pH Regulation of Sterigmatocystin and Aflatoxin Biosynthesis in *Aspergillus* spp.

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ABSTRACT

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Aflatoxin (AF) and sterigmatocystin (ST) are toxic secondary metabolites produced by the same biochemical pathway found in several *Aspergillus* spp. The expression of the homologous ST/AF structural gene, *stcU* in *A. nidulans* and *ver-1* in *A. parasiticus*, was affected by external pH of liquid growth media. Both *stcU* and *ver-1* mRNAs appeared earlier and were expressed at higher levels in cultures grown in acidic media (pH 4 to 6) versus neutral (pH 7) and alkali (pH 8) media. Transcript levels correlated with ST/AF production. Visual and spectrophotometric

analysis of production of the orange ST/AF intermediate, norsolorinic acid (NOR), also paralleled transcript patterns and indicated that the pH effects were operative in different nitrogen- and carbon-based solid growth media. Five- to 10-fold increases in ST, AF, and NOR were measured in cultures grown in pH 4 or 5 versus pH 8 media. An *A. nidulans* strain carrying a mutation resulting in constitutive activity of the pH regulatory factor, PacC, produced 10-fold less ST than did wild type. The *stcU* transcript was not noticeably affected by pH in this strain. The results suggest a general pattern of pH regulation of ST/AF biosynthesis that may override previously noted carbon and nitrogen effects.

Additional keywords: mycotoxin, secondary metabolism.

Aflatoxins (AFs) are toxic and carcinogenic secondary metabolites produced by *Aspergillus flavus*, *A. nomius*, and *A. parasiticus* (18). The AF precursor, sterigmatocystin (ST), has toxic and carcinogenic effects similar to AF B₁ (4,22) and is the end metabolite produced by *A. nidulans* and other *Aspergillus*, *Bipolaris*, and *Chaetomium* spp. (3,7). Both ST and AFs are products of a conserved ST/AF gene cluster (6,27,30) whose biosynthetic genes are regulated by the same ST/AF-specific transcription factor, AflR (29,31). These mycotoxins are highly undesirable in food and feed products and, accordingly, a great deal of effort has been directed towards understanding what nutritional and environmental factors regulate their production. For example, certain carbon and nitrogen sources have been shown to stimulate (e.g., glucose) or inhibit (e.g., nitrate) AF biosynthesis in vitro in *A. parasiticus* and *A. flavus*. Glucose stimulation of AF (1,28,32) is in contrast to glucose repression of other secondary metabolites (10,12). However, a recent study by Luchese and Harrigan (19) hints that glucose itself is not an AF-limiting factor for *A. parasiticus*, provided the fungus is grown in acidic environments (approximate pH of 4 to 5). pH is also mentioned in reports of nitrogen effects on AF production; generally, *Aspergillus* strains acidify ammonium-based media ('AF inducing'), whereas nitrate-based media ('AF repressing') remain pH neutral (5,14). Cotty (8) demonstrated that buffering ammonium media to neutral pH and nitrate media to acidic pH reversed the inducing and repressing effects of nitrogen source on *A. flavus* AF production on solid medium. These results suggest that pH could have a regulatory role in AF production.

Less is known regarding pH, nitrogen, and carbon effects on ST production in *A. nidulans*, but the relationship of these three factors has been clarified in studies of another *A. nidulans* secondary metabolite, penicillin. Ammonium-based medium was originally observed to decrease penicillin output (23), and glucose repression of penicillin synthesis suggested regulation was through CreA, a negative regulator of carbon metabolism (12). Yet *creA*, *creB*, and *creC* loss-of-function mutants only slightly derepressed penicillin gene transcription (11,12), which suggested that a second, *creA*-independent mechanism might be operating on penicillin biosynthesis. It has since been shown that PacC, a transcription factor involved in activation of alkali-expressed genes, is required to activate at least one gene, *ipnA*, in the penicillin pathway and is itself an alkaline-expressed gene (13,26). These studies presented genetic evidence that secondary metabolite gene expression in *A. nidulans* can be controlled through external pH.

