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Repression of the acid ZrfA/ZrfB zinc-uptake system of *Aspergillus fumigatus* mediated by PacC under neutral, zinc-limiting conditions

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Summary. The ZrfA and ZrfB transporters are components of a zinc-uptake system of *Aspergillus fumigatus* that mainly operates under acidic, zinc-limiting conditions. Expression of the genes *zrfA* and *zrfB* is up-regulated by the transcriptional activator ZafA in both acidic and neutral, zinc-limiting media. The transcription of *zafA* is not influenced by PacC, which is the transcriptional regulator involved in regulating pH homeostasis in *Aspergillus*. However, at neutral pH the expression of both *zrfA* and *zrfB* is significantly reduced. In this work, the repression of *zrfA* and *zrfB* in neutral and alkaline, zinc-limiting media was found to be mediated by the transcriptional regulator PacC. [**Int Microbiol** 2009; 12(1):39-47]

Key words: *Aspergillus fumigatus* · zinc-uptake system · zinc · PacC

Introduction

Zinc is an essential micronutrient required for the growth and development of all organisms, but in excess it may prove toxic for cells. Thus, zinc homeostasis must be strictly regulated in order to prevent either a deficiency or an excess of the element [8]. We previously studied the role of the genes *zrfA* and *zrfB* in *Aspergillus fumigatus*. The ZrfA and ZrfB transporters are components of a zinc-uptake system of *A. fumigatus* that mainly operates under acidic, zinc-limiting conditions [19]. ZrfA is an orthologue of Zrt1p, which is the transporter of the high-affinity zinc-uptake system of *S. cerevisiae* [20]. In contrast, the evolutionary relationship between ZrfB and Zrt2p, the latter being the transporter of the low-affinity zinc-uptake system of *S. cerevisiae* [21], is uncertain since the degree of similarity between the two proteins is nearly the same as between ZrfB and Ztr1p [19]. In yeast growing in zinc-limiting medium, *ZRT1* and *ZRT2* are induced by the Zap1 transcriptional activator [22], and the expression of these two genes does not change upon exposure to alkaline pH, as deduced from large-scale expression analyses [9,10,17]. Similarly, in *A. fumigatus* growing in zinc-limiting medium the expression of *zrfA* and *zrfB* is induced by the ZafA zinc-responsive transcriptional activator [13]. However, the transcription of *zrfA* and *zrfB* is significantly reduced upon exposure to neutral or alkaline, zinc-limiting medium [19]. The presence of a PacC-like site (5′-GCCARG-3′) in the promoter of *zrfA* and *zrfB* of *A. fumigatus* suggests that the repression of these genes under neutral or alkaline, zinc-limiting conditions is mediated by an *A. fumigatus* protein orthologous to the *A. nidulans* PacC transcriptional regulator (An-PacC) [18]. The present work shows that the repression of *zrfA* and *zrfB* under neutral, zinc-limiting conditions is exerted through the transcriptional regulator PacC.

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Materials and methods

Strains, media, and culture conditions. The *Aspergillus fumigatus* strains used in this work are listed in Table 1. The strains were routinely grown on PDA (20 g/l potato dextrose agar, 20 g/l sucrose, 2.5 g/l $MgSO_4 \cdot 7\ H_2O$, supplemented with 100 μM Zn^{2+} or on complete AMMH agar medium, which is the base *Aspergillus* minimal medium (0.92 g/l ammonium [+]-tartrate, 10 g/l dextrose, 0.52 g/l $MgSO_4 \cdot 7\ H_2O$, 0.52 g/l KCl, 1.52 g/l KH_2PO_4, pH adjusted to 6.5 with NaOH) supplemented with 1.0 ml of complete Hunter's trace-elements solution (11 g/l H_3BO_3, 22 g/l $ZnSO_47 \cdot H_2O$, 5 g/l $MnCl_2 \cdot 4\ H_2O$, 5 g/l $FeSO_4 \cdot 7\ H_2O$, 1.6 g/l $CoCl_2 \cdot H_2O$, 1.6 g/l $CuSO_4 \cdot 5\ H_2O$, 1.1 g/l $[NH_4]_6Mo_7O_{24} \cdot 4\ H_2O$, 50 g/l Na_2EDTA (pH adjusted to 6.5 with KOH), and 1.5 g/l agar. For specific experiments, the fungal strains were grown on AMMC, SDAE, SDNE, and AMMH buffered agar medium. AMMH buffered agar medium was prepared by buffering AMMH agar medium without KH_2PO_4 with the Na_2HPO_4-citric acid buffer at pH 4, 6, 7, or 8 using the required quantities of 100 mM Na_2HPO_4 and 50 mM citric acid solutions, as described by Cove [4]. AMMC agar medium is the base AMM medium supplemented with 1.0 ml of slightly modified Cove's trace-elements solution [3] (0.04 g/l $Na_2B_4O_7 \cdot 10\ H_2O$, 8.0 g/l $ZnSO_4 \cdot 7\ H_2O$, 0.72 g/l $MnSO_4 \cdot 1\ H_2O$, 1.19 g/l $FeSO_4 \cdot 7\ H_2O$, 0.4 g/l $CuSO_4 \cdot 5\ H_2O$, 0.8 g/l $Na_2MoO_4 \cdot 2\ H_2O$, pH adjusted to 6.5 with NaOH), and 1.5 g/l agar. SDAE zinc-limiting agar medium (pH ~4.5) is the synthetic yeast nitrogen base without ammonium sulfate, dextrose, amino acids, and zinc sulfate (1.7 g/l) (obtained from Q-BIOgene, ref. no. 4029-112) supplemented with dextrose (20 g/l), ammonium sulfate (5 g/l, as nitrogen source), 250 μM Na_2EDTA, and 2 g/l agar. SDNE zinc-limiting agar medium (pH adjusted to 7.5 with NaOH) is synthetic yeast nitrogen base without ammonium sulfate, dextrose, amino acids and zinc sulfate (1.7 g/l), supplemented with dextrose (20 g/l), nitrate (3 g/l, as nitrogen source), 12 μM $FeSO_4 \cdot 7\ H_2O$, 6 μM $CuSO_4 \cdot 5\ H_2O$, 10 μM $Na_2MoO_4 \cdot 2\ H_2O$, 250 mM Na_2EDTA, and 2 g/l agar. The SDAE and SDNE zinc-limiting agar media were supplemented with Zn^{2+} as specified, using a stock sterile solution of 100 mM $ZnSO_4 \cdot 7\ H_2O$ in ultrapure water. Liquid SDA and SDN zinc-limiting media were prepared in the same way as SDAE and SDNE zinc-limiting agar media but omitting EDTA and agar. The total Zn^{2+} concentration in SDA and SDN liquid zinc-limiting medium was 2.3 ± 1.91 μM and 0.18 ± 0.16 μM, respectively, as measured directly by inductively coupled mass spectrometry.

