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ORIGINAL PAPER

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Alkaline pH-induced up-regulation of the *afp* gene encoding the antifungal protein (AFP) of *Aspergillus giganteus* is not mediated by the transcription factor PacC: possible involvement of calcineurin

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Abstract The *afp* gene encoding the antifungal protein (AFP) of *Aspergillus giganteus* has a prototypical alkaline gene expression pattern, which suggests that the gene might be under the control of the ambient pH-dependent zinc-finger transcription factor PacC. This notion is corroborated by the presence in the upstream region of *afp* of two putative PacC binding sites, afpP1 and afpP2, which are specifically recognised by the PacC protein of *A. nidulans* in vitro. However, in this report we provide several lines of evidence to show that pH-dependent up-regulation of *afp* is not mediated by transcriptional activation through PacC. (1) The temporal expression pattern of the *A. giganteus pacC* gene does not parallel the accumulation of the *afp* mRNA during cultivation. (2) Inactivation of afpP1 and afpP2 did not reduce promoter activity under alkaline conditions, as determined from the relative wild-type and mutant *afp::lacZ* reporter activities in *A. nidulans*. (3) Reporter activities in acidity- and alkalinity-mimicking mutant strains are inconsistent with a positive role for PacC in *afp* expression. (4) In *A. giganteus*, the pH-dependent increase in *afp* mRNA and AFP levels can be completely prevented by the calcineurin inhibitor FK506, suggesting that the calcineurin signalling pathway might control the in vivo activation of the *afp* promoter by alkaline pH.

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Introduction

The filamentous fungus *Aspergillus giganteus* secretes a basic, low-molecular weight protein with antifungal activity, called antifungal protein (AFP; Olson and Goerner 1965). The protein inhibits the growth of filamentous fungi, in particular of *Fusarium* and *Aspergillus* species, without affecting the growth of bacteria or yeast (Wnendt et al. 1994; Lacadena et al. 1995; Theis et al. 2003). This restricted susceptibility range makes the protein attractive for medical or biotechnological use in combating fungal infection and contamination. In this regard, several authors have shown that AFP is indeed able to protect rice, geranium and tomato plants against *Magnaporthe grisea*, *Botrytis cinera* and *Fusarium oxysporum* infections, respectively (Vila et al. 2001; Moreno et al. 2003; Theis et al. 2005).

Apart from the biotechnological and medical interest in the protein itself, there is also growing interest from a biological point of view. The antifungal activity is the only function of AFP which has been identified to date. As a consequence, it can be speculated that the protein might confer an ecological advantage on *A. giganteus* over nutrient competitors, and that its expression might therefore be triggered under unfavourable growth conditions. In agreement with this hypothesis, it has been shown that levels of AFP are highest during stationary phase, i.e. when nutrients become limiting, and that *afp* transcription is enhanced by heat shock, osmotic stress, carbon starvation and by the presence of certain co-cultivants (Meyer et al. 2002; Meyer and Stahl 2003). In addition, it is most interesting that the AFP titre is significantly increased under high pH conditions (pH 8), as the pH within soil is mostly alkaline (Meyer et al. 2002). This observation prompted us to investigate further the effects of environmental pH on *afp* expression.

In filamentous fungi, a similar pattern of pH-dependent gene regulation has been described for structural genes required for the production of antibiotics. The expression of genes involved in the biosynthesis of penicillin (*Aspergillus nidulans*, *Penicillium chrysogenum*) and cephalosporin (*Acremonium chrysogenum*) is elevated by alkaline pH and prevented by acidic pH (Espeso et al. 1993; Suárez and Peñalva 1996; Schmitt et al. 2001). Ambient pH regulation has been most extensively investigated in *A. nidulans*, where it is regulated by the zinc finger transcription factor PacC. PacC is proteolytically activated in two steps, the first of which involves the *pal* ambient pH signalling pathway. The processed form of PacC subsequently binds to the consensus sequence 5′-GCCARG-3′ and activates expression of alkaline-expressed genes and prevents expression of acidic-expressed genes (for reviews, see Peñalva and Arst 2002, 2004). Sequence analysis of the 5′ upstream region of *afp* has revealed the presence of two putative PacC binding sites designated afpP1 (5′-GCCAAG-3′; positions −1138 to −1133) and afpP2 (3′-GAACCG-5′; −937 to −932). Gel mobility shift assays with the entire zinc finger region of the *A. nidulans* PacC demonstrated that both of these putative PacC targets are specifically recognised in vitro. These data suggested that PacC might be positively involved in regulation of *afp* (Meyer and Stahl 2002).

However, in this report we provide strong evidence that pH-dependent up-regulation of *afp* is not mediated by transcriptional activation through PacC. (1) The accumulation of the *afp* mRNA does not reflect the expression profile of the *A. giganteus pacC* gene during cultivation under different conditions of ambient pH. (2) Point mutations in the afpP1 and afpP2 sites in the *afp* promoter do not abrogate *afp::lacZ* reporter expression in *A. nidulans* at alkaline pH. (3) Reporter activities in acidity- and alkalinity-mimicking mutant strains are inconsistent with a positive role for PacC in *afp* expression. (4) In *A. giganteus*, the pH-dependent increase in *afp* mRNA and AFP levels can be completely prevented by the calcineurin inhibitor FK506, whereas *pacC* transcription remains unaffected by the compound.

Materials and methods

Strains and plasmids

The wild-type strain of *A. giganteus* used here (0903) and strain TR22-2 have been described by Meyer et al. (2002). *A. nidulans* strains and their genotypes are listed in Table 1. The *Escherichia coli* strains DH5α (Gibco BRL) and Xl10-Gold (Stratagene) were used as hosts for the maintenance of the vectors listed in Table 2 and were transformed by the heat-shock method (Miller 1987).

Growth conditions, genetic techniques, phenotype analysis

Cultivation of the *A. giganteus* strains 0903 and TR22-2 was done in complete medium (C1; 2% malt extract, 1% peptone, 1% glucose) as described by Meyer and Stahl (2002). Standard growth media for *A. nidulans*, genetic techniques and phenotype testing followed those described by Cove (1966), Caddick et al. (1986) and Clutterbuck (1993). *A. nidulans* was transformed by the method described by Tilburn et al. (1995), using regeneration medium buffered at pH 6.5.

