Reduction of aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus* in interaction with *Streptomyces*

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The aim of this study is to investigate aflatoxin gene expression during *Streptomyces–*Aspergillus interaction. Aflatoxins are carcinogenic compounds produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. A previous study has shown that *Streptomyces–*A. flavus interaction can reduce aflatoxin content in vitro. Here, we first validated this same effect in the interaction with *A. parasiticus*. Moreover, we showed that growth reduction and aflatoxin content were correlated in *A. parasiticus* but not in *A. flavus*. Secondly, we investigated the mechanisms of action by reverse-transcriptase quantitative PCR. As microbial interaction can lead to variations in expression of household genes, the most stable [act1, βtub (and cox5 for *A. parasiticus*)] were chosen using geNorm software. To shed light on the mechanisms involved, we studied during the interaction the expression of five genes (aflD, aflM, aflP, aflR and aflS). Overall, the results of aflatoxin gene expression showed that *Streptomyces* repressed gene expression to a greater level in *A. parasiticus* than in *A. flavus*. Expression of aflR and aflS was generally repressed in both *Aspergillus* species. Expression of aflM was repressed and was correlated with aflatoxin B1 content. The results suggest that aflM expression could be a potential aflatoxin indicator in *Streptomyces* species interactions. Therefore, we demonstrate that *Streptomyces* can reduce aflatoxin production by both *Aspergillus* species and that this effect can be correlated with the repression of aflM expression.

INTRODUCTION

Aflatoxins (AFs) are polyketide-derived furanocoumarins. They are produced by fungi of the genus *Aspergillus* (including *Aspergillus flavus* and *Aspergillus parasiticus*) in agricultural foodstuffs (maize, hazelnut, peanut, etc.) (Giorni et al., 2007; Passone et al., 2010). These AFs are toxic and their main adverse effects on humans are hepatocarcinoma (Qian et al., 2007; Passone et al., 1994; IARC, 2014), immune system deficiency (Jiang et al., 2004) and reduced child growth (Gong et al., 2004) and increased risks of stillborn or newborn jaundice (Shuaib et al., 2010). To reduce these multiple effects, many countries have implemented maximum authorized levels of AFs in food and feed (Wu & Guclu, 2012).

AF biosynthesis is coded by a 80 kb long DNA sequence. The latter is a cluster containing 30 putative genes characterized in both *A. flavus* and *A. parasiticus* (Yu, 2012). For structural genes, early (as aflD), medium (as aflM) and late (as aflP) genes are denominated (Fig. S1, available in the online Supplementary Material). The gene aflD encodes a reductase enzyme involved in the conversion of norsolorinic acid to averantin (Papa, 1982); aflM is required for the conversion of versicolorin A to demethylsterigmatocystin (Skory et al., 1992); and aflP encodes a methyltransferase converting sterigmatocystin to O-methylsterigmatocystin (Bhatnagar et al., 1988). Two cluster-specific regulators are also known: aflR encodes a transcription activator that binds a consensus sequence in the promoter regions of AF structural genes (Payne et al., 1993), and AflS is a potential co-activator of AflR (Meyers et al., 1998) (Fig. S1). Schmidt-Heydt et al. (2009) showed that the aflR/aflS ratio can also be used as an indicator of AF biosynthesis. In addition to AflR and AflS, the clustered genes are also regulated by aspecific transcriptional regulators such as LaeA or Ap-1 (Reverberi et al., 2008; Chang et al., 2012).

Microbial interactions with yeast, bacteria or fungi can reduce AF production by aspergilli (Yin et al., 2008). *Streptomyces* are soil-borne bacteria that can develop in crops and that are known to be good biocontrol candidates (Bressan & Figueiredo, 2008). Studies have shown that *Streptomyces* metabolites are sources of AF repressors (Ono et al., 1997; Sakuda et al., 2000). However, until recently no studies have focused on *Streptomyces–*Aspergillus mutual

Abbreviations: AF, aflatoxin; RT-qPCR, reverse-transcriptase quantitative PCR.

