants, an isogenic *pyrG*⁺ strain of CEA17, called AF14, was constructed following the transformation procedure described above by replacing the damaged *pyrG* locus of CEA17 with a wild-type DNA carrying the *pyrG* locus. Revertant *pyrG*⁺ strains were selected on AMMC medium without uracil or uridine. In order to ensure that no ectopic integration events had occurred in addition to the expected one, several *pyrG*⁺ strains were tested by Southern blot using the same DNA fragment as that used for transformation as a probe.

Nucleotide sequence accession numbers. The sequence data of the *zrfA* and *zrfB* genes have been submitted to the DDBJ, EMBL, and GenBank databases under accession numbers AY611771 and AY611772, respectively.

RESULTS

The products encoded by the *zrfA* and *zrfB* genes are membrane zinc transporters. The *zrfA* and *zrfB* genes were identified and cloned as described in Materials and Methods. The *zrfA* gene has an ORF of 1,077 bp. The initiation transcription

site (position +1 in the mRNA) was mapped by rapid amplification of cDNA ends (RACE)-PCR 13 nucleotides upstream from the ATG start codon and 26 nucleotides downstream from the putative TATA box. There are three similar 15-bp sequences in the promoter region that share the same 5'-CAAGGT-3' core. Preliminary data obtained at our laboratory suggested a role of these sequences in the regulation of *zrfA* gene expression at the transcriptional level (unpublished data). We have designated these sequences ZRA1, ZRA2, and ZRA3 (for zinc response *zrfA*), located from positions -60 to -74, -92 to -106, and -142 to -156, respectively (for numbering purposes, we assigned position -1 to the nucleotide preceding the ATG). In addition, from position -94 to -99, located exactly at the middle of the ZRA2 sequence, there is a 5'-GCCARG-3' PacC-like binding motif (30). Interestingly, no introns were identified within the coding region of *zrfA*, in contrast to most *A. fumigatus* genes characterized so far. The ORF of *zrfA* encodes a 359-amino-acid protein with a predicted molecular mass of 38.9 kDa. Analysis of the amino acid sequence of the ZrfA protein using the Dense Alignment Surface method (6) revealed that it may contain up to eight transmembrane domains. Thus, ZrfA seems to be a membrane-embedded protein that crosses the membrane up to eight times. Transmembrane domains III and IV are separated by a region longer than the rest of the spacer regions. The membrane topology of ZrfA, as predicted by the "positive-inside" rule (31), suggests that both the N and C termini are located on the outer surface of the membrane.

Similarly to *zrfA*, the *zrfB* gene has a 1,059-bp ORF not disrupted by introns. It encodes a protein that is structurally similar to ZrfA (353 amino acids and eight transmembrane domains). Interestingly, the initiation transcription site of *zrfB* mRNA was mapped by RACE-PCR 268 nucleotides upstream from the ATG codon and 28 nucleotides downstream from the putative TATA box. Thus, the 5' noncoding region of the mRNA is unusually longer than that of most genes from *Aspergillus*. Also, in the promoter region of *zrfB*, similarly to the *zrfA* gene promoter, there are four 15-bp sequences designated ZRB1, ZRB2, ZRB3, and ZRB4, which are located at positions -346 to -360, -481 to -495, -779 to -793, and -878 to -892, respectively. In addition, there are two PacC-like binding motifs extending from positions -282 to -287 and -982 to -987. The identity of ZrfB to ZrfA is 36.6% while similarity increases to 52.8%.

A search in the GenBank database using the BLASTP algorithm revealed that both proteins ZrfA and ZrfB were similar to other putative membrane metal transporters, although they showed the highest similarity to proteins belonging to the Zrt-, Irt-like protein (ZIP) family of transporters involved in cellular zinc uptake (10). Thus, ZrfA showed the highest identity with the EAA66960 protein from *Aspergillus nidulans* (62.5%), with Zrt1p from *S. cerevisiae* (54.5%), and with protein EAA33619 from *Neurospora crassa* (49.0%). ZrfB showed the highest identity with protein EAA60007 from *A. nidulans* (75.6%), protein EAA52364 from *Magnaporthe grisea* (42.4%), and Zrt2p from *S. cerevisiae* (37.6%). Accordingly, while it seems clear that ZrfA may be considered an orthologue of Zrt1p (differences due to speciation phenomena while conserving function), it would not be as easy to consider ZrfB a true orthologue of Zrt2. In fact, ZrfB/Zrt1p identity is higher

(35.3%) than that of Zrt1p/Zrt2p (28.7%). Finally, neither ZrfA nor ZrfB shows significant similarities (less than 15%) with any human zinc transporter characterized so far.

As predicted by structural features and multiple alignment analysis of the ZrfA and ZrfB proteins against many other putative ZIP transporters, the products of the *zrfA* and *zrfB* genes may actually encode zinc membrane transporters. To obtain further evidence to support this assumption, the *zrfA* and *zrfB* genes were independently expressed in the ZHY3 strain of *S. cerevisiae*. This yeast strain is not able to grow in severely zinc-limiting conditions unless a high amount of zinc (>1,000 μM) is added to the medium because it lacks the *ZRT1* and *ZRT2* genes involved in zinc uptake (34). In order to check whether *zrfA* or *zrfB* was able to complement the lack of genes involved in zinc uptake in *S. cerevisiae*, the ORF of either *zrfA* or *zrfB* was expressed independently in the ZHY3 strain from a centromeric plasmid constructed as described in Materials and Methods. The ability of ZHY3 transformants to grow under zinc-limiting conditions was tested in complete SD medium (without a supplement of zinc) supplemented with 1 mM EDTA and increasing amounts of zinc (from 0 to 1,500 μM). As shown in Fig. 1, both *zrfA* and *zrfB* from *A. fumigatus* were equally able to complement the lack of the *ZRT1* and *ZRT2* genes in *S. cerevisiae*. Thus, this analysis clearly suggested that the proteins encoded by *zrfA* and *zrfB* can function as zinc transporters in the background provided by *S. cerevisiae*, and it supports the idea that the ZrfA and ZrfB proteins may also function as zinc transporters in *A. fumigatus*.