Isolation of the *pacC* gene from *A. giganteus*

A genomic library was constructed from the *A. giganteus* strain 0903 in λGEM-11 (Promega) according to the manufacturer's instructions. Phage plaques were blotted onto nylon filters (Qiagen) and hybridised with the *pacC* gene (2.1 kb) of *A. nidulans*, which was amplified by PCR using primers AnidPac1 and AnidPac2 (see

Table 1 *A. nidulans* strains used in this study

Strain	Genotype	Source
J788	*yA2 argB2 pantoB100*	Imperial college collection
788-12-12	*yA2 argB2* ($argB^{+}$ *afp::lacZ-12*) *pantoB100*	This work
788-1m2-7	*yA2 argB2* ($argB^{+}$ *afp::lacZ-1m2*) *pantoB100*	This work
788-12m-8	*yA2 argB2* ($argB^{+}$ *afp::lacZ-12m*) *pantoB100*	This work
788-2-7	*yA2 argB2* ($argB^{+}$ *afp::lacZ-2*) *pantoB100*	This work
788-2m-1	*yA2 argB2* ($argB^{+}$ *afp::lacZ-2m*) *pantoB100*	This work
EIH	*yA2 argB2 adE20 inoB2 palH72*	Imperial college collection
V32	*yA2 argB2* ($argB^{+}$ *afp::lacZ-12*) *adE20 pantoB100 inoB2 palH72*	This work
HS107	*yA2 argB2 pabaA1 palF15*	Imperial college collection
V34	*yA2 argB2* ($argB^{+}$ *afp::lacZ-12*) *pantoB100 pabaA1 palF15*	This work
2581	($pacC^{c}63$) $pacC^{-}6309$ *inoB2*	Fernández-Martínez et al. (2003)
V48	($pacC^{c}63$) $pacC^{-}6309$ *inoB2 pantoB100 argB2* ($argB^{+}$ *afp::lacZ-12*)	This work
J739	*biA1, argB2, pabaA1, choA1,* $pacC^{c}14$	Imperial college collection
V22	*wA4, biA1, pabaA1, choA1,* $pacC^{c}14$, *argB2* ($argB^{+}$ *afp::lacZ-12*)	This work
2594	*yA2* $pacC^{c}250$ *pabaA1*	Fernández-Martínez et al. (2003)
V59	$pacC^{c}250$ *pabaA1 argB2* ($argB^{+}$ *afp::lacZ-12*)	This work
4D2	*yA2 pabaA1 pyrG89(ΔpacC::pyr4) argB2*	Tilburn et al. (1995)

Table 3). The probe was labelled by random priming using [α–^{32}P]dATP and hybridizations were carried out at 60°C using the Rapid-hyb system (Amersham Biosciences). Positive plaques were purified and characterised by restriction mapping. A 4.58-kb *Apa*I fragment which hybridised to the probe was subcloned into pBluescript SK(+) to yield pBS-PacC2, and subsequently sequenced.

RT-PCR

RT-PCR was performed in order to verify intron positions in the *A. giganteus pacC* gene. Total RNA obtained after growth in C1 medium (pH 8) was treated with DNase I (FPLC pure, Amersham Biosciences) and employed as the template for RT-PCR. First-strand synthesis used primer AgigPac5 (Table 3) and ImProm-II Reverse Transcriptase (Promega). The subsequent PCR using primers AgigPac2-B and AgigPac12-B yielded a full-length *pacC* cDNA, which was then sequenced.

Functional analysis of the *A. giganteus pacC* gene in *A. nidulans*

A 1.9-kb fragment harbouring a truncated *argB* mutant allele was amplified from plasmid pAlc* (a gift from E. Espeso, CSIC, Madrid) by PCR, using primers Arg1 and Arg2 (Table 3). These primers introduced *Eco*RI restriction sites into the 1.9-kb fragment, allowing it to be cloned into *Eco*RI linearised pBS-PacC2. The resulting plasmid pAgPacC30 was used to transform the *A. nidulans pacC* null strain 4D2 (Table 1) according to Tilburn et al. (1995). Homologous integration at the *argB* allele of 4D2 resulted in a functional *argB*$^+$ allele, allowing transformants to be selected for arginine prototrophy (Pérez-Esteban et al. 1993). The presence of a single copy of the desired insertion at the *argB* locus was confirmed by Southern analysis. Growth analysis at 25°C, on molybdate and pH 8 plates, and acid phosphatase staining followed Caddick et al. (1986) and Clutterbuck (1993).

DNA and RNA analyses

DNA was isolated from *A. nidulans* transformants as described by Tilburn et al. (1995). The presence of a single-copy *afp::lacZ* construct integrated at the *argB* locus was confirmed by Southern analysis according to Pérez-Esteban et al. (1993). Primers Arg1 and Arg2 (Table 3) served to amplify the *argB* gene from wild-type *A. nidulans*, which was used as a probe for hybridization studies.

Total RNA was isolated from *A. giganteus* as described by Meyer et al. (2002) and Northern analysis was performed according to Sambrook et al. (1989). The primer pairs AgigPac2-B/AgigPac11-B and OlantiA/OlantiE (Table 3) were used to amplify the *pacC* and *afp* genes, respectively, from *A. giganteus*. The corresponding amplicons were used as probes for hybridization.

Probes were labelled by the random priming method using [α–^{32}P]dATP. Hybridizations were carried out at 65°C (for genomic DNA) and 70°C (for total RNA) using the Rapid-hyb system (Amersham Biosciences). The mRNA bands detected on X-ray film were quantified using the Kodak Image Station 440. mRNA levels were expressed relative to 18S + 28S rRNA.

Construction of *afp::lacZ* transcriptional fusions

Plasmid pBSΔlacZargB$^-$ (Table 2; Espeso and Arst 2000) was used as the reporter gene vector. After line-

Table 2 Plasmids used in this study

Plasmid	Characteristics	Source
pBS-PacC2	pBluescriptSK(+) derivative containing the *pacC* gene of *A. giganteus* as a 4.58-kb *Apa*I fragment	This work
pAgPacC30	pBS-PacC2 derivative containing a mutated *argB* allele which gives rise to a functional *argB*$^+$ after homologous integration into the *argB* gene in *A. nidulans*	This work
pBSΔlacZargB$^-$	Plasmid containing the reporter *lacZ* gene into which promoter fragments can be introduced as *BamH*I-*Spe*I fragments; contains a mutant *argB* allele which gives rise to a functional *argB*$^+$ after homologous integration into the *argB* gene in *A. nidulans*	Espeso and Arst (2000)
pAfpLacZ(1/2)	pBSΔlacZargB$^-$ derivative containing the full-length *afp* promoter (1,139 bp) upstream of *lacZ*	This work
pAfpLacZ(1mut/2)	pBSΔlacZargB$^-$ derivative containing the full-length *afp* promoter (1,139 bp) with an $A_4 \rightarrow T$ mutation in site afpP1 upstream of *lacZ*	This work
pAfpLacZ(1/2mut)	pBSΔlacZargB$^-$ derivative containing the full-length *afp* promoter (1,139 bp) with an $A_4 \rightarrow T$ mutation in site afpP2 upstream of *lacZ*	This work
pAfpLacZ(2)	pBSΔlacZargB$^-$ derivative containing the short *afp* promoter (942 bp) upstream of *lacZ*	This work
pAfpLacZ(2mut)	pBSΔlacZargB$^-$ derivative containing the short *afp* promoter (942 bp) with an $A_4 \rightarrow T$ mutation in site afpP2 upstream of *lacZ*	This work

