2018

Inhibition Of Aflatoxin Biosynthesis With Vibrio Gazogenes

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INHIBITION OF AFLATOXIN BIOSYNTHESIS WITH VIBRIO GAZOGENES

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Submitted in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy in

Environmental Health Sciences

The Norman J. Arnold School of Public Health

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2018

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DEDICATION

I dedicate my research study to my family who helped me find myself

ACKNOWLEDGEMENTS

I thank the Gummadidala and Garimella families for my success completion of my PhD. I thank my committee for guidance and support during my PhD. I thank Dr. Chanda and his lab for giving me a wonderful and friendly work environment for the smooth completion of my dissertational research. I thank the department of ENHS, Arnold School of Public Health and USC for all the funding I have received during my graduate run at the USC.

ABSTRACT

Aflatoxin, a mycotoxin, is one of the world's most potent carcinogen. It contaminates major food products such as milk, grains, nuts, corn, etc., leading to greater than $ 1 billion in economic losses and when ingested causes hepatocellular carcinoma (HCC). It is the primary risk factor for 75% HCC cases in the developing world and 3% HCC cases in developed world. Most common methods used in agriculture to reduce aflatoxin contamination are expensive, time consuming and have low efficiency with limited success rates where as biological controls were proven to be most effective in inhibiting aflatoxins and aflatoxin producing fungi. *Vibrio gazogenes*, a non-pathogenic gram-negative marine bacterium, was proven to synthesize antifungal and antiaflatoxin metabolites. In this research study we have used *Aspergillus parasiticus* and *Aspergillus flavus* – two saprophytic pathogenic fungi as aflatoxin-producing models. Preliminary experimentation by treating *V. gazogenes* with aflatoxin produced aflatoxin responsive metabolites (ARMs) that had the ability to significantly decrease aflatoxin synthesis by inhibiting the aflatoxin genes (aflR, nor-1, ver-1) and global secondary metabolism genes (LaeA, VeA). But the decrease in aflatoxin was only 40%. So we treated the fungal cultures with the cells of *V. gazogenes* and the aflatoxin ELISAs revealed the significant decrease (>99%) in aflatoxin biosynthesis by the fungi. The aflatoxin inhibitory effect was very specific to *V. gazogenes* and not to other gram-positive or gram-negative bacterium. Infecting corn kernels with *A. flavus* in the presence of bacterium significantly decrease the fungal conidial growth by 50% and aflatoxin by 98%. Treating drosophila

flies with *V. gazogenes* prior to *A. flavus* infection increased their survival. Using confocal, scanning electron and transmission electron microscopies we observed the uptake of the bacterium by the fungus into vesicles. RT-PCR assays revealed that live *V. gazogenes* cells significantly up-regulate aflatoxin genes (*aflR, nor-1, ver-1*) and global secondary metabolite genes (*laeA, veA*). The pathway through which *V. gazogenes* inhibits aflatoxin is complicating. But our study had clearly developed a novel tool (*V. gazogenes*) to inhibit the aflatoxin biosynthesis, which is acting at the cellular level rather than at the gene level.

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ARMs ..... Aflatoxin Response Metabolites

ROS ..... Reactive Oxygen Species

SOD ..... Super Oxide Dismutase

CHAPTER 1

INTRODUCTION

1.1 AFLATOXINS

Aflatoxins are one of the most potent and dangerous carcinogens known worldwide (Schmale and Munkvold, 1998). They were discovered in the 1960s when 100,000 turkeys died in Britain due to a toxin found in their peanut meal. The investigation led to the discovery of toxins secreted by *Aspergillus flavus* (Negash, 2018). The term aflatoxin is an acronym of *Aspergillus flavus* toxins (Brase S 2013). Aflatoxins are low molecular weight molecules of secondary metabolism produced by fungi belonging to the genus *Aspergillus* and *Penicillium*, during favorable growth conditions of oxygen, moisture (>7%), warm temperatures (24-35^{o}C) and substrate (sugar) (Williams et al., 2004). Aflatoxins are a group of structurally related compounds consisting of 5 rings – a furofuran moiety, an aromatic ring, a lactone ring and either a pentanone or a lactone ring to complete the structure (Brase S 2013) **(**Figure 1.1**)**. More than 20 known aflatoxins exists of which aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2) are the primary aflatoxins and aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2) are the hydroxylated metabolites of AFB1 and AFB2 (Kumar et al., 2016).

According to the Chicago council on global affairs, 25% of all harvests in USA are contaminated by mycotoxins of which aflatoxin contamination of corn alone causes

losses at the high end of $1 billion (Mitchell et al., 2016). In the USA alone, the number of samples tested positive for aflatoxin increased 6% from 2012 to 2013. The FDA limits for aflatoxin human consumption is 20ppb, animal feeds is 300ppb and for aflatoxin M1 in milk is 0.5ppb (FDA 2011). Aflatoxins contaminate crops, produce, food, nuts, cereal, milk, juices, homes, wood, etc., and can be ingested into intestines and enter systemic circulation. Depending on the amount of dose and length of period of intake, aflatoxin ingestion, inhalation or adsorption causes aflatoxicosis. Large doses for a short period of time lead to acute illness - abdominal pain, vomiting, enlarged liver, liver damage, fever, hemorrhage, pulmonary edema, digestive symptoms, convulsions etc. Chronic sub-lethal doses lead to immunologic suppression, decreased nutritional uptake, decreased growth and underweight in children and promoting liver cancers (Williams et al., 2004). Aflatoxin B1 has been categorized as class 1A human carcinogen by the International Agency of Research on Cancer (IARC) because it causes hepatocellular carcinoma. It is projected that 25,200-155,000 cases of liver cancer worldwide are attributed to aflatoxin exposure (Wu et al., 2011).

Aflatoxin undergoes biotransformation primarily in the liver of both human and animal bodies producing a highly reactive epoxide that can bind to DNA, RNA, and proteins altering mitochondria structures and electron transport, effecting cell division and disrupting protein synthesis (Bbosa et al., 2013). Aflatoxin B1 can be passively absorbed through the intestines and is further metabolized by cytochrome P-450 (CYP1A2, 3A4, 3A5, 3A7) enzymes in liver generating a mixture of metabolites of which aflatoxin-8,9-epoxide is highly reactive forming DNA adducts that are capable of GC to TA mutations inhibiting the tumor suppressor gene p53 (Bbosa et al., 2013) (Wu et

al., 2011) (Gratz et al., 2007) (Carlos A. Muro-Cach 2004). This p53 mutation had been observed in 30-60% of the liver cancers in aflatoxin-exposed cases. The reactive epoxide also binds to proteins in liver inhibiting them causing significant cellular damaging and acute aflatoxicosis in both humans and animals (**REF**). Aflatoxin also crosses placenta and is metabolized by the fetal CYP450 liver enzymes producing the same highly reactive epoxide. Thus aflatoxins are toxic, mutagenic, and carcinogenic (Bbosa et al., 2013).

1.2 *Aspergillus flavus* and *Aspergillus parasiticus*

Aflatoxin is biosynthesized by many Aspergillus species such as *A. flavus, A. parasiticus, A. nominus, A. pseutotamarii, A. bombycis, A. toxicarius, A. parvisclerotigenus, A. minisclerotigenes, A. arachidicola,* and *A. pseudocaelatus*. But primarily aflatoxins are produced in copious amounts by *A. flavus* and *A. parasiticus*, which can cohabit and flourish on practically any crop or food including but not limiting to maize, oilseeds, spices, groundnuts, tree nuts, milk, and dried fruit (Strosnider et al. 2006) (Varga et al., 2011). *Aspergillus* can also synthesize aflatoxin during postharvest handling of storage, transportation and food processing (Wu 2011).

Aspergillus parasiticus is a soil mold that was discovered in 1912 by a pathologist A. T. Speare (Horn et al., 2009). It is a saprophyte, a plant pathogen and an opportunistic pathogen to humans and animals and produces aflatoxins B1, B2, G1 and G2.

The fungus *Aspergillus flavus* is a saprophyte, growing in humid environments with pathogenic ability causing aspergillosis in immuno-compromised humans effecting the skin, oral mucosa and subcutaneous tissues (Hedayati, et al. 2007: 1677-92).

According to the centers for disease control and prevention (CDC) approximately 4.8 million cases of aspergillosis were diagnosed worldwide and *A.flavus* is the second most leading cause. *A.flavus* also infects corn, peanuts and cotton by releasing aflatoxins. Aflatoxin B1, an *A.flavus* secondary metabolite, has been categorized as class 1A human carcinogen by the International Agency of Research on Cancer (IARC) and contaminates crops, produce, food, nuts, cereal, milk, juices, homes, wood, etc., and can be ingested into intestines and enter systemic circulation causing aflatoxicosis and liver cancer.

Most *A.flavus* strains are susceptible to antifungal therapy but the minimum inhibitory concentrations are atleast two fold higher than for other *Aspergillus* species (Krishnan, et al. 2009: 206-22). Furthermore, recent discoveries revealed the presence of active multi drug resistant genes in *A.flavus* strains increasing their potential for drug resistance and pathogenicity (Tobin, et al. 1997: 11-23) (Van Der Linden, et al. 2011: S82-9). Aflatoxin B1 synthesized by *A.flavus* is extremely stable and cannot be detoxified by cooking or autoclaving and thus pollutes many food groups being ingested by humans and animals.

1.3 *Vibrio gazogenes*

Most common methods used in agriculture to reduce aflatoxin contamination are expensive, time consuming and have low efficiency with limited success rates. Novel therapies are required to fight against *A.flavus* strains and inhibit both its pathogenicity and aflatoxin production without affecting the host physiology. In their zeal to find new anti-fungal and anti-aflatoxin agents scientists have turned towards plant and microbe derived compounds especially from organisms that live in aflatoxin induced

environments (Holmes, et al. 2008: 559-72). *Vibrio gazogenes* is a marine gram-negative bacterium notoriously known for its synthesis of antifungal pigments. Studies have shown that when *V.gazogenes* comes in contact with aflatoxin, the toxin induces *V.gazogenes* to synthesize antifungal and anti-aflatoxin compounds (Gummadidala, et al. 2016: 814). Understanding the mechanism by which *V.gazogenes* decreases aflatoxin and inhibits pathogenicity of *A.flavus* will help us further understand how to develop, design and target *A.flavus* pathogen and decrease mortality rates of fungal infected patients and plants. Similarly, fungal bacterial interactions can be used as model systems for generation of new antifungals. Finally polymicrobial (bacterial and fungal) colonies pose a potential problem in clinical setting given their multi-drug resistance capabilities, understanding the molecular pathways that define the fungal bacterial interactions is an important step towards discovering new therapeutic targets.

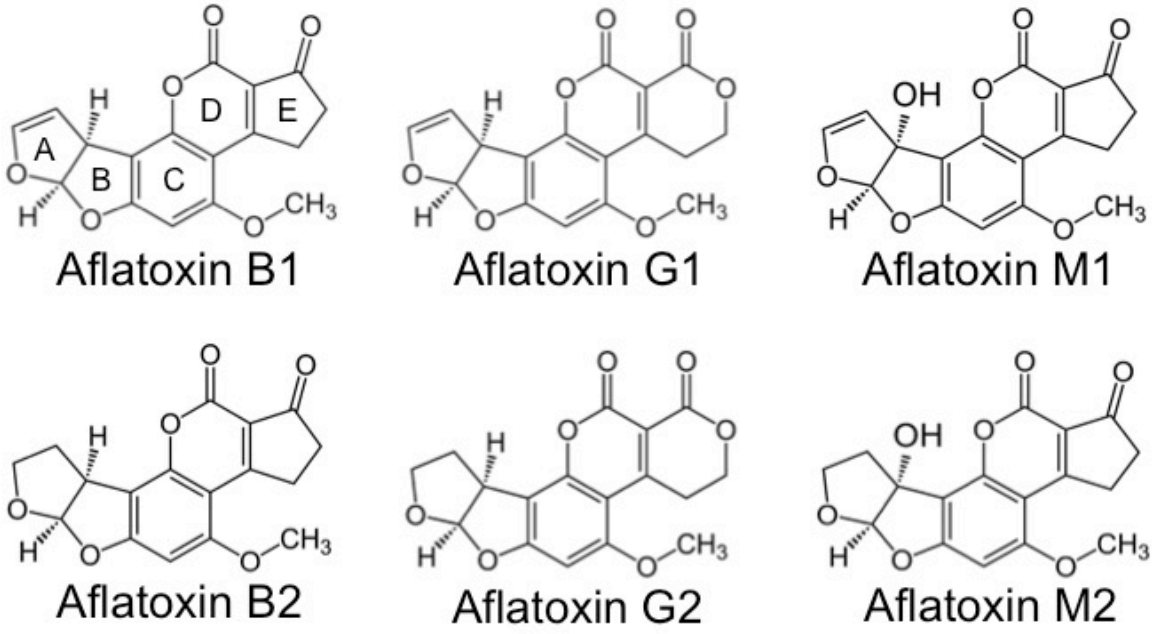


Figure 1.1: Chemical structures of the primary 6 Aflatoxins: The lettering inside the chemical structure of Aflatoxin B1 represents the various rings – A and B make up the furofuran moiety, C is the aromatic ring, D is the lactone ring and E is either a pentone or lactone ring

CHAPTER 2

AFLATOXIN-EXPOSURE OF *Vibrio gazogenes* AS A NOVEL SYSTEM FOR THE GENERATION OF AFLATOXIN SYNTHESIS INHIBITORS[1]

1**Gummadidala** PM, Chen YP, Beauchesne KR, Miller KP, Mitra C, Banaszek N, Velez-Martinez M, Moeller PD, Ferry JL, Decho AW, Chanda A. Front Microbiol. 2016 Jun 3;7:814. Reprinted here with permission of publisher

2.1 ABSTRACT

Aflatoxin is a mycotoxin and a secondary metabolite, and the most potent known liver carcinogen that contaminates several important crops, and represents a significant threat to public health and the economy. Available approaches reported thus far have been insufficient to eliminate this threat, and therefore provide the rational to explore novel methods for preventing aflatoxin accumulation in the environment. Many terrestrial plants and microbes that share ecological niches and encounter the aflatoxin producers have the ability to synthesize compounds that inhibit aflatoxin synthesis. However, reports of natural aflatoxin inhibitors from marine ecosystem components that do not share ecological niches with the aflatoxin producers are rare. Here we show that a non-pathogenic marine bacterium, *Vibrio gazogenes,* when exposed to low non-toxic doses of aflatoxin B_1, demonstrates a shift in its metabolic output and synthesizes a metabolite fraction that inhibits aflatoxin synthesis without affecting hyphal growth in the model aflatoxin producer, *Aspergillus parasiticus.* The molecular mass of the predominant metabolite in this fraction was also different from the known prodigiosins, which are the known antifungal secondary metabolites synthesized by this *Vibrio*. Gene expression analyses using RT-PCR demonstrate that this metabolite fraction inhibits aflatoxin synthesis by down-regulating the expression of early-, middle- and late- growth stage aflatoxin genes, the aflatoxin pathway regulator, *aflR* and one global regulator of secondary metabolism, LaeA. Our study establishes a novel system for generation of aflatoxin synthesis inhibitors, and emphasizes the potential of the under-explored *Vibrio*'s silent genome for generating new modulators of fungal secondary metabolism.

2.2 INTRODUCTION

Aflatoxins are a group of secondary metabolites that are synthesized primarily by food-borne fungi such as *Aspergillus parasiticus* and *Aspergillus flavus*. These *Aspergilli* contaminate a variety of economically important crops such as corn, wheat, peanuts, tree nuts, dried fruits, vegetables, and medicinal plants in tropical and subtropical areas worldwide (Trail et al., 1995, Bennett and Klich, 2003, Chanda et al., 2009, Georgianna and Payne, 2009). Aflatoxin B_1 is the most potent liver carcinogen known and its contamination in food and feed is a significant risk factor of liver cancer risk in humans and animals (CAST, 2003, Liu and Wu, 2010). With liver carcinomas already being the third leading cause of cancer-related mortality worldwide, the global increase in prevalence of hepatitis B virus (HBV) and immunocompromised population has increased the risk of aflatoxin-induced liver cancer (Liu and Wu, 2010). The elimination of aflatoxin accumulation in food and feed, therefore, is of primary importance for reducing its global burden on public health and economy.

Common agricultural approaches used for prevention of aflatoxin contamination in crops include use of fungicides, biocontrol agents and fungi-resistant plants, crop rotation, choice of a plantation time that avoids the aflatoxin-conducive climatic conditions, and control of environmental factors during post-harvest (Kabak et al., 2006, Wu and Khlangwiset, 2010a, Wu and Khlangwiset, 2010b, Cary et al., 2011). However, most of these strategies are expensive, time-consuming and have demonstrated limited success. To complement these conventional strategies, the use of compounds and extracts, collected from plants and microbes that share ecological niches with the aflatoxin producers, are becoming increasingly popular (Holmes et al., 2008). Examples

of these natural compounds include a variety of naturally derived volatile compounds (Greene-McDowelle et al., 1999, Zeringue, 2000, Roze et al., 2004, Roze et al., 2007, Roze et al., 2011). Despite the significant efforts in discovering aflatoxin biocontrol agents, over 55 billion people worldwide still suffer from uncontrolled exposure to aflatoxin (Strosnider et al., 2006), resulting in an *est*. 25,200 to 155,000 liver cancer cases globally (Liu and Wu, 2010). Chronic low-level exposure to aflatoxins and other carcinogenic mycotoxins remains a serious health threat in the US (Kensler et al., 1992) and it is estimated that children in rural areas of the southern US ingest ~40 μg aflatoxin each day through contaminated food; a situation contributing to the significant rise in aflatoxin-induced liver cancer cases (Stoloff, 1976, Van Rensburg, 1977). NIH statistics indicate that 16,600 new cases of aflatoxin-induced liver cancer annually in the US (Kensler et al., 2011). Therefore, the aflatoxin monitoring programs and the destruction and/or decontamination of agricultural commodities, which are adopted to meet aflatoxin levels imposed by regulations from US and Europe for food and feed, remain an expensive and time-consuming process. Hence development of additional novel methodologies and compounds for aflatoxin elimination is essential.

Vibrio gazogenes is an estuarine Gram-negative bacterium that is well-known for its ability to synthesize industrially-relevant proteins such as amylases and proteases (Ratcliffe et al., 1982) and bactericidal and fungicidal pigments, magnesidin A (Imamura et al., 1994), prodigiosins and cycloprodigiosins (Allen et al., 1983). Previous studies have also shown that random mutations in this bacterium with 1-methyl-3-nitro-l-nitrosoguanidine expanded its metabolic output and activated the synthesis of additional bactericidal prodigiosin-related pigments, norprodigiosin and propyl prodigiosin

(Alihosseini et al., 2010). This prompted us to hypothesize that a portion of the bacterium's metabolic potential remains silent under normal growth conditions, and can be activated by genetic and environmental perturbations. In this study, we conducted alterations of metabolism in *V. gazogenes* through exposures to non-toxic doses of the mycotoxin, aflatoxin. While aflatoxin B_1 has been reported to bind to several probiotic bacteria (Kabak et al., 2009) and has also demonstrated the ability to alter bioluminescence responses in *V.* fischeri (Li et al., 2011), there remains a lack of understanding on how interaction of aflatoxin B_1 or other mycotoxins affect fundamental bacterial cell biology. To our surprise, aflatoxin exposure to *V. gazogenes* diminished prodigiosin release into the growth medium, but additionally resulted in the production of a new compound that demonstrated the ability to specifically-inhibit aflatoxin synthesis in the model aflatoxin producer, *A. parasiticus*. Here we report the findings of this study. We establish a novel system for generation of aflatoxin-inhibitors and provide a new avenue in our fundamental understanding of *Vibrio* cell biology.

2.3 MATERIALS AND METHODS

2.3.1 Strains, media, and growth conditions

A. parasiticus, SU-1 (ATCC 56775), a wild-type aflatoxin producer. The strain was grown on 100 mm petri dishes containing potato dextrose agar for 2 weeks. Fresh spores collected from these colonies were used for all the experiments in this study that involved the use of SU-1. In these experiments the fungus was grown in aflatoxin-inducing yeast-extract-sucrose (YES); a rich growth medium (containing 2% w/v yeast extract, 6% w/v sucrose, pH 5.8), by inoculation of 10^4 spores per mL of liquid medium

and incubated in the dark (29°C; shaking at 150 rpm). The bacterium *Vibrio gazogenes* ATCC 43942 (Farmer, Hickman-Brenner et al. 1988), that was originally isolated from sea water, was grown in Difco Marine Broth 2216 (BD Biosciences, Sparks, MD) at 28°C in a shaking incubator (190 rpm).

2.3.2 Growth measurements of *A. parasiticus* and *V. gazogenes*

All fungal growth quantifications were performed using dry weight measurements. Briefly, the mycelia were filtered out of the growth media using a miracloth (Millipore, Billerica, MA) and dried at 75°C for 6 hrs and the final weight was recorded. All *Vibrio* growth measurements were performed using absorbance readings of growth media at 600 nm.

2.3.3 Aflatoxin exposure experiments, extraction and analysis of *Vibrio* metabolites

Aflatoxin B_1 was commercially obtained (Sigma). Three different doses (0.1, 0.2, or 0.3 µg/mL) of aflatoxin B_1 were added to the *Vibrio* growth medium at the start of the culture. In the control flask only the vehicle (70% Methanol) was added. To extract the metabolites from *V. gazogenes* the cells were first harvested by centrifugation and extracted with 60 mL acetone. A portion of the filtrate was concentrated by evaporation under N_2 gas. The concentrate was loaded onto a silica gel column (1.2 x 15 cm) and eluted with dichloromethane : methanol (80:1.5). The fractions were then purified on a silica gel column using chloroform and methanol (50:2). After purification the fractions were concentrated by evaporation under N_2 gas and re-suspended in 1 mL methanol for spectral analysis.

2.3.4 ARMs exposure experiments and aflatoxin comparisons

Comparative semi-quantitative estimations of accumulation of aflatoxin in growth medium was performed using thin layer chromatography (TLC) of the growth medium as described previously (Banerjee, Gummadidala et al. 2014)

2.3.5 Total RNA purification and transcript analysis

Isolation of total RNA from fungal cells exposed to aflatoxin response metabolites from *V. gazogenes* was performed using 30h old cultures. This is a time point that corresponds to the activation of secondary metabolism (hence the expression of aflatoxin genes in *A. parasiticus*) under the growth conditions adopted in this study (Roze, Arthur et al. 2007). Purification of total RNA and preparation of complementary DNA was performed as described previously (Chanda, Roze et al. 2009). Transcript levels were quantified by performing quantitative real-time PCR assays using SsoAdvanced universal SYBR Green supermix (BioRad Laboratories, Hercules, CA) and gene-specific forward and reverse primers (Table 2.1) that were designed using Primer3 online software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Reactions were performed in a CFX96 thermal cycler (Bio-Rad Laboratories, Hercules, CA). As described for previous gene expression studies in *A. parasiticus* (Roze, Arthur et al. 2007, Chanda, Roze et al. 2009), expression value of each gene was obtained from the threshold cycle values were normalized against β-tubulin (the house keeping gene) in each sample. All RT-PCRs were performed in triplicates for each gene per sample. Data analyses were performed using CFX Manager software (Bio-Rad Laboratories).

2.3.6 Statistical analysis

All statistical tests were performed using GraphPad Prism Software (GraphPad, CA, USA). Statistical analyses to determine for statistical significance of differences between control *versus* experimental groups were determined using one-way ANOVA (with sample size 3). An unpaired t-test was used to determine the gene expression effects of ARMs on *A. parasiticus* compared to the untreated samples. Significance was set at $p<0.05$.

2.4 RESULTS

2.4.1 Aflatoxin B_1 exposures do not inhibit *V. gazogenes* growth

As a first step in understanding how *V. gazogenes,* responds to aflatoxin B_1, we investigated the effect of three different doses of aflatoxin B_1 on the growth of *V. gazogenes.* The doses, 10 ppb, 30 ppb and 50 ppb were either below, approximately equal to or 5-fold higher than the highest-allowed aflatoxin level (20 ppb) in food and feed (Mazumder and Sasmal 2001, CAST 2003 , Liu and Wu 2010). Time-course absorbance readings were recorded to compare the growth rates of *V.* gazogenes, in presence of aflatoxin B_1, with untreated-controls. As shown in figure 2.1, none of the aflatoxin B_1 doses demonstrated any significant effect on the growth of *V. gazogenes*.

