Metabolism of Benzo(a)pyrene by *Aspergillus* sp. and the Implications for Human Health

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ABSTRACT

Aspergillus is a genus of ubiquitous fungus that demonstrates enormous resilience and metabolic capabilities in a wide variety of environments. It can be found living in sites contaminated with compounds which are toxic to animals, yet little is known about how this organism is able to survive and reproduce under these conditions. Use of the model filamentous fungus *Aspergillus nidulans* allowed investigation into mechanisms used by the fungus to metabolize the toxic compound benzo(a)pyrene. Benzo(a)pyrene is a contaminant of concern due to its stability in the environment and toxic effects in mammals. *A. nidulans* was able to grow on benzo(a)pyrene and showed higher cell viability than cells grown on control media. Analysis of the transcriptomic response to benzo(a)pyrene showed evidence of cellular growth and energy generation, indicating the fungus can utilize benzo(a)pyrene as a substrate for growth. Using sequence similarity, a cytochrome p450 monooxygenase (CYP) BapA (CYP617D1) was identified and determined to be necessary for benzo(a)pyrene degradation under glucose limiting conditions in *A. nidulans*. This CYP subfamily is conserved in *Aspergillus sp*., and deletion of *bapA* in *A. flavus* demonstrated the same phenotypic inability to degrade benzo(a)pyrene. Further it was shown that *bapA* is controlled by the Nf-κB like transcription factors VeA and VelB in response to carbon limitation. This demonstrates both evolutionary parallels and striking differences in the response of animals and fungi to complex carbon containing molecules like benzo(a)pyrene. The impressive number of CYPs predicted in the *A. flavus* genome (153) led to the hypothesis that additional benzo(a)pyrene degrading CYPs are encoded. Gene deletion of *AFLA_135430* which is contained in a predicted secondary metabolite gene cluster led to reduction of benzo(a)pyrene degradation. Additionally, this deletion led to reduced sporulation, increased sclerotia formation, and imbalance of secondary metabolism demonstrating this CYP may play a dual role in degradation of exogenous compounds like benzo(a)pyrene, and biosynthesis of an endogenous secondary metabolite. Finally, fungal spores are considered indoor pollutants because of their toxicity and pathogenicity within the human respiratory tract. Although *A. fumigatus* is capable of degrading benzo(a)pyrene, uptake of the compound into fungal hyphae leads to transport into developing spores. We were unable to demonstrate benzo(a)pyrene containing spores evade the innate immune response using a zebrafish model. However, because of the known toxicity of benzo(a)pyrene it is likely these spores exhibit some form of negative health impact on humans. This novel discovery may impact how we assess exposure risks to soil contaminants, especially in and around contaminated sites.

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Chapter 1

Introduction:

Bioremediation and microbial metabolism of benzo(a)pyrene

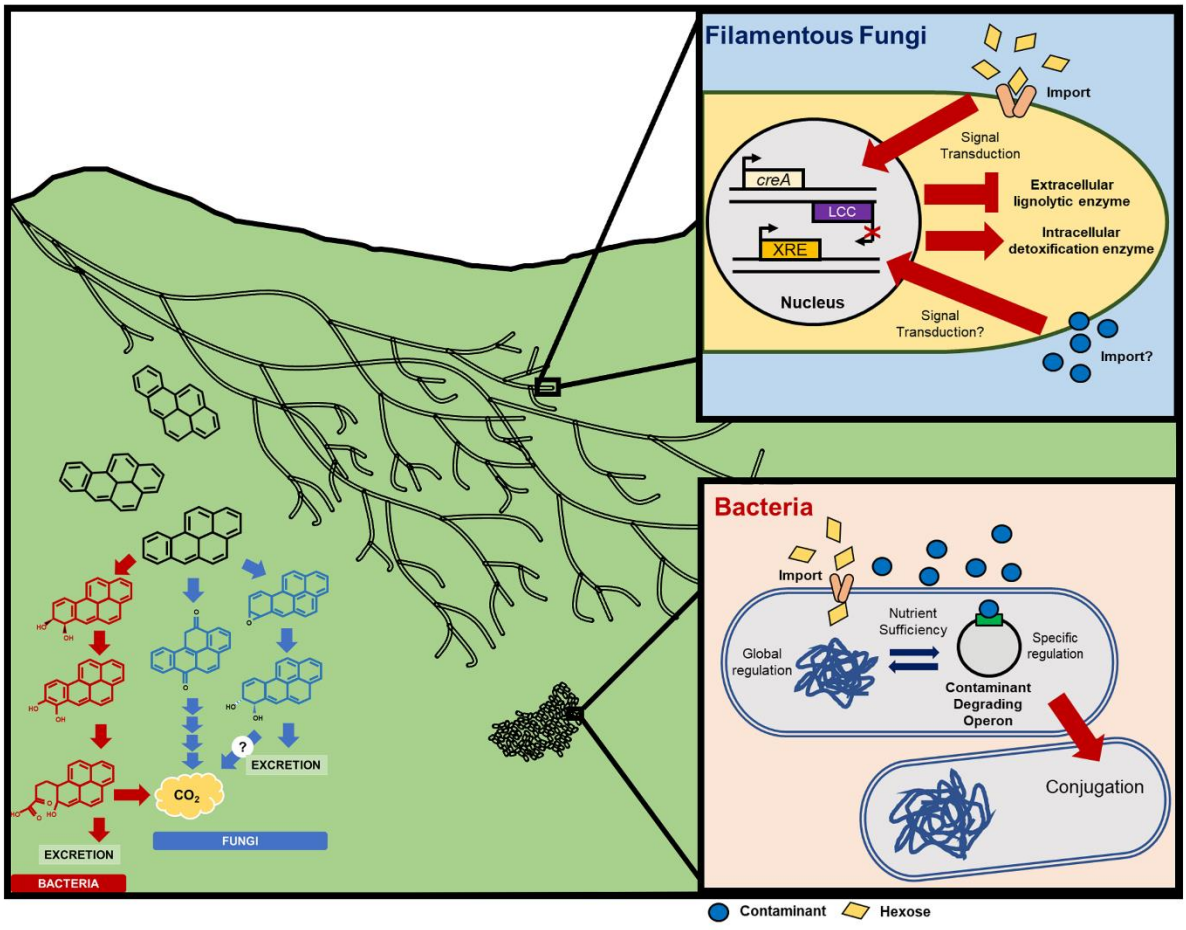
This chapter has been adapted from the following publication:

Ostrem Loss, E. M. and Yu, J. (2018), Bioremediation and microbial metabolism of benzo(a)pyrene. *Molecular Microbiology*. doi:10.1111/mmi.14062

EOL and JHY both contributed to the writing and editing of this chapter.

1.1 Summary

The growing release of organic contaminants into the environment due to industrial processes has inevitably increased the incidence of their exposure to humans which often results in negative health effects. Microorganisms are also increasingly exposed to the pollutants, yet their diverse metabolic capabilities enable them to survive toxic exposure making these degradation mechanisms important to understand. Fungi are the most abundant microorganisms in the environment, yet less has been studied to understand their ability to degrade contaminants than in bacteria. This includes specific enzyme production and the genetic regulation which guides metabolic networks. This review intends to compare what is known about bacterial and fungal degradation of toxic compounds using benzo(a)pyrene as a relevant example. Most research is done in the context of using fungi for bioremediation, however, we intend to also point out how fungal metabolism may impact human health in other ways including through their participation in microbial communities in the human gut and skin and through inhalation of fungal spores.



Graphical abstract: A visual summary of the topics discussed in this review

1.2 Introduction

Understanding the mechanisms used by fungi to degrade organic compounds has applications in many environmental and human health related fields, with bioremediation being the most obvious. The increased release of contaminants into the environment make it inevitable that fungi encounter these compounds more frequently, yet our knowledge of how they interact is largely unknown. In contrast, most research has focused on bacterial degradation of contaminants.

The abundance and diversity of fungi across the planet is astounding, estimated as being the second most abundant kingdom behind arthropods (Ødegaard, 2000; Blackwell, 2011). Fungi make up to 75% of soil biomass (Ritz and Young, 2004), and yet only around 100,000 of an estimated 1.5 million fungal species have been described (Stajich *et al.*, 2017). In addition to understanding how degradation mechanisms in fungi function in the environment, this information might then be applied to understanding how eukaryotic microbes in the human gut and skin metabolize compounds, and the potential impact on human exposure risks.

Bioremediation has been a technique of interest for many years because of its potential low cost, high efficiency removal of toxic contaminants from soil and water. It represents an environmentally friendly solution to the problem of industrial production posing a threat to human health. Bioremediation relies on the metabolism of hazardous compounds by plants and/or microorganisms, leaving by-products in the environment which are no longer toxic. During the process of contaminant removal, microorganisms and plants recolonize soils bringing nutrients and structure back to the soil, which

creates potential for further agricultural and industrial use. Metabolic removal may be driven by the organism's energy needs, detoxification mechanisms, or the fortuitous nature of non-specific enzymes.

Although bioremediation shows a lot of promise, significant advances in sophisticated implementation have not been accomplished. There is some doubt as to whether microorganisms can meet expectations regarding contaminant removal. Not all bacteria and fungi can survive these challenging environments, especially in competition with native colonies that have been selected by fitness, not necessarily by efficient removal of contaminants. Saprophytic fungi are most promising because of their metabolic diversity, and abundance in soils (Harms, Schlosser and Wick, 2011).

