# Aflatoxin detoxification: From identifying degraders and mechanisms to their enhancement

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### Abstract

#### Aflatoxin detoxification: From identifying degraders and mechanisms to their enhancement

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Aflatoxins (AFs) are secondary fungal metabolites that contaminate common food crops and are harmful to humans and animals. The ability to remove AFs from feed commodities will improve health standards and counter the economic drain inflicted by AF contamination. Strategies to mitigate AF contamination fall into three categories: physical, chemical, and biological. In this thesis, I explore the identification of degraders and degradation mechanisms, as well as their enhancement, within the context of chemical and biological strategies.

Known chemical strategies have used strong acids and bases to remove contaminating AF, but these methods often lead to ecological waste issues downstream. Chapter 3 investigates the application of weaker acidic and alkaline conditions to remove two types of AFs, AFB<sub>1</sub> and AFG<sub>2</sub>. I find that a weakly alkaline environment is sufficient to degrade AF, providing an alternative solution for chemical decontamination.

Biodetoxification is a promising solution to AF contamination because of its low cost and few undesired environmental side-effects. Microbes possess a rich potential for removing toxins and pollutants from the environment. Despite the fairly wide availability of this potential, identifying suitable candidates and improving them remain challenging. In Chapter 2, I explore the use of computational tools to discover strains and enzymes that detoxify harmful toxins. Of focus is the detoxification of mycotoxins by biological enzymes. Existing computational tools can be used to address questions in the discovery of new detoxification potential, the investigation the cellular processes that contribute to detoxification, and the improvement of detoxification potential in discovered enzymes. I showcase open bioremediation questions where computational tools that could benefit bioremediation researchers. In Chapter 4, I screen several environmental isolates for their

AF detoxification ability, using AFG<sub>2</sub>. I used different carbon sources (glucose and starch) as isolation and culturing media to examine the effect of the environment on degradation ability. Overall, I find that starch medium expedites the screening process and generally improves the performance of isolates, making this a promising method for identifying new degraders and enhancing their performance. Chapter 5 highlights the characterization of degradation by two promising *Rhodococcus* species, *R. erythropolis* and *R. pyridinivorans*. While previous work has identified their degradation ability, further investigation into degradation mechanisms has been understudied. Here, I explore the characterization of degradation mechanisms toward enzyme identification. Finally, the appendix starts to broach the question of enhancing degradation of known degrading enzymes, the example here is laccase from the fungus *Trametes versicolor*. Using molecular dynamic and quantum mechanics simulations to identify mutations of interest in increasing the affinity of laccase toward AF, I create five mutants to test their degradation against the performance of wildtype. These mutants show a range of improvements against AF and showcase the efficacy of this approach to enhancement.

Together, this body of work highlights the importance of understanding AF degradation for the creation of new strategies of AF mitigation. My thesis provides a framework for developing AF decontamination strategies, from identifying degraders and unlocking their mechanisms to enhancing their performance.

## Dedication

Ad maiorem Dei gloriam

\_\_\_\_\_

"I hope some of this is legible. I am v. tired." - JRR Tolkien

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### Chapter 1 – Detoxification of aflatoxins

Mycotoxins are secondary metabolites produced by a variety of filamentous fungi that contaminate common food crops and cause numerous negative health effects in animals and humans. More than 300 types of mycotoxins have been identified thus far, all of which would be candidates for detoxification (1). Six major types are of importance due to their detrimental health impact and because they routinely contaminate foods and animal feed (2, 3): aflatoxin, ochratoxin, zearalenone, fumonisin, deoxynivalenol, and patulin. There are a variety of food crops that these mycotoxins contaminate, including cereal crops such as wheat, barley, corn, and oats (4, 5), as well as fruit and vegetable products (6). Of these mycotoxins, the major type of particular interest is aflatoxin because of its high prevalence in food and feed, its negative health and economic impacts, and the current lack of effective and efficient decontamination methods, and as such is the focus of this dissertation.

#### 1.1 Aflatoxins

#### 1.1.1 Sources and Prevalence

Aflatoxin (AF) is produced by some species of fungi from the genus *Aspergillus*, whose presence on food crops is often widespread and difficult to control (7). There are many fungal species that produce AF, but the main producers are certain strains of *A. flavus*, *A. parasiticus*, *A. tamarii*, and *A. nomius* (8, 9). AF was first identified as the causative agent of an outbreak of "Turkey X" disease that led to the death of thousands of turkeys in the London area in the early 1960s. The turkeys had all consumed a Brazilian peanut meal that was later found to be contaminated by the mold, *A. flavus* (10). Since then, AF has been intensely studied, including its biosynthesis pathway, health impacts on animals and humans, prevention of contamination, and decontamination in food and feed.

In agriculture, *A. flavus* and *A. parasiticus* are the most common sources of AF. These fungi are found ubiquitously in several food crops, mainly cereal crops (such as corn, wheat, barley, and oats), tree nuts, peanuts (groundnuts), soybeans, oilseeds, and spices. *A. flavus* is prominent in corn and tree nuts and mainly produces AFB<sub>1</sub> and AFB<sub>2</sub>, while *A. parasiticus* is dominant in peanuts and produces AFG<sub>1</sub>, AFG<sub>2</sub>, AFB<sub>1</sub>, and AFB<sub>2</sub> (11).

Aflatoxin contamination is strongly influenced by environmental conditions. High temperatures, high humidity, and drought stress during the growing season contribute to increased fungal growth and AF production. Poor storage conditions, especially in warm and humid environments, can exacerbate contamination in harvested crops.

Aflatoxin contamination is a global concern, but its prevalence varies across regions. Tropical and subtropical climates are particularly conducive to AF-producing mold growth. Regions with hot and humid conditions, such as parts of Africa, Asia, and the southern United States, often experience higher levels of AF contamination.

#### 1.1.2 Structure and Toxicity

#### 1.1.2.1 Types of aflatoxins

To date, there are 20 identified forms of AFs produced by fungi or created as breakdown products that retain toxicity (12). The major types found in food/feed products are  $B_1$ ,  $B_2$ ,  $G_1$ ,  $G_2$ ,  $M_1$ , and  $M_2$ , where B and G types are synthesized naturally by fungi and M types are hydroxylated metabolites of  $B_1$  and  $B_2$  produced by the animal and secreted in their products. Among these major types,  $B_1$  and  $G_1$  are the most frequent contaminators and have greater toxicity (13, 14).

Aflatoxin  $B_1$  (AFB<sub>1</sub>) is of particular interest in bioremediation due to its widespread and highly prevalent rate of contamination on foods along with its harmful acute and chronic effects. AFB<sub>1</sub> is considered the most carcinogenic naturally occurring substance and is classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (IARC), and therefore consumption of large quantities or repeated low dose exposure has led to severe health complications (7). Regardless of the various forms of AF, they share structural similarity in the cyclic coumarin structure (15). B type AFs are characterized by the cyclopentenone ring fused to the lactone ring of the coumarin structure, while G types have a fused lactone ring (Figure 1.1). An additional structural difference exists between  $AFB_1$  and  $AFG_1$ , where the terminal furan ring contains an unsaturated bond at the 8,9 position.



Figure 1.1 Structures of the naturally occurring AFs. Adapted from Guan 2021 (16).

A special characteristic of these toxins is their native fluorescence under UV light, denoted by the letter name given to each type. Accordingly, AFs that fluoresce blue are termed B types, while those that fluoresce green are G types. In fact, this intense fluorescence substantially aided in the initial detection of AF in contaminated feed and its identification and purification soon thereafter (10). Moreover, in the early stages of its discovery as a harmful toxin, AF's ability to fluoresce facilitated a rapid development of monitoring methods in food and feed grains (10). Further study has elucidated that this fluorescence is linked to the lactone ring within the coumarin structure in AF, having been shown that opening of this ring removes the fluorescent property (17).

While there are several types of AFs, for the purposes of the studies described in this dissertation, we will focus on degradation of AFB<sub>1</sub> and AFG<sub>2</sub>. These two AF types were

chosen for their significance in terms of prevalence and toxicity, the sensitivity of their detection in our chosen assays, and to explore toxin-specific variables.

#### 1.1.2.2 Toxic moieties

The toxicity of AF has been linked to two main portions of its structure, the lactone ring and the difuran bond. Studies have shown that cleavage of the lactone ring reduces mutagenicity by 450-fold and toxicity by 18-fold (17). Additionally, the toxicity of  $AFB_1$ and  $AFG_1$  are increased due to the presence of the double bond at the terminal furan ring compared to isoforms lacking this structure (B<sub>2</sub> and G<sub>2</sub>). Therefore, to reduce toxicity, degradation must disrupt these key chemical groups.

#### 1.1.2.3 Mode of action

Aflatoxin exposure can occur in three ways: 1) by ingesting contaminated foods, milk and milk products, or other animal tissues contaminated with AF, 2) by inhaling AF particles from contaminated foods in factories or during food processing, or 3) by absorbing through the skin (18, 19). Since AFB<sub>1</sub> is the most serious in terms of health effects, we discuss the mode of action for AFB<sub>1</sub> in this section and is it summarized in Figure 1.2.

Aflatoxins are readily absorbed into the bloodstream through the gastrointestinal and respiratory tracts. When consumed, AF is transported from the blood to different tissues and into the liver, the latter of which is the main site of metabolism. Here, cytochrome P450s, a superfamily of heme-binding enzymes involved in biotransformation of xenobiotics (like AF), interact for AF metabolic conversion, undergoing reactions such as epoxidation, hydration, and hydroxylation. These reaction products can be less toxic, in the cases of AFM, AFP, and AFQ; however, AFM does retain carcinogenic activity but less than the starting compound. On the other hand, this conversion can be metabolically activated through the formation of the AFB<sub>1</sub>-exo-8,9-epoxide (AFBO).

Cytochrome P450 enzymes, particularly CYP1A2 and CYP3A4, are responsible for the conversion of AFB<sub>1</sub> into its epoxide form (20). Glutathione S-transferases play a crucial role in the subsequent detoxification of the epoxide by conjugating it with glutathione, forming a less toxic mercapturic acid derivative (18). Genetic polymorphisms in these enzymes can influence individual susceptibility to AF-induced toxicity. Aflatoxin-induced

DNA adduct formation is a pivotal event in the initiation of carcinogenesis. The AFBO can react with the N7 position of guanine in DNA, forming stable adducts (20). These adducts can lead to miscoding during DNA replication, resulting in mutations. The p53 tumor suppressor gene is particularly susceptible to AF-induced mutations, contributing to the development of hepatocellular carcinoma (21).



Figure 1.2 Overview of biochemical modes of action for aflatoxin. Adapted from Benkerroum 2020 (20).

Aflatoxins can also induce oxidative stress by promoting the generation of reactive oxygen species (ROS) within cells. While this process is largely poorly understood, recent studies have revealed that AF affects the respiratory chain in macrophages, resulting in the activation of signaling pathways involved in the inflammatory response (22). This oxidative stress can lead to lipid peroxidation, protein damage, and DNA strand breaks. The imbalance between pro-oxidants and antioxidants contributes to cellular damage and the progression of AF-induced pathologies.

#### 1.1.3 Health impacts

AF is one of the most carcinogenic natural substances and is an active inducer of mutations, cancer, hormone disorders, immunodepression, and congenital malformations in humans and animals (7, 12, 13, 23). Acute effects of AF consumption-called aflatoxicosis-range from nausea, vomiting, and abdominal pain to organ failure and death. Aflatoxins have been responsible for several outbreaks resulting in aflatoxicoses in the past 20 years. Namely, in 2004, an outbreak of aflatoxicosis from contaminated maize in Kenya caused a 39% fatality rate among 317 cases (24). Tanzania likewise faced an outbreak in 2016, which posed a significant health risk to consumers and resulted in several reports of acute aflatoxicosis cases. The outbreak prompted government interventions, including the destruction of AF-contaminated maize stocks and increased monitoring of food safety. Other outbreaks have occurred in Ghana and India, with similar effects as those discussed above. These examples illustrate the global nature of AF outbreaks, affecting numerous countries. The negative health effects of AF are rare in high income countries where AFs are strictly regulated, while increased incidence is found in low to middle income countries, especially those in sub-Saharan Africa (21), where regulation on levels in foods for consumption are either not implemented or are not enforced.

Chronic exposure to lower doses of AF can also lead to illness, mainly cancers and immunodepression (25–27). The main reservoir for AF accumulation is the liver, and as such AF is an attributable cause of the large incidence of hepatocellular cancers globally and liver damage ranging from hepatitis to cirrhosis. Aflatoxins have immunosuppressive effects, compromising the body's immune response. This can increase susceptibility to infections and exacerbate the impact of other health conditions. Immunomodulation by AFs further complicates the overall health status of individuals exposed to contaminated food or feed.

In children, AF exposure can lead to growth impairment and malnutrition. Contaminated crops often form a staple part of the diet in affected regions, and chronic exposure can result in stunted growth, delayed development, and nutritional deficiencies. Furthermore, AF exposure has been associated with adverse effects on reproductive health, including

increased rates of spontaneous abortions, stillbirths, and developmental abnormalities. Additionally, these toxins can cross the placental barrier, posing risks to fetal development.

#### 1.1.4 Economic impacts

Due to the serious health implications of AF contamination, economic losses arise in various sectors including agriculture, food production, trade, and public health. Aflatoxins can cause pre-harvest and post-harvest losses in crops such as maize, peanuts, cottonseed, and tree nuts. Infected crops, depending on the level of contamination, may be unsuitable for human and animal consumption, resulting in reduced yields and economic losses for farmers. Strict regulations and standards regarding AF levels are imposed by many countries to protect public health (further explored in Section 1.1.5). Non-compliance with these regulations lead to rejection of exports and the creation of trade barriers, which together severely impact the economies of countries heavily reliant on agricultural exports.

Aflatoxin-contaminated crops must be carefully managed or discarded, leading to increased costs in the food and feed industries. Costs are incurred in monitoring, testing, and implementing measures to reduce AF levels to meet regulatory standards. Governments and industries may need to invest in enhanced monitoring and regulation to ensure compliance with AF standards. This includes testing facilities, surveillance programs, and regulatory enforcement mechanisms, incurring additional costs. On the public health side, AF exposure can lead to acute and chronic health issues, as discussed in the previous section. The associated healthcare costs, including medical treatments, hospitalization, and public health programs, contribute to the economic burden on affected regions. Additionally, AF-contaminated feed negatively impacts livestock health and productivity. Reduced weight gain, lower milk production, and increased susceptibility to diseases contribute to economic losses in the livestock sector. Costs are also acquired in sourcing alternative, uncontaminated feed. It is estimated that AF contamination incurs economic losses of more than \$1.6B annually in the US alone (28)(29). A similar or greater sizable economic burden is faced in agriculture globally and requires efficient and costeffective solutions.

#### 1.1.5 Regulation

The regulation of AF is a critical aspect of ensuring food safety and public health worldwide. At this time, over 100 countries have regulatory limits in food/feed products. Various countries and international organizations have established regulatory frameworks to monitor and control AF levels. These regulations are designed to protect consumers from the harmful effects of AF exposure, particularly its carcinogenic properties. Regulatory authorities set permissible limits, often referred to as maximum allowable levels or tolerance levels, for AFs in different commodities. It is important to note that regulation of acceptable levels in food products is not enforced in all countries. Low to middle income countries have increased risk of exposure to higher levels of AF in their foods since they lack the infrastructure to enforce these limits and the cost of current decontamination methods limit their use (30). This lack of enforcement has led to outbreaks of illness caused by acute exposure to high levels of AF in food products.

Aflatoxin regulations typically include provisions for good agricultural practices (GAP), proper storage conditions, and preventive measures at various stages of the food supply chain. These measures aim to reduce the risk of AF contamination and protect both human and animal health. In the 1970s, the United States experienced AF outbreaks associated with contaminated animal feed. The incidents underscored the need for rigorous monitoring and control measures in the agricultural and food industries. This consequently led to the implementation of regulatory actions, including the establishment of maximum allowable levels for AFs in various feed ingredients.

In the United States, the U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA) collaborate to regulate AF levels. The FDA establishes action levels for AFs in specific food products, including peanuts, tree nuts, and corn. These action levels serve as guidance for industry compliance and are enforced through inspections, sampling, and product testing. The current limit is set at 20  $\mu$ g/kg for total AF (12). These limits are based on average daily consumption of high-risk products and do not account for certain diets with increased exposure to AF contaminated foods.

At the international level, the Codex Alimentarius Commission, a joint initiative of the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), provides global standards for food safety. The Codex has established maximum levels for AFs in various food commodities, contributing to harmonized regulations across borders and facilitating international trade.

In the European Union (EU), the European Commission sets AF regulations to ensure the safety of food and feed products. The EU has established maximum levels for AFs in a range of commodities, and member states enforce these regulations through monitoring programs and inspections. Currently, the European Union has the strictest standards, with AFB<sub>1</sub> at 2  $\mu$ g/kg and total AF (sum of the four major types) at 4  $\mu$ g/kg (12).

#### 1.2 Food decontamination is challenging

Within the food production process, there are multiple stages where contamination can occur, and AFs can build up on food products. The fungi that produce AF are found ubiquitously in the environment and cause contamination in the field or even more post-harvest while in processing and storage. These toxins are stable in the environment and can withstand typical food processing methods such as cooking and baking, making them difficult to eliminate through traditional cooking practices. The buildup of AF on foodstuff necessitates methods of decontamination to supply safe foods for consumption.

#### 1.2.1 Preventative strategies

The best strategy for the removal of AF contamination is its prevention. Since contamination can occur at different stages of the agricultural and food processing chain, several preventive strategies have been implemented to minimize the risk of AF contamination. Among these strategies are adopting good agricultural practices (GAP), proper storage, biocontrol, and resistant crop varieties.

Implementing GAP involves adopting farming practices that minimize the risk of AF contamination in the field. This includes proper crop rotation, maintaining optimal plant density, and ensuring adequate soil fertility. Crop rotation in particular has been reported to be effective in controlling mycotoxin contamination, as in the case of the rotation of

wheat and legume crops or reduced contamination seen in wheat crops after growth on soil previously containing soybean or maize crops (31, 32). While crop rotation does seem to control toxin contamination, it may be less effective in semi-arid areas (31).

Adequate drying of harvested crops is essential to reduce moisture content and inhibit fungal growth. Proper storage conditions, including temperature and humidity control, ventilation, and the use of appropriate containers, help prevent AF formation during postharvest handling.

Biological control methods to mitigate AF contamination involve the use of nonaflatoxigenic strains of *A. flavus* to target AF-producing fungi. Applying biocontrol agents can help by suppressing the growth of toxin-producing fungi in the field. This method has been investigated by multiple researchers (33–35) and the mechanism that these biocontrol strains use to control toxigenic strains is reportedly through competitive exclusion, but the exact mechanism is unknown since other mechanisms such as touch inhibition and chemosensing have been observed (36). In the U.S., the Environmental Protection Agency has approved biological control of AF in crops. Currently, there are two commercial products using non-aflatoxigenic *A. flavus*, Afla-guard® and AF36®, which are applied to crops of peanuts, corn, and cotton seed (11). Research in this field is continually advancing and refining techniques for screening, optimization, and investigation of competitive mechanisms of these fungi (37).

#### 1.2.2 Physical methods for decontamination

Physical decontamination methods have been shown to be effective in some, but not all, cases of AF contamination. One commonly employed method is sorting and removal of contaminated crops, where harvested grains or nuts are inspected, and those that are visibly moldy or damaged are removed. In one study, corn grains were sorted into three groups based on the visual inspection for foreign material and moldy or damaged grains, and the lowest graded grains had the highest AF concentrations (20). This process is effective in eliminating visibly contaminated portions but may not address AF contamination that is not visually apparent. A large issue with this method is the sizable loss of crop yield and unreliability of removing the contaminated foods.

Another physical method involves heat treatment, such as roasting or cooking, which can degrade AFs under certain conditions. Roasting nuts, for example, has been shown to reduce AF levels by 30-60% depending on the length of time employed (38). However, the effectiveness of heat treatment depends on factors such as temperature, duration, and the initial level of contamination, since AFs are relatively heat stable in the range of temperatures used in most food processing (31). The degradation temperature for AFs occurs between 237°C and 306°C (31), and is related to other conditions of the foods, such as moisture content, pH, and ionic strength (39). Along with the specific conditions that need to be met to employ this method, another drawback is the adverse effects heat treatment has on nutritional content of the treated food (40).

Additionally, technologies like irradiation have been explored for their potential to degrade AFs. AF has been shown to be reduced by UV light in the presence of oxygen, but efficiency is limited by the uniformity of radiation exposure to the whole surface of the contaminated product (40). Another successful method is gamma irradiation, which has the potential of reducing not only the AF on contaminated foods, but also the number of AF producing fungi (41, 42).

Other physical methods include dehulling, pulsed electric field, and electrolyzed water (40). While physical methods have been widely used to combat the AF contamination, they possess large limitations to their application, mainly in their high costs, low reproducibility, and low efficiency.

#### 1.2.3 Chemical methods for decontamination

Chemical detoxification methods for AF focus on altering the chemical structure of AFs to render them less toxic or non-aflatoxigenic. Ozonation is a chemical detoxification method that has been explored for its potential to mitigate AF contamination in various food and feed products. Ozone (O3) is a strong oxidizing agent that can react with AFs, leading to their degradation. The ozonation process involves the treatment of contaminated commodities with ozone gas or ozone dissolved in water. Studies have shown that ozone treatment can effectively reduce AF levels in grains, nuts, and other susceptible crops (43, 44). Importantly, ozonation does not leave behind harmful residues, making it an

environmentally friendly option. However, the efficacy of ozonation can be influenced by factors such as the initial level of contamination, treatment duration, and the type of commodity.

One widely studied approach involves the use of chemical agents to bind with AFs and form stable complexes, reducing their bioavailability. Binders such as activated carbon, bentonite, and certain clays have been investigated for their ability to adsorb AFs in contaminated feeds (45–47). This method is limited to use in animal feeds and is not safe for use in foods for human consumption.