The expression of *zrfA* and *zrfB* is regulated in a zinc-dependent fashion. Since *zrfA* and *zrfB* encode membrane zinc transporters, and since there are several 15-bp zinc response (ZR) sequences in their promoter regions, we investigated whether the expression of these genes was regulated by zinc. Thus, a wild-type isogenic strain of *A. fumigatus* (AF14), generated as described in Materials and Methods, was grown for 20 h in complete SD medium and in SD medium without a supplement of either zinc, iron, or copper. Gene transcription was analyzed by Northern blot. In fungal cells grown in complete SD medium, as well as in those grown in SD without a supplement of iron or copper, no transcription of either *zrfA* or *zrfB* was detected (Fig. 2). In contrast, a high level of transcription of both genes was observed in fungal cells grown in SD medium without a supplement of zinc. In addition, no other biological metals, such as Fe^{3+} , Cu^{2+} , Mn^{2+} , Fe^{2+} , Co^{2+} , or Ni^{2+} , were able to mimic the repressor effect of zinc on the transcription of both genes, even when SD medium without a supplement of zinc was supplemented with an excess (250 μM) of those metals. Interestingly, only an excess of cadmium, a nonbiological element, was able to mimic the effect of zinc in turning off the transcription of both genes.

In order to estimate the minimum concentration of zinc initially added to the medium which is required to maintain the transcription of the *zrfA* and *zrfB* genes turned off, *A. fumigatus* (AF14) was grown on SD medium supplemented with increasing amounts of zinc (ranging from 0.0 to 250 μM) for 20 h under the culture conditions described in Materials and Methods, and gene transcription was analyzed by Northern blot (Fig. 3). The initial concentration of zinc required to maintain repressed transcription was 2.0 μM for *zrfA* and 100 μM for

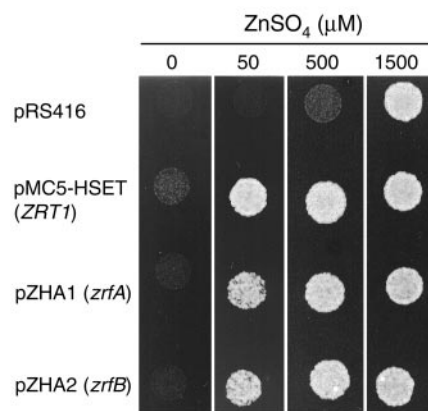


FIG. 1. Complementation analysis of zinc uptake provided by the *A. fumigatus* *zrfA* and *zrfB* genes in the ZHY3 yeast strain. This strain was transformed with the empty pRS416 plasmid, plasmid pMC5-HSET (*ZRT1*), plasmid pZHA1 (*zrfA*), and plasmid pZHA2 (*zrfB*). ZHY3 transformed with pRS416 and pMC5-HSET were included as negative and positive controls, respectively. A total of 10^4 yeast cells were spotted on SD medium supplemented with 1 mM EDTA and increasing amounts of zinc sulfate (0 to 1,500 μM), and plates were incubated for 2 days at 30°C before the picture was taken.

zrfB. This indicates that transcription of *zrfA* and *zrfB* is differentially regulated by the environmental concentration of zinc.

To investigate the stability of the transcriptional product (mRNA) of both genes, *A. fumigatus* (AF14) was grown for 20 h in SD medium without an initial supplement of zinc to ensure that both genes were being transcribed (Fig. 4). At that time, the culture was supplemented with 100 μM zinc, and total RNA was obtained from samples of mycelia taken after 5, 10, 20, and 30 min of culture. No mRNA for either *zrfA* or *zrfB* could be detected after 20 min of incubation as observed by

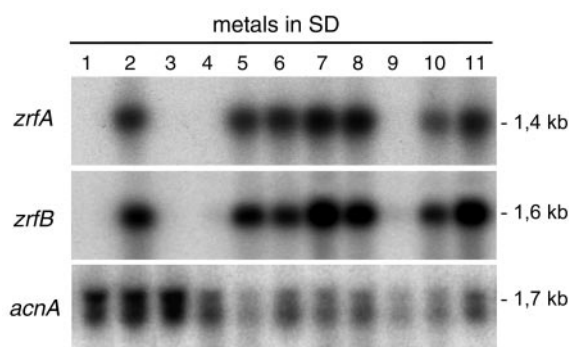


FIG. 2. Northern blot showing the effect of zinc on the transcription of *zrfA* and *zrfB*. Strain AF14 was grown in SD medium supplemented as follows: SD medium plus Zn^{2+} plus Fe^{3+} plus Cu^{2+} (1), SD medium minus Zn^{2+} plus Fe^{3+} plus Cu^{2+} (2), SD medium plus Zn^{2+} minus Fe^{3+} plus Cu^{2+} (3), SD medium plus Zn^{2+} plus Fe^{3+} minus Cu^{2+} (4), SD medium minus Zn^{2+} plus Fe^{3+} plus Cu^{2+} (5), SD medium minus Zn^{2+} plus Fe^{3+} plus Cu^{2+} (6), SD medium minus Zn^{2+} plus Fe^{3+} plus Cu^{2+} plus Mn^{2+} (7), SD medium minus Zn^{2+} plus Fe^{3+} plus Cu^{2+} plus Fe^{2+} (8), SD medium minus Zn^{2+} plus Fe^{3+} plus Cu^{2+} plus Cd^{2+} (9), SD medium minus Zn^{2+} plus Fe^{3+} plus Cu^{2+} plus Co^{2+} (10), and SD medium minus Zn^{2+} plus Fe^{3+} plus Cu^{2+} plus Ni^{2+} (11) (metals in boldface type were added at a final concentration of 250 μM). Probes are indicated to the left of each blot.