arization of the vector with *Bam*HI and *Spe*I, *afp* promoter fragments (including the first five codons of *afp*) were introduced as *Bam*HI-*Xba*I fragments, allowing in-frame fusion to the *lacZ* gene. Native and mutated versions of the *afp* promoter were amplified by PCR using primers with *Bam*HI/*Xba*I overhangs to facilitate cloning into pBSΔlacZargB$^-$ (for oligonucleotide sequences see Table 3). The native full-length *afp* promoter containing both of the putative PacC binding sites afpP1 and afpP2 was amplified using primers Prom1-B and Prom1-X. Primers Prom1-B and Prom1-Xmut were used to obtain the full-length *afp* promoter with an $A_4 \rightarrow T$ transversion at the afpP1 site. A full-length *afp* promoter with an intact afpP1 site but with an $A_4 \rightarrow T$ transversion at afpP2 was constructed by assembly-PCR using the primer pairs Prom1-X/Prom(−2)mut and Prom1-B/Prom(2)mut. A truncated derivative of the *afp* promoter containing only an intact afpP2 site was generated using primers Prom1-B and Prom2-X. The $A_4 \rightarrow T$ transversion in afpP2 was introduced using the primer combination Prom1-B/Prom2-Xmut. All *afp* promoter fragments were sequenced to verify the introduced mutations. The plasmids carrying the different *afp::lacZ* constructs (listed in Table 2) were transformed into the wild type *A. nidulans* strain J788 according to Tilburn et al. (1995). These transforming plasmids harbour a mutant *argB* allele which, after homologous integration by single crossover at *argB*, can give rise to a functional *argB*$^+$ allele and consequently to arginine prototrophy (Pérez-Esteban et al. 1993).

β-Galactosidase reporter assay

4×10^7 conidia of *A. nidulans* strains were inoculated in 20 ml appropriately supplemented liquid minimal medium [Cove 1966; with 1% glucose as carbon source and 5 mM ammonium (+)-tartrate as nitrogen source] and cultivated at 28°C and 200 rpm, for 20–24 h. We chose 28°C (the optimal growth temperature for *A. giganteus*) instead of the usual 37°C for culture of *A. nidulans* because the *afp* promoter of *A. giganteus* is up-regulated by heat shock at 37°C, and we wished to avoid the possibility that cultivation at 37°C might mask pH effects. The pH of the medium was adjusted with NaOH/HCl to 4.5, 6 and 8 and kept constant during cultivation either by buffering with 25 mM citric acid (pH 4.5), 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 6.5) and 25 mM TRIS–HCl (pH 8), respectively, or by adjusting with NaOH/HCl after 10–12 h of cultivation. Protein extraction and determination of β-galactosidase activity followed the protocol of Pérez-Esteban et al. (1993). β-Galactosidase activities were calculated as the mean value obtained from at least three independent cultures for each strain and standard errors are indicated.

β-Glucuronidase reporter assay

5×10^5 conidia of *A. giganteus* TR22-2 were inoculated in 50 ml C1 medium and cultivated at 28°C for up to 48 h. The pH of the medium was adjusted with NaOH/HCl to pH 4.5 or pH 8 and controlled every 10–12 h during growth. Mycelia were harvested at various times, and frozen in liquid nitrogen. Protein extraction and determination of β-glucuronidase activity followed Meyer et al. (2002). β-Glucuronidase activities were calculated as the mean value obtained from three independent experiments and standard errors are indicated.

Determination of the effect of FK506 on *afp* expression

200 ml C1 medium (pH 4.5) was inoculated with 2×10^6 conidia of *A. giganteus* 0903 and cultivated for 30 h at 28°C. Mycelial biomass was filtered, washed with sterile water and divided into six aliquots. One was transferred into C1 medium (pH 8), one to C1 medium (pH 8) supplemented with 50μl of ET [90%

Table 3 Oligonucleotides used in this study

Primer	Sequence (5′ → 3′) or reference
AnidPac1	Meyer and Stahl (2002)
AnidPac2	Meyer and Stahl (2002)
AgigPac2-B	CGGGGATCCATGTCAGAACATCAAGATAAC
AgigPac5	GGGGAATCAATAGTCGTCAAC
AgigPac11-B	CGGGGATCCATGGTTGAGGGCATAATAGAC
AgigPac12-B	CGGGGATCCTGATTAACTGTGCATCTTCGTATC
Arg1	TTTGAATTCCGCCTCATCCGTCATAACG
Arg2	TTTGAATTCGTCGAGGGTTGGGGTGTG
OlantiE	Meyer and Stahl (2002)
OlantiA	Meyer and Stahl (2002)
Prom1-B	AGAGGATCCAGAAACGAACTTCATGATGAATTG
Prom1-X	AGATCTAGAGCCAAGGGGAACGGCCCCGGATG
Prom1-Xmut	AGATCTAGAGCCTAGGGGAACGGCCCCGGATG
Prom(2)mut	AGTACCTAGGCGGCTTAGAGCTG
Prom(−2)mut	CAGCTCTAAGCCGCCTAGGTACTTCATATG
Prom2-X	AGATCTAGAAGTACCTTGGCGGCTTAGAGCTG
Prom2-Xmut	AGATCTAGAAGTACCTAGGCGGCTTAGAGCTG

(v/v) ethanol and 10% (v/v) Tween], and the others into C1 medium (pH 8) supplemented with 50μl portions of FK506 dissolved in ET, yielding final concentrations ranging from 25 nM to 200 nM. FK506 was purchased from Fujisawa (Deerfield, Ill.). Care was taken in order to transfer equal amounts of biomass to the FK506-treated and control media. After cultivation for a further 10 h at 28°C, mycelial samples were harvested and washed with sterile water. Extraction of total RNA and Northern analysis were performed as described above.

AFP was isolated from the culture supernatants by cation exchange chromatography as previously reported (Meyer et al. 2002). AFP levels were determined after SDS-PAGE on 15% (w/v) PAA gels. The experiment was repeated twice.

Results

Cloning and functional analysis of the *A. giganteus pacC*

In order to help determine the role of the transcription factor PacC in pH-dependent regulation of the *afp* gene, a lambda genomic library of *A. giganteus* wild-type strain 0903 was constructed and screened for a *pacC* orthologue using the *pacC* gene of *A. nidulans* as the heterologous probe. A genomic clone that hybridised with the probe was isolated and subjected to restriction mapping and Southern analysis (data not shown). A hybridising *Apa*I fragment was subsequently subcloned and its nucleotide sequence (4,583 bp) determined. A single ORF of 2,265 bp, interrupted by two introns, was identified. The positions of the introns were verified by sequencing the corresponding cDNA. Two PacC consensus binding sequences were identified in the 690-bp region upstream of the predicted translational start