Based on the studies outlined above and the observation that both ST and AF pathway genes are regulated by the same transcription factor (29,31), we hypothesized that pH, regardless of nitrogen or carbon source, would similarly affect ST/AF biosynthesis. In this paper, we demonstrate that ST/AF production and *stcU* and *ver-1* transcript levels (homologous ST/AF pathway genes in *A. nidulans* and *A. parasiticus*, respectively) decrease with increasing pH. This effect was observed using different buffers in ammonium-, nitrate-, and sucrose-based media. Additionally, an *A. nidulans pacC* mutant strain, which constitutively produces active PacC protein and, therefore, mimics growth in alkali conditions (13,26), produces 10-fold less ST than does wild type. These results indicate that pH plays a role in ST/AF regulation, perhaps through PacC or AflR mediation.

MATERIALS AND METHODS

Fungal isolates and growth conditions. All fungi were maintained as silica (room temperature) or glycerol (−80°C) stocks. Isolates used for these studies were *A. nidulans* FGSC26 (*veA1*,

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biA1), *A. nidulans* DOC (*pabaA1*; *inoB2*; *pacC*²⁰², courtesy of H. Arst, Jr.) and *A. parasiticus* ATCC 98106 (*wh*, *nor-1*). *A. parasiticus* ATCC 98106 produces both AF and norsolorinic acid (NOR), an orange-colored intermediate of the ST/AF pathway, which can conveniently be used as a visual tag to assess AF production (15). *A. nidulans* FGSC26 is a wild-type producer of ST. The *pacC*²⁰² mutation results in constitutive expression of the active PacC protein, a transcription factor needed to induce alkali-expressed genes and proposed to inhibit acid-expressed genes (26).

For conidial production, *A. nidulans* strains were grown at 37°C on *Aspergillus* minimal medium supplemented with appropriate nutrients (9), and *A. parasiticus* was grown at 30°C on potato dextrose agar. Conidia were harvested in Tween 80 (0.1%) water and adjusted accordingly to the desired inoculum concentration. Unless stated otherwise, all reagents were purchased from Sigma Chemical Co. (St. Louis) or Fisher Scientific Co. (Pittsburgh).

pH assay. Cultures of *A. nidulans* used for RNA and ST extraction were grown in liquid complete nitrate medium (CNM, using sodium nitrate as nitrogen source) and liquid complete ammonium medium (CAM, using ammonium tartrate as nitrogen source) as modified after Cove (9). Unbuffered CNM (250 ml adjusted initially to pH 6.5 with 10 N NaOH) was inoculated with 1 × 10⁶ conidia/ml and grown for 12 h at 37°C in a rotary shaker at 300 rpm. The mycelia were then harvested onto Miracloth (Calbiochem, La Jolla, CA), washed with CAM, divided into equal portions by weight, and transferred to CAM buffered from pH 4 through 8 or control unbuffered CAM. CAM was buffered with 100 mM succinic acid (approximate pH of 4 to 6), MES (2-[4-morpholino]-ethane sulfonic acid, approximate pH of 5 and 7), or 100 mM sodium phosphate (approximate pH of 7 to 8) and adjusted with 10 N NaOH. Mycelia were collected by vacuum-suction filtration for RNA and ST extraction 24 and 48 h after transfer. Cultures were also grown on oatmeal porridge for ST measurements as previously described (17). Oatmeal porridge was unbuffered, buffered to pH 4 with 100 mM succinic acid, or buffered to pH 7 with 100 mM sodium phosphate.

For *A. parasiticus*, 250 ml of unbuffered peptone mineral salts (PMS) or CNM was inoculated with 1 × 10⁶ conidia/ml and grown in a rotary shaker at 30°C for 36 h at 300 rpm (24). The mycelia were then harvested, washed, and transferred into CAM as in the *A. nidulans* experiment. CAM was buffered with 100 mM succinic acid (approximate pH of 4 to 6), MES (approximate pH of 5 and 7), 100 mM sodium phosphate (approximate pH of 7 to 8), or Tris-HCl (approximate pH of 8) and adjusted with 10 N NaOH. Controls included unbuffered PMS, CNM, and CAM. At

3, 6, 24, and 36 h after transfer, mycelia were collected for RNA extraction, and the media was saved for pH determination and AF extraction.