One supplementary table and two supplementary figures are available with the online Supplementary Material.

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interactions and their impact on AF production and AF gene expression.

Recently, we found that Streptomyces (27 strains)—A. flavus (NRRL 62477) mutual interaction on contact can reduce the concentration of AF B1 (AFB1) and AF B2 (AFB2) in vitro by up to 4.4% (remaining concentration) (Verheecke et al., 2014).

In this study, six of the Streptomyces strains previously used were chosen for further investigation. Our preliminary goal was to verify the interaction impact on an AF G producer, namely A. parasiticus. Our main objective was to study the impact of these interactions on AF gene expression. The methodology was applied to A. flavus and A. parasiticus on expression of five targeted genes (aflD, aflM, aflP, aflR and aflS).

**METHODS**

**Fungal and Streptomyces strains.** The fungal strains used were A. flavus NRRL 62477 and A. parasiticus Aflc3. The six actinomycete strains were selected on ISP-2 medium after 10 days at 28 °C, mainly based on the results from Verheecke et al. (2014): antagonism on contact with A. flavus, reduction of AFs concentration under 17% versus control and growth on ISP-2 medium (unpublished data). Their 16S rRNA genes were sequenced according to the method described by Zitouni et al. (2005). The six strains were identified as Streptomyces roseolus S06, Streptomyces calvus S13, Streptomyces thinghervisensis S17, Streptomyces sp. S27, Streptomyces griseoplanus S35 and Streptomyces caerulescens S38. Streptomyces were kept at -20 °C in cryotubes in ISP-2 medium with 20% (v/v) glycerol.

**Interaction method and AF quantification.** Pre-cultures for both Aspergilli (on yeast extract peptone dextrose medium) and for Streptomyces (on ISP-2) were made for 7 days at 28 °C as previously described by Verheecke et al. (2014). The culture conditions are based on Verheecke et al. (2014) with slight modifications: a sterile 8.5 cm diameter Petri dish (Hutchinson) was dropped on ISP-2 (Shirling & Gottlieb, 1966) prior to inoculum and two streaks (instead of one) of cellophane sheet (Hutchinson) was dropped on ISP-2 (Shirling & Gottlieb, 1966) prior to inoculum and two streaks (instead of one) of cellophane sheet for measurement of biomass was removed from the cellophane sheet for measurement of (avoiding taking bacterial biomass). At day 7 (set two), the fungal mouldy cellophane was removed and used for RNA extraction from the bacterial biomass. Using a scalpel and with the naked eye, the fungal biomass was separated in cryotubes in ISP-2 medium with 20% (v/v) glycerol. One day 4 (set one), the fungal biomass was separated from the bacterial biomass. Using a scalpel and with the naked eye, the mouldy cellophane was removed and used for RNA extraction (avoiding taking bacterial biomass). At day 7 (set two), the fungal biomass was removed from the cellophane sheet for measurement of dry weight (after drying: 18 h at 80 °C). In the remaining media, three agar plugs (6 mm) were removed from the fungal growth area for AF quantification (Verheecke et al., 2014). The experiment was done twice in triplicate.

AF quantification was done as previously described (Verheecke et al., 2014). Briefly, methanol (1 ml) was added to agar plugs during a 30 min incubation period (shaken three times). This was then centrifuged for 15 min at 12470 g and the supernatant was filtered (0.45 mm, 4 mm PVDF; Whatman) into vials. AF quantification was done on an Ultimate 3000 system ( Dionex- Thermo Electron) with all the R5 series modules. A C18 pre-column and column were used (Phenomenex, Luna 3 mm, 200 x 4.6 mm). Detection of AFs was done according to instructions for the Coring Cell analysis system (Coring System Diagnostica). Quantification was realized with Chromeleon software, using AFB1 and AFB2 (Sigma-Aldrich) (detection limit: 0.5 p.p.b.) as standards. Statistical analyses were made using ‘nparcomp’ R (version 2.15.2).

