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RESEARCH ARTICLE

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New insights in the regulation of the afp gene encoding the antifungal protein of *Aspergillus giganteus*

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Abstract The antifungal protein (AFP) secreted by the mould *Aspergillus giganteus* is a small, highly basic polypeptide with antifungal activity. Previous work has shown that transcription of the corresponding *afp* gene is regulated by ambient pH, being suppressed under acidic and strongly induced under alkaline conditions. This observation suggested that the *afp* gene is regulated by the wide-domain transcriptional factor PacC. Here, we show that two putative PacC binding sites within the *afp* promoter, denoted afpP1 and afpP2, are efficiently recognised in vitro by a PacC fusion protein of *A. nidulans*. In addition, we found that phosphate, which was used as a buffering agent during cultivation, plays an important role in regulating *afp* expression. AFP production was nearly completely inhibited in the presence of external phosphate. The results of Northern analysis indicate that the inhibitory effect of phosphate is mediated at the transcriptional level.

Keywords *Aspergillus giganteus* · *afp* regulation · PacC · Phosphate inhibition

Introduction

Aspergillus giganteus is an imperfect ascomycete fungus characterised by its ability to produce very long conidiophores. The mould was found to secrete a 5.8-kDa protein with antifungal activities, named the antifungal protein (AFP; Olson and Goerner 1965). AFP inhibits the growth of filamentous fungi, in particular *Fusarium* and *Aspergillus* species, but does not affect the growth of bacteria and yeast (Wnendt 1994; Lacadena et al. 1995). The restricted host range of AFP makes the protein attractive for use as an inhibitor of fungal plant pathogens. For example, *Fusarium* contamination of grain during plant development or seed storage could be prevented without affecting non-target organisms. Knowledge of the signals involved in AFP expression and secretion could offer a way to increase AFP production and could accelerate such an application.

Analysis of the transcriptional regulation of the corresponding *afp* gene revealed that *afp* expression is regulated by different environmental conditions, e.g. changes in ambient pH, the presence of different stress-inducing agents and developmental cues (Meyer et al. 2002). As shown by reporter gene analysis, *afp* transcription is highest under alkaline pH conditions and lowest at acidic pH. Moreover, AFP titres are significantly enhanced under alkaline conditions, confirming that pH regulation is physiologically relevant.

Similar pH-dependent gene regulation has been described for certain fungi, including *A. nidulans* and *Penicillium chrysogenum*. Biosynthetic genes involved in the production of secondary metabolites, such as penicillin, cephalosporin and aflatoxin, are repressed by acidic pH and induced by alkaline pH (Espeso et al. 1993; Denison 2000; Schmitt et al. 2001). Ambient pH regulation is controlled by the wide-domain zinc finger transcription factor, PacC, which acts as a transcriptional activator for alkaline-expressed genes and as a repressor for acidic-expressed genes in *A. nidulans* (Tilburn et al. 1995). PacC is activated by the *pal* signal-transduction pathway under alkaline conditions, due to the removal of a C-terminal inhibitory region. The truncated PacC protein subsequently induces the expression of genes containing the core sequence 5′-GCCARG-3′ in their promoter regions (Orejas et al. 1995; Espeso et al. 1997, 2000; Mingot et al. 1999.

Sequence analysis of the *afp* 5′ upstream region revealed the presence of two putative PacC binding sites, designated afpP1 (3′-GAACCG-5′) and afpP2 (5′-GCCAAG-3′), which suggested that *afp* transcription might be regulated by PacC (Meyer et al. 2002). In order

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to verify this supposition, band shift assays were performed with a glutathion *S*-transferase (GST)::PacC fusion protein, containing the entire zinc finger region of the *A. nidulans* PacC protein. Here, we demonstrate that both putative PacC targets are specifically recognised by the *A. nidulans* fusion protein. Moreover, an intragenic putative PacC binding site, named afpP4 (3′-GAACCG-5′), was found to interact with GST::PacC.

In addition, we observed that phosphate, which has been used as a physiological buffer for adjustment and control of ambient pH during liquid cultivation of *A. giganteus*, strongly inhibits AFP production. The data indicate that excess phosphate provokes a delayed induction of *afp* transcription, leading to a decrease in AFP titres.

Materials and methods

Strains and plasmids

A. giganteus strain IfGB 15/0903 and *A. nidulans* DSM 969 were selected from our laboratory stock of micro-organisms. *Escherichia coli* strain DH5α (Gibco BRL) was used as a host for the maintenance of pGEX-PacC(69-168) (Espeso et al. 1997) and was transformed by the heat-shock method (Miller 1987).