To measure NOR levels, *A. parasiticus* was grown on either CAM, CNM, YES (0.2% yeast extract and 2.0% sucrose), or YEL (0.2% yeast extract and 2.0% lactose) agar. Media were buffered from pH 5 to 8 with 100 mM succinic acid or 100 mM sodium phosphate with control left unbuffered (unbuffered CNM or CAM was adjusted initially to pH 6.5 with 10 N NaOH). For each treatment, filter discs (three 1 cm²) were dipped in a conidial suspension (1 × 10⁶ conidia/ml) and placed equidistantly on an 8-cm-diameter petri dish. Cultures were incubated at 30°C for 2, 3, and 4 days, at which time filter discs (three/time point) were collected and extracted for NOR.

mRNA analysis. Total RNA was extracted as previously described (6) and separated (20 µg/lane) by electrophoresis in a 1.5% formaldehyde–1.2% agarose gel before transfer to Hybond (Amersham Corp., Arlington Heights, IL) nylon membrane overnight in 10× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Membranes were dried, cross-linked under UV light, and prehybridized in a sodium dodecyl sulfate (SDS)–phosphate solution (7.0% SDS, 200 mM NaH₂PO₄, and 1 mM EDTA, pH 7.2) at 60°C for 1 to 3 h, at which time random primed [α -³²P]dCTP-labeled probes were added to the buffer. A 0.70-kb *KspI/SmaI* fragment from pRB7 was used to detect *stcU* transcript (31), and a 0.65-kb *BamHI/EcoRI* fragment from pBSV2 was used to detect *ver-1* transcript (24). *stcU* and *ver-1* are homologs encoding a reductase necessary for the conversion of the ST/AF precursor versicolorin A to demethylsterigmatocystin in *A. nidulans* and *A. parasiticus*, respectively (17,24). A 1.1-kb *HindIII/EcoRI* fragment from pucHH(458) (provided by M. Peñalva, Centro de Investigaciones, Biológicas, Madrid, Spain) was used to detect *ipnA* message in *A. nidulans*. Membranes were washed for 30 min each at 60°C with 2× SSC/0.1% SDS, 0.5× SSC/0.1% SDS, and 0.1× SSC/0.1% SDS prior to exposure to X-ray film (Jersey Lab Supply, Livingston, NJ). Membranes were stripped by submerging in boiling 0.1% SDS and agitating for 10 min.

Quantification of mycotoxins. The common practice of measuring AF B₁ rather than all four AFs (B₁, G₁, B₂, and G₂) was followed throughout this study. AF B₁ is produced in higher amounts than any other of the AFs (15).

Enzyme-linked immunosorbent assay (ELISA) detection of AF B₁. AF B₁ was analyzed from the filtrate by indirect competitive ELISA as described previously (21).

High-pressure liquid chromatography (HPLC) detection of AF B₁. In a separate experiment, AF B₁ was also extracted from growth medium of *A. parasiticus* by adding an equal volume of

TABLE 1. pH effects of four buffers on aflatoxin production by *Aspergillus parasiticus*

Treatment ^a	Aflatoxin B ₁ (ng/ml)			Actual pH ^b		
	12 h	24 h	36 h	12 h	24 h	36 h
Unbuffered	3.0	241	394	4.2	2.5	2.7
Succinic acid						
pH 4.0	1.0	276	1,062	4.1	4.0	3.9
pH 5.0	ND ^c	219	808	5.1	4.9	4.8
pH 6.0	ND	2	19	5.8	5.8	5.7
MES						
pH 5.0	13.0	446	851	4.9	4.8	4.8
pH 7.0	ND	14	30	6.6	6.2	6.2
Na ₂ HPO ₄						
pH 7.0	ND	77	244	6.8	6.6	6.2
Tris HCl						
pH 8.0	ND	5	22	7.2	6.8	6.5
LSD _{0.05} ^d	3	90	253	0.1	0.1	0.1

^a *Aspergillus* complete ammonium medium was either unbuffered or buffered as indicated for a total of eight treatments.