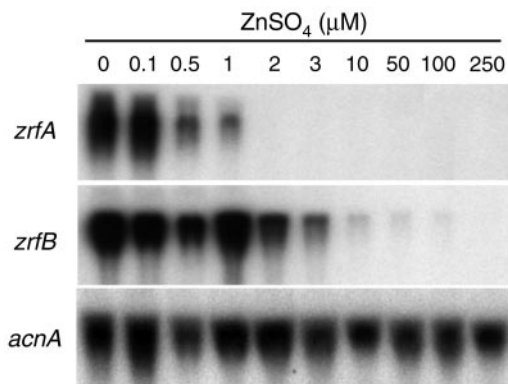


FIG. 3. Repression of transcription of *zrfA* and *zrfB* by increasing amounts of zinc. Strain AF14 was grown for 20 h at 37°C in complete SD medium supplemented with zinc sulfate as indicated above each lane. Note that supplement of zinc refers to the amount of zinc initially added to the medium over traces of zinc already present in the medium as a contaminant. However, zinc consumption as a consequence of fungal growth reduced the amount of zinc available in the medium, such that after 20 h of culture, the minimal amount of zinc available in the medium required to repress transcription of each gene is unknown. Probes are indicated to the left of each blot.

Northern blot which shows that mRNA is being degraded rapidly as soon as the transcription of new mRNA molecules stops. Interestingly, identical results were obtained when repression was triggered with a concentration of zinc in the medium as low as 1 μM (data not shown), which suggests that the transcriptional mechanism which controls transcription of either *zrfA* or *zrfB* is sensible to any sudden slight increase of zinc in the environment. In sum, these results suggest that the control of the transcription of the *zrfA* and *zrfB* genes must lie in the first stage of a tightly regulated mechanism involved in zinc uptake.

***zrfA* and *zrfB* are required for optimal growth of *A. fumigatus* under zinc-limiting conditions.** In order to confirm the function of these genes in *A. fumigatus*, a mutant strain for each gene and a double *zrfA**zrfB* Δ mutant strain were constructed by the gene replacement technique described in Materials and Methods. Briefly, a 381-bp fragment of the *zrfA*-encoding region was replaced by the *neo-pyrG-neo* cassette in the CEA17 *pyrG*⁻ strain to generate strains AF01, AF02, and AF03 (Fig. 5A). Similarly, a 750-bp fragment containing almost the entire open reading frame of the *zrfB* gene was replaced by the *hisG-pyrG-hisG* cassette in strain CEA17 to generate strains AF04, AF05, and AF13 (Fig. 5B). The AF06, AF10, and AF11 double *zrfA**zrfB* Δ mutant strains were constructed by replacing the *zrfB* gene by the *hisG-pyrG-hisG* cassette in strain AF09, a spontaneous *zrfA* Δ *pyrG*⁻ strain obtained from AF01 as described in Materials and Methods. The recombination events occurring in the AF09 strain were identical to those taking place when the *zrfB* gene was replaced in strain CEA17. All strains harbored the correct integration event at the expected locus, as verified by Southern blot analyses (Fig. 5C). In order to more clearly assess the function of each gene over the growth of *A. fumigatus*, and following a strategy similar to that used to construct either *zrfA* Δ - or *zrfB* Δ -null mutants, a revertant strain for each gene was generated after reintroducing independently a wild-type copy of either gene *zrfA* or *zrfB* at its

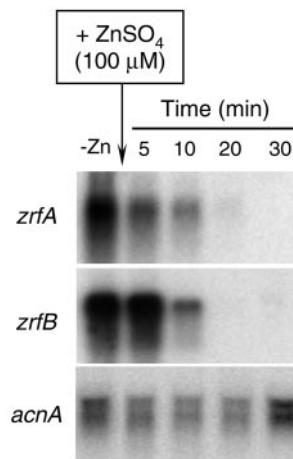


FIG. 4. Life span (turnover) of *zrfA* and *zrfB* mRNA after repression of transcription by zinc. Strain AF14 was grown in complete SD medium without a supplement of zinc for 20 h, and a sample was taken (lane 1, -Zn). At this point, the culture was supplemented with 100 μM zinc and samples were collected and immediately frozen in liquid nitrogen after 5, 10, 20, and 30 min. Probes are indicated to the left of each blot.

original locus into the strain AF15, a spontaneous *zrfA**zrfB* Δ *pyrG*⁻ strain obtained from AF10 (data not shown). The gene *zrfA* was reintroduced in AF15 using the *zrfA-pyrG-neo* cassette to generate strains AF36 and AF37, two independent *zrfB* Δ mutant strains that should grow identically to any single *zrfB* Δ mutant strain. The gene *zrfB* was reintroduced into AF15 using the *zrfB-pyrG-hisG* cassette to generate strains AF38 and AF39, two independent *zrfA* Δ mutant strains that should present growth identical to that of any single *zrfA* Δ mutant strain.

Phenotypic analysis was performed for all the *pyrG*⁺ mutant strains. The three independent *zrfA* Δ mutant strains (AF01, AF02, and AF03) grow identically under all conditions tested as described below, as do the three independent *zrfB* Δ mutant strains (AF04, AF05, and AF13). Similarly, the three independent *zrfA**zrfB* Δ mutant strains (AF06, AF10, and AF11) as well as the two independent *zrfA* revertant strains (AF36 and AF37) and the two independent *zrfB* revertant strains (AF38 and AF39) also grew identically under all conditions tested. Thus, for simplicity, only mutant strains AF01 (*zrfA* Δ), AF13 (*zrfB* Δ), AF10 (*zrfA**zrfB* Δ), AF36, and AF39 will be mentioned from now on.