DNA isolation and Southern analysis

A. giganteus was grown in 50 ml YPG medium (0.3% yeast extract, 1% peptone, 2% glucose, pH 4.5) for 65 h at 28 °C. Genomic DNA was isolated as described by Yelton et al. (1984). Southern analysis was performed according to Sambrook et al. (1989). DNA was transferred to a Qiabrane Nylon membrane (Qiagen) using a Posiblot pressure blotter (Stratagene) and was UV-crosslinked. A 2.1-kb hybridisation probe from the *A. nidulans pacC* gene was generated by PCR, using primers AnidPac1 and AnidPac2 (Table 1). A 330-bp hybridisation probe from the *A. giganteus afp* gene was generated by PCR, using primers OlantiA and OlantiE (Table 1). DNA probes were labelled by random primer labelling, using ^{32}P-labelled dATP and hybridisations were carried out according to the manufacture's instructions (Amersham Pharmacia Biotech).

Band shift assay

For the gel shift assay, double-stranded oligonucleotides were used as the DNA template (Table 1). Double-stranded oligonucleotides were obtained by annealing two homologous single-stranded oligonucleotides: the two oligonucleotides were mixed in the molar ratio 1:1 in TEN buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl, pH 8.0), incubated for 10 min at 95 °C and slowly cooled down to room temperature. The oligonucleotide duplex was end-labelled with [γ-^{32}P]dATP, using T_4-kinase (Amersham Pharmacia Biotech) according to Sambrook et al. (1989).

The binding reaction was performed according to the instructions of Roche (DIG gel shift kit for non-radioactive band shift assays). The binding reaction mixture (20 µl final volume) was performed in binding buffer (100 mM Hepes, pH 7.0, 5 mM EDTA, 50 mM $(NH_4)_2SO_4$, 5 mM dithiothreitol, 1% Tween 20, 150 mM KCl) and contained 100 fmol labelled template DNA, 0.1–10 pmol unlabelled DNA as specific or non-specific competitor, 1 µg poly(dI-C) and either GST (40 ng) or GST::PacC(69-168) fusion protein (5–80 ng). Binding reactions were incubated for 15 min at room temperature and the DNA and protein–DNA complexes, respectively, were separated on 7% native polyacrylamide gels. Gels were exposed to an X-ray film 2–4 h.

Purification of AFP

A. giganteus was cultivated at 28 °C in 50 ml C1 medium (2% malt extract, 1% peptone, 1% glucose) for the indicated times. AFP isolation from culture supernatants was performed by cationic exchange chromatography, as reported by Meyer et al. (2002). AFP amounts were analysed by SDS-PAGE. The polyacrylamide concentration in the separating gel was 15% (w/v).

Preparation of the GST proteins

Plasmid pGEX-2T (Amersham Pharmacia Biotech) contains the coding sequence for GST. Plasmid pGEX-PacC(69-168), containing the PacC zinc finger region, drives the expression of the GST::PacC fusion protein in *E. coli* DH5α (Espeso et al. 1997). Proteins were expressed in *E. coli* and purified by glutathione affinity chromatography, according to the MicroSpin GST purification module (Amersham Pharmacia Biotech). A sufficient yield of both proteins was obtained when expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1–3 h and the elution buffer contained an additional 0.5 M NaCl.

Table 1. Oligonucleotides used in this study. The position of each oligonucleotide in the promoter and coding region of the *afp* gene is given. Numbering is from the first codon of translation. The nucleotides GCG AAT TCA at the 5′ end of EI do not correspond to the *afp* promoter. Putative PacC binding sites are shown *in italics*