Fungi have a unique set of ecological features which make them promising candidates for use in bioremediation strategies. In this review we intend to give an overview of the potential of using fungi for bioremediation, and by specifically focusing on remediation of benzo(a)pyrene we highlight the current research gaps in biochemical and genetic control of this contaminant's removal from the environment. We also address how this knowledge may be applied to other fields, such as the growing awareness of eukaryotic organisms which reside on and in us.

1.2 Bioremediation techniques

The United States Environmental Protection Agency (U.S. EPA) has implemented bioremediation techniques for various contaminated sites for over 20 years, with mostly positive results (United States Environmental Protection Agency, 2001). Technologies

have changed over the years to increase efficiency and reduce costs. Strategies differ depending on the extent and characteristics of contaminating compounds. Techniques range in extent of intervention from simply waiting for native colonies to degrade contaminants, to adding water, nutrients and even non-native organisms to encourage robust growth and contaminant degradation. Techniques also range in cost often positively correlating with level of intervention (**Figure 1.1**).

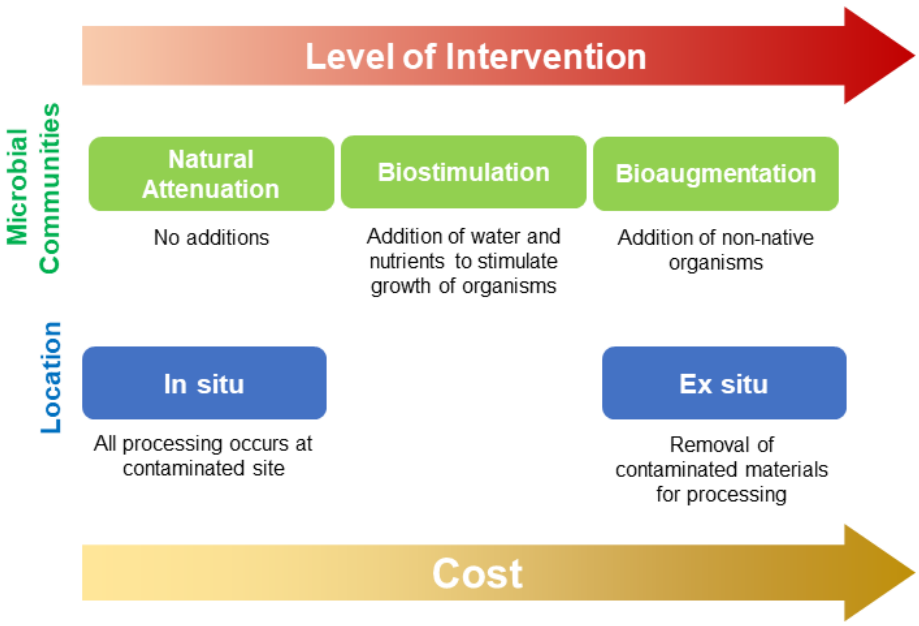


Figure 1.1: A schematic of various bioremediation techniques on a scale representing cost and level of intervention required to execute each.

The site of removal has changed over time from predominantly *ex situ* to *in situ* remediation methods (Kensa, 2011). *Ex situ* bio-stimulation remediation involves removal and containment of contaminated material which is turned and/or composted to allow aeration and addition of amendments while, when necessary, capturing toxic, volatile intermediates. This method costs more in labor and equipment with projects costing an average of $300/cubic yard. *In situ* methods are designed to pump oxygen

and nutrients directly into the contaminated site, and generally cost less, with projects costing less than $40/cubic yard (**Figure 1.1**; United States Environmental Protection Agency, 2001).

Bioaugmentation strategies are becoming more appealing because they allow for control of microbial communities, which are more metabolically active towards a given contaminant. Bioaugmentation is the addition of specific organisms to a site which allows for catering to the given characteristics of the contaminant and the site (**Figure 1.1**). This method shows promise, especially since advances in “omics” research approaches have become more available and allow us to comprehensively understand the mechanisms of degradation abilities (El Amrani *et al.*, 2015). Research methods like transcriptomics, genomics, proteomics, and metabolomics not only reveal if a contaminant can be degraded by a given organism, but they also reveal complex mechanisms and consequent products of metabolism. These insights can yield important proteins and molecular mechanisms, which can be optimized to further improve efficiency and reduce cost. Additionally, the complex interactions of microbes in contaminated sites can be more fully understood with ‘omics’ approaches yielding cooperative communities of organisms which may more efficiently degrade contaminants.

1.3 Unique characteristics of fungi

Despite not yet fully understanding the complexities of microbial metabolism, many microorganisms are well suited to metabolize complex organic compounds in the

environment. Organic waste, including natural sources such as dead plant material, are mainly degraded biotically by saprophytic bacteria and fungi, which play a critical role in carbon cycling (Mäkelä, Donofrio and de Vries, 2014). Although saprophytic fungi and bacteria play similar roles in the environment, the methods they employ are very different.

Bacteria are predominantly single celled organisms, which reproduce by cell division, rapidly expanding into their environment in order to utilize available nutrients. Filamentous fungi on the other hand, grow as interconnected networks of cells called hyphae, which structurally resemble the growth of plant roots (**Figure 1.2).** Although their growth tends to be slower than bacteria, their connected nature allows them to grow across areas of low water activity and nutrient depletion (Harms, Schlosser and Wick, 2011). Individual cells measure only 2 ~10 μm, but as a whole network can extend over hundreds of acres. For instance, the largest known living organisms is a network of *Armillaria solidipes* which extends 3.8 km in the Blue Mountains in Oregon (Ferguson *et al.*, 2003). The pressure inside of a hyphal cell growing 20 μm/min is up to 600 kPa , approximately the same pressure inside a bike tire, is maintained by a biochemically unique cell wall made of chitin and glucans (Lew, 2011). Rigidity in bacterial cell walls are provided by peptidoglycans, and can maintain slightly less turgor pressure estimated to be between 100-300 kPa (Cayley, Guttman and Record, 2000; Deng, Sun and Shaevitz, 2011). The amount of pressure within fungal cell walls allow for penetration into rock surfaces, granting access to compounds sorbed to organic surfaces and assists in shaping soil structure (Bornyasz, Graham and Allen, 2005). Fungi and bacteria both produce biosurfactants which can assist in accessing

compounds sorbed to organic surfaces, but fungi tend to produce more perhaps because high concentrations of biosurfactants can disrupt the less robust cell wall of bacteria (Cooper and Paddock, 1984; Desai, 1987).

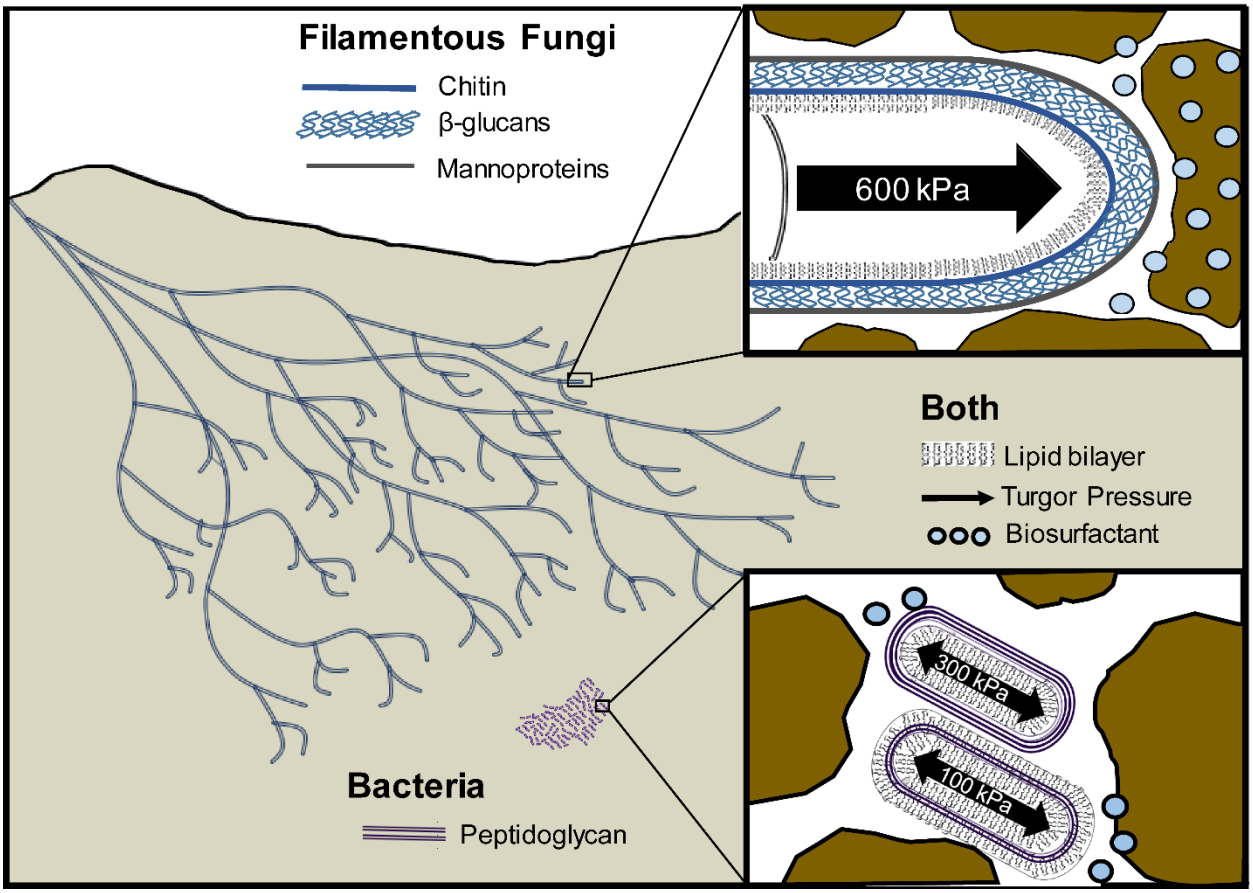


Figure 1.2: An illustration of the morphological and biophysical differences between filamentous fungi and bacteria growing in soil.

