MECHANISM OF STERIGMATOCYSTIN BIOSYNTHESIS REGULATION BY PH IN *ASPERGILLUS NIDULANS*

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ABSTRACT

External pH constitutes one of the most important environmental factors that control growth, metabolism and differentiation in microorganisms, including fungi. We have analyzed the effect of external pH on sterigmatocystin biosynthesis in *Aspergillus nidulans*. It was observed in repeated experiments that alkaline pH, in opposition to acid pH, increased sterigmatocystin production and the transcript levels of *aflR*, the master gene that regulates expression of the sterigmatocystin cluster in *A. nidulans.*

It is known that pH effects in fungi operate mostly through the Pal/Pac signaling pathway, originally described in *Aspergillus nidulans.* Accordingly, we studied the role of this signaling pathway in ST biosynthesis. It was observed that *aflR* transcript levels were increased in the *"alkalinity mimicking"* mutant *pacC^c 14* and were minimal in the *"acidity mimicking"* mutant *palA1*. No sterigmatocystin was produced by *palA1* or *pacC-* mutants at neither acid or alkaline pH of incubation. Finally, *fluG* and *flbA,* genes known to regulate both conidiation and sterigmatocystin synthesis upstream in the regulatory cascade, were up-regulated at alkaline pH.

Key words: *Aspergillus nidulans*; pH; sterigmatocystin; *pacC*

INTRODUCTION

Mycotoxins are a group of toxic secondary metabolites produced by fungi, mainly from the genera *Aspergillus*, *Penicillium* and *Fusarium* (22). They are frequently found contaminating a wide variety of food and feed, representing a serious threat to human and animal health and causing severe economic losses (8). Aflatoxins (AF) are among the most studied mycotoxins. Aflatoxins are produced mainly by *Aspergillus flavus* and *A. parasiticus*. Aflatoxin B_1 is not only toxic but also teratogenic and mutagenic; being the most potent carcinogenic compound found in nature (15).

Studies using the model organism *A. nidulans*, which produces the AF precursor sterigmatocystin (ST), have led to a better understanding of the biosynthetic steps involved in AF production (4). The fact that the mycotoxin gene clusters from *A. nidulans* and *A. parasiticus* have high homology (25) has also been helpful in the study of AF/ST synthesis and regulation.

Among the most important physiological determinants that regulate AF/ST production in *Aspergilli* are the carbon and nitrogen sources, and the pH of the medium. Simple carbohydrates, such as glucose and sucrose, favor AF biosynthesis in *A. parasiticus* and ST biosynthesis in *A.*

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nidulans (19). It is also known that while ammonium favors AF biosynthesis in *A. parasiticus*, it inhibits ST production in *A. nidulans* (13). However, studies on the effect of pH on mycotoxin synthesis have often produced complex and at times contradictory results; *e.g.* data in the literature have reported either a positive (9, 11, 18) or no effect (5) of acid pH in aflatoxin biosynthesis by *A. flavus* and/or *A. parasiticus*, but no information exists on the mechanism involved (for a discussion see (7)). It has also been reported that ST biosynthesis was stimulated by acid pH in *A. nidulans* (18).

Regulation of fungal metabolism by pH is mostly effected through the Pal/Pac signaling pathway, described for the first time in *A. nidulans* (6). PacC is a transcription factor activated at alkaline pH by the products of six *pal* genes that sense external pH and transduces the activating signal. PacC activation may lead to repression of genes expressed preferentially under acidic growth conditions, and up-regulation of genes transcribed preferentially under alkaline growth conditions, including *pacC* itself (23). Loss of function mutations in any of the *pal* genes or *pacC*, lead to an *"acidity mimicking"* phenotype; i.e., display a pattern of gene expression similar to that of the *wild-type* strains grown under acidic conditions, independently of ambient pH. In contrast, *pacC* constitutive mutants show an *"alkalinity mimicking"* phenotype, displaying a gene expression pattern similar to that of the *wild-type* strains grown under alkaline conditions, irrespective of ambient pH (see (20, 21) for helpful reviews). A *pacC* null mutant from *A. nidulans* displays an *"acidity mimicking"* phenotype, and exhibits poor growth and almost null conidiation, leading to the hypothesis that PacC might also be involved in development (23). Interestingly, Guzman-de-Peña and Ruiz-Herrera (12) found a correlation between AF biosynthesis and sporulation in *A. parasiticus*; and between both sexual and asexual sporulation and ST production in *A. nidulans* (13), thus linking mycotoxin synthesis and differentiation in *Aspergilli*. Further on, several reports that proposed a model G-protein/cAMP/PKA signaling pathway that connected growth, conidiation and ST production in *A. nidulans* have been published (3, 14).