Strains AF01, AF13, and AF10 of *A. fumigatus* and the wild-type AF14 isogenic strain were grown in SD liquid medium under both zinc-replete and zinc-limiting conditions (Fig. 6 and Table 3). All strains showed similar growth rates (calculated as described in Materials and Methods) and reached similar maximum dry weights when they were grown under zinc-replete conditions (SD medium plus 100 μM zinc). In contrast, under zinc-limiting conditions (SD medium without a supplement of zinc), strains AF14, AF01, and AF13, as well as revertant strains AF36 and AF39, equally reduced (about four-fold) their maximum dry weight, while strain AF10 reduced it by 16-fold. Similarly, under zinc-limiting conditions, the growth rates of strains AF14, AF01, AF13, AF36, and AF39 were similar, although slightly reduced (about 1.1-fold), in compar-

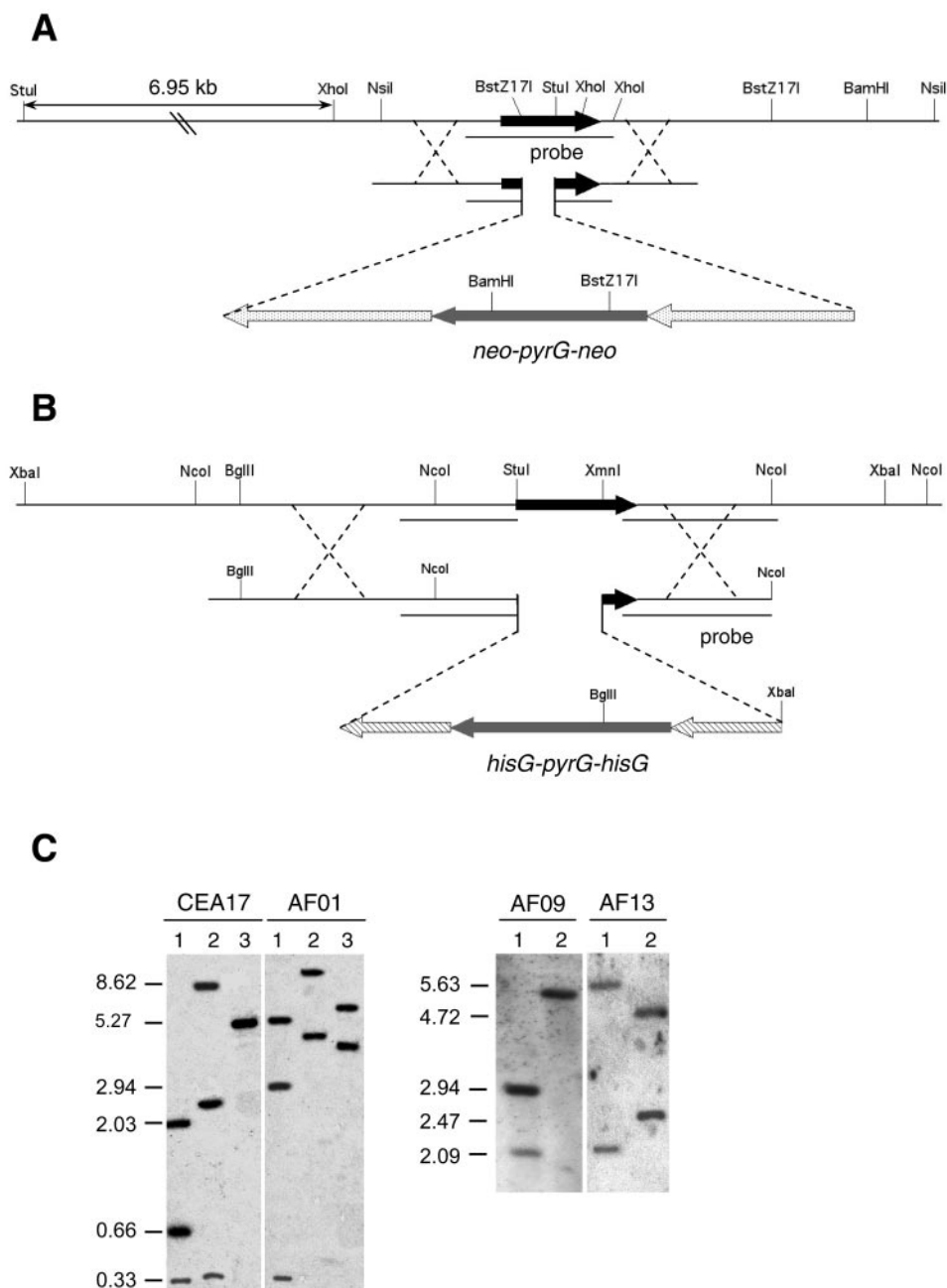


FIG. 5. (A) Schematic representation of the integration of the *neo-pyrG-neo* cassette used to disrupt the *zrfA* gene by a deletion-substitution mutation. (B) Schematic representation of the integration of the *hisG-pyrG-hisG* cassette used to disrupt the *zrfB* gene by a deletion-substitution mutation. Only relevant restriction sites have been indicated in both schemes. (C) Southern blots confirming that the integration event had taken place as expected. Genomic DNA from strains CEA17 (as a control) and AF01 was digested with XhoI/BstZ171 (lane 1), StuI/BstZ171 (lane 2), and NsiI/BamHI (lane 3) and hybridized with the probe indicated in panel A. Similarly, genomic DNA from strains AF09 (as a control) and AF13 was digested with NcoI (lane 1) and XbaI/BglII (lane 2) and was hybridized with the probes indicated in panel B. The exact sizes (in kb) of the hybridizing DNA fragments are indicated to the left of each blot.

ison with growth in the presence of zinc. However, the growth rate of strain AF10 under zinc-depleted conditions was so low that it could not be measured accurately. This indicates that either the *zrfA* or *zrfB* gene is required for optimal growth of *A. fumigatus* in liquid medium under zinc-limiting conditions.