Both saprophytic fungi and bacteria contain a plethora of metabolically diverse enzymes, which are able to transform complex organic molecules into smaller, and often times less toxic compounds. Understanding the genetic control of these metabolizing enzymes is an important factor in effectively using microbes for bioremediation in order to produce of enzymes on a large scale, or to manipulate strains

to more efficiently degrade contaminants. Advances in molecular techniques have increased our knowledge of bacterial and fungal genetic regulation and have demonstrated differences between these organisms.

Bacterial genomes tend to be small, between 100 and 15,000 kbp (Ochman and Caro-Quintero, 2016), so fitness instead relies on the transfer of plasmid DNA in order to quickly adapt to environmental stresses and diverse carbon sources (Norman, Hansen and Sørensen, 2009). Plasmid DNA can be transferred between bacteria of different species, and in extreme cases different kingdoms, through conjugation (**Figure 1.3**). This allows fast transfer of DNA encoding contaminant degrading enzymes in a contaminated site. Regulation of these catabolic pathways occur specifically or globally. Specific regulation occurs when a compound or group of structurally similar compounds directly interact with regulatory proteins encoded in the plasmid itself in order to induce transcription (Carbajosa and Cases, 2010). However, this is not always a simple event, as regulatory proteins may act synergistically or antagonistically with other pathway containing plasmids. Interaction with the host physiological response constitutes a global regulatory mechanism. This global transcriptional regulation incorporates the host's nutrient status and stress response proteins into regulatory binding sites that promote or repress the expression of contaminant degrading operons (Cases and de Lorenzo, 2001, 2005). The competitive contaminated site encourages newly evolved metabolic pathways to respond to the hosts nutrient and stress status in order to allocate energy efficiently, from constitutive to conditional expression (Cases and de Lorenzo, 2001). For example, the regulatory protein ClcR which activates the degradation of 3-Cl catechol, which can be used as a carbon source, is directly inhibited

by the TCA cycle intermediate fumarate, in order to limit expression of degradative machinery to environments where there isn't a more favorable source of energy (McFall *et al.*, 1997). This is probably quite common of organic contaminant degrading operons, as only a few mutations of the regulatory protein are required in order to respond to TCA cycle intermediates (Cases and de Lorenzo, 2001).

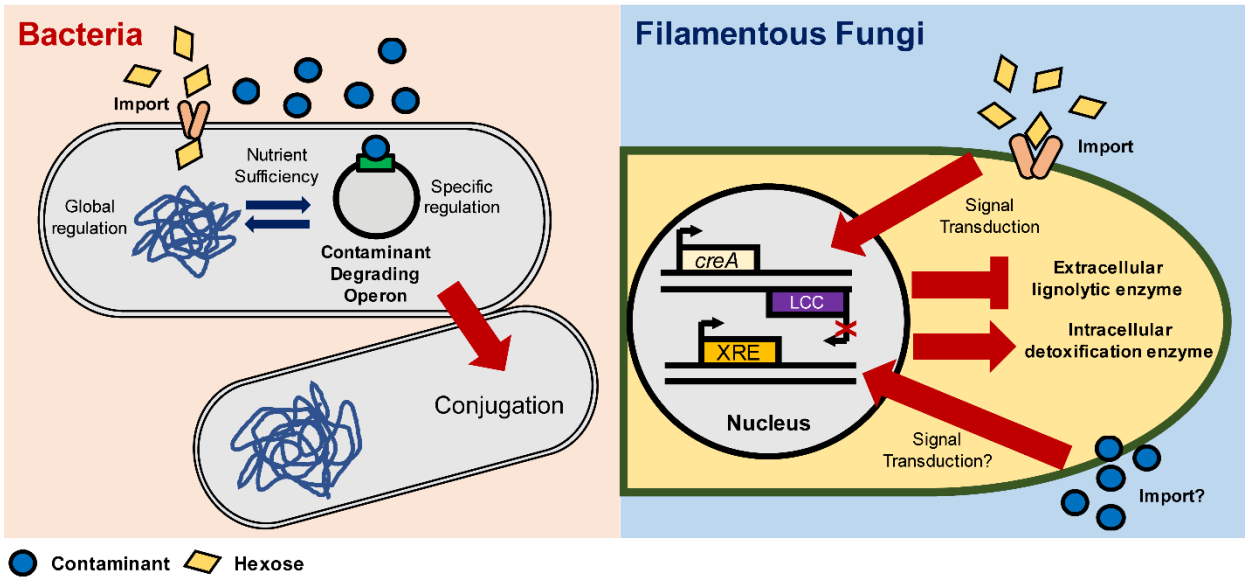


Figure 1.3: A diagram comparing the genetic regulation of contaminant degrading enzymes in bacteria and filamentous fungi. Filamentous fungal degradation is represented by mechanisms studied in ligninolytic fungi where CreA represents carbon catabolite repression, LCC is extracellular enzyme production and XRE is xenobiotic response element.

Fungal genomes tend to be larger than bacterial genomes, 8,000-180,000 kbp (Mohanta and Bae, 2015) in length and contain more transcription factors and controllable steps which are critical to survival in a diverse set of environments. Survival in harsh conditions relies on the tight orchestration of expressing a diverse set of genes (**Figure 1.3**). These survival mechanisms include resilience to environmental stressors, efficient reproduction, and stringently regulated carbon utilization. Hierarchical levels of

transcription regulatory elements, from pathway specific $Zn(II)_2Cys_6$ transcription factors like AraR for arabinose utilization (Battaglia, Zhou and de Vries, 2014) to Cys_2His_2 zinc finger global transcription factors such as CreA for carbon signaling (Dowzer and Kelly, 1989, 1991) are both needed to balance appropriate expression of degrading enzymes.

Extracellular peroxidase and laccase enzymes degrade a broad set of contaminants, including those larger hydrocarbons that are much more recalcitrant and resistant to bacterial degradation since they do not require uptake by the organism. The genetic regulation of these enzymes has been extensively studied because of the potential to produce them on an industrial scale for use in contaminated sites. Manganese peroxidase and lignin peroxidase, produced by white rot fungi, are responsible for catalyzing lignin degradation, something that is unique to these types of fungi. Lignin, like some organic contaminants, is composed of cyclic hydrocarbons that are resistant to abiotic and microbial oxidation. Peroxidase enzymes have a very high redox potential (1.0-1.5V) and catalyze the oxidation reaction of the contaminant with H_2O_2 (Hofrichter *et al.*, 2010). These enzymes are non-specific and can hence degrade many different compounds (Hammel, 1995; Hofrichter *et al.*, 2010). The limiting step is the reliance of the reaction on H_2O_2, which is inhibited by concentrations too low, and also by concentrations too high (Asgher *et al.*, 2008; Wong, 2009). These enzymes are produced by basidiomycetes exclusively which are not as amenable to genetic manipulation, hence production of these enzymes in a cost efficient heterologous or homologous system is not simple. Laccases, on the other hand, have a lower redox potential (0.4-0.8V) but are produced by both basidiomycetes, and ascomycetes (Baldrian, 2006; Couto and Toca-Herrera, 2007; Singh Arora and Dumar Sharma, 2010)

. The physiological purpose is to degrade lignin as well as to contribute to other processes such as morphogenesis, pigmentation, and pathogenesis (Singh Arora and Dumar Sharma, 2010; Ruhl, 2011; Forootanfar and Faramarzi, 2015). Expression of laccase genes is differentially regulated in response to medium composition, such as metal ions, xenobiotics and nutrient concentrations. Various *cis*-acting responsive elements have been identified in laccase promoter regions. These include metal response element (MREs), xenobiotic response elements (XREs), ACE1 copper-responsive transcription factor binding sites (ACE1), antioxidant response elements (AREs), heat shock response elements (HSEs), CreA binding sites (CreA), and NIT2 binding sites (NIT2; **Figure 1.3**; Janusz, Kucharzyk, Pawlik, Staszczak, & Paszczynski, 2013; Piscitelli, Giardina, Lettera, Pezzella, & Faraco, 2011).