Taking into consideration all these data, we proceeded to analyze the regulation of ST production by ambient pH in *A. nidulans*, and whether the Pal/Pac signaling pathway was or not involved in this process.

MATERIALS AND METHODS

Strains and Growth Media and Conditions

The strains used for these studies were *Aspergillus nidulans* FGSC26 (*biA1, veA1*), *A. nidulans* MAD002 (*biA1, veA1*), *A. nidulans* MAD134 (*biA1, vea1, palA1, wA3*), *A. nidulans* MAD135 [*biA1,veA1, pacC^c14 (5-492)*] and MAD812 (*pantoB100, veA1, pacC^{+/-}209*). *A. nidulans* FGSC26 and *A. nidulans* MAD002 are both good ST producers. *A. nidulans* MAD134 is a mutant strain unable to process the inactive form of PacC, and therefore mimicks growth in acidic conditions regardless of the pH of the medium (*"acidity mimicking"* phenotype). On the other hand, the *A. nidulans* mutant strain MAD135 produces only a pH-independent activated form of PacC, mimicking growth in alkaline conditions, also regardless of the pH of the medium (*"alkalinity mimicking"* phenotype); and strain MAD812 has a stringent loss of function mutation. All MAD strains were kindly donated by Miguel A. Peñalva (Centro de Investigaciones Biológicas, Madrid, Spain).

All strains were kept at 4 °C as silica stocks. For conidia production, *A. nidulans* strains FGSC26, MAD002, MAD135, and MAD812 aliquots from the stocks were transferred to solid Kafer medium (16) with sodium nitrate as the sole nitrogen source and supplemented with 5% sucrose as carbon source, and grown for 5 days at 37 °C. *A. nidulans* MAD134 was grown at 28 °C on solid Kafer medium with ammonium tartrate and 5% sucrose (acidic growth conditions) in order to avoid extragenic suppressors of the *palA* mutation (M. A. Peñalva, personal communication).

Spore suspensions

Conidia were harvested by adding 10 ml of a Triton X-100 solution (0.01%) to each Petri dish, counted with a hemocytometer and adjusted to the desired inoculum concentration with sterile distilled water. Before inoculation conidia suspensions were kept overnight at 4 °C to ensure an even germination.

Growth conditions

Conidia were inoculated into 100 x 15 mm Petri dishes containing 50 ml of liquid ($1x10^7$) or solid ($1x10^6$) Kafer medium (see Results) supplemented with 5% sucrose and 70 mM sodium nitrate as carbon and nitrogen sources respectively, and incubated under static conditions at 37 °C for variable periods of time. In some experiments, pH of the media was adjusted with 100 mM citrate buffer.

At the end of the incubation period, in liquid cultures the medium was separated from the mycelium by filtration. Mycelia were transferred to a Petri dish, washed with 10 ml of distilled water containing 0.01% Triton X-100, and gently shaken to release the spores. This procedure was repeated twice. Mycelia were then dried at 75 °C for 24 h, stored at room temperature in desiccators with silica gel as drying agent for another 24 h, and weighed. The residual growth medium was processed immediately or stored at 4 °C until used. When mycelia were used for RNA extraction, they were collected by vacuum filtration, frozen with liquid nitrogen and stored at -70 °C until used. When solid medium was used, after spore recovery ST was extracted as described below, agar was melted in a microwave oven, mycelia were recovered with the use of tweezers, washed with hot water, dried and weighed as above.