To test the function of the *zrfA* and *zrfB* genes on the growth of *A. fumigatus* in solid medium, all strains were cultured in SD

agar, which was converted to a severely zinc-limiting medium by adding 250 μ M EDTA (SDE medium). In this medium, maximal growth was reached after 6 days of incubation regardless of the zinc supplement added to medium, although normal growth in all strains (including the wild type) could be achieved only at a concentration of zinc of 250 μ M or higher in the medium (Fig. 7A). Interestingly, while the wild-type (AF14)

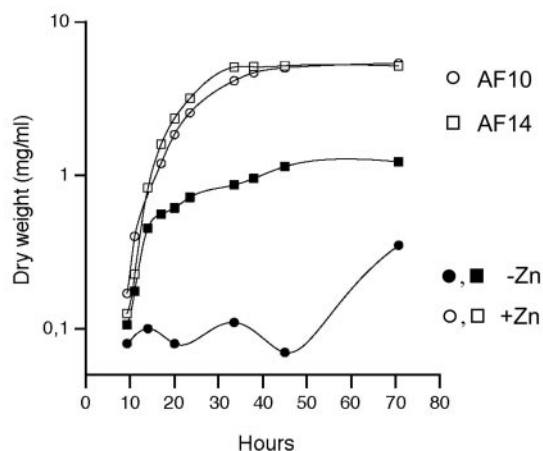


FIG. 6. For clarity, only the growth curves of the isogenic AF14 wild type (squares) and the double mutant AF10 *zrfA zrfB*Δ strain (circles) are represented. These strains were grown in complete SD medium supplemented with 100 μM zinc sulfate (open symbols) or without a zinc supplement (closed symbols) for 70 h at 37°C.

and *zrfA*Δ (AF01) strains grew identically in SDE medium supplemented with 10 μM zinc, strain *zrfB*Δ (AF13) grew generating cottony colonies formed by narrow but long extended and low branched hyphae in which a delay in sporulation was observed at the edge of the colony. Interestingly, this pattern of growth resembles that of the AF14 wild-type strain grown on solid SDE medium without a supplement of zinc. The *zrfA zrfB*Δ (AF10) strain was unable to grow. In addition, a *zrfB*Δ strain in which a wild-type copy of the gene *zrfA* was reintroduced at its original locus (AF36) grew identically to strain AF13 (*zrfB*Δ) (Fig. 7B). Similarly, a *zrfA*Δ strain in which a wild-type copy of the gene *zrfB* has been reintroduced at its original locus (AF39) grew identically to strain AF01 (*zrfA*Δ) (Fig. 7C). Although the growth defects observed in strains AF13, AF10, and AF36 are probably due to the sequestering of zinc by EDTA, it is still possible that some other essential metal ion could also be sequestered by EDTA, lowering its concentration to a suboptimal level for the normal growth of these strains. Thus, to preclude this possibility, we tested whether the SDE medium with the addition of 10 μM zinc supplemented with other metals (Cu²⁺, Fe³⁺, Co²⁺, Ni²⁺, or Mn²⁺) at the same molar rate as EDTA (i.e., 250 μM) could improve the growth of strains AF13, AF10, and AF36 to a

TABLE 3. Growth data for *A. fumigatus* strains in liquid SD medium

Strain	α (1/h)		Max. dry wt (mg/ml) ^a	
	SD + Zn	SD - Zn	SD + Zn	SD - Zn
AF14	0.33 ± 0.04	0.30 ± 0.03	5.18 ± 0.2	1.22 ± 0.2
AF01	0.31 ± 0.03	0.27 ± 0.01	5.07 ± 0.3	1.23 ± 0.1
AF13	0.33 ± 0.07	0.27 ± 0.05	5.51 ± 0.1	1.47 ± 0.1
AF10	0.30 ± 0.01	ND ^b	5.28 ± 0.2	0.32 ± 0.1
AF36	0.32 ± 0.02	0.28 ± 0.01	5.20 ± 0.1	1.25 ± 0.1
AF39	0.30 ± 0.02	0.28 ± 0.02	5.20 ± 0.1	1.22 ± 0.1

^a Determined after 65 h of culture in liquid SD medium with or without a supplement of zinc.

^b ND, not determined.

similar extent to that of either the AF14 or the AF01 strain. However, only zinc allowed all strains to reach their maximal growth (data not shown), confirming the notion that the alteration of growth in AF13, AF10, and AF36 would be due to a loss of function of these mutants in taking up zinc from the medium.

In sum, the analysis of growth of the mutants in SDE medium may be interpreted as that the expression of *zrfA* only is required for *A. fumigatus* to grow (at a suboptimal level) in a zinc-limiting medium in the absence of *zrfB*. However, if it is taken into account that (i) either *zrfA* or *zrfB* encodes proteins required for optimal growth under zinc-limited conditions as shown by the yeast complementation experiments (Fig. 1), (ii) the expression of either *zrfA* or *zrfB* is detected by Northern blot in zinc-limiting SD medium (Fig. 3), and (iii) the *zrfB*Δ strain (AF13) does not grow as well as the *zrfA*Δ strain (AF01) or the wild-type strain (AF14) (Fig. 7A), we should conclude that both genes are actually required for optimal growth and differentiation of the fungus in solid medium in which zinc is found in limited amounts, even though *zrfA* makes a smaller contribution.