2.4.2 Aflatoxin B_1 exposures do not inhibit prodigiosin synthesis

Next, we investigated the effect of aflatoxin B_1 exposures on the production of prodigiosins by *V. gazogenes*. The prodigiosin fraction was obtained from cells (either untreated control cells or cells exposed to aflatoxin B_1) using our optimized laboratory

protocol (see methods). Since the prodigiosins exhibit an absorbance peak at 530 nm (figure 2.2a), this wavelength was used to compare prodigiosin levels between experimental treatments and controls at three different time-points of growth (12h, 18h and 42h). Our results (figure 2.2b) demonstrated that although cells exposed to aflatoxin B_1 showed a minor increase in absorbance values compared to the untreated samples, the difference was not statistically significant.

2.4.3 Additional *V. gazogenes* metabolite fraction obtained by bacterial exposure to aflatoxin B_1: aflatoxin response metabolites (ARMs)

While growth and prodigiosin production by *V. gazogenes* was not affected in presence of aflatoxin B_1, we observed that exposure to aflatoxin B_1 resulted in a distinct alteration of color in the growth medium (figure 2.3a) suggesting the presence of a different metabolite compared to untreated cells. Based on the 'blue-shift' in color of the growth medium (bright red to orange) upon addition of aflatoxin B_1, we hypothesized that the bacterium synthesizes an additional metabolite fraction under these conditions with a corresponding absorbance lower than that of the prodigiosin fraction. To test this, we performed UV-Vis spectral analysis on the metabolite fractions of aflatoxin B_1-treated samples. The *Vibrio* metabolite fractions obtained from aflatoxin B_1 treated samples revealed a new absorbance peak at 470 nm, in addition to the prodigiosin peak at 530 nm (Figure 2.3b). This suggested that aflatoxin B_1 exposure affects the cellular metabolism of *V. gazogenes* resulting in a different metabolite profile, compared to the untreated control. Here, we denote this additional metabolite fraction in response to aflatoxin B_1 exposure as 'aflatoxin response metabolites (ARMs)'.

2.4.4 ARMs do not inhibit *A. parasiticus* growth but inhibits aflatoxin synthesis

Next we proceeded to investigate whether ARMs affect the aflatoxin synthesis in the model aflatoxin B_1 producer, *A. parasiticus*. The activation of ARM production by *Vibrio* occurred upon addition of aflatoxin B_1 to their growth medium. Therefore, we envisioned this alteration of metabolite profiles as a defensive response from *Vibrio* cells. We hypothesized that ARMs will have a specific inhibitory effect on aflatoxin synthesis in the producer cells. To test this we studied the growth and aflatoxin production by *A. parasiticus* in presence of two different doses of the ARMs metabolite fraction (1 µg and 2 µg per mL of growth medium); the doses were chosen arbitrarily. To compare the levels of aflatoxin biosynthesis in *A. parasiticus* exposed to ARMs exposed with the untreated cells, we adopted a semi-quantitative approach in which we compared the intensities of aflatoxin B_1 and aflatoxin B_2 bands on the thin-layer chromatography (TLC) plates (see methods). As predicted, our TLC results generated from 40h cultures of *A. parasiticus,* demonstrated that ARMs applied at the concentration of 2 µg per mL of growth medium inhibited both aflatoxin B_1 and aflatoxin B_2 by approximately 2-fold (figure 2.4a). Since the drop in aflatoxin synthesis could also have resulted from the inhibition of *A. parasiticus* growth, we next compared the dry-weights of the *A. parasiticus* mycelia exposed to 1 and 2 µg per mL of ARMs extract with the untreated control mycelia. As shown in figure 2.4b, addition of ARMs to the growth medium did not result any significant change in *A. parasiticus* dry weight, suggesting that inhibition of aflatoxin synthesis in *A. parasiticus* by ARMs was a direct effect and not a growth dependent effect.

2.4.5 ARMs metabolite fraction displays a different HPLC trace compared to prodigiosin fraction

Our UV-Vis spectral analysis suggested that ARMs were synthesized by *V. gazogenes* upon exposure to aflatoxin. We then proceeded to confirm that this fraction (peak absorbance at 470 nm) was composed of metabolites of molecular masses that are different from the *Vibrio*'s prodigiosin fraction (peak absorbance at 530 nm). As shown in figure 2.5, HPLC traces showed that the prodigiosin fraction predominantly demonstrated the expected molecular weight of 324 D, corresponding to the known prodigiosin. The HPLC trace of ARMs on the contrary was clearly different, with a predominantly displayed molecular mass 232 D, which demonstrate that the metabolite fraction of ARMs was chemically different from the *Vibrio'*s prodigiosin fraction. These results suggest that the differential metabolite profile in response to aflatoxin exposure can occur either due to synthesis of new metabolites by *V. gazogenes* or due to breakdown of prodigiosins resulting in novel smaller molecules with aflatoxin synthesis inhibitory activity.

2.4.6 ARMs inhibit A. parasiticus aflatoxin biosynthesis at the level of transcript accumulation

The fungal growth and aflatoxin results then prompted us to investigate whether aflatoxin biosynthesis was inhibited at the level of transcript accumulation of aflatoxin genes. To conduct this analysis we performed a quantitative comparison of transcript accumulation of two genes *nor*-1, and *ver-1* that encode two enzymes, Nor-1, Vbs and Ver-1 respectively involved in the aflatoxin biosynthetic pathway (Chanda, Roze et al.

2009). Activation of these genes in *A. parasiticus* occurs at 24h, and transcripts of all aflatoxin enzymes accumulate by 30h, when the fungus is grown in YES growth medium (Roze, Arthur et al. 2007). Hence we chose to examine the effects of ARMs extract on *A. parasiticus* at three different time-points, 24h, 30h and 40h, a time-point when aflatoxin is synthesized by the fungus at peak levels (Roze, Arthur et al. 2007). In addition to these genes, we also compared the transcript accumulation of the aflatoxin pathway regulator, *aflR,* at the same time points. As shown in figure 2.6, *nor-1, ver-1* as well as the *aflR* genes transcript levels demonstrated ≥5 fold reduction in presence of ARMs extract compared to the vehicle control by 30h. Hence, our results suggest that ARMs extract reduces aflatoxin synthesis at the level of transcript accumulation.

2.4.7 ARMs inhibit transcript accumulation of the secondary metabolism global regulator, *laeA* but not *veA*

Since the regulatory network of the aflatoxin biosynthesis pathway is integral to the global network of secondary metabolism in *A. parasiticus* as described in a recent review by Brakhage (Roze, Arthur et al. 2007), we also proceeded to investigate whether, ARMs target the global regulation of secondary metabolism. One key global regulatory complex of fungal secondary metabolism is the VeA complex (Bayram, Krappmann et al. 2008). Central to this complex is the cross-talk between the two global regulators, LaeA, a methyltransferase that is key to the epigenetic regulation of aflatoxin biosynthetic pathway (Bok and Keller 2004), and VeA, a light responsive regulator that migrates from cytoplasm to the nucleus in absence of light to form the VeA complex with LaeA and other components in the complex (Bayram, Krappmann et al. 2008). In this study we investigated whether ARMs affect transcript accumulation of either *laeA* or *veA* genes.

To our surprise we found that, while no significant changes occurred in *veA* transcripts, the *laeA* transcript accumulation was reduced by ~2 fold by 30h and ~4 fold by 40h (figure 2.7), suggesting that ARMs inhibit aflatoxin biosynthesis at least in part, through inhibition of LaeA.

2.5 DISCUSSION

Here we demonstrate the feasibility of a novel system for generation of aflatoxin biosynthesis inhibitors, a concept that is analogous to the generation of antibodies upon antigen exposure. Our data reveal that the estuarine bacterium *V. gazogenes*, upon aflatoxin exposure, produces a metabolite profile that is chemically different from untreated-cells. Upon isolation of the ARMs and applying them on the aflatoxin producer cells, we found that the metabolites inhibit aflatoxin biosynthesis at the levels of transcript accumulation. Based on our current study we propose two possible explanations underlying this inhibition (illustrated in the schematic in figure 2.7). One possible mechanism of inhibition is through the regulation of the *laeA* gene activation. The *laeA* transcripts dropped by 2-4 fold during 30h to 40h time points suggesting that ARMs inhibit the formation of the *Velvet* complex, a protein complex comprising LaeA protein that regulate fungal secondary metabolism (Bayram, Krappmann et al. 2008). Alternatively, it is also possible that in addition to *laeA* mediated inhibition ARMs inhibit the activation of aflatoxin genes directly. Fungal growth was not inhibited during the ARMs-mediated inhibition of aflatoxin biosynthesis, suggesting that the metabolites target secondary metabolism specifically. Future studies will identify the molecule(s) within ARMs that results in the aflatoxin inhibition. From our current preliminary studies, we postulate that two or more compounds generated in response to aflatoxin

exposure act either complementarily or synergistically to inhibit aflatoxin synthesis inhibition. These collaborative effects will be determined in those functional characterization studies with the purified compounds.

It is important to emphasize that specific aflatoxin inhibitory natural products that have been characterized to-date were reported primarily from terrestrial organisms whose ecological domains likely overlap with those of the aflatoxin producers. Examples include natural products and volatiles from plants (Cleveland, Carter-Wientjes et al. 2009, Roze, Koptina et al. 2011, Chitarrini, Nobili et al. 2014), fungi (Ono, Sakuda et al. 1997, Yoshinari, Noda et al. 2010, Hua, Beck et al. 2014) and bacteria (Jermnak, Chinaphuti et al. 2013, Wang, Yan et al. 2013, Kong, Chi et al. 2014). Our study provides the first evidence, to the best of our knowledge, of an organism that demonstrates the ability of synthesizing aflatoxin inhibitors, while not sharing ecological niches with aflatoxin producers at all. Also this is the first report, to the best of our knowledge, of a *Vibrio*-producing metabolite(s) that specifically inhibit aflatoxin biosynthesis without affecting fungal growth. It is possible that mycotoxin triggered synthesis of mycotoxin inhibitors is a phenomenon that is conserved in the *Vibrio* species. Alternatively, it is also possible that *Vibrio gazogenes* is a chemically-gifted organism that has genetically evolved with the rising mycotoxin levels in the environment with global changes in climate (Kolpin, Schenzel et al. 2014, Rangel, Alder-Rangel et al. 2015).

The effect of ARMs mediated down-regulation of *laeA* gene, but not *veA* gene suggests that the metabolites target cellular signaling receptors that specifically regulate *laeA* gene expression. Since LaeA is a global regulator of secondary metabolism and influences several mycotoxin biosynthetic pathways (Keller, Turner et al. 2005), we

anticipate that aflatoxin inhibitor within ARMs will inhibit other mycotoxins as well. Hence, for our follow-up studies we will categorize these as secondary metabolism specific inhibitors instead denoting these as specific inhibitors against aflatoxin biosynthesis.

Current investigations in our laboratory reveal that other fungal secondary metabolites trigger synthesis of metabolite fractions in *V. gazogenes* that demonstrate different HPLC traces compared to either prodigiosins or ARMs fractions. These results implicate the need to examine the regulation of *Vibrio* genes under different environmental signals. It appears from our studies that many areas of the *Vibrio* genome remain silent under standard laboratory growth conditions and can be activated as needed to generate metabolites that are relevant to the public health. Our future studies will shed light on these silent areas of the *V. gazogenes* genome that encode the biosynthesis of the secondary metabolism modulatory metabolites; the knowledge will enable us to clone these areas on plasmids and engineer them as needed with the goal of purifying these compounds in large quantities.

Table 2.1: List of PCR primers used for this study

Genes	Primer sequences
(1) *nor-1*	F 5′-CACTTAGCCAGCACGATCAA-3′
	R 5′-ATGATCATCCGACTGCCTTC-3′
(2) *ver-1*	F 5′-AACACTCGTGGCCAGTTCTT-3′
	R 5′-ATATACTCCCGCGACACAGC-3′
(3) *β-tubulin*	F 5′-TCTCCAAGATCCGTGAGGAG-3′
	R 5′-TTCAGGTCACCGTAAGAGGG- 3′
(4) *aflR*	F 5′-ACCTCATGCTCATACCGAGG-3′
	R 5′-GAAGACAGGGTGCTTTGCTC- 3′
(5) *veA*	F 5′-TCCAGCTATCCCAAGAATGG-3′
	R 5′-TAATCCCCCGATAGAGCCTT-3′
(6) *laeA*	F 5′-ATGGGGTGTGGAAGTGTGAT-3′
	R 5′-ATCGGTAAAACCAGCCTCCT-3′

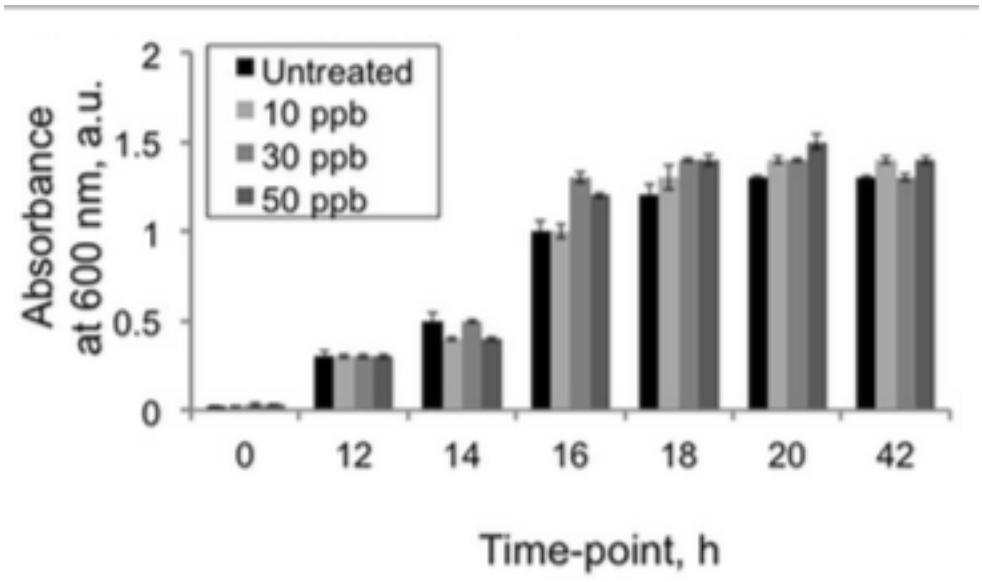


Figure 2.1: Effect of Aflatoxin B1 exposure on *Vibrio gazogenes* growth: Growth comparisons were performed using comparisons of 600 nm absorbance values between untreated *V. gazogenes* cultures and cultures were supplemented with 10, 30, and 50 ppb of aflatoxin B1. Statistical significance of two-tailed *p*-values were determined using an unpaired *t*-test with sample size of 3 and significance set as $p < 0.05$.

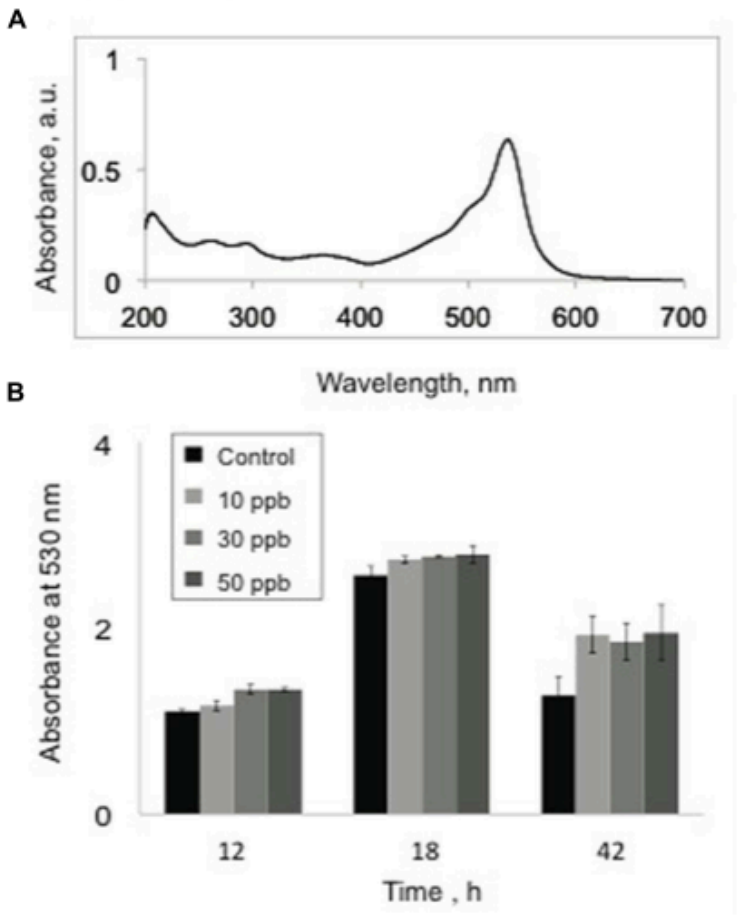


Figure 2.2: Effect of aflatoxin B1 exposure on prodigiosin production: (A) UV-Vis spectral profile of a prodigiosin-rich metabolite fraction demonstrating peak absorbance at 530 nm. (B) Comparison of absorbance values at 530 nm, of methanol extracts from untreated *V. gazogenes* cultures and cultures were supplemented with 10, 30, and 50 ppb of aflatoxin B1. Statistical significance of two-tailed *p*-values were determined using an unpaired *t*-test, with n=3, and $p < 0.05$ as significance level.

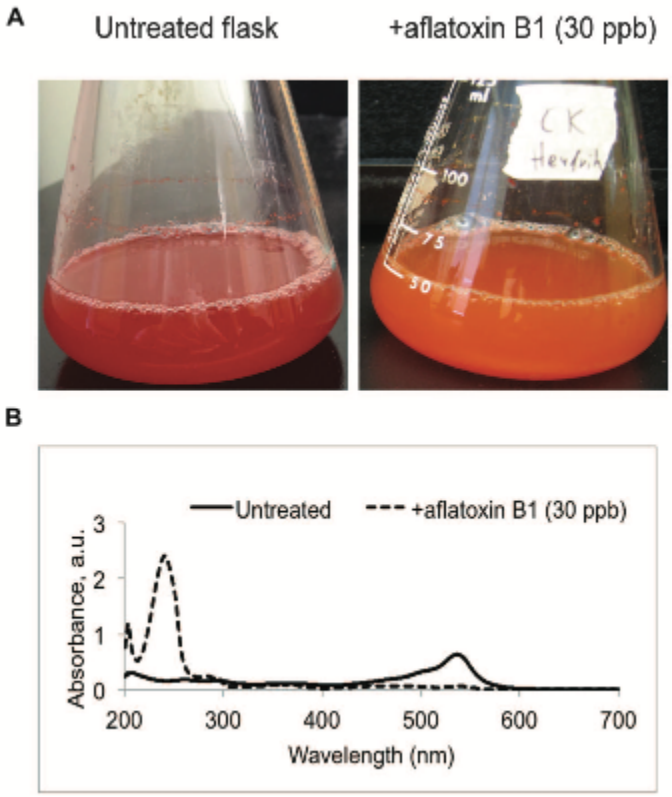


Figure 2.3: Aflatoxin-response metabolites (ARMs) produced by the bacterium *V. gazogenes* during exposure to aflatoxin B1 (AFB1). (A) Representative flasks demonstrating the differences in appearance of untreated *V. gazogenes* cultures and the aflatoxin B1 supplemented cultures. (B) Comparison of UV-Vis profiles of the methanol extracts from untreated and supplemented *V. gazogenes* cultures.

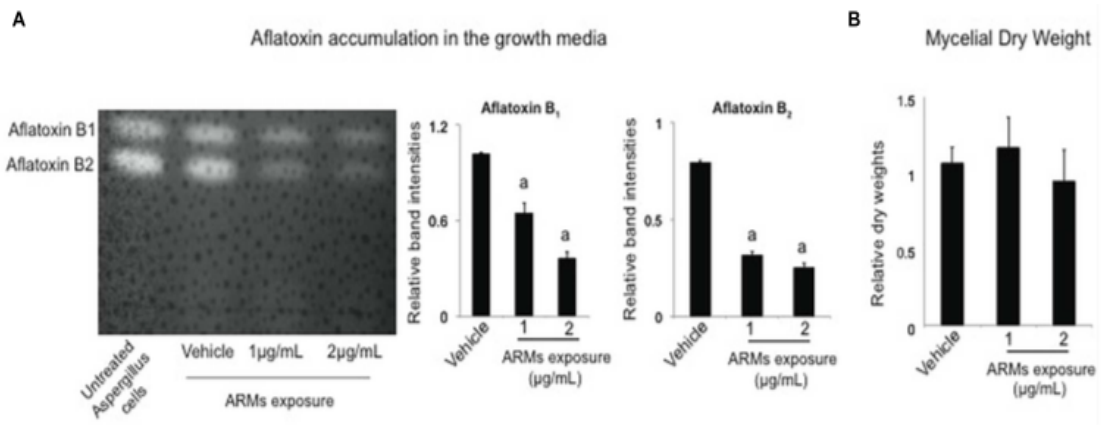


Figure 2.4: Effect of ARMs on aflatoxin biosynthesis and fungal growth. (A) Effect on aflatoxin accumulation in the growth media: Left panel, a representative TLC plate providing a qualitative comparison of aflatoxin accumulation in the untreated culture and cultures that were supplemented with 1 and 2 µg/mL ARMs extract and the vehicle (DMSO). Right panel, semi-quantitative comparative comparisons of band intensities of aflatoxin B1 and aflatoxin B2. a, significant difference in band intensity compared to the vehicle control. (B) Effect on growth: Comparison of dry-weight measurements. Bars represent measurements relative to the dry-weight of untreated cells. Statistical significance of two-tailed *p*-values were determined using an unpaired *t*-test, with sample size of $n = 3$ and $p < 0.05$ set as level of significance.

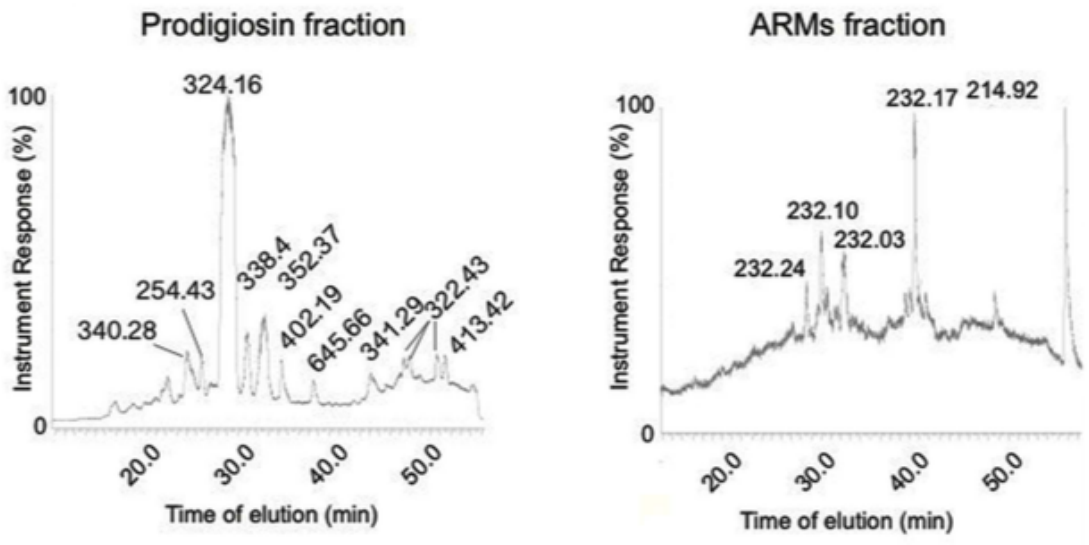


Figure 2.5: Comparison of HPLC traces of ARMs extract and the prodigiosin fraction of *V. gazogenes*.