Conidia from *A. fumigatus* strains grown in either PDA supplemented with 100 μM Zn^{2+} or complete AMMH agar medium were used as inoculum for both solid and liquid media. Pre-warmed SDA and SDN liquid media were inoculated to a density of 5×10^5 spores/ml and incubated at 37°C with shaking at 200 rpm.

Table 1. *Aspergillus fumigatus* strains used in this study

Strain	Genotype	Reference
CEA17	*pyrG1* (auxotrophic *pyrG*$^-$)	[5]
AF14	Prototrophic wild-type (isogenic to CEA17)	[19]
AF58	*pyrG1 pacC*$^{D1598\rightarrow2215}$*::pyrG* (phenotype *pacC*^{c})	This study
AF60	*pyrG1 pacC*$^{D1598\rightarrow1861}$*::pyrG* (phenotype *pacC*$^{+/-}$)	This study

Construction of plasmids used for *Aspergillus fumigatus* transformation. The plasmids and oligonucleotides constructed and used during the course of this study are listed and described briefly in Tables 2 and 3, respectively.

The plasmids pPAC4 and pPAC7 carried a DNA fragment designed to generate a prototrophic strain expressing either an alkalinity-mimicking or an acidity-mimicking PacC protein. Firstly, a 2.23-kb fragment containing the coding sequence of the gene *pacC* (obtained by high-fidelity PCR using oligonucleotides JA276 and JA277 as primers and genomic DNA from strain AF14 as template) was subcloned into pGEM-T-easy (Promega) to generate plasmid pPAC9 and then sequenced. The nucleotide sequence of *pacC* perfectly matched the DNA sequence corresponding to the map element AFUB037210 of the A1163 strain. Indeed, this was expected, since both AF14 and A1163 are CEA17 derivatives [7,19]. The pPAC4 plasmid was constructed as follows: (i) a 1.61-kb DNA fragment containing a partial coding sequence of *pacC* (nucleotides 1–1597) was obtained by high-fidelity PCR using plasmid pPAC9 as template and oligonucleotides JA161/JA162 as primers and cloned into pGEM-T-easy to generate the pPAC2 plasmid. (ii) The 1.64-kb *Sac*II–*Spe*I fragment from pPAC2 was inserted into the pPYRG plasmid digested with *Sac*II/*Xba*I to generate pPAC3. (iii) A 2.31-kb DNA fragment containing the sequence downstream from *pacC* was obtained by high-fidelity PCR using genomic DNA from the *A. fumigatus* AF14 strain as template and oligonucleotides JA152/JA153 as primers, digested with *Nhe*I/*Spe*I, and ligated to *Spe*I-digested pPAC3 to generate pPAC4. Plasmid pPAC7 was constructed by ligating a *Stu*I/*Nco*I-digested PCR fragment of 382 bp containing the 3′ coding sequence of *pacC* (nucleotides 1861–2215) obtained by high-fidelity PCR (using pPAC9 as template and oligonucleotides JA177 and JA178 as primers) to pPAC4 digested with *Hpa*I/*Nco*I. This ligation adds two serine codons. Therefore, pPAC7 carries a deleted *pacCΔ*$^{1598\rightarrow1861}$ gene that encodes a PacC$^{+/-}$ protein without residues L471–H556. Plasmids pPAC4 and pPAC7 were linearized by digestion

Table 2. Plasmids used in this study

Plasmid	Description	Reference
pACTF1	Carries the *actG* gene of *A. fumigatus*	This study
pPAC2	Carries a partial coding sequence of *pacC* (nucleotides 1–1597)[a]	This study
pPAC3	pPAC2 with the *pyrG* gene from *A. niger*	This study
pPAC4	pPAC3 with a 2.31-kb sequence downstream from *pacC*	This study
pPAC7	pPAC4 with a 382-bp fragment containing the 3′ coding sequence of *pacC* (nucleotides 1861–2215)[a]	This study
pPAC9	Carries a 2.23-kb fragment containing the *pacC* coding sequence of *A. fumigatus*	This study
pPYRG	Carries the *pyrG* gene from *A. niger* and flanking regions	This study
pZRF1g	Carries a 3.09-kb fragment containing the *zrfA* gene of *A. fumigatus*	[19]
pZRF24g	Carries a 4.24-kb fragment containing the *zrfB* gene of *A. fumigatus*	[19]
pZAF14	Carries the cDNA sequence of *zafA*	[13]

[a]Nucleotides are numbered using as a starting point the A nucleotide (position 1) of the ATG start codon.