**RNA extraction and quantification.** In total, 60 mg of mycelium was crushed in liquid nitrogen to a fine powder. The powder was then stored at -80 °C until RNA isolation. Total RNA was isolated using an Aurum Total RNA kit (Bio-Rad). The manufacturer’s instructions for eukaryotic and plant cell materials were followed, except for two modifications: DNase I digestion was extended to 1 h and elution was done at 70 °C for 2 min in the elution buffer. Total RNA was eluted into 80 μl and stored at -20 °C. Then, 1 μl of total RNA of each sample was loaded into an RNA StSens chip (Bio-Rad) and quantified on a Nanodrop 2000 spectrophotometer (Thermo Scientific) according to the manufacturer’s instructions. Samples with RNA Quality Indicator >7, A260/230 >2 and A260/230 >1.3 were selected for further analysis.

**Reverse-transcriptase quantitative PCR (RT-qPCR).** Reverse transcription was carried out with an Advantage RT-PCR kit (Clontech) with Oligo (dT)18 primer according to the manufacturer’s instructions (RNA concentration: 1 μg total RNA), with one modification: incubation at 42 °C was extended to 4 h. RT-qPCR was performed in duplicate using a CFX96 Touch instrument (Bio-Rad) using SsoAdvancedTM SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instructions (annealing temperature, 59 °C; concentrations: primers, 500 nM and cDNA, 100 ng). Primer pairs and associated efficiencies were validated (85–115%) (Table S1).

**Validation of reference genes.** Based on the literature, six candidate genes (act1, β-tub, cox5, efl, gpdA and tbp) were studied as potentially suitable reference genes (Radonić et al., 2004; Bohle et al., 2007). For identification of the optimal number of reference genes and stability, eight samples (randomly selected among the different conditions) were tested in triplicate. The measures of gene stability V’ (gene pairwise variation) and M (V of a gene with other genes) were calculated using geNorm software (Vandesompele et al., 2002). M values are represented in Fig. S2 for A. flavus and A. parasiticus, according to the geNorm software in standard configuration. This led to the choice of act1 and β-tub (for A. flavus) and act1, β-tub and cox5 (for A. parasiticus) as optimal reference genes.

**Relative quantification.** Relative quantification was determined compared with the chosen reference genes. Calculation of gene expression was via qbase+ software as well as statistical analysis (Hellemans et al., 2007).

The correlations between fungal dry weight, AF content and gene expression were determined using Pearson correlation (r, asterisks indicate statistically significant differences at P<0.05).

**RESULTS**

**Interaction of Streptomyces with A. parasiticus and A. flavus**

Interaction between Streptomyces and both Aspergillus species was monitored in Petri dishes over 7 days. On day 7, all the tested Streptomyces strains showed a mutual antagonism on contact with the aspergilli. For A. parasiticus, compared with the control dry weight (100%), in interaction with the bacterial strains, the fungal residual dry weight (RDW) ranged from 24.7% (S06) to 57.2% (S17) (Table 1). For A. flavus (Table 2), RDW ranged from 60.7% (S35) to 92.7% (S27) of the control dry weight (100%) when treated with the same bacterial strains.
Reduction of AF concentration

On day 7, the production of AFs by *A. parasiticus* and *A. flavus* was reduced in contact with the six *Streptomyces* strains tested. For *A. parasiticus*, AFB1 and AFG1 production was monitored (Table 1). S17 showed lower reductions of 13 and 6.2% of the concentration in the medium as a percentage of the control, respectively. S27 and S38 showed the greatest reductions, with no AFB1 or AFG1 detected. S06, S13 and S35 reduced to the greatest extent, with no AFB1 or AFG1 detected. For *A. flavus*, AFB1 and AFB2 production was monitored (Table 2). S17 showed lower reductions of 13 and 6.2% for AFB1 and 2.9% for AFG1. S06, S13 and S35 reduced to the greatest extent, with no AFB1 or AFG1 detected. S38 were the greatest reducers, with no AFB1 or AFB2 detected. Pearson correlation was also applied.