Primer	Sequence	Source	Name	Position
Pac30	5′-GAG CGC ATA TGA AGT AC*C TTG GC*G GCT TAG-3′	*afp* promoter	afpP1	From –956 to –927
Pac(–)30	5′-AGC TCT AAG CC*G CCA AG*G TAC TTC ATA TGC-3′	*afp* promoter	afpP1	From –923 to –952
Pac20	5′-TGG TTT A*GC CAA G*GG GAA CG-3′	*afp* promoter	afpP2	From –1145 to –1126
Pac(–)20	5′-CGT TCC C*CT TGG C*TA AAC CA-3′	*afp* promoter	afpP2	From –1126 to –1145
Pac22	5′-GAT GAT CAG CCA ACT GAA AAC A-3′	*afp* promoter	afpP3	From –1120 to –1099
Pac(–)22	5′-TGT TTT CAG TTG GCT GAT CAT C-3′	*afp* promoter	afpP3	From –1099 to –1120
EI	5′-GCG AAT TCA CAG TTA CGG ACA ATC GAT TG-3′	*afp* promoter	–	From –1100 to –980
Pro10(–)	5′-TCT CTG CAA TGA ACA AGG AAC-3′	*afp* promoter	–	From –884 to –904
Pac18	5′-GCT GCC *CTT GGC* GCG GTT-3′	*afp* gene	afpP4	From 43 to 60
Pac(–)18	5′-AAC CGC *GCC AAG* GGC AGC-3′	*afp* gene	afpP4	From 60 to 43
OlantiA	5′-GCC ACA TAC AAT GGC GTA GGT-3′	*afp* gene	OlA	From 130 to 150
Olanti(–)A	5′-ACC TAC GCC ATT GTA TGT GGC-3′	*afp* gene	OlA	From 150 to 130
OlantiE	5′-CTA GCA GTA GCA CTT CCC CTT-3′	*afp* gene	–	From 432 to 412
AnidPac1	5′-GGG GCG GCT GCG ACA GCT GCT GTG G-3′	*pacC* gene, *Aspergillus nidulans*	–	–
AnidPac2	5′-AGG CAT TTT GGA GTC TCC ATC CTC ATC-3′	*pacC* gene, *A. nidulans*	–	–

RNA isolation and Northern analysis

Mycelia were harvested from 50 ml of culture at certain points in time and total RNA was isolated as described by Meyer et al. (2002). Upon separation on 1.5% agarose gels containing 2% formaldehyde for 16 h at 3 V/cm, the RNA was transferred to a Qiabrane Nylon membrane (Qiagen), using a Posiblot pressure blotter (Stratagene) and was UV-crosslinked. The RNA samples were balanced according to their content of 28S and 18S rRNA. A 330-bp PCR amplicon of the *afp* coding region generated with primers OlantiA and OlantiE (see Table 1) was labelled by random primer labelling, using [α-^{32}P]dATP, and was used as the probe for Northern analysis. Hybridisation was carried out according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Results

Recognition of the afpP1 and afpP2 targets by a PacC fusion protein

Southern analysis was performed with genomic DNA from *A. giganteus* and a full-length *A. nidulans pacC* gene as the probe. Cross-hybridisation showed that *A. giganteus* contains a *pacC* homologue, thus providing an additional indication that the *afp* gene regulation might be controlled by PacC (Fig. 1).

In order to confirm that the two putative binding sites, afpP1 and afpP2, within the *afp* promoter (from –937 to –932 and from –1138 to –1133, respectively) were recognised by the PacC transcription factor, band shift assays were performed in vitro. For this purpose, a purified GST::PacC fusion protein was used. The fusion protein contains the entire zinc finger region of PacC from *A. nidulans* involved in DNA binding. The corresponding vector pGEX-PacC(69-168) (Espeso et al. 1997) was transferred to *E. coli* DH5α. Heterologous expression of GST::PacC under control of the *tac* promoter was induced with 0.1 mM IPTG and the fusion protein was purified, using glutathione affinity chromatography (Fig. 2).

Incubation of the purified GST::PacC fusion with double-stranded oligonucleotides encompassing sites afpP1 and afpP2, respectively, resulted in complexes with reduced gel mobility (Fig. 3a). Strong binding was detected with the afpP2 site, even at the lowest concentration of protein tested (5 ng). Binding to the afpP1 site was considerably weaker, indicating that the afpP2 site represents a higher affinity site for PacC, compared with afpP1. As a control, binding assays were performed with the GST protein alone (Fig. 3b). Here, no protein–DNA interaction was detected, confirming that the observed binding of the heterologously expressed GST::PacC to afpP1 and afpP2 is specific for PacC.

Further confirmation of the binding specificity to the afpP1 and afpP2 sites was obtained by competition analysis, using either site afpP1 or afpP2, respectively, as a specific competitor or using a synthetic double-stranded oligonucleotide from the coding region of the *afp* gene (OlA) as an non-specific competitor (Fig. 4). As shown in Fig. 4a, formation of the protein–afpP1 complex was inhibited by increasing amounts of unlabelled afpP1 as a specific competitor. The complex disappeared when a 50-fold excess of unlabelled afpP1 was added. In contrast, complex formation was not affected by the addition of a 100-fold excess of the non-specific competitor, OlA. Comparable results were obtained when competition experiments were performed with the afpP2 site. Here, excess cold double-stranded oligonucleotide afpP2 resulted in reduced protein–afpP2 complex formation, whereas the addition of the unlabelled competitor, OlA, had no such influence (Fig. 4b). Remarkably, the protein–afpP2 complex did not completely disappear until a specific competitor in 100-fold excess was used, again indicating that afpP2 under in vitro conditions is more strongly bound by the fusion protein than by afpP1. From these data, we conclude that sites afpP1 and afpP2 are specifically recognised by