Table 3. Oligonucleotides used in this study

Oligonucleotide	Sequence[a] (5′ → 3′)	Restriction site
JA67	GGATCCCCGGGATCCCATTGCTAGCGG	–
JA113	TCGCTTGCCGAGCAAACAGCTTGCC	–
JA152	GTTAACTAAATCGTGGGTTATTTG	*Hpa*I
JA153	ACTAGTCCGGAGCCTCCGCGTC	*Spe*I
JA161	CCCGGGCACAATGTCTGAACACCAAG	*Sma*I
JA162	CCATGGTTAACTGTCGGCCATCGTGGTTG	*Nco*I-*Hpa*I
JA177	CTGCACTAGGCCTCCAGCCCGTCTGACGCCG	*Stu*I
JA178	TAACCATGGATTTAGTGGTGCATCTTGG	*Nco*I
JA276	GGTACCATGTCTGAACACCAAGATACCG	*Kpn*I
JA277	ACCCGGGATTTAGTGGTGCATCTTGG	*Sma*I

[a]Restriction sites introduced to facilitate the cloning procedures are underlined.

with *Not*I and were used to transform the CEA17 strain, as described previously [19].

Standard molecular biology procedures. Most of the DNA manipulations were carried out following standard molecular biology protocols [16]. Plasmid DNA was extracted and purified using the Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions. To obtain DNA and RNA, the mycelium of *A. fumigatus* was harvested by filtration through a filter paper, rinsed with cold sterile water, vacuum-dried, weighed, frozen in liquid nitrogen, and grinded carefully.

To purify genomic DNA from *A. fumigatus*, about 500 mg of mycelia powder was resuspended in 1 ml of lysis buffer (20 mM HEPES, 1 M sucrose, 20 mM EDTA, 0.9% SDS), incubated at 65ºC for 15 min, and diluted 1:2 with dilution buffer (50 mM Tris-HCl, 20 mM EDTA, pH 8.0). The mixture was extracted carefully with phenol:chloroform:IAA, avoiding vigorous agitation. The genomic DNA was precipitated, resuspended in 0.2 ml of 10 mM Tris-HCl (pH 8.0), and incubated in the presence of 10 µg of RNAse (DNAse-free) for 30 min at room temperature. For Southern blot analyses, approximately 4 µg of DNA per lane was loaded in a 0.8% agarose gel and transferred by capillary action to nylon membranes (Hybond-N^+, Amersham Biosciences). The probe used for Southern blotting analyses was a mixture of a 2.31-kb fragment (obtained by PCR using oligonucleotides JA152/JA153) and a 1.6-kb *Spe*I–*Hpa*I fragment (obtained from pPAC2). Probes were digoxigenin-labeled by random priming (DIG DNA Labeling Kit, Roche), hybridized, and detected using the chemiluminescence method of the DIG Nucleic Acid Detection Kit (Roche) as indicated in the supplied protocol.

Total RNA from *A. fumigatus* was isolated according to the protocol provided with the RNeasy Plant Mini kit (Qiagen). Twenty µg per lane were loaded onto 1% formaldehyde agarose gels and transferred by capillary action to nylon membranes (Hybond-N, Amersham biosciences). For Northern analysis, probes were labeled by random priming using the DNA Labeling Beads (-dCTP) kit and [^{32}P]-dCTP (3000 Ci mmol^{-1}) (Amersham Biosciences). The DNA fragments used as probes were the following: (i) for *zrfA*, a 697-bp *Eco*RI–*Kpn*I fragment obtained from the pZRF1g plasmid; (ii) for *zrfB*, a 970-bp *Stu*I–*Sac*I fragment obtained from the pZRF24g plasmid; (iii) for *pacC*, a 1605-bp *Hpa*I–*Sma*I fragment obtained from the pPAC2 plasmid; (iv) for *zafA*, a 1389-bp fragment obtained by PCR using the pZAF14 plasmid as template and oligonucleotides JA113 and JA67 as primers; (v) for the γ-actin (*actG*) gene, a 926-bp *Eco*RI–*Eco*RI fragment obtained from the pACTF1 plasmid. The probes were allowed to hybridize overnight at 65°C in hybridization buffer (5× SSC, 5× Denhardt's, 0.5% SDS, 0.1 mg denatured herring sperm/ml) and then washed according to standard procedures. Films were scanned using a densitometer and the signal intensity measured (as OD units/mm^2 of a selected area) using the volume analysis report tool of the Quantity One program (version 4.6; BioRad).

Construction of *pacC* mutants of *A. fumigatus*. To analyze whether the transcription of *zrfA* and *zrfB* under zinc-limiting conditions was PacC-regulated in response to environmental pH, the uridine-uracil-auxotrophic CEA17 strain [5] was transformed with a DNA fragment designed to generate a prototrophic strain expressing either an alkalinity-mimicking PacC protein truncated at position L471 (AF58) or an acidity-mimicking PacC protein without residues L471 to H556 (AF60) (Fig. 1). These strains would, respectively, resemble the finely characterized *pacC^{c}14* and *pacC$^{+/-}$20205* mutations of *A. nidulans* [11,18]. Hence, for consistency with the previous nomenclature, coined to reflect the phenotype of *pacC* mutants [14], the *A. fumigatus* mutants were denoted as *pacC^c* (AF58) and *pacC$^{+/-}$* (AF60).

Results

Identification of the *pacC* gene. The *pacC* gene was identified by searching the DNA genomic database of *A. fumigatus* through the GenBank non-redundant database, using the BLASTP algorithm and the PacC protein (residues 5–678) from *A. nidulans* [15,18] as a query.

The PacC protein of *A. fumigatus* was encoded by the AFUB037210 locus of *A. fumigatus* A1163 [7], which formally corresponded to the *A. fumigatus pacC* gene. The proteins Af-PacC (676 residues) and An-PacC (674 residues) showed an identity of 66.5% and a similarity of 71.6% (Fig. 1A).