Intracellular mechanisms are also used by fungi in order to degrade exogenous compounds. Cytochrome p450 mono-oxygenases (CYPs) are generally more abundant in eukaryotic fungi than in prokaryotes and animals, although in all kingdoms are more abundant in organisms which are pathogenic or live in soil (**Table 1.1**). The function of many in fungi are not yet known (Kelly and Kelly, 2013), although some have been shown to oxidize polycyclic aromatic hydrocarbons (PAHs) and dioxins (Cerniglia & Sutherland, 2010b; Kasai et al., 2010). CYPs are known to demonstrate substrate promiscuity (Nath and Atkins, 2008), so these enzymes may help both ligninolytic and non-ligninolytic fungi metabolize a wide range of xenobiotics. Some fungi are capable of producing both extracellular and intracellular enzymes for degradation, so which mechanism the fungi utilizes is dependent on environmental signals. The model white rot fungus *Phanerochaete chrysoporium* seems to preferentially express peroxidase

enzymes in nutrient limited media, and CYPs in nutrient sufficient media (Syed *et al.*, 2010). The regulatory mechanisms which guide this process will be important for understanding how fungi will behave in a given contaminated site, but this has not yet been investigated.

Table 1.1: **Abundance of encoded cytochrome p450 monooxygenases in various organisms**

	Organism	Lifestyle	Number of encoded CYPs[a]	Total encoded proteins[b]	%
Prokaryotes (most have no encoded CYPs (Kelly, Kelly, Jackson, Warrilow, & Lamb, 2005))	*Escherichia coli*	Human GI tract, Facultative anaerobic bacterium	0	5008	0
	Methanosarcinia barkeri	Cow rumen, methane producing anaerobe	1 (Kelly et al., 2005)	3535	0.028
	Sulfolobus tokodaii	Thermophilic and acidophilic archaeon	1 (Kelly et al., 2005)	2770	0.036
	Sinorhizobium meliloti	Nitrogen fixing bacterium	3 (Kelly et al., 2005)	3663	0.082
	Bacillus subtilis	Aerobic bacterium in soil and GI tract	8 (Kelly et al., 2005)	4042	0.198
	Mycobacterium tuberculosis	Human pathogen	20 (Kelly et al., 2005)	4094	0.449
	Streptomyces avermitilis	Soil bacterium	33 (Kelly et al., 2005)	7445	0.443

Fungal Eukaryotes (Most have at least one encoded CYP ((Kelly *et al.*, 2005))	*Saccharomyces cerevisiae*	Baker's yeast	3	5404	0.0555
	Schizosaccharomyces pombe	Fission yeast	2	5132	0.039
	Candida albicans	Opportunistic pathogenic yeast	11	6271	0.175
	Neurospora crassa	Bread mold (saprophytic fungus)	41	11390	0.360
	Penicillium chrysogenum	Soil dwelling filamentous fungus	98 (Chen et al., 2014)	11198	0.875
	Magnaporthe grisea	Rice blast fungus (plant pathogen)	135 (Chen et al., 2014)	12860	1.050
	Aspergillus flavus	Plant and human pathogen	153 (Chen et al., 2014)	13340	1.147
	Phanerochaete chrysosporium	Wood rotting fungus	149	11777 (Martinez *et al.*, 2004)	1.270
Animals (Most have at least one encoded CYP (Kelly et al., 2005))	*Homo sapiens*	Terrestrial vertebrate	57	63744	0.089
	Mus musculus	Terrestrial vertebrate	102	61940	0.165
	Takifugu rubripes	Aquatic vertebrate	54	31052	0.174
	Drosophila melanogaster	Terrestrial invertebrate	84	30482	0.276
	Caenorhabditis elegans	Soil dwelling invertebrate	74	28310	0.261

[a] Cytochrome p450 number was obtained from the cytochrome p450 homepage (Nelson, 2009) unless otherwise referenced

[b] Genome size was obtained from NCBI unless otherwise referenced

Because the literature is too extensive to fully describe and compare mechanisms used by fungi and bacteria to degrade all environmental contaminants, one particular compound, benzo(a)pyrene (BaP) will be discussed as a case study. BaP represents an abundant contaminant whose prevalence and toxicity make it a contaminant of concern.

1.4 A case study: Benzo(a)pyrene

The amount of literature on BaP degrading microorganisms is extensive, with numerous reviews summarizing the organisms and degradation products of microorganisms, as well as the major enzymatic pathways utilized by bacteria. This section is intended to highlight the major trends in the literature, as well as mention some gaps in our knowledge that may be important in understanding environmental and human health.

BaP is a five ring PAH, which is considered a high molecular weight (HMW) PAH. BaP is formed by the incomplete combustion of organic compounds. Although there are natural sources of BaP like forest fires and volcanoes, increasing industrial use and combustion of petroleum has led to more BaP formation. The International Agency for Research on Cancer (IARC) lists BaP as a known cancer causing compound in humans, causing the EPA to name BaP in the top ten contaminants of concern at Superfund sites (U.S. Centers for Disease Control Agency for Toxic Substances and Disease Registry (ATSDR), 1995; U.S. Centers for Disease Control Agency for Toxic Substances and Disease Registry (ATSDR) and Division of Toxicology and Human Health Sciences, 2013). Humans are also exposed to BaP through their diet and cosmetic use, especially foods which have been cooked over an open flame or smoked

(Saito *et al.*, 1978), and beauty products which contain mineral oil (Grob *et al.*, 1991). These sources are likely to interact with bacterial and fungal communities found on the skin and in the gut, yet the consequences are not known.

BaP is relatively resistant to degradation due to its low bioavailability and the high oxidation state of carbon. BaP has a large octanol water partition coefficient, i.e., it is preferably bound to organic matter in soils rather than in aqueous solution (Sims and Overcash, 1983). This lowers the bioavailability of the compound, although as mentioned biosurfactant producing microbes gain access more readily. The carbon in BaP also has a high oxidation state making it less preferable to be used as a source of energy by microorganisms due to the large amount of energy needed to break carbon-carbon bonds (Rentz, Alvarez and Schnoor, 2008). Most microorganisms that degrade BaP hence tend to co-metabolize the compound with the addition of another carbon source needed for production and induction of necessary enzymes.

1.4.1 - Bacteria

Reports have been published on the ability of bacterial strains to degrade BaP since the 1970s (Barnsley, 1975). To our knowledge, degradation of BaP has not been studied in any model bacterial species, but instead focus on strains which have been isolated from sites contaminated with PAHs, indicating there is strong selective pressure in the environment for PAH degradation. The research question that remains is how ‘willing’ are bacteria to degrade large PAHs like BaP, as these compounds are more recalcitrant

and require more oxidizing energy to transform. Although bacteria are capable of utilizing low molecular weight PAHs as a carbon and energy source, HMW PAHs are generally more resistant to microbial degradation (Kanaly and Harayama, 2000). It was previously recognized that bacteria cannot utilize BaP as a substrate for growth, although two reports now show evidence that bacterial strains isolated from a contaminated site and human skin can (Kaushish Lily *et al.*, 2009; Sowada *et al.*, 2014). It is more well known that bacteria can co-metabolize BaP with the addition of salicylate, succinate, other low molecular weight (LMW) PAHs, or other complex nutritional supplements to the medium. These additions are necessary for carbon source and growth; also to replenish electrons in NADH coenzymes and induce PAH degrading enzymes (Rentz, Alvarez and Schnoor, 2008). Although additional carbon sources are needed, some of these bacterial species can mineralize BaP producing non-toxic CO_2 as a byproduct. **Table 2.1** is a summary of some of the more well studied BaP bacterial degraders, including inducing conditions, and metabolites formed. Although minor deviations exist, the general pathway for bacterial degradation consists of the rate limiting initial oxidation by dioxygenase to cis-dihydrodiol (Cerniglia, 1992), the formation of catechols by *cis*-dihydrodiol dehydrogenase, which becomes a substrate for other dioxygenases that form ring cleavage products (**Figure 1.4**; Atlas 1981; Gibson and Subramanian 1984). These products could then be either excreted or shuttled into pathways that metabolize smaller PAHs which are eventually converted into succinic, fumaric, pyruvic and acetic acids and aldehydes, all of which are utilized by the micro-organism for the synthesis of cellular constituents and energy (Wilson and Jones, 1993).

Table 1.2. Bacterial metabolism of BaP.

Bacterial species	Inducer/growth substrate	Products of metabolism	Notes	References
***Sphingomonas yanoikuyae* JAR02**	Induced with salycilate and succinate	CO_2 Ring cleavage: Pyrene-8-hydroxy-7-carboxylic acid Pyrene-7-hydroxy-8-carboxylic acid		(Rentz, Alvarez and Schnoor, 2008)
***Mycobacterium vanbaalenii* PYR-1**	Induced with phenanthrene YE, peptone, soluble starch Sediment water microcosm	CO_2 Dihydrodiols: *trans*-Benzo(*a*)pyrene-11,12-dihydrodiol *cis*-Benzo(*a*)pyrene-11,12-dihydrodiol *cis*-Benzo(*a*)pyrene-4,5-dihydrodiol Ring cleavage: 10-Oxabenzo(*def*)chrysene-9-one		(Heitkamp & Cerniglia, 1989; Moody, Freeman, Fu, & Cerniglia, 2004)
***Mycobacterium* sp. strain RJGII-135**	Induced with pyrene	Diol: Benzo(*a*)pyrene-7,8-dihydrodiol Ring cleavage:		(Schneider *et al.*, 1996)

		cis-4-(8-Hydroxypyrene-7-yl)-2-oxobut-3-enoic acid or *cis*-4-(7-hydroxypyrene-8-yl)-2-oxobut-3-enoic acid 4,5-Chrysene-dicarboxylic acid 7,8-Dihydro-pyrene-7-carboxylic acid or 7,8-dihydro-pyrene-8-carboxylic acid		
***Sphingomonas yanoikuyae* B8/36**	Induced with biphenyl, *m*-xylene, or salicylate	Dihydrodiols No ring cleavage		(Gibson, 1999) (R Mahaffey, Gibson, & Cerniglia, 1988)
***Bacillus subtilis* BMT4i (MTCC 9447)**	No inducing/sole carbon source	Not determined	Determined by CFU on BaP media	(Kaushish Lily *et al.*, 2009)
***Pseudomonas saccharophila* P15**	Induced with salycilate	CO_2		(Chen & Aitken, 1999)
***Sphingomonas paucimobilis* EPA505**	Grown on phenanthrene	CO_2	Demonstrated reduced carcinogenicity	(Ye, Akmal Siddiqi, Maccubbin, Kumar, & Sikka, 1995)
***Stenotrophomonas maltophilia* VUN 10,010**	Grown on pyrene	CO_2		(Boonchan, Britz and Stanley, 2000)

