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# Culture conditions for zinc- and pH-regulated gene expression studies in *Aspergillus fumigatus*

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**Summary**. In *Aspergillus fumigatus*, the regulation of zinc homeostasis is strongly influenced by environmental pH. Thus, the study of zinc-regulated gene expression in *A. fumigatus* requires controlling variations in culture pH, as this may affect zinc availability. However, depending on the nitrogen source, the pH of the culture can change dramatically over time. In addition, due to the ubiquitous distribution of zinc and that it is an essential micronutrient required in minute amounts for optimal fungal growth, neither buffering of the culture media to prevent pH variations nor the use of chelating agents is advisable if mycelium is to be used for expression analyses. In this work, the growth of *A. fumigatus* in several culture media was examined in order to determine the conditions yielding mycelia suitable for gene expression analyses in acid and neutral media, regardless of zinc availability. Our results showed that a zinc-limiting synthetic basal medium could be readily converted into a zinc-replete one and subsequently into acid or neutral medium by using, respectively, ammonium or nitrate as nitrogen source. [**Int Microbiol** 2007; 10(3):187-192]

**Key words:** *Aspergillus fumigatus* · nitrogen sources · pH variations · zinc availability

## Introduction

Investigation of the influence of environmental pH and zinc concentration on gene expression in *Aspergillus fumigatus* requires culture conditions that allow mycelium to be harvested at either the mid- or the late-exponential phase of fungal growth, while both parameters stay controlled. Fungal growth is severely affected by zinc availability [24], which is in turn influenced by pH, since the solubility of zinc decreases gradually around neutral pH [2]. Therefore, zinc availability and pH must be well-defined in order to study the transcription of genes whose expression is dually regulated by these environmental factors.

The traces of zinc present in the complex liquid media typically used to grow *Aspergillus* in most investigations about this fungus are high enough to render these media zinc-replete environments. Furthermore, the pH values of complex media (particularly those containing yeast extract or similar components) change dramatically during sustained fungal growth [6]. For this reason, complex media are usually buffered with non-physiological buffers (e.g., bis-Tris propane, Tris-HCl, or Hepes), although their effects on fungal growth have been largely ignored. Consequently, such media are useless to simultaneously control pH and zinc availability. Instead, a defined liquid medium is needed that could be easily converted from zinc-limiting to zinc-replete,

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with pH maintained within a neutral or acid range without the use of non-physiological buffers.

In the present study, the standard *Aspergillus* minimal medium [16], a zinc-replete defined media traditionally used to culture *A. nidulans* in many pH investigations [5], was chosen as starting medium. It can easily be made zinc-limiting by omitting zinc sulfate from the trace-element solution used in its preparation. In addition, the *Aspergillus* minimal medium is highly versatile since it can be buffered over a broad range of pH values (from 3 to 8) by using $Na_2HPO_4$-citric acid buffer and supplemented with different nitrogen sources (e.g., nitrate or ammonium) [7]. Additionally, both the capability of *Aspergillus* to use different nitrogen sources and the effect of pH on nitrogen uptake have been investigated [3,7]. However, the effect of either ammonia or nitrate uptake by *Aspergillus* on pH variations of the culture medium has never been reported. Herewith, we describe the effects of these nitrogen sources and of zinc availability on culture pH. Interestingly, this aspect of *Aspergillus* physiology can easily be exploited during fungal growth to control the pH of either zinc-replete or zinc-limiting media.