Growth analysis of PacC mutants. To determine whether the AF58 and AF60 strains indeed had the *pacC^c* and *pacC$^{+/-}$* phenotypes, respectively, the effect of pH on fungal growth was tested in AMMH agar medium buffered at different pH values (Fig. 2A) as well as in SDAE and SDNE media (Fig. 2B), which provide the optimal culture conditions for

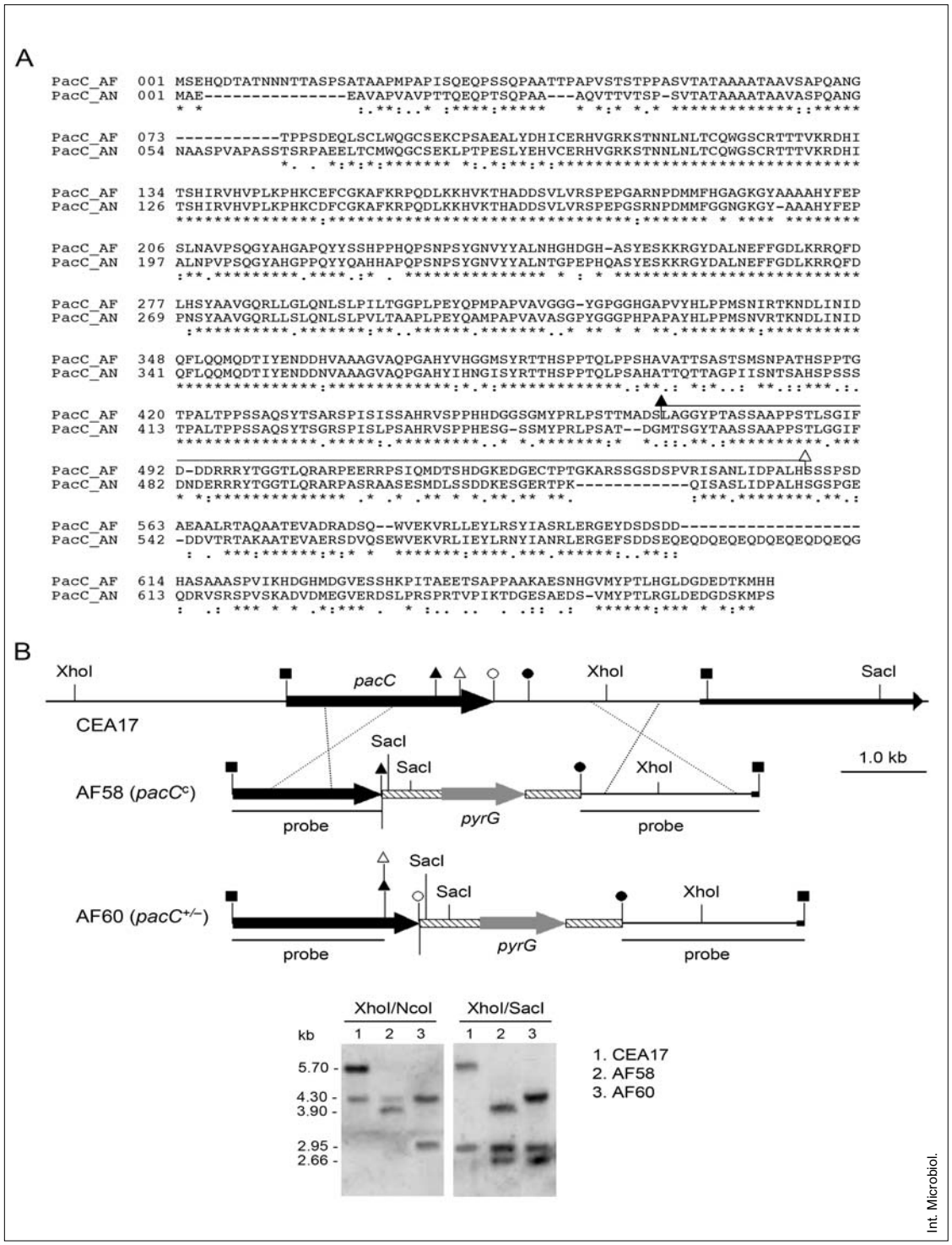

Fig. 1 (**A**) Comparison of the *Aspergillus fumigatus* and *A. nidulans* PacC proteins. (**B**) Construction of the *A. fumigatus pacC* mutant strains. Squares delimit the DNA fragment used to transform *A. fumigatus pyrG1* uracil-uridine-auxotrophic strain CEA17 into a prototrophic strain expressing an alkalinity-mimicking *pacC*^{c} gene or an acidity-mimicking *pacC*$^{+/-}$ gene. The *pacC*^{c} gene encodes a PacC^{c} protein truncated at position L471 (indicated by a closed triangle). The *pacC*$^{+/-}$ gene encodes a PacC$^{+/-}$ protein that lacks residues L471–H556 (the overlined sequence delimited by a closed and an open triangle). The *pyrG* gene of *A. niger* was used as a selection marker (gray arrow indicates the coding sequence of *pyrG*; the flanking dashed rectangles shows the adjacent intergenic regions). Only relevant restriction sites are indicated. The source of the genomic DNA, restriction enzymes used in the digestions, and sizes of the fragments detected that matched the expected sizes are indicated in each panel. The thinner arrow indicates a putative open reading frame downstream of the *pacC* gene.