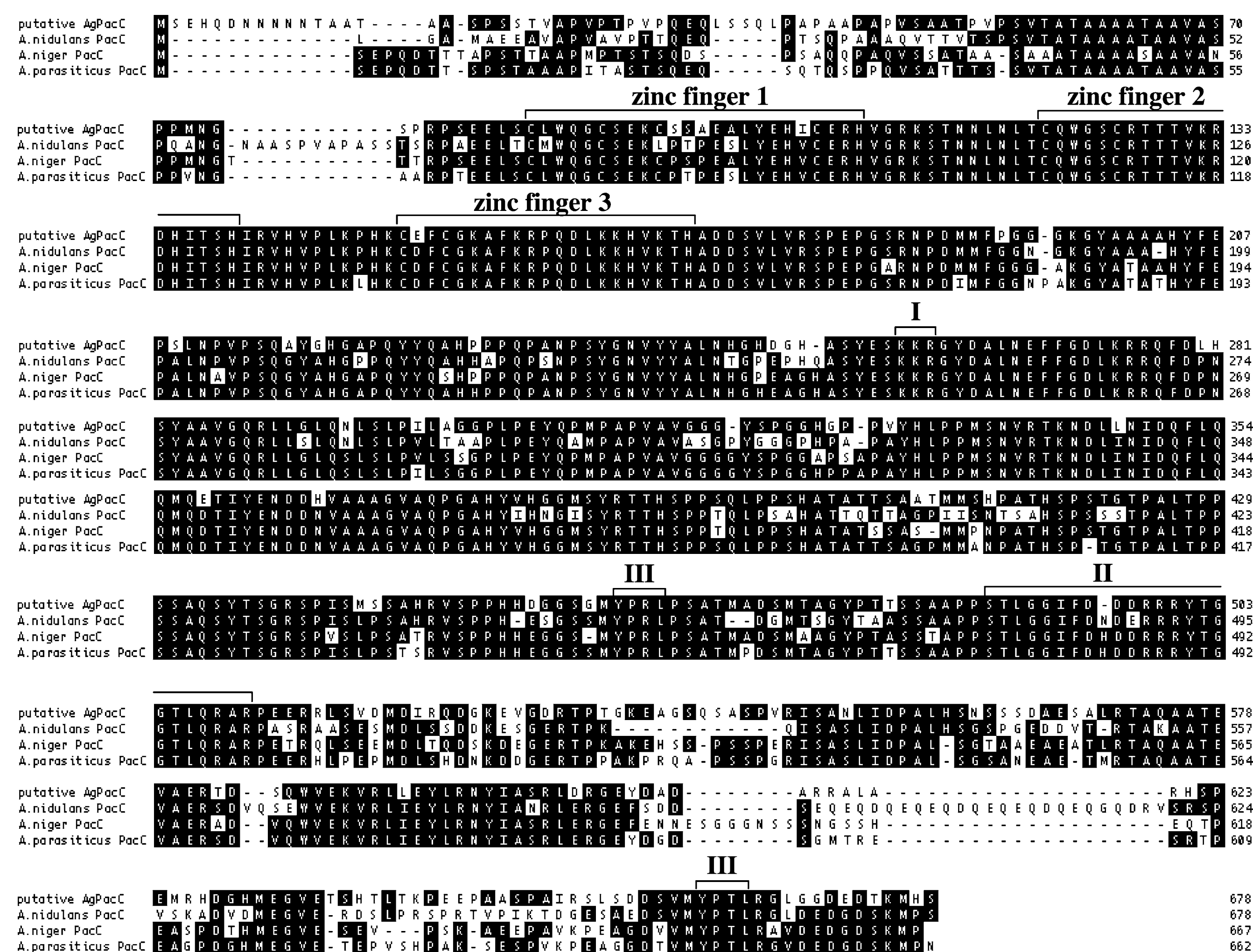

Fig. 1 Alignment of PacC sequences from *Aspergillus nidulans*, *A. niger* and *A. parasiticus* with the putative transcription factor AgPacC from *A. giganteus*. Conserved residues are marked in *black*. The positions of the three zinc fingers, the processing site(s) (*I*), the 'signalling protease box' (*II*) and the PalA-binding motifs (*III*) are indicated. The nucleotide and derived amino acid sequences of the putative *A. giganteus* PacC have been deposited in the NCBI/GenBank database under the Accession No. AY763122. Entrez protein accession numbers are as follows: *A. nidulans*, S54308; *A. niger*, S63587; *A. parasiticus*, AAK98616