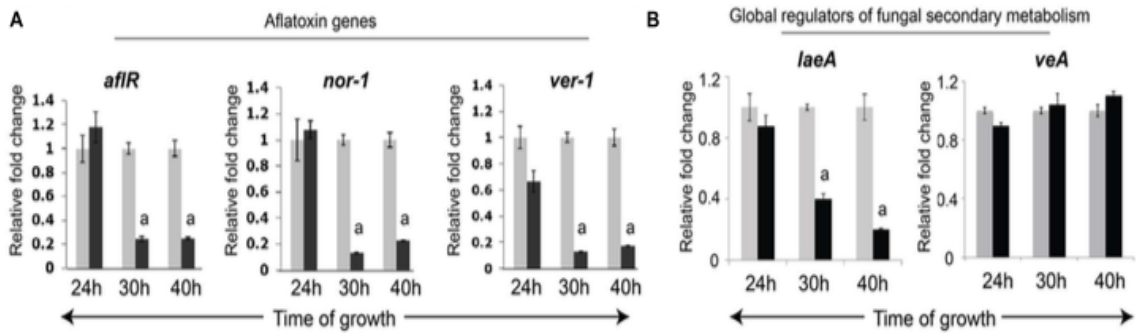


Figure 2.6: Effects of ARMs on *Aspergillus parasiticus* gene expression. (A) Comparison of transcript accumulation of aflatoxin-synthesis regulatory genes in *A. parasiticus*. mRNA levels for each gene were observed at 24 h (aflatoxin synthesis start point), 30 and 40 h time points (aflatoxin synthesis is activated and reaches peak levels by 40 h). Black bars, cells grown in presence of ARMs (2 µg/mL), Gray Bars, DMSO (vehicle) control. (B) Comparison of transcript accumulation of two global regulators of secondary metabolism, *veA* and *laeA* at the same time-points. Statistical significance of difference in transcript accumulation between control and ARMs-treated cells were determined using an unpaired *t*-test with sample size of 3 and two tailed $p < 0.05$ set as level of significance. a, $p < 0.05$.

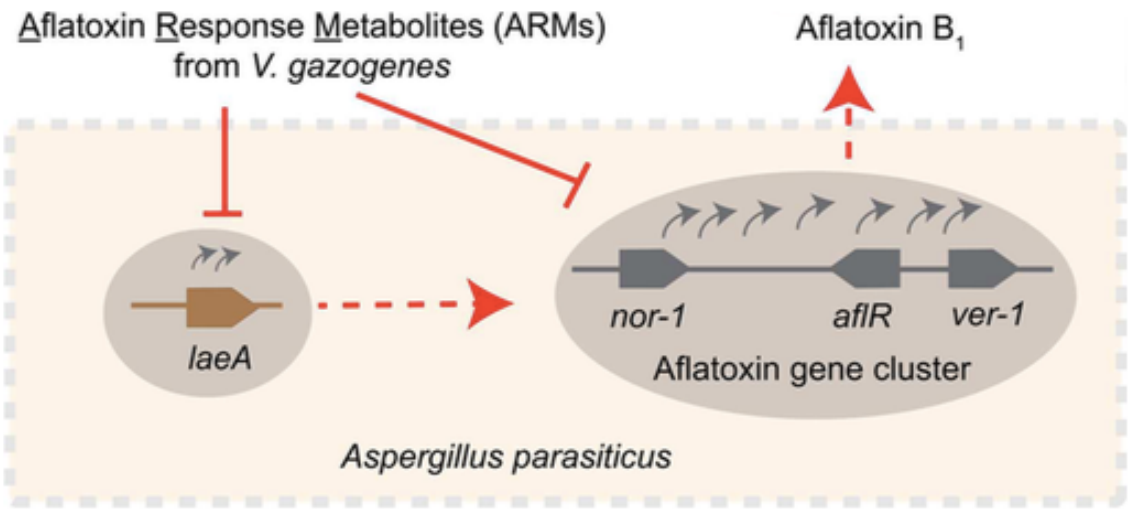


Figure 2.7: Schematic representation of the inhibitory effect of ARMs on aflatoxin biosynthesis: Current study demonstrates that ARMs inhibit aflatoxin biosynthesis in *A. parasiticus* at the level of gene expression. We hypothesize that the inhibition of aflatoxin genes as exemplified by the decreased *nor-1*, *ver-1*, and *aflR* transcripts in presence of ARMs can be the effect of one or both of the following: (1) inhibition of *laeA* expression, which in turn can have inhibitory impact on the activation of the aflatoxin genes, or (2) a dual inhibition caused by direct inhibition on aflatoxin gene cluster activation along with a *laeA* mediated inhibition. Red dotted arrows, regulatory roles established in previous studies, red solid lines, inhibitory effect, gray curved arrows, gene activation, gray solid line, schematic of the aflatoxin gene cluster showing relative positions of *nor-1*, *ver-1,* and *aflR* in the cluster, brown solid line, *laeA* gene.

CHAPTER 3

COMPLETE GENOME SEQUENCE OF *Vibrio gazogenes* ATCC 43942[1]

[1]Gummadidala PM, Holder ME, O'Brien JL, Ajami NJ, Petrosino JF, Mitra C, Chen YP, Decho AW, Chanda A. Genome Announc. 2017 Jul 27;5(30). Reprinted with permission from publisher.

3.1 ABSTRACT

Vibrio gazogenes ATCC 43942 has the potential to synthesize a plethora of metabolites in response to environmental triggers, which are of clinical and agricultural significance. The complete genomic sequence of *Vibrio gazogenes* ATCC 43942 is reported herein contributing to the knowledgebase of strains in the *Vibrio* genus.

3.2 INTRODUCTION

Vibrio gazogenes is an estuarine Gram-negative bacterium that is known for its ability to synthesize industrially relevant proteins such as amylases and proteases (Ratcliffe, Sanders et al. 1982), and bactericidal and fungicidal pigments, magnesidin A (Imamura, Adachi et al. 1994), prodigiosins, and cycloprodigiosins (Allen, Reichelt et al. 1983).

V. gazogenes ATCC 43942 was recently studied by our laboratory for its response to aflatoxin, a hepatocarcinogen and a mycotoxin that is produced from a group of filamentous fungi under the genus *Aspergilli.* The bacterium demonstrated the ability to generate a group of metabolites (named *aflatoxin response metabolites*, denoted as ARMs) that were able to inhibit aflatoxin synthesis in the aflatoxin producer, *Aspergillus parasiticus* (Gummadidala, Chen et al. 2016). Also, in our ongoing (unpublished) studies, we have consistently observed the ability of this *Vibrio* strain to degrade mycotoxins and generate a unique set of antibiotics that are active against multiple antibiotic resistant bacterial strains. These observations prompted us to categorize this bacterium as clinically and agriculturally significant, and have provided the rationale for sequencing its genome.

Genomic DNA extraction (10 – 20 μg) was performed using PureLink genomic DNA minikit (Invitrogen). The extracted DNA was quantified using Nanodrop 1000 (Thermo Scientific) and quality of the DNA was assessed by running a 1% agarose gel with the DNA gel stain SYBR safe (Life Technologies) and visualized in a ChemiDoc MP system (Bio-Rad). DNA sequencing was performed on the Pacific Biosciences RS II platform. One SMRT cell, yielding 73,434 post-filtered polymerase reads and having an N50 read length of 26,245 bases and a mean read length of 16,358 bases, was used for assembly in Pacific Biosciences's SMRT Analysis v2.3.0 package using the RS_HGAP_Assembly.2 protocol[5]. Quiver was subsequently used to polish the assembly. The finished genomic sequences were annotated with NCBI's Prokaryotc Genome Annotation Pipeline. A high-quality finished version of the *V. gazogenes* genome is reported here as two circular chromosomes and one circular plasmid with a mean coverage of 185x with features as follows:

(1) Chromosome 1 (denoted as Chr_1): size 3,471,064 bp; GC% 45.5; proteins 2,988; rRNA 25; tRNA 87; ncRNA 4; Genes 3153; Pseudogenes 49,

(2) Chromosome 2 (denoted as Chr_2): Chr_2; size 1,303,572 bp; GC% 44.9; proteins 1,102; tRNA 4; Genes 1,138; Pseudogenes 32, and

(3) Plasmid (denoted as P_1): size 11,916 bp; GC% 45.2; proteins 22; Genes 23; Pseudogene 1.

The utility of prodigiosins that are synthesized by *V. gazogenes* ATCC43942 coupled with its ability to produce unique antibiotics and mycotoxin inhibitors under custom designed environmental settings make this strain 'chemically gifted'. In this

context, its finished genomic sequence provides a necessary point of comparison with other *V. gazogenes* strains and bacterial species within the *Vibrio* genus for elucidation of the molecular factors that govern its unique metabolic profile.

3.3 Nucleotide sequence accession numbers(s)

The sequence of *V. gazogenes* ATCC 43942 has been deposited in NCBI GenBank under the accession no(s) that are as follows: CP018835, CP018836 and CP018837.

CHAPTER 4

ACTIVATION OF AFLATOXIN BIOSYNTHESIS ALLEVIATED TOTAL ROS IN *Aspergillus parasiticus*[1]

[1]Kenne GJ*, Gummadidala PM*, Omebeyinje MH, Mondal AM, Bett DK, McFadden S, Bromfield S, Banaszek N, Velez-Martinez M, Mitra C, Mikell I, Chatterjee S, Wee J, Chanda A.Toxins (Basel). 2018 Jan 29;10(2). *Co-first authors. Reprinted with permission from publisher.

4.1 ABSTRACT

An aspect of mycotoxin biosynthesis that remains unclear is its relationship with the cellular management of reactive oxygen species (ROS). Here we conduct a comparative study of the total ROS production in the wild-type strain (SU-1) of the plant pathogen and aflatoxin producer, *Aspergillus parasiticus*, and its mutant strain, AFS10, in which the aflatoxin biosynthesis pathway is blocked by disruption of its pathway regulator, *aflR*. We show that SU-1 demonstrates a significantly faster decrease in total ROS than AFS10 between 24 h to 48 h, a time window within which aflatoxin synthesis is activated and reaches peak levels in SU-1. The impact of aflatoxin synthesis in alleviation of ROS correlated well with the transcriptional activation of five superoxide dismutases (SOD), a group of enzymes that protect cells from elevated levels of a class of ROS, the superoxide radicals (O_2^-). Finally, we show that aflatoxin supplementation to AFS10 growth medium results in a significant reduction of total ROS only in 24 h cultures, without resulting in significant changes in SOD gene expression. Our findings show that the activation of aflatoxin biosynthesis in *A. parasiticus* alleviates ROS generation, which in turn, can be both *aflR* dependent and aflatoxin dependent.

4.2 INTRODUCTION

Filamentous fungi synthesize and release a diverse array of secondary metabolites into their environment, many of which have profound impacts on agriculture, industry, environmental sustainability, and human health (Keller et al., 2005). Many compounds are used as medicines, including statins, penicillin, and other antibiotics. Many others, like aflatoxins and fumonisins, can be life threatening to humans and animals. Aflatoxin

B_1 (AFB_1), a highly carcinogenic secondary metabolite synthesized by a group of Aspergilli, is a life-threatening toxin causing significant morbidity and mortality worldwide, as well as billions of dollars in annual economic losses (Magnussen et al., 2013). Due to the significant human and agricultural impacts of aflatoxin (AF), its biosynthetic pathway is one of the most characterized and widely studied models for understanding fungal secondary metabolism (Roze et al., 2011).

The aflatoxin biosynthesis process is activated by several environmental cues and orchestrated by a complex regulatory network of more than 25 genes and 17 enzymatic steps (Brakhage et al., 2013; Yin et al., 2011; Yu et al., 2004; Chanda et al., 2009). The operation of this network is governed by the interactions of a set of global transcription factors, including LaeA and VeA (Brakhage et al., 2013; Kale et al., 2008; Bayram et al., 2008; Calvo et al., 2004; Calvo et al., 2008; Duran et al., 2007). Upon receiving signals from cell surface receptors, these global transcription factors communicate with pathway-specific transcription factors [examples include AflR (Cary et al., 2006) and GliZ (Scharf et al., 2012; Bok et al., 2006)] to activate specific aflatoxin biosynthesis genes. Many of the enzymes synthesized by this pathway then localize to specific vesicles known as toxisomes (Chanda et al., 2009; Chanda et al., 2010; Roze et al., 2011; Lim et al., 2014; Menke et al., 2013), which provide a platform for the completion of biosynthesis, sequestration, and export of aflatoxin to the environment (Chanda et al., 2009; Chanda et al., 2010; Roze et al., 2011; Lim et al., 2014; Menke et al., 2013).

To manipulate secondary metabolism in fungi for the benefit of public and environmental health, it is essential to understand the motivation for a fungal cell to preserve such an energy-consuming metabolic process with enormously complex

molecular and cellular organization throughout the course of evolution. One of the most commonly hypothesized functions of fungal secondary metabolites is defense against other organisms in the same ecological niche. Antibacterial properties of secondary metabolites like penicillin and other beta-lactam antibiotics are well established in literature (Van Krimpen et al., 1987). Beyond antibacterial properties, reports from Rohlfs et al. (Rohlfs et al., 2007) suggest that aflatoxin and sterigmatocystin protect fungal cells from pests and insects. These studies all suggest that secondary metabolism provides fungi with a survival mechanism in nature.

Several recent studies suggest that secondary metabolism is integrated with primary metabolism and its associated cellular mechanisms (Roze et al., 2011; Chanda et al., 2009; Linz et al., 2012; Roze et al., 2010), which implies that secondary metabolism may have a regulatory impact on other fungal cellular processes as well. One cellular process that appears to be associated with secondary metabolism in fungi is oxidative stress response. Recently, several basic leucine zipper (bZIP) transcription factors in filamentous fungi have been reported in the literature that not only regulate antioxidant genes participating in oxidative stress response, but are also associated with the regulation of secondary metabolism (Roze et al., 2011; Baidya et al., 2014; Hong et al., 2013; Hong et al., 2013; Montibus et al., 2013; Reverberi et al., 2012; Montibus et al., 2015; Yin et al., 2013). These reports are in line with previous reports (Jayashree et al., 2000; Narasaiah et al., 2006; Reverberi et al., 2006; Reverberi et al., 2008) suggesting that oxidative stress induces aflatoxin synthesis in *Aspergillus parasiticus.*

While these lines of evidence collectively demonstrate that the two cellular processes (aflatoxin biosynthesis and intracellular oxidative stress management)

communicate at different regulatory nodes and are co-regulated, the effect of aflatoxin on oxidative stress remains unclear. In this study we address this knowledge gap through a comparative study of total reactive oxygen species (ROS) output between the wild-type *A. parasiticus* and its mutant, AFS10, in which the aflatoxin pathway regulator gene, *aflR*, is disrupted (Cary et al., 2002; Ehrlich et al., 1999). In addition to measuring ROS, we also conducted a comparative assessment of superoxide dismutase (SOD) gene expression. SODs are conserved in eukaryotes and are synthesized in response to intracellular (O_2^-) radicals (a type of ROS) generated as a byproduct of primary cellular functions (Fridovich 1975). To differentiate the aflatoxin-dependent effect on ROS generation from the possible genetic effects (of *aflR* disruption) we also conducted aflatoxin supplementation studies on AFS10. The results of this work provide direct evidence in support of the regulatory role of aflatoxin synthesis on total ROS output and explain the rationale for the co-regulation of oxidative stress with aflatoxin synthesis.

4.3 MATERIALS AND METHODS

4.3.1 Strains, media and growth conditions

Aspergillus parasiticus wild type strain SU-1 (ATCC56775) and the *aflR* disrupted mutant, AFS10 (Ehrlich et al., 1999; Roze et al., 2007), were used for this study. Yeast extract sucrose (YES) (2% yeast extract, 6% sucrose; pH 5.8) was used as the liquid growth medium for the entire study for both strains. Fungal cells were grown for 24 h and 48 h by inoculating 10^7 spores per 100 mL of growth medium and incubating the cells at 29°C in a dark orbital shaker at 150 rpm.

4.3.2 Quantification of ROS

Comparison of ROS concentrations between SU-1 and AFS10 was conducted spectrophotometrically using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) based on a previously described protocol (Chang et al., 2011). Equal weight (0.5 g) of mycelia from a 24 and 48 h culture was placed into 1 mL of freshly made 1 μM DCFH-DA in phosphate buffered saline (PBS). After 4 h of incubation in the dark at room temperature (25°C), the fluorescent yield of the DCFH-DA oxidation product, dichlorofluorescin (DCF), was measured using a Victor™ X3 2030 Multilabel Reader (PerkinElmer, Waltham, MA, USA) with an excitation/emission wavelength of 490/525 nm.

4.3.3 Identification of Superoxide Dismutase genes

Since functional characterization of the SOD genes in *A. parasiticus* has not yet been completed, a bioinformatics analysis was performed to identify SOD gene sequences to allow for a comparative expression analyses to address our hypothesis. The SOD genes analyzed in this study were identified by searching for "superoxide dismutase" in the accessible genome database (Yu et al., 2008) of *A. flavus*, a close relative of *A. parasiticus* that exhibits ~98–100% amino-acid sequence identity with *A. parasiticus* proteins that have been sequenced (Roze et al., 2011). The search rendered five annotated amino-acid sequences which were then queried in the PROSITE database (Sigrist et al., 2013) against the 390 available SOD genes to investigate whether they contained (a) the conserved functional domains typical of SODs, or (b) motifs with a high probability of occurrence that are commonly present in the SOD genes. Details of these sequences and queries can be found in table 4.1.

4.3.4 RNA extraction, purification and cDNA synthesis

Total RNA was extracted from cells harvested using a TRIzol-based (TRIzol Reagent; Invitrogen, Carlsbad, CA, USA) method previously described (Chanda et al., 2009). Within 24 h of extraction, RNA cleanup was performed using a Qiagen RNEasy Cleanup Kit (Qiagen, Valencia, CA, USA), and samples were stored at −80°C. Total RNA was then reverse transcribed to cDNA using iScript™ cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA). All samples were checked for concentration and purity after each step using a NanoDrop 2000 Spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA). All cDNA samples were stored at −20°C until subsequent PCR quantification.

4.3.5 Quantitative PCR Assays

Expression of SOD genes was examined by quantitative PCR assays (qPCR) using SsoAdvanced Universal SYBR Green Supermix (BioRad Laboratories, Hercules, CA) and gene specific forward and reverse primers (table 4.2) designed using Primer3 online software (Ye et al., 2012). Reactions were performed per BioRad SYBR Green protocol guidelines and quantified using a CFX96 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA).

The 18s ribosomal DNA was used as a reference in the gene expression experiments, with β-tubulin used as a positive control rather than a reference gene. This use of β-tubulin in this manner provided proof of consistent quantification across all experiments and revealed an expected range of variation within the protocol. Expression of each SOD gene was obtained from the threshold cycle values normalized against 18s

rDNA in each sample. All RT-PCRs were performed in triplicate for each gene per sample. For quantitative comparison of gene expression, the expression values for each target gene at the early stationary phase (48 h) were expressed as the fold change relative to the 24 h time point to reflect changes associated with the initiation of aflatoxin biosynthesis, which begins at 30 h (Roze et al., 2015). All data analysis was performed using CFX Manager software (Version 3.1, Bio-Rad Laboratories, Hercules, CA, USA, 2012).

4.3.6 Aflatoxin supplementation experiments

For aflatoxin supplementation studies, 0.5 g of AFS10 mycelia were collected from YES media at 24 and 48 h and each placed in 12-well trays containing 1 mL of their culture media. Total aflatoxin (in 70% methanol solution) isolated from an SU-1 culture using our standard chloroform-methanol isolation procedure (Gummadidala et al., 2017) was added to each sample well at a final concentration of 50 ppm. The control mycelia were supplemented with an equal volume of 70% methanol solution. After a 4 h incubation, mycelia were transferred to 1 mL of 1 μM DCFH-DA in PBS substrate for an additional 1 hour incubation in the dark before being measured (in triplicate) for DCF fluorescence. Aflatoxin uptake into the mycelia during the incubation period was quantified by measuring total percent removal of aflatoxin from the medium every hour until 4 h and by measuring the total accumulation of aflatoxin in the mycelium in parallel, after 4 h. Percent removal of aflatoxin from the medium was calculated as follows: Percent removal = ((Initial total aflatoxin in the supplementation medium − total aflatoxin in the medium at a time point)/Initial total aflatoxin in the supplementation medium) × 100. Aflatoxin was quantified in the medium as described below. Aflatoxin

accumulation in the mycelium was measured after washing three times with PBS buffer followed by extracting aflatoxin from the mycelium using a chloroform: methanol procedure as described previously (Roze et al., 2007). Aflatoxin in the extract was then measured using an enzyme-linked immunosorbent assay (ELISA). Dead cells of AFS10 obtained upon autoclaving at 121°C for 15 min were used in the uptake experiments as controls for free diffusion systems. Loss of viability in these cells was confirmed prior to experimentation by confirming their inability to grow in fresh growth medium.

4.3.7 Aflatoxin quantification

Qualitative comparisons of aflatoxin accumulation in the growth media were performed using thin-layer chromatography (TLC) as described previously (Hong et al., 2008). Quantification of aflatoxin for the aflatoxin uptake experiments was performed using a Veratox for Aflatoxin ELISA kit (Neogen Food Safety, Lansing, MI, USA) and measured on a Stat Fax 4700 Microstrip Reader (Awareness Technologies, Palm City, FL, USA).

4.3.8 Satistical analysis

Statistical analyses for this study were conducted using the GraphPad Prism Software (GraphPad, CA, USA). The statistical significance of two-tailed p-values were determined using an unpaired *t*-test, using $n = 3$ and $p < 0.05$. For the gene expression studies, a two-fold increase or decrease in transcript level was our cutoff for comparing expressions between two genes.

4.4 RESULTS

4.4.1 SU-1 demonstrates a significantly larger decrease in total ROS compared to AFS10 between 24h and 48h

Aflatoxin biosynthesis is activated in SU-1 at 24 h under our culture conditions and reaches peak levels by the start of the stationary phase at 48 h (Skory et al., 1993; Chanda et al., 2009). Under these conditions aflatoxin biosynthesis is not activated in AFS10. As shown in figure 4.1a, during the 24h – 48h time window, aflatoxin accumulation in the growth medium was observed and aflatoxin genes were activated in SU-1 but not in AFS10. The genes *nor-1* and *ver-1* were chosen as representative aflatoxin genes that demonstrated drastic increases in expression similar to previously reported semi-quantitative analysis of transcript and protein analysis (Roze et al., 2007). Quantitative comparison of total ROS (Dichlorodihydrofluorescein [DCF] fluorescence measurements shown in Figure 4.1b) shows that at 24 h both strains demonstrate similar levels of total ROS, but by 48 h the total ROS decreased at a significantly higher rate in SU-1 than in AFS10. This demonstrated an association between the activation of aflatoxin biosynthesis and a decrease in total ROS, which may be attributable to either the presence of aflatoxin or the regulatory role of *aflR*.

4.4.2 Higher total ROS in AFS10 compared to SU-1 at 48h associates with significant differences in SOD gene expression

4.4.2.1 Bioinformatics analysis of SOD genes

Since SOD genes are synthesized in eukaryotes in response to intracellular O_2^- radicals (a type of ROS) generated as a byproduct of primary cellular functions

(Fridovich 1975), we investigated whether higher ROS at 48 h in AFS10 is correlated with the transcriptional activation of SOD genes. As a first step to do so we initiated a search for SOD genes within the available genome database of a closely related species, *A. flavus* (Yu et al., 2008) and identified five amino acid sequences (table 4.1). Out of these five sequences, two different sequences of copper–zinc SOD genes are annotated in the database as CuZn*sod1* and cytosolic CuZn*sod*, two sequences of iron SOD are annotated as Fe*sod* and Fe*sodA*, and one manganese SOD is annotated as Mn*sod*. These five sequences were queried against the PROSITE database (Sigrist et al., 2013) to verify whether they contained any of the conserved functional domains or patterns that are present in the well-characterized SODs within the database.

As shown in table 4.2A, two of these sequences contained superoxide dismutase (SOD) signatures. CuZn*sod*1 had two typical CuZn SOD signatures. The conserved sequence (AFHVHQfGDnT) matched with the consensus pattern, [GA]-[IMFAT]-H-[LIVF]-H-[S]-x-[GP]-[SDG]-x-[STAGDE], for signature 1**,** where 2 *H*'s are copper ligands. Similarly, conserved sequence (GNAGaRpACgvI) matched with the consensus pattern, G-[GNHD]-[SGA]-[GR]-x-R-x-[SGAWRV]-C-x(2)-[IV], for signature 2**,** where *C* is involved in a disulfide bond. Mn*sod* contained the conserved sequence, DmWEHAYY, corresponding to manganese and iron SOD signature. This signature matched with the consensus pattern, D-x-[WF]-E-H-[STA]-[FY](2), where *D* and *H* are manganese/iron ligands.

The PROSITE database was then used to investigate whether the three other sequences that did not contain typical SOD motifs contained regions that have high probability of occurrence (frequent patterns) in SODs. The remaining three amino acid

sequences displayed the four patterns (an *N*-myristoylation site, a Casein kinase II phosphorylation site, and *N*-glycosylation site, and a Protein kinase C phosphorylation site) that are the most frequently present within the 390 SOD genes available in PROSITE database, suggesting strongly that these are SOD sequences (table 4.2B).