Another chemical method involves the use of ammoniation, where ammonia gas is applied to commodities like corn to chemically transform AFs into less toxic derivatives (48, 49). Ammoniation also has been used to treat milk for the reduction of AFM<sub>1</sub> contamination and showed efficacy of 79-90% (50). However, this method may affect the nutritional quality of the treated product.

Additional chemical methods have been discovered and implemented, namely the use of acidic and alkaline conditions to decrease AF content, which is further explored in Chapter 3.

#### 1.3 Biodegradation by microorganisms

Bioremediation, or the use of biological entities to degrade or remove toxins in the environment, is a promising alternative to current decontamination methods. Bioremediation offers the benefits of lower costs, fewer undesired environmental side-effects, and potentially higher efficiency and reliability (51, 52). The use of microbes is a particularly attractive choice in bioremediation, offering diversity of metabolism and enzymes, faster activity, and the feasibility of strain evolution and engineering for improved performance (53). There are six key factors that make a good bioremediator: 1) fast and efficient at degradation, 2) safe degradation products, 3) non-pathogenic to plants, animals, or humans, 4) not detrimental to the quality of the food/feed, 5) applicable outside of lab settings, and 6) applicable to multiple pollutants (51). Several species of bacteria and fungi have been shown to degrade AF. Among identified degraders, none effectively

encompassed all six factors, with speed and efficiency often being subpar. A vast majority of known degraders have yet to be analyzed for their reduction in toxicity of AF byproducts, limiting their potential for application. The differences in degradation potential and toxicity reduction between bioremediatory organisms imply there are different mechanisms of degradation and/or levels of affinity toward the target toxin. Additionally, the mechanisms of degradation by these identified microorganisms are often unknown or understudied, limiting the ability to improve upon the native degradation performance. Therefore, identifying new species that possess AF degradation ability and elucidating the mechanisms of degradation are necessary to ensure this capability is effective and commercially viable.

#### 1.3.1 Modes of biological detoxification

In the context of microbial interventions for removing mycotoxins, the two main modes of detoxification are adsorption and biotransformation (Figure 1.3).

#### 1.3.1.1 Adsorption

In adsorption, AFs are physically bound to the outer structures—polysaccharides and proteins—of the bioremediatory microbes to reduce bioavailability of the toxins upon consumption (54–56). This process is reversible and does not result in chemical changes to the substrate. Microbial adsorption of AFs, namely AFB<sub>1</sub>, has been shown to occur through a stable, yet reversible mechanism in *Lactobacillus* strains (57, 58). Yeast cell wall from *Saccharomyces cerevisiae* has also shown wide adsorption affinity for a variety of mycotoxins, including AF (59, 60). Kabak *et al.* report reversible adsorption of mycotoxins to *Lactobacillus* and *Bifidobacterium* (61). Denaturation of surface proteins by heat in *Lactobacillus* results in increased adsorptive property, suggesting mycotoxins bind to protein moieties or polysaccharides whose surface areas are increased by denaturation (58, 62).

#### 1.3.1.2 Biotransformation

Biotransformation utilizes microbes and their enzymes to convert mycotoxins into nontoxic compounds (63, 64). Biotransformation can be further divided into three subcategories of action (schematically shown in Figure 1.3): secretion of enzymes (extracellular degradation), uptake of the toxin into the cell (intracellular degradation), and expression of enzymes on the cell surface (cell surface-mediated degradation). Cell-surface mediated degradation poses more challenges for utilization downstream, necessitating the expression of the enzyme in a generally recognized as safe (GRAS) organism before use in food processing. Intracellular degradation of toxins more closely follows normal metabolic processing of molecules by microbes inside the cell. Microbes that mitigate mycotoxins through extracellular degradation are more likely to produce stable enzymes that can be isolated and used in practice; this has been the strategy for several existing commercial products (65–67).



Figure 1.3 Simplified representation of the cellular machinery involved in biological detoxification. Created with BioRender.

#### 1.3.2 Detoxification of AFs by bacteria and fungi

Several species of bacteria and fungi have been shown to degrade AF. Each species displays a different efficiency (time and completeness of degradation) and level of reduction in toxicity after degradation. A vast majority of known degraders have yet to be analyzed for their reduction in toxicity of AF byproducts, limiting their potential for

application. These differences in degradation potential and toxicity reduction imply different mechanisms of degradation or levels of affinity toward the toxin between bioremediatory species. Here, we explore some examples of identified degraders. This section is not exhaustive but highlights the knowledge of microbial degraders in the field.

Numerous studies have investigated probiotic lactic acid bacteria (LAB), commonly known for their role in food fermentation processes, as AF bioremediators that mainly use adsorption mechanisms to remove AF. The majority of these LAB are *Lactobacillus* strains and studies suggest that the binding efficiency of LAB to AF is strain- and toxin-dependent (68). LAB are commonly used as starter cultures in the fermentation of dairy products, and their potential to mitigate AF contamination in milk products is of considerable interest: one study showed that *Lb. bulgaricus* was able to bind AFM<sub>1</sub> in contaminated yogurt by 60% in 6 hours (69). Studies have also shown that LAB strains can effectively bind to AFs in the digestive system of animals, reducing the bioavailability of these toxins and offering a potential strategy for AF detoxification in livestock (70). While LAB hold promise as AF degraders, the application of these bacteria in practical settings and across various food matrices requires further research.

Certain bacteria, particularly from the genera *Bacillus*, have demonstrated the ability to modify or degrade AFs. For instance, a strain of *Bacillus subtilis* degraded AFB<sub>1</sub> by 54% in 120 min when used during a washing step of maize (71). A *Bacillus licheniformis* strain has also been found to reduce AFB<sub>1</sub> into less toxic metabolites by 89% in a 120 hour testing period (72). Similarly, *Bacillus amyloliquefaciens* WF2020 detoxified AFB<sub>1</sub>, reaching degradation levels *in vitro* between 80-90% in 96 hours. This strain also caused a defect in fungal growth and inhibited AFB<sub>1</sub> production when co-incubated with *A. flavus*, indicating a good potential in biocontrol as well. The precise mechanisms and enzymes underscoring these observed detoxification successes are areas of active investigation.

Other bacteria such as *Pseudomonas* and *Rhodococcus* species have been explored for their potential to degrade AF. Some strains of *Pseudomonas* have exhibited enzymatic activities capable of degrading AFs (73–76). Similarly, certain *Rhodococcus* strains have demonstrated the ability to transform AFB<sub>1</sub> into less toxic compounds. Interestingly, in a screening of *Rhodococcus* species, investigators found that while the majority of the 42

tested strains could degrade  $AFB_1$  to some degree, two strains, *R. kunmingensis* JCM 15626 and *R. jostii* JCM 11615, could not reduce toxin levels (77). This indicates that AF degradation ability is not a primary or essential function in these organisms. Additionally, there was a large amount of variability in reduction levels, from less than 50% to greater than 90% (77), resulting from these *Rhodococcus* strains.

Yeast and fungi have also been explored for AF degradation, leading to the discovery of promising strains for biological detoxification. Fungal strains, such as *Trichoderma* sp. 639, *Phoma* sp., *Rhizopus* sp. 668, *Sporotrichum* sp. ADA, and *Alternaria* sp., may degrade between 65% and 99% of AFB<sub>1</sub> in 5 days at 28°C (31). Interestingly, certain species of *Aspergillus* have even been identified as AF degraders. In one study, *A. niger* ND-1 could remove AFB<sub>1</sub> by 26% in 48 hours during the initial screen, while after optimization of fermentation conditions, degradation improved to 58% in only 24 hours (78). Another study has looked at non-aflatoxigenic *Aspergillus* strains used in biocontrol and they also possess varying degrees of degradation, all with levels >44% in 7 days (79).

The diversity of bacteria and fungi with AF degradation capabilities indicate a microbial richness that could be leveraged for developing environmentally friendly and sustainable strategies to reduce AF contamination in various crops. Understanding the specific enzymes and pathways involved in microbial detoxification processes is crucial for unlocking the full potential of these microorganisms in mitigating the adverse effects of AFs on food and feed safety. At present, there are no identified wild type variants with adequate detoxification efficiency to be incorporated in the food production chain as a bioremediator. To achieve the required efficiency, optimization of detoxification performance (through artificial selection or other means) is likely required.

#### 1.3.3 Degradation by enzymes

Enzymatic degradation has been suggested in a number of studies (15, 16, 71, 80); however, identification of the degrading enzymes has proven difficult so many studies limit their focus to strains that degrade AF. Sangare *et al.* showed a *Pseudomonas* species capable of degrading AFB<sub>1</sub> from cell-free culture supernatant, suggesting that an extracellular enzyme is responsible for the degradation (81). Similar extracellular AF degradation has been reported for *Rhodococcus* spp., *Stenotrophomonas* spp., and *Myxococcus* spp. (15, 82, 83). An extracellular enzyme from *Myxococcus fulvus* was isolated and tested to reveal high activity against AFB<sub>1</sub> over wide temperature and pH ranges (84). Another AF-degrading enzyme was isolated from a strain of *Pantoea* sp. and identified as outer membrane protein A (85). Other enzymes with AF degrading abilities include laccase, manganese peroxidase, FDR-A, and AF oxidase (86–89). Table 1.1 outlines the bacterial and fungal enzymes that have been found to degrade AFs.

The use of enzymes for reducing the threat of mycotoxins has reached industrial applications, even if only in a few cases. The Mycofix® line of products (67) combine different modalities, including biotransformation and adsorption to remove several mycotoxins from feed. FUMzyme® is a commercially available fumonisin esterase produced in a genetically modified strain of *Komagataella pastoris* (90) that has shown success in removing the contamination from feed (66). However, in the case of AF, there are no commercially available enzyme products yet so more research is needed to address this.

Table 1.1 Representative view of identified bacterial and fungal enzymes with the capability to degrade A	F.
Those hypothesized but not yet confirmed are marked by an asterisk (*).	

ENZYME FAMILY	ORGANISM	AFLATOXINS DEGRADED	SOURCE
Myxococcus AF- degrading enzyme	Myxococcus fulvus	$B_1, G_1, M_1$	Zhao <i>et al.</i> 2011 (84)
Outer membrane protein A	Pantoea sp. T6	B1	Xie <i>et al.</i> 2019 (85)
Reductase	Mycobacterium smegmatis	$B_1, B_2, G_1, G_2$	Taylor <i>et al.</i> 2010 (88)
Manganese peroxidase	Phanerochaete sordida Irpex lacteus Pleurotus ostreatus	B <sub>1</sub>	Wang <i>et al.</i> 2011 (91) Wang <i>et al.</i> 2019 (92) Yehia <i>et al.</i> 2014 (93)
Laccase (oxidase)	<i>Trametes versicolor</i> <i>White rot fungi</i>	Bı	Scarpari <i>et al.</i> 2014 (94) Alberts <i>et al.</i> 2009 (86)

Pseudomonas AFB <sub>1</sub> -	Pseudomonas	$\mathbf{B}_1$	Song et al. 2019 (95)
degrading enzyme	aeruginosa		
Bacillus AF-degrading	Bacillus shackletonii	$B_1, B_2, M_1$	Xu et al. 2017 (96)
enzyme			
Aflatoxin oxidase (AFO)	Armillariella tabescens	<b>B</b> <sub>1</sub>	Cao <i>et al.</i> 2011 (89)

#### 1.3.4 Types of bioremediator implementation

Within the food production process, there are multiple sites where contamination can occur, and AF can accumulate on food products. The Aspergillus fungi that produce AF are found ubiquitously in the environment. Although they can cause pre-harvest contamination, most of the contamination occurs post-harvest while in processing. Thus, only a single site of bioremediator implementation is not sufficient. The first place of implementation is infield bioaugmentation: the addition of pollutant degrading microorganisms to the site of contamination (54). This process introduces a species, usually a genetically engineered microorganism (GEM), to the environment for its ability to actively and continually degrade the pollutant on site (97). GEMs are used mainly because natural degraders are often insufficient, lacking high affinity for the pollutant leading to slower degradation times. Improved degradation capability is achieved through genetically altering rate-limiting steps of known metabolic pathways, making knowledge of this process crucial to bioaugmentation. Another implementation strategy is through feed additives to degrade pollutants in the gastrointestinal tract of animals. Additives in this strategy are enzymes of microbes that possess degradation capacity (98). The enzymes, identified and purified, are mixed with contaminated feed and work in the gut to minimize the effects of the toxin on animal health (98). This strategy requires information concerning the optimum conditions of these enzymes to ensure application success. Overall, practical bioremediation implementation necessitates research regarding the mechanisms microbes use to degrade pollutants.

#### 1.4 Detection methods of AFs and their degradation byproducts

#### 1.4.1 Overview of common detection methods

Various methods have been developed to detect and quantify AFs in diverse matrices, employing both traditional and advanced techniques. Commonly, AFs have been detected using their photophysical properties, since they have characteristic absorption and emission spectra. Due to this characteristic absorption, a variety of chromatographic and spectrophotometric techniques can be employed to aid in detection. Conventionally, thin-layer chromatography (TLC) was a standard detection method that provided a cost-effective option for qualitative analysis. TLC has largely been replaced by other techniques due to the frequency of inaccurate results from analysis procedure errors and laboratory conditions (99). Currently, common methods include high-performance liquid chromatography (HPLC) and liquid chromatography coupled with mass spectrometry (LC-MS, discussed more below), which offer high sensitivity and precision. HPLC is the official reference method for AFs since it offers good reproducibility and shorter analysis times. It is, however, costly and requires specialized operation, which can prohibit adoption and reliance on this technique (99–101).

Immunoassay-based techniques are widely employed due to their simplicity and rapidity, making them suitable and advantageous for routine screening (102–104). One such method is the enzyme-linked immunosorbent assay (ELISA), that allows for higher sample throughput and qualitative screening, as well as the ability to purchase kits for AF detection. However, ELISA is hampered by its possible cross reactivity with related mycotoxins and increased susceptibility to false positives/negatives (99). An alternative immunoassay-based method is lateral flow immunoassay (LFIA), which is used due to its simplicity and cost-effectiveness. Additionally, this technique is extremely useful in low to middle income countries since it does not require specialized personnel for operation (105). Despite these advantages, LFIA has poor reproducibility and sensitivity and is limited to detecting a single target analyte at a time.

Emerging technologies, like biosensors, offer promising alternatives for specific and sensitive AF detection (99). Biosensors use the specific binding of the analyte of interest

to the complementary biorecongnition element, resulting in a change of physico-chemical properties, such as pH, electron transfer, mass, or heat transfer (106). These methods are usually highly sensitive, selective, and have lower cost; however, there remains concerns of low reproducibility.

The common detection methods discussed in this section are summarized in Table 1.2. There are many advantages and disadvantages to each of these methods and often the choice of method depends on factors such as the required sensitivity, sample matrix, and available resources.

 Table 1.2 Representative list of detection methods with their advantages and disadvantages for use. Adapted from Yan 2020 (107).

METHOD	ADVANTAGES	DISADVANTAGES
TLC	Simple equipment, low cost, and easy	Cumbersome steps, poor sensitivity,
	operation	high detection limit, and harmful
		reagents
HPLC	Good repeatability, low detection limit,	Needs derivation, complex operation,
	and high sensitivity	and high instrument cost
UPLC	Fast detection speed, short experimental	High instrument cost
	period, no derivative, and high	
	sensitivity	
LC-MS	Simple pretreatment, high sensitivity,	Complex equipment operation and high
	and multi-component analysis	instrument cost
ELISA	Higher throughput, high sensitivity and	Short reagent life and higher false
	accuracy, does not require extensive	positive rates
	sample cleanup	
LFIA	Fast detection speed, low cost, easy	Poor repeatability, difficult to quantify,
	operation, simple equipment, and short	and poor sensitivity
	experimental period	
Biosensors	High sensitivity, low production cost,	Low reproducibility
	easy modification, and good stability	

#### 1.4.2 Fluorescence assay

To determine AF degradation levels, our lab has developed a fluorescence assay to detail the temporal dynamics of degrader species growth and degradation. Normal methods of determining AF degradation rely on chromatography, such as high-performance liquid chromatography (HPLC), which limits the throughput of samples analyzed. As previously stated, AF toxicity has been linked to a portion of its structure–the lactone ring–which also provides for the native fluorescence of this molecule (7). Therefore, fluorescence and toxicity are linked, and loss of fluorescence correlates with loss of toxicity. By utilizing a microplate reader, we can probe detailed temporal dynamics of growth and degradation of cell cultures and analyze samples at a higher throughput. Our assay has reliable and validated (by MS) calibration curves that can be used to convert fluorescence readings to toxin concentrations (108).

#### 1.4.3 Liquid Chromatography/Mass Spectrometry

The detection of AFs using Liquid Chromatography-Mass Spectrometry (LC-MS) represents a highly sophisticated and sensitive analytical technique. LC-MS is widely employed for its ability to simultaneously separate and quantify multiple AF compounds with high precision (109, 110). This method involves chromatographic separation of AF types in a liquid phase, followed by their identification and quantification using mass spectrometry. LC-MS allows for the determination of specific AF types, providing detailed information on the composition of AF contamination. The technique offers exceptional sensitivity, enabling the detection of AFs at low concentrations, often at the parts per billion (ppb) level (111). LC-MS is favored for its accuracy and selectivity, making it a preferred choice for regulatory authorities, research laboratories, and industries involved in food safety. Additionally, analysis by LC-MS offers the opportunity to identify degradation products when studying detoxification mechanisms by physical, chemical, or biological means (112-114). This product identification can be crucial in the determination of the safety of particular detoxification methods. While this method can be very expensive, requires specialist expertise, and relies on ionization for sensitivity (99), its capability to handle complex sample matrices and provide reliable results makes LC-MS a key tool in
the comprehensive analysis and monitoring of AF contamination in various agricultural commodities.

### Chapter 2 – Computational biology tools can aid in developing microbial detoxification systems

The content of this Chapter is adapted from the following publication:

N. Sandlin, D. Russell Kish, J. Kim, M. Zaccaria, and B. Momeni, "Current and emerging tools of computational biology to improve detoxification of mycotoxins," *Appl Environ Microbiol.* 88, 3, e0210221 (2022).

### 2.1 Introduction

How can we effectively remove mycotoxins using biological organisms? Conceptually, we break down this search into two steps: (1) finding organisms that have this capability, and (2) optimizing their performance by modifying the environmental conditions, the detoxifying strain, or the target enzymes. We next survey existing computational tools that facilitate this process (Figure 2.1). We focus our discussions on genomic and structural biology tools, and acknowledge that there are other useful tools, —including proteomics— and *in silico* modeling, that can offer additional insights, but are beyond the scope of this work. Additionally, we use examples of different mycotoxins to show the versatility of this workflow for the development detoxification systems.

#### Enzymatic removal of toxins: Questions addressed by computational tools





### 2.2 Finding candidate organisms: who can do the job?

Discovering organisms that can degrade mycotoxins poses challenges that can be met both through experimental and computational approaches. In terms of enzymatic degradation, there are three challenges to be addressed. First, organisms must have the genes necessary to produce enzymes and possibly cofactors involved in degradation. Second, organisms must have favorable regulatory mechanisms for these enzymes. Third, the method of obtaining and isolating the enzymes must be favorable to the end use case. One can describe the search space as being largely defined by these characteristics that may be specific to the use cases but are still conceptually similar among different cases.

From the experimental front, high-throughput screening may be used both to identify candidate organisms as well as explore mutations for optimizing degradation potential. Environmental isolates are a traditional source for identifying mycotoxin degraders. Isolates can be cultivated and tested for degradation, especially when high-throughput screening is possible. Similarly, screening may be used for optimizing the environmental conditions or the enzyme itself. However, unless feasible high-throughput assays are available, this process is resource and time exhaustive. Therefore, looking to computational methods to screen for new organisms is beneficial.

As an example, there is a known, highly specific two-step enzymatic process in the detoxification of fumonisin (FUM), which involves a carboxylesterase and an aminotransferase (115). This becomes a useful bottleneck in the search space, as candidate organisms must contain both enzyme-encoding genes to be viable degraders. Therefore, tools such as BLASTp (116) can be utilized in cases where genome sequences are available. Simply put, the presence of these two genes largely dictates if an organism is a FUM degrader. Alternatively, in the example of AF degradation, many species possess hydrolases or oxidases related to those that are known to degrade AF (86, 117, 118). The search space is instead constrained on a separate manifold involving the specificity and affinity of the hydrolase for AFs indicating the presence of the same hydrolase gene may not be sufficient to identify degradation potential, since it may be optimized for a different substrate. The sequence-to-function relationship then becomes critical, which is not guaranteed to be captured by sequence similarity through BLASTp. This shortcoming can be thought of as a signal to noise ratio, where key amino acids involved in the active site mechanism are sparse signals, and the rest of the sequence functions primarily to provide the correct structural shape and may be noisy in this regard. This is witnessed in the work by Dellafiora and colleagues (119), where two related, AF-degrading oxidases shared only 72% sequence similarity, despite using the same mechanism for degradation. In a more extreme example, a recently identified carboxylesterase that degrades FUM showed only around 34% sequence similarity to previously reported FUM-degrading carboxylesterases (120).

Sequence similarity may be used to imply functional similarity; however, such a predicate does not include enzymes that share functional similarity without sequence similarity. High sequence similarity among closely related species might not fully overlap with functional similarity either. Therefore, searches should be conducted on a sequence-to-function relationship model. While this method loses the high-throughput optimizations of BLAST-based sequence similarity, it may be modeled via a reductive filter pipeline to maintain reasonable complexity. It also loses the generalizability of sequence similarity, and instead pipelines must be custom designed for each case. In the example of AFs, initial work has

filters. Furthermore, this model does not necessarily require a labeled, positive enzyme to seed the search, rather it only requires characteristics to build the filters. Prior research by Risa and colleagues (77) has identified that excreted enzymes can be responsible for degrading AFs. SignalP predicts protein excretion in bacteria and can be used as an initial filter to narrow down proteomes. These sequences can be passed through both size and sequence-based enzyme classification filters based on facile experimental determinations to further reduce the candidate pool. From there, 3D structures may be built, the binding pockets predicted, and mycotoxins (*i.e.*, AF) docked to identify high affinity interactions that then may be confirmed experimentally. These computational processes will be explained in further detail below. The reductive filter model uses low-complexity tools at its head, increasing in complexity towards the tail to ensure efficiency. Similarly, its modular nature allows for easy insertion or upgrading of components as advances occur in each domain.

