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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.*, 1994; IARC, 2014), immune system deficiency (Jiang *et al.*, 2005), reduced child growth (Gong *et al.*, 2004) and increased risks of stillborn or newborn jaundice (Shuaib *et al.*, 2010). To reduce those 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.

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* Afc5. 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 thinghirensis* S17, *Streptomyces* sp. S27, *Streptomyces griseoplanus* S35 and *Streptomyces caeruleatus* 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 cellophane sheet (Hutchinson) was dropped on ISP-2 (Shirling & Gottlieb, 1966) prior to inoculum and two streaks (instead of one) of *Streptomyces* culture were inoculated in parallel 2 cm away from *Aspergillus* inoculation (centre of the Petri dish). Two sets of plates (three Petri dishes each) were inoculated: one set for RNA extraction at 90 h (day 4) and one at 7 days for analysis of fungal growth and AF concentration. 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 (ϕ 9 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 μm, 4 mm PVDF; Whatman) into vials. AF quantification was done on an Ultimate 3000 system (Dionex- Thermo Electron) with all the RS series modules. A C18 pre-column and column were used (Phenomenex, Luna 3 μm, 200 × 4.6 mm). Detection of AFs was done according to instructions for the Coring Cell analysis system (Coring System Diagnostix). 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, $A_{260/280}$ >2 and $A_{260/230}$ >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*, *βtub*, *cox5*, *ef1*, *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 *βtub* (for *A. flavus*) and *act1*, *βtub* 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.

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.

Strain	Fungal growth (%) (day 7)	Effect on AF accumulation (% MC) in co-culture (day 7)		Effect on gene expression (day 4)					
		AFB1	AFG1	*aflD*	*aflM*	*aflP*	*aflR*	*aflS*	Ratio *aflR*/*aflS*
Control	103.5 ± 0.9^{a}	108.3 ± 5.8^{a}	101.3 ± 10.9^{a}	1.00	1.00	1.00	1.00	1.00	0.8
S06	24.7 ± 26.4^{c}	ND^{c}	ND^{c}	0.7	0.01*	0*	0.1*	0.07*	1.2
S13	$35.2 \pm 11.6^{b,c}$	ND^{c}	ND^{c}	0.67	0.13*	0.08	0.2	0.16*	1.2
S17	57.2 ± 6.6^{b}	13 ± 3.5^{b}	$6.2 \pm 0.3^{b,c}$	1.56	2.61	2.28	1.05	0.64	1.4
S27	35.2 ± 17^{b}	4.1 ± 0.5^{b}	$2.9 \pm 0.2^{b,c}$	0.84	0.41	0.1	0.39	0.44	0.8
S35	32.9 ± 2.9^{c}	ND^{c}	ND^{c}	0.50	0.03*	0.01*	0.27	0.42	0.5
S38	$44.3 \pm 12^{b,c}$	$4.5 \pm 0.7^{b,c}$	$4.0 \pm 0.3^{b,c}$	0.64	0.28	0.11	0.5	0.44	1.5

*Significant difference ($P<0.05$).

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) for AFB1 and AFG1, respectively. S27 and S38 showed higher reduction of 4.1 and 4.5 % for AFB1 and 2.9 % and 4.0 % for AFG1. 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 the least reduction, with 24 and 5.3 % concentration in the medium for AFB1 and AFB2, respectively. S13 showed higher reduction of 15.6 and 9.3 % for AFB1 and AFB2, respectively. S06, S27, S35 and 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

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.

Strain	Fungal growth (%) (day 7)	Effect on AF accumulation (% MC) in co-culture (day 7)		Effect on gene expression (day 4)					
		AFB1	AFB2	*aflD*	*aflM*	*aflP*	*aflR*	*aflS*	Ratio *aflR*/*aflS*
Control	100.0 ± 15.4^{a}	100.0 ± 13.9^{a}	100.0 ± 17.3^{a}	1.00	1.00	1.00	1.00	1.00	0.9
S06	64.6 ± 8.6^{b}	2.3 ± 4.5^{c}	ND	0.69	0.25	1.57	2.37	0.40	2.9
S13	81.3 ± 16.2^{a}	15.6 ± 9.2^{b}	9.3 ± 20.8^{b}	1.60	0.45	0.41	0.82	0.70	0.5
S17	77.7 ± 11.2^{a}	24.0 ± 19.8^{b}	5.3 ± 11.9^{b}	0.95	0.26	3.03	1.53	0.39	1.8
S27	92.7 ± 18.3^{a}	8.1 ± 5.1^{b}	ND	1.42	0.26	0.39	0.88	0.96	0.5
S35	60.7 ± 11.4^{b}	0.2 ± 0.5^{c}	ND	0.50	0.12*	1.02	0.63	0.24	1.3
S38	62.4 ± 15.2^{b}	3.1 ± 5.3^{c}	ND	1.44	0.14*	0.21*	0.69*	0.62	0.5

*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 mediumand *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 *Lentinula 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 shoown). 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 *Aspergilli* 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 griseoplanus* S35 appears to be the best biocontrol candidate for further testing on maize.

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