AF gene expression

Gene expression was determined on day 4 with *A. flavus* and *A. parasiticus* alone (controls) and in interaction with the six *Streptomyces* strains. Five genes (*aflD, aflM, aflP, aflR* and *aflS*) were investigated relative to two reference genes (*act1* and *βtub*) for *A. flavus* and three reference genes (*act1, βtub* and *cox5*) for *A. parasiticus*.

For *A. parasiticus*, *aflM* expression was slightly impacted by S13 (7.7-fold), moderately by S35 (33.3-fold) and very highly by S06 (100-fold) (Table 1). S35 and S06 also reduced *aflP* expression 83- and 250-fold, respectively. Regarding *aflS* and *aflR*, S13 significantly reduced *aflS* expression (6.25-fold) and S06 repressed the expression of both *aflS* (10-fold) and *aflR* (14.3-fold). The interaction did not significantly impact *aflD* expression.

For *A. flavus*, S35 repressed the expression of *aflM* (8.4-fold) and *aflR* (1.5-fold) (Table 2). S38 repressed the

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### Table 1. Impact of *Streptomyces* strains on *A. parasiticus* AFs and gene expression

Data with the same letter are not significantly different (*P*<0.05). MC, Concentration in the media as a percentage of the control; ND, not detected. Mean values are given ± SD.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fungal growth (%) (day 7)</th>
<th>Effect on AF accumulation (% MC) in co-culture (day 7)</th>
<th>Effect on gene expression (day 4)</th>
<th>Ratio aflR/aflS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AFB1</td>
<td>AFG1</td>
<td>aflD</td>
</tr>
<tr>
<td>Control</td>
<td>103.5 ± 0.9a</td>
<td>108.3 ± 5.8a</td>
<td>101.3 ± 10.9a</td>
<td>1.00</td>
</tr>
<tr>
<td>S06</td>
<td>24.7 ± 26.4c</td>
<td>NDc</td>
<td>NDc</td>
<td>0.7</td>
</tr>
<tr>
<td>S13</td>
<td>35.2 ± 11.8b,c</td>
<td>NDc</td>
<td>NDc</td>
<td>0.67</td>
</tr>
<tr>
<td>S17</td>
<td>57.2 ± 6.6b</td>
<td>13 ± 3.5b</td>
<td>6.2 ± 0.3b,c</td>
<td>1.56</td>
</tr>
<tr>
<td>S27</td>
<td>35.2 ± 17b</td>
<td>4.1 ± 0.5b</td>
<td>2.9 ± 0.2b,c</td>
<td>0.84</td>
</tr>
<tr>
<td>S35</td>
<td>32.9 ± 2.9c</td>
<td>NDc</td>
<td>NDc</td>
<td>0.50</td>
</tr>
<tr>
<td>S38</td>
<td>44.3 ± 12b,c</td>
<td>4.5 ± 0.7b,c</td>
<td>4.0 ± 0.3b,c</td>
<td>0.64</td>
</tr>
</tbody>
</table>

*Significant difference (*P*<0.05).

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### Table 2. Impact of *Streptomyces* strains on *A. flavus* AFs and gene expression

Data with the same letter are not significantly different (*P*<0.05). MC, Concentration in the media as a percentage of the control; ND, not detected. Mean values are given ± SD.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fungal growth (%) (day 7)</th>
<th>Effect on AF accumulation (% MC) in co-culture (day 7)</th>
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<th>Ratio aflR/aflS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AFB1</td>
<td>AFB2</td>
<td>aflD</td>
</tr>
<tr>
<td>Control</td>
<td>100.0 ± 15.4a</td>
<td>100.0 ± 13.9a</td>
<td>100.0 ± 17.3a</td>
<td>1.00</td>
</tr>
<tr>
<td>S06</td>
<td>64.6 ± 8.6b</td>
<td>2.3 ± 4.5c</td>
<td>ND</td>
<td>0.69</td>
</tr>
<tr>
<td>S13</td>
<td>81.3 ± 16.2a</td>
<td>15.6 ± 9.2b</td>
<td>9.3 ± 20.8b</td>
<td>1.60</td>
</tr>
<tr>
<td>S17</td>
<td>77.7 ± 11.2a</td>
<td>24.0 ± 19.8b</td>
<td>5.3 ± 11.9b</td>
<td>0.95</td>
</tr>
<tr>
<td>S27</td>
<td>92.7 ± 18.3a</td>
<td>8.1 ± 5.1b</td>
<td>ND</td>
<td>1.42</td>
</tr>
<tr>
<td>S35</td>
<td>60.7 ± 11.4b</td>
<td>0.2 ± 0.5c</td>
<td>ND</td>
<td>0.50</td>
</tr>
<tr>
<td>S38</td>
<td>62.4 ± 15.2b</td>
<td>3.1 ± 5.3c</td>
<td>ND</td>
<td>1.44</td>
</tr>
</tbody>
</table>