### 2.3 Community-level detoxification: when the task needs to be divided

Mycotoxin degradation may require multiple reactions to reach byproducts with complete or significantly decreased toxicity. There are several examples where a single enzyme is insufficient for complete degradation and two or more enzymatic steps are required for the detoxification process. In such cases, we need to better understand how multiple enzymes from the same, or even different, species are required for degradation of a single mycotoxin. While this increases the difficulty and cost of searching for degrading enzymes that can work together, the outcome of complete degradation and reduced toxicity is desirable for application in agriculture where mycotoxin levels must fall under set regulatory limits. For degradation of FUM B<sub>1</sub> by *Sphingopyxis* sp. MTA144, Heinl *et al.* found that two enzymes were involved (115). A carboxylesterase facilitated the initial deesterification step to form a hydrolyzed FUM B<sub>1</sub>, which is less active in its known ceramide synthase inhibitory pathway but still possesses significant toxic effect (115, 121). A second enzyme, an aminotransferase, deaminated the hydrolyzed byproduct of the first reaction resulting in complete degradation and loss of toxic effects (115). Similarly, Carere *et al.* elucidated a two component enzymatic pathway involved in the epimerization of deoxynivalenol (DON) by *Devosia mutans* 17-2-E-8 (122, 123). The enzymes, designated DepA and DepB, first oxidized DON into 3-keto-DON (DepA) (122) and subsequently reduced 3-keto-DON into 3-epi-DON (DepB) (123), significantly reducing toxicity. These examples highlight the need to understand all the enzymes playing a role in complete degradation.

In some instances, mycotoxin biotransformation does not lead to complete detoxification (124). For example, the DON degradation mentioned above led to end products that are less toxic than the starting substrate, but still retained some toxicity. In biotransformation of zearalenone (ZEA), there have been cases where microbial breakdown results in byproducts,  $\alpha$ -zearalenol and  $\beta$ -zearalenol, that are even more toxic than ZEA itself (125–127). In such cases, it is necessary to identify additional species or enzymes that can take byproducts such as these and convert them into non-toxic compounds in a multi-step process.

Multi-step degradation underscores the possible need to look beyond single microorganisms and employ microbial consortia to complete the job. For example, Wang *et al.* discovered a microbial consortium that utilizes multiple species across various taxa working in unison to transform ZEA to non-toxic byproducts (128). Bioinformatic searches for identifying multiple enzymes necessary for a particular case would be an extension of the single-enzyme searches discussed in the previous section, using similar tools (*i.e.*, searching for individual organisms that carry two or more necessary enzymes that have previously been identified in multiple species/strains).

## 2.4 Regulation: even when the detoxification capability exists in an organism, its availability may be under regulation

Even after organisms have been identified that are capable of detoxifying target pollutants, the availability of the relevant enzymes depends on whether the environmental context induces the relevant genes of enzyme production and secretion effectively. These considerations require one to explore the internal regulation of the production and secretion of detoxifying enzymes. Microorganisms respond to cellular and environmental changes through regulatory decisions that could impact the availability of degradation machinery

for target pollutants (129). Production of enzymes is regulated through different mechanisms, such as transcription factors binding in and around promoter regions that contribute to the amount of enzyme produced by the cell. These mechanisms are likely influenced by nutrient availability and overall conditions of the cell (*i.e.*, growth phase) (130). Secreted enzymes have an added layer of regulation due to the high energy cost of secretion. While these enzymes have beneficial effects, often being employed to breakdown macromolecules in the environment for cellular uptake, they also incur an energy/biomass cost (131). Therefore, certain enzymes targeted for secretion are up- or down-regulated by the presence of nutrients in the environment that respectively do or do not require extracellular breakdown.

Here, we primarily emphasize the existing native potential as the starting point, even though ultimately the deployment likely happens in a safe and tractable host organism. Our discussion on regulation and the detoxification machinery in the native context has two purposes: 1) it reveals the preferred conditions for the expression of the detoxification machinery to enable more effective screening for functions of interest; and 2) it allows us to better understand the diversity of possibilities and the ideal machinery to be transferred to a host organism. Understanding the influence of regulation on production and secretion of the enzyme is also necessary for strain optimization to factor in the cost-benefit balance of increased enzyme production and secretion.

Several existing bioinformatic tools can help us uncover aspects of bacterial gene regulation, such as promoter and DNA binding sites, operon regions, and secretion signals, which are mentioned in later sections. The usefulness of these tools in the context of bioremediation is that they allow researchers to uncover possible mechanisms of regulation that control the detoxification process. Insight from regulation (*e.g.*, similarity to a known catabolic pathway) can also be used to choose suitable environmental conditions or infer the mechanism of degradation.

### 2.4.1 Promoter Prediction

Identifying promoter regions and DNA binding sites are important in that transcription initiation is the most frequently regulated step in gene expression. Promoters contain an intrinsic strength that governs the amount of transcription a gene undergoes and when that transcription occurs according to environmental factors such as nutrient availability (130). It is important to properly regulate gene expression to ensure the degrading enzyme is sufficiently expressed, but only when the particular substrate is present to limit wasteful production of enzymes that are disadvantageous to the cell without the substrate (132). By uncovering promoters associated with genes/enzymes of interest in bioremediation, we can understand how the cell naturally regulates its expression and better manipulate it toward improved expression for application in agriculture. There are several existing tools for predicting and cataloging promoter regions in different organisms, such as phiSITE (133, 134), SAPPHIRE (135), PRODORIC2 (136), BacPP (137), and PPCNN (138). We will expand on the latter three here.

PRODORIC2 is a transcription factor binding site (TFBS) database that possesses one of the largest collections of DNA binding sites in prokaryotic organisms (136). In 2018, its most recent update, PRODORIC2 expanded its database to host the genomic information of 2274 bacterial strains and their 5191 replicons (136). This database is curated to only include experimentally validated binding sites, limiting the expanse of bacterial species it contains but ensuring accuracy in its TFBS inventory. De Avila e Silva et al. created the bioinformatic tool, BacPP, to predict promoter sequences in Escherichia coli strains through neural network simulations (137). BacPP is able to recognize and predict promoter sites with varying levels of accuracy (all above 83%) across the different sigma factors crucial for prokaryotic transcription initiation (137). Additionally, BacPP has 76% prediction accuracy among other enterobacteria species (137). The advantage of this method is its ability to classify promoter sequences by sigma factor, an important feature that was seen as a shortcoming of previous tools of this type. However, BacPP is currently limited to *E. coli* and, to a lower accuracy, enterobacteria. Another promoter prediction tool is the Promoter Prediction Convolutional Neural Network (PPCNN), developed for both eukaryotic and prokaryotic prediction and implemented into the CNNProm program. This approach uses deep learning neural networks for its prediction models (138). For prokaryotes, PPCNN was trained on *E. coli* and *Bacillus subtilis*, offering insight into both Gram-positive and Gram-negative bacteria. A highlight of this method is its applicability to other sequenced species because it predicts promoters without prior knowledge of specific promoter features (138).

### 2.4.2 Operon Prediction

Metabolically- or functionally-related genes within prokaryotic genomes are often arranged in contiguous segments called operons that are co-transcribed along the same messenger RNA (139). This organization imparts an added layer of regulation on the genes within the operon. Specifically, in the context of bioremediation, if an enzyme of interest is encoded within an operon, it reveals new genes that could help play a role in degradation, either functionally or through regulation. As an example, Heinl et al. identified two FUM degrading enzymes that were held within a gene cluster organized in two operons. They subsequently determined other genes in the operon held importance to transcriptional regulation and transport of the degrading enzymes, as well as additional enzymes that might play a role in further breakdown on the degradation byproducts. Additionally, downstream utilization of the enzyme-encoding gene(s) can be affected by its placement within an operon. For example, Altahli and El-Deeb transferred ZEA degradation capability in *Pseudomonas putida* into *E. coli* via a plasmid encoding detoxification genes (125). Multiple genes were shown to be expressed for detoxification; however, they were unable to separate these genes due to their organization in operons. Therefore, understanding the genomic organization of these genes within operons can aid in their use for degradation. Identifying operons computationally has been a field of interest for a number of years, leading to tools such as Operon DataBase (140, 141), OperomeDB (142, 143), Operon Hunter (144), and Operon-mapper (145, 146), with recent advances in de novo prediction of operons from genomic data, which is explained further below.

Operon-mapper, a web-based server for operon prediction, was developed in 2018 and is the first publicly available tool for operon prediction that only requires genome sequences as the input (145, 146). Operon-mapper uses a five step procedure: (1) open reading frame (ORF) prediction using Prokka software (147, 148); (2) homology gene determination using the hmmsearch program based on Hidden Markov Models (145, 148); (3) intergenic distance evaluation using a custom program (145); (4) operon prediction using an artificial neural network with intergenic distance and a score defining functional relatedness of protein products as the input arguments (145, 149, 150); and (5) gene function assignment using the DIAMOND algorithm (151). The accuracy of this method in predicting operons was ~90% across eight tested genomes with varying size and GC content, and outperformed other algorithms in a recent evaluation of correlation to experimentally validated operons (152). Operon-mapper also has the advantage of providing ORF identification and functional annotation of proteins (145).

### 2.4.3 Secreted Protein Prediction

A signal peptide (SP) is a sequence of amino acids in a newly synthesized protein that moves the protein into or across the membranes in the cell (153). Determining whether and how an enzyme is secreted outside the cell enables better utilization of the degradation machinery (schematically represented in Figure 1.3). To predict secreted proteins, several algorithms to identify SPs within a proteome have been developed: SignalP (154), Psort (155), Pred-Tat (156), and TatP (157).

Of note, SignalP uses these secretion signals and distinguishes between the types of secretion pathways. The current version, SignalP 5.0, uses deep neural networks in combination with conditional random field classification and optimized transfer learning to determine SPs in prokaryotes, eukaryotes, and archaea (154). This update builds upon previous versions based on artificial neural networks (158), with added improvements of hidden Markov models (159), enhanced cleavage site predictions (160), and discrimination of signal peptides and transmembrane helices (161). For prokaryotes, there are two main secretion pathways, Sec and Tat, each with three enzymes that are signal peptidases (SPase I-III), needed to cleave proteins for secretion. SignalP 5.0 is able to distinguish between three types of SPs: Sec substrates cleaved by SPase I, Sec substrates cleaved by SPase II, and Tat substrates cleaved by SPase I (154). Unfortunately, due to limited training data sets, SignalP 5.0 is unable to predict Sec substrates processed by SPase III or Tat substrates processed by SPase II. However, the current ability to determine between the three secretion pathways is important in understanding how the protein will be secreted and the regulation of the secretion process. SignalP 5.0 is available either through a webserver or

as a standalone package, making it an accessible tool for secreted protein prediction. SignalP has already been used in the context of determining mycotoxin degrading enzymes. Carere *et al.* utilized this predictive power in conjunction with an experimental approach to narrow down gene candidates for the identification of DepA in the DON degradation pathway by *D. mutans* (122). This example highlights the usefulness of this tool to mycotoxin degradation research.

## 2.5 Sub-optimal enzymes: naturally evolved enzymes may not be the best match

Enzymes found capable of degrading mycotoxins may not be naturally optimized for targeting the mycotoxin of interest. Importantly, some of the detoxifying enzymes belong to common categories such as oxidases and hydrolases; however, it is not well understood what features of the potential enzymes separate efficient detoxifiers from nonefficient ones. Thus, there is a need to better understand what aspects determine the efficacy of the enzymes and how they can be improved. Enzyme optimization often involves adaptation of a wild-type isolate to a new substrate or reaction environment. New reaction environments often involve changes of temperature, pH, and solvent conditions, all of which non-trivially affect the structure and activity of the enzyme. One technique that is agnostic to fundamental understanding of these effects is directed evolution (162–164). In directed evolution, genetic diversity is introduced via random mutations and the resultant mutant proteins are screened/selected for improved performance. There is some evidence that restricting directed evolution to residues close to the active site leads to a higher probability of displaying meaningful contributions to its activity (165). However, it remains unclear how such a process is achieved through traditionally structure-agnostic in vitro mutagenesis. Often, directed evolution is applied iteratively to further improve strong performing mutants (166). Though directed evolution conveniently creates a black-box optimization method, it does so at the cost of efficiency, where screening for fitness can become a major bottleneck in the process (167). As an alternative, a variety of computational tools have been developed for targeted enzyme engineering (e.g., those reviewed in (168, 169)).

Protein sequence activity relationship (ProSAR) models can assist the search algorithm by creating a statistical model that links the protein sequence to its activity (*i.e.*, fitness) (170, 171). ProSAR relies on a library generated from mutagenesis with a constraint of constant protein sequence length, along with the corresponding activities of interest (catalytic constant, thermostability, etc.). A statistical model is built that links the presence or absence of individual mutations to a contribution to the activity, from which some subset of the highest contributing mutations can be fixed for the next round of mutagenesis. Unlike the close mutations described earlier by Morely *et al.* (165), this method is able to link individual mutations to activity contributions without explicit knowledge of the 3D structure. The traditional statistical methods for ProSAR involved partial least square regression and genetic algorithm, while more recently traditional statistical methods could be replaced with Recurrent Neural Network architectures (172).

Focused evolution, where targeted mutations are introduced based on rational mutation hypotheses, can increase the efficiency of optimization by narrowing the search space; however, current robust methods require 3D structures of the enzyme. When optimizing for known properties such as thermostability and where reasonable 3D models are available, such as homology models, a small subset of rational mutations can feasibly be explored through computational methods and the final mutations evaluated experimentally. Rational mutation methods rely on heuristic evaluation methods like FoldX (173) to predict changes in Gibbs free energy from mutations, or predictive methods like DbD2 (174), which predicts mutations to introduce disulfide bonds that potentially have stabilizing effects on the protein for given conditions. Potential mutations identified via heuristic methods are then commonly evaluated as a narrow combinatorial library. Although not strictly necessary, to reduce cost and labor for the in vitro experiments, the mutated proteins are often computationally evaluated for stability to further narrow down viable mutations. Because of their heuristic nature, it is always necessary to be able to introduce the mutations in vitro and evaluate them experimentally under the target conditions to confirm the mutated protein is improved.

 Table 2.1 Representative examples of applications of computational biology tools for usages outlined in the previous section.

 Tools used for bioremediation are marked by an asterisk.

CATEGORY	FUNCTION	TOOL	EXAMPLES OF USE
Functional-gene level/community level	Protein sequence homology search	BLASTp	He <i>et al.</i> 2017 (175)* Carere <i>et al.</i> 2018 (176)* Lyagin and Efremenko 2019 (177)* Sun <i>et al.</i> 2020 (178)*
Regulation	Promoter prediction	BacPP PRODORIC2	Millacura <i>et al.</i> 2017 (179) Kernan <i>et al.</i> 2017 (180) Ibraim <i>et al.</i> 2019 (181)
		PPCNN	Wuisan <i>et al.</i> 2021 (182)
	Operon prediction	Operon-mapper	Martinez-Amador <i>et al.</i> 2019 (184) Grünberger <i>et al.</i> 2019 (185)
	Secretion prediction	SignalP	Otero <i>et al.</i> 2017 (186)* Carere <i>et al.</i> 2018 (176)*
Sub-optimal enzymes	Optimizing existing enzymes	Response-Surface- Methodology	Khatoon and Rai 2020 (187)* Zaveri <i>et al.</i> 2021 (188)*
		MD/QM Studies	Lonsdale <i>et al.</i> 2012 (189) Yang <i>et al.</i> 2019 (190) Wang <i>et al.</i> 2018 (191)
	Discovery of	Biopanning	Frietze <i>et al.</i> 2016 (192)
	novel enzymes	ML Generative Models	Nian <i>et al.</i> 2010 (193)
		Directed Evolution	Ang et al. 2009 (194)*
		ProSAR	Yang et al. 2007 (195)
		TD-MS/Shotgun MS	Fornelli et al. 2013 (196)

### 2.6 Future outlook

The computational biology tools we discussed above—although not comprehensive represent a range of traditional applications for better understanding the mechanisms and ultimately improving the performance of toxin biodegradation. Some of these tools have already been used in this context, whereas others have the potential to yield helpful insights. Table 2.1 captures the current landscape, using representative examples from the literature. Next, we explore ongoing and future advancements in computational methods that would further facilitate answering pertinent questions in the field of mycotoxin bioremediation.

### 2.6.1 Taking the next step: combining machine learning with high-throughput experimentation

Both the use of machine learning and automated, high-throughput laboratory experiments are becoming increasingly prevalent for enzyme optimization. Enzyme engineering may become a useful tool for the optimization of known degrading enzymes, especially when only sequences, rather than solved crystallographic structures, are known (197). Models for directed evolution can be experimentally realized in parallel and incrementally updated, moving toward an optimal sequence. Like directed evolution, biopanning assays, also known as phage display assays, are a technique often used to determine novel antibodies with high affinity to some known antigen (198, 199). Biopanning involves washing a random peptide library over a target ligand immobilized on some substrate. The nonbinding peptides may be washed away, after which the peptides with high affinity remain bound to the ligand and can be separately identified. Like a genetic algorithm, these peptides form the seed for the next round of mutation and panning. While this technique does not offer per-sequence performance metrics, we did obtain partitioned sequence datasets. Such partitioned datasets have been used in unsupervised, autoregressive sequence models for nanobodies to generate novel sequences that overlap with the highaffinity partition without needing to perform additional physical experiments (200, 201). While further evaluation is needed to obtain specific performance estimates for these novel sequences, the method aims to narrow the search space needed in optimization. Biopanning has been previously shown to optimize TEM-1 beta-lactamase and biotin ligase, indicating this method may be feasible to use in optimizing mycotoxin degrading enzymes (202–205).

Complemented by high-throughput assays, machine learning approaches are increasingly used to bring out patterns, similarities, and dependencies; —for example, in sequence-function relation of an enzyme family that may otherwise be too cryptic, or in situations when an a priori model does not exist.

### 2.6.2 Computational chemistry can further advance our understanding of enzymatic processes

To achieve understanding of enzymatic mechanisms, advancements in quantum mechanics (QM) and molecular mechanics (MM, atomistic) studies will be vital for characterizing reaction mechanisms and exploring the chemical space available via mutations (206). Additionally, crystallographic structures can be slow and expensive to solve; therefore, recent advances in protein 3D structure prediction will be instrumental to develop high-throughput pipelines.

Molecular mechanics provides a view of a system at the atomic level. It is often used for molecular dynamics (MD) simulations, where a system (*e.g.*, a protein-substrate interaction) is studied using Newtonian physics, often at nanosecond to microsecond timescales. For some protein systems, this timescale is sufficient to study the relevant mechanisms, such as in the case of using steered MD simulations to characterize an aflatoxin oxidase enzyme isolated from *Armillariella tabescens* as a member of the dipeptidyl peptidase III (DPP III) family of enzymes. However, for larger proteins, or proteins involving large conformational shifts, extensive computation may be needed. For these systems, a coarse-grained approach is taken where moieties in the system are combined to reduce the total atom count, reducing the computational cost (207, 208). Some examples are coarse-grained water models, as well as proteins where the side chains are often reduced to a single pseudo-atom. Coarse-grained models face issues in faithfully reproducing the system, and current research is focused in this area (208).

Atomistic models allow some insight into the interaction between the protein and the toxin. Such models are often sufficient to determine if the toxin will sterically fit in the binding pocket, and may also help to determine pose, electrostatic favorability of the binding, and conformational changes of the protein-ligand complex (209, 210). Unlike the more common use for MM in evaluating non-covalent inhibitors, some difficulty emerges in the inherent covalent nature of detoxification, which cannot be captured by an atomistic view (211). This issue may preclude some energetic effects brought about by the changes in electronic structure, raising concerns about how realistic such a model is. This concern may be partially solved by using QM/MM methods, where part of the system is partitioned into a QM region, and the rest remains in MM views (212). The QM region then can model electronic changes, and the rest can remain in lower cost MM regions. However, the QM region cannot be too large, which precludes cases that require large, complex QM regions (*e.g.*, in metalloenzymes like laccases). Additionally, the QM region adds computational cost and cannot be well-integrated into microsecond timescale calculations.

At a relatively high computational cost, QM calculations provide a detailed and comprehensive view of the electronic state of the system. They can provide information about covalent and electronic changes, often necessary for detoxification studies. An example of this is calculating the Fukui function of a molecule, which describes the change in a frontier orbital as the molecule undergoes a redox reaction. Fukui functions have been used to identify the location of redox in an AF-laccase system (213). QM may also be used to study electron transfer in the protein. As a tool for microbiologists, however, QM remains prohibitively expensive, both in computational cost and learning curve and is often used for fine-grained mechanistic studies in collaboration with a QM expert.

# Chapter 3 – Weakly alkaline conditions degrade aflatoxins through lactone ring opening

The content of this Chapter is adapted from the following publication:

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### 3.1 Introduction

A known chemical strategy for AF removal is through the application of strong alkaline or acidic conditions (20, 214). For example, Mendez-Albores *et al.* found that the application of 1 N aqueous citric acid converted AFB<sub>1</sub> into less toxic byproducts (215). Alternatively, KOH treatment (pH 12) by Vidal *et al.* significantly reduced AF to below the limits of detection (216). Additionally, in the context of alkaline conditions to eliminate AF from foodstuffs, there are current treatment practices such as alkaline electrolyzed water, alkaline cooking, and nixtamalization (217–219). Nixtamalization is a common and long-used processing technique used in the preparation of masa from corn. This process involves cooking the corn in boiling water containing lime (Ca(OH)<sub>2</sub>) to alkalinize the environment to a pH higher than 10 (220). While some chemical processing methods reduce the

availability of nutrients in food, the process of nixtamalization actually increases it. Nixtamalization effectively reduces the levels of AF as well as prepares the maize for downstream use; however, on the industrial scale, it produces large quantities of wastewater, polluted with organic matter and high in pH, that is difficult to discard (219). These examples support the removal of AF contamination from foods employing strong alkaline and acidic conditions. However, considerations to be made include ensuring a) the full removal of chemicals after processing, b) the unaltered nutritional value of the food, and c) limited generation of ecological waste.