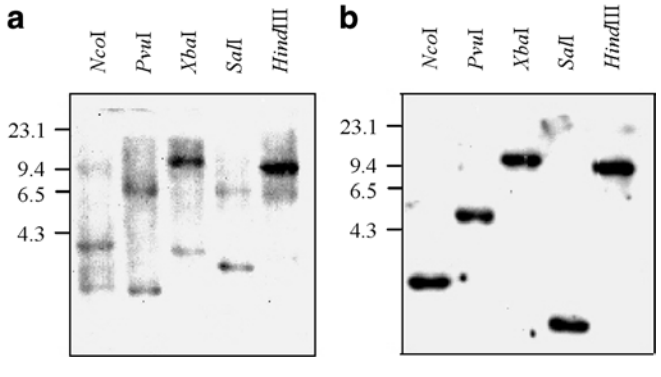

Fig. 1a, b. Southern blot analysis of *Aspergillus giganteus* with *pacC* and *afp* genes as probes. Genomic DNA isolated from *A. giganteus* was restricted with the enzymes indicated and then blotted and hybridised. **a** The *pacC* gene from *A. nidulans* was used as probe. Hybridisation was performed at 56 °C. **b** The *afp* gene from *A. giganteus* was used as a probe. Hybridisation was performed at 65 °C. The molecular weights of marker λ/*Hin*dIII are *indicated on the left* (kilobases)

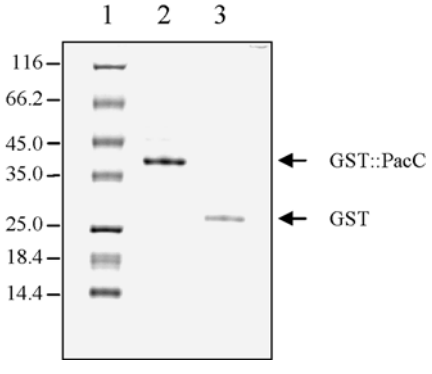

Fig. 2. Heterologous expression of glutathione *S*-transferase (GST)::PacC and GST protein in *E. coli* DH5α. Expression of the proteins was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside and allowed to proceed for 1 h. Purification by glutathione affinity chromatography followed. Proteins were separated in a 15% SDS-PAGE. *Lane 1* Molecular size marker, *lane 2* GST:PacC fusion protein (1 μg), *lane 3* GST protein (0.5 μg). The predicted molecular mass of GST:PacC is about 40 kDa

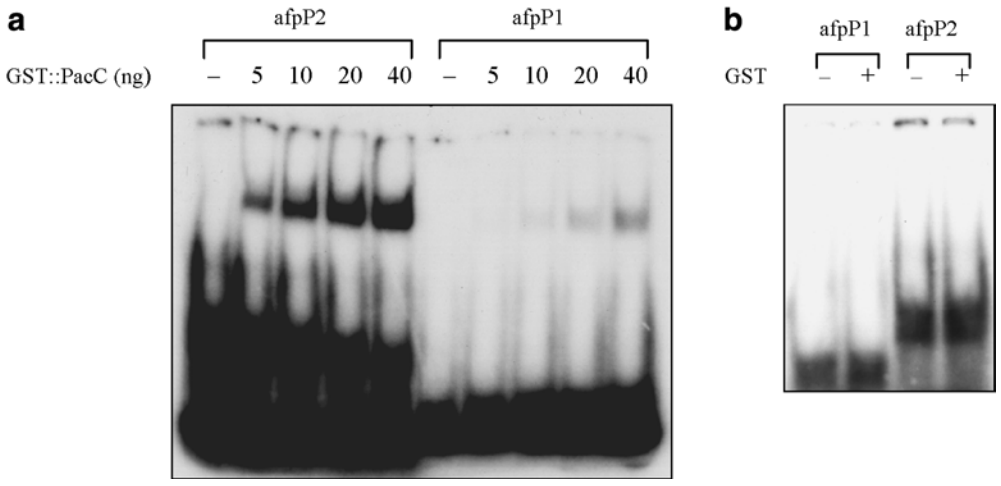

Fig. 3a, b. Gel retardation assay using the putative PacC binding sites, afpP1 and afpP2. Radiolabelled double-stranded oligonucleotides containing site afpP1 or afpP2 were used as target DNA (100 fmol per binding assay; for sequences of oligonucleotides see Table 1). **a** Analysis was performed with different amounts of purified GST::PacC(69-168), as indicated. **b** Reactions were performed with purified GST (40 ng)

the PacC fusion protein and that both sites differ in their relative affinities to PacC.