4.4.2.2 Expression profiles of SOD genes

The gene expression of all five SODs was examined in both SU-1 and AFS10 at 24 h and 48 h post-inoculation in yeast extract sucrose (YES). Quantitative comparison of the transcript levels between 24 h and 48 h, with levels normalized to 24 h (raw expression data relative to 18s rRNA shown in **Figure S1**) are shown in figure 4.2 and the list of primers used are mentioned shown under table 4.3. The data suggest that SOD expression profile in this fungus is growth phase dependent. Hence, while the expressions of Fe*sod* and CuZn*sod1* are higher in 24 h cultures (corresponding to the exponential growth phase) the Mn*sod* expression is significantly higher in the 48 h cultures (corresponding to the stationary growth phase). As seen in figure 4.2, AFS10 displayed a significantly larger increase in Mn*sod* expression from 24 h to 48 h (~70-fold increase in AFS10 versus a ~40 fold increase in SU-1). Additionally, CuZn*sod* expression that remained constant in SU-1 showed a significant increase from 24 to 48 h in AFS10. No significant difference was observed between SU-1 and AFS10 for genes Fe*sod* and CuZn*sod1*. Our results, therefore, demonstrate an association between higher ROS levels in AFS10 (compared to SU-1) and absence of aflatoxin biosynthesis during the 24 h–48 h time window in AFS10 with the significantly larger increases (compared to SU-1) in Mn*sod* and CuZn*sod* transcripts from 24 h to 48 h.

4.4.3 Aflatoxin supplementation to AFS10 growth medium changes total ROS output without changing the SOD transcript levels

The significantly larger decrease in total ROS in SU-1 compared to AFS10 could either be aflatoxin dependent, *aflR* dependent, or both. To examine if total ROS production is in-part aflatoxin dependent, we investigated whether aflatoxin supplementation to AFS10 impacts the total ROS levels. The results from this experiment are shown in figure 4.3. A 4 h supplementation of 24 h mycelia with total aflatoxin isolated from an SU-1 growth medium resulted in a significant decrease of total ROS (figure 4.3a). In contrast, the 4 h aflatoxin supplementation to 48 h AFS10 mycelia significantly increased the total ROS. To understand this differential effect of aflatoxin supplementation on the 24 h and 48 h AFS10 cultures, we conducted an examination of aflatoxin uptake by the mycelium during the 4 h time-period. As shown in figure 4.3b, the percentage removal of aflatoxin per unit mass of mycelium by the end of 4 h was significantly higher for 48 h cultures than 24 h cultures. This data also agreed with the aflatoxin accumulation in the mycelia, which demonstrated a significantly higher accumulation of aflatoxin in 48 h cultures than in 24 h cultures. To examine whether the aflatoxin accumulation was a free diffusion versus an active uptake mechanism by the mycelium, we conducted a similar experiment with equal masses of dead AFS10 cultures obtained upon autoclaving the cultures. Our results demonstrate that while the free diffusion of aflatoxin from the medium to the immersed dead cells resulted in a faster removal of aflatoxin from the medium, the aflatoxin could not be retained in the dead mycelia unlike the live cells, when taken out of the medium and washed. Collectively the gradual increase in aflatoxin removal from the medium (unlike the dead cells) and the

ability of retaining the aflatoxin in the mycelium suggests an active uptake mechanism of aflatoxin by the cells. The significantly higher uptake of aflatoxin in 48 h cultures than the 24 h cultures suggest that the differential effects of the aflatoxin supplementation on total ROS in the 24 h versus 48 h cultures are associated with the differential levels of aflatoxin uptake by the mycelia of these ages.

Finally, we also examined whether aflatoxin supplementation resulted in changes in the expression levels of the SOD genes either in 24 h or 48 h cultures. Contrary to the total ROS readings, there were no significant changes in SOD expression that were attributable to AF supplementation (figure 4.3c), thereby suggesting the possibility that aflatoxin supplementation induced changes in the total ROS are acute biochemical effects.

4.5 DISCUSSION

This study provides the first direct demonstration of the regulatory role of a secondary metabolite on a cellular process of the producer's oxidative stress management. It also can now explain the previous reports on the cross-talk between oxidative stress and secondary metabolism (Jayashree et al., 2000; Narasaiah et al., 2006; Reverberi et al., 2006; Reverberi et al., 2008). Based on our current findings and previously published literature, we propose here a ROS management model for aflatoxin producers (illustrated in figure 4.4). According to this model, aflatoxin biosynthesis protects cells against ROS accumulation from at least three different sources: (a) primary metabolic processes, (b) secondary ROS generated from aflatoxin biosynthesis, as proposed previously by Roze et al. (Roze et al., 2015), and (c) ROS generated upon

aflatoxin uptake by cells during the stationary phase of growth (aflatoxin supplementation data from 48 h AFS10 cultures in the current study). The aflatoxin-dependent protection occurs in one or a combination of the following ways: (a) utilization of ROS in the biochemical steps of the biosynthesis pathway (Narasaiah et al., 2006), (b) aflatoxin-dependent reduction of ROS in cells at exponential growth phase (aflatoxin supplementation data from 24 h AFS10 cultures in the current study) and (c) *aflR*-dependent reduction of ROS (current study) possibly through its gene regulatory impacts outside the aflatoxin pathway gene cluster (Price et al., 2006; Yin et al., 2012). Our data support the likelihood that disruption of *aflR* blocks all the three modes of aflatoxin-dependent protection, leading to a higher accumulation of super-oxide radicals in AFS10 compared to SU-1. This can explain the increased demand for SOD activation and the higher SOD transcript levels in AFS10 than in SU-1.

To address the direct effect of aflatoxin on total ROS, we designed a 4 h supplementation experiment to compare the individual effects of the supplementation on the 24 h and the 48 h AFS10 cultures. We understand based on previous literature (Roze et al., 2007; Banerjee et al., 2014; Chanda et al., 2009) that 24 h cultures and 48 h cultures (under our standard growth conditions), are very different physiological systems; 24 h cultures demonstrate no secondary metabolite synthesis and in 48 h cultures secondary metabolite synthesis occurs at peak levels. The 4 h time was optimized from initial uptake experiments in which we noticed no significant increase in the growth of the mycelia until 4 h under the given experimental conditions (data not shown). We reasoned that supplementation beyond 4 h would result in adaptation of fungal cells and that would not allow us to observe the acute effects as described in this study.

It is speculated that fungal toxisomes, which are sites for the synthesis and compartmentalization of secondary metabolites (Chanda et al., 2009; Roze et al., 2011), receive input from peroxisomes and mitochondria as well as from the secretory and Cvt vesicle transport pathways (Roze et al., 2011). A significant increase in the mitochondrial SOD, MnSOD, at 48 h suggests that it is primarily responsible for dismutating the superoxides during the stationary phase. Previous proteomic data on fungal toxisomes in *A. parasiticus* (Linz et al., 2012) demonstrated an enrichment of superoxide dismutases, especially MnSOD, within the toxisomes as well. Catalases also present in the tosixomes then convert the hydrogen peroxide product of the dismutation reactions into oxygen and water. The data shown here correspond increased MnSOD with ROS levels after the initiation of aflatoxin biosynthesis support the possibility that superoxides are compartmentalized into fungal toxisomes in addition to the mitochondria, and become available for incorporation into secondary metabolite biosynthetic pathways, including aflatoxin synthesis, in addition to dismutation by SODs. We emphasize here that while the SOD expression profiles are closely and independently associated with total ROS and the activation of aflatoxin biosynthesis, our data (figure 4.3c) do not support aflatoxin as a direct regulator of SOD gene transcription, thereby suggesting that additional regulator(s) work in concert with AflR to regulate SOD gene expression. An example of such a regulator is the bZIP transcription factor AtfB (Roze et al., 2011; Wee et al., 2017), which is in part one regulator of the SODs and the cellular response to intracellular oxidative stress (Hong et al., 2013; Hong et al., 2013; Wee et al., 2017) that binds to *aflR* gene promoter and physically interacts with the AflR (Miller et al., 2005; Roze et al., 2004).

One limitation of this study is the lack of an appropriate methodology for clean biochemical measurements specific for superoxide radicals (O_2^-) within Aspergillus cells. Commercially available small molecules like DMPO, that can successfully trap O_2^- within mammalian and yeast cells, have conventionally been used for such O_2^- quantifications. However, these small molecules fail to enter Aspergillus cells (data not shown). Within the cell, toxisomes are very dynamic systems that are continuously exporting protein and metabolite contents to the extracellular environment (Chanda et al., 2010), at which time any present superoxide radicals would be detectable by molecules such as DMPO. Therefore, unless the extremely unstable O_2^- radicals are incorporated into the location of aflatoxin synthesis within toxisomes, as in case of SU-1 (but not in AFS10), commercial cellular stains like MitoSOX or CellROX cannot provide a true overall quantification of the total O_2^- radicals or total ROS through cellular imaging experiments as done for many mammalian cells, and will lead to inaccurate interpretations. The protocol used in these experiments is based on a methodology previously established by Chang et al. (Chang et al., 2011). The method allows the substrate DCFH-DA to react with the total ROS generated within mycelia and form the fluorescent marker DCF that can then be quantified spectrophotometrically. While we acknowledge the technical limitations of the DCFH-DA probe in providing an accurate quantification of superoxides and total ROS (Kalyanaraman et al., 2012), we reason that our experimental design, being dependent of relative ROS levels rather than accurate ROS quantifications, was able to circumvent these challenges and therefore our interpretations on relative ROS levels were not impacted.

In conclusion, our findings establish the foundation for a long-term study that will investigate the molecular, cellular, and biochemical mechanisms underlying the differential effects of aflatoxin on ROS accumulation in cells that are in an exponential growth phase versus those in a stationary phase. We hypothesize based on these findings that secondary metabolites have a regulatory role in the cellular coordination of secondary metabolism and oxidative stress response in filamentous fungi. Our future studies will shed more light on revealing the complexity of such coordination and thereby help identify novel targets for the manipulation of secondary metabolism.

Table 4.1: Amino acid sequences of the SODs analyzed in the study. The names of the SODs as annotated in the gene bank database and their accession numbers are mentioned above each sequence within the shaded rows.

FeSOD (Gene bank accession number EED58116.1)
MLPRFLRPQSTLRAVSSLTQKPASALPRFQTRGLHRVPQLTHDTHFKNNGIQELLSPEAFDFAWTQYQTLLID KLNLLTQDTVDADAKPGELLVKYSRRPEMASVFNYASMAHNNHFFFNCLSPTPTQIPDKFAKDIVDTCSSIES LKLDFLATANAMFGPGFVWLAKNLEREGLMHIFCTYNAGSPYPAAHSRRQPVDMATHSPDAPLGNQFAGAMGA HSANQKSLAPGAVDVQPILCVNTWEHVWMMDYGIGGKAEYLERWWDRINWEVVFDNYNAVS SMKGTRHAANRNRSLSML
FeSODA (Gene bank accession number EED55486.1)
MAASLIRTSARTALRAGASATPKAAGVAGLTFARGKATLPDLAYDYGALEPSISGKIMELHHKNHHQTYVNSY NTAIEQLQEAVAKEDITTQINLKPLINFHGGGHINHTLFWENLAPKSQGGGEPPSGALAKAIDESFGSLGEFQ SKMNAALAGIQGSGWAWLVKDKQTGNIGIKTYAVSSSLTRTLSLVSSSLFSVLMLGSTPTTFNTRTARLSTSA PSGTSSTGRRLRSASRKRAKVGWITCSRSIPAGSGKLIFLVWDPLRPLRIFFPHLTSQAINSLEMSAESPGEK RGGFRAFFAGALRPKKSRQVLRKASTPNLKEGLQSKDDVPAMPSLTPLEAHRLKYREVNLQKDTQLGETHDHT AMLHSIGVGELDPSDPHAQLHEFDNRPPGEPMIASLTSDLWAKVTEYLNPAERASLAFSSRTLYARLGREPWI TINLPENHDYKADFLISQDRLLPHHLLCFPCGKYHRRTQEGYEKLQPADIINPLFDCPNARNNALPAPRHRIT HGRVLYFTFHQLVMRAYRFGPRYGISADSLSRRWRRDGWSHQTRYHIHQGRLLMRVVSTCFAEPGLSASQQRL LLYSRDDYWPYFSVCAHWRDGELMNVCKCALGHIPVPRTTNGLQGLEHRAKDMYHRREHNPNALASLCGKCRP MRRCPECPSEYLVEVKLTEDRSGSHRNLFRHAIVVTRWSDLGDGRSPRLSKEWAAINGDEAGEGYDSFEKIGK RAISGIFESAITDDTLPGQRILSMNPKERSWVRLGIIGIEVPYLYFALGVICGGKLGVLSGVIFCIILYYTRV GVWVGWVGWVGWVGWVGWVGWVGWVGWIGLCGFI
CuZnSOD1 (Gene bank accession number EED46237.1)
MVKAVAVLRGDSKISGTVTFEQADANAPTTVSWNITGHDANAERAFHVHQFGDNTNGCTSAGPHFNPFGKEHG APEDENRHVGDLGNFKTDAEGNAVGSKQDKLIKLIGAESVLGRTLVIHAGTDDLGRSEHPESKKTGNAGARPA CGVIGIAA
Cytosolic CuZn SOD (Gene bank accession number EED49986.1)
MLTKSLFAGAALGLSLSSAVAHEAPVVEGNEPQTVYEAVLQDKDNTTVRGTFTTHGAEDGIGIQFRVALTGVP KDTFLNYHIHDNPVPKDGNCYATGGHLDPYKRGDQPPCNTTVPQTCQVGDISGKHGPVWTADGNFEVLYRDFF LSNVEDTIAFFGNRSVVVHLPDNKRINCGNFHLVSDGEEKKKKEEAKEDQGC
MnSOD (Gene bank accession number EED56070.1)
MATTFSLPPLPYAYDALEPVICKQIMEIHHQKHHQTYITNLNAALSAQSTALAANNIPQLINLQQKIKFNGGG HINHSLFWKNLAPHASPETNIDQAAPVLKAAIEAQYGSVEKFKEAFGATLLGLQGSGWGWLVANGPGGKLEIV STKDQDPVTDKVPVFGVDMWEHAYYLQYFNNKASYVEGIWKVLNWRTAEDRFKNGVEGSALLKL

Table 4.2: A bioinformatics analysis of the SOD annotated amino acid sequences. (A) Results from a search of the conserved domain signatures of SODs. Two sequences, CuZnSOD1 and MnSOD (shaded cells) show the typical SOD signatures. (B) (i) Results from a study of the detection of the most frequent patterns of the SODs available in the PROSITE database. A total of 390 SOD sequences were analyzed. The cells with the four most frequent patterns are highlighted in the table. (ii) Results from the analysis of the four most frequent patterns within the sequences (CuZnSOD cytosolic, FeSOD, FeSODA) that did not show conserved domain signatures.

(**A**) Presence of signatures of conserved domains.

Gene	Conserved Domain	Conserved Sequence
FeSOD	None	None
FeSOD A	None	None
CuZnSOD1	CuZn superoxide dismutase signature 1 CuZn superoxide dismutase signature 2	AFHVHQfGDnT GNAGaRpACgvI
CuZnSOD cytosolic	None	None
MnSOD	Manganese and iron superoxide dismutases signature	DmWEHAYY

(**B**) Analysis of SOD frequency patterns in FeSOD, FeSOD A and CuZnSOD cytosolic.

(i). Frequency data for the presence of frequency patterns in the 390 SODs within the PROSITE database

ID	Patterns	Sites corresponding to the Patterns	Frequency
PS00001	ASN_GLYCOSYLATION (pattern)	*N*-glycosylation site	302
PS00004	CAMP_PHOSPHO_SITE (pattern)	cAMP- and cGMP-dependent protein kinase phosphorylation site	47
PS00005	PKC_PHOSPHO_SITE (pattern)	Protein kinase C phosphorylation site	273
PS00006	CK2_PHOSPHO_SITE (pattern)	Casein kinase II phosphorylation site	331
PS00007	TYR_PHOSPHO_SITE (pattern)	Tyrosine kinase phosphorylation site	37
PS00008	MYRISTYL (pattern)	N-myristoylation site	387
PS00009	AMIDATION (pattern)	Amidation site	35
PS00016	RGD (pattern)	Cell attachment sequence	38
PS00017	ATP_GTP_A (pattern)	ATP/GTP-binding site motif A (P-loop)	14
PS00342	MICROBODIES_CTER (pattern)	Microbodies C-terminal targeting signal	34
PS50310	ALA_RICH (profile)	Alanine-rich region profile	2
PS50321	ASN_RICH (profile)	Asparagine-rich region profile	1
PS50324	SER_RICH (profile)	Serine-rich region profile	1

(ii). Frequency of occurrence of the most frequent patterns in FeSOD, FeSOD A and CuZnSOD cytosolic

4 most frequent sites in SODs (from table above)	Consensus Pattern	Frequency of occurrence in		
		CuZnSOD cytosolic	FeSOD	FeSODA
N-myristoylation site	G-{EDRKHPFYW}-x(2)-[STAGCN]-{P}	5	4	15
CK-2 phosphorylation site	[ST]-x(2)-[DE]	3	1	7
N-glycosylation site	N-{P}-[ST]-{P}	3	1	1
Protein kinase C phosphorylation site	[ST]-x-[RK]	2	6	11

Table 4.3: List of primers used in the study.

NO.	Genes	Primer Sequences
1	*nor-1*	F 5′-CACTTAGCCAGCACGATCAA-3′ R 5′-ATGATCATCCGACTGCCTTC-3′
2	*ver-1*	F 5′-AACACTCGTGGCCAGTTCTT-3′ R 5′-ATATACTCCCGCGACACAGC-3′
3	*β-tubulin*	F 5′-TCTCCAAGATCCGTGAGGAG-3′ R 5′-TTCAGGTCACCGTAAGAGGG- 3′
4	FeSOD	F 5′-GAGATGGCCTCCGTATTCAA-3′ R 5′-CATCAATCCTTCCCTCTCCA-3′
5	FeSOD A	F 5′-CCAAGGAGGACATCACCACT-3′ R 5′-GCATAGGTCTTGATGCCGAT-3′
6	CuZnSOD1	F 5′-CACCAGTTCGGTGACAACAC-3′ R 5′-GTGTTCACTACGGCCAAGGT-3′
7	CuZnSOD cytosolic	F 5′-CCTCCTTGCAATACAACCGT-3′ R 5′-GTCTTCCTTCGCCTCTTCCT-3′
8	MnSOD	F 5′-CCACATCAACCACTCCCTCT-3′ R 5′-TCCTGATCCTTCGTCGAAAC-3′

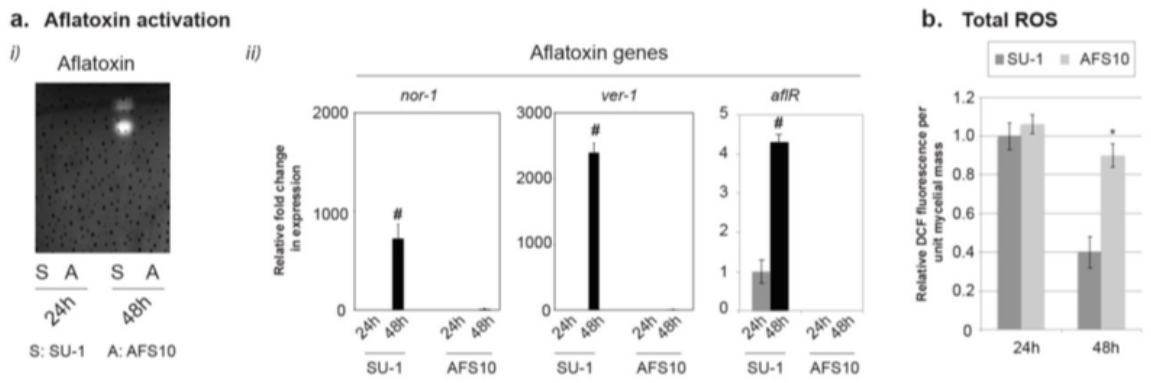


Figure 4.1: Decrease of total ROS during activation of aflatoxin biosynthesis. (**a**) Comparison of Figure 1. Decrease of total ROS during activation of aflatoxin biosynthesis. (a) Comparison of (i) aflatoxin accumulation and (ii) Gene expression levels relative to 24 h of three aflatoxin pathway aflatoxin accumulation and (ii) Gene expression levels relative to 24 h of three aflatoxin pathway genes in SU-1 and AFS10. (**b**) Comparison of total ROS at 24 h and 48 h. The error-bars represent genes in SU-1 and AFS10. (b) Comparison of total ROS at 24 h and 48 h. The error-bars represent standard error of the mean. The two-tailed *p*-value was determined using unpaired *t*-test (GraphPad standard error of the mean. The two-tailed p-value was determined using unpaired t-test (GraphPad statistical software). #, Significant difference of transcript levels between 24 h and 48 h (*p*-value < 0.05, statistical software). #, Significant difference of transcript levels between 24 h and 48 h (p-value < 0.05, $n = 3$); * Significant difference of total ROS between SU-1 and AFS10 (*p*-value < 0.05, $n = 3$).

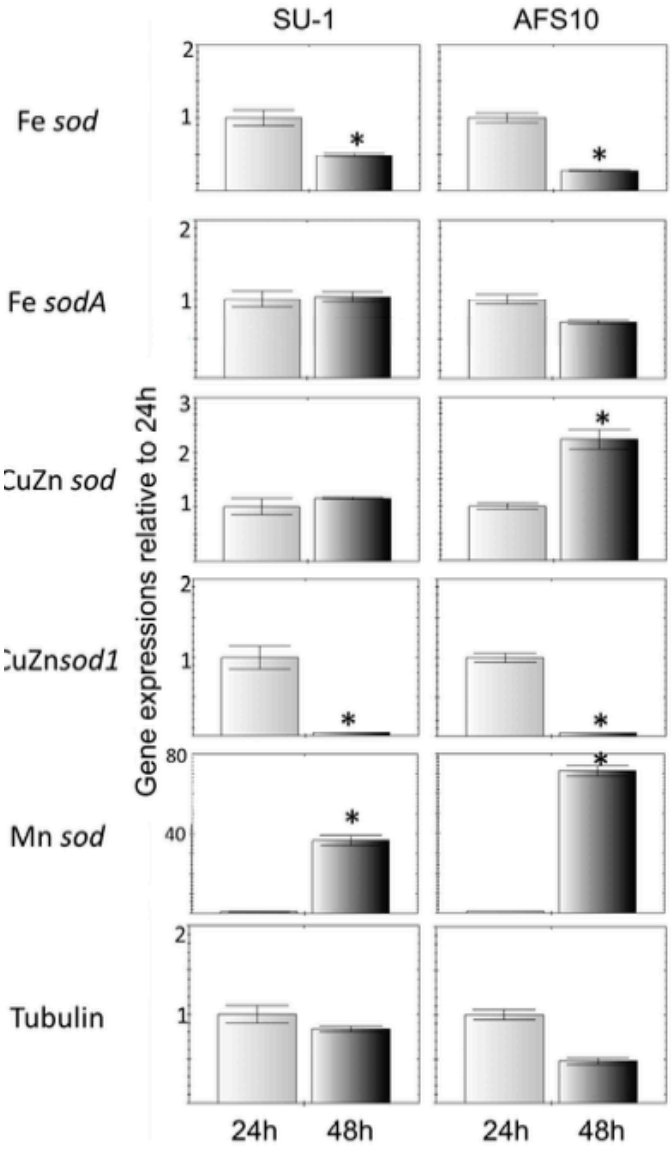


Figure 4.2: Comparison of SOD gene expression in SU-1 and AFS10. Quantitative PCR (qPCR) comparison of SOD gene expression in the two strains at 24 and 48 h of culture growth. All expression quantification were conducted in triplicate. For each gene the expression value was normalized against the 18s rRNA reference gene and compared to a beta-tubulin control. The expression values for each target gene at early stationary phase (48 h) were expressed as the fold change relative to 24 h time point. Fold changes ≥2.0 were considered up- or down- regulated. All data and statistical analysis (Student's *t*-test) were performed using CFX Manager software (Bio-Rad Laboratories). Compared to 24 h gene expression, FeSOD showed a significant decrease in both the wild-type (2.1-fold; $p = 0.003$) and AFS10 (3.9-fold; $p < 0.001$); Fe*sodA* showed no significant change for either strain; CuZn*sod* expression did not change in the WT, but showed a 2.1-fold

increase (p = 0.003) in AFS10; CuZn*sod*1 showed a large, significant decrease in expression for both the WT (22.4-fold; p = 0.001) and AFS10 (26.4-fold; $p < 0.001$); Mn*sod* had a dramatically significant 36.2-fold increase in gene expression in the WT ($p < 0.001$), and an even greater 69.8-fold increase in AFS10 ($p < 0.001$) compared 24 h expression. (Raw gene expression data is included as Figure 4.5). * Indicates statistically significant difference from respective 24 h gene expression; $p \leq 0.05$.