We delve into the effects of weaker acidic and alkaline environments on the elimination of AFs, particularly AFB<sub>1</sub> and AFG<sub>2</sub>. We test the degradation levels of buffered medium in the range of pH 4.0-9.0, using the native fluorescence of AFs and mass spectrometry as detection methods. The fluorescence of AF is strongly linked to its lactone ring moiety, also the main contributor to AF's toxicity (17). It has been discovered that opening the lactone ring significantly reduces toxicity and fluorescence, making this a reliable proxy for AF detoxification (17). Additionally, we identify the degradation products and toxicity of degradation by pH 9 buffered medium as we look to include weaker alkaline environments as a mean to remove AF from foods during processing. Further, we consider the influence pH has on biological methods of degradation, their performance and detection.

### 3.2 Results

### 3.2.1 Increasing the pH of the medium leads to loss of AF fluorescence

We first quantify how different pH values affect the fluorescence of AF (see *fluorescence degradation assay* in Section 3.5.5). Standard culture defined medium is buffered to pH 4-6 in 0.1 M citrate buffer, 7-8 in 0.1 M MOPS, and 8-9 in 0.1 M Tris-HCl. Buffered medium is then supplemented with AFB<sub>1</sub> or AFG<sub>2</sub> (at an initial concentration of 15  $\mu$ g/mL) and degradation measured using our fluorescence assay. The fluorescence of AF in these buffered media is monitored during 48 hours of incubation time. The results for the full range of pH tested are shown in Figure 3.1A and B, where there is a gradual increase in the

intensity of toxin fluorescence reduction as the pH increases. In the following sections, we explore more closely the extremes of the pH range as well as a neutral pH for comparison. A neutral pH of 7 has little effect on the fluorescence of AFB<sub>1</sub>, while the extremes of the pH range show increased fluorescence at acidic conditions and decreased fluorescence at basic conditions (Figure 3.1). Particularly, pH 4 buffered medium displays a rapid increase in the fluorescence of AFB<sub>1</sub>, but not AFG<sub>2</sub> (Figure 3.1). However, pH 9 buffered medium decreases fluorescence by 40% for AFB<sub>1</sub> and by ~90% for AFG<sub>2</sub> (Figure 3.1), suggesting a loss of toxin concentration.



Figure 3.1 The pH in a buffered medium influences fluorescence readout in AF degradation assays. A)  $AFB_1$  and B)  $AFG_2$  were incubated with buffered medium at pHs within the range of 4.0 to 9.0 over 36 hours with readings for fluorescence of AF taken periodically over the incubation period. pH 4.0-6.0 was buffered in 0.1 M citrate buffer, pH 7.0 was buffered in 0.1 M MOPS, and pH 8.0-9.0 was buffered in 0.1 M Tris-HCl. RFU has been normalized to initial fluorescence.

Additionally, to ensure that the effect is independent of the buffer used in the experiment, a medium altered to pH 9 using four different buffers is tested and shows consistent results of decreased AF fluorescence over the testing period (Figure 3.2). This consistency indicates that the pH of the environment, and not the specific buffer used, is the contributing factor for this observation.



**Figure 3.2 Different buffers affect AFB**<sub>1</sub> **and AFG**<sub>2</sub> **fluorescence similarly at pH 9.** A) AFB<sub>1</sub> and B) AFG<sub>2</sub> were incubated with medium buffered at pH 9 in either 0.1 M Tris-HCl, sodium tetraborate, glycine-NaOH, or sodium phosphate for 48 hours with readings for fluorescence of AF taken periodically over the incubation period. RFU has been normalized to initial fluorescence.

### 3.2.2 Loss of fluorescence by pH 9 medium is proportional to loss of toxin concentration

To test if the trends seen via our fluorescence assay represent AF degradation, we examine the changes in AF concentrations (initial concentrations set at 15 µg/mL) for pH 4 and pH 9 using an extraction/fluorescence method and LC-MS. The extraction method (see aflatoxin extraction in Section 3.5.3) uses an ethyl acetate liquid/liquid extraction protocol to remove AF from the surrounding aqueous environment. Such extraction essentially eliminates any transient effect of the pH on the toxin. Pairing this extraction with fluorescence analysis allows for an accurate reading of AF levels independent of the shortterm effects of the pH environment. After extraction at three time points, 0, 24, and 48 hours, pH 4 samples show insignificant changes of AF levels for both AFB<sub>1</sub> and AFG<sub>2</sub> (Figure 3.3A and B), indicating that the increases to the fluorescence in the initial tests (Figure 3.1C) were transient effects of the pH on AFs. In contrast, pH 9 samples display sustained degradation of AFB1 and AFG2 by roughly 60% and 100%, respectively (Figure 3.3A and B). Additionally, an LC-MS analysis of the pH 9 conditions (see *LC-MS assay* in Section 3.5.4) shows similar results in the remaining levels of AFB<sub>1</sub> (Figure 3.3C) and AFG<sub>2</sub> (Figure 3.3D) after 48 hours incubation, similar to the extraction/fluorescence method of detection. First, this further confirms that pH 9 buffered medium degrades AFs.

Second, these two methods taken together show that the loss of AF fluorescence in pH 9 conditions is directly proportional to a decrease in toxin concentration.



**Figure 3.3 pH 9 buffered medium degrades AFs.** Normalized fluorescence of A) AFB<sub>1</sub> and B) AFG<sub>2</sub> from ethyl acetate extraction after incubation buffered medium at time points (TP) 0, 24, and 48 hours, shows a decrease at pH 9, but not at pH 4. Data are the mean of three replicates. Error-bars show the standard deviation. Sample analysis through LC-MS of C) AFB<sub>1</sub> and D) AFG<sub>2</sub> levels at TP 0 (black) and 48 (blue or green) hours of incubation in pH 9 buffered medium corroborates the extraction/fluorescence results.

### 3.2.3 Degradation of AFG<sub>2</sub> in a pH 9 buffered condition leads to lactone ring opening

Since the pH 9 buffered medium displays promising AF degradation, we investigate potential byproducts of the degradation reaction. Based on the loss of fluorescence after incubation in pH 9 buffered medium, and because of the link between the lactone ring and the fluorescence of AF (17), we hypothesize the lactone ring moiety to be broken open. The LC-MS assay finds that as  $AFG_2$  in the sample is removed over the 48-hour incubation period, a second peak in the spectrum increases at m/z 305. Using the exact mass, a

potential structure of the degradation byproduct is produced with opening of the lactone ring and a decarboxylation event (Figure 3.4).



Figure 3.4 LC-MS analysis of AFG<sub>2</sub> after incubation in pH 9 buffered medium reveals potential byproducts of degradation. Chromatograms for time points (TP) 0 and 48 hours of incubation of AFG<sub>2</sub> in pH 9 buffered medium, highlighting m/z 331 (AFG<sub>2</sub>) and m/z 305 (potential byproduct). The corresponding m/z value is shown for each identified peak. Structures of AFG<sub>2</sub> and the proposed structure of the byproduct are shown next to their respective peak.

A byproduct peak is not found in the  $AFB_1$  spectra, despite the decrease in toxin after incubation in the pH 9 buffered medium (Figure 3.5). This indicates that  $AFB_1$  is likely degraded to a further extent than what is detectable through our analysis. However, due to the loss of fluorescence in  $AFB_1$  after incubation, we can infer that its lactone ring was targeted similarly to the one in  $AFG_2$ .



Figure 3.5 AFB<sub>1</sub> degradation by pH 9 buffered medium does not show potential byproducts in the spectrum. The full chromatogram for time points (TP) 0 and 48 hours of incubation of AFB<sub>1</sub> in pH 9 buffered medium, showing reduction of AFB<sub>1</sub> (m/z 313) and no increased mass peak at the 48-hour time point. Retention times are in minutes. The corresponding m/z value is shown for each identified peak.

### 3.2.4 Byproducts of degradation in a pH 9 buffered condition have reduced toxicity

While it is confirmed that AF levels are decreased or diminished in the pH 9 buffered condition, this alone does not confirm that toxicity has also been decreased. To test the toxicity of the byproducts of degradation in this condition, an SOS ChromoTest for genotoxicity is implemented using the S9 rat liver enzyme induction (see *genotoxicity assay* in Section 3.5.6), since AF undergoes induction by cytochrome P450 post-consumption that activates its toxicity (221). Aflatoxin B<sub>1</sub> is incubated in the pH 9 condition for 48 hours prior to testing. We use controls of toxin levels in a neutral pH medium (pH 7) that do not show degradation, according to our fluorescence assay (Figure 3.1) and LC-MS analysis (Figure 3.8). Standard interpretation of this assay considers an IF of >1.5 as genotoxic. In our results, the AFB<sub>1</sub> control condition has an IF of ~2.6 at the highest concentration tested (15  $\mu$ g/mL), indicating genotoxicity. The same starting concentration of AFB<sub>1</sub> after incubation in the pH 9 condition reduced the IF to below 1.5, suggesting decreased toxicity (Figure 3.6). Compared to controls, the pH 9 treated samples have reduced toxicity. Likely, the remaining toxicity in this sample comes from the

residual, undegraded  $AFB_1$  seen in fluorescence and MS experiments and not the byproducts of the degradation reaction. The toxicity of  $AFG_2$  byproducts was not investigated since the SOS ChromoTest is not sensitive for  $AFG_2$ , but byproducts of the pH 9 condition show loss of fluorescence and lactone ring opening, which have been confirmed to correlate to reduce toxicity (17).



**Figure 3.6 Byproducts of AFB**<sup>1</sup> **degradation in pH 9 buffered medium have reduced toxicity.** SOS-ChromoTest kit for genotoxicity was used to assess the toxicity of byproducts after pH 9-mediated degradation. AFB<sup>1</sup> was incubated in pH 9 buffered medium for 48 hours prior to ethyl acetate extraction and resuspension in methanol. Serial 2-fold dilutions were used to obtain a trend line. AFB<sup>1</sup> incubated in pH 7 medium was used as a control (no degradation) and run through the same extraction protocol. Values of IF over 1.5 are interpreted as genotoxic.

### 3.2.5 The pH of the environment has implications in the detection and characterization of biological AF degraders

It is well known and studied that some fungal and bacterial species degrade AFs. Within the process for identification, initial screening for degradation takes place, where during the growth of the microbe, degradation levels are measured. As microbes grow, they can change the pH of their environment. Due to this change, if the conditions are right, there may be false positive identification of degraders, namely those that increase the pH during growth. Additionally, when further characterizing these degraders for degradation mechanisms, this pH change can provide inaccurate results. As an example, we found that *Rhodococcus erythropolis* (further discussed in Chapter 5) initially showed an extracellular degradation mechanism, where cell-free filtrate was able to degrade  $AFB_1$  and  $AFG_2$  to completion when cell cultures were grown unbuffered (Figure 3.7B). However, when cultured in medium buffered to pH 7, cell-free filtrate no longer degraded AF (Figure 3.7B), despite live cells in the buffered medium retaining degradation ability (Figure 3.7A). When measuring the final pH of the filtrate from unbuffered conditions, we found that *R. erythropolis* alkalinizes the environment during growth to around pH 8.5 from an initial pH of 7.2. Thus, we conclude that the pH changes of the environment during growth resulted in the perceived extracellular degradation. This example showcases the influence pH can play in the characterization of biological degraders.



**Figure 3.7 pH influences the interpretation of biological degradation by** *Rhodococcus* **species.** A) Growth (orange) and degradation (blue) curves for live cultures of *R. erythropolis* grown in pH 7 buffered medium. B) Degradation curves for *R. erythropolis* cell-free filtrate from cultures grown in unbuffered (red) and buffered (black) medium. Data are the mean of 3 replicates. RFU has been normalized to initial fluorescence.

#### 3.2.6 A neutral pH has negligible effects on AF levels

Since our fluorescence assay is of use in determining the degradation levels by microbial degraders, we found that buffered medium at pH 7 does not significantly affect the fluorescence of the either  $AFB_1$  or  $AFG_2$  (Figure 3.1). While there is slight decrease in the fluorescence of  $AFG_2$  in this condition, we find that toxin concentration, analyzed via LC-MS, is not diminished over the testing period (Figure 3.8B). By the same analysis, we show

that AFB<sub>1</sub> levels are also not affected by the pH 7 environment (Figure 3.8A). Thus, testing via our fluorescence assay for degradation in a pH 7 buffered medium is a solution to the issue of pH interference when characterizing microbial degraders, since it neither significantly decreases the toxin concentration nor fluorescence.



Figure 3.8 Neutral pH of 7 does not significantly affect AF levels. LC-MS analysis of A) AFB<sub>1</sub> and B) AFG<sub>2</sub> levels at time points (TP) 0 and 48 hours of incubation with pH 7 buffered medium shows that the AF concentrations are maintained over time. Retention times are in minutes. The corresponding m/z value is shown for each identified peak.

### 3.2.7 Degradation of AF is successful by pH 9 buffered medium when tested in a food matrix

To test the applicability of this degradation method, we subjected cornmeal to artificial contamination by AF prior to incubation in the pH 9 environment. Artificial contamination was achieved through two methods: 1) a liquid suspension of AF was added to the cornmeal and then dried, and 2) a liquid suspension of AF was added to the cornmeal, incubated overnight, washed with sterile water, and then dried. The first method achieves a higher level of contamination and tests surface-level contamination, while the second ensures contamination penetrates the food matrix. We find that compared to controls of baseline cornmeal (not artificially contaminated) both the dry and wash methods result in AF contamination, with the dry method showing 4-fold higher toxin concentration than the wash method for AFB<sub>1</sub> (Figure 3.9A) and 12-fold higher concentration for AFG<sub>2</sub> (Figure 3.9B). After incubation in the pH 9 buffered medium, AF levels decreased significantly for

the dry method conditions (Figure 3.9), indicating that surface-level contamination can be targeted by this method. The wash method conditions also showed a significant decrease in AF concentration after pH 9 incubation (Figure 3.9), highlighting the ability for this method to decontaminate toxin that has penetrated the food matrix.



Figure 3.9 pH 9 buffered medium can degrade AF in a food matrix. Commeal was artificially contaminated with A) AFB<sub>1</sub> and B) AFG<sub>2</sub> prior to degradation testing using incubation in pH 9 buffered medium. Controls are uncontaminated commeal. Dry conditions are samples where AF suspended in water was dried down on the commeal. Wash conditions are samples where AF was incubated with commeal before being washed in water and dried. pH 9 conditions were incubated in pH 9 buffered medium for 48 hours. All samples underwent AF extraction and FL reading for toxin concentration. Data are the mean of 3 replicates and error is standard deviation. \* = p<0.005, Student t-test.

### 3.3 Discussion

We investigate the ability to chemically detoxify prevalent crop contaminants, AFs, through the application of weakly basic and acidic buffered medium conditions. We find that alkaline environments have the potential for AF degradation, consistent with previous literature (222, 223). Namely, a pH 9 buffered environment displayed degradation in our fluorescence assay to levels of 60% for AFB<sub>1</sub> and 95% for AFG<sub>2</sub> within 48 hours. Further, we show that the buffer used for pH 9 conditions does not significantly impact the degradation efficiency. Additionally, to our knowledge, we are the first to identify and test toxicity of degradation byproducts from alkalinized AF degradation. Lastly, we show that

this pH 9 buffered medium method is sufficient to decontaminate AF from cornmeal, a representative food matrix for contamination.

From our findings, we propose that the alkaline environment enables the degradation of AFs through the mechanism of lactone ring opening, potentially a hydrolysis event. Alkaline hydrolysis is consistent with the byproducts of AF conversion in pH 9 conditions, in our study. Other microbe-produced molecules with lactone rings, such as quorum sensing molecules, are also known to degrade in alkaline environments (224, 225). However, to formally confirm the mechanism, further experimentation needs to be performed.

While our findings highlight the potential of pH manipulation as a method to degrade AFs, it is important to consider the practical implications and limitations of this approach. Chemical methods of AF decontamination have been widely studied and used in the agricultural industry. Yet, they are still limited by the arduous downstream clean up after treatment, especially full removal of chemicals and reduced ecological waste. Also, pH adjustment may not always be feasible in certain food processing or storage conditions, as it can impact the properties of the product or interfere with other desired chemical reactions. Previously used methods that implement alkaline environments have particularly struggled with the waste produced due to the strong bases used at large scales. However, these previous methods established a building block for our proposed strategy. Based on other applications of alkalis to remove AFs, this method of pH 9 buffered medium could be readily implemented in food processing at the stages of food washing (226). With the opening of the lactone ring by the alkaline conditions, the compound becomes more water soluble and the AFs can be removed during washing with water. Since the pH of the method outlined in this study is less harsh downstream than previous alkaline applications, we foresee potential issues of excessive wastewater and other ecological pollutants being reduced.

Another consideration is combining chemical alkaline methods with emerging biological means of decontamination. It is known that some bacterial and fungal species are capable of degrading AFs, for example, by enzymatic conversion of AFs to non-toxic byproducts. Some of these species may alter their environment to be more basic or achieve higher

degradation efficacy in a more alkaline environment. An interesting future study would be to assess combined AF degradation effect utilizing an alkaline medium that supports the growth of one or more microbial AF degraders.

As a word of caution, we encourage future studies to use a neutral pH buffered environment and document the pH when screening for biological organisms that are capable of degrading AFs. This is critical for distinguishing the enzymatic degradation from alkaline degradation of AFs by different organisms. The absence of information about the pH in some of the previous reports of AF degradation makes the interpretation of the degradation mechanism difficult.

In conclusion, our study demonstrates that pH manipulation can be an effective strategy for the degradation of AFs. Alkaline conditions promote the hydrolysis of AFs, as seen in the proposed degradation product, and render them more susceptible to degradation mechanisms. The efficacy of pH-based degradation may vary depending on the AF type as seen in the differences between AFB<sub>1</sub> and AFG<sub>2</sub>. The findings from this study contribute to our understanding of AF degradation mechanisms and offer insights for the development of effective strategies to reduce AF contamination in the food and feed industries.

### 3.4 Future directions

Further research is needed to optimize pH manipulation approaches for different food and feed commodities and to evaluate their feasibility and practicality. One of the gaps that was not evaluated was the nutrient content of the food after this degradation approach. Some chemical methods deplete the nutrients in food, which is a negative aspect. Testing the nutritional properties of the food, such as the cornmeal used in our testing, is a necessary step before application of our alkaline treatment method, since reduced nutritional value is not desirable. Some potential markers of nutrition that can be experimentally analyzed are proximate, minerals, xanthophylls, and phenolic acids content (227). Additionally, chemical methods often are linked with arduous water waste. Before this method could be proposed as a better alternative to current standards of decontamination, the waste products should be analyzed. While our MS analysis revealed that AF is depleted and our genotoxicity assay revealed that the remaining byproducts are less toxic, the waste from

incubation with pH 9 buffered medium should be tested to determine the downstream ecological safety of this method.

### 3.5 Methods

### 3.5.1 Reagents

AFB<sub>1</sub> and AFG<sub>2</sub> (Cayman Chemical) are dissolved in LC-MS grade methanol to the final concentration of 1 mg/mL for stock solutions.

#### 3.5.2 Medium and pH buffering

Minimal medium is used as the base for all pH tested:  $KH_2PO_4$  (1.5 g/L),  $K_2HPO_4x$   $3H_2O$  (3.8 g/L),  $(NH_4)_2SO_4$  (1.3 g/L), sodium citrate dihydrate (3.0g/L), FeSO\_4 (1.1 mg/L), 100x vitamin solution (1 mL), 1000x trace elements solution (1 mL), 1 M MgCl<sub>2</sub> (5 mL), 1 M CaCl<sub>2</sub> (1 mL), 100x amino acid stock (10 mL), and glucose (4.0g/L). Stock solutions of citrate buffer, MOPS, and Tris-HCl are made at a concentration of 1 M prior to addition to the base minimal medium at a final concentration of 0.1 M.

### 3.5.3 Aflatoxin extraction

For each indicated time point, liquid-liquid extraction is used to stop the reaction and extract aflatoxin and potential byproducts. Ethyl acetate is added (v/v) to each sample in microcentrifuge tubes and vortexed vigorously for 2 min. Samples are centrifuged at  $11,000 \times g$  for 2 min. to separate the phases. The top organic layer is transferred to a new microcentrifuge tube. This extraction is repeated twice per sample to increase efficiency. Ethyl acetate is left to evaporate at 65°C. The aflatoxin precipitate is resuspended in 100-200 µL of LC-M grade methanol.

#### 3.5.4 LC-MS assay

For the analysis we employ Kinetex 2.6  $\mu$ m EVO C18 column (100 × 2.1 mm). Mobile phase A: Water 5 mM Ammonium Acetate, 0.5% Acetic Acid. Mobile phase B: Methanol

5 mM Ammonium Acetate, 0.5% Acetic Acid. Flow rate is 350  $\mu$ L/min. UV detection wavelength is set at 354, 360 nm. The following gradient method is used in all runs:

Time (min)	%A	%B
Initial	90	10
3	90	10
10	30	70
10.1	10	90
12	10	90
12.1	90	10
15	90	10

The eluent from the column is directed into the electrospray source of an Agilent 6230 TOF mass spectrometer operated in positive ionization mode. Data are converted into the mzML file format and analyzed using the MZMine software.