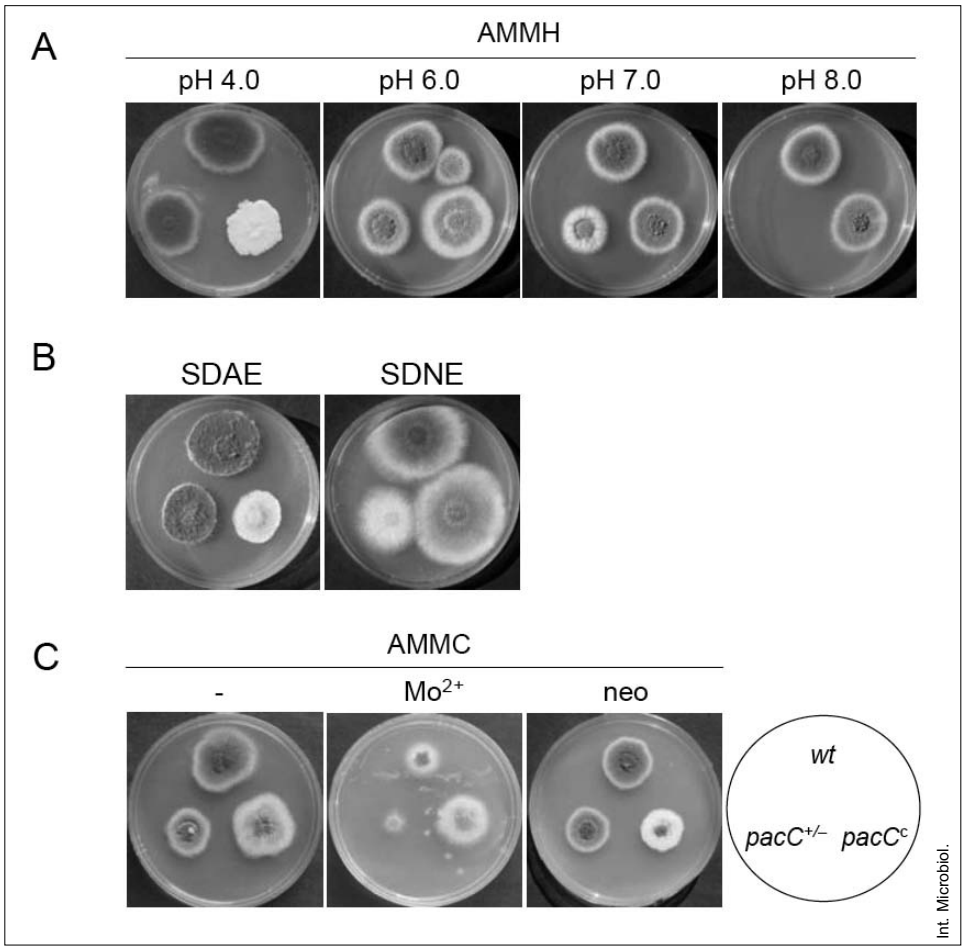

Fig. 2. Growth ability of the *pacC*^{c} (AF58) and *pacC*$^{+/-}$ (AF60) mutant strains in different zinc-replete media (i.e., supplemented with 100 μM Zn^{2+}). (**A**) AMMH medium buffered with Na_2HPO_4-citric acid at pH 4, 6, 7, or 8. (**B**) SDAE (pH 4.5) and SDNE (pH 7.5) media. (**C**) AMMC medium (pH 6.5) with no supplements or supplemented with either 30 mM Mo^{2+} (as $Na_2MoO_4 \cdot 2\ H_2O$) or neomycin (at a final concentration of 2 mg/ml). As indicated in the adjacent scheme, 10^3 spores of each strain were spotted onto the same plate; the plates were then incubated at 37ºC in a humidified atmosphere. Fungal growth reached a maximum after 3 days of incubation in AMMC (with no supplements or supplemented with neomycin), AMMH, and SDNE but only after 5 days of incubation in AMMC supplemented with Mo^{2+} and SDAE.

zinc- and pH-regulated gene expression studies in *A. fumigatus* [12]. In addition, the effect of a high concentration of either Mo^{2+} or neomycin on the growth capacity of these strains was tested in AMMC agar medium (pH 6.5) (Fig. 2C). Although the effect of Mo^{2+} and neomycin on *Aspergillus* growth remains unknown, it has been largely proven that they are useful for the isolation and phenotypic testing of *Aspergillus* mutants with altered pH regulation, as described by Peñalva and Arst [14]. In AMMH, growth of the *pacC*$^{+/-}$ mutant was identical to that of the wild-type at acid pH but the growth capacity was gradually reduced as pH increased, and at pH 8.0 the mutant did not grow at all. In contrast, the *pacC*^{c} mutant grew poorly at acid pH but its growth capacity improved as pH increased such that it grew similarly to the wild-type in neutral and in alkaline medium (Fig. 2A). In SDAE medium (pH ~4.5), the *pacC*$^{+/-}$ and *pacC*^{c} mutants grew as in AMMH (pH 4.0), whereas in SDNE (pH 7.5) medium their growth was similar to that observed in AMMH (pH 7.0) (Fig. 2B). The *pacC*$^{+/-}$ mutant hardly grew in AMMC supplemented with Mo^{2+} but it grew similarly to the wild-type in medium supplemented with neomycin. In contrast, the *pacC*^{c} mutant grew better than the wild-type in Mo^{2+}-supplemented medium but its growth was less than either the wild-type or the *pacC*$^{+/-}$ mutant in neomycin-supplemented medium (Fig. 2C). Thus, under all conditions tested, the growth capacity of the *pacC*$^{+/-}$ (AF60) and *pacC*^{c} (AF58) strains was affected exactly as expected for an acidity- and alkalinity-mimicking mutant strain, respectively.