***zrfA* and *zrfB* are acid-expressed genes in zinc-limiting conditions.** Just as a relationship was seen between the existence of ZR motifs in the promoter regions of the *zrfA* and *zrfB* genes and their expression, depending on the environmental concentration of zinc, a relationship may also exist between the existence of PacC-like sequences in their promoters and the regulation of their expression in a pH-dependent manner at the transcriptional level. In addition, the apparently strategic localization of PacC-like sequences in the promoter regions of both *zrfA* and *zrfB* (a PaC-like sequence in the *zrfA* promoter is located exactly at the middle of its ZRA2 sequence, while in the *zrfB* promoter, a PacC-like sequence is located just downstream from its TATA box) led us to suspect that the transcription of these genes might be regulated in response to two different environmental stimuli, such as the concentration of zinc and pH. To address this issue, the AF14 wild-type strain was grown in SD medium without a supplement of zinc for 24 h in order to enable the expression of both genes *zrfA* and *zrfB*, after which the culture was split into three aliquots that were buffered at either acid pH (3.5), neutral pH (6.5), or alkaline pH (7.5), as described in Materials and Methods. Next, each buffered aliquot of the culture was divided in two flasks, one of them supplemented with an excess of zinc (200 μM), while the other was not. Analysis of gene expression under these new conditions revealed that transcription of both genes was progressively repressed as the pH of the medium turned alkaline. Transcription of *zrfA* and *zrfB* in neutral or alkaline medium was equally reduced but only under zinc-limiting conditions, since in zinc-replete media, the expression of both genes was repressed regardless of pH (Fig. 8). These results clearly showed that the *zrfA* and *zrfB* genes are acid-expressed genes under zinc-limiting conditions. In addition, these results suggest that for these genes, the regulatory mechanism of gene expression that controls zinc availability prevails over the one that responds to changes in the environmental pH. Thus, pH-dependent regulation must play a secondary role in the transcriptional expression of both genes.

To further investigate the relationship between the expression of the *zrfA* and *zrfB* genes and the growth of *A. fumigatus*

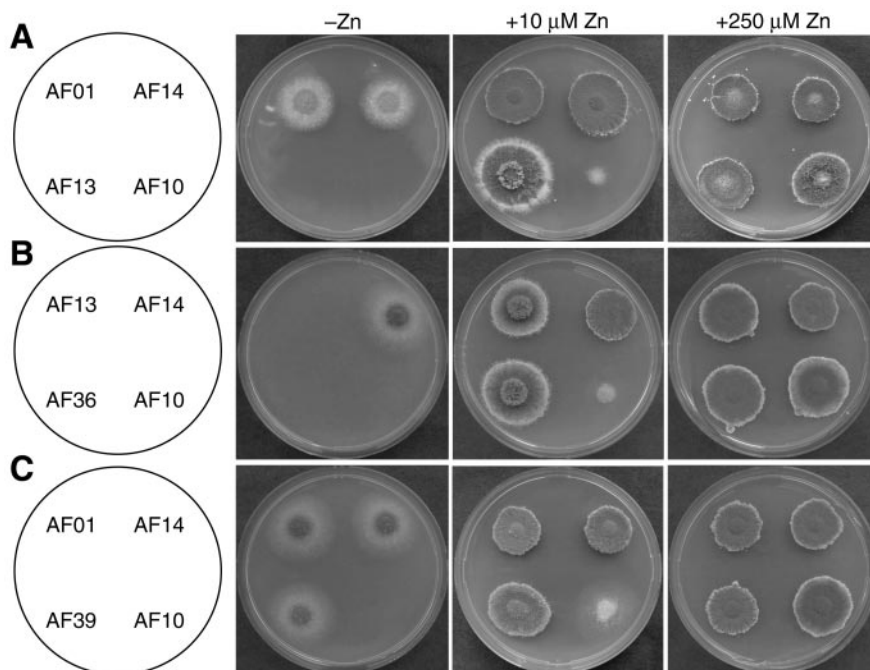


FIG. 7. (A) Growth of the wild-type strain and mutants in SDE zinc-limiting medium without a supplement of zinc or with a supplement of either 10 or 250 μM zinc sulfate. (B) Comparison of growth of the *zrfA* revertant strain AF36 to that of the *zrfB* mutant strain AF13 in the same medium used in the upper panel. (C) Comparison of growth of the *zrfB* revertant strain AF39 to that of the *zrfA* mutant strain AF10 in the same medium used in the upper panel. In all cases, 10^3 spores of each strain were spotted onto the same plate and arranged as indicated in the scheme, and the inoculated plates were incubated for 6 days at 37°C in a humid atmosphere before the picture was taken.

depending on the environmental pH, all strains were grown in alkaline CDDTE (pH 8.7) and in acid CDDTE (pH was lowered to 4.5 with HCl) agar media (Fig. 9A). The CDDTE medium is an extremely zinc-limiting medium since it does not have a supplement of zinc and contains 250 μM EDTA. All fungal strains grew equally well in alkaline CDDTE medium without a supplement of zinc. In contrast, in acid CDDTE medium without a supplement of zinc, the *zrfB*Δ AF13 strain showed a slightly reduced growth capacity, while the *zrfA**zrfB*Δ AF10 strain did not grow at all. Interestingly, an excess of zinc (250 μM) improved the growth of strains AF13 and AF10 to a level similar to that seen for AF01 and AF14 strains, while an excess amount of other metals, such as copper or iron, was completely unable to improve the growth of either the AF13 or AF10 strain (data not shown). This shows that, as for growth in acid SDE medium (Fig. 8), and although we cannot appreciate a defect in the growth of the AF01 *zrfA*Δ strain culture on acid, zinc-limiting CDDTE medium, the optimal growth of the fungus under this conditions can be achieved only when expression of both genes occurs.

Since *A. fumigatus* is an opportunistic pathogen, we also tested the growth of these mutants in fetal bovine serum (FBS) agar. FBS has a neutral or slightly alkaline pH (pH 7.2 to 7.5) that does not decline during incubation and remains alkaline (12). In addition, even though metal cations do not remain soluble in water as the pH becomes neutral or alkaline, metals do not precipitate since the main pool of each metal is usually tightly bound to chelating proteins present in the serum. FBS was also supplemented with 250 μM EDTA (FBSE) (pH 7.5) to chelate traces of free zinc and to render the medium more

zinc limiting. All *A. fumigatus* strains grew healthily in FBSE without a supplement of zinc (Fig. 9B). In contrast, when FBSE was acidified with HCl to a pH value of 4.5, strains AF14, AF01, and AF13 were able to grow (strain AF13 grew more slowly), while AF10 did not grow at all. However, when acid FBSE was supplemented with zinc, all strains improved their growth to a level identical to that of the wild type, including strain AF10, which regained its full growth ability. In addition, it was observed that the inability of the AF10 strain to grow in acidified FBSE was specifically linked to a defect in