*Significant difference (*P*<0.05).
expression of *aflP* (4.8-fold) and *aflR* (1.45-fold). S06 enhanced the expression of *aflR* (2.37-fold). Expression of *aflD* and *aflS* was not significantly impacted by the six strains.

The ratio *aflR/aflS* was monitored in both producing strains. Both positive controls were close to 1:0.8 for *A. parasiticus* and 0.9 for *A. flavus*. This ratio was above 1 for *A. parasiticus* in interaction with S06 (1.2), S13 (1.2), S17 (1.4) and S38 (1.5) and for *A. flavus* in interaction with S06 (2.9), S17 (1.8) and S35 (1.3). Ratios for the other interactions were below 1.

**Assessment of correlation**

Independently of the *Streptomyces* tested, Pearson correlations were done between RDW and AF concentration. For *A. parasiticus*, the reduction of AFB1 and AFG1 concentration in the medium was correlated (*r*=0.94*" and 0.91*) with RDW reduction. For *A. flavus*, AFB1 and AFB2 concentration were not correlated with RDW reduction.

Pearson correlations were also applied to gene expression versus RDW or AF concentration in the medium. For *A. parasiticus*, all gene expressions were correlated with RDW reduction. The strongest correlation was obtained for expression of *aflP* (*r*=0.97*"*). Correlations were also identified between the reduction of AFB1 concentration in the medium and *aflD*, *aflM* and *aflP* repression (*r*=0.91*", 0.92* and 0.86*, respectively). For *A. flavus*, RDW and AFB1 and AFB2 concentrations were only correlated with *aflM* expression (*r*=0.86*, 0.86* and 0.83, respectively).

**DISCUSSION**

Six *Streptomyces* strains had their impact confirmed on *A. flavus* and tested for *A. parasiticus*. They all showed mutual antagonism on contact as described by Magan & Lacey (1984). This type of interaction has already been studied in Petri dishes (Sultan & Magan, 2011; Verheecke et al., 2014). The latter showed that after 10 days at 28 °C on ISP-2 medium, 27 of 37 actinomycete strains showed mutual antagonism on contact with *A. flavus* and were able to reduce AF accumulation (residual concentration below 38%). Here, after 7 days, the interaction with both *Aspergillus* species and the six chosen bacterial strains led to mutual antagonism on contact impacting fungal growth and resulting in residual AF concentration in the medium below 24%.

In our study, for *A. parasiticus*, RDW reduction was correlated with AF concentration reduction. This correlation is generally observed in the literature (reviewed by Holmes et al., 2008; Bluma et al., 2008a, b). However, exceptions to this rule are also found. Indeed, Reverberi et al. (2008) studied the effect of *Lentimula edodes* CF42 filtrate (2%, w/v) on *A. parasiticus* after 9 days at 30 °C in potato dextrose broth. The results showed 1.90% AF concentration while no impact on fungal growth was detected. In our study, we highlight another example in another *Aspergillus* species. Indeed, for *A. flavus*, RDW reduction was not correlated with AF concentration reduction. In conclusion, we observed different responses to the *Streptomyces* interaction depending on the *Aspergillus* species studied. Regarding *A. flavus*, the results described here demonstrate that bacterial interaction did not impact AF concentration in the medium just by fungal growth reduction.