ST quantitation

ST was extracted from the mixture of culture medium, dry mycelium (see above) and conidia, with 50 ml acetone for 30 min, followed by 50 ml chloroform by further 30 min (17). The organic phase was separated, filtered through anhydrous sodium sulfate and evaporated in a fume hood in a water boiling bath. The residue was resuspended in 500 µL HPLC grade methanol and filtered through C-18 columns (Alltech).

Analysis and quantitation were performed by HPLC as previously described (13). The detection limit was 3 ng ST in 20 µl samples.

RT-PCR analysis of the expression of genes encoding proteins involved in growth and ST synthesis

Total RNA extraction was performed using TriZOL (Invitrogen). RNA purification was achieved using RNeasy mini Kit (Qiagen). RNA integrity was confirmed by electrophoresis in a 1.5% agarose gel in TAE 1X. The expression levels of the genes *fluG, flbA, laeA, pacC* and *aflR,* were evaluated semi quantitatively by RT-PCR. First-strand cDNA was obtained using 1 µg of total RNA, adding 1 µl Oligo dT (Invitrogen), 1 µl dNTP mix (10 µM each) and enough RNase-free water to adjust to 12 µl, heating the mix at 65 °C for 15 min and transferring it immediately to ice. 4 µl of 5x RT Buffer, 2 µl of DTT 0.1 M and 1 µl RNase OUT (Invitrogen) were added to each reaction, gently mixed and incubated at 42 °C for 2 min. After that, 1 µl Superscript II Reverse Transcriptase (Invitrogen) was added to each reaction for a final volume of 20 µl. RT conditions were: an initial incubation at 42 °C for 50 min, followed by 15 min at 70 °C. The cDNA derived from 0.1 µg of total RNA was used as a template. PCR conditions were as follows: After an initial incubation at 94 °C for 3 min, 28 cycles of 94 °C for 45 s, 54 °C for 1 min and 72 °C for 1 min were performed, followed by a final incubation at 72 °C for 7 min.

The PCR primers for each gene were as follows: 5'-ACCCTAATGTTTATTTGGAT-3' (forward) and 5'-TGGATAGGTCTGGTATAAGG-3' (reverse) for *fluG;* 5'-, TCCCTCAAATTCTCTCAATCGAACCGG-3' (forward) and 5'-GTAGAATGACAGGTTTTCTTCGCAGA-3' (reverse) for *flbA;* 5'-GGTGACGATTTGTATAGTCC-3' (forward) and 5'-CTCTTCATGAAACTGGTTTC-3' (reverse) for *laeA;* 5'-GACTGACGGTATGACTTCTG-3' (forward) and 5'-GTTGGCAATGTAGTTACGTA-3'

(reverse) for *pacC*; 5'-GCCATCCTGTCTCCGAATAC-3' (forward) and 5'-CGAACCTCTACGACTGTCTTG-3' (reverse) for *aflR*; and 5'-CCAAGGCCAACCGCGAGAAGATGAC-3 (forward) and 5'-AGGGTACATGGTGGTGCCGCCAGAC-3' (reverse) for γ-actin (control gene). Transcription levels were normalized by comparing the UV absorption intensity of the bands to that of γ-actin using ImageJ software (http://rsb.info.nih.gov/ij/).

Statistical analyses

Each experiment was repeated three to five times, and values presented in this paper are means of these replicates. Differences between means were evaluated by Tukey's Test (p=0.05) using SAS (version 6.12; SAS Institute, Cary, NC, USA). Coefficients of determination (r^2) and regression data are included in the corresponding results.