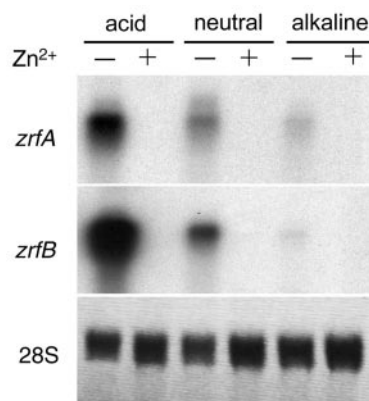


FIG. 8. Regulation of the expression of the *zrfA* and *zrfB* genes at the transcriptional level in response to changes in environmental pH under either zinc-replete (SD medium plus 200 μM Zn²⁺) or zinc-limiting conditions. Probes are indicated to the left of each blot.

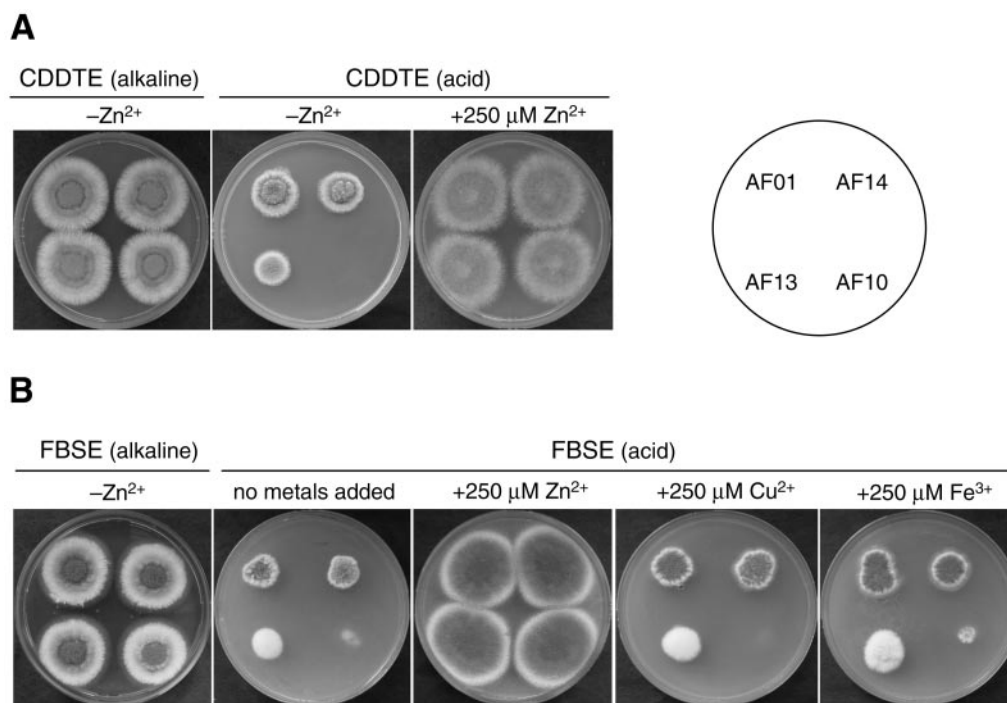


FIG. 9. Effect of zinc and pH on growth of *A. fumigatus* strains. (A) Strains were grown in alkaline CDDTE medium (without a supplement of zinc), acid CDDTE medium (without a supplement of zinc), and acid CDDTE medium supplemented with 250 μM zinc. (B) Strains were grown in FBSE medium (pH 7.5) without a supplement of zinc, in acid FBSE medium (pH 4.5) without a supplement of any metal, and in acid FBSE medium supplemented with 250 μM zinc sulfate, copper sulfate, or ferric chloride. In all cases, 10³ spores of each strain, arranged as indicated in the scheme, were spotted onto the same plate. Plates were incubated for 3 days at 37°C in a humid atmosphere before the picture was taken.

zinc uptake under zinc-limiting conditions, since all strains improved their capacity to grow only when they were supplemented with an excess amount of zinc but not with similar amounts of either copper or iron. Revertant strains AF36 and AF39 grew identically to strains AF13 and AF01, respectively, in either CDDTE or FBSE medium under all growth conditions tested (data not shown). These results show that the *zrfA* and *zrfB* genes are irrelevant for zinc uptake and growth in normal serum even under zinc-limiting conditions.

In summary, the *zrfA* and *zrfB* genes are required for optimal growth under acid, zinc-limiting conditions, but they are not apparently required for growth under alkaline, zinc-limiting conditions. In addition, provided that pH and zinc depletion conditions are maintained, the patterns of *zrfA* and *zrfB* gene expression do not depend on the medium used, since growth was identical in media as different as CDDTE medium, a synthetic defined medium, and fetal bovine serum, a complex medium that somehow resembles the environment that may find the fungus when it grows in living tissues.

DISCUSSION

We reported for the first time that the in vitro expression of immunodominant antigens from *Aspergillus* spp. is up-regulated by a low environmental concentration of zinc (27). This result prompted us to conjecture that the acquisition of zinc by *A. fumigatus* from a zinc-limiting environment, such as a living animal, must be one of the main difficulties that this fungal pathogen must overcome to survive and grow under these