## Materials and methods

**Fungal strains and culture media.** The *A. fumigatus* wild-type strain AF14 and the *zafAΔ* null mutant AF17 were used in this study [12,24]. These fungal strains were routinely grown in PDA complex medium (20 g potato dextrose agar/l, 20 g sucrose/l, 2.5 g $MgSO_4 \cdot 7H_2O$/l). For specific experiments, they were grown in different culture media containing the AMMH or SD basal medium as the zinc-limiting component. The zinc-limiting basal medium AMMH is the *Aspergillus* minimal medium (AMM) without a nitrogen source (10 g dextrose/l, 0.52 g $MgSO_4 \cdot 7H_2O$/l, 0.52 g KCl/l, 1.52 g $KH_2PO_4$/l; pH adjusted to 6.5 with NaOH) and supplemented with 1.0 ml of Hunter's trace-elements solution without zinc sulfate (11 g $H_3BO_3$/l, 5 g $MnCl_2 \cdot 4H_2O$/l , 5 g $FeSO_4 \cdot 7H_2O$/l, 1.6 g $CoCl_2 \cdot H_2O$/l, 1.6 g $CuSO_4 \cdot 5H_2O$/l, 1.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$/l, 50 g $Na_2EDTA$/l; pH adjusted to 6.5 with KOH) [8,9,16]. The AMMH basal medium does not contain a vitamin supplement. The zinc-limiting basal medium SD is the synthetic yeast nitrogen base (YNB) without a nitrogen source and zinc sulfate (1.7 g/l; Q-BIO, gene, reference no. 4029-112), but supplemented with dextrose (20 g/l), 12 μM $FeSO_4 \cdot 7H_2O$, 6 μM $CuSO_4 \cdot 5H_2O$ and 10 μM $Na_2MoO_4 \cdot 2H_2O$. The SD basal medium also carries a supplement of B vitamins ($B_1$, $B_2$, $B_3$, $B_5$, $B_6$, $B_7$, $B_8$, and $B_9$) and *p*-aminobenzoic acid (PABA) as components of YNB [21]. To prepare the complete but zinc-limiting AMMHa and AMMHn media, AMMH basal medium was, respectively, supplemented with 5 mM ammonium (as 0.92 g ammonium [+]-tartrate/l) or 70 mM nitrate (as 6 g $NaNO_3$/l).The initial pH of both AMMHa and AMMHn was around 4–5. Thus, to prepare acid AMMHa medium, it was not necessary to adjust the pH. By contrast, the pH was adjusted to 7.0 or 6.5–6.8 with NaOH to prepare the neutral non-buffered AMMHa or AMMHn medium, respectively. The zinc-limiting SDA and SDN media were prepared by supplementing SD basal medium with, respectively, 38 mM ammonium (as 5 g $[NH_4]_2SO_4$/l) or 35 mM nitrate (as 3 g $NaNO_3$/l). Since the initial pH of the SDA medium is around 4.2, the pH was not adjusted, whereas neutral SDN medium (initial pH around 4–5) was prepared by adjusting the pH to 7.5 with NaOH. The $Zn^{2+}$ concentrations in SDA and SDN liquid media were 2.3 ± 1.91 and 0.18 ± 0.16 mM, respectively, as measured directly by inductively coupled mass spectrometry in a Perkin-Elmer Elan 6000 ICP-MS instrument.

**Culture conditions and fungal growth.** In all cases, spores grown in PDA were used to inoculate 200 ml of pre-warmed liquid media dispensed into 1-liter flasks to a density of $5 \times 10^5$ spores/ml. All cultures were incubated at 37°C with shaking at 200 rpm. To construct growth curves, 8-ml samples of culture were taken at each sampling time and filtered through Whatman GF/C filters to harvest mycelia. The wet cake was dried at 60ºC in a drying oven for several hours and then weighed. The net dry weight was expressed as mg of mycelium per ml of culture.

**Northern-blot analyses.** According to our standardized culture conditions, a culture of *A. fumigatus* must reach a dry weight ≥ 0.5 mg/ml isolate enough RNA for Northern-blot analyses. Total RNA was obtained according to the protocol provided with the RNeasy Plant Mini Kit (QIAGEN). RNA samples (20 μg of total RNA per lane) were loaded onto 1% formaldehyde agarose gels, and transferred by capillarity to neutral nylon membranes, and hybridized according to standard protocols [18]. DNA fragments containing most of the coding sequence from each gene were labeled by random priming using the DNA Labeling Beads (-dCTP) kit and [$\alpha^{32}$P]-dCTP (3000 Ci/mmol) (Amersham Pharmacia Biotech) and used as probes.