^b pH values of medium at respective time points.

^c ND = none detected.

^d Means of triplicates were analyzed using Fisher's protected least significant difference at *P* = 0.05.

TABLE 2. Ability of buffers to maintain external pH in *Aspergillus* liquid shake cultures

Treatment ^b	pH ^a						
	<i>A. nidulans</i>		<i>A. parasiticus</i>				
	CNM		PMS		CNM		
	24 h	48 h	3 h	6 h	36 h	6 h	24 h
Unbuffered	5.3	4.8	6.3	6.0	3.7	4.9	3.3
Succinic acid							
pH 4	4.0	4.1	4.0	4.1	4.0	4.0	4.1
pH 5	5.0	4.9	5.0	5.1	5.0	5.1	5.2
pH 6	5.9	5.9	5.9	6.0	5.7	6.0	5.9
Na ₂ HPO ₄							
pH 7	6.9	6.8	6.7	6.8	6.5	6.5	6.3
pH 8	7.8	7.0	7.0	7.0	6.7	6.7	6.6

^a The pH of the complete ammonium medium was assessed at 3, 6, 24, 36, or 48 h after shift.

^b *A. nidulans* and *A. parasiticus* were grown in complete nitrate medium (CNM) or peptone mineral salts medium (PMS, *A. parasiticus* only) and then shifted to complete ammonium medium that was either unbuffered or buffered from pH 4, 5, and 6 (succinic acid), to pH 7 and 8 (sodium phosphate).

acetone followed by an equal volume of chloroform to 10 ml of growth media in a separatory funnel. The aqueous layer was back-extracted twice with chloroform. The organic phase containing the toxins was separated in a separatory funnel, collected, and dried in a fume hood. The residue was redissolved in 1 ml of chloroform and used for HPLC analysis as described previously (15).

Spectrophotometric detection of NOR. For NOR analysis, three colonized filter discs/petri dish were immersed in 5 ml of acetone for ~1 h. The discs were then removed, the acetone was evaporated in a fume hood, and the residue was then resuspended in 5 ml of 90:10 methanol/1 N NaOH (approximate pH of 10.0; NOR is a pH indicator and is purple at pH 10, which allows for a spectrophotometric assay of NOR at 595 nm). The resulting crude extract was diluted 10- to 50-fold depending on color intensity, and a 200- μ l aliquot was measured on a 96-well-titer ELISA plate reader with 200 μ l of methanol/1 N NaOH in the control well. This data was normalized to a NOR standard previously purified in our laboratory.

HPLC detection of ST. ST was extracted from 100 mg of lyophilized mycelium of *A. nidulans* by agitating mycelium in 2 ml of acetone for 20 min, followed by adding an equal volume of chloroform and continued agitation for another 20 min. The resulting slurry was centrifuged at $\sim 3,000 \times g$, and the supernatant was collected and allowed to dry in a fume hood. The residue was redissolved in 1 ml of chloroform and used for HPLC analysis. Oatmeal cultures were similarly extracted, but used 15 ml each of chloroform and acetone. Conditions for HPLC analysis were as described previously (15).

Statistical analysis. Each treatment was represented by three replicates, and values presented in this paper were means of these replicates. ELISA quantifications of AF were analyzed by two-way analysis of variance. Differences between means were evaluated by Fisher's protected least significant difference. HPLC analyses of AF and ST, as well as spectrophotometer analyses of NOR, were quantitated after combining extracts from three replications. Coefficients of determination (r^2) and regression models were calculated for these data.

RESULTS

Acidification of growth media. The pH of unbuffered control CAM medium decreased over time in both *A. nidulans* and *A. parasiticus* cultures (Tables 1 and 2). In *A. nidulans*, pH in the unbuffered control dropped from 6.5 to 4.8 after 48 h (Table 2). The pH of the buffered media treatments were maintained for 24 h, but declined slightly for media buffered at pH 7 and 8 in 48-h *A. nidulans* cultures.