Regulation of the BaP pathway is not completely known, and there is some question as to whether or not genes responsible for HMW PAH degradation are found on large mega-plasmids or chromosomally (Obayori and Salam, 2011). One study showed BaP degrading genes from *Bacillus subtilis* BMT4i are contained chromosomally, although it is not known how common this is in HMW PAH degrading bacteria (Kaushish Lily, Bahuguna and Dangwal, 2010). Alternatively, PAH-catabolic genes from *Pseudomonas* species, called the nah-like genes have been extensively studied. These genes are responsible for utilizing low molecular weight (LMW) PAHs (4 rings or less) as sources of carbon and include catabolic genes which incorporate metabolites into primary metabolism (Habe and Omori, 2003). BaP degrading dioxygenases are described in *Mycobacterium vanbaalen*ii PYR-1 leading to initial ring cleavage products (Moody *et al.*, 2004). If bacterial species are capable of utilizing BaP as a source of carbon, these ring cleavage metabolites may be shuttled into pathways responsible for LMW PAH degradation. It is also likely that some bacteria transform BaP into metabolites which can then be excreted.

Another interesting finding which may start a new area of study was bacteria isolated from human skin, predominately *M. luteus,* is capable of utilizing BaP as a carbon source, with no indication if the intermediates may cause oxidative damage, or carcinogenic effects to the host (Sowada *et al.*, 2014). It is unknown if products of BaP degradation with *cis* conformations are less toxic than those metabolized by eukaryotes, so questions about human exposure to toxic metabolites from bacteria residing on the skin remain.

1.4.2 - Fungi

Much less is known about fungal degradation of benzo(a)pyrene, but it is clear fungi utilize enzymes which are different from bacteria in order to degrade HMW PAHs like BaP. Many papers published on BaP degrading fungal species are isolated from contaminated sites indicating that selective pressure also acts on fungal degradation mechanisms. It is also clear, however that the model white rot fungus *P. chrysosporium* has the innate ability to degrade BaP perhaps given its ecological niche as a lignin degrader (Syed and Yadav, 2012). The distinguishing features of BaP degradation by fungi are whether degradation occurs extracellularly by peroxidase enzymes, or whether it is degraded intracellularly by monooxygenases. The former pathway leads to BaP mineralization (Bumpus *et al.*, 1985) while the latter pathway leads to water soluble metabolites which are more susceptible to abiotic degradation and biotic degradation by other microorganisms like bacteria (Cerniglia, Mahaffey, & Gibson, 1980). Since fungi are required to genetically respond to environmental signals in order to orchestrate nutrient acquisition, fungal species like *P. chrysosporium* are able to metabolize BaP by multiple mechanisms depending on the culturing conditions (Syed *et al.*, 2010). **Table 3.1** summarizes some BaP metabolizing fungal species, as well as the culturing methods used, and metabolites formed. While this is not a comprehensive list, it provides give examples of fungal BaP metabolism.

Table 1.3. Fungal metabolism of BaP.

Fungal species	Culture conditions	Enzyme	Metabolites	Notes	References
Phanerochaete chrysoporium	Nitrogen limited media veratryl alcohol	Lignin peroxidase	Benzo(a)pyrene-1,6-dione Benzo(a)pyrene-3,6-dione Benzo(a)pyrene-6,12-dione CO_2		(Barclay, Farquhar and Legge, 1995;Bumpus et al., 1985;Hammel, 1995)
Phanerochaete chrysoporium	Nitrogen sufficient media	P450	3-hydroxy-benzo(a)pyrene		(Syed *et al.*, 2010)
Trametes versicolor	2,2(prm1)-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) or 1-hydroxybenzotriazole (HBT)	Laccase	Benzo(a)pyrene-1,6-dione Benzo(a)pyrene-3,6-dione Benzo(a)pyrene-6,12-dione		(Collins *et al.*, 1996; Majcherczyk, Johannes and Hüttermann, 1998)
Pleurotus ostreatus	Wheat straw	N/D	CO_2	Isolated from contaminated site	(Wolter *et al.*, 1997)
Saccharomyces cerevisiae	semi-anaerobically grown cells	Purified CYP61	3-hydroxy-benzo(a)pyrene	Sterol 22-desaturase, cytochrome P45061	(Steven, Kelly, Lamb, & Kelly, 1997)
Fusarium solani	Sole carbon source		Benzo(a)pyrene-1,6-dione Benzo(a)pyrene-3,6-dione CO_2	Stores BaP in vesicles	(Rafin *et al.*, 2000; Veignie *et al.*, 2002)
Cunninghamella elegans		Saboraud	epoxides	Presumed CYP activity	(Cerniglia et al., 1980; Cerniglia,

		dextrose broth	water soluble sulfate and glucuronide conjugates		1992; Cerniglia and Gibson, 1979)
Aspergillus ochraceus	Induction by BaP		Benzo(a)pyrene-9,10-diol Benzo(a)pyrene-4,5-diol Benzo(a)pyrene-7,8-diol Benzo(a)pyrene-1,6 -dione Benzo(a)pyrene-3,6 -dione 3-hydroxy-benzo(a)pyrene 9-hydroxy-benzo(a)pyrene		(Datta and Samanta, 1988)

CYPs in fungi are of particular interest because they play a role in many important areas including synthesis of secondary metabolites and degradation of antimicrobials. The genomes of soil dwelling fungi tend to encode many more CYP genes than higher eukaryotes (**Table 1.1**), the function of many are not yet know (Yadav, Doddapaneni and Subramanian, 2006). Interestingly, only two studies have shown the direct role of a specific CYP, by induction with BaP and *in vitro* metabolism (Kelly et al., 1997; Syed et al., 2010). The utilization of CYPs to degrade BaP produce *trans*-dihydrodiol metabolites similar to those produced by higher eukaryotes including humans. These metabolites are more toxic than the parent compound so there is concern that fungal metabolism may increase risks to human health. These metabolites are conjugated to compounds such as sulfuric and glucuronic acids, which suggests detoxification

mechanisms similar to humans indicating the conserved function as part of a detoxification system (Cerniglia et al., 1980). In light of bacterial degradation of BaP on skin, it is possible fungi residing on skin or in the gut may also be metabolizing BaP, and hence expose humans to more cancer-causing compounds than previously thought.

Another area of research which points to potential negative health consequence is the role of BaP uptake and sporulation by fungi. BaP is known to alter the immune response in mammalian systems (Silkworth, Lipinskas and Stoner, 1995), and effective clearance of fungal spores relies on an correctly balanced adaptive immune response (Rivera, Hohl, & Pamer, 2006). Filamentous fungi produce billions of spores that are inhaled by humans and are known to cause invasive growth and sensitization to allergic responses (Osborne *et al.*, 2006; Badiee and Hashemizadeh, 2014). Uptake of BaP into hyphae was shown in *Fusarium solani* (Fayeulle *et al.*, 2014), yet the fate in vegetative cells and possible transport into reproductive bodies has not been explored. If the relatively metabolically inactive spores contain BaP from the environment, humans who live near contaminated areas may be introduced to spores which may be more damaging. These questions have not yet been addressed, and insights could lead to necessary changes in contaminant management.