RESULTS

Relationship between initial pH, growth and ST production

A. nidulans MAD002 (*wild-type* strain) was grown in Kafer medium at three different initial pH values for 72 h. Growth, conidiation and ST synthesis were evaluated approximately every 12 h. When initial pH was 4.61, it was observed that only basal levels of ST were produced until 58 hours of incubation, ST synthesis being activated after this time (Table 1). Activation of ST synthesis occurred earlier when the initial pH value was raised (58 h at initial pH= 5.22, and 46 h at pH= 6.90) (Table 1). As it may be seen in this table, time of activation of ST synthesis occurred when pH reached a value above 7.5. The amount of ST produced and mycelial growth at 72 h of incubation were also linearly increased as initial pH value of the medium increased (r^2 = 0.977 and r^2 = 0.993, respectively).

**Table 1.** Rate of growth and ST synthesis in *A. nidulans* MAD002 (*wild-type*).

Initial pH	Incubation Time (h)	Final pH	Growth (mg DW)	Total Sterigmatocystin (ng)
4.61	22	5.4	45.9 A	0
	34	6.2	62.8 A	35 A
	46	6.8	104.8 A	38 A
	58	7.2	211.2	43 A
	72	7.7	483.9	1,972
5.22	11	5.4	13.9 A	65 A
	23	5.9	26.5 A	83 A
	34	6.3	49.4 A	38 A
	46	6.9	93.1 A	201
	58	8.3	190.7	2,247
	72	8.4	525.3	12,779
6.90	10	6.9	23.2 A	275 B
	22	7.0	38.9 A	26 B
	34	7.4	80.3 A	49 B
	46	7.6	232.5	5,896 A
	58	8.8	364.2	3,699 A
	72	9.0	605.0	28,727

Cultures were obtained in unbuffered liquid Kafer medium incubated under static conditions, and growth and ST formation were measured as described in Materials and methods. Statistical analysis of samples (n=5) proceeded by the Tukey test. Values in the same experimental block (same initial pH) marked with the same letter in the same column are statistically similar (p= 0.05).

Transcription of regulatory genes through time of incubation

The previous experiment suggested a correlation between pH and ST production. Taking into consideration that both phenomena are regulated in *Aspergilli* by known pathways (6, 25), we decided to investigate the correlation existing between the drift in external pH during incubation, and the expression of the corresponding regulatory genes. *A. nidulans* MAD002 was incubated in Kafer medium pH = 6.60 for 83 h. Mycelia were collected at different intervals, and levels of transcripts of the regulatory gene *aflR* was evaluated (Fig. 1). *aflR* expression levels increased through time linearly ($r^2 = 0.922$) as pH of the medium was increased, raising the hypothesis that alkaline pH promotes ST biosynthesis.
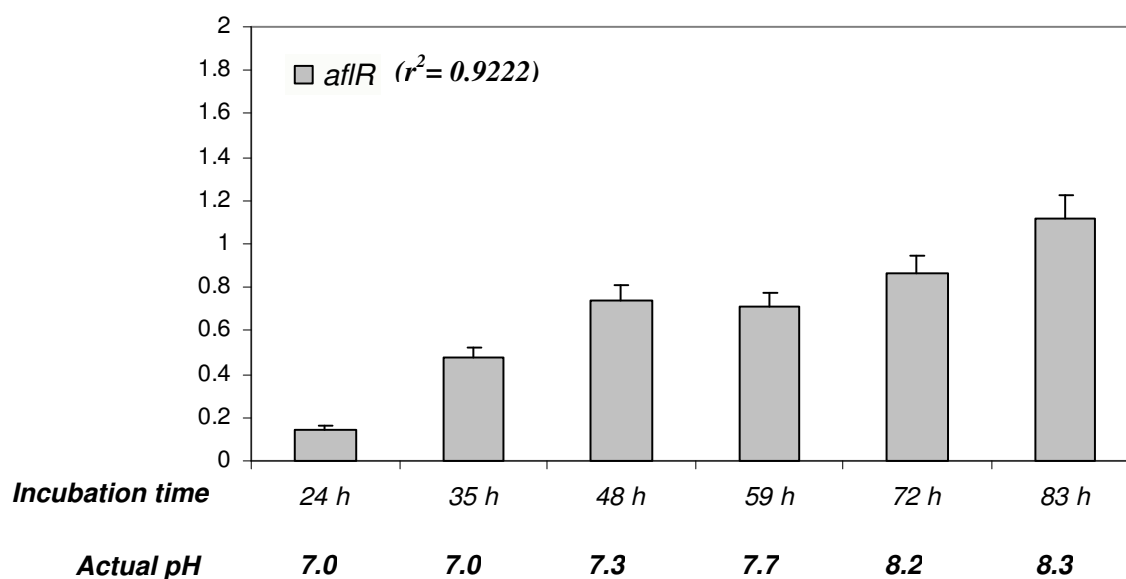
Figure 1. Effect of pH on the transcription of the regulatory gene *aflR* in the *wild-type* strain MAD002 through incubation time. Cultures were incubated in large Petri dishes containing liquid pH= 6.5 unbuffered Kafer medium under static conditions at 37 °C. At intervals, sets of dishes were recovered, RNA was extracted and transcription levels were measured by semi-quantitative RT-PCR. γ-actin was used as an endogenous reference. The amount of each mRNA was normalized to the amount of γ-actin mRNA in each sample. Data are means ± SD (n= 3). Time of incubation and actual pH of the different samples are indicated.