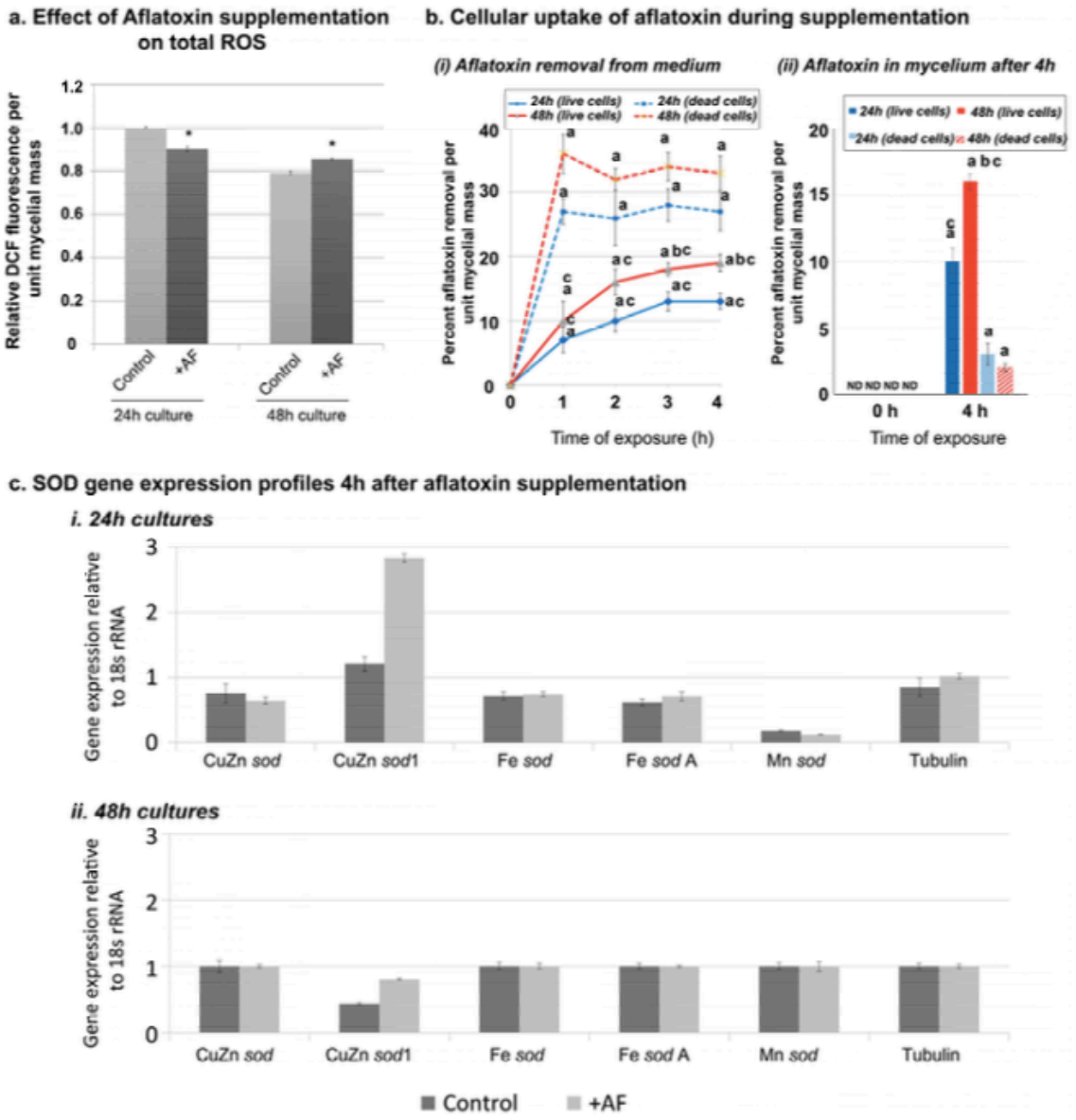


Figure 4.3: Aflatoxin supplementation to AFS10. (**a**) Effect on total ROS. A quantitative comparison of ROS in AFS10 supplemented with 50 ppm aflflatoxin (in 70% methanol) and a 70% methanol control was conducted. Total ROS was quantiffiied at 24 h and 48 h of growth + 4 h of incubation in 1 μμM 20,70-dichlorofluorescein diacetate (DCFH-DA) in phosphate buffered saline (PBS) substrate with 2′,7′-dichlorofluorescein diacetate (DCFH-DA) in phosphate buffered saline (PBS) substrate with the the corresponding AF concentration. Error-bars represent SEM. (*) denotes statistically significant corresponding AF concentration. Error-bars represent SEM. (*) denotes statistically significant difference ($p < 0.05$; $n = 3$) in ROS compared to the 70% methanol control for the corresponding growth difference ($p < 0.05$; $n = 3$) in ROS compared to the 70%

methanol control for the corresponding time. (**b**) Cellular uptake of aflatoxin during aflatoxin supplementation. (i) Percent removal of aflatoxin growth time. (b) Cellular uptake of aflatoxin during aflatoxin supplementation. (i) Percent removal from the supplementation medium in live cells of 24 h and 48 h AFS10. The percent removal was of aflatoxin from the supplementation medium in live cells of 24 h and 48 h AFS10. The percent calculated at every hour until 4 h to compare the aflatoxin removal pattern by live cells with the dead removal was calculated at every hour until 4 h to compare the aflatoxin removal pattern by live cells cells that allow free diffusion from the medium into the cells. (ii) Percent aflatoxin accumulation in with the dead cells that allow free diffusion from the medium into the cells. (ii) Percent aflatoxin the mycelium of 24 h and 48 h cultures. Aflatoxin in the mycelia of live cells was compared to the accumulation in the mycelium of 24 h and 48 h cultures. Aflatoxin in the mycelia of live cells was dead cells. Error-bars represent SEM. a, statistically significant difference ($p < 0.05$; $n = 3$) in aflatoxin compared to the dead cells. Error-bars represent SEM. a, statistically significant difference ($p < 0.05$; n levels with 0 h, b, statistically significant difference ($p < 0.05$; $n = 3$) in aflatoxin levels between 24 h = 3) in aflatoxin levels with 0 h, b, statistically significant difference ($p < 0.05$; $n = 3$) in aflatoxin levels and 48 h cultures, c, statistically significant difference ($p < 0.05$; $n = 3$) in aflatoxin levels between between 24 h and 48 h cultures, c, statistically significant difference ($p < 0.05$; $n = 3$) in aflatoxin levels live and dead cells at a particular time-point. (**c**) Comparison of SOD gene expression in aflatoxin between live and dead cells at a particular time-point. (c) Comparison of SOD gene expression in supplemented and control AFS10. qPCR comparison of SOD gene expression in the control and 4 h aflatoxin supplemented and control AFS10. qPCR comparison of SOD gene expression in the control aflatoxin supplemented cells. The gene expression values were normalized against the 18s rRNA and 4 h aflatoxin supplemented cells. The gene expression values were normalized against the 18s reference gene. Fold changes 2.0 were considered up- or down-regulated. All data and statistical analysis (Student's *t*-test) were performed using CFX Manager software (Bio-Rad Laboratories).

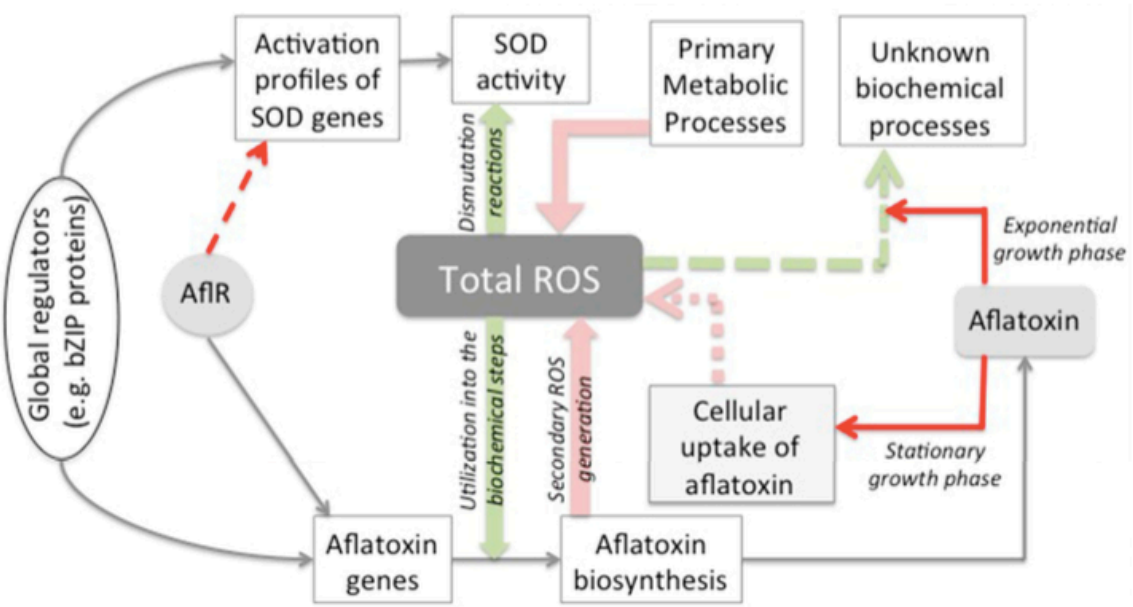


Figure 4.4: Proposed model for total ROS management in *A. parasiticus*. Based on our current findings and previous reports we propose that aflatoxin-dependent protection occurs in one or a combination and previous reports we propose that aflatoxin-dependent protection occurs in one or a combination of the following ways: (a) utilization of ROS in the biochemical steps of the biosynthesis pathway (Narasaiah et al., 2006), (b) aflatoxin-dependent reduction of ROS in cells at exponential growth phase (current study) of the following ways: (a) utilization of ROS in the biochemical steps of the biosynthesis pathway (b) aflatoxin-dependent reduction of ROS in cells at exponential growth phase (current study) and (c) aflR- dependent reduction of ROS (current study) possibly through its gene regulatory impacts outside the aflatoxin pathway gene cluster (Price et al., 2006; Yin et al., 2012). Aflatoxin dependent biochemical processes that sequester ROS still remain uncharacterized (green dashed arrow). Pink arrows indicate the sources of ROS accumulation. These include ROS generation from primary metabolic processes, secondary ROS generated from aflatoxin biosynthesis (Roze et al., 2015), and ROS generated upon aflatoxin uptake by cells during stationary phase of growth (based on aflatoxin supplementation data from 48 h AFS10 cultures in the current study). The mechanisms that result in ROS accumulation upon cellular uptake of aflatoxin remains uncharacterized (pink dashed arrow). The model can now explain the physiological need of the cells to co-regulate secondary metabolism (in this case, aflatoxin biosynthesis) and oxidative stress response through the bZIP proteins (Roze et al., 2011; Baidya et al., 2014; Hong et al., 2013; Hong et al., 2013; Montibus et al., 2013; Reverberi et al., 2012; Montibus et al., 2015; Yin et al., 2013). Red arrows indicate the contributions of the current study. The molecular mechanism aflR-mediated regulation of SOD genes remains uncharacterized (red dashed arrow) and will be investigated in our follow up studies.

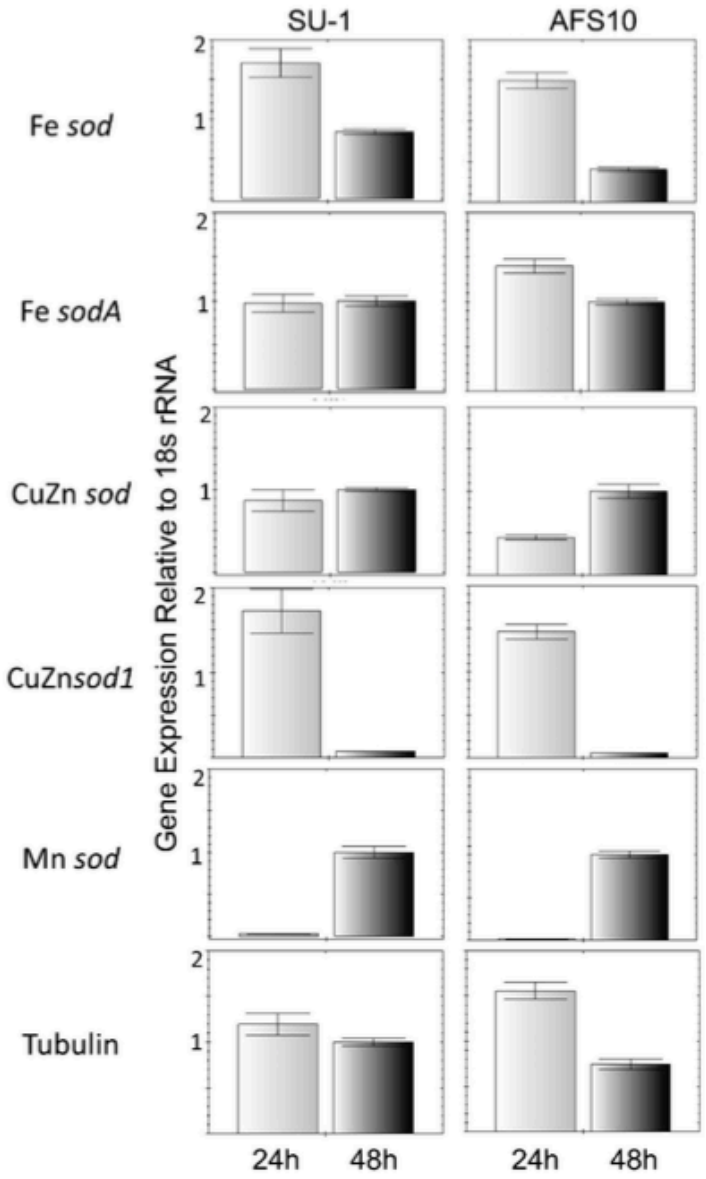


Figure 4.5: Raw expression data of the SOD genes in SU-1 and AFS10. qPCR comparison of SOD gene expression in the two strains at 24 h and 48 h of culture growth. All expression quantifications were conducted in triplicate. For each gene the expression value was normalized against and 18s rRNA reference gene and compared to a β-tubulin control

CHAPTER 5

Vibrio gazogenes: A NOVEL TOOL TO COMBAT THE PATHOGEN, *Aspergillus flavus*[1]

[1]Gummadidala PM., Omebeyinje M., Deo T., Chanda A. To be submitted to *Science*.

5.1 ABSTRACT

Aspergillus flavus is the most virulent and second most important *Aspergillus* species causing human infections ranging from hypersensitivity reactions to invasive infections (Hedayati et al., 2007). The Aspergillosis caused by *A. flavus* has a 90% mortality rate primarily due to the development of multi drug resistance. *A. flavus* also produces aflatoxins that contaminate food supplies globally and which when consumed lead to hepatocellular carcinoma. Biological controls were proven to be most effective in inhibiting aflatoxins and aflatoxin producing fungi. *Vibrio gazogenes* was proven to synthesize antifungal and antiaflatoxin metabolites. In this study we treated *A. flavus* cultures with 16 million *V. gazogenes* cells and observed >99% significant decrease in aflatoxin levels in the first generation and ~40% decrease of aflatoxin levels in the second generation of *A. flavus* cultures. The dead and live *V. gazogenes* cells have similar aflatoxin inhibitory effects that were specific to *V. gazogenes* and not to other gram-positive or gram-negative bacterium. Infecting corn kernels with *A. flavus* in the presence of bacterium significantly decreased the fungal conidial growth by 80% and aflatoxin by >98%. Treating drosophila flies with *V. gazogenes* prior to *A. flavus* infection increased their survival (~40%). Using confocal laser, scanning electron and transmission electron microscopies we observed the uptake of the bacterium by the fungus into endosome like compartments. RT-PCR data revealed controversial gene expressions of aflatoxin pathway genes and global secondary metabolite regulatory genes in the presence of live and dead *V. gazogenes*. These data suggest that the live and dead *V. gazogenes* aflatoxin inhibitory mechanisms are different. The data also uncover the yet unstudied concept that *V. gazogenes* mechanism of aflatoxin inhibition is not at the gene level but is at the

cellular level. Finally our study has discovered a novel tool (*Vibrio gazogenes*) to inhibit the aflatoxin production and pathogenicity of plant and human pathogen *Aspergillus flavus*.

5.2 INTRODUCTION

The fungus *Aspergillus flavus* is a saprophyte, growing in humid environments with pathogenic ability causing aspergillosis in immuno-compromised humans effecting the skin, oral mucosa and subcutaneous tissues (Hedayati et al., 2007). Furthermore, *A. flavus* is the most virulent and second most important *Aspergillus* species causing human infections ranging from hypersensitivity reactions to invasive infections (Hedayati et al., 2007). According to the centers for disease control and prevention (CDC) approximately 4.8 million cases of aspergillosis were diagnosed worldwide and *A. flavus* is the second most leading cause. Most *A. flavus* strains are susceptible to antifungal therapy but the minimum inhibitory concentrations are atleast two fold higher than for other *Aspergillus* species (Krishnan et al., 2009). Furthermore, recent discoveries revealed the presence of active multi drug resistant genes in *A. flavus* strains increasing their potential for drug resistance and increased pathogenicity (Tobin et al., 1997) (Van Der Linden et al., 2011).

A. flavus is also a plant pathogen and releases aflatoxins that are secondary metabolites and aflatoxin B1 had been categorized as class 1A human carcinogen by the International Agency of Research on Cancer (IARC). Aflatoxins contaminate a wide range of crops, produce, food, nuts, cereal, milk, juices, homes, wood, etc., and can be ingested into intestines and enter systemic circulation causing aflatoxicosis and liver cancer. Aflatoxin B1 synthesized by *A. flavus* is extremely stable (Garcia et al., 1994) at

temperatures greater than 150^{O}C maintained for 30 minutes (Raters and Matissek 2008) and so cannot be detoxified by cooking or autoclaving. Therefore aflatoxins pollute many food groups that are ingested by humans and animals. Most common methods used in agriculture to reduce aflatoxin contamination are expensive, time consuming and have low efficiency with limited success rates. Novel therapies are required to fight against *A. flavus* strains and inhibit both its pathogenicity and aflatoxin production without affecting the host physiology. In their zeal to find new anti-fungal and anti-aflatoxin agents scientists have turned towards plant and microbe derived compounds especially from organisms that live in aflatoxin induced environments (Holmes et al., 2008).

Vibrio gazogenes is a marine gram-negative bacterium notoriously known for its synthesis of antifungal pigments (Darshan and Manonmani 2015). Studies have shown that when *V. gazogenes* comes in contact with aflatoxin, the toxin induces *V. gazogenes* to synthesize antifungal and anti-aflatoxin metabolites termed aflatoxin responsive metabolites (ARMs). ARMs are responsible for decreasing 60% of aflatoxin by inhibiting the aflatoxin biosynthetic pathway at the gene level (Gummadidala et al., 2016). But various studies have shown that when the bacterium and fungus (*A. flavus* or *A. parasiticus*) have been co-cultured then the aflatoxin production was inhibited by greater than 95% (Chang and Kim 2007, Wang et al., 2013). Also *Lactobacillus pentosus* and *Lactobacillus beveris* bacteria have been successfully used to eliminate aflatoxin B1 from contamination of milk via the binding of aflatoxin B1 to the bacteria (Hamidi et al., 2013).

These studies have prompted us to hypothesize that co-culturing *V. gazogenes* bacterium with *A. flavus* fungus will have significant inhibitory effects on the aflatoxin

biosynthesis of *A. flavus*. In this study, we treated *A. flavus* with live and dead *V. gazogenes* cells and observed the decrease in aflatoxin with no effect on fungal mycelial growth. We further conducted microscopic imaging and observed the uptake of *V. gazogenes* into endosome like compartments in *A. flavus*. To understand the mechanism of action of *V. gazogenes* we performed transcript accumulation analysis and surprisingly concluded that *V. gazogenes* aflatoxin inhibitory activity is not at the gene level but possibly at the cellular level. Finally our tests to understand the effect of *V. gazogenes* on pathogenicity of *A. flavus* reveal the decrease of aflatoxin and conidial formation in corn and increase of survival in drosophila that were infected with *A. flavus*. Definitively we report the discovery of a novel tool (*V. gazogenes*) to combat the aflatoxigenicity and pathogenicity of *A. flavus*.

5.3 METHODS AND MATERIALS

5.3.1 Strains, media and growth conditions

The fungus *Aspergillus flavus* strain CA14PyrG.1 (acquired from USDA) and bacteria *Vibrio gazogenes* (ATCC29988), *Staphylococcus aureus* (**ATCC**), and *Escherichia coli* (**ATCC**) were used for this study. Yeast extract sucrose (YES) (2% yeast extract, 6% sucrose; pH 5.8) was used as the liquid growth medium and potato dextrose agar (PDA) and YES agar (YESA) were used as the solid growth media, for *A. flavus*. Fungal cells were grown for 72 h (as required by experiments) by inoculating $0.5x10^6$ spores per 50 mL of liquid growth medium and incubated at 29°C in a dark orbital shaker at 150 rpm. For growth of fungal mycelia on solid media, $2x10^4$ spores

were center inoculated on 100 mm petri dishes containing 10 mL of PDA/YESA and incubated in the dark at 29°C.

Difco Marine broth (cat # 2216, BD Biosciences, Sparks, MD) was used, as liquid growth media for *V. gazogenes* and the bacterial cells were grown for 24 h by inoculating 10^5 colony forming units (cfu) /100 ml of growth medium. The cultures were incubated at 29°C in a dark orbital shaker at 150 rpm. Tryptic soy broth (TSB, cat # 211822, BD Biosciences, Sparks, MD) was used as liquid growth media for *S. aureus*, and *E. coli*. Small inoculum were grown with 10^5 cfu/5 ml liquid growth media and incubated overnight at 37°C in a dark orbital shaker at 150 rpm. At the end of the incubation time, 100,000 cells were taken from the small inoculum and further used to inoculate 100 ml TSB and incubated for 24 h at 37°C in a dark orbital shaker at 150 rpm.

5.3.2 *A. flavus* treatment with *V. gazogenes*, *S. aureus*, and *E. coli*

The fungus *A. flavus* was treated with both live (*V. gazogenes*) and dead bacteria (*V. gazogenes*, *S. aureus*, and *E. coli*). The 24 h bacterial cultures were spun down at 4000 rpm for 15 min at room temperature and resuspended using YES media. With the help of absorbance measurements at 600 nm, 4, and 16 million cells from a 24 h bacterial culture (live or made unviable by boiling at 100^{O}C for 10 min in a hot plate) were sorted out and added to the 50 ml YES liquid media simultaneous with $5x10^5$ *A. flavus* spores and incubated at 29°C in a dark orbital shaker at 150 rpm for 24 h, 48 h and 72 h. At the end of incubation, *A. flavus* mycelia were harvested by filtering the mycelia through a miracloth (Millipore, Billerica, MA) and the cells subjected to appropriate processing for various growth measurements, aflatoxin analysis and gene expression analysis.

Comparably 4 or 16 million *V. gazogenes* cells (viable or unviable) were spread out as a monolayer on the YESA or PDA 10 cm media plates. The bacterial cells were allowed to dry in the biosafety cabinet and $2x10^4$ spores of *A. flavus* were center inoculated. The hyphal growth of the fungus was recorded daily and at the end of the incubation period the YES and PDA media was chopped up and used appropriately for further processing of aflatoxin analysis.

5.3.3 Growth measurements of *A. flavus*, *V. gazogenes*, *S. aureus*, and *E. coli*

All fungal growth in liquid media was quantified by using dry weight measurements. Briefly, the mycelia were filtered out of the growth media using a miracloth (Millipore, Billerica, MA) and dried in an oven at 80°C for 6 h and the weight difference before and after drying was recorded. To estimate growth of *A. flavus* on PDA and YESA media plates, the spread of mycelial colony was measured daily. After 9 days of incubation time, spores were manually collected from PDA plates using 1xPBS with 0.01% tween and resuspended in 50% glycerol. Spores were counted using haemocytometer. All bacterial growth measurements were performed using absorbance readings of growth media at 600 nm. An absorbance of 1.2 on the UV/Vis spectrophotometer was considered as 10^6 cells/ml bacterial cellular density and calculations were performed for 4 and 16 million bacterial cells appropriately.