## Results and Discussion

**Physiological control of pH in zinc-limiting and zinc-replete media.** *Aspergillus fumigatus* (AF14) was grown in both acid and neutral, non-buffered AMMH medium supplemented with ammonia as sole nitrogen source (AMMHa) under zinc-replete or zinc-limiting conditions. In the acid medium, the pH of the culture remained acidic, and mycelia from either zinc-replete or zinc-limiting media could be harvested at the exponential phase of growth, i.e., after 20–24 h of incubation (data not shown). In contrast, exponential-growing mycelia from neutral media must be harvested after 20 h of culture in zinc-replete media or after 30 h in zinc-limiting media. However, at these harvesting times, the pH of the culture had already become acidified (Fig. 1A). Thus, to prevent a significant variation in the pH of the culture media as a consequence of fungal growth, the AMMHa medium was buffered with $N_2HPO_4$-citric acid at either acid or neutral pH values (i.e., 4.0 and 7.0, respectively) as previously described [7]. It was observed that fungal growth in the acid-buffered media was identical to that in the non-buffered media, regardless of zinc availability (data not shown). However, in the neutral-buffered AMMHa (Fig. 1B), growth was remarkably delayed such that mycelia should be harvested after 40 h of culture in zinc-limiting media. Furthermore, the buffering capacity of the $N_2HPO_4$-citric acid buffer was not strong enough to maintain pH around neutral pH after 20 h of fungal incubation in zinc-replete media. Thus, buffering the AMMHa medium with $N_2HPO_4$-citric acid was not appropriate for our purposes.

We have previously shown that, when *A. nidulans* is cultured in zinc-replete AMM medium supplemented with nitrate as sole nitrogen source, the pH of the culture remains around neutral and then becomes alkaline as the culture progresses [6]. Thus, the AMMH medium does not need to be