A third putative binding site within the *afp* promoter, afpP3, is located from –1107 to –1112. This site matches the PacC binding motif, except for the last base (5′-GCCAAC-3′). No binding of the fusion protein to the altered afpP3 site could be detected (data not shown), indicating that afpP3 is not recognised by PacC. Interestingly, a fourth site, named afpP4, which is located within the ORF at position +49 of the *afp* gene (3′-GAACCG-5′; for nucleotide sequence see GenBank accession number X60771) was found to interact with PacC (Fig. 5). A retardation complex was weakly formed with 5 ng or 10 ng GST::PacC, but was clearly established when 20 ng of the fusion protein were used (Fig. 5a). Competition experiments with up to 100-fold molar excess of either the cold afpP4 target or the non-specific competitor OlA were performed. Results showed that excess unlabelled OlA did not affect the retardation complex, whereas unlabelled afpP4 diluted the formation of the protein–DNA complex (Fig. 5c), demonstrating that the complex is specific to afpP4.

Effect of phosphate on *afp* expression

Liquid cultivation of *A. giganteus* resulted in pH changes of the cultivation medium, which occurred independently of the initial pH value. The medium was acidified in the first 24 h of cultivation, due to glucose utilisation, followed by an increase in pH (data not shown). Therefore, additional buffering was necessary in order to keep the initial pH values of the cultivation medium constant. However, when *A. giganteus* was cultivated in C1 medium with 50 mM sodium citrate (pH 4) and 50 mM Tris-HCl (pH 8), the buffering capacity of Tris was insufficient to fully prevent pH changes due to glucose utilisation (final pH 5.6). Suárez and Peñalva (1996) recommended physiological buffers, e.g. sodium phosphate buffer, in order to prevent pH changes resulting from metabolic activity. When *A. giganteus* was

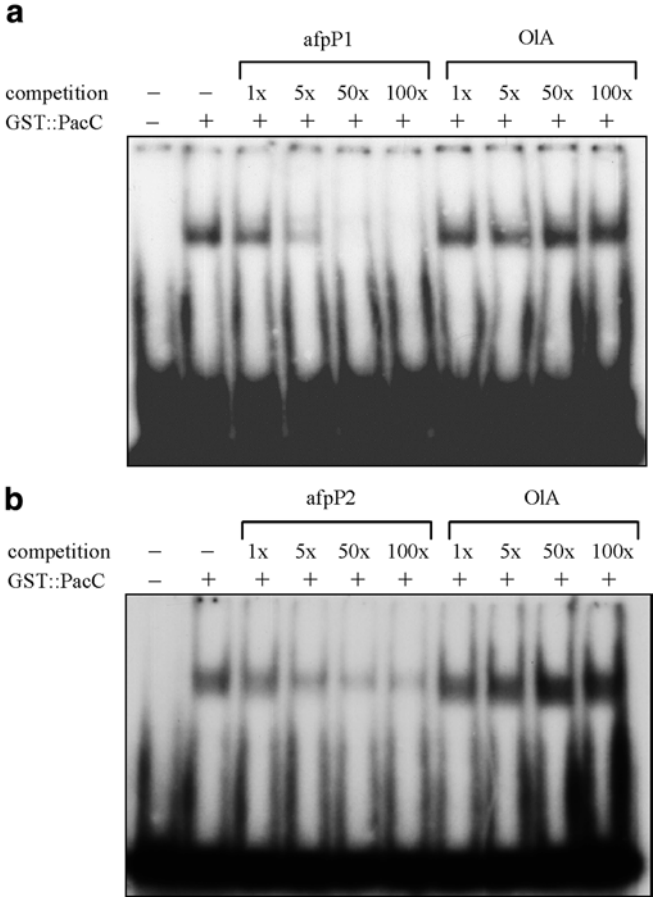

Fig. 4a, b. Competition experiments of PacC–DNA complexes with unlabelled oligonucleotides. Radiolabelled double-stranded oligonucleotides (100 fmol) containing site afpP1 or afpP2 were used as target DNA and incubated with purified GST::PacC(69-168). **a** Analysis was performed with a labelled afpP1 site and 40 ng of the PacC fusion protein in the absence (–) or presence (+) of the indicated molar excess of either unlabelled afpP1 or an oligonucleotide which did not contain a PacC binding site (*O1A*). **b** Analysis was performed with a labelled afpP2 site and 10 ng of the PacC fusion protein in the absence (–) or presence (+) of the indicated molar excess of either unlabelled afpP2 or O1A