In *A. parasiticus* cultures, acidification of unbuffered media differed based on whether the fungus was originally grown in PMS or CNM media. A greater decrease in pH in the unbuffered controls was seen in cultures transferred from CNM (Table 2). In contrast,

there were no significant PMS/CNM effects on the ability of buffers to maintain pH. The pH of media buffered from pH 4 to 6 was maintained within 0.2 pH units in *A. parasiticus* cultures, but dropped considerably in media buffered at pH 7 and 8 at 24 and 36 h, regardless of preshift medium. Neutral and alkali pHs were poorly maintained over time, regardless of buffer or fungal strain.

***stcU* and *ver-1* mRNA accumulates in acid pHs.** In liquid shake culture, *stcU* transcript was present at 24 h in *A. nidulans* cultures grown at pH 4 and 5 (Fig. 1A). By 48 h, transcript was detected in all but the pH 8 treatment, but it was still most strongly expressed at pH 4. Liquid stationary cultures buffered with MES expressed *stcU* transcript at pH 5, but expression was negligible at pH 7 (Fig. 1B). The decrease in *stcU* expression in neutral and basic environments (pH 7 and 8) was in contrast to that of *ipnA*. The penicillin structural gene was only expressed at pH 7 and 8 at 24 h (data not shown) in a manner similar to other reports (13,26).

A. parasiticus ver-1 expression was first observed at 6 h (Fig. 1C). Highest expression was observed in cultures grown in acidic media (pH 4 to 6), and transcription was not detected at pH 8. This trend was maintained through 36 h (Fig. 1C).

Mycotoxin production parallels *stcU/ver-1* expression. AF B₁ production by *A. parasiticus* significantly increases in acidic environments (Table 1), regardless of buffer. For example, AF B₁ concentrations after 24 and 36 h at pH 5.0 were similar in media buffered with MES or succinic acid. A separate experiment using succinic acid and phosphate buffer (Table 2) also demonstrated a negative linear relationship between AF B₁ levels and increasing pH of the external media at 24 h ($y = 3.898 - 0.496x$, $r^2 = 0.914$) (Fig. 2B). In this experiment, only the culture grown in pH 4 produced detectable AF at 6 h (80 ng/ml).

A similar trend was observed with ST production in *A. nidulans*. At 24 h after transfer, only *A. nidulans* cultures grown in control and pH 4 and 5 treatments produced detectable amounts of ST (Fig. 2A). At 48 h after transfer, cultures grown in media buffered from pH 5 to 8 produced modest amounts of ST, and the pH 4 treatment produced the highest amount. The pH of the control culture had decreased from 6.5 to 4.8 by 48 h (Table 2) and was producing a comparable amount of ST as did the pH 4 treatment at this time point. The coefficients of determination for ST concentration versus pH were $r^2 = 0.667$ and $r^2 = 0.638$ for the 24- and 48-h treatments, respectively (Fig. 2A).

NOR concentrations were dependent on media and pH and showed a similar negative linear regression to increasing pH as did AF B₁ and ST (Fig. 2C, D, and E). NOR was not detected for any treatment on YEL agar, although slight amounts, below the limits of spectrophotometric detection, were visualized in the pH 5 treatment (data not shown). For the three other media, CAM, CNM, and YES, the highest NOR values for buffered treatments were measured at pH 5 (Fig. 2C, D, and E). Cultures grown on unbuffered agar (by day 4, approximate pH of 3.5 in CAM and

Fig. 1. *Aspergillus nidulans* and *A. parasiticus stcU* and *ver-1* expression in different pH regimes. **A,** *stcU* expression in succinic acid and sodium phosphate buffers. Total RNA (20 μ g/lane) of *A. nidulans* wild-type strain FGSC26 grown in different pH regimes was probed with *stcU* at 24- and 48-h time points. pH values of this data are represented in Table 2. ST values are presented in Figure 2A. **B,** *stcU* expression in MES buffer. Total RNA (20 μ g/lane) of *A. nidulans* wild-type strain FGSC26 grown at pH 5 and 7 was probed with *stcU* at 24- and 48-h time points. **C,** *ver-1* expression in succinic acid and sodium phosphate buffers. Total RNA (20 μ g/lane) of *A. parasiticus* ATCC 98106 grown in different pH regimes was probed with *ver-1* at 6- and 36-h time points. pH values of this data are represented in Table 2. AF B₁ values are presented in Figure 2B. Bottom of lanes are labeled by pH treatment in which C is unbuffered complete ammonium medium and 4 through 8 describe pH of the buffered media.