Determination of the optimum pH for growth and ST production in the *wild-type* and mutant strains affected in the Pal/Pac pathway

A. nidulans mutants affected in the Pal/Pac pathway were included in these experiments because of the general observation on the role of this pathway in the regulation of different phenomena by pH in *Aspergilli*. All strains were grown in Kafer (liquid or solid) medium containing 100 mM citrate buffer at different pH values. It was observed that mycelial growth of *wild-type* strains (FGSC26 and MAD002) was higher at alkaline pH. ST levels were about 30-70-fold higher at alkaline pH as compared to acidic or neutral conditions in both strains. In contrast to these results, the *A. nidulans* "acidity mimicking" mutant MAD134 (*palA1*) grew poorly at these three different pH values compared to the *wild-type* strains grown at similar pH values and time.

Interestingly, this mutant was unable to produce ST at any of the pH values tested. On the other hand, the MAD135 (*pacC^{c}14*) mutant produced ST regardless of the pH of the medium. Nevertheless, as previously reported (18), its levels of ST production were well below those from the *wild-type* strains. These results are shown in Table 2. Finally, contrary to the behavior of strain MAD135, a *pacC* negative mutant (MAD812) was unable to synthesize ST at neither acid nor alkaline pH (Table 3).

Table 2. Growth and ST synthesis in *A. nidulans* strains grown in buffered medium.

Strain	Initial pH	Final pH	Growth[a] (mg mycelium)	Total Sterigmatocystin (ng)
FGSC26 *(wt)*	3.0	3.2	175 A	7,683 A
	6.5	6.4	186 A	7,477 A
	8.0	8.7	406	508,799
MAD002 *(wt)*	3.3	4.1	515 A	4,249 A
	6.2	7.4	356	2,158 A
	8.0	8.6	562 A	141,097
MAD134 (*palA1*)	3.3	3.5	70.4	0
	6.1	6.1	57.3 A	0
	7.9	7.6	35.6 A	0
MAD135 (*pacC^{c}14*)	3.3	3.5	130.5 A	463 A
	6.1	6.2	76.6 A	229 A
	7.9	8.2	298.4	325 A

Cultures were obtained in buffered (100 mM citrate) liquid Kafer medium incubated under static conditions, and growth and ST formation were measured as described in Materials and methods. Statistical analysis of samples (n=5) proceeded by the Tukey test (p=0.05). Statistical significance of the differences in the same experimental block (same strain and medium) were treated as described for Table 1.

Table 3. Growth and ST synthesis of *A. nidulans* MAD002 and MAD812 strains grown in solid buffered medium.