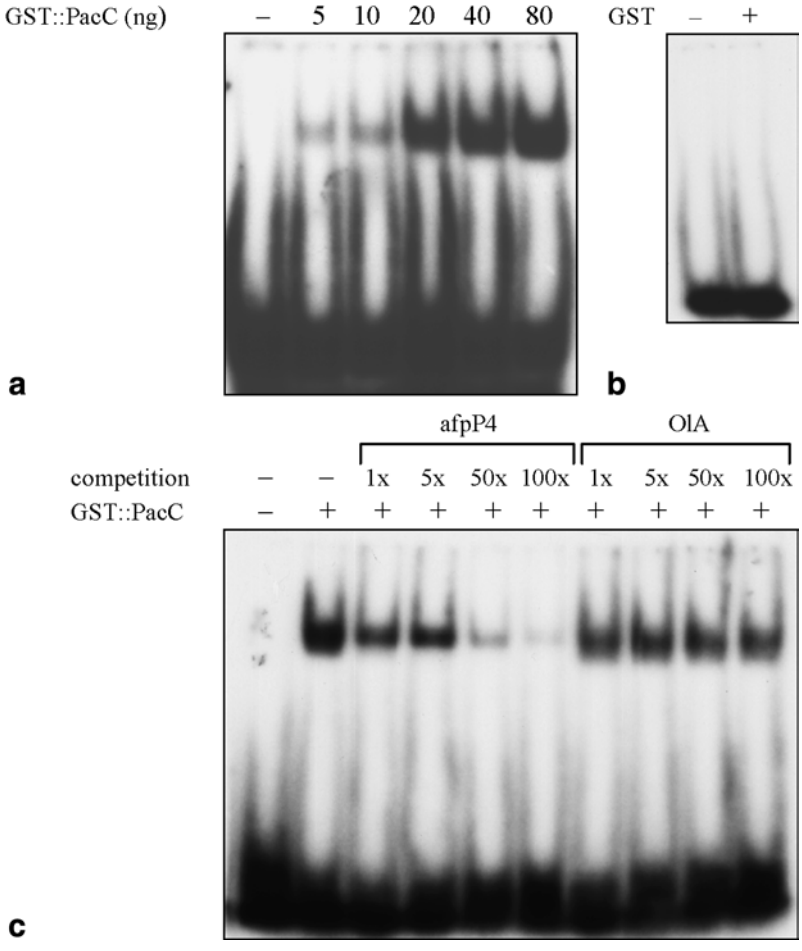

Fig. 5a–c. Binding of GST:PacC to the intragenic afpP4 site. The radiolabelled afpP4 site (100 fmol; Table 1) was used as target DNA and incubated with purified GST:PacC69-168 or GST. **a** Analysis was performed with different amounts of the PacC fusion protein, as indicated. **b** A control reaction was carried out with purified GST (40 ng). **c** Competition experiments were conducted with 40 ng of the PacC fusion protein and with either unlabelled afpP4 or unlabelled O1A, as described in Fig. 4

cultivated with an additional 100 mM sodium phosphate buffer (alkaline broth was adjusted to pH 8.0 and acidic broth to pH 5.6, respectively), the pH values were kept constant. However, the presence of phosphate resulted in a strong reduction of AFP in the culture broth. This decrease was independent of the initial pH value and, moreover, was observed for all basic proteins secreted by *A. giganteus* (Fig. 6). The biomass accumulated under both pH conditions and in the presence or absence of phosphate was the same (data not shown).