### 3.5.5 Fluorescence degradation assay

Buffered medium is aliquoted into sterile microcentrifuge tubes and AF added according to desired final concentration (15  $\mu$ g/mL) per well. Samples are arrayed in black glassbottom 96-well plates (Nunc<sup>TM</sup> #165305 96-Well Optical Bottom) at a final volume of 150  $\mu$ L per well. Standard control of no toxin (medium alone) was used. A BioTek Synergy Mx multi-mode microplate reader is used to monitor optical density at 600 nm (to account for contamination) and fluorescence of AF at an excitation of 380 nm and emission of 440 nm, with a gain of 50 for AFG<sub>2</sub> and a gain of 65 for AFB<sub>1</sub>. Reads are taken at 5 min intervals over 48 hours (unless otherwise noted). Output is exported as a text file for downstream analysis and visualization. Typically, 2-3 replicates are used per condition. Sterile water is placed at the peripheral wells of the 96-well plate to contain evaporation.

For experiments testing fluorescence after extraction, a similar protocol is followed but with single point reads taken in triplicate.

#### 3.5.6 Genotoxicity assay

The genotoxicity assay is performed with metabolic activation using S9 rat liver extract, according to the protocol for SOS-ChromoTest<sup>™</sup> kit from Environmental Bio-detection Products (Mississauga, ON, Canada). For the assay, the negative control is composed of a

10% dimethyl sulfoxide (DMSO) solution in sterile water, and the positive control is 2aminoanthracene (2-AA). Toxin samples are prepared by adding AFB<sub>1</sub> to pH 9 buffered medium at a concentration of 15  $\mu$ g/mL and incubating at 28°C for 48 hr. A positive control of AFB<sub>1</sub> in a pH 7 buffered medium (no degradation) incubated under the same conditions is used. After incubation, samples undergo extraction via the method (described above) prior to serial 2-fold dilutions, per the assay protocol. Kit-provided bacterial suspension (*E. coli*) is added to the toxin samples and incubated at 37°C for 2 hours, with shaking. Color development is quantified on a BioTek Synergy Mx multi-mode microplate reader with readings at 420 nm to measure cell survival through alkaline phosphatase, and at 600 nm to measure SOS system induction via  $\beta$ -galactosidase activity. SOS-induction factor (IF) is then calculated according to the provided analysis protocol.

### 3.5.7 Commeal AF degradation assay

Cornmeal (Indian Head Stone Ground Yellow Cornmeal) is weighed at 300 mg per condition and transferred to 1.5 mL microcentrifuge tubes for sterilization by autoclave. To create our baseline contaminated conditions, sterile cornmeal is artificially contaminated with 1 ml of 15  $\mu$ g/mL AFB<sub>1</sub> or AFG<sub>2</sub> in sterile water. The Dry cornmeal amendment conditions involved the addition of 15  $\mu$ g/mL of AFB<sub>1</sub> or AFG<sub>2</sub> in sterile water, which are immediately dried down overnight on a heat block set to 65°C. The Washed amendment conditions involve cornmeal being incubated in AF solution (15  $\mu$ g/mL AFB<sub>1</sub> or AFG<sub>2</sub> in sterile water) overnight at room temperature, then being centrifuged at 5,000 x g for 5 min and the supernatant removed. The amended cornmeal is then washed in sterile water three times, and again dried down overnight on a heat block set to 65°C. Each amended cornmeal is then treated with 1 mL of pH 9 buffered medium for 48 hours at 28°C. After incubation, the cornmeal is once more dried down overnight on a heat block set to 65°C. The baseline cornmeal and each type of AF-amended cornmeal are compared to their respective pH 9 treatments. All conditions are performed in duplicate.

All commeal conditions undergo AF extraction using ethyl acetate. Ethyl acetate (750  $\mu$ L) is added to each sample in microcentrifuge tubes and vortexed vigorously for 2 min. Samples are centrifuged at 11,000 ×g for 2 min. to separate the phases. The top organic

layer is transferred to a new microcentrifuge tube. This extraction is repeated twice per sample to increase efficiency. Ethyl acetate is left to evaporate at 65°C. The AF precipitate is resuspended in 500  $\mu$ L of LC-M grade methanol. Fluorescence of the extractions is read according to Section 3.5.5 with single point reads taken in triplicate.

### 3.5.8 Statistical analysis

To compare the reduction in toxin concentration between cornmeal treatment conditions (Figure 3.9), we use Student t-test using the Matlab function ttest.

# Chapter 4 – Starch can expedite the screening for bacterial aflatoxin degraders

The content of this Chapter is adapted from the following manuscript:

N. Sandlin and B. Momeni. Starch can expedite the screening for bacterial aflatoxin degraders. *Applied and Environmental Microbiology*. Submitted.

Author contributions: Conceptualization, N.S. and B.M.; methodology, N.S.; formal analysis, N.S.; investigation, N.S. and B.M.; data curation, N.S.; writing—original draft preparation, N.S.; writing—review and editing, N.S. and B.M.; visualization, N.S.; supervision, B.M.; funding acquisition, N.S. and B.M.

### 4.1 Introduction

A number of bacterial and fungal species have been identified as AF degraders (228–231), yet none have been effectively implemented for commercial use. The issues currently faced in using these species is in their strict requirements to work (*e.g.*, temperature and nutrient requirements) and unknown degradation mechanisms (232, 233). Therefore, it is highly beneficial to find AF degrading species that can degrade under a broader range of conditions at greater rates and with greater efficiency. The process of finding better AF degrading species has previously been achieved through large-scale screening of environmental isolates either on the target toxin itself (*e.g.*, AF) or compounds with a similar structure, such as coumarin (229). However, such screens can be costly and still result in limited numbers of potential degraders.

The different carbon compounds that bacteria encounter in their natural environment is numerous and it is known that many bacterial species have the ability to utilize a diverse array of carbons that elicit various responses in their growth and metabolite production (234). Hacking this ability could potentially allow for enhancement to phenotypes of interest, such as AF degradation. The presence of starch in the medium, compared to other carbon sources such as glucose, has been shown to improve the AF degradation performance by the fungus *Aspergillus niger* (78) and bacterium *Myroides odoratimimus* (235). Previous work in our lab has also shown that simply changing microbes from a medium with glucose as the sole carbon source to a medium with starch as its sole carbon source increased degradation performance (236). Here, we sought to implement a new screen that allows a degrader microbe to grow on a more complex carbon source that combines starch with another complex carbon, AF. Growth on starch is a good choice because starch is safe to handle, cost-effective, and allows rapid screening of isolates.

Building on this background, we first screened environmental bacterial isolates from several sources for their ability to grow on starch defined medium before being tested for AF degradation on this medium. In parallel, we screened isolates on glucose defined medium prior to testing for degradation to determine differences in the strains obtained from different carbon sources. We used the native fluorescence of aflatoxin G<sub>2</sub> (AFG<sub>2</sub>) to quantitatively characterize AF degradation by our examined isolates. This degradation assay showed that a higher percentage of strains isolated on starch medium had degradation capability compared to those isolated on glucose medium. Additionally, the degradation performance of glucose isolates improved when transferred to a starch environment in the degradation assay. These results indicate that starch can be utilized as a cost-effective screening tool for AF degraders and that environmental conditions such as carbon source can play a significant role in degradation efficiency.

### 4.2 Results

### 4.2.1 Selection on starch identified a greater percentage of efficient AF degraders

Environmental samples were taken from various locations in the surrounding area to broadly screen different species and environments for natural AF degraders: soil, leaf, sidewalk, doorknobs, and phone screens. After initial culturing on a rich solid (agar) medium, individual colonies (distinguished by different colony morphologies) were inoculated in a defined medium containing either starch or glucose as the sole carbon source (Figure 4.1A). Out of 50 isolates tested for growth in starch, 25 were culturable and we next subjected them to our fluorescent AF degradation assay (Table 4.1). Of the 25 starch isolates, 24 showed some degree of degradation, four of which had degradation efficiency >50% (Figure 4.1B). Among glucose isolates, a larger percentage of our tested isolates grew (55 of 67); however, eight were unable to degrade AFG<sub>2</sub> and only one (GI-38) had degradation efficiency >50% (Table 4.1 and Figure 4.1B), indicating that fewer active degraders performed well in the glucose screen. The difference in the numbers of efficient degraders that arose from the two screens is significantly favorable for the starch screen based on Fisher's exact test, p = 0.031 (Figure 4.1B).

Selection	# of	Growth in	Degradation performance		
medium	isolates	defined medium	None	Poor (<50%)	Good (>50%)
Starch	50	25	1	20	4
Glucose	67	55	8	46	1

Table 4.1	Starch	and	glucose	screen	results.
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Figure 4.1 Profile of isolates from starch and glucose screens exhibits superior  $AFG_2$  degradation performance for those selected in media containing starch as the main carbon source. A) Experimental design schematic showing workflow from sample collection to isolate testing for AF degradation (Created with BioRender.com). B) Breakdown of degradation performance of environmental isolates based on selection medium. Blue bars show isolates that were able to degrade greater than 50% AF in the degradation assay and orange bars show isolates with less than 50% AF degradation. p = 0.031, Fisher's exact test, comparing the number of good degraders arising from total isolates able to grow in glucose versus starch medium.

# 4.2.2 Newly identified AF degrading species arise from the starch screen

After testing for AF degradation by isolates in their isolation medium, 15 isolates from each screen were semi-randomly selected for further analysis to understand the general trends of the species that arose from each screen. Selected strains were chosen to represent the spectrum of degradation profiles, with representatives of the best, worst, and average performers. These selected isolates had their DNA extracted and PCR amplified for 16S rRNA sequencing to determine strain identity (Table 4.2). Of the glucose isolates analyzed, species of *Pseudomonas, Staphylococcus, Bacillus*, and *Pantoea* were present, all genera having previously been identified AF degrader genera were present, we also found species of *Citrobacter* and *Acinetobacter* which have not been previously implicated as AF degraders. To our knowledge, this is the first report of species in these two genera to possess AF degradation ability, although species from these two genera have been shown to degrade other mycotoxins. For example, an *Acinetobacter* sp. also isolated from soil degraded

deoxynivalenol (238). Some of our identified isolates match previous literature on toxin degraders, but only at the genus level because there are few to no identifications to species level in the literature. Therefore, we are not able to confirm or refute if our identified species are the same as those previously reported to be AF-degraders in the literature. Overall, the starch screen resulted in newly identified degrader species while the glucose screen did not.

 Table 4.2 Isolate identification and degradation profile. Isolates were identified through 16S rRNA

 sequencing. Identifications shown in the table are closest match through BLASTn. Order of strains is based on efficiency of AF degradation in starch medium.

	Isolate	16S rRNA identification	Isolated	AF degrad	AF degradation (%)	
	Isolate		from	In starch	In glucose	
Starch Isolates	SI-C4	Stenotrophomonas maltophilia	Soil	64	13	
	SI-B3	Stenotrophomonas	Soil			
		cyclobalanopsidis		63	28	
	SI-C3	Citrobacter cronae	Soil	59	11	
	SI-B2	Stenotrophomonas lactitubi	Soil	53	39	
	SI-E10	Acinetobacter oleivorans	Soil	31	13	
	SI-C2	Enterobacter asburiae	Soil	29	27	
	SI-C5	Pseudomonas fulva	Soil	22	29	
	SI-D4	Pseudomonas faucium	Tree trunk	20	8.1	
	SI-B10	Klebsiella aerogenes	Soil	20	17	
	SI-B9	Klebsiella aerogenes	Soil	20	17	
	SI-G9	Acinetobacter geminorum	Sidewalk	18	13	
	SI-D6	Pseudoxanthomonas putridarboris	Doorknob	16	31	
	SI-C8	Pseudomonas urethralis	Soil	11	11	
	SI-B4	Stenotrophomonas pavanii	Soil	10	25	
	SI-C6	Comamonas sediminis	Soil	3.8	19	
Glucose Isolates	GI-38	Rhodococcus erythropolis	Soil	84	82	
	GI-55	Bacillus xiamenensis	Leaf	72	30	
	GI-1	Pseudomonas oryzihabitans	Phone	47	34	
	GI-5	Staphylococcus epidermidis	Phone	40	0.0	
	GI-6	Priestia flexa	Doorknob	39	35	

	GI-9	Pseudomonas baltica	Leaf	39	33
	GI-56	Bacillus altitudinis	Soil	38	35
	GI-50	Pseudomonas umsongensis	Snow	35	32
	GI-33	Bacillus sanguinis	Soil	34	32
	GI-37	Pantoea agglomerans	Soil	33	33
	GI-17	Bacillus aerius	Leaf	33	32
	GI-14	Pseudomonas glycinis	Leaf	32	28
	GI-25	Pantoea cypripedii	Snow	31	28
	GI-16	Bacillus clarus	Leaf	28	11
	GI-51	Pseudomonas mandelii	Snow	19	29

We inferred the phylogenetic relationships among the AF-degrading taxa that we found in our screening assays. Most isolates (22 out of 30) belonged to the phylum Pseudomonadota. The second most prevalent group of degraders were Firmicutes, due to the number of *Bacillus* species that were identified (5 out of 30). The other phyla represented were Bacillota and Actinomycetota. Overall, the distribution of isolates across many phyla indicates a broad range of bacteria able to degrade AF, without specific taxabased indications for this ability. Notably, species that showed the strongest AF degradation performance in starch did not group together and were dispersed throughout the phylogenetic tree (Figure 4.2, green). However, the only isolate that showed no degradation ability in starch was phylogenetically distant from other isolates (Figure 4.2, red).

Tree scale: 0.01



**Figure 4.2 Good degraders are not phylogenetically distinct from poor degraders**. Phylogenetic tree of glucose and starch isolates retrieved from GenBank database based on 16S rRNA sequences is shown. Green highlighted isolates are considered good degraders in starch medium, while red highlighted isolates are non-degraders in starch medium. GI and SI prefixes indicate those isolated in glucose and starch media, respectively.

# 4.2.3 Starch medium, compared to glucose, improves the degradation performance of isolates

To understand how the environmental carbon source influences degradation, isolates that had undergone 16S rRNA sequencing were tested for AF degradation performance in the opposite medium from their isolation. Starch isolates had significantly lower degradation efficiency when tested in glucose medium (Figure 4.3C, blue), and glucose isolates had significantly increased degradation efficiency when tested in starch (Figure 4.3C, red). Additionally, it is important to look at the growth of these species when understanding degradation capacity to ensure that changes in the degradation efficiency are not the result of enhanced or inhibited growth. When looking at growth characteristics for isolates between the two medium types, growth rates remained similar for both glucose- and starch-isolates (Figure 4.3A). While carrying capacity remained the same for starch isolates in both media, glucose isolates had significantly lower carrying capacity in glucose compared

to starch (Figure 4.3B). Taken altogether, these dynamics of growth and degradation indicate that lower cell density is not the cause of decreased degradation for the starch isolates in glucose medium, and that for glucose isolates, a lower cell density in starch outperform the higher cell density of glucose culturing. This increase in performance is likely the result of a metabolism shift when moved to a more complex carbon environment rather than impact on growth since growth rates and carrying capacity remained similar.



**Figure 4.3** AFG<sub>2</sub> degradation is improved for glucose-isolated strain when tested in starch medium. Isolates were tested for their growth and AF degradation efficiency when grown in starch and glucose defined media. Starch isolated strains (SI) are shown in blue and glucose isolated strains (GI) are shown in red. Testing in starch medium is indicated by DIS and testing in glucose medium is indicated by DIG. A) Growth rates. B) Carrying capacity. C) Degradation efficiency, shown as percent AF degraded in 48 hours. In A-C, the marked triangles indicate the group's median, while the marked dash indicates the group's mean. D) Degradation efficiencies for each isolate in both media. The dotted line represents the same efficiency between the two media. Each dot is the mean of 2 replicates per culturing condition. \* = p < 0.05, Mann-Whitney U test.

Looking closer at individual isolates in glucose and starch media, we see the effect that testing in a starch medium has on degradation capacity. As an example, isolate GI-5 showed no degradation when tested in glucose but showed about 40% degradation in starch (Figure 4.3D, highlighted). Additionally, isolate SI-C3, a newly identified AF degrader, decreased its degradation from 60% to 28% when moved into glucose (Figure 4.3D, highlighted), which indicates that in a screen using glucose, this new degrader would likely not have been identified. Overall, the fraction of strains that showed higher degradation in starch versus glucose was 73% (11 out of 15) among starch isolates and 93% (14 out of 15) among glucose isolates (Figure 4.3D).

# 4.2.4 Isolates also show degradation ability on AFB<sub>1</sub>

One key function of a good AF degrader is the ability to degrade different AF types. Previous data focused on AFG<sub>2</sub> since its stronger fluorescence is more reliably detected in our degradation assay. Here, we tested both starch and glucose isolates for their AFB<sub>1</sub> degradation efficiency to understand the relationship between degradation of these two AF types. For both sets of isolates in starch medium, there is a linear relationship between AFG<sub>2</sub> and AFB<sub>1</sub> degradation, with higher efficiency of AFG<sub>2</sub> degradation (Figure 4.4). This trend is less apparent in the glucose medium for starch isolates, with  $R^2 = 0.1799$  (Figure 4.4B). For glucose isolates tested in glucose medium, the relationship between AFG<sub>2</sub> and AFB<sub>1</sub> degradation is much stronger,  $R^2 = 0.7528$  (Figure 4.4A). Generally, we find that the best degraders of AFB<sub>1</sub> are the same as the best AFG<sub>2</sub> degraders (Figure 4.5).

Additionally, when comparing the overall performance of isolates on  $AFB_1$  in starch and glucose media, a similar trend to  $AFG_2$  is seen in that testing in starch significantly increases degradation efficiency of isolates compared to glucose (Figure 4.5A). We see this trend further confirmed when looking at individual isolates for their degradation in starch versus glucose, where isolates mainly fall in favor of starch for higher degradation efficiency (Figure 4.5B). The ability of these isolates to degrade both types of AF in a linear association confirms that the use of  $AFG_2$  in our assays and screens is adequately representative of  $AFB_1$  degradation performance.



**Figure 4.4 AFB**<sub>1</sub> **degradation by isolates correlates linearly to AFG**<sub>2</sub> **degradation efficiency.** Isolates were tested for their degradation efficiency on two types of aflatoxin, AFB<sub>1</sub> and AFG<sub>2</sub>, when grown in starch and glucose defined media. Degradation efficiency is shown as percent AF degraded in 48 hours for A) glucose isolated strains and B) starch isolated strains. Dots (•) represent testing in starch medium and crosses (×) represent testing in glucose medium. Each point is the mean of 2 replicates per culturing condition.



**Figure 4.5 AFB**<sub>1</sub> **degradation is improved when tested in starch medium.** Isolates were tested for their AF degradation efficiency when grown in starch and glucose defined media. Starch isolated strains are shown in blue and glucose isolated strains are shown in red. A) Degradation efficiency, shown as percent AF degraded in 48 hours, grouped by isolation and testing medium. Testing in starch medium is indicated by DIS and testing in glucose medium is indicated by DIG. Degradation efficiency is shown as percent AF degraded in 48 hours. The marked triangles indicate the group's median, while the marked dash indicates the group's mean. B) Degradation efficiencies for each isolate in both media. The dotted line represents the same efficiency between the two media. Each dot is the mean of 2 replicates per culturing condition. \* = p < 0.05, Mann-Whitney U test.

# 4.3 Discussion

We investigated the possibility of using starch (instead of glucose) as the main carbon source in the growth medium to identify AF degraders from environmental samples. In this process, we identified new degrader species and found that starch in the environment resulted in an improved degradation phenotype for most isolates. Degradation levels varied in each isolate; however, generally, starch led to higher degradation levels compared to glucose. Additionally, the starch screen allowed for a more streamlined identification of AF degrader species, where growth on starch as the sole carbon source primed candidates for degradation of AF and facilitated the screening for better degraders.

Of importance in the data shown is how environmental carbon source can change the degradation profiles of certain species. The improvement to degradation potentials by isolates when only moved into a more complex carbon environment implicates a possible regulatory change and/or metabolic shift in the microbes that helps facilitate AF degradation. Using a more complex carbon source as a screen for AF degradation is an intuitive approach. By supplying the cells with a complex carbon as its sole carbon source, we are steering strains that can switch/adapt their metabolism toward complex carbons, and in the process, those that can break down AF. Further studies into the mechanisms behind this process are needed to control and improve this function for practical implementation.

Tested isolates were collected from areas that were not at predisposed risk for AF contamination and they likely did not have prior exposure to AF in the natural environment. The ability of these isolates to degrade AF indicates that the degradation capacity is not necessarily rare among bacterial species. Additionally, the taxonomic breakdown of the analyzed isolates shows a fairly diverse array of species that possess AF degradation ability, further indicating that AF degradation ability can arise in many species of bacteria.

To identify new species of AF degraders, our findings indicate using growth on starch medium is a good screening method due to its low cost, higher percentage of good degrader strains, and better outcome of strains with broader environmental working conditions. Downstream, utilizing a starch screen for samples that have a higher probability of preexposure to AFs will be beneficial in finding new degrader strains that possess a high degradation ability.

# 4.4 Future directions

In addition to identifying new degrader strains, it will be important for the application of this degradation capacity to further explore the underlying mechanisms of degradation, such as metabolic pathways, enzyme identification, and degradation by-product analysis. The results of this study highlight a few promising candidates for further characterization; particularly, GI-38 and SI-C3 are great candidates. GI-38, a *Rhodococcus* sp., showed the highest levels of degradation regardless of culturing medium. It would be beneficial to understand the mechanisms of degradation, such as enzyme identification, to utilize this degradation capacity in food processing. Alternatively, SI-C3, a *Citrobacter* sp., showed high degradation in starch but a significant reduction in glucose, as well as being a newly identified degrader species. For SI-C3, we can use the large distinction in degradation efficiency between starch and glucose to understand the metabolic implication of the process. Elucidating the metabolic pathways would help for downstream enhancement to degradation and potentially be used to identify other degraders that possess similar degradation machinery through computational analysis.