Strain	Initial pH	Final pH	Growth (mg[1])	Total ST
MAD002 *(wt)*	3.1	4.4	726.9 A	29,076
	7.8	8.9	747.0 A	2'530,700
MAD812 *(pacC$^{+/}$209)*	3.1	4.0	707.3	0
	7.8	8.3	260.6	0

Cultures were obtained in buffered (100 mM citrate) solid Kafer medium, and growth and ST formation were measured as described in Materials and methods. Statistical analysis of samples (n=5) proceeded by the Tukey test (p=0.05). Statistical significance of the differences in the same experimental block (same strain and medium) were treated as described for Table 1.

Determination of the expression of regulatory genes in *wild-type* and mutants affected in the Pal/Pac pathway

In order to analyze the effect of pH on the expression of the regulatory genes (*pacC and aflR*) in *wild-type* and Pal/Pac affected mutants, we used buffered medium of different pH values as described above. It was observed that expression levels of *pacC* and *aflR* in strain FGSC26 increased linearly as pH of the medium increased ($r^2 = 0.981$ and $r^2 = 0.997$, respectively). Expression levels of *pacC* in strain MAD134 (*palA1*) remained low and constant at all pH values. *aflR*

levels were also basal, in agreement with the absence of ST production at any pH value. Transcript levels from strain MAD135 (*pacC^c14*) contrasted with those of strain MAD134 (*palA1*). Levels of *pacC* transcript in *pacC^c14* were twice higher than those from *palA1* mutant, and its *aflR* transcript levels were high regardless of the pH of the medium, correlating with ST production in this strain (these results are shown in Fig. 2).
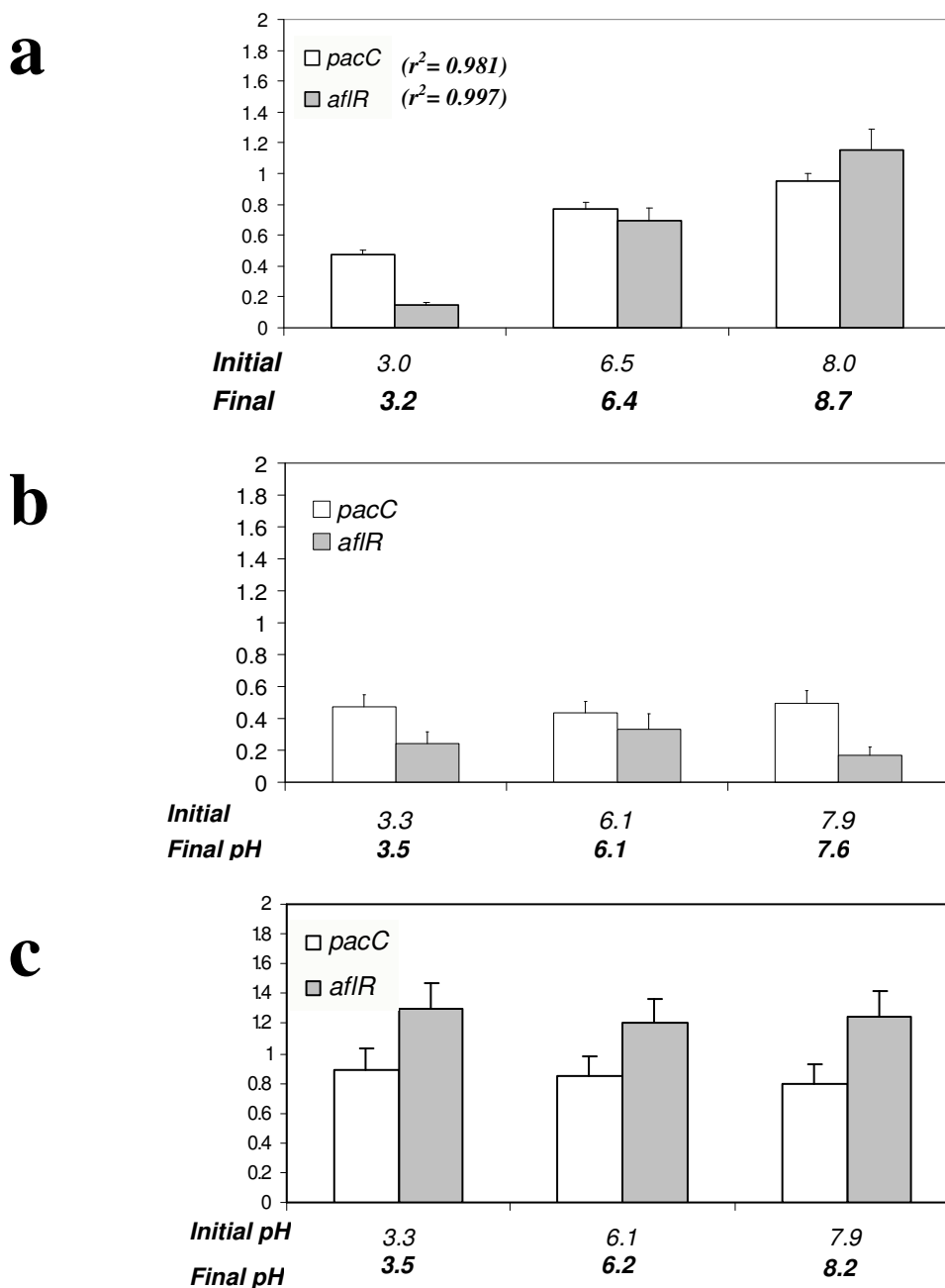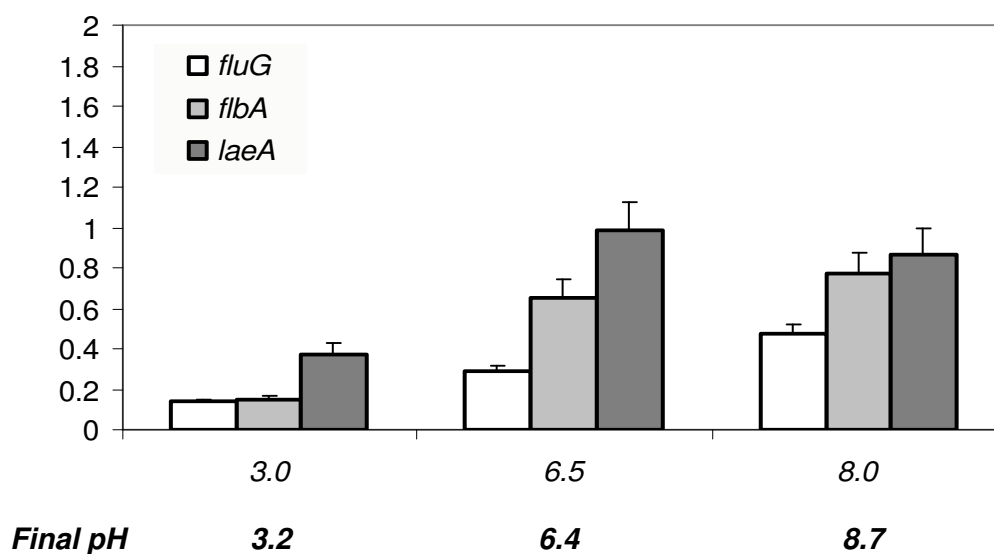