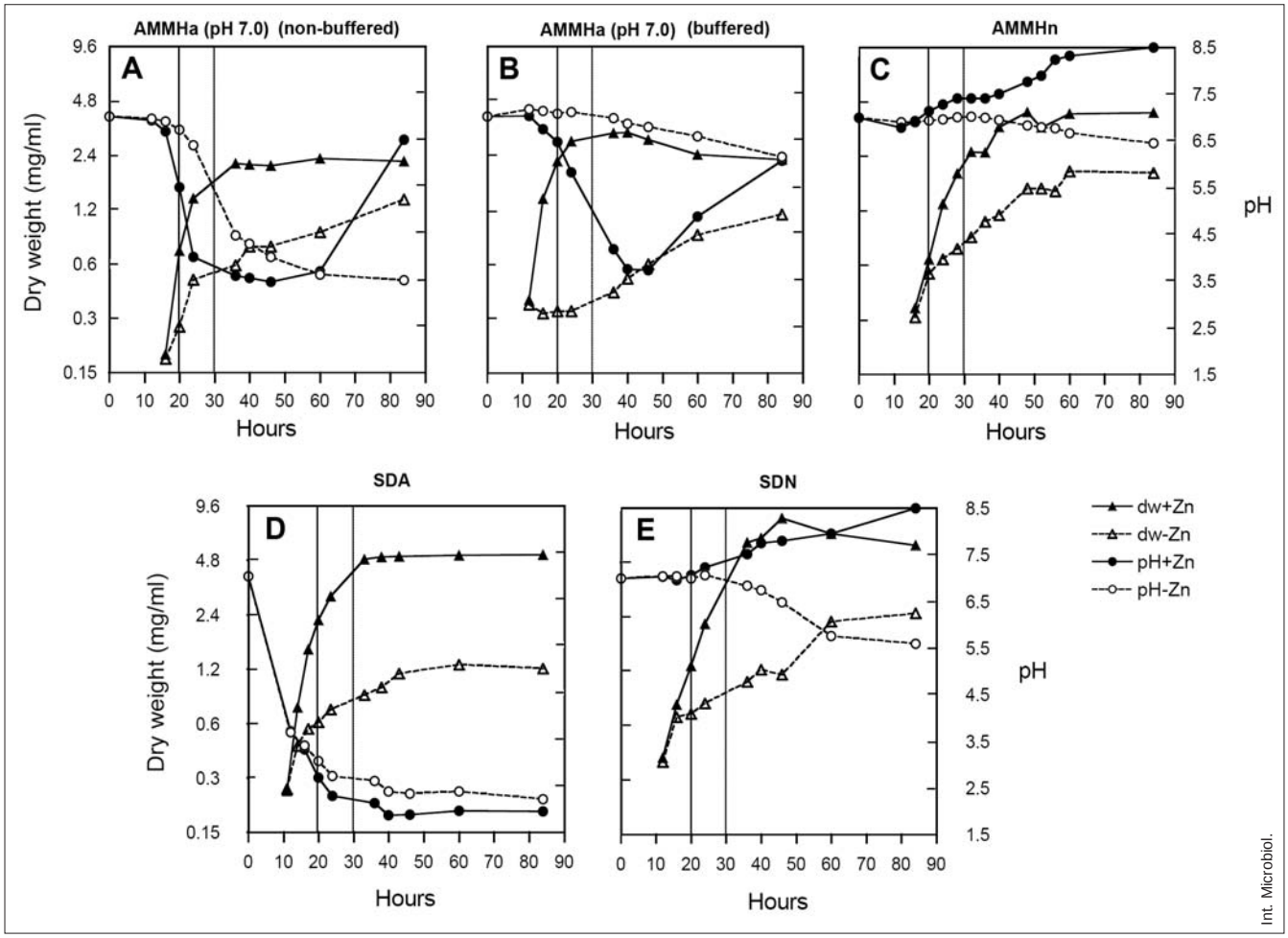


**Fig. 1.** Growth (as dry weight, triangles) and pH variation (circles) in media supplemented with 100 M $Zn^{2+}$ (filled symbols) and without a supplement of zinc (open symbols) of a wild-type strain of *Asperigillus fumigatus* (AF14). The pH of the non-buffered AMMHa medium was adjusted to 7.0 with NaOH. Lines have been drawn at 20 and 30 h of incubation to rapidly visualize the phase of fungal growth within this period of time. Each graph value is the mean of three independent experiments (error bars have been omitted for claritity).

buffered since the culture pH is maintained within a neutral or acid range simply by the use of nitrate or ammonium as sole nitrogen source. The decrease in pH in AMMHa medium independent of zinc availability (Fig. 1A,B) was most likely due to the fact that the *A. fumigatus* ammonium (AMT)/methylamonium (MEP) permeases (MeaA, MepA, MepB) recruit ammonium ions ($NH_4^+$) that enter the cell as ammonia ($NH_3$) which, in turn, acidify the extracellular environment [10,11,17]. That the pH of the AMMHn medium remained unchanged during the first stages of culture (up to 20 h) (Fig. 1C) can be explained by the molecular mechanisms that underlie nitrate and zinc uptake, as it has been proposed for the CrnA nitrate transporter of *A. nidulans* [26]. Thus, during the lag and early-exponential phases of growth, when the nitrate concentration of nitrate is relatively high, the CrnA nitrate transporter of *A. fumigatus* allows some nitrate slippage. However, as nitrate is gradually consumed, its transport would become gradually coupled to $H^+$ intake, resulting in alkalinization of the medium.

It has been proposed that the molecular mechanism responsible for zinc uptake under zinc-limiting conditions is a $Zn^{2+}/H^+$ antiporter system [25]. Therefore, protons translocated along with nitrate into fungal cells growing in AMMHn zinc-limiting medium might be efficiently extruded by the coupling of their transport to zinc uptake. However, growth in zinc-limiting media is reduced due to zinc starvation. Consequently, the rate of nitrate uptake is reduced accordingly, resulting in a slight acidification of the medium (Fig. 1C). This is not the case in AMMHn zinc-replete medium, where fungal growth is not restricted by zinc availability. In addition, since zinc is required in much smaller amounts than nitrate, nitrate uptake in zinc-replete media might be higher

than in zinc-limiting media, leading to alkalinization of the medium (Fig. 1C). In any case, these culture conditions meet all requirements to investigate the effects of pH and zinc availability on gene expression, even though the amounts of fungal biomass that can be harvested from these media after 20 h of incubation are somewhat scarce. For this reason, AMMHa and AMMHn media were replaced by SDA and SDN media, respectively.