# 4.5 Methods

# 4.5.1 Environmental isolates and culture media

Environmental samples were collected from in and around the Chestnut Hill area in Massachusetts. These samples include soil, snow, leaf, tree trunk, doorknob, and phone screen swabs. Sterile DI water was added to the soil to create a suspension. All samples were streak inoculated on standard LB agar and incubated for 1-3 days at room temperature and 28°C. Individual colonies were then inoculated in either glucose or starch defined medium (medium screens performed from separate environmental samples) in a 96-well plate. Isolates were tested for growth via absorbance at OD600 on a BioTek Synergy Mx microplate reader.

The defined media for this study was comprised of 1.5 g/L KH<sub>2</sub>PO<sub>4</sub>, 3.8 g/L K<sub>2</sub>HPO<sub>4</sub> (x 3H<sub>2</sub>O), 1.3 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 3.0g/L sodium citrate (x 2H<sub>2</sub>O), 20.9 g/L MOPS, 1.1 mg/L FeSO<sub>4</sub>, 1 mL/L mixed vitamin solution (2 mg/L of biotin, 2 mg/L of folic acid, 10 mg/L of pyridoxine-HCl, 5 mg/L of thiamine-HCl ×2H2O, 5 mg/L of riboflavin, 5 mg/L of nicotinic acid, 5 mg/L of D-Ca-pantothenate, 0.1 mg/L of vitamin B12, 5 mg/L of p-aminobenzoic acid, and 5 mg/L of lipoic acid), 1 mL/L SL-10 trace elements solution (10 mL/L of HCl (25%; 7.7 M), 1.5 g/L of FeCl2 ×4H2O, 70 mg/L of ZnCl2, 0.1 g/L of MnCl2 ×4H2O, 6 mg/L of H3BO3, 0.19 g/L of CoCl2 ×6H2O, 2 mg/L of CuCl2 ×2H2O, 24 mg/L of NiCl2 ×6H2O, and 36 mg/L of Na2MoO4 ×2H2O), 1 M MgCl<sub>2</sub> (5 mL), 1 M CaCl<sub>2</sub> (1 mL), and 10 mL/L mixed amino acid stock (1.6 g/L of alanine, 1 g/L of arginine, 0.4 g/L of asparagine, 2 g/L of aspartic acid, 0.05 g/L of cysteine, 6 g/L of glutamic acid, 0.12 g/L of glutamine, 0.8 g/L of glycine, 1 g/L of histidine monohydrochloride monohydrate, 2 g/L of isoleucine, 2.6 g/L of leucine, 2.4 g/L of lysine monohydrochloride, 0.6 g/L of methionine, 2 g/L of phenylalanine, 2 g/L of proline, 1 g/L of serine, 0.7 g/L of threonine, 0.3 g/L of tryptophan, 0.25 g/L of tyrosine, 2 g/L of valine, 2 g/L of adenine hemisulfate salt, and 2 g/L of uracil). Either glucose (4.0 g/L) or starch (4.0 g/L) was added as the two possible carbon sources. Both defined media were buffered to pH 7 in MOPS.

To assess for AF degradation, each defined medium was amended with pure  $AFG_2$  and  $AFB_1$  (Cayman Chemical) that had been dissolved in LC-MS grade methanol to the final concentration of 1 mg/mL. Each mycotoxin amendment resulted in a final AF concentration of 15 ug/mL.

# 4.5.2 Fluorescence degradation assay

This method was described in Section 3.5.5, with the following modifications for this study: live cell conditions had a starting OD of 0.01 and were continuously shaking between reads.

# 4.5.3 PCR and 16S rRNA gene sequencing

Isolates underwent colony PCR for 16S rRNA gene amplification. Cells were taken from defined glucose agar plates, suspended in 10 µL Milli-Q, and lysed at 98°C for 15 min.

Universal primers used for amplification and sequencing were: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR product was sent for Sanger sequencing, both forward and reverse, at Eurofins Genomics. Resulting sequences were analyzed for the consensus sequence between the forward and reverse sequences using the sequence alignment function of BLASTn (NCBI) and then by megablast through BLASTn for closest sequence match for strain identification.

# 4.5.4 Phylogenetic tree

Based on BLASTn analysis and the closest match for each isolate, we used the GenBank reference sequences for the creation of the phylogenetic tree through multi-sequence alignment. We created the phylogenetic tree file using the Matlab functions multialign, seqpdist, and seqlinkage. The tree file was annotated using Interactive Tree of Life (iTOL) (239).

# 4.5.5 Data analysis

Raw data from the aflatoxin degradation assay was processed using Matlab generated codes to measure growth and degradation characteristics. Background fluorescence (no toxin negative control) is subtracted from the readings to remove fluorescence from sources other than the toxin. The readouts are also normalized to fluorescence data from no cell controls to remove the effect of fluorescence loss due to bleaching or other causes over time with an additional normalization to account for fluorescence loss due to cell scattering. To convert the fluorescence readout to the corresponding toxin concentration, we employ a calibration curve based on measurements of a set of known toxin concentrations (240). After normalization, degradation efficiency is calculated as the percentage of toxin removed during the testing period (48 hours).

# 4.5.6 Statistical analysis

To compare the odds ratio between good degraders and poor or non-degraders obtained from the starch versus glucose isolations (Figure 4.1), we used Fisher's exact test using the Matlab function fishertest. To compare the growth rates, carrying capacities, and AF degradation efficiency between starch-versus glucose-isolates (Figure 4.3 and Figure 4.5), we used Mann-Whitney U test using the Matlab function ranksum.

# Chapter 5 – Characterization of aflatoxin detoxification by *Rhodococcus* species

# 5.1 Introduction

Improving microbial bioremediation efficacy requires a better understanding of how degradation takes place. Characterizing microbial degradation potential, is an important and valuable first step. Considering the potentials of synthetic biology for evolving or engineering more efficient AF degraders, additional mechanistic insight is needed to guide the process.

Here, we will focus on two *Rhodococcus* species, *R. pyridinivorans* (*R. pyr*) and *R. erythropolis* (*R. ery*), both of which have shown to be capable of degrading AF (82, 241). *Rhodococcus* species, Gram-positive Actinobacteria, are promising candidates for bioremediation because of their relatively high levels of degradation potential across hydrocarbons, production of safe degradation byproducts, versatile metabolism, and optimum growth at 28°C, all of which are important factors for practical bioremediator application (242). These species are found across diverse niches, including soil and water, and can thrive in harsh ecological conditions, such as the arctic, desert, and heavily contaminated sites (242, 243). Species in this genus are also known to possess wide metabolic diversity due to their relatively large genomes and the presence of multiple homologues of enzymes in catabolic pathways, thereby increasing their versatility (242). Additionally, *Rhodococcus* species can be non-pathogenic, environment-friendly (244, 245), and amenable to genetic manipulations (246, 247). Despite these advantages, progress towards real-world applications of these strains is hampered by lack of insights into the underlying mechanisms of detoxification.

*R. pyr* and *R.* ery have been explored as AF degraders. In a previous screen of *Rhodococcus* species, multiple strains of *R. pyr* and *R. ery* showed varying levels of AF degradation in the range of 70-100% via HPLC analysis (241). Previously in 2005, Teniola et al. investigated one strain of R. ery cell-free extract and found that ~90% of AFB<sub>1</sub> was degraded after 4 hours (248). Further, Alberts et al. looked at proteinase K treated cell-free filtrate of *R. ery* and found that treated filtrate retained AF degradation (82). This previous research has shown that enzymes secreted extracellularly facilitate the AF degradation process for *R. ery*, however further elucidation of the degradation machinery for this species or other *Rhodococcus* spp. has not been studied. Although *Rhodococcus* spp. have been a focus of multiple AF degradation studies, they are still underexplored for how they are able to degrade AF. A thorough, mechanistic characterization of the detoxification process would provide a solid basis to guide engineering or evolution efforts for enhancing the detoxification performance. In this study, we use the native fluorescence of AFB1 and  $AFG_2$  to quantitatively characterize how these strains degrade AF. We have chosen two species of *Rhodococcus* and two variants of AF to explore interspecies variations and toxin-specificity.

# 5.2 Results

# 5.2.1 Live cultures of *R. ery* and *R. pyr* degrade AFB<sub>1</sub> and AFG<sub>2</sub>

*Rhodococcus* species have been shown to hold ample AF biodegradation potential. We tested the degradation of fluorescence associated with AFB<sub>1</sub> and AFG<sub>2</sub> by our *R. pyr* and *R. ery* strains (Figure 5.1). After 48 hours, *R. pyr* cultures showed 38% AFB<sub>1</sub> degradation, while *R. ery* showed 60% (Figure 5.1B). Conversely, both *R. ery* and *R. pyr* were able to degrade AFG<sub>2</sub> to near completion in about 36 hours (Figure 5.1D). Our data exhibits a toxin specific difference in degradation capacity for both strains, suggesting that degradation machinery has stronger affinity for one AF type over another, or that degradation machinery between the two AF types is different.



Figure 5.1 Growth and degradation curves of *Rhodococcus* sp. on AFB<sub>1</sub> and AFG<sub>2</sub>. Growth curves of *R. ery* (blue) and *R. pyr* (red) when grown in the presence of A) AFB<sub>1</sub> and C) AFG<sub>2</sub>. Cell density is measured by OD 600 over 48 hours. Degradation curves for B) AFB<sub>1</sub> and D) AFG<sub>2</sub> by live cell cultures of *R. ery* and *R. pyr* using FL assay, where toxin concentration is calculated from RFU and calibration curves. Data are the mean of 3 replicates.

# 5.2.2 *R. ery* and *R. pyr* do not detoxify AF through extracellular enzymes

Previous literature on *R. ery* has pointed to AF degradation as an extracellular enzymatic process. Alberts *et al.* tested cell-free filtrate (CFF) and showed that it was the CFF fraction that retained degradation activity (82). Additionally, they treated CFF with proteinase K and SDS which resulted in reduced activity, indicating involvement of enzymatic activity (82). As previously explored in Section 3.2.5, we showed that environmental pH can affect the detection and characterization of degradation by microbes. For our lab strains, we sought to confirm extracellular activity after adjusting to a buffered environment at pH 7 that does not display background AF degradation. CFF of *R. ery* and *R. pyr* were subjected

to degradation testing using the extraction/fluorescence method, where CFF is incubated with AF and extracted at time points 0, 24, and 48 hours prior to measuring fluorescence. After incubation for 48 hours, CFF for both strains did not significantly reduce AF compared to controls incubated in GMM7 (Figure 5.2). Thus, our strains do not degrade AF through an extracellular process.



Figure 5.2 Cell-free filtrates do not show AF degradation activity. Cell-free filtrate of *R. ery* (blue) and *R. pyr* (red) incubated with A) AFB<sub>1</sub> and B) AFG<sub>2</sub> for 0, 24, and 48 hours prior to extraction with ethyl acetate and fluorescence reading for AF levels. Toxin incubated in GMM7 prior to extraction was used as a control (black). Data are the mean of three replicates.

# 5.2.3 R. ery and R. pyr detoxify AF through intracellular enzymes

In order to elucidate the active fraction of cells that facilitates degradation, we looked at various cell components: whole viable cells in PBS as a control (VC), CFF that was preexposed to AF to check if production of the degradation machinery needs to be induced (EC-PE), cell lysate for intracellular degradation (IC), cells treated with Proteinase K to shave the cell surface of proteins and check for cell-surface mediated degradation (PKC), and heat-killed cells to observe adsorption mechanisms (H). Cell components were incubated with AFB<sub>1</sub> and AFG<sub>2</sub> in our FL assay to uncover the mechanism of degradation. After 48 hours, VC of *R. ery* and *R. pyr* were able to degrade AFB<sub>1</sub> fluorescence by 76% and 62%, respectively, and AFG<sub>2</sub> fluorescence by 99%, the normally observed degradation efficiencies for these strains (Figure 5.3). EC-PE, even though cells were grown up in medium supplemented with AF, still did not display AFG<sub>2</sub> degradation to significant levels for both strains (Figure 5.3B). When tested on AFB<sub>1</sub>, EC-PE was more effective, up to 40% degradation for *R. ery*, but it was not the most active fraction (Figure 5.3A). Since activity is not seen to similar levels as the control, we can confirm that extracellular degradation is not the main mechanism by these strains; however, since there was some activity in the EC-PE on AFB<sub>1</sub>, these two AF types may not be degraded by the same mechanisms. Heatkilled cells lost their degradation potential (Figure 5.3); taken together with the knowledge that AF undergoes biotransformation by these strains, as indicated by the loss of FL, an adsorption mechanism can be ruled out. The PKC conditions, in which the cell-surface expressed proteins were removed, for both strains on AFG<sub>2</sub> and *R. ery* on AFB<sub>1</sub> showed similar levels of activity compared to the controls (Figure 5.3), however, the PKC of *R. pyr* with AFB<sub>1</sub> had reduced activity. We take this to mean that *R. pyr* requires some cellsurface proteins for the degradation of AFB<sub>1</sub> but not AFG<sub>2</sub>. Finally, IC of both strains is the most active fraction of cells on AFB<sub>1</sub> and AFG<sub>2</sub>(Figure 5.3), indicating that the degradation mechanism is intracellular.





Since the intracellular component of both *Rhodococcus* species facilitates degradation, we explored whether this activity is enzymatic. Cell lysate was treated with proteinase K, known to be a non-specific inactivator of enzymes, and then subjected to our FL degradation assay. Untreated lysate of both *R. ery* and *R. pyr* were active on AFB<sub>1</sub> and AFG<sub>2</sub>, to varying degrees (Figure 5.4). That same lysate, after proteinase K treatment, displayed a significant reduction in activity from 81% and 43% to 20% on AFB<sub>1</sub> for *R. ery* and *R. pyr*, respectively (Figure 5.4A). Similarly, for AFG<sub>2</sub>, activity was completely lost after treatment (Figure 5.4B). These results indicate that degradation activity for these strains is enzymatic.



**Figure 5.4 Effects of Proteinase K treatment on activity of cell lysate.** Activity of cell lysates of *R. ery* (ERY, blue) and *R. pyr* (PYR, red) before and after treatment with proteinase K (+ ProK) on A) AFB<sub>1</sub> and B) AFG<sub>2</sub>. Data are the mean of 3 replicates. Toxin concentration is normalized to initial concentration to report as percent remaining.

# 5.2.4 Degrading enzymes between *Rhodococcus* species exhibit variable efficiencies

To further characterize the degrading enzymes, we explored the effects of various treatments on the cell lysate when testing on  $AFG_2$ . First, lysate was treated with 10 mM DTT, a thiol reducing agent, to check if the activity is an oxidation reaction. Treated lysate of *R. ery* showed slight reduction in activity, from 98% to 80% (Figure 5.5A), while activity of *R. pyr* lysate was reduced from 60% to 46% after treatment (Figure 5.5B). This

reduction in activity indicates the possibility of an oxidation reaction; however, the incomplete reduction in activity points to the possibility of additional enzymes that do not function through oxidation. Secondly, lysate was treated with Chelex to remove metal ions and certain cations. Here, we see distinct results between the two strains, where Chelex treatment has no effect on the activity of *R. ery* lysate (Figure 5.5A), but increases the activity of *R. pyr* lysate to 85% (Figure 5.5B). The degrading enzyme of *R. pyr* is inhibited by the presence of these ions in the testing environment while *R. ery* degrading enzyme is not.



**Figure 5.5 Degrading enzymes between species have different properties.** Cell lysates of A) *R. ery* and B) *R. pyr* were treated with 10 mM DTT (solid) and Chelex (dashed) prior to testing degradation of AFG<sub>2</sub> through FL assay for degradation. Data are mean of 3 replicates. Toxin concentration is normalized to initial concentration.

The effect of the metal ions on the activity of the degrading enzymes was further investigated by the supplementation of individual ions after Chelex treatment. The metal ions (Fe, Zn, Mn, Co, Cu, and Ni) and cations (Mg and Ca) were added at the initial concentration found in the growth medium for these strains. Similar to the Chelex treatment on *R. ery* degrading enzyme activity, the supplementation of ions did not impact degradation (Figure 5.6A). For *R. pyr*, the degrading enzyme activity was not affected by the addition of the metal ions or Mg, as similar levels as the Chelex treated control are seen in those supplemented (Figure 5.6B). However, the addition of Ca did have significant

inhibitory effect, but not to the same levels as seen in the untreated lysate (Figure 5.6B). A possible explanation could be the requirement of multiple ions to impart the inhibitory effect, however combinations were not tested.



Figure 5.6 Effects of ions on AFG<sub>2</sub> degradation by cell lysates. Cell lysates of A) *R. ery* and B) *R. pyr* were treated with Chelex (CH) prior to the supplementation of individual metal ions (Fe, Zn, Mn, Co, Cu, and Ni) and cations (Mg and Ca). Treated cell lysate was tested for degradation efficiency (%) of AFG<sub>2</sub> through FL assay for degradation. Data are the mean of 3 replicates. \* = p < 0.05, \*\* = p < 0.005, Student t-test.

# 5.2.5 LC-MS confirms removal of AF by live cells and lysates

We investigate potential byproducts of the degradation reaction. Based on the loss of fluorescence in our *Rhodococcus*-incubated samples, and because of the link between the lactone ring and the fluorescence of AF (17), we hypothesize the lactone ring moiety to be broken open. The LC-MS assay finds that while AFB<sub>1</sub> and AFG<sub>2</sub> in the sample is removed over the 48-hour incubation period, there are no apparent byproduct peaks (Figure 5.7). This could indicate that AF is degraded beyond what is detectable through our analysis or that byproducts are not able to be processed in the same way as AF, with ethyl acetate extraction and LC-MS in positive ionization mode. While byproducts were not found through this analysis, we can confirm with these results that AF is removed by live cell cultures and cell lysates, as was seen in our FL assay. Of note, we see that live cells remove AF to near completion whereas cell lysate has residual, undegraded AF (Figure 5.7). This occurs more strongly for AFB<sub>1</sub> treated with *R. pyr* cell lysate, where residual levels of toxin

are much higher than AFG<sub>2</sub> (Figure 5.7C and D). The distinctions in degradation of the two types of AF by the same active fraction further provides evidence for the variances in affinity for different AFs as a substrate.



Figure 5.7 LC-MS analysis of AF degradation by live cell culture and lysates. Chromatograms from LC-MS for A and B) *R. ery* and C and D) *R. pyr* after 48 hours of incubation with A and B) AFB<sub>1</sub> and C and D) AFG<sub>2</sub>. AF was incubated with either live cell cultures or cell lysates prior to extraction of residual AF and byproducts. The corresponding m/z value is shown for each identified peak, highlighting m/z 331 (AFG<sub>2</sub>) and m/z 313 (AFB<sub>1</sub>). Control samples are toxin in GMM7 medium.

# 5.2.6 Byproducts of degradation are less genotoxic

While reduction of AF levels by the *Rhodococcus* strains is confirmed through LC-MS and FL data, toxicity reduction of residual byproducts has not been established. To test the toxicity of the degradation byproducts, we use an SOS ChromoTest for genotoxicity. This assay uses the S9 rat liver enzyme induction (see *genotoxicity assay* in Section 3.5.6), since AF undergoes induction by cytochrome P450 post-consumption that activates its toxicity

(221). AFB<sub>1</sub> was incubated with live cell cultures of *R. ery* and *R. pyr* in GMM7 for 48 hours prior to extraction of undegraded AF and its byproducts for testing. We use controls of toxin incubated in GMM7. Standard interpretation of this assay considers an IF of >1.5 as genotoxic. In our results, the AFB<sub>1</sub> control condition has an IF of ~2.6 at the highest concentration tested (15  $\mu$ g/mL), indicating genotoxicity. The same starting concentration of AFB<sub>1</sub> after incubation with *R. ery* and *R. pyr* reduced the IF to roughly 0.75, signifying diminished toxicity (Figure 5.8). Compared to controls, the pH 9 treated samples have reduced toxicity. The toxicity of AFG<sub>2</sub> byproducts was not investigated since the SOS ChromoTest is not sensitive for AFG<sub>2</sub>. However, *Rhodococcus* condition show loss of fluorescence and lactone ring opening, which have been confirmed to correlate to reduce toxicity (17).



Figure 5.8 Byproducts of AFB<sub>1</sub> degradation by Rhodococcus sp. have reduced toxicity. SOS-ChromoTest kit for genotoxicity was used to assess the toxicity of byproducts after live cell-mediated degradation. AFB<sub>1</sub> was incubated with *R. ery* (blue) and *R. pyr* (red) in GMM7 for 48 hours prior to ethyl acetate extraction and resuspension in methanol. Serial 2-fold dilutions were used to obtain a trend line. AFB<sub>1</sub> incubated in GMM7 was used as a control (no degradation) and run through the same extraction protocol. Values of IF over 1.5 are interpreted as genotoxic.

# 5.2.7 Rhodococcus species show improved AF degradation when grown in starch medium

We sought to explore the potential for enhancing the degradation capacity for these *Rhodococcus* strains by utilizing growth on a more complex carbon source. The principle behind the use of starch has been explored previously in Chapter 4, where we see the increased degradation capacity by environmental isolates when grown on starch medium. Here, we grew *R. ery* and *R. pyr* in glucose and starch media prior to testing degradation through the FL assay on both AFB1 and AFG2. We see similar levels of growth in both media types for both strains, showing the ability of R. ery and R. pyr to grow on the more complex carbon (Figure 5.9A and C). R. ery has improved degradation of both AF types when in starch medium, where the time to degrade AF is reduced but similar overall levels are seen compared to glucose (Figure 5.9B and D). For AFG<sub>2</sub>, *R. ery* grown in starch removes 90% of toxin in 18 hours compared to 24 hours in glucose (Figure 5.9D), displaying enhanced efficiency in degradation. Alternatively, R. pyr only shows slight improvement, with 80% AFG<sub>2</sub> degradation in glucose compared to 90% in starch within 36 hours (Figure 5.9D). Additionally, *R. pyr* does not have the same improvement on AFB<sub>1</sub> when grown in starch (Figure 5.9B), alluding to differences in degradation mechanisms between AFB<sub>1</sub> and AFG<sub>2</sub>.