AF inhibition can occur through gene repression (Yu, 2012; Alkhayyat & Yu, 2014). Thus, we developed a methodology to monitor AF gene expression. Our preliminary work identified maximum gene expression at 90 h (data not shown). Based on those results, we monitored gene expression under the same conditions. Reference genes were then chosen based on geNorm software and the data matched the MIQE guidelines (Bustin et al., 2009). In our study, we tested six candidates genes for their stability during *Aspergillus–Streptomyces* interaction and the most stable genes were identified (Radonić et al., 2004). Nevertheless, *cox5* was less stable than expected (fifth out of seven for *A. flavus*) and *gapdh* was more stable than described in the literature for other organisms (Dheda et al., 2004; Bohle et al., 2007; Radonić et al., 2004).

In particular, we monitored the expression of three structural genes, *aflD* (early), *aflM* (medium) and *aflP* (late), and two regulator-coding genes, *aflR* and *aflS*. The expression of *aflM* was mostly repressed (between 2.2- and 100-fold) under the conditions tested. A disruption of the *aflM* homologue in *Aspergillus nidulans* (*verA*) led to a reduction of sterigmatocystin production by 200- to 1000-fold (Keller et al., 1994) and versicolorin A accumulation. Here, we showed that repression of *aflM* expression was highly correlated with AFB1 concentration reduction in both *Aspergillus* species. Thus, the measure of *aflM* expression could be an indicator of AF concentration in our experimental conditions.

For *A. parasiticus*, gene expressions were correlated with growth reduction. This could be linked to a delay in fungal growth impacting gene expression. For *A. flavus*, RDW reduction was not correlated with gene expression. The latter were differentially modulated depending on the bacterial strain. Similar results were obtained for *A. flavus* with caffeic acid addition to the medium: *aflD* (6.6-fold), *aflM* (7.1-fold), *aflP* (9.1-fold) and *aflS* (1.5-fold) were repressed without affecting fungal growth (Kim et al., 2008). In our case, the same range of repression was observed in the *Streptomyces–Aspergillus* interaction.

With regard to regulators, expression of *aflR* was differently impacted. It was enhanced 2.37-fold by S06 for *A. flavus* and repressed up to 10-fold by S06 for *A. parasiticus*. Variation of *aflR* expression was also observed in *A. parasiticus* after addition of *Trametes versicolor* filtrate in the medium. Indeed, after 3 days, *aflR* expression was enhanced by more than 10-fold in Czapek–Dox broth solidified with agar while AF content was reduced (Zjalic et al., 2006). In the present study, *aflR* expression was...
enhanced in S06 interaction with A. flavus and AF production was also reduced. In the S06 interaction, aflR expression was not representative of AflR function on aflD, aflM or aflS expression.

Depending on the fungal and bacterial strains, the ratio aflR/aflS was differently impacted. It ranged for A. flavus from 2.9 by S06 to 0.5 by S35 and for A. parasiticus from 1.5 by S38 to 0.5 by S35. This ratio was first studied under various activity of water and temperatures, and a ratio above 1 would lead to an activation of AFB1 biosynthesis (Schmidt-Heydt et al., 2009). In our study, a ratio above 1 was found under most conditions but was not correlated with high AF accumulation.

Moreover, the repression of aflM expression was highly correlated with AFB1 concentration in the medium in both Aspergillus species. A further indicator besides the aflR/aflS ratio could be aflM expression in relation to AF accumulation in the interaction with Streptomyces.

In conclusion, we have shown that mutual antagonism on contact between Streptomyces species and species of the genus Aspergillus led to a reduction of AF accumulation by A. flavus and A. parasiticus. The AF reduction of the latter was correlated with fungal growth reduction whereas no correlation was observed for A. flavus. Here, Streptomyces species bacterial interactions mainly led to the repression of aflM and aflS but had a different impact on aflP and aflR expression. Expression of aflM was correlated with AF accumulation in both Aspergillus species and could be an indicator of AF content in the interaction with Streptomyces. Based on this, Streptomyces griseoflavus S35 appears to be the best biocontrol candidate for further testing on maize.

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