Figure 2. Effect of pH on the transcription of *pacC* and *aflR* genes in *wild-type* and mutant strains of *A. nidulans* grown at three different pH values. Culture conditions proceeded as described for Fig. 1, but medium was added of 100 mM citrate buffer at the indicated pH values, and incubation lasted for 72 h. Gene transcription levels were analyzed as described for Fig. 1. Data are means ± SD (n= 3). a) *wild-type* strain FGSC26; b) "a*cidity mimicking*" mutant MAD134 (*palA1*); c) "*alkalinity mimicking*" mutant MAD135 (*pacC^c14*). Initial and final pH values of the different samples are indicated.

Effect of pH on the regulatory mechanism of ST synthesis

We further proceeded to determine the probable level at which external pH affects ST production. It has been suggested that *fluG* and *flbA* encoded proteins have a positive role in ST production (17). In this model, *laeA* transcript product also activates ST formation (3). Transcript levels of these genes were evaluated in FGSC26 and MAD134 (*palA1*) strains grown at three different pH values in citrate buffered medium for 72 h (Fig. 3). It was noted that *fluG* (r^2= 0.998 in *wild-type* and $r^{2=}$ 0.971 in MAD134 strains) and *flbA* (r^2= 0.889 and $r^{2=}$ 0.976, respectively) were preferentially expressed at alkaline pH in both strains. On the other hand transcript levels of *laeA* were higher at neutral and alkaline pH in the *wild-type* strain (Fig. 3), but in the MAD134 (*palA1*) strain an opposite trend in expression was observed, decreasing with an increase in pH (data not shown).

Figure 3. Effect of pH on the transcription of *fluG, flbA* and *laeA* genes in the *wild-type* strain FGSC26 grown at three different pH values. Culture conditions proceeded as described for Fig. 2, and transcription levels were analyzed as described for Fig. 1. Data are means ± SD (n= 3).

DISCUSSION

The formation of natural products resulting from the secondary metabolism of plants and microorganisms is subjected to different environmental cues, such as temperature, humidity, light, pH, nutrient source, etc. Aflatoxins (AF), probably the most important and studied mycotoxins, do not escape to this rule. Accordingly, it has been described that temperature and nitrogen source affect AF production in *A. parasiticus* and ST synthesis in *A. nidulans*, but in different ways (11). It was demonstrated that nitrate as the sole nitrogen source promoted ST synthesis in *A. nidulans*, while ammonium promoted AF production in *A. parasiticus*. It was also observed that AF production by *A. parasiticus* was best at 27 °C, while ST was maximally produced by *A. nidulans* when incubated at 37 °C (11).

pH is also an important factor in the production of aflatoxins. Acid pH has been described to stimulate AF synthesis in *A. parasiticus* (10, 18). According to the results described in this paper, we may conclude that pH of the medium does have an effect on ST synthesis in *A. nidulans*, with alkaline pH promoting mycotoxin production. These data are in contradiction with a previous communication reporting stimulation of ST biosynthesis at acid pH (18). The

reasons for this discrepancy are difficult to pin point. Possibly, conditions of growth, time of incubation and the determination only of ST present in the mycelium (contrary to our protocols) may be partially responsible for this behavior.

Among our data that support the conclusion that it is alkaline pH the one that promotes ST production, we may cite the following: 1) the repeated observation that higher levels of ST are produced at alkaline pH in the *wild-type* strains; 2) the observation that expression levels of *aflR* (the transcriptional regulator of the gene cluster encoding proteins involved in ST synthesis (24)) in the *wild-type* strain of *A. nidulans* were high under alkaline conditions, and very low at acidic pH; 3) the observation that an *"acidity mimicking" palA* mutant did not produce ST at any pH value; 4) the observation that a PacC constitutive *"alkalinity mimicking"* mutant produced similar levels of ST at all the pH values tested, although as reported (18), at lower levels than the *wild-type* strain; 5) the fact that a *pacC* negative mutant did not form ST at any pH value. These last results also meet the criteria for recognizing gene regulation by pH through the Pal/Pac signaling pathway (1); and finally 6) it is also important to point out that the regulation of other genes involved in the common control of ST biosynthesis (*laeA, fluG* and *flbA*) are in agreement with a positive regulation by alkaline pH.

It is accepted that LaeA is a global regulator of secondary metabolism in *A. nidulans* (2). Levels of transcription of *laeA* gene were higher at neutral to alkaline growth conditions in the *wild-type* strain.

fluG and *flbA* are known to be involved in a G-protein/cAMP/PKA signaling pathway that connects ST synthesis and development in *A. nidulans* (3, 14). The observation that transcription of both genes was stimulated by alkaline pH of the medium agrees with the general observation that sterigmatocystin production is stimulated at alkaline pH.

In summary, based on our results, we may suggest that alkaline pH regulates the signaling pathway that controls ST synthesis in *A. nidulans* at the level of *aflR* by the Pal/Pac signaling pathway.

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