5.3.4 Aflatoxin measurements

5.3.4.1 Aflatoxin ELISA analysis

Aflatoxin was extracted from *A. flavus* cultured YES liquid media by adding equal volume of chloroform in a separating funnel and collecting the organic layer.

Chloroform was evaporated from the organic layer and the residual aflatoxin was resuspended in 1 ml of 70% methanol. Aflatoxin was extracted from *A. flavus* cultures grown in/on YES and PDA media plates by chopping the agar media and vigorously shaking it with equal volume of chloroform. Again the chloroform layer was collected, evaporated and the residual aflatoxin was resuspended in 1 ml of 70% methanol. The resuspended aflatoxin extract was spun down at 15,000 rpm for 1 min at room temperature to remove extra debris from the media and mycelia. The clear aflatoxin extract was used for analyzing aflatoxin using the Neogen Veratox Aflatoxin ELISA kit (cat # 8030) from Neogen (Lansing, MI, USA) and measured on a Stat Fax 4700 Microstrip Reader (Awareness Technologies, Palm City, FL, USA) as per kit's protocols. The ELISA has 2 ppb and 50 ppb as lower and upper limits of detection respectively. Therefore highly concentrated aflatoxin samples were diluted to fit within the detection range.

5.3.4.2 Metabolite analysis using UPLC system

The Food and Feed Safety Research Unit at USDA performed metabolite analysis using UPLC (Ultra high pressure liquid chromatography). Cultures of *A. flavus* (with/without *V. gazogenes*) (50 ml) were lyophilized and then extracted twice with 5% methanol / 95% ethyl acetate + 0.1% formic acid (15 ml) overnight with shaking at room temperature. The 2 extractions were pooled and concentrated in vacuo. The dried extract was redissolved in methanol at 5 mg/ml and centrifuged (14,000 rpm, 2 min) to remove particulate prior to analysis. Samples were analyzed using a Waters Acquity UPLC system (40% methanol in water, BEH C18 1.7 µm, 2.1 x 50 mm column) using fluorescence detection (Ex= 365 nm, Em= 440 nm). Samples were diluted 10-fold if the

aflatoxin signal saturated the detector. Analytical standards (Sigma-Aldrich, St. Louis, MO) were used to identify and quantify aflatoxin B1 (AFB1, retention time = 4.60 min.). Aflatoxin concentrations are expressed in ng aflatoxin / ml culture or ng aflatoxin / g mycelium.

Simultaneously samples were also analyzed for Cyclopiazonic acid, on a Waters Acquity UPLC system using PDA UV and Qda mass detection with the following gradient solvent system (0.5 ml/min, solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile): 5% B (0-1.25 min.), gradient to 25% B (1.25-1.5 min.), gradient to 100% B (1.5-5.0 min.), 100% B (5.0-7.5 min.), then column equilibration 5% B (7.6-10.1 min.). Cyclopiazonic acid was identified using an authentic standard purchased from Sigma Aldrich (CPA, retention time = 4.10 min, M+H = 337.2 *m/z*). CPA concentrations are expressed in ng CPA / ml culture or ng CPA / g mycelium.

5.3.5 RNA extraction, purification and cDNA synthesis

Total RNA was extracted from fungal cells harvested using a TRIzol-based (TRI Reagent®; cat # T9424, Sigma, Carlsbad, CA, USA) method. The harvested mycelia were ground with a mortar and pestle in liquid nitrogen. The cold powdered mycelia was mixed with TRI Reagent® and chloroform and the mix was spun down at 10,000 rpm for 10 min at room temperature. The organic layer was mixed with equal volumes of isopropanol and incubated on ice for 15 min and later centrifuged at 10,000 rpm for 15 min at room temperature. The precipitated crude RNA was washed with 70% ethanol and resuspended in RNAse/DNAse free water (Sigma, Carlsbad, CA, USA). Within 24 h of extraction, RNA cleanup was performed using a Qiagen RNEasy Cleanup Kit (Qiagen,

Valencia, CA, USA), as per kit's instructions and samples were stored at −80°C. Total RNA was then reverse transcribed to cDNA using iScript™ cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA) as per kit's instructions. All samples were checked for concentration and purity after each step using a NanoDrop 2000 Spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA). All cDNA samples were stored at −20°C until subsequent RTPCR quantification.

5.3.6 Quantitative PCR Assays

Expression of global secondary metabolism genes (*laeA, veA, AtfB*), aflatoxin pathway genes (*aflR, nor-1, ver-1*), pathogenic genes (SAP) and superoxide dismutase (SOD) genes (MnSOD, CuZnSOD) was examined by quantitative PCR assays (qPCR) using SsoAdvanced Universal SYBR Green Supermix (BioRad Laboratories, Hercules, CA). Gene specific forward and reverse primers were designed using Primer3 online software (Ye et al., 2012). Reactions were performed as per BioRad SYBR Green protocol guidelines and quantified using a CFX96 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). All RT-PCRs were performed in triplicate for each gene per sample. The 18s ribosomal DNA was used as a reference gene in the gene analysis. The gene expression values of *A. flavus* obtained from the threshold cycle values were normalized to the 18s rDNA of each sample. We choose 24, 30 and 40 h to study the transcripts of the genes (explained later in results). For quantitative comparison of gene expressions, the values for each target gene at 30 h and 40 h were expressed as fold change relative to the 24 h time point of that specific treatment condition. Data analysis was performed using CFX Manager software (Version 3.1, Bio-Rad Laboratories,

Hercules, CA, USA, 2012). Significant change in gene expression was considered if fold change was ≥ 2 and p-value was < 0.01.

5.3.7 Imaging of fungus and bacteria

5.3.7.1 Confocal microscopy

A. flavus was grown on a cover slip and treated with *V. gazogenes* for 6 h. The fungal mycelia were fixed using 4% formaldehyde and washed using 1xPBS and 0.05% tritionX-100. The resulting mycelia were studied and imaged using Leica TCS SP5 confocal microscope at 20x magnification.

5.3.7.2 Scanning electron microscopy

A. flavus was treated with 16 million live *V. gazogenes* cells for 48 h in YES liquid medium and the mycelia were harvested and fixed using 3% glutaraldehyde and 2% osmium tetroxide, dehydrated in ethyl alcohol, and dried using LADD critical point dryer. Samples were later coated with gold using Denton Vacuum Desk II sputter coater. The coated samples were loaded onto TESCAN Vega-3 SBU scanning electron microscope and studied at 17k magnification to understand the effect of *V. gazogenes* by *A. flavus* hyphae.

5.3.7.3 Transmission electron microscopy

Briefly *A. flavus* was treated with 16 million live *V. gazogenes* cells for 48 h in YES medium. Mycelia were harvested, fixed using 3% glutaraldehyde and 2% osmium tetroxide, dehydrated in ethyl alcohol, and made into blocks using resin mix. The resin blocks were trimmed and cross-sectioned into 80 nm thick sections using a diamond

knife (Micro Star Technology Inc., Huntsville, Texas) loaded onto a Sorvall Porter-Blum MT2-B Ultra-Microtome. The sections were loaded onto copper grids, and later stained with lead stain and 5% uranyl acetate. The stained grids were then loaded onto Hibachi H8000 scanning transmission electron microscope. Pressure was maintained at 10^{-7} Torr, accelerating voltage at 200kV and images were taken at low magnification (10,000x) and at high magnification (20,000x) and the localization of the bacterium in *A. flavus* cells was studied.

5.3.8 Corn treatments

Corn kernels were infected with *A. flavus* in the presence and absence of live *V. gazogenes*. Briefly fresh commercial packaged corn was bought from grocery store and kernels were separated. The kernels were then poked with a toothpick or needle to make a microscopic hole to mimic an insect bite. 10 kernels were placed in each set and 3 sets were in each treatment condition – corn with no infection (negative control), corn with *V. gazogenes* (positive control for bacteria), corn with only *A. flavus* (positive control for fungal infection), and corn with *A. flavus* and live *V. gazogenes* (the treatment set). A monolayer of 400,000 live *V. gazogenes* cell suspended in YES media was applied to the kernels and semi dried. 10,000 *A. flavus* spores were inoculated on top of the bacterial layer for the treatment conditions. Microscopic images using a Leica dissection microscope were taken to observe and record the effect of *V. gazogenes* on *A. flavus* infection. The percentage of infection was estimated (after 48 h of incubation) by comparing the number of infected kernels in the presence and absence of *V. gazogenes*. After 5 days of incubation the kernels were ground in chloroform and filtered. The filtrate was evaporated and residual extract was rinsed out with 100% methanol. Methanol was

evaporated and extracted aflatoxin was resuspended in 1 ml of 70% methanol. The resuspended aflatoxin extract was spun down at 15,000 rpm for 1 min at room temperature to remove extra debris from the corn and mycelia. The clear aflatoxin extract was used for analyzing aflatoxin using the Neogen Veratox Aflatoxin ELISA kit (cat # 8030) from Neogen (Lansing, MI, USA) and measured on a Stat Fax 4700 Microstrip Reader (Awareness Technologies, Palm City, FL, USA) as per kit's protocols. The ELISA has 2 ppb and 50 ppb as lower and upper limits of detection respectively. Thus highly concentrated aflatoxin samples were diluted to fit within the detection range.

5.3.9 Drosophila fly treatment

Drosophila melanogaster female flies of 3-5 days old were treated with *A. flavus* in the presence and absence of live *V. gazogenes* and the survival of the flies were recorded. Method used was published by Ramírez-Camejo et al., in 2014. Drosophila flies were anaesthetized using carbon dioxide and placed on a PDA 10 cm agar plate containing *A. flavus* colony with spores. The flies were rolled on the plates by agitation for 1 min to make them inoculated and then transferred to tubes containing food. (To quantify the number of spores, the inoculated flies were vortexed in sterile water containing 0.01% Tween 80 and centrifuged to spin down the spores, which were then counted using haemocytometer – 2 to 4 $x10^4$ spores attached/fly). The flies were transferred to fresh media tube after 1 h to loose any extra spores. For control or untreated fly treatments 25 flies per tube of 3 tubes were used. For *V. gazogenes* treatments 29 flies per tube of 3 tubes were used. For *V. gazogenes* treatments the drosophila flies were starved for 8 h and then given feed containing *V. gazogenes* for next 24 h. The flies were anaesthetized using carbon dioxide and rolled on PDA 10 cm plates

grown with *A. flavus* similar to the control flies. These infected flies were placed back on *V. gazogenes* containing feed and the feed was replaced with fresh feed containing *V. gazogenes* every 24 h. At the end of 7 days the survival of the flies as compared to controls (negative control had no *A. flavus* infection and positive control had *A. flavus* infection but no *V. gazogenes*) was observed and recorded.

5.3.10 Statistical analysis

All experiments were performed in triplicate. Microsoft Excel was used to generate graphs, perform statistical analysis and calculate significance for dry weight measurements and aflatoxin analysis. Significance was considered when p value was less than 0.01. RT-PCR statistical analysis was performed using CFX manager software with parameters set for significance at p-value <0.01 and gene expression fold change ≥ 2.

5.4 RESULTS

5.4.1 *V. gazogenes* inhibits aflatoxin production of *A. flavus* without effecting growth of the fungus

To understand the effect of *V. gazogenes* on *A. flavus* growth and aflatoxin synthesis, we initially performed a dose response of multiple cellular concentrations of *V. gazogenes* on *A. flavus*. The fungus was treated with 4 and 16 million *V. gazogenes* cells in YES liquid media for 48 h. The dry weight analysis at the end of the incubation period revealed that the mycelial growth of *A. flavus* did not differ with either 4 or 16 million *V. gazogenes* cells as compared to the untreated control (Figure 5.1a). On the other hand, the aflatoxin levels analyzed using ELISA technique show that there were nearly

undetectable levels of aflatoxin in the YES liquid media in the presence of *V. gazogenes* (irrespective of cellular concentration) as compared to the untreated control (Figure 5.1b).

Most surfaces in the environment on which *A. flavus* thrives are solid. Thus we performed similar experiments on YESA solid media. *A. flavus* was treated with different bacterial cellular concentrations of 4 and 16 million *V. gazogenes* cells on the YESA media plates and the spread of the fungal colony on the media was measured and recorded daily. The graph in figure 5.1c reveals that *V. gazogenes* decreases the *A. flavus* growth by 25% regardless of the bacterial cellular concentration. Aflatoxin analysis at the end of 9 days of incubation showed that 16 million *V. gazogenes* cells significantly decreased aflatoxin production to nearly undetectable levels where as 4 million *V. gazogenes* cells only made a 25% difference as compared to the untreated control (figure 5.1d).

These data clearly state that 16 million *V. gazogenes* cells consistently and significantly decrease aflatoxin production (>99%) in both solid and liquid media. The aflatoxin inhibitory effect of 16 million *V. gazogenes* cells was further validated by UPLC (table 5.2). The ultra HPLC shows the complete loss of aflatoxin B1 and aflatoxin B2 by *A. flavus* during *V. gazogenes* treatments. We also observed the complete loss of Cyclopiazonic acid (another major mycotoxin belonging to ergoline alkaloids) of *A. flavus* during *V. gazogenes* treatments. Accordingly for further experimentation we choose to use 16 million *V. gazogenes* cells.

5.4.2. *V. gazogenes* decreases *A. flavus* aflatoxin production over time

To understand the effect of *V. gazogenes* on aflatoxin production beyond the normal 48 h, we treated *A. flavus* (in YES liquid media) with 16 million *V. gazogenes* cells for 72 hours, collecting media and mycelia at 24, 48 and 72 h time points. Dry weight analysis revealed the no difference in the growth of the fungi between treated and untreated samples (figure 5.2a) for that specific time point. On the other hand the aflatoxin analysis revealed that *V. gazogenes* decreases aflatoxin to undetectable levels even at 72 h time point (figure 5.2b). The data shows that the aflatoxin inhibitory effect of *V. gazogenes* does not stop at the early stationary phase (48 h) where the fungal cells were thriving but also that the *V. gazogenes* effectively inhibits aflatoxin when the fungal cells were over crowded and starting to form a biofilm (72 h).

5.4.3 *V. gazogenes* aflatoxin inhibitory effect was carried on to the second generation of *A. flavus*

The classical potato dextrose agar media was used to study the growth of *A. flavus* spores in the presence of *V. gazogenes*. Two different bacterial cellular concentrations of 4 and 16 million *V. gazogenes* cells were spread on the agar plate and fungal colony growth was observed for 9 days. Observation of the growth of fungal biofilm in the presence of the bacterium tells us that the growth was significantly slower in the beginning but eventually catches up to the control with no bacteria (figure 5.3a). The aflatoxin analysis shows the same pattern of aflatoxin inhibition (~75% for 4 million cells and undetectable levels for 16 million cells) in the presence of *V. gazogenes* (figure 5.3b). But surprisingly the spore count for the fungal colony in the presence of 16 million cells

was >50% as compared to its no bacterial control (figure 5.3c). The gram-negative bacterium *V. gazogenes* encourages spore generation in *A. flavus*.

To test the viability and aflatoxin inhibitory effects of the spores generated in the presence of *V. gazogenes*, we collected the spores from bacterial treated (4 and 16 million cells) fungal colonies and re-plated/inoculated them on fresh PDA plates. We called these the second-generation spores. The second-generation spores had growth pattern and spore formation similar to controls (figure 5.3d). The second-generation spores generated from 4 million bacterial cells treated fungi did not carry on the aflatoxin inhibitory characteristics but the spores generated from the 16 million bacterial cells treated fungi inhibited aflatoxin by 40% (figure 5.3e). Surprisingly the second-generation spores carried their aflatoxin inhibition characteristics. This tells us that the *V. gazogenes* aflatoxin inhibitory effects can be carried through generations.

5.4.4 Dead *V. gazogenes* cells inhibit aflatoxin levels in *A. flavus*

Dead bacterial biomass had proven to be better and safer bioadsorbent for contaminants in the environment and is much preferred due to lack of nutrients and cultural conditions (Zeroual et al., 2006). To test the supposition that dead *V. gazogenes* cells were equally capable of decreasing aflatoxin levels in *A. flavus* cultural media, we boiled the bacteria at 100^{O}C for 10 min in a hot plate to make them unviable. (Bacterial cells were tested by inoculation into fresh marine broth media and observed for growth using spectrophotometric analysis over the next 48 h and confirmed the no growth of the *V. gazogenes* cells). 4 and 16 million dead *V. gazogenes* cells were used to treat *A. flavus* and the mycelial growth and aflatoxin synthesis were observed. After 48 h the fungal

mycelial mass did not change (regardless of bacterial cellular concentrations) but the aflatoxin levels significantly decrease with 16 million dead *V. gazogenes* cells (figure 5.4) similar to live *V. gazogenes* cells. The UPLC data in table 5.2 shows that dead *V. gazogenes* cells cause complete loss of aflatoxin B1, aflatoxin B2 and cyclopiazonic acid similar to live *V. gazogenes* cells. This shows us that the unviable and viable *V. gazogenes* cells equally inhibit mycotoxin production.

5.4.5 Aflatoxin inhibition of *A. flavus* is specific to *V. gazogenes*

To understand if the aflatoxin inhibitory effect was limited to *V. gazogenes*, we treated *A. flavus* with a gram-positive bacterium, *Staphylococcus aureus* and a gram-negative bacterium, *Escherichia coli* and analyzed the fungal growth and aflatoxin levels. 16 million dead bacterial cells were added to *A. flavus* and the fungus was harvested after 72 h. Dead bacteria cells were used to reduce the pathogenic interaction between the *A. flavus*, *S. aureus* and *E. coli*. The growth of the *A. flavus* did not change in the presence of either *S. aureus* or *E. coli* and the aflatoxin levels did not show any significant change either (figure 5.5). This data points out that there is a high possibility that the aflatoxin inhibitory effect of *V. gazogenes* is exclusive to itself.

5.4.6 *A. flavus* uptake of *V. gazogenes*

Bacterial-fungal interactions exist via various physical associations. In the bacterial-fungal biofilms, one form of association is the internalization of bacteria by fungi altering the fungal physiology (Frey-Klett et al., 2011). *V gazogenes* synthesizes prodigiosins, which are red in color giving the bacterial cells a red color. *A. flavus* cultures (colorless or white) in YES liquid medium were treated with live *V. gazogenes*

(red color) and every 6 h the bacterial presence in YES was observed and recorded using spectrophotometry reading at 600 nm. We observed that the *V. gazogenes* optical density decreased over time and the fungal mycelia turned reddish pink (as attributed to the red pigment in the bacterial cells). The figure 5.6a shows that over a time period of 42 h the bacteria were completely depleted from the YES media and the media turned back to its original yellow. To understand the interactions between *A. flavus* and *V. gazogenes* we fixed *A. flavus* hyphae in the presence of live *V. gazogenes* and studied them using a Leica confocal microscope. At 20x magnification we observed the presence of bacteria inside the fungal walls (figure 5.6b) confirming the uptake of *V. gazogenes* by *A. flavus* hyphae.

5.4.7 Live *V. gazogenes* increases aflatoxin biosynthesis pathway genes of *A. flavus* over time

Previously, Gummadidala et al (Gummadidala et al, 2016) had shown that *V. gazogenes* metabolites decrease aflatoxin biosynthesis by inhibiting the aflatoxin genes. After understanding the effect of *V. gazogenes* on growth and aflatoxin production of *A. flavus* and observing the uptake of *V. gazogenes* by *A. flavus* hyphae, we hypothesized the aflatoxin inhibitory effect was happening at the transcript level. A quantitative comparison of transcript accumulation of two aflatoxin genes (*nor-1* and *ver-1*) and the aflatoxin pathway regulator gene (*aflR*) was performed. Norsolorinic acid is the first stable compound in the 17-step aflatoxin biosynthesis pathway, which is synthesized by the Nor-1 reductase enzyme that is encoded by *nor-1* gene (Jiujiang Yu, 2012). Versicolorin A (VER A) is the last compound synthesized prior to the making of the intermediates that lead to the final aflatoxin products. VER A is converted to the first

intermediate by a reductase, Ver-1 that is encoded by *ver-1* gene (Jiujiang Yu, 2012). The aflatoxin biosynthesis genes get activated by 24 h with transcript accumulation at 30 h and by 40 h the fungus is producing copious amounts of aflatoxin in YES liquid growth media (Roze et al., 2007a). Thus we choose 24, 30 and 40 h to study the transcripts of *nor-1*, *ver-1* and *aflR* genes. The 30 h and 40 h samples were compared to the 24 h sample of that specific treatment condition to calculate relative fold change in gene expressions. Figure 5.7 shows 3 graphs of fold change of *aflR*, *nor-1* and *ver-1* gene expressions. The *aflR* (2-3 fold change), *nor-1* (3-7 fold change) and *ver-1* (2-9 fold change) gene expressions of untreated control samples of *A. flavus* were significantly upregulated from 24 h to 40 h, which is in accordance with multiple published studies. The *aflR* (~7 fold change), *nor-1* (~25 fold change) and *ver-1* (14-17 fold change) gene expressions of live *V. gazogenes* treated samples of *A. flavus* were significantly upregulated from 24 h to 40 h as compared to their specific controls. On the contrary the for the *A. flavus* treated with dead *V. gazogenes* cells, *aflR*, and *nor-1* show no significant change and *ver-1* decreased at 30 h and increased at 40 h to reach get back to the regular levels. The data leads us to hypothesize that the *V. gazogenes* aflatoxin inhibitory mechanism is different for live and dead *V. gazogenes* cells. The compilation of the data shows that both live and dead *V. gazogenes* cells decrease aflatoxin but do not inhibit the aflatoxin pathway genes. This leads to the conclusion that the aflatoxin inhibitory effect of *V. gazogenes* was not occurring by regulation of the aflatoxin pathway genes.

5.4.8 Effect of *V. gazogenes* on global secondary metabolism regulatory genes of *A. flavus*

The two global secondary metabolism regulatory genes *laeA* and *veA* have been found to be involved in regulation of aflatoxin synthesis in *A. flavus* and the deletion of these genes leads to complete loss of aflatoxin (Cary et al., 2018). To understand if any of the upstream positive or negative regulators of aflatoxin pathway were affected we studied the transcript accumulation of *laeA*, *veA* and *AtfB* in the presence of live/dead *V. gazogenes* cells at the 24 h, 30 h and 40 h time points of *A. flavus* growth (figure 5.8). The 30 h and 40 h samples were compared to the 24 h sample of that specific treatment condition. The live *V. gazogenes* cells inhibit the *laeA* and increase the *veA* gene expressions from a 24 h to 40 h time period, which was opposite to what happened in the untreated control samples. *laeA* is a negative regulator of *veA* (Amaike and Keller 2009) so its not surprising that one transcript accumulation increases while the other deceases. The pattern of *laeA* and *veA* gene expression was similar during dead *V. gazogenes* treatments with increase in their gene expression at 40 h time point. We would like to point out once again that the pattern of gene expression is not explaining the decrease in aflatoxin levels. This draws the conclusion that live or dead *V. gazogenes* aflatoxin inhibitory effect is not due to regulation of aflatoxin pathway genes or global secondary metabolite regulatory genes. Rather the bacterial inhibitory effect might be at the cellular level.

5.4.9 *V. gazogenes* was endocytosed into endosomal like compartments by the *A. flavus*

To understand the effect of *V. gazogenes* on *A. flavus* at a cellular level we used electron microscopy to study the surface (SEM) and inside (TEM) of the fungal hyphae in the presence of live *V. gazogenes* cells. The scanning electron microscope (SEM) pictures in figure 5.9 shows images of the surface of *A. flavus* hyphae untreated/treated with live *V. gazogenes* cells. At 17k magnification the wrinkled effected surface of *V. gazogenes* treated hyphae can be appreciated. The SEM magnified image of the treated hyphae shows the entry of the bacteria (yellow arrows) into the *A. flavus* probably via endocytosis like process. The transmission electron microscopy (TEM) pictures in figure 5.10 illustrate cross-sectional images of the *A. flavus* hypha exposing different internal cellular structures. The 10k and 20k magnified TEM images show the presence of the *V. gazogenes* in endosomal like compartments (white arrows) in the *A. flavus* hypha. Similarly uptake of nano particles by fungi also altered the fungal physiology by decreasing aflatoxin biosynthesis (Mitra et al., 2017). These microscopic images state that *V. gazogenes* enter the *A. flavus* hyphae leading to decrease in aflatoxin biosynthesis. Therefore we state that the *V. gazogenes* aflatoxin inhibitory effect was happening at the cellular level.