Figure 5.9 Degradation of AF is improved in starch versus glucose culturing medium. Growth curves in the presence of A) AFB<sub>1</sub> and C) AFG<sub>2</sub> of *R. ery* (blue) and *R. pyr* (red) when grown in medium containing glucose (solid) or starch (dashed) as the sole carbon source. Cell density is measured by OD 600 over 48 hours. Degradation curves for B) AFB<sub>1</sub> and D) AFG<sub>2</sub> by live cell cultures of *R. ery* and *R. pyr* using FL assay, where toxin concentration is normalized to the initial concentration. Data are the mean of 3 replicates.

# 5.3 Discussion

Bioremediation of AF contaminated food is an important area in food safety with global economical and health impacts. Our results suggest that bacterial species such as *R. ery* and *R. pyr* have the capability to degrade AF; however, limitations of their use exist in unknown degradation mechanisms and practical limitations of their use. Here, we explore

the characterization of degradation by these species, with a focus on uncovering how degradation is facilitated to apply the AF degrading capacity in food processing.

Previous literature has pointed to *R ery* and *R .pyr* as potential AF degraders, with some elucidation of the degradation mechanism for AFB<sub>1</sub> as extracellularly produced enzymes (82). In our study, we find that CFF is not active against either AF type tested, regardless of pre-exposure of the cells to AF during growth. Instead, our results show that cell lysate is the active fraction, and that degradation is facilitated through intracellular enzymes. A possible explanation for this contrasting result is that we test degradation in a neutrally buffered medium, since pH, particularly more alkaline pH, can affect the degradation assay (see Chapter 3). *R. ery* and *R. pyr* have been shown to increase the pH of their environment during growth, to around pH 8.5. This shift is likely the cause of CFF in unbuffered medium reporting as active.

Further characterization of the degradation process implicates the potential for multiple enzymes. After DTT treatment, lysate only saw a slight reduction in activity. This reduction points to the requirement of oxidation in the reaction, but since DTT did not completely inhibit degradation, it suggests that an alternative reaction is occurring that can proceed in the presence of DTT. The possibility of multiple enzymes is not unlikely, as degradation could necessitate multiple reactions for the complete breakdown of AF. Additional studies are needed to confirm this theory.

An interesting result of this study is the differences in degradation between the two species of *Rhodococcus*; where we see similar levels of degradation by live cells, but the characterization reveals distinctions in the degradation process. *R. ery* degradation was not impacted by Chelex treatment, whereas *R. pyr* displayed significant inhibition, indicating the degrading enzymes between the two species are distinct. Further, when *R. pyr* live cells were treated with proteinase K, we saw that AFB<sub>1</sub> degradation was impacted, whereas AFG<sub>2</sub> degradation by *R. pyr* and degradation of both toxins by *R. ery* were not impacted. This suggests that *R. pyr* may need cell-surface proteins to degrade AFB<sub>1</sub> while *R. ery* does not. It also suggests differences in the degradation mechanism of the two AF types.

To enhance bacteria with improved bioremediation performance, we need to understand how they degrade AF, which is why characterization is an important step. We can apply enhancements such as the starch environment, that potentially increases the rate of production of the degrading enzyme, without full understanding of the degradation mechanism, but this is only a small improvement. This enhancement does give us further insight into the degradation process, where the degrading enzymes could be involved in metabolism of complex carbons, which is why we see increased degradation when grown on starch. However, to enhance degradation to levels necessary for application, further investigation into the degradation machinery is necessary.

# 5.4 Future directions

Progress in making bioremediation more efficient depends on better understanding of the underlying mechanisms. We emphasize that identifying, isolating, and characterizing the detoxifying enzyme should be a fruitful next step. One path to this identification is through fractionation of the cell lysate, such as using Fast Protein Liquid Chromatography (FPLC), and subsequent proteomics of the active fraction. These results will also enable more directed strain engineering efforts in the future that uncover the important machinery that regulates the production of the detoxifying enzymes. Alternatively, they allow potential transfer of the detoxifying enzymes to tractable organisms and/or to generally regarded as safe (GRAS) organisms for practical deployment.

# 5.5 Methods

# 5.5.1 Bacterial strains and growth conditions

*Rhodococcus erythropolis* (DSM 43066) and *Rhodococcus pyridinivorans* (DSM 44555) were grown at 28°C with continuous shaking (240 rpm) for 24 hours in GMM7 (glucose) or SMM7 (starch): composition of media found in Section 4.5.1.

To assess for AF degradation, each defined medium was amended with pure AFG<sub>2</sub> and AFB<sub>1</sub> (Cayman Chemical) that had been dissolved in LC-MS grade methanol to the final

concentration of 1 mg/mL. Each mycotoxin amendment resulted in a final AF concentration of 15 ug/mL.

# 5.5.2 Fluorescence degradation assay

This method is described in Section 3.5.5, with the following modifications for this study: live cell conditions had a starting OD of 0.01 and were continuously shaking between reads.

# 5.5.3 Lysate extraction

Cultures of *R. ery* and *R. pyr* (100 mL) were grown overnight in GMM7 (glucose) to exponential phase, OD ~0.6-1.0. Cells were centrifuged at 3500 x g for 5 minutes and washed in 1x PBS twice before a final resuspension in 10 mL of 100 mM MOPS buffer, pH 7.0. Aliquots of 1.5 mL were transferred into screwcap microcentrifuge tubes containing 0.1 mm silica beads. Cells were lysed using a Mini-BeadBeater 16 (Biospec Products), with 8 min of total lysing time in 1 min increments. Cell debris was centrifuged down at 10,000 x g for 3 min and the supernatant was filter sterilized using 0.22  $\mu$ m, PVDF Whatman Uniflo syringe filters (Cytiva). The lysate was used for AF degradation assays.

# 5.5.4 Lysate treatments

Proteinase K: Cell lysate was treated with 1 mg/mL of Proteinase K (Sigma Aldrich, Recombinant PCR Grade) at 37°C for 3 hours. After incubation, treated lysate underwent degradation testing via fluorescence degradation assay.

DTT: Cell lysate (1 mL aliquot) was incubated with 10 mM Dithiothreitol (DTT, Thermo Scientific) for 1.5 hours at 37°C. After incubation, lysate was filter sterilized using 0.22  $\mu$ m, PVDF syringe filters.

Chelex: Cell lysate (1 mL aliquot) was incubated with 0.07 g of Chelex mesh (Sigma-Aldrich) for 1.5 hours at 37°C. Lysate was filter sterilized using 0.22  $\mu$ m, PVDF syringe filters to remove Chelex mesh after incubation.

# 5.5.5 Metal ion and cation supplementation

Stocks of metal ions and cations were made at the following concentrations: 1.5 g/L of  $FeCl_2 \times 4H_2O$ , 70 mg/L of  $ZnCl_2$ , 0.1 g/L of  $MnCl_2 \times 4H_2O$ , 0.19 g/L of  $CoCl_2 \times 6H_2O$ , 2 mg/L of  $CuCl_2 \times 2H2O$ , 24 mg/L of  $NiCl_2 \times 6H_2O$ , 1 M MgCl<sub>2</sub>, and 1 M CaCl<sub>2</sub>. To Chelex-treated cell lysate (0.5 mL aliquots), 1 µL of the metal ion stock solution was added. Samples were incubated at room temperature for 30 min prior to use in FL degradation assay.

# 5.5.6 Aflatoxin extraction

Method described in Section 3.5.3.

# 5.5.7 LC-MS assay

Method described in Section 3.5.4.

# 5.5.8 Genotoxicity assay

Method described in Section 3.5.6.

# 5.5.9 Data analysis

Raw data from the aflatoxin degradation assay was processed using Matlab generated codes to measure growth and degradation characteristics. Background fluorescence (no toxin control) is subtracted from the readings to remove fluorescence from sources other than the toxin. The readouts are also normalized to fluorescence data from no cell controls to remove the effect of fluorescence loss due to bleaching or other causes over time with an additional normalization to account for fluorescence loss due to cell scattering. To convert the fluorescence readout to the corresponding toxin concentration, we employ a calibration curve based on measurements of a set of known toxin concentrations (240). After normalization, degradation efficiency is calculated as the percentage of toxin removed during the testing period (48 hours).

# 5.5.10 Statistical analysis

To compare the degradation efficiency between cell lysates supplemented with different metal ions and cations (Figure 5.6), we use Student t-test using the Matlab function ttest.

# Chapter 6 – Discussion

# 6.1 Summary and significance

This thesis offers new insights into the process of AF detoxification through the exploration of degradation mechanisms and their enhancement. Through the identification of new AF biological degraders, degradation mechanisms, and understanding the ability to improve degradation efficiency, this work offers a comprehensive overview of AF detoxification toward application in agriculture and food processing.

# 6.1.1 Computational biology tools can aid in developing microbial detoxification systems

Chapter 2 explored the use of computational tools to discover strains and enzymes that detoxify harmful compounds, particularly focusing on mycotoxins and biological enzymes. We discussed the use of established and novel computational tools to complement existing empirical data, providing a unique look into the role that computational tools can play in understanding the often unknown and underexplored processes of mycotoxin detoxification.

By utilizing the diverse, readily available computational tools, researchers can delve deeper into the mechanisms of degradation that can be arduous or complex to explore through wet-bench experimentation alone. The push to use the highlighted tools, as well as others, in the bioremediation field is needed to make the desired progress for biological decontamination methods. Understanding the mechanisms of degradation is often the limiting step in the application of biological degraders. Using computational tools in the characterization of biological degradation, in sectors such as identifying new potential degraders or degrader communities, gaining insight into the genetic organization of the degradation machinery, and uncovering regulatory steps, allows clarity in the subsequent steps toward application. By implementing these computational approaches alongside experimental approaches, there is greater potential for desirable outcomes within a shorter timeframe. Additionally, the enhancement of the degradation process can be aided by information acquired through computational methods. For example, the use of molecular dynamic or quantum mechanics simulations offers insight into interaction strengths between the amino acid residues of the enzyme active site and the toxin substrate, as explored in the Appendix. This information can help identify potential amino acid mutations that may increase binding affinity and therefore enhance degradation. This insight is crucial to the actual enhancement of the process.

In the future, it will become important for more collaboration between researchers in the fields of computational biology and bioremediation. Ideally, this collaboration would culminate in better tools and software that are created with the questions in the bioremediation field in mind. Ultimately, a pipeline could be created that utilizes the tools described in Chapter 2 to streamline the process from identification of AF degraders to uncovering their mechanisms to their enhancement. Then, these results can be experimentally validated. Such a pipeline would augment current approaches looking at screening methods for new degraders and the characterization of degradation by individual species. Ultimately, with more knowledge on how individual degraders facilitate degradation of a certain pollutant, we can better gauge general principles of degradation that will serve to provide a solution to the AF contamination issue.

# 6.1.2 Weakly alkaline conditions degrade aflatoxin through lactone ring opening

In Chapter 3, I explored the role of pH and its influence on AF degradation. The results from this study show that weakly alkaline conditions, namely pH 9, can have significant degradation on both  $AFB_1$  and  $AFG_2$ , by 60% and 95%, respectively. Elucidation of this mechanism reveals that the lactone ring structure of AF is opened, as shown in the loss of fluorescence of AF and the proposed byproduct of  $AFG_2$  degradation. While the exact mechanism of the ring opening is not clarified in this study, it is likely through a hydrolysis event which would be consistent with the proposed byproduct. Further, when this method was tested in a food matrix of cornmeal, the pH 9 condition retained the degradation ability of both AF types, indicating the applicability of this method in controlling contamination of foodstuff. Lastly, since no significant degradation effects are found at pH 7, this condition is proposed as a neutral environment for degradation testing in other systems, such as biological, that will not impact the observed degradation effects.

Previous work on chemical decontamination methods of AF have used the application of strong alkaline or acidic conditions (20, 214). The use of strong acids and bases have posed issues in terms of safety of the method, downstream ecological waste, and practical limits of use on foodstuff. My work has shown that weakly alkaline conditions can impart significant levels of AF degradation, which should be safer to use in food processing for user and consumer, as well as potentially reduce downstream waste, since less harsh chemicals are needed. Conceptually, the ability of weaker alkaline conditions to degrade AF poses an interesting question of the stability of AF. While research shows high environmental stability, where normal food processing such as cooking does not mitigate its effects, a weak alkaline environment is able to breakdown AF into a less toxic byproduct.

While these findings highlight the potential of using pH manipulation to degrade AFs, it is important to consider the practical implications and limitations of this approach. This study was able to successfully degrade AF from a food source, cornmeal; however, additional investigation should be done to examine the nutritional content of the food after decontamination to ensure loss of nutrients is not significant. Previous chemical methods have been criticized for the nutrient depletion effects on foods since it decreases the value of the food (249). Additionally, other foods should be tested with this method to understand its range of application, especially on seeds/grains prone to AF contamination (*e.g.*, intact corn kernels, tree nuts or ground nuts, etc.), and naturally contaminated sources should be investigated to ensure the applicability of this method outside of a lab environment. Here, I tested in a small laboratory scale that does not directly translate to the scale used in food processing. Further testing on foodstuff in the scale seen in food processing is needed to optimize this method.

Another consideration for this method is where in the food processing chain can it be implemented. I propose that the best point of application with minimal interference to operation would be during the washing step of food processing, where the wash could be supplemented at pH 9 to remove AF prior to subsequent steps. A similar framework has been used with other chemical methods. While this would not mitigate the contamination that arises during post-processing storage, it would deplete contamination that occurs up until the processing step and other good agricultural practices can be used to prevent additional contamination.

Beyond the use of pH as a chemical method for AF degradation, pH has important implications in the context of biological assays. Since pH plays a significant role in the observed degradation in two different detection methods, fluorescence and MS, I emphasize the need to include a buffered medium when testing biological samples. As microbes grow, it is common for the pH of the environment to change as certain metabolites are produced. This change can cause interference in the interpretation of results, particularly when screening for new degraders (as false positives may arise) or when characterizing known degraders where degradation is attributed to the wrong cellular fraction. These pH-related artifacts can delay the identification of degradation mechanisms and enzymes for downstream use. From my data, pH 7 has little impact on observed degradation and should be used as the baseline testing environment for biological systems to counteract pH effects, or at the very least, researchers should report the pH of testing conditions.

# 6.1.3 Starch can expedite the screening for bacterial aflatoxin degraders

Identifying new degrader species is highly beneficial in that it can offer alternatives to overcome the limitations of existing biodegraders, such as narrow working conditions and low degradation rates. In Chapter 4, I screened several environmental isolates for their AF detoxification ability, using AFG<sub>2</sub>. I used different carbon sources (glucose and starch) as isolation and culturing media to examine the effect of the environment on degradation ability. Starch is a good choice for this screen since it is cost-effective, easy to obtain, and has been shown to enhance AF degradation by certain fungal and bacterial species (78, 235). In my study, strains isolated in medium with starch as the primary carbon source showed a higher percentage of good AF degraders, 16% compared to 2% when glucose was the primary carbon source. Additionally, most species isolated in glucose medium exhibited improved degradation efficiency when moved into starch medium, with one

isolate improving degradation levels from 30% to 70%. The starch screen also revealed two previously unidentified AF degrader bacterial species, while the glucose screen only isolated bacterial strains of previously identified degrader genera. Of note, good AFG<sub>2</sub> degraders also appeared to perform well against AFB<sub>1</sub>, indicating the ability of these strains to degrade multiple types of AF. Overall, the use of starch medium for assessing AF degradation expedited the screening process and generally improved the performance of isolates.

While this study did not explore the mechanisms of degradation by these isolates or how starch was imparting improved degradation performance, I can infer that growth on a more complex carbon source is likely shifting the metabolism of these isolates and promoting the increased production of the degradation machinery. This is a feasible reason since we see that growth rates and carrying capacity are not increased in the starch environment. Therefore, the increase in degradation capacity is not the result of an increased number of cells facilitating degradation, but rather an enhancement to the degradation by individual cells. This suggests that there is regulation of the degradation machinery, where under normal conditions of simple carbon availability, lower levels of production or possibly secretion occur, while the more complex environment shifts the cells to produce more of the machinery. If regulation is the limiting step to enhanced degradation, once the machinery is fully elucidated, we can manipulate the rate-limiting step through cloning under a constitutive promoter. Additionally, this information can be used to computationally identify new degraders by searching for homologous metabolic pathways. An interesting avenue of research would be to take a comprehensive view at degraders enhanced under starch conditions and determine commonalities between the machinery and pathways involved in degradation.

The diversity of degrader species arising from this screen indicates that the AF degradation ability is not a rare occurrence or limited to certain taxa. We found species from various genera that exhibited a wide range of degradation levels, where phylogenetically grouped species did not always perform similarly nor did the best degraders group together. Additionally, the environmental samples used were sourced from around the Boston College campus, an area that is not at high risk for AF contamination. Thus, the isolates found in this screen were probably not pre-exposed to AF or at least not in sufficient quantities to necessitate degradation ability. This, paired with AF having a low fitness impact on the isolates, indicate that the degradation machinery may not be designated for AF, but rather degradation of AF is a happy accident. Since the machinery is not necessarily selected for AF degradation, enhancing the affinity of degradation enzymes involved in this process is a real possibility.

Since this study focused on the development of a new screening method for AF degraders, it did not examine the degradation mechanisms of individual species. Further research is necessary to elucidate the mechanisms used by species of interest that arose in this screen before they can be used as bioremediators. Additionally, it is important to determine if the degradation machinery, removed from the pressure of the starch condition, will retain improved degradation or if it is simply a regulatory issue. A deeper focus on mechanisms and their characterization will hopefully resolve that question. To better understand the pressure supplied by starch, it would be worthwhile to investigate more complex carbon sources, such as carboxymethyl cellulose, to see where the limit is on manipulating this metabolic shift in favor of AF degradation. Starch isolates can be grown on these more complex carbons to test if the degradation enhancement can be pushed or is limited to starch.

From the results of this study, I suggest that using starch as the carbon source is a promising means to identify new AF degraders in the environment. The species identified here did not come from samples at risk for AF exposure. In the future, I propose utilizing the framework of this screen on environmental samples from higher risk sources, like crop soil and crop leaves. Subsequent screens implementing these sources will provide a greater chance for identifying new degraders with promising degradation capability.

# 6.1.4 *Rhodococcus* species are promising aflatoxin degraders with distinct characteristics of degradation between species

Chapter 5 focused on the characterization of AF degradation by two promising degraders, *R. ery* and *R. pyr*. Live cultures of *R. ery* and *R. pyr* were shown to degrade AFB<sub>1</sub> by 60% and 38%, respectively, while both strains degraded AFG<sub>2</sub> to completion. A closer look into

how these strains degrade showed that degradation occurs through an intracellular enzymatic process, where the enzymes between species exhibit variable efficiencies. *R. ery* cell lysate was unaffected by Chelex treatment while *R. pyr* cell lysate displayed improved degradation after the removal of metal ions by Chelex. Further examination revealed that the addition of Ca had a slight inhibitory effect, however, not the extent seen in non-Chelex treated lysate. The byproducts of the degradation reaction were tested for toxicity and were found to not have retained toxicity compared to AFB<sub>1</sub> control, ensuring the safety of this degradation method. Additionally, *Rhodococcus* strains were grown on a starch medium and were generally found to improve their degradation efficiency, with *R. ery* showing more improvement than *R. pyr*.

By investigating degradation using two related species and two types of AF, we examine the species-specific and toxin-specific characteristics of degradation. Between species of *Rhodococcus*, there is a distinct difference in degradation phenotypes and the characteristics of degradation. One of the key distinctions is the influence of Chelex treatment on cell lysate (the fraction facilitating degradation), where *R. ery* is not affected and R. pyr shows significant increase in degradation after removal of metal ions and cations. This indicates that the degrading enzymes of R. pyr and R. ery are inhibited in different ways and are likely different types of enzymes. Additionally, the influence of growth on starch is very distinct between the two species. R. ery shows marked improvement in starch versus glucose, particularly in shortening of the time to degradation. This improvement occurs on both toxin types. Alternatively, R. pyr only shows slight improvement for AFG<sub>2</sub> degradation, moving from 80% to 90% in the starch condition. R. pyr does not show improvement on AFB<sub>1</sub> when grown in starch, highlighting not only a difference between the species but also between degradation of different AF types. The variance in degradation stresses the possibility of different degradation mechanisms between the two species of *Rhodococcus*.

Between toxins, there is a rather large difference in degradation efficiency, where  $AFG_2$  is mostly degraded to completion (by live cells) while  $AFB_1$  has markedly less degradation in the same testing period, when looking at FL assay data. However, results from MS analysis reveal that in live cell conditions there is depletion of both toxin types. This
observation, taken together with the complete loss of toxicity of  $AFB_1$ , signifies that degradation of both toxins goes to or near completion; however, the difference lies in the mechanism. The FL degradation assay is limited in its detection to lactone ring opening, whereas MS can detect even small changes to the toxin. Here, I believe that it is possible the degradation of  $AFG_2$  is through lactone ring opening, whereas for  $AFB_1$ , cells are targeting alternative sites on AF in addition to the lactone ring.

To fully understand the differences between the degradation of AF types and the two *Rhodococcus* species, additional research is necessary on the mechanisms and pathways involved. The first step would be the identification of the degrading enzymes and then identification of the genes involved in the production and regulation of the degrading enzyme. Two possible approaches to this would be through random mutagenesis and sequencing of the two species and through transcriptomics using the pressure of starch to look for changes in transcript levels. A limit of genetic work on these species is that they are understudied, and their genomes are not well annotated. However, this future work would aid in better understanding these promising degraders, that are not only important for AF, but generally important in the bioremediation field.