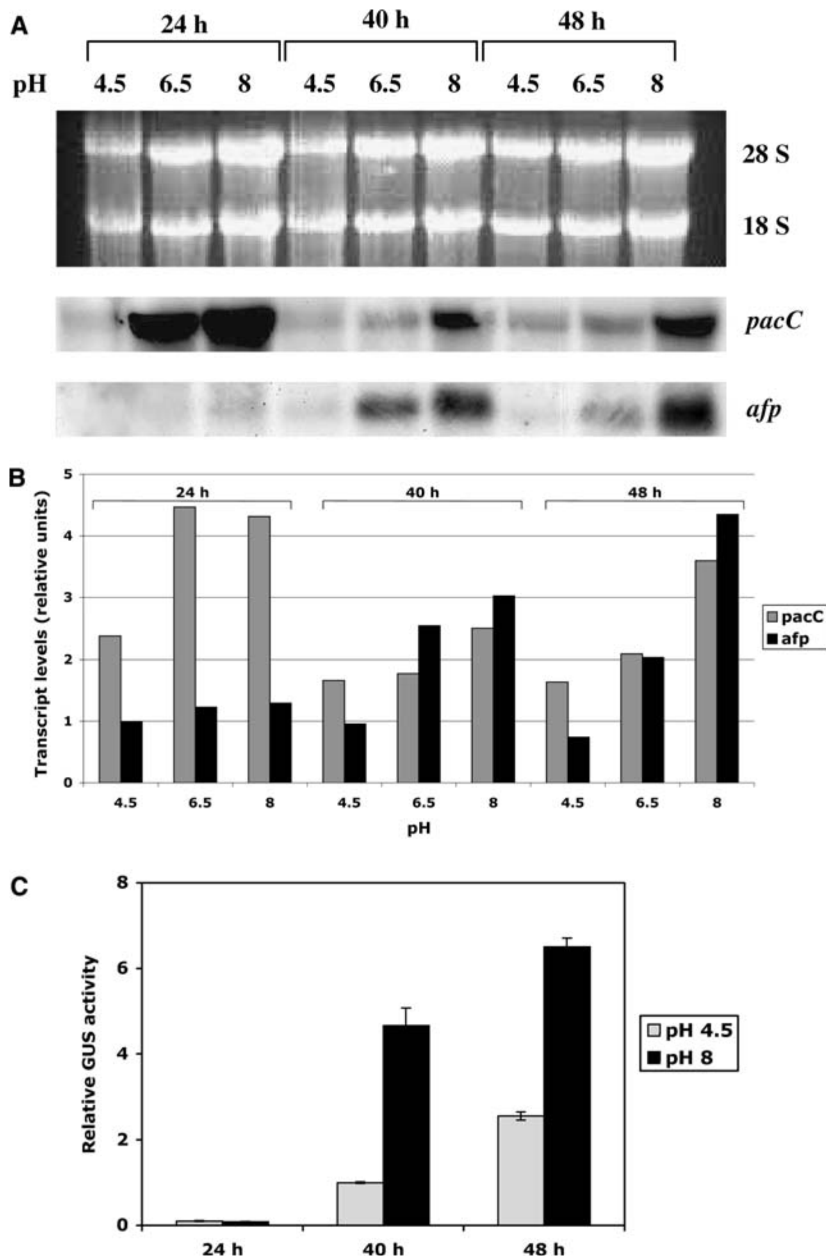

Fig. 2 Effect of pH on the accumulation of *afp* and *pacC* transcripts in *A. giganteus*. **a, b** The *A. giganteus* strain 0903 was grown in C1 medium at pH 4.5, 6.5 and 8, respectively. The pH of the medium was monitored and, when necessary, adjusted to the initial pH value with NaOH or HCl. Total RNA was isolated at the times indicated. The filter was simultaneously hybridised with *pacC* and *afp* probes. The 28S and 18S rRNAs, stained with ethidium bromide, were used as the loading control. Transcript levels were quantified by densitometric analysis of the Northern blot shown in **a**. For calibration, rRNA was used as standard. Levels of *pacC* (*shaded bars*) and *afp* (*black bars*) transcripts are expressed relative to that of the *afp* mRNA after cultivation at pH 4.5 for 24 h. **c** The *A. giganteus* strain TR22-2 was assayed for GUS expression as a function of the pH of the growth medium. Mycelia were grown in C1 medium (at 28°C) at pH 4.5 (*shaded bars*) and pH 8 (*black bars*), respectively. At the indicated times, mycelial samples were harvested and assayed for *β*-glucuronidase activity. Each *bar* represents the means of three independent experiments. Activity is expressed as relative to that measured in cultures grown at pH 4.5 at 40 h. Standard deviations were less than 8%

codon. The deduced 678 amino acid sequence of the product is 72.9–77.7% identical to the PacC proteins of *Aspergillus nidulans*, *A. niger* and *A. parasiticus* (Fig. 1). It can therefore be concluded that the gene indeed codes for the PacC orthologue in *A. giganteus*. The *A. giganteus* PacC zinc finger region shows 88.2–95.5% identity to the corresponding domains of the other PacC homologues. In particular, fingers 2 and 3, which make contact with the DNA (Espeso et al. 1997), are nearly identical in all four proteins, indicating that the *A. giganteus* PacC is likely to bind to the same consensus binding site. Finger 1, which does not bind DNA but interacts with finger 2 (Espeso et al. 1997; Fernández-Martínez et al. 2003), is less conserved, although it still shows a high degree of identity.

To determine the function of the *A. giganteus pacC* gene, a *pacC* null mutant strain of *A. nidulans* (strain 4D2, in which the *pacC* coding region has been deleted; Table 1) was transformed with a plasmid that contained the *A. giganteus pacC* gene under the control of its own promoter (plasmid pAgPacC30). Two single-copy transformants were identified by Southern hybridization (data not shown) and phenotypically analysed. These transformants showed slightly reduced growth compared to *pacC* wild-type strains, and produced fewer conidiophores. However, like *pacC* wild-type strains and in contrast to the recipient, they were able to grow at alkaline pH and 25°C, were no longer molybdate sensitive and showed an almost wild-type pattern of colony staining for acid phosphatase (data not shown). Thus,

these data indicate that *A. giganteus pacC* gene is expressed in *A. nidulans* and can substitute, at least to some extent, *A. nidulans pacC* .

Transcription of the *A. giganteus pacC* and *afp* genes

In order to analyse the impact of culture pH on *afp* and *pacC* transcription, the wild-type *A. giganteus* strain 0903 was cultivated in liquid C1 medium adjusted to acidic (pH 4.5), neutral (pH 6.5) or alkaline (pH 8) conditions. Cultures were sampled after 24, 40 and 48 h (these times correspond approximately to the early, mid-exponential and early-stationary growth phases, respectively), and Northern analyses were performed. As shown in Fig. 2a and b, both *pacC* and *afp* are less expressed under acidic growth conditions and their transcript levels are progressively elevated as the ambient pH is raised, demonstrating that expression of both genes is pH-dependent. The *afp* transcript is mainly detected during mid-exponential and early-stationary growth phases, indicating preferential transcription during later stages of growth. In contrast, the *pacC* transcript reaches its maximum in the early growth phase, when levels of the *afp* mRNA are low. This suggests that, at least under the conditions tested, PacC is not positively involved in *afp* transcription.

The pH dependence of *afp* expression was confirmed by reporter gene analysis using the *A. giganteus* strain TR22-2. This strain contains an ectopically integrated single-copy of an *afp::uidA* construct, consisting of the 1.2-kb *afp* promoter fragment fused to a reporter gene (*uidA*) encoding β-glucuronidase (GUS) (Meyer et al. 2002). Strain TR22-2 was grown at pH 4.5 and pH 8, mycelia were harvested after 24, 40 and 48 h and the GUS activity of mycelial extracts was determined. Figure 2c shows that there is almost no reporter expression in young mycelium (24 h), whereas reporter activity markedly increases with the age of the culture and is generally higher at pH 8.

Analysis of *afp::lacZ* expression using *A. nidulans* as host

To assess whether PacC is physiologically relevant for pH-dependent regulation of the *afp* gene, we used *A. nidulans* as a heterologous system in which, in contrast to *A. giganteus*, gene-targeting systems as well as a variety of pH mutant strains are available. We constructed *lacZ* fusions with full-length and truncated *afp* promoter fragments (1,139 bp and 942 bp, respectively) and mutated versions thereof. A single point mutation ($A_4 \rightarrow T$ transversion) that prevents PacC binding (Tilburn et al. 1995; Espeso et al. 1997) was introduced into sites afpP1 and afpP2 by PCR. If PacC binding to the *afp* promoter is physiologically relevant, the targeted mutations in afpP1 and afpP2 should result in impaired promoter activity under alkaline conditions. The respective *afp::lacZ* constructs, denoted 12, 1m2, 12m, 2 and 2m (Fig. 3a), were introduced into the *A. nidulans argB2* strain J788, and arg^+ transformants were analysed by Southern hybridization to confirm the presence of a single-copy integration at the *argB* locus (data not shown; strains are listed in Table 1). To determine the levels of β-galactosidase activity of *A. nidulans* transformants in the mid-exponential growth phase, the strains were grown at pH 4.5 for 20 h and then the pH was adjusted to pH 8 by the addition of NaOH (equivalent to 16 mM Na^+ at most) or kept at pH 4.5, and the cultures were incubated for a further 4 h. Note that the level of Na^+ necessary to affect *afp* promoter activity is at least 100 mM Na^+ (Meyer et al. 2002). Figure 3b shows that β-galactosidase activity of the native, full-length *afp* promoter (construct 12) at pH 8 is 1.4 fold higher than that at pH 4.5. This difference is very subtle, indicating that expression of the β-Gal reporter in *A. nidulans* partially mimics the alkaline induction of the *afp* gene in *A. giganteus*. However, this pH effect occurred consistently, suggesting that *A. nidulans* can nevertheless be used as a heterologous system to analyse whether pH-dependent regulation of the *afp* promoter is mediated by PacC. Mutation of site afpP2 in the full-length *afp* promoter (construct 12m) had essentially no effect on reporter expression compared to the native *afp* promoter, indicating that site afpP2 is not bound by PacC in vivo. This result is corroborated by analysis of construct 2m, which, despite the mutation of site afpP2, still responded to the pH shift. Interestingly, the point mutation in site afpP1 (construct 1m2) reduces β-galactosidase activity generally, raising the possibility that an additional binding site for a positive regulatory factor might exist in this region, which is eliminated by the $A_4 \rightarrow T$ transversion. Construct 1m2 still displayed a higher reporter activity at pH 8 than at pH 4.5, suggesting that the putative PacC binding site afpP1 is also unlikely to be involved in pH-dependent up-regulation of the *afp* promoter.