5.4.10 Effect of *V. gazogenes* on SOD genes

Previous studies in our lab support the hypothesis that reactive oxygen species output is regulated by aflatoxin biosynthesis (Keene et al., 2018). To understand the cellular processes involved in aflatoxin inhibitory effect of *V. gazogenes* we studied the genes involved in reactive oxygen species (ROS) regulation. Superoxide dismutases are

synthesized in response to reactive oxygen species. We studied the genes MnSOD and CuZnSOD in the presence of live/dead *V. gazogenes* cells at the 24 h, 30 h and 40 h time points of *A. flavus* growth (figure 5.11). The 30 h and 40 h samples were compared to the 24 h sample of that specific treatment condition. The CuZnSOD gene expression was decreased as compared to its specific control during both live and dead *V. gazogenes* treatments. This is similar to the already existing data stating the decrease in CuZnSOD from 24 h to 40 h. The MnSOD gene expression increased as compared to its specific control during both live and dead *V. gazogenes* treatments following the same pattern as untreated control samples. The only difference was that in the live V. gazogenes treatments the increase in MnSOD over time is double as compared to the untreated controls. Keene et al propose a model for aflatoxin biosynthesis protecting the cells against the toxic effects of ROS (Keene et al., 2018). Therefore more aflatoxin means less ROS. Thus the increase in MnSOD in presence of live *V. gazogenes* cells might be hypothesized that there might be increased levels of ROS as the aflatoxin levels had been all but completely inhibited.

5.4.11 *V. gazogenes* decreases aflatoxin in corn and delays infestation of corn by *A. flavus*

A. flavus effects plant health and crop produce by not only producing the mycotoxin aflatoxin but also by acting as a pathogen. Data already showed the aflatoxin inhibitory effects of *V. gazogenes* so now we would like to understand the effects of *V. gazogenes* on *A. flavus* pathogenicity. We pricked corn kernels to mimic insect bites since it was proved that insect bites make the corn more susceptible to *A. flavus* infestations (Cardwell et al., 2000). Three sets of 10-kernels/each set were used and data

was shown in figure 5.12 where the top panel shows pictures of kernels taken using Leica dissection microscope. The kernels having no *A. flavus* or *V. gazogenes* did not have any bacterial or fungal contamination. The positive controls for *V. gazogenes* did not have any fungal contamination or bacterial infestation suggesting that *V. gazogenes* might not be a plant pathogen. The positive controls for *A. flavus* had plenty of mycelial growth and spore generation proving the already known fact that *A. flavus* infests maize. The kernels treated with live *V. gazogenes* cells prior to *A. flavus* infestation showed only 20% conidial formation, which is almost 80% less than *A. flavus* positive controls. The aflatoxin production in the *V. gazogenes* treated kernels was <98% as compared to the positive controls. These data show that *V. gazogenes* has a high possibility to become anti-*A. flavus* pesticide for plants.

5.4.12 *V. gazogenes* increases survival of Drosophila flies infected with *A. flavus*

Drosophila melanogaster is a well-studied and well-established model organism for understanding human diseases. Here we used the common fruit fly as a model to study the effects of *V. gazogenes* on *A. flavus* human pathogen. We used atleast 25 flies/tube of 3 sets per condition and data was shown in figure 5.13. Drosophila flies with no *A. flavus* or *V. gazogenes* had survival of 100% at the end of 7 days. Drosophila flies with only *V. gazogenes* also had survival of 100% at the end of 7 days suggesting that *V. gazogenes* might not be a Drosophila fly pathogen. The positive controls with Drosophila having infected with only *A. flavus* had survival of 0 at the end of 8 days since all flies died. Surprisingly the flies treated with *V. gazogenes* in their feed and then infected with *A. flavus* had survival of 40% at the end of 7 days. Finally we studied the pathogenic gene SAP (serine alanine protease) since it was considered as a marker for A. flavus

pathogenicity. In the untreated samples SAP gene expression increased at 40 h but in live *V. gazogenes* treated samples the SAP gene expression increase early on at 30 h and maintain high through 40 h. The dead V. gazogenes cell treatments to not effect SAP gene expression as compared to their control at 24 h. The data show that *V. gazogenes* increase survival in Drosophila during *A. flavus* infections and the mechanism of action might not be at the gene level.

5.5 DISCUSSION

This research study shows the discovery of a novel tool (*Vibrio gazogenes*) to inhibit aflatoxin production and pathogenicity of human and plant pathogen *Aspergillus flavus*. Researchers had previously shown the inhibition of aflatoxin production in *A. flavus* or a close sister species *A. parasiticus* by lactic acid bacteria such as *Lactobacillus casei* (Chang and Kim 2007), gram positive bacteria such as *Bacillus subtilis* (Farzaneh et al., 2017) and other soil bacteria. Most studies show a decrease in aflatoxin levels and the mechanism of action of the bacterium was primarily by inhibiting the aflatoxin biosynthesis pathway genes. Here for the first time we use a marine bacterium *V. gazogenes* and the mechanism of aflatoxin inhibition was not at the gene level (we observe an increase in aflatoxin and secondary metabolite regulatory genes) but at the cellular level thereby breaking the previously established paradigm.

Both viable and unviable *V. gazogenes* cells inhibition of aflatoxin is >98% with out effecting the growth of *A. flavus* in both solid and liquid growth medium. On the other hand live *V. gazogenes* cells significantly increase spore production of *A. flavus* on classical potato dextrose media. Spores are the means by which the fungi disperse and

find new and favorable environment to grow and flourish on. The second-generation spores generated due to *A. flavus* treatment with *V. gazogenes* carried over the aflatoxin inhibition properties and consequently decreased aflatoxin by 40%. It is new to know that the V. gazogenes aflatoxin inhibitory effects can transcend generations.

The gene data of *A. flavus* of various genes up- and down- regulated in the presence of live and dead *V. gazogenes* does not follow the same pattern. This leads us to conclude that live and dead *V. gazogenes* cells do not follow the same mechanism of aflatoxin inhibition in *A. flavus*. *V. gazogenes* were made nonviable or dead by heating which causes protein denaturation but might not break the structural integrity of the peptidoglycan structure of the bacterial cell wall (Carolyn A. Haskard, et al., 2001). Lactic acid bacteria made nonviable by heating effectively removed aflatoxin B1 from the media suggesting binding rather than metabolism (Carolyn A. Haskard et al., 2001). Therefore the dead *V. gazogenes* might be removing aflatoxin from media via cell wall binding.

The aflatoxin inhibition by live *V. gazogenes* was through the uptake of the live bacterium by fungus into endosomal like compartments, which we had observed using confocal, SEM and TEM microscopies. Previously published data show the internalization of the bacteria via endocytosis like mechanism (Guerra-Tschuschke et al., 1991). Anindya et al isolated protoplasts and performed feeding experiments concluding that aflatoxin synthesis was happening in vesicles termed aflatoxisomes, which are endosomal like compartments (Chanda et al., 2009). Thus we suggest that live *V. gazogenes* cells were entering the *A. flavus* through endocytosis like mechanism (into endocytosis like compartments) probably into the aflatoxisomes (that contains all the

enzymes required for aflatoxin synthesis) and inhibiting the aflatoxin biosynthesis at the cellular level. Researchers propose that during the fungal growth, a shift in the media components might activate the hydrolase and laccase enzymes, which have the capacity to degrade the lactone ring of the aflatoxin (Fatemeh Siahmoshteh et al., 2016). Considering the existing data we hypothesize that live *V. gazogenes* cells might be degrading the aflatoxin in the aflatoxisome. Since there was no aflatoxin released into the growth media during live *V. gazogenes* treatments (undetected using ELISA) but an increase in aflatoxin pathway genes was observed we predict that the fungal cells were making increased transcript to generate more aflatoxin in the fungal cells.

The relationship between the live *V. gazogenes* and *A. flavus* is not conclusive from the data obtained. Further experimentation is required to determine the symbiotic relationship's existence and nature between the two microorganisms. We do not yet know if the bacterium is still viable inside the fungus. Bacteria are hard to kill and as such there is a strong possibility for bacterial-fungal interaction within the fungal cell.

Understanding the mechanism by which *V. gazogenes* decreases aflatoxin and inhibits pathogenicity of *A. flavus* will help us further understand how to develop, design and target *A. flavus* pathogen and decrease mortality rates of fungal infected patients and plants. This research establishes a novel concept for combating Aspergillosis infections that are very common in homes impacted by weather events such as hurricanes and are common in immunocompromised individuals (Krishnan et al., 2009). Finally polymicrobial (bacterial and fungal) colonies pose a potential problem in clinical setting given their multi-drug resistance capabilities. This study has initiated a novel research direction that can elucidate the molecular details that regulate bacterial uptake in fungal

pathogens. Such interactions are critical in designing probiotic supplements for preventing release of virulence factors and secondary metabolites that are key in fungal pathogenesis.

Table 5.1: List of primers used in this study.

Genes	Primer Sequences
aflR	F 5'-ACCTCATGCTCATACCGAGG-3' R 5'-GAAGACAGGGTGCTTTGCTC-3'
nor-1	F 5'-CACTTAGCCAGCACGATCAA-3' R 5'-ATGATCATCCGACTGCCTTC-3'
ver-1	F 5'-AACACTCGTGGCCAGTTCTT-3' R 5'-ATATACTCCCGCGACACAGC-3'
AtfB	F 5'-CCGGTTTCGTGAGGTATCCA-3' R 5'-GCATGGGAGAAACCAGATCG-3'
laeA	F 5'-ATGGGGTGTGGAAGTGTGAT-3' R 5'-ATCGGTAAAACCAGCCTCCT-3'
veA	F 5'-TCCAGCTATCCCAAGAATGG-3' R 5'-TAATCCCCCGATAGAGCCTT-3'
MnSOD	F 5'-CCACATCAACCACTCCCTCT-3' R 5'-TCCTGATCCTTCGTCGAAAC-3'
CuZnSOD-1	F 5'-CACCAGTTCGGTGACAACAC-3' R 5'-GTGTTCACTACGGCCAAGGT-3'
18s	F 5'-GCTGAAAACCTCGACTTCGG-3' R 5'-CCTAATTCCCCGTTACCCGT-3'
Tubulin	F 5'-TCTCCAAGATCCGTGAGGAG-3' R 5'-TTCAGGTCACCGTAAGAGGG-3'
SAP	F 5'-GAATTCTCGTGGACGTAGCG-3' R 5'-GACGTCGGTCCTTCTTCTCC-3'

Table 5.2: Data of Aflatoxin B1, Aflatoxin B2 and Cyclopiazonic acid obtained from UPLC

Sample	Aflatoxin B1 ppb (ng/g mycelia)	Aflatoxin B2 ppb (ng/g mycelia)	Cyclopiazonic acid ppb (ng/g mycelia)
A. flavus (no *V. gazogenes*)	24.51	3.27	7298.41
A. flavus + 16 million live *V. gazogenes* cells	0	0	0
A. flavus + 16 million dead *V. gazogenes* cells	0	0	0

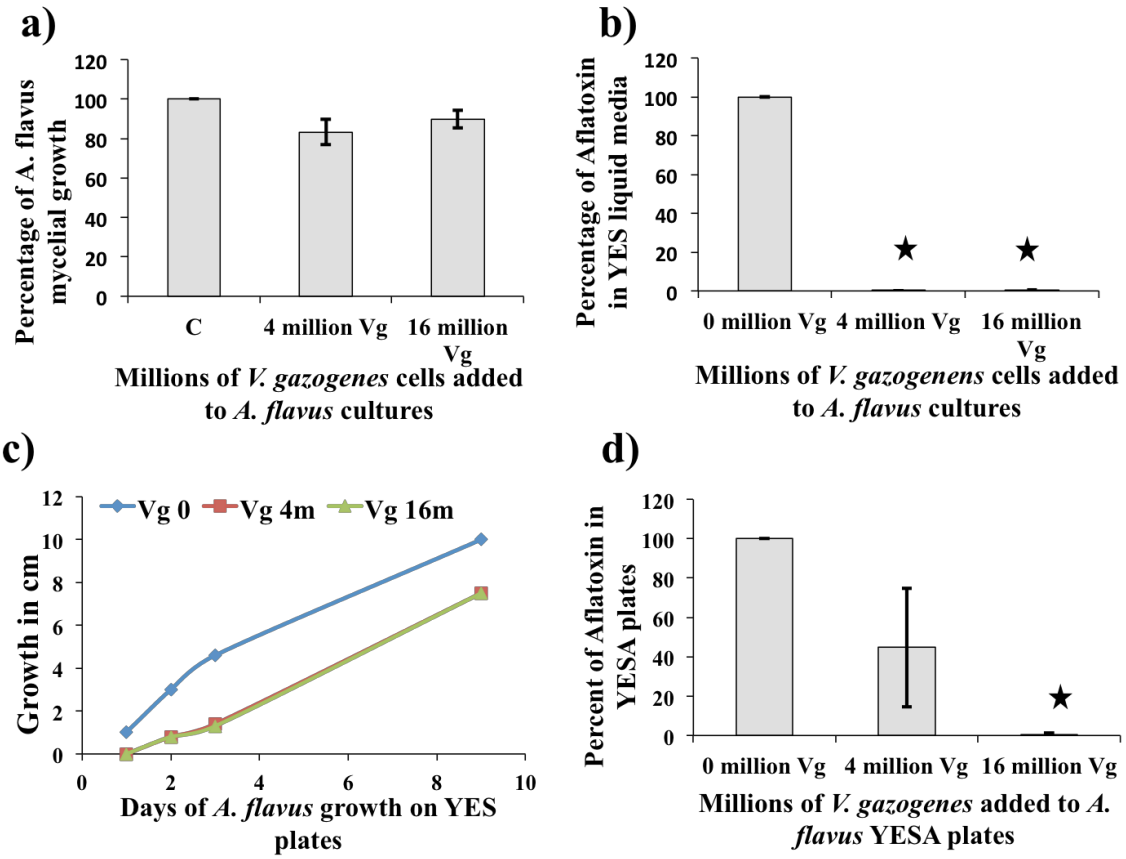


Figure 5.1: Effect of *V. gazogenes* on aflatoxin biosynthesis and growth of *A. flavus* mycelial growth: a) & b) *A. flavus* co-cultured with 4 and 16 million *V. gazogenes* cells in YES liquid media and the dry weight of fungal mycelia was measured and aflatoxin levels analyzed after 48 h of incubation. The percentage of *A. flavus* mycelial weight was calculated and plotted on y-axis. Bars represent measurements relative to the dry weight of untreated (a). Percent aflatoxin accumulation in media of 48 h cultures (b). p-value <0.01 and n=3 c)&d) *A. flavus* co-cultured with *V. gazogenes* on YES agar media plates and the colony growth was measured and the aflatoxin was analyzed after 9 days of incubation. The growth of fungal colony in cm was measured and plotted on y-axis (c) (n=2). Percent aflatoxin accumulation in agar media of the fungal colonies was plotted (d) (n=4). p-value <0.01. Statistical significance of two-tailed *p*-values were determined using an unpaired *t*-test. Error-bars represent SEM. Star indicates significance.

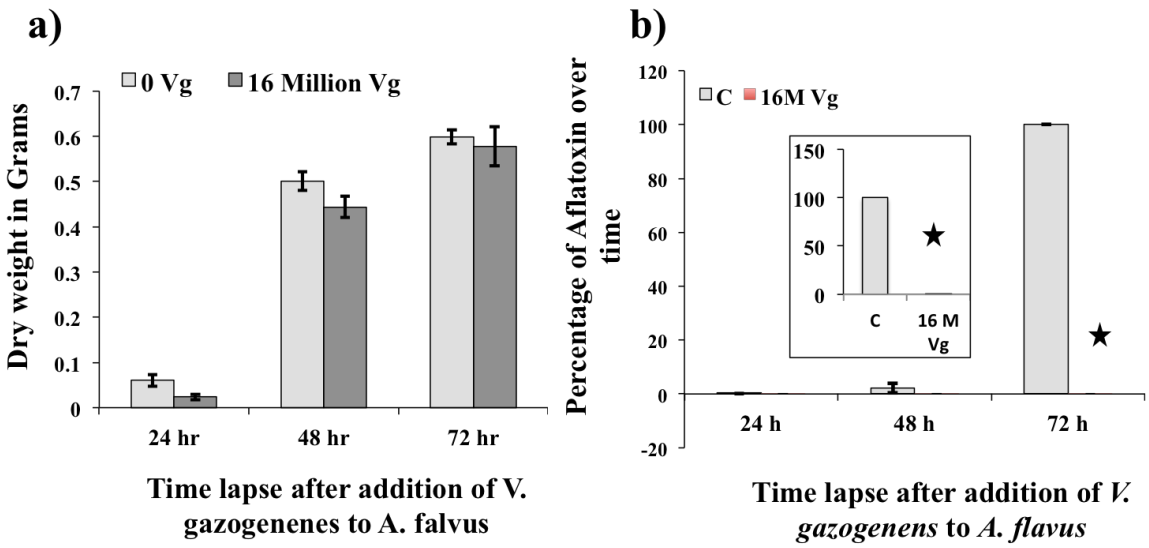


Figure 5.2: Effect of *V. gazogenes* on *A. flavus* aflatoxin production over time: *A. flavus* was co-cultured with 16 million *V. gazogenes* cells in YES liquid media and the mycelial growth of fungus was calculated and aflatoxin analyzed at 24, 48 and 72 h. a) Dry weight of mycelia was plotted on y-axis and bars represent the measurements in grams. b) Percentage of aflatoxin was plotted on y-axis considering no treated (C) 72 h control aflatoxin levels as 100% and the aflatoxin in rest of samples plotted relative to the 72 h C. Insert shows the accumulation of aflatoxin at 48 h. p-value <0.01. Statistical significance of two-tailed *p*-values were determined using an unpaired *t*-test for n=3. Error-bars represent SEM. Star indicates significance.

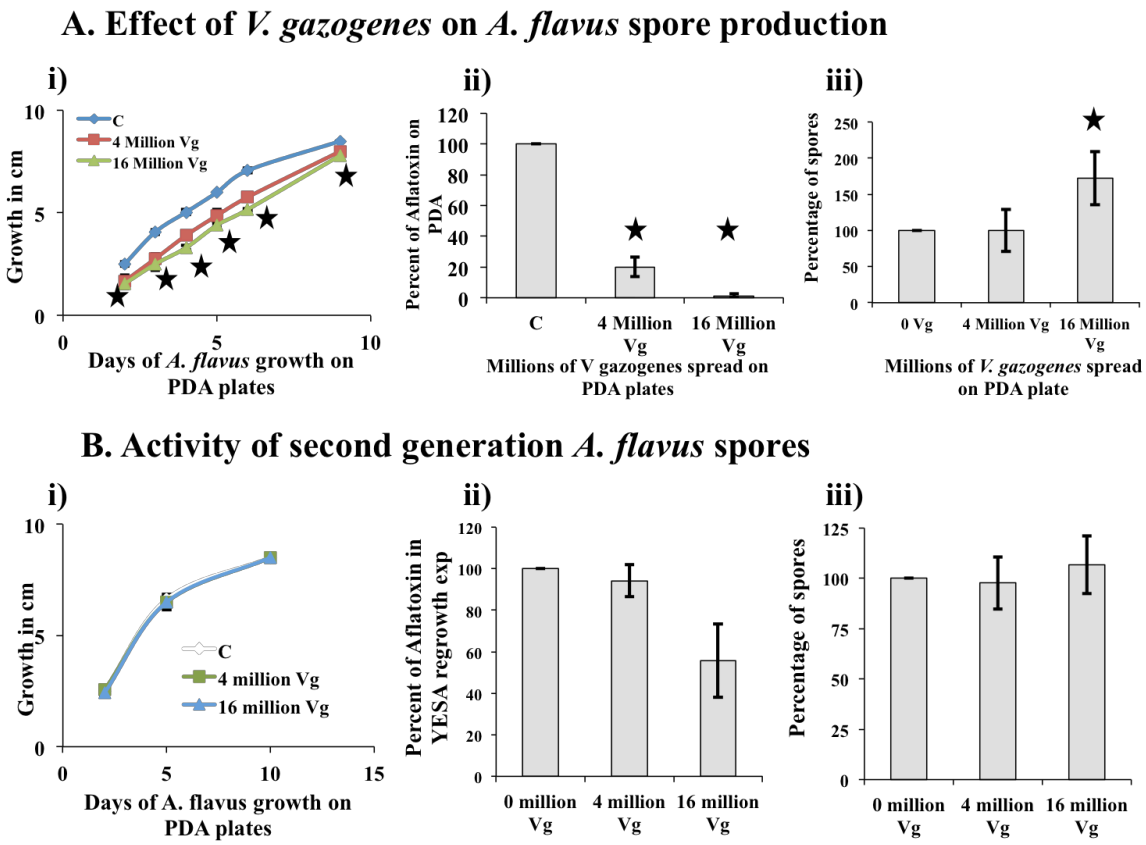


Figure 5.3: A) Effect of *V. gazogenes* on *A. flavus* spore generation: *A. flavus* was co-cultured with *V. gazogenes* on 10cm PDA media plates and the colony growth was measured and the aflatoxin was analyzed after 9 days of incubation. i) The growth of fungal colony in cm was measured and plotted on y-axis. ii) The bars represent percent aflatoxin accumulation in agar media of the fungal colonies as compared to the untreated control. iii) Percent of spores per colony was counted and plotted with the bars representing the number of spores as compared to the untreated control (n=4). B) Activity of second-generation *A. flavus* spores. Spores generated from bacterial treatment were re-plated on fresh PDA plates (without *V. gazogenes*) and colony growth was observed for 9 days. i) The growth of fungal colony in cm was measured and plotted on y-axis. ii) The bars represent percent aflatoxin accumulation in agar media of the fungal colonies as compared to the untreated control. iii) Percent of spores per colony was counted and plotted with the bars representing the number of spores as compared to the untreated control (n=2). Statistical significance of two-tailed *p*-values were determined using an unpaired *t*-test. Error-bars represent SEM. Star indicates significance.

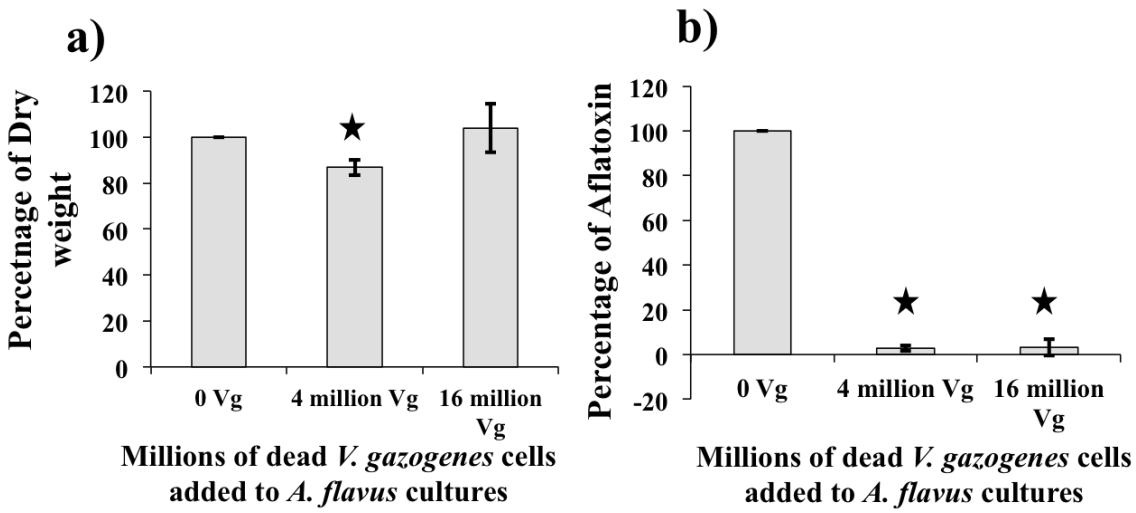


Figure 5.4: Effect of dead *V. gazogenes* cells on *A. flavus* growth and aflatoxin production: *A. flavus* co-cultured with dead 4 and 16 million *V. gazogenes* cells in YES liquid media and the dry weight of fungal mycelia was measured and aflatoxin levels analyzed after 48 h of incubation. a) The percentage of *A. flavus* mycelial weight was calculated and plotted on y-axis. Bars represent measurements relative to the dry weight of untreated. b) Percent aflatoxin accumulation in media of 48 h cultures. p-value <0.01 and n=2. Statistical significance of two-tailed *p*-values were determined using an unpaired *t*-test for n=3. Error-bars represent SEM. Star indicates significance.