## 6.2 Broader impacts

#### 6.2.1 A more complete look at AF detoxification

The culminative impression of this work is understanding more completely the process of AF degradation. The framework of Chapter 2 provides a streamlined computational exploration of degradation mechanisms from finding new degraders to improving their degradation performance. This pipeline, paired with experimental approaches in Chapters 4, 5, and the appendix, offers a more complete view of degradation by microbes. The work of Chapter 3 aids this view by providing insight into alternatives to biological methods and giving crucial information on the role pH plays when investigating AF degraders. Taken together, I offer a clear pathway from the identification of new degraders and mechanisms to their enhancement and eventual application.

General conclusions that can be drawn from these studies come from the distinctions in degradation by different microbes. I find that although many strains were shown to degrade AF, the levels of degradation between species, especially taxonomically similar species, were not always consistent. This reveals that not all mechanisms used by degraders will be the same; however, there is room to explore the similarities between them. By examining both the similarities and differences between degradation mechanisms, we can begin to understand the complexity of the AF degradation process. I think these distinctions also reveal that most of these mechanisms are not optimized for AF but concomitantly degrade AF while performing their main functions. Also, degradation of different AF types may be proportional, as seen in the starch study, but not always the same or by the same mechanism, as seen in the *Rhodococcus* study. What can be inferred from this is that a solution to AF contamination might not be one size fits all, but it will be necessary to approach different AFs through various routes. I believe that the work within this thesis can provide future researchers with guidelines to tackle AF detoxification and help to answer pressing questions within the field.

#### 6.2.2 Future outlook

For the research presented in this thesis, the next steps need to focus on uncovering the specific mechanisms of AF degradation by environmental isolates of interest that appeared in the starch screen and by *Rhodococcus* species. I foresee the combinatorial use of experimental and computational methods to simplify this process. From an experimental approach, this means further characterization of degradation in terms of optimization of working conditions and isolating enzymes that facilitate degradation. From a computational approach, this means identifying metabolic pathways or regulatory elements and applying methods such as QM for enzyme enhancement. The combined use of computational and experimental approaches will allow for a complete look at the AF degradation process.

Once multiple degradation mechanisms are revealed, one specific avenue of research that I am interested to see is understanding the differences between degraders at the genus, species, and strain levels. At the genus level, are there similarities between different organisms that can reveal general principles of AF degradation such as metabolic pathways or classes of enzymes that can be more widely searched for in safe-to-use bacteria and fungi? At the species level, what dictates whether species within the same genus will both have AF degradation capability? At the strain level, why do some strains degrade AF better than others? To answer these questions, future studies can contrast degradation in environmental isolates (genus level), the *Rhodococcus* lab strains (species level), and the *R. ery* lab strain and *R. ery* environmental isolate (strain level).

Beyond these initial steps, future studies should look toward preparing these strains and their degrading enzymes for application in the food and feed industry. An approach to this task is through strain engineering, where microbes that possess AF degradation machinery undergo enhancement through genetic manipulations to increase performance. This enhancement can be through overriding limiting steps, such as production or regulation. Alternatively, when degrader strains are not able to be used in food and feed, due to their pathogenicity or poor growth conditions that do not align with application, the degradation machinery can be transferred into generally recognized as safe (GRAS) organisms. Most strains discussed in this work are not able to be applied directly to food and feed due to their status as non-GRAS organisms. GRAS strains engineered with the degradation machinery can overcome this issue and would be especially beneficial if the method of application desired to use whole cells, but it could also allow for safer and more efficient extraction of the active fraction or enzymes. The limit to reaching this step is the identification of the degradation machinery and pathways involved in its expression.

In addition to strain engineering, enzyme engineering is a promising approach, with focus on optimizing enzymes through rational design or directed evolution. Rational design involves performing chosen mutations in the coding sequence, usually based on structural and functional information about the enzyme. QM is a promising way to gain this information and has already been shown to be beneficial in the enhancement of AF degrading enzymes, as seen with laccase in the Appendix. Rational design depends on reliable structural information of the target enzyme, which is often not available. However, recent advances have provided computational methods for protein structural determination, of note is AlphaFold (250), which would be beneficial once a degrading enzyme is identified. Directed enzyme evolution bypasses the need for structural information of the target enzyme and focuses on the principles of natural evolution in a laboratory setting. Directed evolution subjects a gene of interest to iterative rounds of mutagenesis and screening for desired phenotypes. There are many methods for the creation and screening of mutants, and recent reviews cover them extensively (251, 252). These approaches rely on identification of the degrading enzyme, so only known degradation mechanisms can be optimized in this way. Once degrading enzymes of the *Rhodococcus* species are isolated and identified, I would look toward utilizing enzyme engineering to enhance their performance.

An alternative approach to optimize strains, that removes the need for prior knowledge of the degradation pathway, is through artificial selection. Selection would look to improve the degradation phenotype by a degrader strain through iterative rounds under a selective pressure. A barrier to this approach for our strains is that AF has little to no fitness impact, so there is nothing to drive the selection. Recent studies have looked to partner assisted artificial selection as a way to overcome this setback (253). By utilizing a microbe that derives a fitness impact from AF and is beneficial to the growth of a degrader strain, selection can be driven despite the previous limitation. Work in this area to identify the assisting strains is needed, but this area of research would allow for enhancement of AF degradation without the need to explore the underlying mechanisms.

Within the AF bioremediation field, future work is needed to better understand the current gap to application of AF biological decontamination methods. To date, multiple strains and enzymes have been identified as AF degraders. While a known limit to their use lies in low efficiency, little has been done in the field toward improving degradation rates or performance for application. Other mycotoxins have commercially available enzyme solutions that are in use, but AF is behind on this front. I believe that research often stops at the point of identification and rarely moves toward enhancement. In coming years, a shift needs to occur that looks more toward improving known degraders and their enzymes so that there will be a solution to AF contamination that is more cost-effective. Resolution to this issue may come with more collaborative efforts between microbiologists, geneticists, biochemists, and plant/food safety biologists.

## 6.3 Concluding Remarks

The work presented in this thesis aims to provide a comprehensive look at AF detoxification through the identification of degraders and degradation mechanisms and their potential enhancement. Through these different investigations that focus on either identification, enhancement, or the bridging of the two, I believe this work can offer researchers in this field an overview on the necessary steps in finding AF detoxification methods that are suitable for application. While this work does not conclude with an AF degrader that is ready for implementation in food processing, I was able to categorize new degradation methods and mechanisms, as well as offer insight into the enhancement of biological degradation. Overall, this greater understanding of AF detoxification will significantly improve the existing approaches to finding and improving AF detoxification methods.

# Appendix Site-directed mutagenesis of laccase for the enhancement of AF degradation efficiency

This work was done in collaboration with the Reverberi and Bonaccorsi Labs at Sapienza University of Rome and William Dawson at RIKEN Center for Computational Science in Kobe, Japan.

## A.1 Introduction

Laccase is an enzyme that is of interest in AF biodegradation (86, 94, 119) and generally in biotechnology (254). Laccases are multicopper containing enzymes capable of performing one electron oxidation of a broad range of substrates. Several bacterial and fungal laccases have been identified to interact with AFs (86, 255–257); however, most isoforms do not have satisfactory performance in terms of time and cost efficiency for their application in food and feed industries.

Previous work combined experimental and computational approaches to pave the way to a rational optimization of laccase as an AF bioremediator, using laccase from *Trametes versicolor* (TV) (240). Our lab along with our collaborators used a molecular dynamics simulation to sample the conformational space of both AFB<sub>1</sub> and AFG<sub>2</sub> within the active pocket of laccase. The poses found were scored using a quantum mechanical model and the strongest interacting poses were extracted for detailed analysis. They found that AFG<sub>2</sub> had one pose that stood out as being low energy: G2-10-0. For AFB<sub>1</sub>, two competing low energy poses were B1-2-2 and B1-15-2. One other pose that stood out was B1-1-0. While

B1-1-0 does not have a strong interaction energy, it looks the most similar to G2-10-0, with the furan ring deep inside the pocket. In Figure A.1, we show a heatmap of amino acid interactions of the laccase active pocket and each of those poses. From this interaction map, we can predict specific single amino acid residues that are sub-optimal for AF degradation and focus on three residues of interest: LEU164, ASN264, and THR430.



Figure A.1 Interaction heat map between amino-acids and different toxin poses. Figure reproduced from Zaccaria 2023 (240). Darker is stronger.

Our goal is to determine which amino acids interact strongly with the toxins to use as a substitute for the sub-optimal residues. For computational efficiency, our collaborators used a simplified quantum mechanical model. They started with one of the AFs in a box, and randomly added the side chain of one of the many amino acids into the box. They optimized the geometry, evaluate the interaction energy, and repeated the procedure for several sidechains. Finally, they stored the value for the strongest positive interaction. From these simulations, two interactions stand out immediately: those between the toxins and the positively charged amino acids, arginine and lysine (Figure A.2). Likely, these residues want to form a hydrogen bond with the oxygens of laccase. In the pocket of laccase, however, there appear to be no positive residues, so approaching site-directed mutagenesis using these residues could have drawbacks. The other strong interaction is phenylalanine (Figure A.2), where  $\pi$ - $\pi$  stacking may be involved.



Figure A.2 Interaction strengths (positive is stronger) between side chains and toxins. Figure is reproduced from unpublished work by William Dawson.

Using this information, we propose to experimentally test two different types of mutations: disabling mutations (for verifications) and enhancing mutations. First, we want to probe whether our understanding from our computational model is correct. To do so, we can look at the strong interactions identified in Figure A.1 and "turn them off". Here, we can use the concept of alanine scanning. The second type of mutation is to try to enhance the activity of TV laccase based on the interactions we currently see. Looking at the heat map, we identify strongly interacting residues, then filter for ones that are sub-optimal according to our supplementary analysis. Then, we replace the sub-optimal residue by a new residue which interacts stronger in the suitable position.

## A.2 Results and Discussion

Five TV laccase mutants were created to test the validity of the QM modelling, two disabling mutants (N264A and T430A) and three enhancing mutants (N264F, T430F, and L164F). These mutants were chosen based on the interaction strength of the three residues with the various tested poses of AF. For enhancing mutations, phenylalanine was selected to replace each residue due to its high level of interaction with both AFB<sub>1</sub> and AFG<sub>2</sub> (Figure A.2). After cloning (Methods, A.4.1), expressing (Methods, A.4.2), and purifying mutant

TV laccase (Methods, A.4.3), we performed degradation assays to quantify their performance against wildtype (WT) (Methods, A.4.4-A.4.6).

## A.2.1 Mutant laccases are able to degrade AFB<sub>1</sub> better than WT

When testing degradation using LC-MS for detection, we find that after 24 hours two mutants, T430A and T430F have an increased degradation phenotype compared to WT (Figure A.3A). WT is able to remove roughly 44% of AFB<sub>1</sub> in the testing period, while T430A and T430F remove 55% and 54%, respectively, displaying the best degradation efficiency of the mutants (Figure A.3A). N264F and L164F do not have increased degradation ability, but remain similar to WT. Alternatively, N264A displays a reduction in degradation efficiency at 38% (Figure A.3A). This reduced performance is interesting since N264A and T430A were predicted to be disabling mutants, where decreased degradation efficiency was expected. While N264A behaves as predicted in this assay, T430A does not. It is possible that the increase in performance of T430A compared to WT comes from the higher interaction strength reported for alanine compared to threonine (Figure A.2).

In contrast to the MS analysis, analysis using a fluorometric assay shows N264A, T430A, and L164F with the most reduction in fluorescence (Figure A.3B), indicating higher efficiency in degradation of AFB<sub>1</sub>. Additionally, this assay finds that all mutants have an increased degradation phenotype compared to WT after 48 hours (Figure A.3B). WT is able to remove roughly 33% of AFB<sub>1</sub> in the testing period, while N264A, T430A, and L164F remove 44%, 42% and 45%, respectively (Figure A.3B). N264F and T430F have an increased degradation performance compared to WT with 40% removal for N246F and 36% removal for T430F, but do not degrade to the same higher levels seen by their alanine mutant counterparts (Figure A.3B). The difference in results between these two assays for detection could be explained by what each method is able to detect. The fluorometer assay will only look at opening of the lactone ring as a measure of degradation, but MS is able to detect other changes to AF. Likely, AFB<sub>1</sub> is targeted at additional sites during degradation leading to the higher rate of efficiency in MS compared to the fluorometric assay and differences in the profile of degradation by these mutants.



**Figure A.3 Mutant laccases tend to degrade AFB**<sub>1</sub> **better that WT**. A) AFB<sub>1</sub> degradation was tested through detection by LC-MS after 24 hours of incubation with WT or mutant laccase. Percent toxin reduction calculated compared to a control of toxin alone. B) AFB<sub>1</sub> degradation was tested through detection by fluorometer, with data taken every 2 hours for the first 6 hours and then at timepoints 24 and 48 hours. Data is normalized to a control of toxin alone at each timepoint.

## A.2.2 AFG<sub>2</sub> degradation is distinct from AFB<sub>1</sub> degradation

To understand how different laccase mutants perform on other AF types, we conducted further testing of their performance against AFG<sub>2</sub>. Here, we use our fluorescence assay for detection over a 72-hour testing period and exclude L164F due to difficulties in expression. In contrast to AFB<sub>1</sub>, we observe different patterns of degradation compared to WT (59% toxin removal) when tested on AFG<sub>2</sub> (Figure A.4). We find that on AFG<sub>2</sub> T430A has higher efficiency than T430F, which is a significant increase compared to WT (Figure A.4). This pattern is comparable to results on AFB<sub>1</sub> for these two mutants (Figure A.3). A big distinction, however, is that N264F outperforms the other mutants on AFG<sub>2</sub>, with a degradation efficiency of 92% (Figure A.4); while on AFB<sub>1</sub>, N264F performed similarly to WT (Figure A.3A). This difference in degradation phenotype suggests that mutations made to laccase can affect the degradation of different AFs in distinct ways.



**Figure A.4 Mutant laccases tend to degrade AFG<sub>2</sub> better that WT**. AFG<sub>2</sub> degradation was tested via FL assay, with data taken over 72 hours. Reported degradation efficiency is the percent of AF degraded within the testing period. Data are the mean of 3 replicates.

## A.3 Conclusions

This work aimed to validate computational modelling data that proposed specific amino acid mutations that would potentially lead to an increase in AF degradation efficiency by TV laccase. We find that, generally, mutations do enhance the degradation performance of laccase, however, not in the ways that the model suggested. Alanine mutants were expected to "turn off" degradation but our results showed an enhancement in some cases, particularly for T430A. Further, phenylalanine substitutions were expected to enhance degradation efficiency. While we find some improvement in these mutants, they can be outperformed by alanine mutants on AFB<sub>1</sub>; however, N264F does perform the best on AFG<sub>2</sub>. Overall, we find that amino acid residues that were selected based on computational modelling do improve degradation efficiency using the predicted optimal amino acid substitution, but degradation still remains sub-optimal. Further study is needed to examine the degradation performance by these mutants in order to understand the implication of the model for predicting enzyme enhancement.

## A.4 Methods

## A.4.1 Plasmids, primers, and cloning

Mutant plasmid constructs were made using overlap extension PCR. Mutant inserts were made using primers containing point mutations (Table A.1) and pIB5 Tv lac plasmid (Figure A. 5) containing the WT laccase sequence as a template. Assembled and purified PCR products from each assembly and plasmid vector pIB5 were digested with XhoI and EcoRI in 10x Buffer R (ThermoFisher Scientific). Digested products were ligated into vector pIB5 using Ligase from Takara for 30 minutes at 18°C then transformed into competent 2x TOP10 cells. Transformants were then plated onto LB + ampicillin agar medium plates and incubated overnight at 37°C. Product insertion was verified through PCR and the sequence verified through sanger sequencing.



Figure A.5 Plasmid map of pIB5-TvLaccase

## Table A.1 Primers

Name	Sequence (5'->3')
N264A_for	AGAGCGAATCCGGCTTTTGGCACGGTCGGG

N264A_rev	CCCGACCGTGCCAAAAGCCGGATTCGCTCT
N264F_for	AGAGCGAATCCGTTCTTTGGCACGGTCGGG
N264F_rev	CCCGACCGTGCCAAAGAACGGATTCGCTCT
T430A_for	GTAGTTTCAACTGGGGGCTCCTGCTGCTGGCGAT
T430A_rev	ATCGCCAGCAGCAGGAGCCCCAGTTGAAACTAC
T430F_for	GTAGTTTCAACTGGGTTCCCTGCTGCTGGCGAT
T430F_rev	ATCGCCAGCAGCAGGGAACCCAGTTGAAACTAC
L164F_for	GGACCTCGTTTCCCATTCGGTGCAGACGCAACC
L164F_rev	GGTTGCGTCTGCACCGAATGGGAAACGAGGTCC

## A.4.2 Strains and transformation

Laccase mutants were expressed in *Pichia pastoris* GS115. Electrocompetent cells were made by the following method: Culture (100 mL) was grown overnight at 30°C with shaking in YPD. Cells were spun down at 2000xg for 5 minutes at 4°C. Pellet was washed twice in 100 mL of ice-cold sterile water. Cells were spun down again and washed in 20 mL of ice-cold 1M sorbitol. Cells were spun down a final time and resuspended in 0.5 mL ice-cold 1M sorbitol.

Plasmid constructs containing laccase mutations (5  $\mu$ L) were linearized using SalI and 10x Buffer O (ThermoFisher Scientific) and were transformed into 40  $\mu$ L competent cells of *P. pastoris* using BioRad gene pulser at 1.5 kV. Transformants were spread on defined minimal medium agar plates consisting of 2% glucose and 10 mL YNB stock (10.2 g yeast nutrient broth and 30 g ammonium sulfate in 300 mL deionized water) per 200 mL. Plates were incubated at 30°C for 2-3 days.

Gene insertion was checked through colony PCR using lithium acetate-SDS method for genomic DNA extraction. Briefly, a cell colony was transferred into 100  $\mu$ L of 0.2 M lithium acetate- 1% SDS solution. The mixture was incubated at 70°C for 15 minutes. To

the mix, 300  $\mu$ L of 100% ethanol was added and then centrifuged at 15,000xg for 3 minutes. The supernatant was removed and pellet resuspended in 100  $\mu$ L of TE buffer. Mixture was centrifuged again and then 1  $\mu$ L was used in the PCR reaction. Primers used were Lac\_for and L164F\_rev.

Transformants were patched onto minimal medium supplemented with 100 mM copper (II) sulfate and 0.1 mg/mL ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) to check for laccase activity via colorimetric change (example, Figure A.1). Mutant strains displaying activity were chosen for protein purification.



Figure A.6 ABTS plate containing Tv laccase mutants. Blue color indicates laccase activity against ABTS.

## A.4.3 Protein isolation

<u>Growth and supernatant preparation</u>: Cultures of GS115 TV laccase were grown overnight in baseline defined minimal medium consisting of 2% glucose, 12.5 mL YNB stock, 12.5 mL 1M potassium phosphate buffer pH 6.8, and 62.5  $\mu$ L 0.4 M copper (II) sulfate per 250 mL. Cultures were centrifuged at 2,000xg for 10 min and the supernatant filtered using a 0.22  $\mu$ m filter. pH was adjusted to 7.4 using 10 M NaOH.

<u>Chromatography</u>: ConA resin was filled in a chromatography column and pre-equilibrated with 25 mM MOPS/ 100 mM NaCl, pH 7.4. 500 mL of prepared culture supernatant was

added to the column. 20 mL of 250 mM methylmannoside in 25 mM MOPS/ 100 mM NaCl, pH 7.4 was added and incubated at room temperature for 1 hour with agitation to the column. The eluant was collected and subjected to a second round of chromatography on Q-sepharose, pre-equilibrated with 25 mM MOPS/ 100 mM NaCl, pH 7.4. The flow through from this column was collected.

<u>Sample concentration</u>: Using a 10 kDa ultrafiltration column, the sample was centrifuged for concentration at 3500xg in 20-minute cycles until a volume of about 2 mL was obtained. Concentration of protein was measured using Qubit.

## A.4.4 LC-MS

A stock of AFB<sub>1</sub> was made at 10  $\mu$ g/mL using 25.74 mL 0.1 M acetate buffer, pH 6.8 and 260  $\mu$ L of AFB<sub>1</sub> (from a 1 mg/mL stock).

Reaction samples were combined to a final volume of 900  $\mu$ L consisting of AFB<sub>1</sub> stock and laccase mutants, added in the concentration of ~6  $\mu$ g, calculated based on concentration from A280 to normalize concentration between samples. Samples were extracted at time points 0- and 24-hours using ethyl acetate (as described in Section 3.5.3). Samples were incubated at 28°C. Two replicates were used per laccase mutant. Control of toxin alone in acetate buffer was used.

Samples were run on HPLC-MS/MS using the MRM method, modified from Safari et al (258).

#### A.4.5 Fluorescence degradation assays

For Figure A.3B, the following method was used: A stock of AFB<sub>1</sub> was made at 0.3 µg/mL using 12.6 mL 0.1 M acetate buffer, pH 6.8 and 3.78 µL of AFB<sub>1</sub> (from a 1 mg/mL stock).

Reaction samples were combined to a final volume of 900  $\mu$ L consisting of AFB<sub>1</sub> stock and laccase mutants, added in the concentration of ~6  $\mu$ g, calculated based on concentration from A280 to normalize concentration between samples. Each reaction condition started 5 minutes apart to account for reading time. Reads were taken every 2 hours for the first 6 hours and at 24 and 48 hours. Excitation was set to 380 nm and emission was set to 400600 nm, with counts per second (CPS) at 440 nm extracted for plotting. Between reads, samples were incubated at 28°C. Two replicates were used per laccase mutant. As controls, toxin alone samples in an acetate buffer were used.

For Figure A.4, the method is found in Section 3.5.5, with the following modifications: Laccase used was normalized between WT and mutants based on total protein concentration measured via Qubit, buffer used for suspension was acetate buffer at pH 6.5, and reading was over 72 hours.

## A.4.6 Statistical analysis

To compare the degradation efficiency between laccase mutants (Figure A.4), we use Student t-test using the Matlab function ttest.

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