Analysis of *afp::lacZ* expression in acidity- and alkalinity-mimicking mutant strains of *A. nidulans*

To determine whether PacC might be indirectly involved in *afp* regulation by functioning as an activator or repressor of another regulatory mechanism, we crossed the wild-type *pacC* strain (strain 788-12-12) containing the native *afp* promoter upstream of *lacZ* (construct 12) with *pacC* mutant strains that display either an acidity-mimicking (*palH72*; *palF15*; *pacC⁻6309*) or an alkalinity-mimicking (*pacC^c14*; *pacC^c250*) phenotype. The relevant genotypes of the respective strains are given in Table 1. Progeny of the crosses were screened for the respective mutations by assaying for sensitivity/resistance to neomycin and molybdate, growth at pH 8 and at 25°C, and colony staining for acid phosphatase and alkaline phosphatase (data not shown). The presence of a single copy of *afp::lacZ* at the *argB* locus was verified

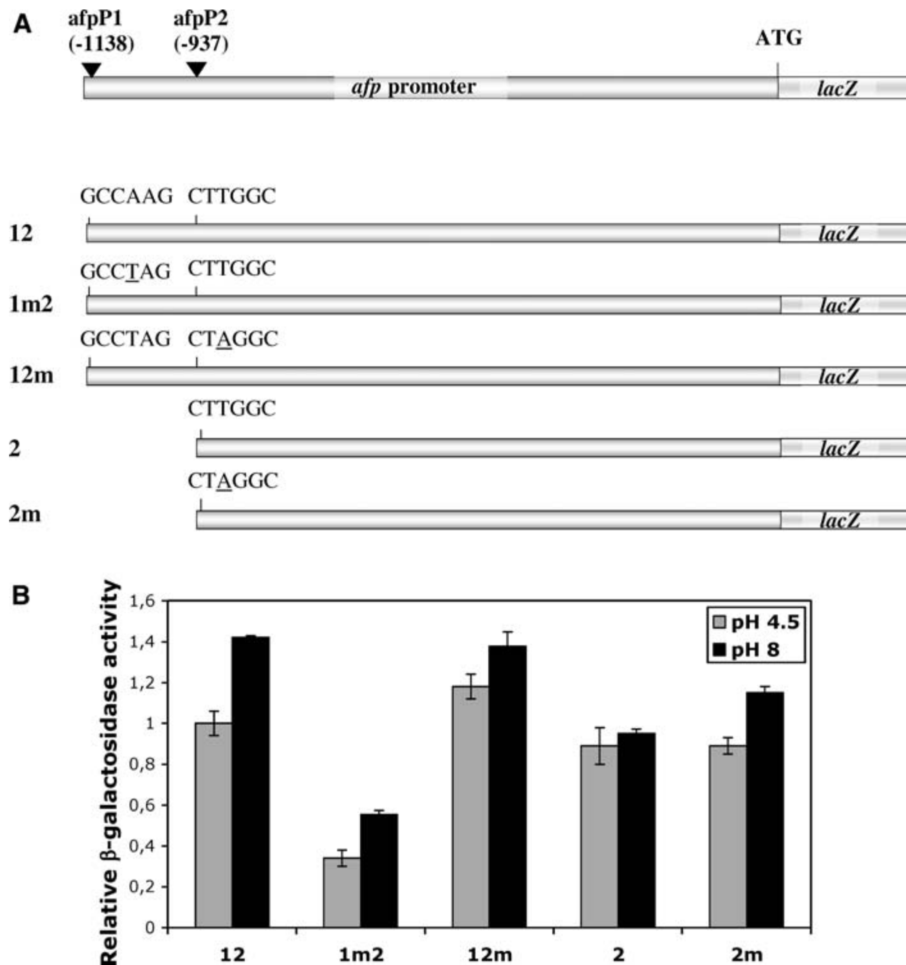

Fig. 3 Effects of $A_4 \rightarrow T$ mutations in sites afpP1 and afpP2 on *afp::lacZ* activity in *A. nidulans*. **a** Schematic representation of the *afp* promoter fragments fused to the *lacZ* reporter gene. The positions of the afpP1 and afpP2 sites are indicated relative to the translational start codon. Note that site afpP2 is located on the complementary strand. The base change in the consensus PacC binding site (GCCAAG to GCCTAG) introduced by PCR is *underlined*, and all the mutations were confirmed by DNA sequencing (see Materials and methods). The primers used to mutagenise the two sites are listed in Table 3. The reporter constructs were named as follows: 12: native, full-length *afp* promoter (1,139 bp); 1 m2: full-length *afp* promoter with a single point mutation in site afpP1; 12 m: full-length *afp* promoter with a single point mutation in site afpP2; 2: truncated *afp* promoter (942 bp) with intact site afpP2; 2 m: truncated *afp* promoter with mutated site afpP2. **b** The *lacZ* activity of single-copy, targeted *A. nidulans* transformants containing constructs 12, 1 m2, 12 m, 2, and 2 m, respectively, was measured. Strains were cultivated for 20 h at pH 4.5 and then kept at pH 4.5 (*shaded bars*) or shifted to pH 8 (*black bars*) and cultured for a further 4 h. β-Galactosidase activity is expressed relative to that of the wild-type promoter (construct 12) in cultures grown at pH 4.5. There was no further increase in reporter activity, when cultures growing at pH 8 were monitored for up to 9 h (data not shown). Values represent the means of six independent experiments

by Southern analysis in all cases (data not shown). Strains were grown for 24 h at pH 4.5 and 8, respectively, and β-galactosidase activity was determined. Table 4 shows that a constitutively active PacC, which is present in the alkalinity-mimicking strains *pacC*^{c}*14* and *pacC*^{c}*250* irrespective of the ambient pH, leads to a strong decrease in *afp::lacZ* expression when the strains were grown at acidic pH. Consistently, reporter expression in the acidity-mimicking mutant strain, in which PacC is not present (*pacC*$^{-}$*6309* mutant), was higher as compared to the *pacC* wild-type strain. In the acidity-mimicking mutant strains, in which the *pal* signalling pathway is not activated (*palH72* and *palF15* mutants), similar reporter activities to those in the *pacC* wild-type strain were observed when the strains were grown at pH 6.5 (data not shown). As both *pacC* null and *pacC* constitutive mutations have pronounced effects on growth and morphology (Tilburn et al. 1995 and references therein), these data raise the possibility that PacC functions only very indirectly as a negative regulator of *afp* expression.