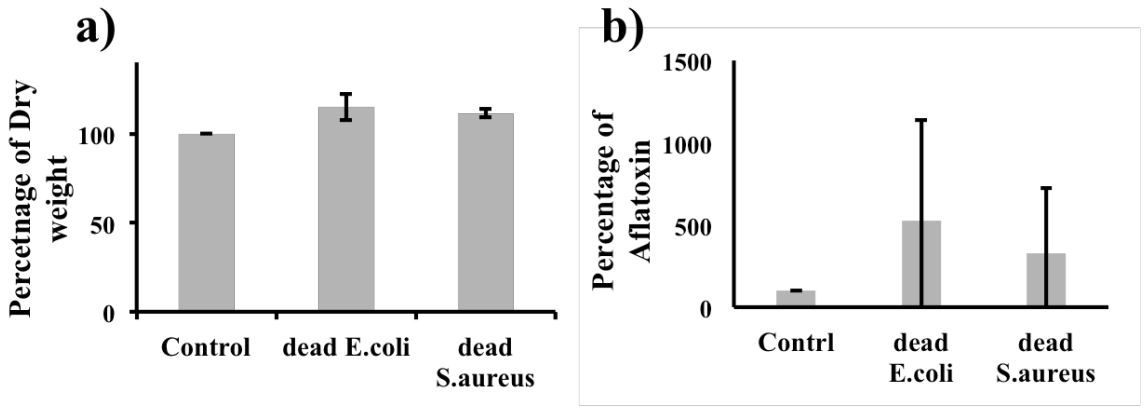


Figure 5.5: Effect of dead *S. aureus* and *E. coli* on *A. flavus* growth and aflatoxin production: *A. flavus* co-cultured with dead 16 million *S. aureus* and *E. coli* cells in YES liquid media and the dry weight of fungal mycelia was measured and aflatoxin levels analyzed after 72 h of incubation. a) The percentage of *A. flavus* mycelial weight was calculated and plotted on y-axis. Bars represent measurements relative to the dry weight of untreated. b) Percent aflatoxin accumulation in media of 48 h cultures. p-value <0.01 and n=2. Statistical significance of two-tailed *p*-values were determined using an unpaired *t*-test for n=2 revealing that there was no statistical difference between control and treated samples. Error-bars represent SEM.

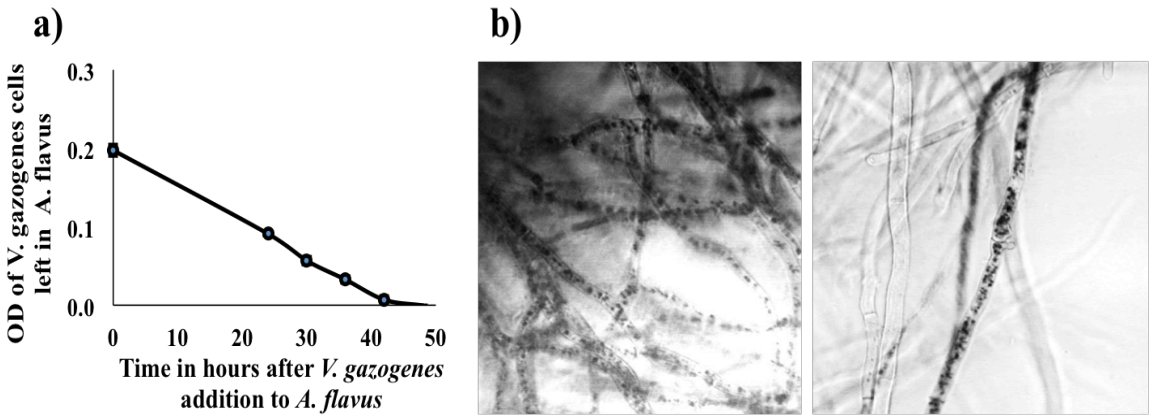


Figure 5.6: Uptake of *V. gazogenes* by *A. flavus*: a) *V. gazogenes* and *A. flavus* were co-cultured in YES liquid media. Every 6 h the OD of the bacteria at 600 nm was measured and plotted on y-axis with incubation time in the x-axis (n=3). b) The confocal microscopy of *A. flavus* hyphae in the presence of *V. gazogenes* at 20x magnification. This is a representation of n=3 experiments.

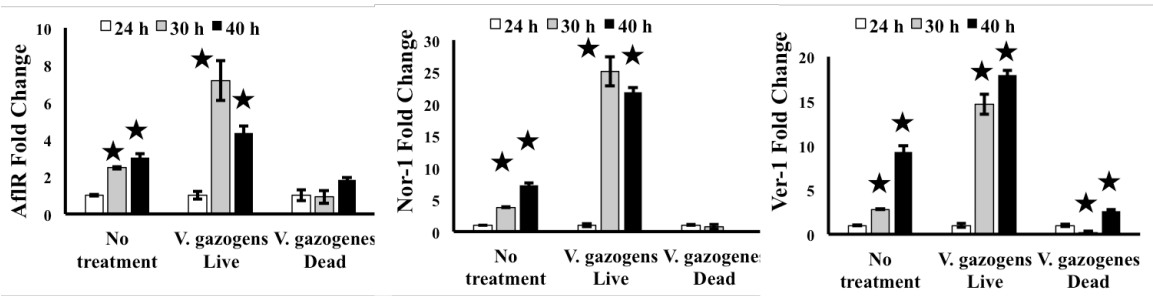


Figure 5.7: *V. gazogenes* increases aflatoxin pathway genes: Quantitative PCR (qPCR) comparison of aflatoxin pathway gene (*aflR, nor-1, ver-1*) expressions in *A. flavus* during the two treatment conditions of live and dead *V. gazogenes* at 24 h, 30 h and 40 h time points of culture growth. Each individual gene expression was normalized to the housekeeping gene 18s which was used as reference gene. The 30 h and 40 h samples were compared to the 24 h sample of that specific treatment condition to calculate relative fold change in gene expressions. Fold changes ≥2.0 with p-value <0.01 were considered significantly up or down regulated. Star indicates statistically significant fold change as compared to the 24 h control of that specific treatment.

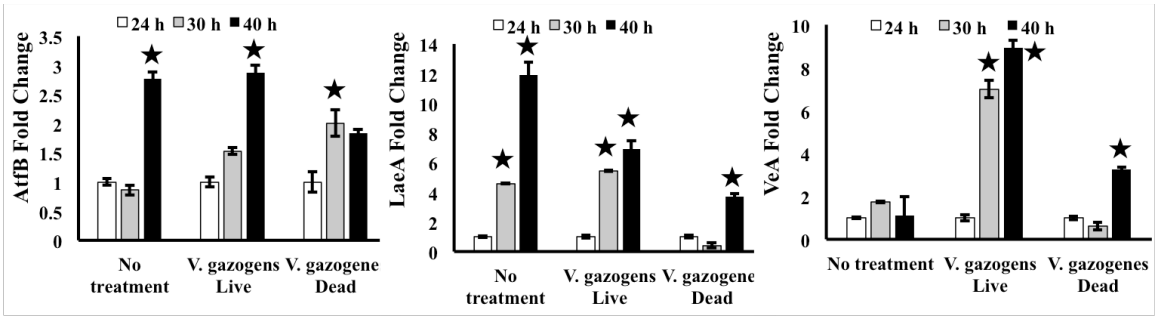


Figure 5.8: *V. gazogenes* increases global secondary metabolite regulatory genes: Quantitative PCR (qPCR) comparison of global secondary metabolite regulate (*AtfB, laeA, veA*) gene expression in *A. flavus* during the two treatment condition of live and dead *V. gazogenes* at 24 h, 30 h and 40 h time points of culture growth. Each individual gene expression was normalized to the housekeeping gene 18s which was used as reference gene. The 30 h and 40 h samples were compared to the 24 h sample of that specific treatment condition to calculate relative fold change in gene expressions. Fold changes ≥2.0 with p-value <0.01 were considered significantly up or down regulated. Star indicates statistically significant fold change as compared to the 24 h control of that specific treatment.

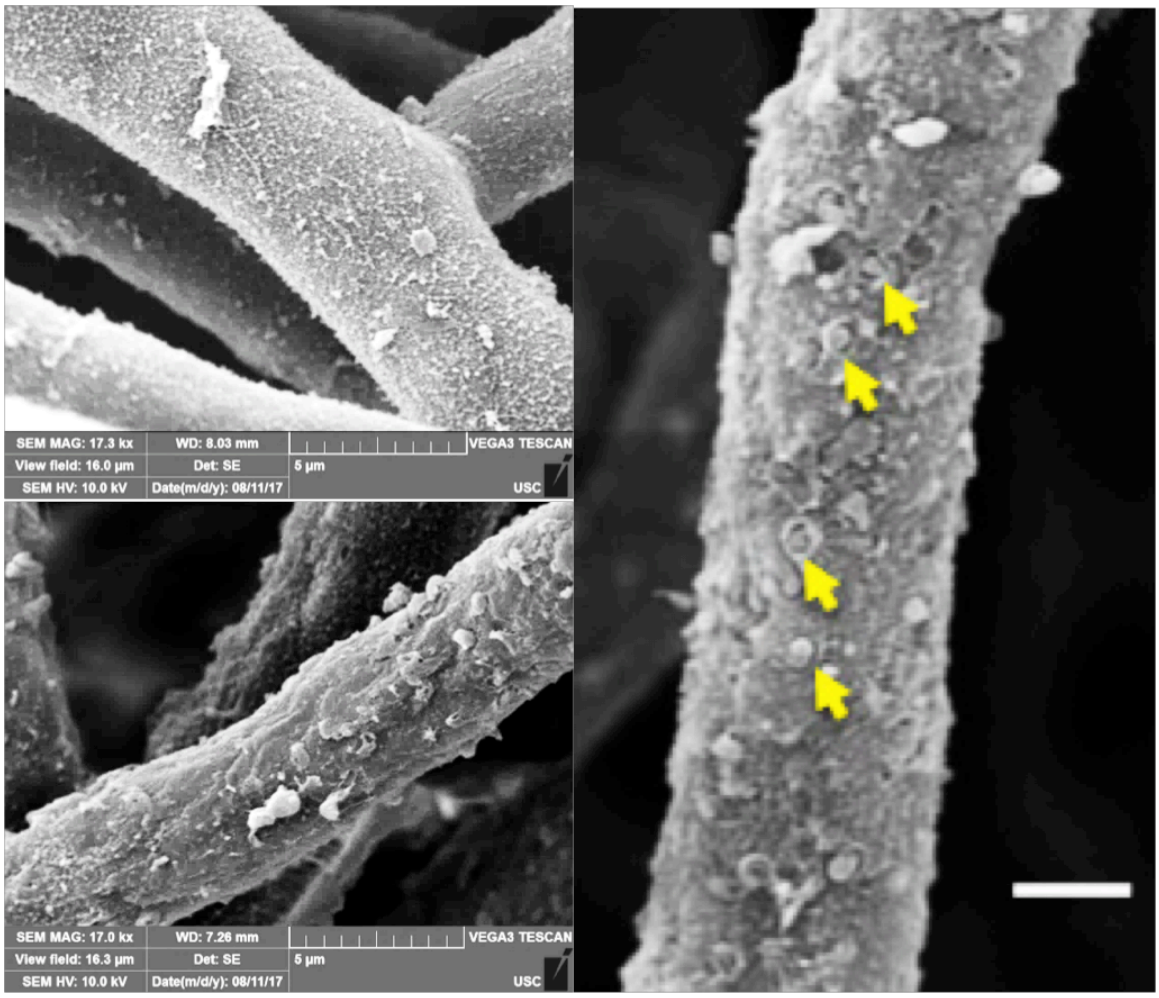
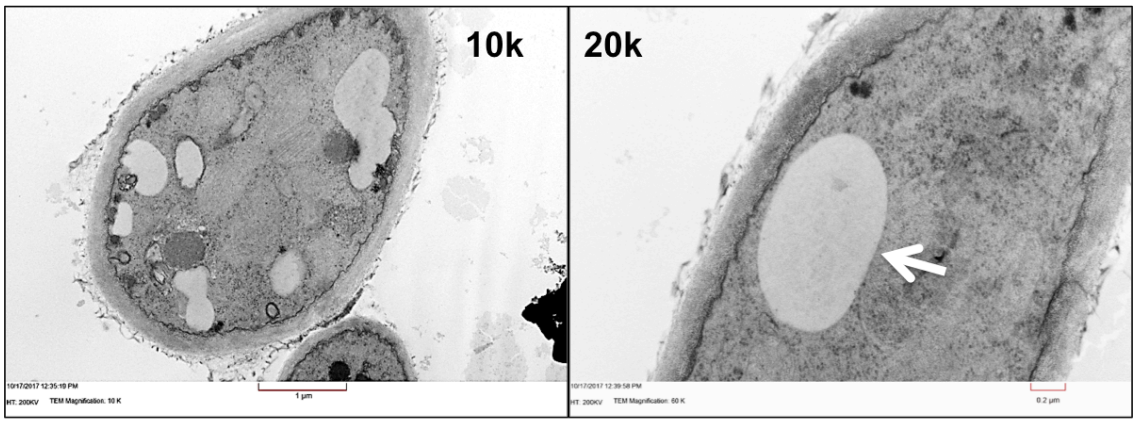
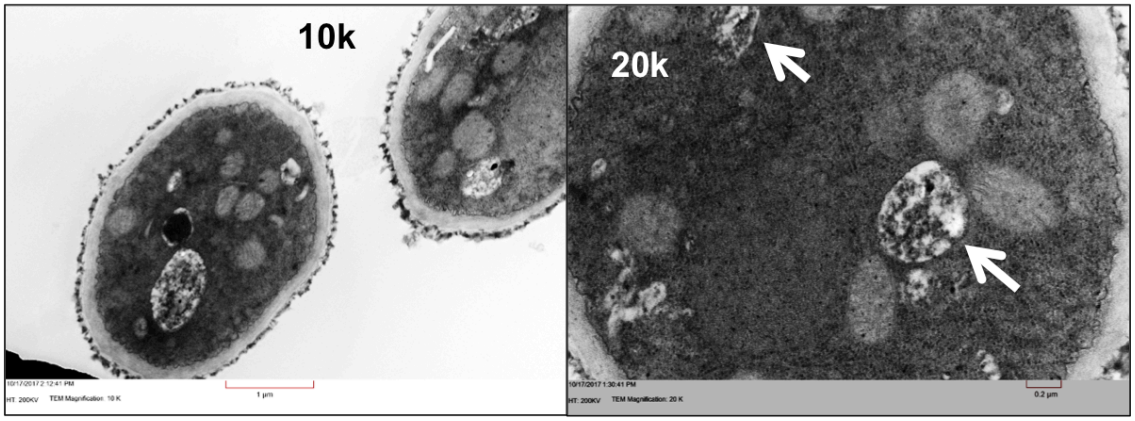


Figure 5.9: Scanning electron microscopy of *A. flavus* uptake of *V. gazogenes*: Scanning electron microscopy of *A. flavus* hyphae treated with *V. gazogenes* at 17k magnification. The top left image is of control hypha and the left bottom image is of *A. flavus* in presence of *V. gazogenes*. The magnified image is of a hypha with arrows pointing to the bacteria being endocytosed. The scale is 5 µm.



Untreated *A. flavus* hyphae



***V. gazogenes* treated *A. flavus* hyphae**

Figure 5.10: Transmission electron microscopy of *A. flavus* uptake of *V. gazogenes*: Transmission electron microscope's cross sectional images of untreated control *A. flavus* hyphae (top images) and *V. gazogenes* treated hyphae (bottom images) with 10k magnification (left images) and 20k magnification (right images). The arrows point to endosomal compartments. This is a representation of n=3 experiments.

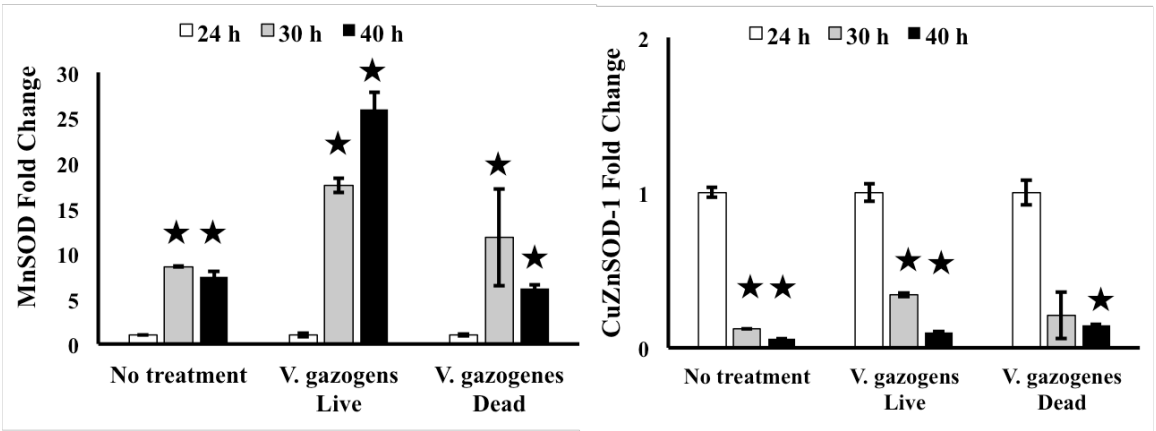


Figure 5.11: *V. gazogenes* increases SOD genes: Quantitative PCR (qPCR) comparison of SOD gene expression in *A. flavus* during the two treatment condition of live and dead *V. gazogenes* at 24 h, 30 h and 40 h time points of culture growth. Each individual gene expression was normalized to the housekeeping gene 18s which was used as reference gene. The 30 h and 40 h samples were compared to the 24 h sample of that specific treatment condition to calculate relative fold change in gene expressions. Fold changes ≥2.0 with p-value <0.01 were considered significantly up or down regulated. Star indicates statistically significant fold change as compared to the 24 h control of that specific treatment.

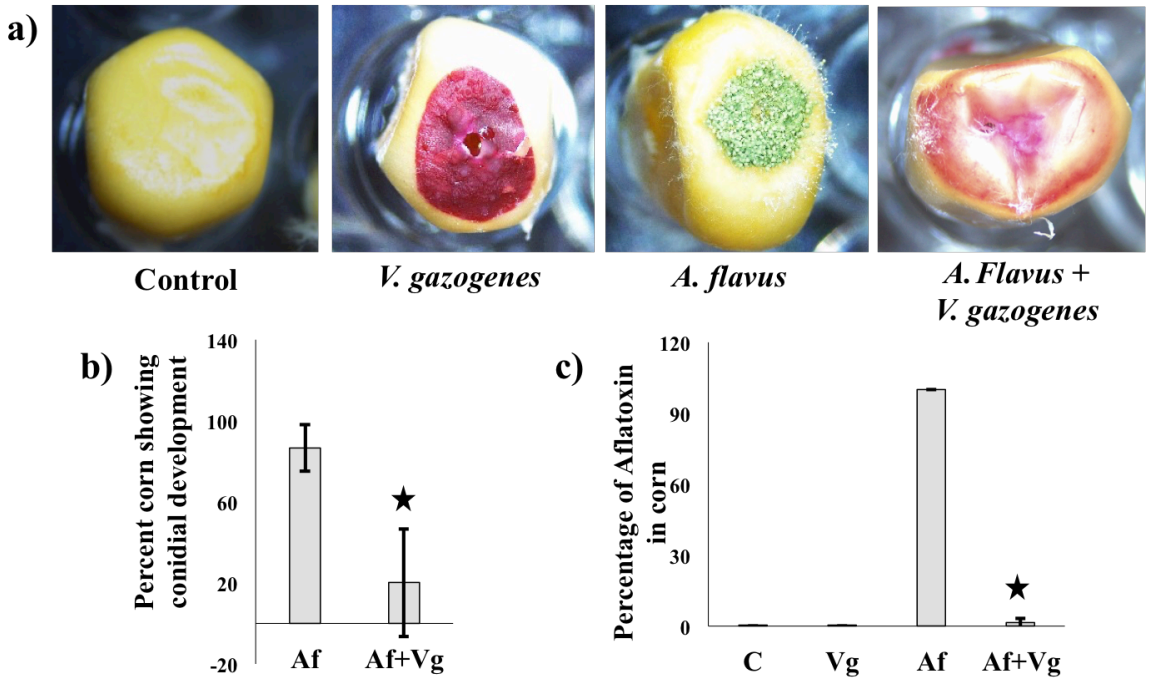


Figure 5.12: Effect of *V. gazogenes* on corn infestation of *A. flavus*: Packaged corn kernels were used with 10-kernels/treatment set and the sets were done in triplicate. Control (C) was a negative control with no *A. flavus* or *V. gazogenes*. *V. gazogenes* (Vg) was a positive control for the bacteria having only *V. gazogenes*. *A. flavus* (Af) was a positive control for the fungus having only *A. flavus*. *A. flavus* + *V. gazogenes* (Af+Vg) was the treatment condition where kernel was infested with *A. flavus* in the presence of *V. gazogenes*. a) The top panel shows images taken using Leica dissection microscope on the 5th day on incubation. b) The graph represents the percent of conidia/spores formation on the corn kernels in the presence and absence of *V. gazogenes* during *A. flavus* infestation. c) The bars represent the percentage of aflatoxin present in the kernels as compared to the A. flavus positive control (Af). p-value <0.01. Statistical significance of two-tailed *p*-values were determined using an unpaired *t*-test for n=3. Error-bars represent SEM. Star represents the difference as compared to the control was statistically significant.

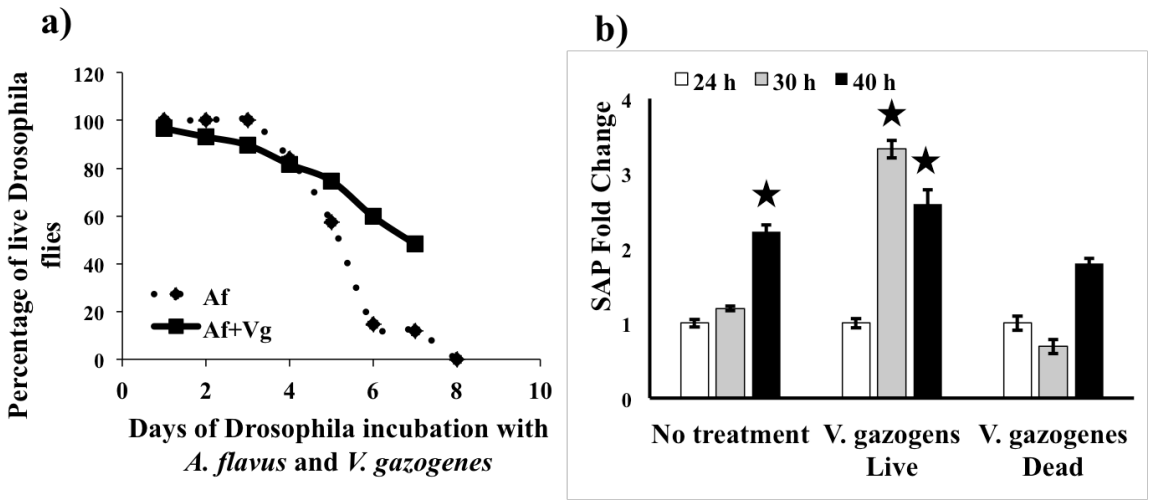


Figure 5.13: Effect of *V. gazogenes* on Drosophila infection by *A. flavus*: a) *Drosophila melanogaster* flies were infected with *A. flavus* in the absence (Af) and presence (Af+Vg) of live *V. gazogenes* cells. We used 3-5 day old female flies, 25 flies/tube (for Af) and 29 flies/tube (for Af+Vg) in sets of 3 (method was describes earlier). The percentage of flies survived per day per tube was calculated and plotted as a line graph. b) Quantitative PCR (qPCR) comparison of SAP (pathogenic gene) gene expression in *A. flavus* during the two treatment 2condition of live and dead *V. gazogenes* at 24 h, 30 h and 40 h time points of culture growth. The gene expression was normalized to the housekeeping gene 18s which was used as reference gene. The 30 h and 40 h samples were compared to the 24 h sample of that specific treatment condition to calculate relative fold change in gene expressions. Fold changes ≥2.0 with p-value <0.01 were considered significantly up or down regulated. Star indicates statistically significant fold change as compared to the 24 h control of that specific treatment.

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