In order to analyse whether the impact of phosphate acts at the level of *afp* transcription, the *A. giganteus* wild-type strain was cultivated in liquid C1 medium at pH 8 with added 50 mM or 100 mM sodium phosphate; and Northern and protein analyses were performed. The amounts of *afp* mRNA and secreted AFP were determined after 24, 48 and 72 h of cultivation. As can be seen in Fig. 7a, *afp* promoter activity is actually modulated in response to external phosphate. In non-phosphate medium, transcription of the *afp* gene was highest after 48 h and decreased towards 72 h. In contrast, *afp* mRNA could barely be detected after 48 h and increased towards 72 h when *A. giganteus* was cultivated in C1 medium with additional 50 mM phosphate. However, in the presence of 100 mM phosphate, *afp* transcription was more strongly inhibited than with 50 mM

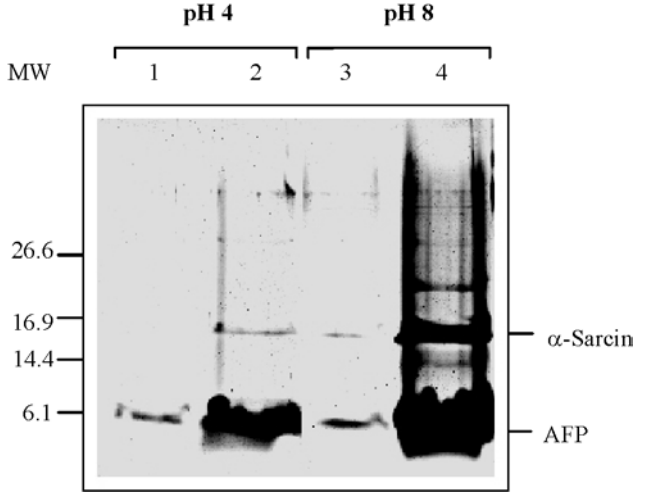

Fig. 6. Effect of phosphate on *afp* expression. *A. giganteus* was cultivated in C1 medium (see Materials and methods) at pH 4 or pH 8, in the presence or absence of 100 mM sodium phosphate. Basic proteins in the culture broth were isolated after 65 h of cultivation, using cationic exchange chromatography, and were separated on a 15% SDS-PAGE. *Lanes 1, 3 A. giganteus* was cultivated in the presence of 100 mM sodium phosphate, *lanes 2, 4 A. giganteus* was cultivated in the absence of sodium phosphate. The pH of the media was adjusted every 12 h with NaOH or HCl to maintain the initial pH value of the culture broth. In addition to the antifungal protein, *A. giganteus* secretes a second basic protein called α-sarcin (Olson and Goerner 1965)

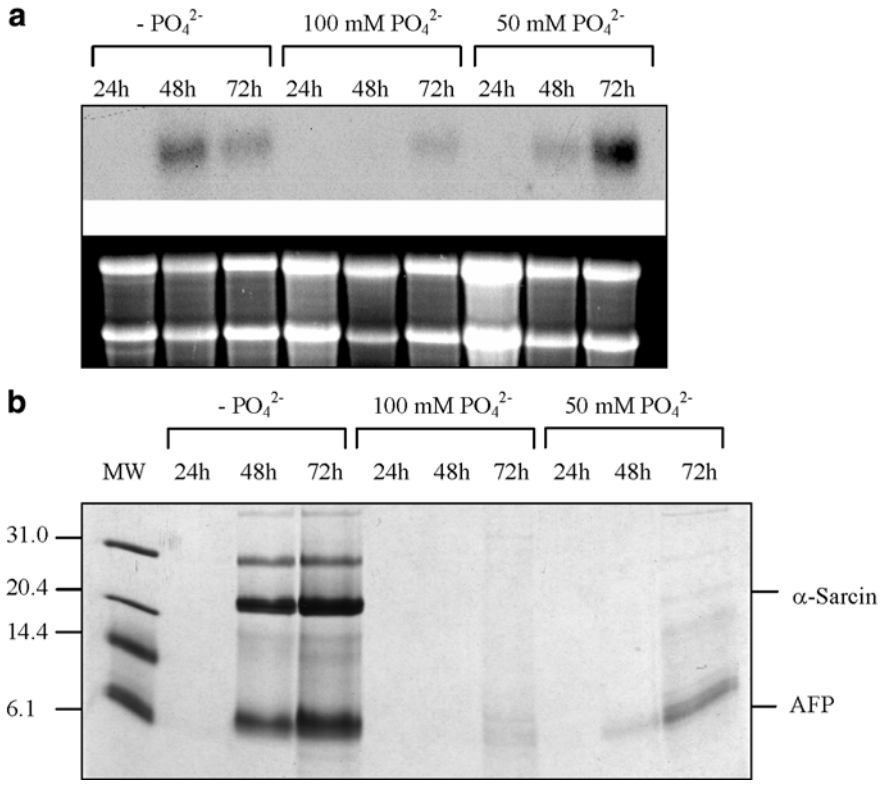

Fig. 7a, b. Effect of phosphate on *afp* mRNA and protein accumulation. *A. giganteus* was cultivated in C1 medium at pH 8 in the absence or presence of 50 mM or 100 mM sodium phosphate. The pH of the non-phosphate C1 medium was controlled every 12 h and adjusted with NaOH or HCl to the initial pH value of the culture broth. Total RNA and basic proteins in the culture broth were isolated at the times indicated. **a** Total RNA was hybridised with an *afp* probe. Ethylene bromide-stained RNA showing 28S and 18S rRNA was used as the loading control. **b** Basic proteins were separated on a 15% SDS-PAGE. *MW* Molecular size marker