afp expression in response to inhibition of the calcineurin signalling pathway

As our results revealed that alkaline up-regulation of the *afp* promoter is not mediated by binding of PacC to sites afpP1 and afpP2, we looked for an alternative mechanism which might account for this response. In *Saccharomyces cerevisiae* and *Candida albicans*, the

Table 4 Effects of acidity- and alkalinity-mimicking *pacC* mutations on *afp::lacZ* expression

Strain	Relevant mutation	β-Galactosidase activity[a]	
		pH 4.5	pH 8
788-12-12	None	1.00 ± 0.03	1.42 ± 0.01
V48	*pacC*$^{-}$*6309*	2.57 ± 0.06	1.97 ± 0.07
V22	*pacC*^{c}*14*	0.09 ± 0.03	0.83 ± 0.04
V59	*pacC*^{c}*250*	0.28 ± 0.02	1.36 ± 0.01

[a] The reporter construct 12, which contains the wild-type *afp* promoter, was introduced into different genetic backgrounds in *A. nidulans* by crossing strain 788-12-12 with *pacC*^{c}, and *pacC*$^{-}$ strains (see Table 1). Mycelia were grown in appropriately supplemented and buffered minimal medium adjusted to pH 4.5 and 8, respectively, for 24 h. The specific activities of β-galactosidase obtained in three independent experiments (± SD) are given. Activities were normalised to that of strain 788-12-12 grown at pH 4.5

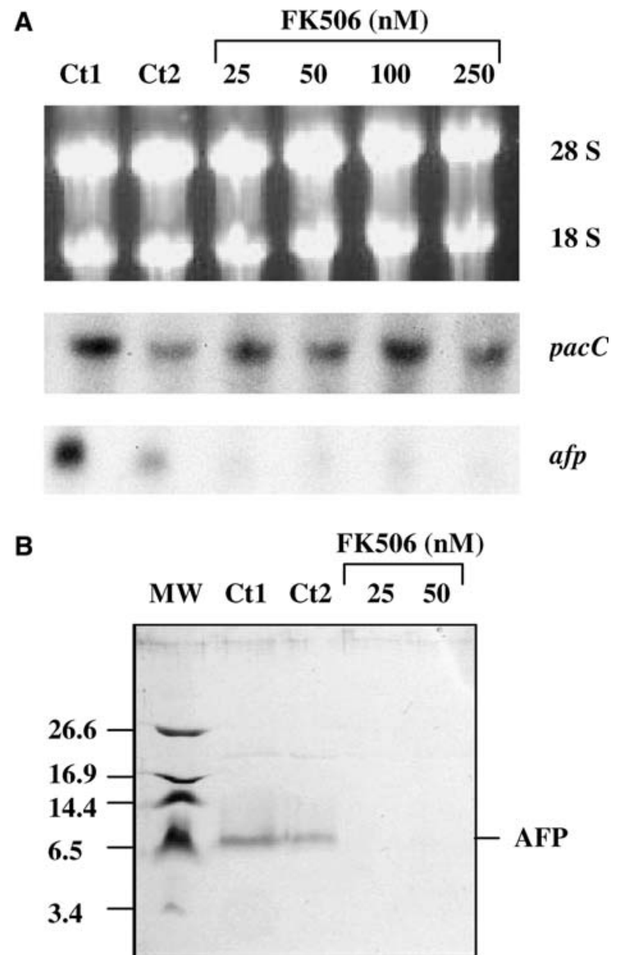

Fig. 4 Effect of FK506 on the levels of the *afp* transcript and its protein product in *A. giganteus*. *A. giganteus* strain 0903 was grown in C1 medium (pH 4.5) for 30 h. Mycelia were then harvested and transferred into C1 medium (pH 8) containing the indicated concentrations of FK506 [dissolved in 50µl of ethanol/Tween (ET)]. After cultivation for 10 h, RNA was isolated from mycelial extracts, and basic proteins were purified from the culture supernatant. *A. giganteus* grown in the absence of FK506 (Ct1) and *A. giganteus* supplemented with 50µl ET (Ct2), respectively, served as controls. **a** Total RNA was simultaneously hybridised with *pacC* and *afp* probes. The 28S and 18S rRNAs were visualised by staining with ethidium bromide, and used as the loading control. **b** Basic proteins in the culture broth were fractionated on a 15% SDS-PA gel. *MW* molecular size marker (Biorad)

transcriptional response to alkaline pH involves various mechanisms and the calcineurin pathway is a relevant component of the response (Mendizabal et al. 2001; Lamb et al. 2001; Davis et al. 2002; Serrano et al. 2002; Viladevall et al. 2004). This prompted us to examine the pH-related physiological role of calcineurin in *A. giganteus*. We cultivated *A. giganteus* at pH 4 in C1 medium and, after the culture had reached the early-exponential growth phase, we transferred the mycelium to C1 medium buffered at pH 8 and characterised *afp* expression at the RNA and protein levels after the culture had reached mid-exponential phase. To examine whether the calcineurin signalling pathway is involved in *afp* regulation, we added different concentrations of FK506, which is commonly used as a specific inhibitor of calcineurin, to the pH 8 medium. 25 nM FK506 was sufficient to block *afp* transcription (Fig. 4a) and hence AFP production (Fig. 4b) completely. Assuming that FK506 specifically inhibits calcineurin in *A. giganteus*, these results suggest that the calcineurin signalling pathway might mediate the up-regulation of the *afp* gene at alkaline pH. Interestingly, *pacC* transcript levels were not affected by FK506, providing additional evidence that PacC is not positively involved in *afp* regulation. We also tested whether pH-dependent up-regulation of *afp::lacZ* in *A. nidulans* is affected by FK506. Strain 788-12-12 was grown at pH 4.5 for 20 h and then shifted to pH 8 for 4 h. The addition of 100 nM FK506 was found to substantially reduce expression of the *afp::lacZ* reporter (data not shown).

Discussion

Expression of the *afp* gene is markedly increased under alkaline conditions, suggesting that the gene might be under the control of the transcriptional regulator PacC, the activity of which is dependent on ambient pH. This notion is supported by the presence of two in vitro PacC binding sites (afpP1 and afpP2) in the 5′UTR of the *afp* gene (Meyer and Stahl 2002). We therefore obtained a *pacC* orthologue from *A. giganteus* in order to test whether PacC is physiologically relevant to the pH-dependent regulation of the *afp* gene. The *A. giganteus* PacC protein shows 72.9–77.7% identity to the PacC proteins of *A. nidulans*, *A. niger* and *A. parasiticus*. The four PacC proteins are most conserved in the zinc finger DNA binding domains, the 'signalling protease boxes' (Díez et al. 2002), the processing sites (Mingot et al. 1999) and the PalA binding motifs (Vincent et al. 2003). Moreover, the *A. giganteus pacC* gene expressed in *A. nidulans* under control of its own promoter is, at least partially, able to complement an *A. nidulans pacC* null mutation.