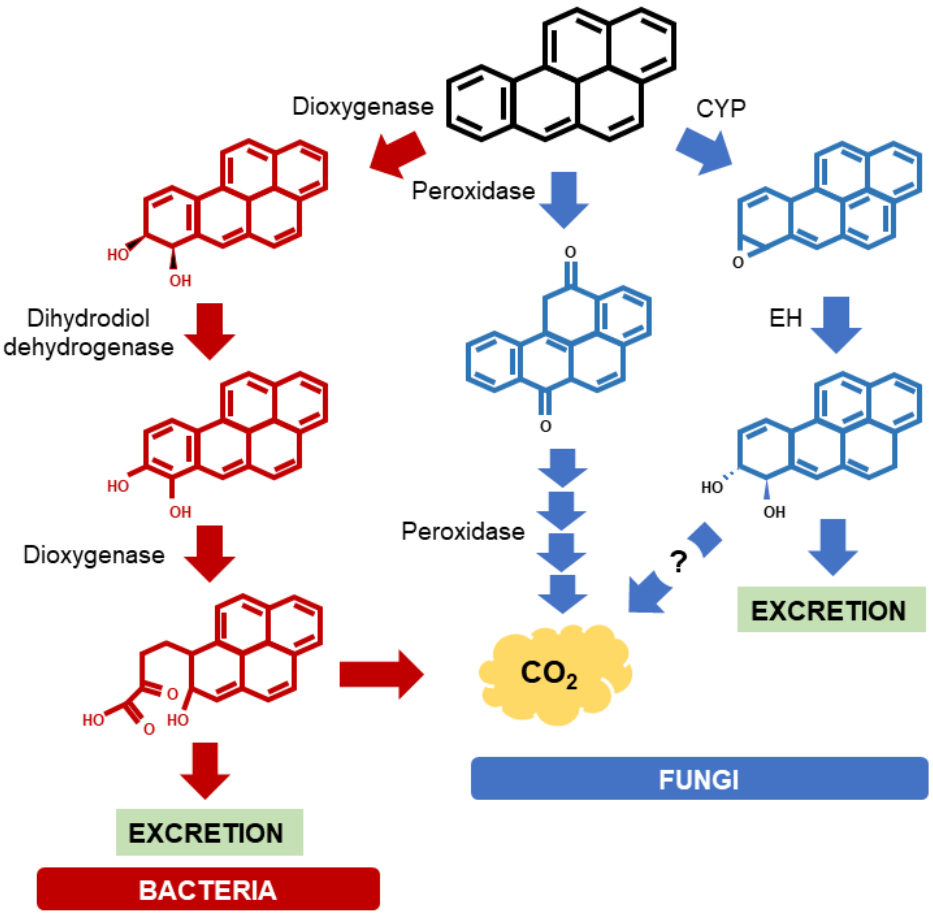


Figure 1.4: Metabolism of benzo(a)pyrene by bacteria and fungi. Fungal metabolism is depicted using two possible mechanisms; intracellularly through cytochrome p450 (CYP) and epoxide hydrolase (EH) or through extracellular peroxidase formation. Peroxidase formation is unique to ligninolytic basidiomycetes whereas CYP mediated degradation is widely distributed in the fungal kingdom.

1.5 Conclusion

Bacteria and fungi are both capable of metabolizing a wide range of organic compounds, yet the mechanisms they use, metabolites they produce, and efficiency of degradation are very different. This is demonstrated by degradation of a known toxic contaminant, BaP (**Figure 1.4**). Bacteria need sufficient growth substrates in order to metabolize BaP efficiently, yet the metabolites of degradation tend to be less toxic to humans. Fungi are capable of metabolizing BaP using several mechanisms depending on growth conditions. The metabolites produced by degradation are similar to those produced by mammalian systems, which are known carcinogens. How this might impact human health is still unclear, and how microbes and the products of their metabolism interact with one another is being revealed thanks to 'omics' approaches. Synergy is seen in communities of degrading microorganisms, as cross kingdom communities are more effective at complete removal of contaminants like BaP (Boonchan, Britz and Stanley, 2000). Additionally, the use of genetically engineered organisms which have enhanced degradation mechanisms (Kumar *et al.*, 2013), and expression of degradation pathways in heterologous expression systems also hold promise for faster and more efficient degradation (Copley, 2009). Both of these techniques require further investigation in order to implement them in contaminated sites.

Removal of BaP from the environment is undoubtedly beneficial, yet metabolism by communities residing in the microbiome may expose humans to toxic metabolites more frequently than previously thought. Human populations who live near contaminated sites may be exposed to additional pollution due to fungi or bacteria which uptake

contaminants and then reproduce through sporulation which contain sequestered contaminants. More research is needed to address these concerns.

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Chapter 2:

Cytochrome P450 monooxygenase mediated metabolic utilization of benzo(a)pyrene by fungi

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2.1 Abstract

Soil dwelling *Aspergillus* fungi possess the versatile metabolic capability to utilize complex organic compounds which are toxic to humans, yet the mechanisms they employ remain largely unknown. Benzo(a)pyrene is a common carcinogenic contaminant, posing a significant concern for human health. Here, we report that *Aspergillus* fungi can degrade benzo(a)pyrene effectively. In *Aspergillus nidulans*, exposure to benzo(a)pyrene results in transcriptomic and metabolic changes associated with cellular growth and energy generation, implying that the fungus utilizes benzo(a)pyrene as a food. Importantly, we identify and characterize the conserved *bapA* gene encoding a cytochrome P450 monooxygenase that exerts the first step in the degradation of benzo(a)pyrene. We further demonstrate that the fungal NF-κB-type global regulators VeA and VelB are required for benzo(a)pyrene degradation in *A. nidulans*, which occurs through expression control of *bapA* in response to nutrient limitation. Our study illuminates fundamental knowledge of fungal benzo(a)pyrene metabolism and provides novel insights into enhancing bioremediation potential.

2.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are major soil pollutants, and exposure to PAHs has long been recognized as a significant health risk to humans. The five-ring PAH benzo(a)pyrene is a contaminant formed by the partial combustion of organic matter (U.S. Centers for Disease Control Agency for Toxic Substances and Disease Registry (ATSDR) and Division of Toxicology and Human Health Sciences, 2013). The increased use of hydrocarbons for energy during the past century has consequently increased the deposition of benzo(a)pyrene, making it an abundant pollutant found in the environment (U.S. EPA, 2017).

Organisms have varied ways of metabolizing benzo(a)pyrene depending on their ecological niche (**Figure 2.1**). Saprophytic bacteria metabolize benzo(a)pyrene resulting in ring cleavage, indicating that they attempt to degrade this carbon containing molecule into usable non-toxic fragments (Cerniglia, 1992). Humans are equipped with cytochrome P450 monooxygenase (CYPs) enzymes to transform and excrete benzo(a)pyrene, but this process results in the creation of reactive intermediates, which cause adduct formation and oxidative stress in cells (Kim *et al.*, 1998; Tsuji *et al.*, 2011). This makes benzo(a)pyrene an especially harmful compound and has been implicated in consequences ranging from cancer to immune dysregulation (U.S. EPA, 2017). In addition, its high molecular weight and high octanol water partition coefficient make benzo(a)pyrene stable in the environment and relatively inaccessible to microbes for

degradation (U.S. Centers for Disease Control Agency for Toxic Substances and Disease Registry (ATSDR) and Division of Toxicology and Human Health Sciences, 2013).

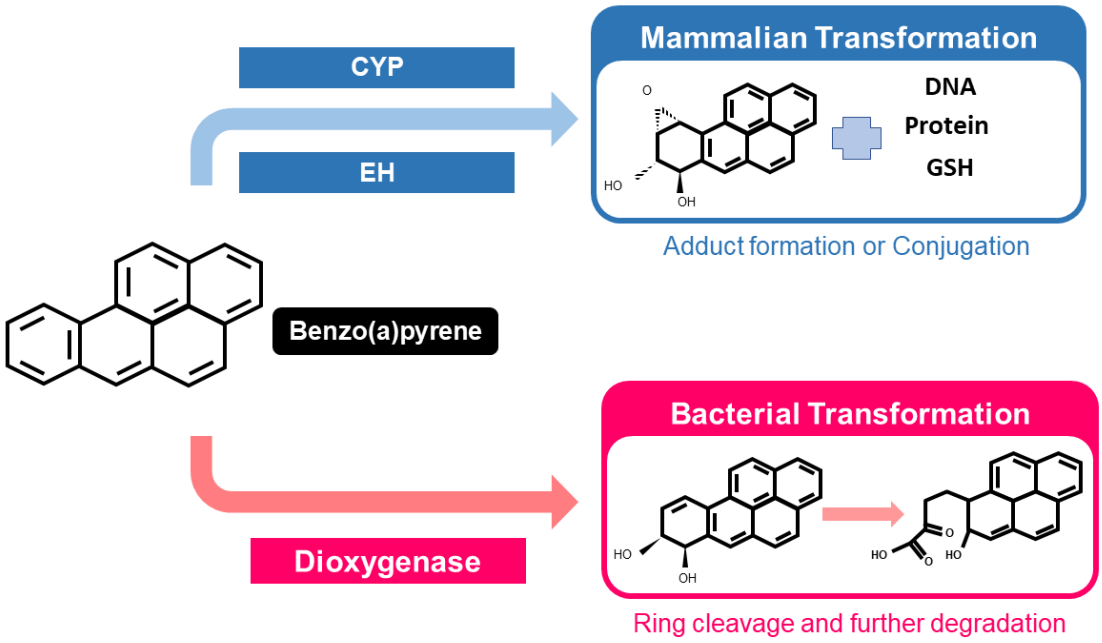


Figure 2.1: Benzo(a)pyrene transformation enzymes and products in different organisms. Partial pathways of benzo(a)pyrene transformation in mammals and bacteria. The mammalian pathway is represented by cytochrome P450 monooxygenase (CYP) and epoxide hydrolase (EH) mediated activation to (±)*trans*-7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene, although other metabolic pathways also occur forming alternative metabolites.