phosphate. Here, *afp* mRNA could only be detected at 72 h. These results indicate that excess phosphate leads to a decelerated induction of *afp* transcription and therefore to a reduced AFP level in the culture broth (Fig. 7b).

Discussion

Transcription of the *afp* gene of *A. giganteus* is enhanced by alkaline pH and decreases under acidic conditions (Meyer et al. 2002). The presence of two putative PacC binding sites within the *afp* promoter (afpP1, afpP2) suggested that the *afp* gene might be under the control of the wide-domain transcriptional factor PacC found in various filamentous fungi. Suárez and Peñalva (1996) showed that the *P. chrysogenum* PacC transcriptional factor fully complements the *A. nidulans pacC* null mutation, indicating that homologous PacC proteins are functionally interchangeable in filamentous fungi. Therefore, we performed band shift assays with a GST::PacC fusion protein of *A. nidulans* in order to examine whether the putative afpP1 and afpP2 targets of *A. giganteus* are recognised by the heterologous PacC. As shown here, both targets were found to interact in vitro with the PacC fusion protein, strongly supporting the hypothesis that the observed ambient pH-dependent regulation of the *afp* gene is mediated through the transcriptional factor PacC. Interestingly, PacC shows a higher affinity to the afpP2 site, as compared with the afpP1 site. However, it has been demonstrated for the *ipnA* gene of the penicillin gene cluster of *A. nidulans* that the site mediating the highest affinity for PacC in vitro contributes only modestly to in vivo promoter activation (Espeso and Peñalva 1996). Therefore, it has to be proven in vivo which of the two binding sites within the *afp* promoter actually contributes to PacC-mediated pH regulation. Remarkably, the afpP4 site located within the ORF of the *afp* gene is recognised by the PacC fusion protein in vitro. Regulatory sites within coding sequences are widely recognised in higher eukaryotes, but only a few examples have been described for *Saccharomyces cerevisiae* genes (Wenz et al. 2001; see references therein). As far as we are aware, none have been reported for filamentous fungi. Thus, it would be interesting to test in future experiments whether site afpP4 displays an intragenic regulatory element.

In the second part of this paper, the influence of phosphate on *afp* expression was investigated. Sodium phosphate was used to adjust the pH and to prevent medium acidification during growth. However, the presence of phosphate was found to decrease the amount of secreted AFP considerably. There are few data regarding phosphate inhibition of gene expression in filamentous fungi. For example, Zhang et al. (1988) reported that excess phosphate inhibits cephalosporin production in *Cephalosporium acremonium*, by inhibiting the formation of the biosynthetic enzymes. However, this effect has not been studied in detail at the physiological or molecular levels. High concentrations of phosphate are generally found to be critical in regulating secondary metabolism in microorganisms. Phosphate is

usually exhausted during the exponential growth phase before synthesis of secondary metabolites is initiated. Some intermediates, especially those of antibiotics, are phosphorylated, but the end-products are not. Phosphatases, which cleave these intermediates, are inhibited by phosphate feed-back inhibition or repression. Hence, excess of phosphate results in the inhibition of enzymatic activities and therefore in the inhibition of secondary metabolism (Weinberg 1978). In addition, phosphorylation and dephosphorylation are crucial steps in turning signal transduction cascades on or off, regulating e.g. fungal development, differentiation and other processes (Kronstad et al. 1998; Lengeler et al. 2000). Moreover, changes in external phosphate concentration have been shown to be involved in the mycelial lipid composition of *Streptomyces* (Hanel et al. 1985). Consequently, the amount of phosphate in the cultivation medium has quite complex influences on physiological processes in pro- and eukaryotes. Regarding the phosphate regulation of *afp* expression in *A. giganteus*, we determined that an increase in phosphate concentration resulted in a delayed induction of *afp* transcription. It is interesting to note that the amount of all basic proteins in the culture broth was decreased by increasing concentrations of phosphate. Therefore, it can be speculated that expression of these proteins could be co-ordinately regulated by phosphate.

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