Regulation of *pacC* transcription. Expression of the *pacC* gene was analyzed in a wild-type strain of *A. fumigatus* as well as in the *pacC*^{c} (AF58) and *pacC*$^{+/-}$ (AF60) strains, grown in either acid (SDA) or neutral (SDN), zinc-limiting or zinc-replete liquid medium using the culture conditions described previously [12]. The *pacC* gene was expressed under neutral growth conditions at a level slightly higher than under acidic growth conditions (Fig. 3A), similar to what has been reported for *pacC* of *A. nidulans* [18]. In addition, *pacC* expression in *A. fumigatus* was not influenced by zinc availability (Fig. 3A). As expected, the *pacC*$^{+/-}$ gene in strain AF60 was transcribed into an mRNA that was 200 nucleotides (nt) shorter than the wild-type *pacC* mRNA, whereas the *pacC*^{c} gene in strain AF58 was transcribed into an mRNA approximately 600

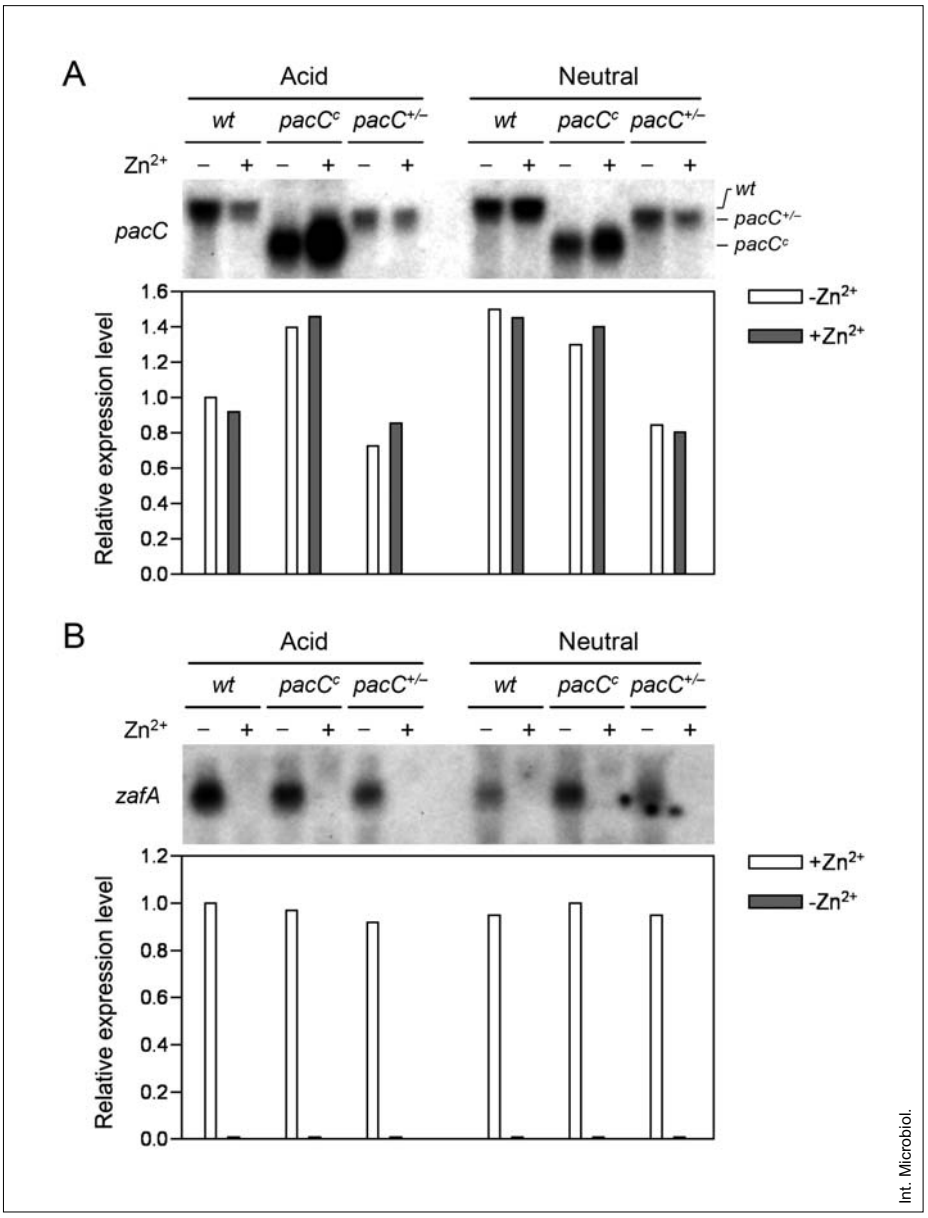

Fig. 3. Transcriptional analysis of (**A**) *pacC* and (**B**) *zafA* by Northern blotting of RNA from wild-type (AF14), *pacC*^{c} (AF58), and *pacC*$^{+/–}$ (AF60) strains grown in acidic SDA or neutral SDN liquid medium for 20 h at 37ºC. The media were supplemented with 100 μM Zn^{2+} (+) or without a supplement of Zn^{2+} (–), as indicated at the top of each lane. Each blot membrane was also hybridized with a probe for the γ-actin (*actG*) gene. The relative expression values for each signal detected by Northern blotting were normalized with respect to the expression of the *actG* gene and then referenced to a value of 1.0, which was assigned arbitrarily to the signal obtained for the wild-type strain grown in acidic, zinc-limiting medium.

nt shorter than the wild-type *pacC* mRNA. Moreover, under all conditions tested expression of the *pacC*$^{+/–}$ gene in AF60 was quantitatively similar to that of the wild-type strain under acidic growth conditions, whereas under all conditions tested the expression of *pacC*^{c} in AF58 was quantitatively similar to that of the wild-type strain under neutral growth conditions.