conditions. Thus, based on insight gained from the investigation of the mechanisms of zinc uptake in *S. cerevisiae* (33, 34), we assessed the role of zinc uptake in the growth of *A. fumigatus*. First, we identified two genes in *A. fumigatus* (*zrfA* and *zrfB*) that encoded proteins ZrfA and ZrfB, which are structurally similar to proteins Zrt1p and Zrt2p, respectively, from *S. cerevisiae*. All these proteins belong to the ZIP family of zinc transporters (10). In the promoter regions of the *A. fumigatus* genes, there were several 15-bp ZR sequences that are probably involved in their regulation at the transcriptional level, as suggested by preliminary data obtained in our laboratory. Interestingly, the core sequence found in all *A. fumigatus* 15-bp ZR sequences is also found at the 3' end of the zinc response elements identified in yeast promoters whose expression is somehow regulated by zinc (32). In fact, we observed that only a high concentration of zinc down-regulated their expression and that no metal other than Cd²⁺ affected the transcriptional expression of these genes. Interestingly, it has repeatedly been reported that Cd²⁺ mimics Zn²⁺ in its ability to bind biological substrates (21, 24), probably due to the very similar electronic conformation that exists between the two elements. In any case, cadmium is a toxic element, since even though it can replace Zn²⁺ structurally, it cannot replace it functionally (16). In addition, we also observed that the expression of *zrfA* and *zrfB* was differentially down-regulated at the transcriptional level by concentrations of environmental zinc as low as 2 to 10 μM. Transcription of *zrfB* was totally turned off at a concentration of zinc approximately 50-fold higher than that required to repress transcription of *zrfA*. Interestingly, a similar mech-

anism of differential transcriptional regulation which depends on the environmental zinc concentration has also been described for the *ZRT1* and *ZRT2* genes from *S. cerevisiae*. A *zrt1* mutant strain of *S. cerevisiae* showed a marked reduction in growth, while a *zrt2* mutant grew as a wild-type yeast strain under zinc-limiting conditions. This can be explained in terms of the notion that the *ZRT1* and *ZRT2* genes encode the high- and low-affinity zinc uptake systems, respectively, of *S. cerevisiae* (33, 34). Similarly to *S. cerevisiae* strains deficient in zinc uptake, the growth (i.e., maximal dry weight) of a *zrfAzrfBΔ A. fumigatus* mutant was reduced by fourfold compared to that of a wild-type strain. However, even though the ZrfA and ZrfB proteins of *A. fumigatus* seem to be orthologues of Zrt1p and Zrt2p from *S. cerevisiae*, respectively, the growth of *A. fumigatus zrfAΔ* mutants was nearly identical to that of the wild type, whereas the growth of *zrfBΔ* mutants had an effect on the growth of *A. fumigatus* that strongly resembled that of the *zrt1* mutation on the growth of *S. cerevisiae*. This suggests one of the following possibilities. First, despite the overall similarity between Zrt1p/ZrfA and Zrt2p/ZrfB, *A. fumigatus* proteins would not be true orthologues of the yeast proteins, such that ZrfA and ZrfB would be responsible for a low- and high-affinity zinc uptake system, respectively. Second, ZrfA and ZrfB could be true orthologues of yeast proteins, such that they would show high- and low-affinity zinc transport activity, respectively, but a higher level of expression of *zrfB* might compensate for its low zinc transport activity, making it the most relevant system operating in zinc uptake in *A. fumigatus*. Third, both ZrfA and ZrfB would be transporters with a similar zinc transport activity, which would be closer to that exhibited by Zrt1p. This last assumption is supported by the fact that both genes improved the growth of the ZHY3 yeast strain to an equal extent under severe zinc-limiting conditions (SD medium plus 1 mM EDTA plus 50 μ M Zn²⁺). In any case, since the transcription of *zrfB* was repressed at a higher concentration of zinc than the transcription of *zrfA*, the expression of *zrfB* may be the main system for acquisition of zinc in *A. fumigatus* cells under a broader range of environmental zinc concentrations.

The existence of PacC-like sequences in the promoter region of the *zrfA* and *zrfB* genes prompted us to test whether the transcriptional expression of *zrfA* and *zrfB* might somehow also be regulated by the ambient pH. In addition, since the PacC-like sequence of the *zrfA* promoter overlaps the 15-bp ZR sequence, alkaline pH may directly repress the transcription of *zrfA* by blocking transcriptional induction as a result of a competitive binding mechanism as suggested previously by other investigators (9). However, it is also possible that at alkaline pH, the binding of the PacC active form to the binding site located just 9 nucleotides downstream from the *zrfB* TATA box might interfere with the efficient binding of RNA polymerase and the transcription of *zrfB*. Whichever mechanism is in fact operating, the transcription of these genes will be up-regulated in an acid environment, whereas it will be down-regulated in an alkaline, zinc-limiting environment. On the other hand, since the transcription of these genes is also regulated by pH, it is still possible that the ZrfA and ZrfB proteins may function when a zinc-limiting environment turns acid. This is unlikely, however, because *S. cerevisiae* strains expressing the *A. fumigatus* genes grew identically at both acid and alkaline

pH (data not shown), suggesting that the ZrfA and ZrfB proteins in the yeast background function in a pH-independent manner.

Serum stimulates the growth of *A. fumigatus*, even though serum contains metal-binding proteins that sequester free-ion metals, affording a concentration of free-ion metals too low to support the growth of many microorganisms, including most pathogenic fungi (12). Thus, pathogens must have developed mechanisms to obtain metals from serum. One of these mechanisms that has been developed by *A. fumigatus*, as well as by other pathogens, is the production of siderophores to scavenge iron from serum (17). However, this system would not be efficient unless an iron uptake system were coupled to it. In fact, specific transporters for siderophores have recently been characterized in *A. nidulans*, which indicates that orthologues probably exist in *A. fumigatus* (8, 14, 15). Likewise, even though no system has been described for zinc scavenging to date, a zinc uptake system would be required by pathogens, including *A. fumigatus*, to incorporate zinc into cells. The results obtained by us show that *zrfA* and *zrfB* are important genes for zinc uptake but only under acidic conditions. Accordingly, another system must exist in addition to this one, which would be used by fungi to obtain zinc under alkaline conditions. In fact, we have identified another zinc transporter-encoding gene (named as *zrfC*) that is expressed only under alkaline conditions (unpublished data). This system as well as the transcriptional regulatory mechanism that underlies the expression of all zinc-expressed genes are currently under active investigation at our laboratory.

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