The AMMHa medium closely resembles the SDA medium (pH 4.5), and can be easily and consistently prepared as zinc-limiting or zinc-replete [24]. Indeed, the main difference between the two types of media is that the latter contains twice the amount of dextrose as carbon source, a 7.5-fold higher amount of ammonium as nitrogen source, and a vitamin supplement. These modifications greatly improve the growth capacity of *A. fumigatus* and increase culture yields (Fig. 1D). In addition, SDA medium buffered at pH 7.0 with a phosphate buffer supplemented with the specific zinc chelator TPEN (200 mM $PO_4^{3-}$, 400 mM $K^+$, and 57 mM TPEN) has been reported to be useful for pH-shifting experiments [14,24]. However, the TPEN/$PO_4^{3-}$/$K^+$ buffer did not buffer at neutral pH after sustained fungal growth. Moreover, it added a stress that impaired the growth of *A. fumigatus* in zinc-limiting SDA medium. In contrast, when the ammonium in zinc-limiting and zinc-replete SDA media was replaced by nitrate as sole nitrogen source, neutral pH could be maintained in the resulting media (SDN, pH 7.4) and culture yields at the late-exponential phase of growth were better than those in AMMHn medium (Fig. 1E). Thus, SDA and SDN media provided ideal culture conditions to investigate expression of zinc- and pH-regulated genes under acid or neutral pH, respectively, and each medium under either zinc-limiting or zinc-replete conditions.

**Nitrogen source and the regulation of zinc and pH homeostasis.** In *A. nidulans*, the transcription of genes involved in the utilization of most nitrogen sources requires two transcriptional activators: (i) a transcription factor with a zinc finger of the $Cys_6$ class (i.e., a Zn binuclear cluster) specific for each pathway, and (ii) the general regulator AreA, a GATA factor with a zinc finger of the $Cys_2$-$Cys_2$ class required for the transcription of almost every gene involved in the utilization of nitrogen sources (except ammonium and glutamine) [19]. Accordingly, in *A. nidulans*, nitrate uptake by CrnA and nitrate reduction by nitrate (NiaD) and nitrite (NiiA) reductases are subject to induction by nitrate and repression by ammonium [13]. The expression of the *crnA-niiA-niaD* gene cluster of *A. nidulans* depends on binding of NirA and AreA to CTCCGHGG and HGATAR motifs, respectively; both motifs are located in the promoter regions of these genes [13,23]. The expression of *nirA* is constitutive [4], while transcription of *areA* is autoregulated and repressed by ammonium [19]. Similarly, expression of the highly conserved *crnA-niiA-niaD* gene cluster in *A. fumiga-*