Fungi are one of nature's most resourceful organisms accounting for up to 75% of the soil microbial biomass, and greatly contribute to the biogeochemical reactions in PAH contaminated sites (Harms, Schlosser and Wick, 2011). Previously it has been reported that many fungi including the most common soil fungi *Aspergillus* species prevail in various contaminated sites, and can metabolize certain PAHs (Cerniglia and Sutherland, 2010). As a soil saprophyte *Aspergillus* fungi harbor abundant and diverse

enzymatic systems, which allow them to metabolically utilize complex organic molecules that are highly toxic to animals (Klich, 2007; Mukherjee, 2016). As they are closely related to animals, there is evidence that fungi may utilize enzymatic mechanisms similar to those used in humans (Cresnar and Petric, 2011). However, utilization of specific genes involved in metabolism of benzo(a)pyrene *in vivo* in endogenous fungus remain to be revealed.

Part of the metabolic armory harbored by *Aspergillus* sp. is over 100 CYPs encoded in the genome (Chen *et al.*, 2014). These enzymes participate in a variety of physiological activities that allow the fungus to adapt to new ecological niches. Soil is a hostile and competitive environment so these CYPs play a role in the synthesis and degradation of various defensive and/or toxic compounds. *A nidulans* contains 119 predicted CYPs, of which 13 have functions determined experimentally and 32 of which are near key secondary metabolite synthases suggesting their potential biosynthetic role (Kelly *et al.*, 2009). Therefore, a large number of CYPs have no known or predicted function.

CYPs in the white rot fungus *Phanerochaete chrysosporium* are grouped into 16 clusters, and as shown by differential regulation, may exist to facilitate adaptation to environments with diverse carbon sources and xenobiotic detoxification (Yadav, Doddapaneni and Subramanian, 2006). Because *Aspergillus spp.* fill a similar saprophytic niche, it is likely that *A. nidulans* also utilizes CYPs to adapt to new carbon sources and/or protect itself from toxins.

Many studies have focused on fungal species which have been isolated from contaminated sites in order to investigate the ability to degrade benzo(a)pyrene, which makes a detailed genetic approach more difficult because of limited genetic tools (Cerniglia and Sutherland, 2010). In this study, for the first time, we show that many, if not all, *Aspergillus* species can effectively use benzo(a)pyrene as a carbon source for growth, indicating drastic transformation of the chemical structure. We also uncover key aspects of cellular degradation of benzo(a)pyrene by *A. nidulans* using comprehensive genetic, genomic, and biochemical approaches. Importantly, we identify a gene (*bapA*) predicted to encode CYP617D1, and show that *bapA* is necessary for degradation of benzo(a)pyrene *in vivo* in two *Aspergillus sp*. This critical finding further allows us to investigate the upstream regulatory events associated with benzo(a)pyrene metabolic degradation in fungal cells. Our study illuminates knowledge of fungal benzo(a)pyrene metabolism and provides novel insight into designing and implementing enhanced bioremediation strategies.

2.3 Materials and Methods

Strains, media and culture conditions

Aspergillus strains used in this study are listed in **Table 2.1**. All media used in this study were prepared as previously described (Kafer, 1977). Briefly, 10^6 spores/mL were added to 400 mL minimal medium with 1% glucose in 2 L flasks and incubated 18 hours in 220 rpm at 37°C. The mycelial aggregates were then collected on sterile Miracloth (Sigma-Aldrich), rinsed, and transferred to 100 mL MM with 0.1% glucose in 250 mL

Erlenmeyer flasks. To account for any non-metabolic sources of non-recovery of benzo(a)pyrene autoclaved fungal cells which were autoclaved on liquid cycle at 121°C for 20 minutes were used as controls. A 100mM stock solution benzo(a)pyrene (Sigma-Aldrich) in dimethyl sulfoxide (DMSO) was added to the cultures to a final concentration of 200 μM; the same volume DMSO was added to controls. All flasks were further incubated at 220 rpm at 37°C for designated time. *Escherichia coli* DH5α cells were grown in Luria–Bertani medium with ampicillin (100 μg/mL) for plasmid amplification.

Table 2.1: *Aspergillus* strains used in this study

Strain	Species/Genotype	Source/Reference
FGSC4	*A. nidulans* Wild Type, *veA*$^{+}$	FGSC [a]
NRRL 3357	*Aspergillus flavus* Wild Type	FGSC [a]
AF293	*Aspergillus fumigatus* Wild type	(Brookman and Denning, 2000)
M2040	*Aspergillus oryzae* Wild Type	KACC[b]
RJMP1.59	*A. nidulans pyrG89*; *pyroA4*; *veA*$^{+}$	(Shaaban *et al.*, 2010)
TMK6	*pyrG89*; *pyroA4*; Δ*bapA*:: *AfupyrG*$^{+}$;*veA*$^{+}$	This study
TEO2	*pyrG89*; *pyroA*::*bapA(p)*::*bapA*::FLAG$_{3x}$::*pyroA*[c]; Δ*bapA::AfupyrG*$^{+}$; *veA*$^{+}$	This study
THS15	*pyrG89*; *pyroA4*; Δ*vosA*::*AfupyrG*$^{+}$; *veA*$^{+}$	(Park *et al.*, 2012)
THS16	*pyrG89*; *pyroA4*; Δ*velB*::*AfupyrG*$^{+}$; *veA*$^{+}$	(Park *et al.*, 2012)
THS11	*pyrG89*; *pyroA4*; ΔvelC::*AfupyrG*$^{+}$; *veA*$^{+}$	(Park *et al.*, 2014)
THS17	*pyrG89; pyroA4;* Δ*veA::AfupyrG*$^{+;}$ *veA*$^{+}$	(Bayram *et al.*, 2008)

[a] Fungal Genetic Stock Center

[b] Korean Agricultural Culture Collection

[c] The 3/4 *pyroA* marker causes targeted integration at the *pyroA* locus.

Extraction and HPLC analysis

The extraction of benzo(a)pyrene was optimized to recover all benzo(a)pyrene adhered and uptaken by cells, but not bio-transformed. Cell cultures were extracted using 100 mL 1:1 hexane:ethyl acetate with pyrene (Sigma-Aldrich; final concentration = 200 μM) as an internal standard to correct for extraction efficiency. The entire mixture was sonicated using Sonic dismembrator model 100 (Fisher Scientific) with ¼ inch probe on full power for 6 min to ensure disintegration of hyphal pellets. 1 mL solvent was removed and centrifuged to remove particulate matter, and diluted 100x in 1:1 solvent A (30 mM acetate buffer pH 4.7, 10% acetonitrile): solvent B (acetonitrile). Benzo(a)pyrene and pyrene were quantified by high-performance liquid chromatography using an Agilent 1260 HPLC system equipped with a 3 x 50 mm Poroshell 120 EC-C18 2.7 μm column. A linear gradient that ramped from 55% B to 90% B over 10 min at a flow rate of 0.75 mL/min was used, followed by fluorescence detection ($\lambda_{excitation}$ = 248 nm, $\lambda_{emission}$ = 465 nm). All standard curves were linear and the detection limits were <0.1 μM for pyrene and benzo(a)pyrene.

AlamarBlue reduction assay

Cell viability was determined by percent AlamarBlue (Bio-Rad) reduction as described previously (Lee *et al.*, 2014) with the following exceptions. Cells were prepared as described for benzo(a)pyrene degradation with solvent (DMSO) only as a control, and 0.45 mL were added to 0.45 mL fresh 0.1% glucose MM and 100 μL AlamarBlue and incubated for 2 hours at 37°C.

RNA preparation and qRT-PCR

Fungal cells from the submerged cultures were collected at designated time points, squeeze-dried, flash frozen in liquid nitrogen, and stored at -80°C until subject to RNA preparation. Total RNA isolation was done using Trizol as described previously(Yu *et al.*, 2004). cDNA was prepared using AMV reverse transcriptase kit (NewEngland Biolabs) with oligo-dT primer. Reverse transcriptase quantitative PCR (RT-qPCR) was performed with iTaq universal SYBR green supermix (Bio-Rad, Hercules, CA) on a Bio-Rad CFX96 real-time PCR detection system. mRNA was normalized using $2^{\Delta\Delta C}$t method(Livak and Schmittgen, 2001). Primers used are listed in **Table 2.2**. Total RNA was extracted and submitted to ProteinCT Biotechnologies (Madison, WI) for library preparation and RNA sequencing.