The *A. giganteus pacC* and *afp* genes are expressed predominantly at pH 8 and only weakly at pH 4.5, demonstrating that both are alkaline-expressed genes. However, *pacC* transcript levels are highest during the early growth phase, whereas transcription of the *afp* gene is elevated during exponential and stationary growth phases. This suggested that the alkaline induced up-regulation of the *afp* gene might be independent of PacC. This was confirmed by the observation that inactivation of both sites did not prevent the increase in promoter activity normally seen under alkaline conditions, as measured using the corresponding *afp::lacZ* fusion constructs as reporters in *A. nidulans*. Thus, although PacC binds to both putative PacC binding sites in vitro, a physiological role for these sites cannot be established, indicating that PacC is probably not a direct activator of *afp* expression. We caution however, that PacC-mediated pH regulation might nevertheless be present but obscured by the absence of some necessary factor in growth conditions and/or by the absence in *A. nidulans* of a pathway-specific regulator present in *A. giganteus*.

The finding that DNA-protein interactions occur under in vitro conditions but not in vivo is not unusual. For example, the bidirectional promoter lying between the divergently transcribed *ipnA* and *acvA* genes of the penicillin gene cluster of *A. nidulans* contains five PacC consensus sequences (ipnA1–ipnA4B), all of which are recognised in vitro. However, site ipnA3 seems to be the major site for PacC activation of both genes in vivo, which suggests that context might also influence PacC binding (Tilburn et al. 1995; Espeso and Peñalva 1996; Then Bergh and Brakhage 1998). Hence, it is possible that sites afpP1 and afpP2 are not positioned favourably for in vivo PacC binding. The ipnA2 site that has the highest affinity for PacC in vitro only modestly contributes to in vivo promoter activation of the *ipnA* gene (Espeso and Peñalva 1996). As the ipnA2 site is the site most distal from the *ipnA* transcriptional start point (−593 bp), Espeso and Peñalva (1996) suggested that specific contacts between PacC and the general transcription machinery at the transcription start site are less favoured. A similar situation might apply to sites afpP1 and afpP2 in the *afp* promoter, as they are located at positions −1,133 and −932 bp respectively, upstream of the ATG (the transcriptional start point is as yet undetermined).

Interestingly, *pacC* alkalinity-mimicking mutations (*pacC*^{c}*250*, *pacC*^{c}*14*) decreased *afp::lacZ* activity in *A. nidulans*, whereas a *pacC* acidity-mimicking mutation (*pacC*$^{-}$*6309*) resulted in elevated *afp::lacZ* activities, possibly suggesting that PacC is indirectly involved in the inhibition of *afp* expression. In filamentous fungi, it has been demonstrated that PacC regulates not only genes coding for secreted enzymes and proteins involved in the synthesis of exported metabolites, but also genes for proteins such as transporters involved in ion homeostasis, salt tolerance and iron uptake (for review, see Peñalva and Arst 2002; Caracuel et al. 2003; Van Kuyk et al. 2004; Eisendle et al. 2004). There are therefore numerous ways in which PacC might affect the *afp* promoter indirectly. Moreover, in view of the lack of effect of *palF*15 and *palH*72 on reporter expression, any role of PacC is likely to be very indirect.

The transgenic expression studies in *A. nidulans* revealed that the $A_4 \rightarrow T$ transversion in site afpP1 reduced reporter activity to 40% of that seen with the wild-type promoter. Analysis of the surrounding nucleotides uncovered a purine-rich inverted repeat overlapping site afpP1 (5′-GCCAAGGGGAAC-3′; the inverted repeat is underlined) andwhich has been disrupted by the $A_4 \rightarrow T$ transversion 5′ - GCCTAGGGGAAC - 3’

One possible explanation for the reduction in promoter activity could be that this motif serves as a binding site for an as-yet unknown transcription factor. It is also possible that the sequence 5′-AGGGG-3′ within the inverted repeat, which is homologous to the STRE found in many genes with stress-related functions in *Saccharomyces cerevisiae* (reviewed by Estruch 2000), might be involved in *afp* regulation, and that the $A_4 \rightarrow T$ mutation negatively affected binding of a *trans*-acting factor to it.

In *S. cerevisiae*, Lamb et al. (2001) have shown that pH-responsive genes can be classified into three groups, defined by their relationship to the Rim101p (PacC) pathway: (1) genes which are fully dependent on Rim101p action, (2) genes whose expression is partially dependent on Rim101p, and (3) genes which are up-regulated in a Rim101p-independent way. Our work gives some indication that the *afp* gene of *A. giganteus* falls into the third class. Moreover, we show here that pH-induced up-regulation of the *afp* promoter can be efficiently blocked in *A. giganteus* by FK506, which is known to inhibit calcineurin function in *S. cerevisiae* and *Schizosaccharomyces pombe* (Yoshimoto et al. 2002; Sio et al. 2005). In *S. cerevisiae*, calcineurin activity is activated by specific environmental conditions, such as high pH, high levels of Ca^{2+} and Na^{+}, and elevated temperature (Cyert 2001; Mendizabal et al. 2001; Yoshimoto et al. 2002; Serrano et al. 2002). Viladevall et al. (2004) have recently demonstrated that exposure of *S. cerevisiae* to alkaline pH induces a transient increase in cytosolic Ca^{2+} levels which triggers a calcineurin-mediated response. Calcineurin is a Ca^{2+}/calmodulin-dependent protein phosphatase, which, after activation, dephosphorylates the transcriptional activator Crz1p, allowing its translocation to the nucleus. Crz1p acts by binding to promoters containing calcineurin-dependent response elements (CDREs; 5′-GNGGC(G/T)CA-3′; Yoshimoto et al. 2002). *S. cerevisiae* genes that are up-regulated by exposure to alkaline pH via Crz1p include *ENA1*, which codes for an Na^{+}/Li^{+} -efflux pump and PHO89 encoding a Na^{+} -phosphate symporter (Mendizabal 2001; Serrano et al. 2002; Viladevall et al. 2004). Interestingly, Juvvadi et al. (2003) have reported that calcineurin activity increases when *A. oryzae* is grown at high pH, indicating that the calcineurin signalling pathway might also be used in filamentous fungi as an

adaptive response to alkalinization. Computational analysis of the 5′ upstream region of the *afp* gene revealed that it contains five putative CDREs, each with seven of eight nucleotides conserved. Thus, it is conceivable that the calcineurin signalling pathway might be responsible for the alkaline up-regulation of the *afp* gene. However, this speculation awaits experimental validation. In view of the fact that the *afp* promoter is upregulated by heat shock and osmotic stress, in addition to alkalinization (Meyer et al. 2002), it will be of special interest to examine the possible involvement of the calcineurin signalling pathway in *afp* regulation.

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