Transcription of *zafA* and PacC function. The *zafA* gene encodes a zinc-responsive transcriptional activator that up-regulates the expression of *zrfA* and *zrfB* under zinc-limiting conditions [13]. However, *pacC* expression is not influenced by the environmental concentration of zinc and hence by ZafA, since the gene is expressed at the wild-type level in a *zafAΔ* null mutant [12]. However, this is not surprising since no zinc response (ZR) elements (i.e., 15-bp consensus sequences with a common 5′-CAAGGT-3′ core) [13,19] are present in the *pacC* promoter region of *A. fumigatus*. In contrast, in the promoter region of *zafA*, there is a PacC-like site (at position –557 with respect the ATG start codon) that potentially binds PacC to modulate *zafA* expression. Hence, to ascertain whether *zafA* expression was influenced by ambient pH through the PacC transcriptional regulator, its expression was analyzed in the *pacC*$^{+/–}$ and *pacC*^{c} strains under either acidic or neutral, zinc-limiting conditions (Fig. 3B). As shown in Fig. 3B, the expression of *zafA* was not influenced by ambient pH in a PacC-mediated fashion.

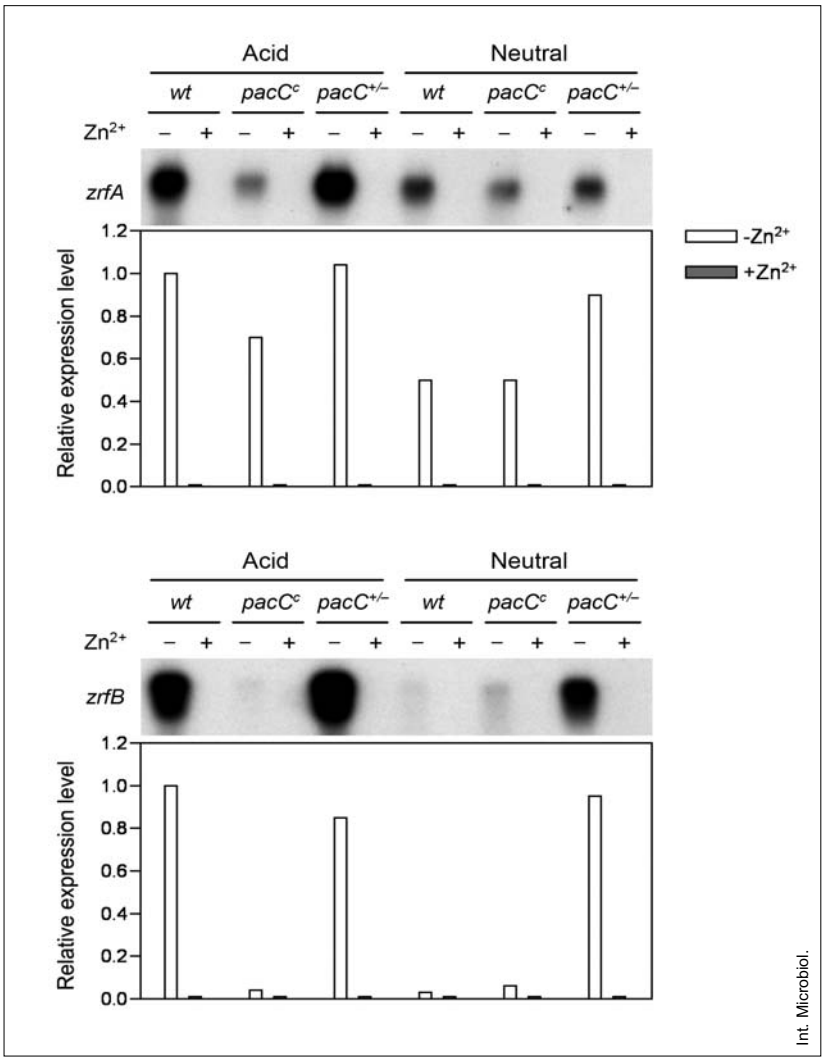

Fig. 4. Transcriptional analysis of *zrfA* and *zrfB* by Northern blotting in the wild-type (AF14), *pacC*^{c} (AF58), and *pacC*$^{+/-}$ (AF60) strains grown as described in Fig. 3. Each blot membrane was also probed for the *actG* gene and the relative expression values for each *zrfA* and *zrfB* signal were calculated as in Fig. 3.

Partial repression of *zrfA* and *zrfB* transcription by PacC only under neutral or alkaline growth conditions. The transcription profile for the *zrfA* and *zrfB* genes was also analyzed in the *pacC*$^{+/-}$ and *pacC*^{c} mutants (Fig. 4). The expression of these genes by the *pacC*^{c} mutant under acidic and neutral growth conditions was similar to that of a wild-type strain grown in neutral medium. In contrast, expression of the *zrfA* and *zrfB* genes by the *pacC*$^{+/-}$ mutant under acidic and neutral growth conditions was similar to that of a wild-type strain grown in acidic medium. Therefore, in neutral or slightly alkaline medium, the transcription of *zrfA* and *zrfB* is repressed through the transcription factor PacC.

Discussion

In the *zrfA* promoter region there is one PacC-like binding site and three ZR elements, designated ZRA1–3 [19]. In previous work carried out in our laboratory, the ZRA sequences were found to be cis-acting elements required for regulating the expression of *zrfA* at the transcriptional level (unpublished data), most likely through the binding of the zinc-responsive transcriptional activator ZafA [13]. Note that the only PacC-like binding site in the *zrfA* promoter was located within the ZRA2 cis-acting sequence (Fig. 5). Hence, the low-level repression of *zrfA* transcription detected in neutral, zinc-limiting medium might have resulted from the displacement of ZafA bound to ZRA2 by PacC, according to the same model proposed for other genes repressed by PacC in acidic medium [6]. However, the ZRA1 and ZRA3 sites in the *zrfA* promoter would in this case still remain available for ZafA binding regardless of pH. This would explain the reduced, although noticeable, expression of *zrfA* under neutral, zinc-limiting conditions. In contrast, in the promoter region of *zrfB* there is a PacC-like binding site located between the TATA box and the transcription initiation site [19], whereas we found that all four ZR elements of the gene (ZRB1–4)