YES and approximate pH of 7 in CNM) produced the highest amounts of NOR.

ST production in an *A. nidulans* PacC constitutive mutant. Although both cultures appeared to grow equally well, oatmeal

cultures of *A. nidulans* DOC produced approximately 10-fold less ST than did FGSC26 over a 5-day period (Fig. 3A). Attempts to buffer oatmeal cultures to pH 4 were unsuccessful (Table 3), but it was noted that FGSC26 produced less ST in the pH 7 treatment.

Fig. 2. Mycotoxin production in different pH regimes. Coefficients of determination (r^2) and linear regression models were calculated for mycotoxin concentrations versus pH in all experiments. Coefficients of determination are shown in upper right hand corner for all experiments. **A**, Sterigmatocystin (ST). *Aspergillus nidulans* FGSC26 ST production at 24 and 48 h. The y axis is measurement of ST in micrograms per milligram of mycelium and the x axis is pH treatments. The linear regressions of ST versus pH at 24 and 48 h are $y = 0.732 - 0.099x$ and $y = 6.584 - 0.855x$, respectively. **B**, Aflatoxin (AF). *A. parasiticus* ATCC 98106 AF production at 24 h. The y axis is measurement of AF in micrograms per milliliter and the x axis is pH treatments. The linear regression of AF B_1 versus pH is $y = 3.898 - 0.496x$. **C**, Norsolorinic acid (NOR) produced in complete ammonium medium. *A. parasiticus* ATCC 98106 NOR production at 2, 3, and 4 days. The y axis is measurement of NOR in nanograms per disc and the x axis is pH treatments (C = control, unbuffered medium). The linear regressions of NOR versus pH for the 2-, 3-, and 4-day treatments are $y = 1276.3 - 166.74x$, $y = 1278.5 - 162.88x$, and $y = 1284.7 - 160.88x$, respectively. **D**, NOR produced in yeast extract sucrose medium, as in C. The linear regressions of NOR versus pH for the 2-, 3-, and 4-day treatments are $y = 214.48 - 28.42x$, $y = 257.04 - 33.91x$, and $y = 307.99 - 38.51x$, respectively. **E**, NOR produced in complete nitrate medium, as in C. C = unbuffered medium in all panels. The linear regressions of NOR versus pH for the 2-, 3-, and 4-day treatments are $y = 137.52 - 18.68x$, $y = 185.82 - 25.18x$, and $y = 199.20 - 27.00x$, respectively.

Fig. 3. Sterigmatocystin (ST) production and *stcU* transcript in wild-type and a PacC mutant of *Aspergillus nidulans*. **A**, ST production in total micrograms from oatmeal cultures of *A. nidulans* FGSC26 and *A. nidulans* DOC. Oatmeal cultures were either unbuffered or buffered to pH 4 or 7, pH values are shown in Table 3. **B**, Total RNA (20 $\mu\text{g}/\text{lane}$) of *A. nidulans* FGSC26 and *A. nidulans* DOC (e.g., *pacC*^{c202}) probed with *stcU* at 24- and 48-h time points.

When grown in unbuffered CAM medium, FGSC26 *stcU* transcript usually peaked at 48 h (Figs. 1A and 3B). Transcript level was greater at 24 than 48 h in *A. nidulans* DOC grown in similar conditions (Fig. 3B). Buffering of external medium had no discernible effect on *stcU* expression in this strain (data not shown).