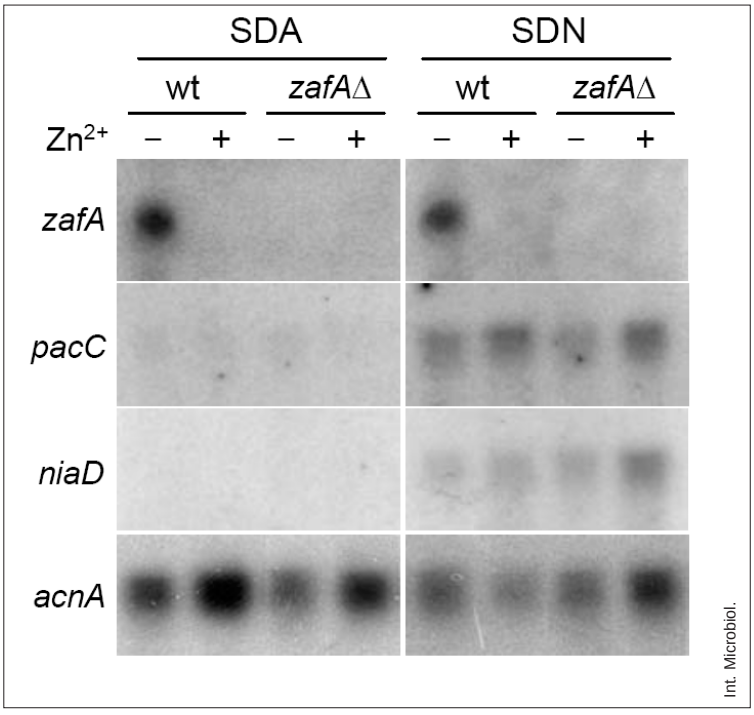


**Fig. 2.** Expression analyses by Northern-blot of the genes *zafA*, *pacC*, and *niaD* in *Aspergillus fumigatus* AF14 wild-type strain and in a *zafA*Δ null strain (AF17). Both strains were grown in SDA (ammonium as nitrogen source and acid pH) and SDN (nitrate as nitrogen source and neutral pH) media under zinc-replete and zinc-limiting conditions for 20 h at 37ºC. The γ-actin gene of *A. fumigatus* (*acnA*) was used as internal control for RNA loading and quality.

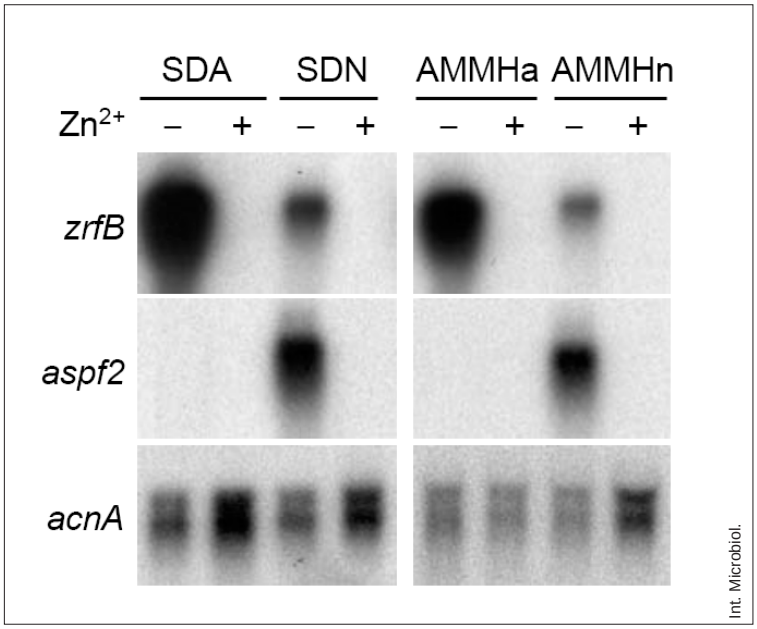


**Fig. 3.** Expression of the genes *zrfB* and *aspf2* in the *Aspergillus fumigatus* AF14 wild-type strain grown in SDA, SDN, AMMHa and AMMHn media under zinc-replete and zinc-limiting conditions. The fungus was incubated at 37ºC in the SD-based media for 20 h and in the AMMH-based media for 30 h. The γ-actin gene (*acnA*) was used as internal control for RNA loading and quality.

*tus* is induced by nitrate and repressed by ammonium [1]. Furthermore, as inferred by the similarity between the highly conserved *crnA-niiA-niaD* gene clusters of *A. nidulans* and *A. fumigatus*, including the conserved binding motifs for NirA and AreA, it is very likely that regulation of nitrate utilization is identical in the two species.