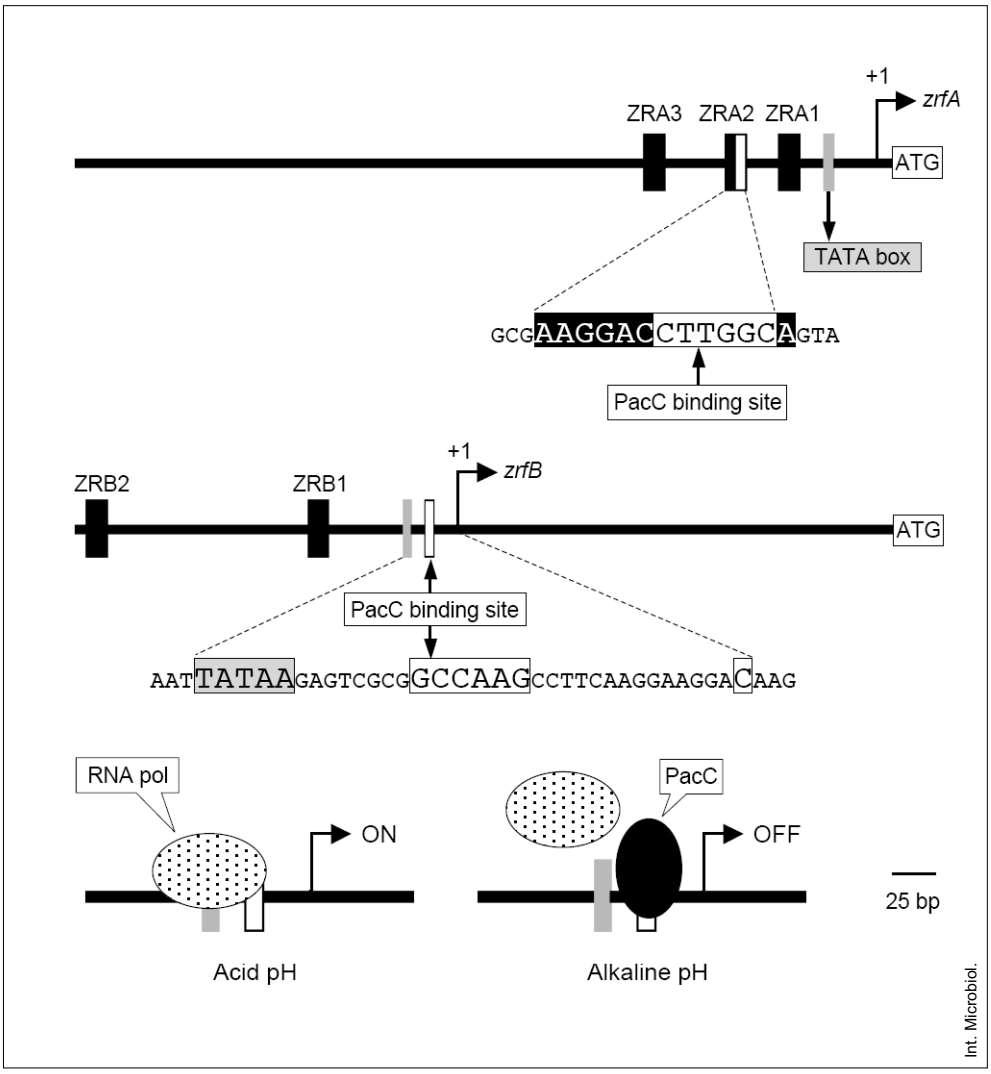

Fig. 5. Schematic representation of the *zrfA* and *zrfB* promoter regions and the repression model proposed for *zrfB*. The ZRB3 and ZRB4 binding sites for ZafA are not shown in the promoter region of *zrfB*. Only the relevant sequences are shown.

[19] were located upstream of the TATA box (Fig. 5). The particular location of the only PacC-like binding site in the *zrfB* promoter suggests that the strong repression of *zrfB* transcription detected under neutral, zinc-limiting conditions results from a blockade of efficient binding of RNA polymerase by PacC (Fig. 5). To our knowledge, this mechanism of gene repression has thus far not been described for any gene repressed at acidic pH, although a similar mechanism operates in the repression of other genes by transcriptional activators that may also function as repressors (e.g., the repression of *ZRT2* by Zap1 in *S. cerevisiae*) [2].

In summary, our results show that the PacC transcription factor exerts transcriptional repression of both *zrfA* and *zrfB* in *A. fumigatus* to various extents. The biological significance of this observation is not clear. Nevertheless, it is known that when the pH of a mild, zinc-limiting medium (i.e., a medium with a low concentration of free Zn^{2+} available for microorganisms) changes from acid to neutral, the medium becomes highly zinc-limiting (i.e., with an extremely low concentration of free Zn^{2+}) since the solubility of zinc decreases around neutral pH [1]. Hence, one possibility is that the ZrfA and ZrfB zinc transporters evolved to function optimally under the milder zinc-limiting environment provided by acidic growth conditions, such that transcription of the respective genes is reduced under neutral conditions in order to economize cellular resources. However, *A. fumigatus* grows well in neutral and in alkaline zinc-limiting medium [19]. Therefore, *A. fumigatus* must be equipped with at least a zinc transporter that functions under neutral or alkaline conditions. In this regard, we recently identified a zinc-transporter-encoding gene (*zrfC*) that is only expressed under alkaline conditions (unpublished data). This new gene, as well as the transcriptional regulatory mechanism that underlies its expression, is currently being investigated in our laboratory.

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