DISCUSSION

Specific fungal secondary metabolites are usually found to be restricted to a limited set of species or genera. In particular, the mycotoxins ST and AF and related compounds are most commonly produced by *Aspergillus* spp. and a few other ascomycetes or deuteromycetes (2,3,7,16). Results from several laboratories have now shown that ST biosynthesis in *A. nidulans* and AF biosynthesis in *A. flavus* and *A. parasiticus* is conserved through the preservation of a large gene cluster (6,27,30) that is regulated by the same binuclear zinc transcription factor, AflR (29,31). Logic suggests that conserved factors regulating the expression of these two clusters could be involved in regulatory responses to nutritional and environmental parameters including nitrogen sources, carbon sources, and pH. This has been true for penicillin production in *Penicillium chrysogenum* and *A. nidulans*. Biosynthesis of this secondary metabolite is regulated by both the carbon catabolite repressor, CreA, and PacC, although not to the same degree in the two species (11–13,25,26).

In contrast to penicillin, ST/AF production increases in glucose- and ammonium-based media and decreases in nitrate-based media (1,5,14). A previous hypothesis proposed that changes in pools of nitrate assimilation enzymes and associated cofactors could be one reason for nitrate suppression of AF (20). Cotty (8) reported that pH changes might partially explain the nitrogen effects on AF production in *A. flavus*, as unbuffered ammonium medium becomes acidified over time, whereas unbuffered nitrate medium remains near pH 7.0 and buffering eliminated this effect. We observed similar pH changes in *A. parasiticus* and *A. nidulans* nitrogen cultures. By buffering ammonium growth media in a range from pH 4 to 8, we observed an approximately fivefold decrease in mycotoxin production in both *Aspergillus* spp. with increasing pH. Solid culture extractions of NOR (a common precursor to ST and AF) indicated that the pH effect was conserved when *A. parasiticus* was grown on different nitrogen and carbon media. These results are supportive of observations of pH effects on AF production in *A. flavus* (8). However, the fact that *A. parasiticus* still produced more NOR in buffered ammonium than in buffered nitrate medium (Fig. 2C and E) suggests that other, pH-independent mechanism(s) also regulate AF and ST biosynthesis.

The potential regulatory role of pH is suggested by the differential induction of *ver-1* and *stcU* transcripts in buffered media. *stcU* and *ver-1* transcript levels first appeared in acidic pH treatments and only later, if at all, in neutral and alkali conditions. This

does not appear to be a buffer effect, as the same trend was noted using different buffers (Table 1, Fig. 2). The separation of pH effect from any buffer effect is also seen for *A. flavus* AF production (8) and in penicillin studies in which the role of pH regulation is well established (13,25). It is striking that pH affects ST production and *stcU* transcription inversely as compared with penicillin production and *ipnA* transcription in *A. nidulans* (26). Notably, the constitutive *pacC* genotype (e.g., *pacC^{c202}*) produces elevated levels of penicillin and repressed levels of ST (13). PacC is a Cys₂His₂ zinc finger transcription factor mediating pH regulation of gene expression in *A. nidulans* and *Penicillium chrysogenum* (25,26). PacC binds to several regions in the *ipnA* promoter and is proposed to activate transcription of other alkaline-expressed genes and to repress transcription of acid-expressed genes (26). We have identified putative PacC consensus motifs in the ST and AF gene clusters, and further studies are aimed at determining whether there is a direct or indirect interaction between PacC expression and ST/AF biosynthesis.

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TABLE 3. Ability of buffers to maintain external pH in *Aspergillus nidulans* oatmeal cultures

Strain ^b	Treatment	pH over 5 days ^a					
		0	1	2	3	4	5
FGSC26	Unbuffered	6.5	6.3	6.4	6.3	6.1	6.4
	pH 4	4.0	5.6	7.0	7.1	7.0	7.3
	pH 7	7.0	7.0	7.0	7.0	7.1	6.9
DOC	Unbuffered	6.5	6.2	5.6	6.3	6.8	6.6
	pH 4	4.0	5.1	5.4	6.1	6.7	5.7
	pH 7	7.0	7.1	6.7	6.7	6.2	6.2

^a The pH of the colonized oatmeal porridge was assessed after 0, 1, 2, 3, 4, and 5 days of growth.

^b *A. nidulans* FGSC26 (e.g., wild-type PacC) and *A. nidulans* DOC (constitutive PacC activity) were grown in oatmeal porridge that was either unbuffered, buffered to pH 4 (succinic acid), or buffered to pH 7 (sodium phosphate).

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