The SDA and SDN media can be used to investigate gene expression at acid or neutral pH under zinc-limiting and zinc-replete conditions only if the nitrogen source does not interfere with the regulation of gene expression by pH and/or zinc availability. To address this issue, we analyzed whether the expression of *zafA*, encoding the transcriptional activator involved in the regulation of zinc homeostasis in *A. fumigatus* [12], was altered in a wild-type strain (AF14) grown with either ammonium or nitrate as sole nitrogen source and under zinc-replete or zinc-limiting conditions. The results showed that *zafA* expression was influenced neither by nitrogen source nor by culture pH (Fig. 2). In addition, the possibility that post-transcriptional events affected ZafA activity depending on the nitrogen source was precluded since this protein induced gene expression under zinc-limiting conditions in the presence of either ammonia or nitrate (Fig. 3). Additionally, we analyzed whether the expression of *pacC*, which encodes the PacC transcriptional activator that regulates pH homeostasis in *A. fumigatus* (unpublished data), as does its orthologue in *A. nidulans* [15], was influenced by nitrogen source. The results showed that expression of *pacC* was up-regulated in SDN (i.e. at neutral pH) compared to SDA (i.e. at acid pH) (Fig. 2). To show that the higher expression of *pacC* in SDN was due to culture pH rather than to nitrogen source, the expression of *pacC* was analyzed in *A. fumigatus* cultured for 20 h in buffered AMMHa (pH 7.0) and AMMHn media. The results showed that transcription of *pacC* was unaffected by nitrogen source (data not shown). Therefore, the higher expression level in SDN is due to the neutral pH of the medium, as for *pacC* of *A. nidulans* [22]. Furthermore, we showed that the transcription profile of *pacC* remained unaltered in a *zafAΔ* null mutant under both zinc-limiting and zinc-replete conditions (Fig. 2). This suggests that ZafA does not regulate the expression of *pacC*. Finally, to test whether cross-talk occurred between the regulation of nitrogen and zinc homeostasis, *niaD* expression was analyzed. This gene was chosen because it is a good reporter of the regulatory mechanism that underlies nitrogen metabolism [13]. It would be expected that, in the absence of an interactive transcriptional mechanism regulated by nitrogen and zinc, transcription of *niaD* should be repressed by ammonium (i.e., in SDA medium) and induced by nitrate (i.e., in SDN medium), both in a *zafAΔ* null strain and in a wild-type strain grown under either zinc-replete or zinc-limiting conditions. Indeed, we observed that *niaD* expression is repressed in SDA but induced in SDN (Fig. 2), which strengthens the idea that the regulation of nitrogen metabolism and zinc homeostasis are not linked. However, it does not preclude the possibility that in media in which zinc is severely limited, many metabolic pathways, including those involved in the assimilation of nitrogen, might be impaired as a result of a zinc deficit (e.g., AreA, as well as other zinc finger transcription factors, requires $Zn^{2+}$ for stability of its zinc finger).

**Gene expression regulation by both zinc and pH can be readily investigated in *A. fumigatus* grown in media SDA and SDN.** The gene *zrfB* of *A. fumigatus* encodes a zinc membrane transporter whose transcription is chiefly induced in acid, zinc-limiting media but repressed in neutral, zinc-limiting media [24]. In contrast, the gene *aspf2* encodes an immunodominant antigen that is only expressed under neutral, zinc-limiting conditions [20]. Therefore, *zrfB* and *aspf2* of *A. fumigatus* could be considered as prototypic examples of genes that, under zinc-limiting conditions, are expressed at either acid or neutral pH, respectively. Thus, in order to validate the use of SDA and SDN media for gene expression analyses with respect to pH and zinc availability, *zrfB* and *aspf2* expression in fungus cultured in these media was analyzed. To rule out the possibility that qualitative or quantitative differences between SD and AMMH basal media affected the consistency of the results, the expression of these genes was analyzed in *A. fumigatus* cultured in AMMHa and AMMHn media as well. The transcriptional profiles of *zrfB* and *aspf2* were those expected if these media provided the appropriate environmental conditions regarding pH and zinc availability (Fig. 3). Therefore, culture media that use either SD or AMMH as the basal medium provide optimum physiological culture conditions to control of pH and of zinc availability and harvest mycelia from *A. fumigatus* suitable for gene expression analyses. However, the fungus grows faster in SD media than in AMMH media. In the former, the exponential phase of growth is reached after 20 h of incubation, at which time a large amount of fungal biomass can be harvested. In AMMH media, 30 h are required to achieve the same results. Therefore, SDA and SDN are the media of choice for gene expression investigations in which zinc availability and pH must be strictly controlled.

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