Table 2.2: **Primers used in this study**

Name	**Sequence (5' - 3')** [a]	**Purpose**
oMK-299	CGTCGTCATATCACCCTTTG	*bapA*-qPCR 5'
oMK-300	TTCGAATAGACTCGGGCTTT	*bapA*-qPCR 3'
oMK-331	GGCAACATCGTTATGTCTGG	*Aniactin*- qPCR 5'
oMK-332	CCGATCCAGACGGAGTATTT	*Aniactin* qPCR 3'
oEO132	**CAGCTG**GGACTTCGCCGCCATCAA	*bapA* complementation 2kb F' (For △bapA)+PvuII

oEO133	**GGATCC**TTACCACCCATCCAATGGCGTC	*bapA* complementation R' (For △*bapA*)+BamHI
oMK-55	TGACATCTTCTTGACTGCCAACG	*AnibapA* flanking region 5'
oMK-58	ATATGCCTCCAGCAGCTACCG	*AnibapA* flanking region 3'
oMK-56	*GCTTTGGCCTGTATCATGACTTCA*GTTGAGATTGGGCCTCGACGAAG	*AnibapA* 3' with *AfupyrG* tail
oMK-57	*ATCGACCGAACCTAGGTAGGGTA*TCTCACTCTGTGTCCATCGAAC	*AnibapA* 5' with *AfupyrG* tail
oMK-59	GAGAACCGTTTCATCGGTATC	*AnibapA* flanking region 5' nested
oMK-60	TCGCCAGTAACTGGCGCAAAC	*AnibapA* flanking region 3' nested
oEO1	ACCCAGAATTTATTTACGCGGAG	*AflbapA* flanking region 5'
oEO2	CATGCATTCGCAGCTTGTGGC	*AflbapA* flanking region 3'
oEO3	*TTTGGCCTGTATCATGACTTCAGC*TCGTGAGCAGAGGCTGCGG	*AflbapA* 3' with *AfupyrG* tail
oEO4	*ATCGACCGAACCTAGGTAGGGTA*ATTGCCCCCGAAATTATCGGTAA	*AflbapA* 5' with *AfupyrG* tail
oEO5	CGGAGCGGTTCAAGAGA	*AflbapA* flanking region 5' nested
eEO6	GGGGACTGGAGTTCATGGATG	*AflbapA* flanking region 3' nested
oEO-62	TTCGAGATGGAGCTGGCA	Plasmid sequence promoter region 5’
oEO-70	CTATTACTTGTCATCGTCATCCT	Plasmid sequence 3x flag 3’
	[a] Tail sequences are shown in italics. Restriction enzyme sites are in bold	

RNA sequencing

Sequencing was done as previously described (Alkahyyat *et al.*, 2015). The library was constructed and purified, then sequenced (SE100bp) using the Illumina HiSeq2500, and over 20 million high-quality reads per sample were achieved. All RNA-seq data files are

available from the NCBI Gene Expression Omnibus database (Accession number: GSE116804).

Data QC and analysis

Verification of the quality of reads, alignments, gene annotation and differential expression analysis were performed as described previously (Alkahyyat *et al.*, 2015).

Functional enrichment analysis (KEGG)

KEGG PATHWAY database was used to search against *A. nidulans* (ani) KEGG pathway maps in order to identify *A*. *nidulans* metabolic pathways which were upregulated after exposure to benzo(a)pyrene on February 20, 2018 (Kanehisa *et al.*, 2016).

Metabolomics of amino acid and primary metabolites

Fungal tissue was prepared as described for benzo(a)pyrene degradation with DMSO control and extraction of cellular components was performed as previously described (Wang *et al.*, 2016) with the following exceptions. Hyphal mats were filtered and squeezed dry, noting the mass after removing liquid, 1 day after transfer to benzo(a)pyrene containing media. Tissue was flash frozen in liquid nitrogen and stored at -80°C. 2 mL extraction solvent (Wang *et al.*, 2016) was added and samples were sonicated using ¼ inch probe for 3 minutes, then centrifuged to remove cell debris. Additional sample prep and analysis was performed as described previously (Wang *et al.*, 2016).

Protein alignment

CYP sequences similar to PC-PAH1 and PC-PAH3 in *Aspergillus* sp. were identified using BLASTp (Altschul *et al.*, 1997). Proteins sequences were found using NCBI and protein alignment was calculated using Clustal Omega at EMBL-EBI output ClustalW with character counts (McWilliam *et al.*, 2013). Phylogentic tree was created using Jalview nearest neighbor joining (Waterhouse *et al.*, 2009).

Distribution, phylogenetic and structure analysis of BapA families.

BapA (AN1884) was assigned to CYP617D1 (Kelly *et al.*, 2009). According to the rules of the International P450 Nomenclature Committee, any two CYPs with amino acid sequence identity greater than 40% belong to a single CYP family, and greater than 55% belong to a subfamily (Nelson, 2006). BapA protein sequence was used to query FungiDB (Stajich *et al.*, 2012) and homologs with over 40% identity were collected as CYP617 family members.

CYP617 members were aligned and the phylogenetic tree was constructed as previously described (Bamal *et al.*, 2018).

Generation of Δ*bapA* and complemented strains

Double-joint PCR was used to generate the deletion construct of *AnibapA* (AN1884) and *AflbapA* (*AFLA_036020*; Yu et al., 2004). Briefly, the deletion constructs containing the *A*. *fumigatus pyrG* marker with 5' and 3' flanking regions of *bapA* were introduced into the recipient strain RMJP1.59 or 3357.5. Three independent Δ*bapA* strains in *A. nidulans* (TMK6- #1, #35, and #47) and *A. flavus* (TEO1 #2, #8, and #9) were isolated, which all failed to degrade benzo(a)pyrene. To generate complemented

strains of Δ*AnibapA*, a *bapA*$^{+}$ gene region including its upstream 2 kb region was introduced to pHS13 (Park *et al.*, 2012) with 3xFLAGs and ampicillin marker and transformed into *E. coli* DH5α competent cells. These plasmids were screened using primers listed in **Table 2**, and sent to UW Gene Expression Center for sequence verification of the insert. The purified plasmid was then introduced into the recipient Δ*bapA* strain (TMK6). Three independent complemented strains (C'AN1884 #12, #16, and #17) were also generated and they all behaved identically to one another as well. TMK6 (Δ*AnibapA*), TEO2 (C' Δ*AnibapA)* and TEO1 (Δ*AflbapA*) were randomly chosen as the testing strains for further experiments.

Microsome isolation and activity

Cells were prepared as described without benzo(a)pyrene treatment to capture peak *bapA* expression (**Figure 2.S2**). After 1 day incubation, cells were filtered, washed, squeezed dry, and flash frozen in liquid nitrogen. Frozen tissue was ground in liquid nitrogen with the addition of glass beads in a mortar and pestle to a fine powder. The powder was resuspended in 30 mL homogenization buffer (0.1 M KPO_4, pH 7.25, 0.1 M KCl, 10mM EDTA, pH 8, 0.25 mM PMSF, 0.1 mM DTT) and kept on ice. A 1/8 inch sonication probe was used on full power for 30 seconds to fully homogenize cells and form microsomal structures. Large debris was filtered using Miracloth and the supernatant was centrifuged for 20 min at 20,000X g to remove large organelles. The supernatant was then centrifuged for 60 min at 105,000X g. This supernatant was removed and pellets were resuspended in 200 μL dilution buffer (0.25 M KPO_4, pH 7.25, 20% (v/v) glycerol, 10 mM EDTA, pH 8, 0.25 mM PMSF, 0.1 mM DTT), flash frozen in

liquid nitrogen, and stored at -80°C for a maximum 1 week. Protein concentration was determined with Coomassie protein assay (ThermoFischer). Benzo(a)pyrene metabolism was measured by incubating 2 mg/mL microsomal protein, 1 μM benzo(a)pyrene, and 1 μM NADPH in up to 1 mL 50 mM phosphate buffer at pH 7.5 at 37°C for 1 h. Protein was denatured by boiling for 20 minutes prior to incubation as a control. Metabolites were extracted by adding 2 mL of 2:1 acetone:ethyl acetate and vortexing for 2 min. Solvent was removed and centrifuged at 13,000 for 10 min and dried under N_2. Samples were resuspended in 50 μL 1:1 HPLC solvent A: solvent B and analyzed by HPLC.

Statistics

Statistical significance was determined using student's t test with 2-tailed distribution, and two-sample unequal variance.

2.4 Results

2.4.1 *Aspergillus* fungi can degrade benzo(a)pyrene effectively

To test our initial hypothesis that *Aspergillus* fungi are able to degrade benzo(a)pyrene, we employed four distantly related representative *Aspergillus* species: *A. nidulans*, *A. flavus*, *A. oryzae*, and *A. fumigatus*. These fungi are as distantly related to each other as human to chicken or fish (i.e., up to 450 million years apart), thus covering a broad range of *Aspergillus* species (Samson *et al.*, 2014). The amount of benzo(a)pyrene recovered was significantly lower in the living cells than that of controls (**Figure 2.2A**),

indicating that benzo(a)pyrene was degraded or transformed in all species tested. The chromatogram showed no additional fluorescent peaks, suggesting that the degraded products were either water soluble and/or non-fluorescing at the same wavelengths of benzo(a)pyrene and the internal standard pyrene (**Figure S1**). *A. nidulans* and *A. oryzae* were able to remove 92% ± 4.9% and 95% ± 3.5% of added benzo(a)pyrene, respectively. As *A. nidulans* is a well-studied genetic model, we used it for further uncover the genetic and biochemical mechanisms of benzo(a)pyrene degradation.

2.4.2 Benzo(a)pyrene increases fungal cell viability

To understand the physiology behind the effective degradation of benzo(a)pyrene by fungi, the viability of fungal cells upon addition of benzo(a)pyrene was determined. Benzo(a)pyrene is a group 1 carcinogen and its transformation in animals produces reactive metabolites that lead to DNA adducts and other oxidative stresses (U.S. EPA, 2017). If benzo(a)pyrene causes similar cellular damage to *A. nidulans*, benzo(a)pyrene exposed fungal cells would be less viable than control (DMSO). Conversely, if cells are able to metabolize benzo(a)pyrene to a product which can be used as a source of cellular growth and energy, an increase in cell viability would be observable. AlamarBlue® assay showed that cells with benzo(a)pyrene had significantly higher viability than the DMSO treated controls, including timepoints after the addition of fresh medium to supply cells with the additional nutrients for the continuous proliferation (**Figure 2.2B**). The prolonged viability of the cells with benzo(a)pyrene was observable with the presence of a small amount of glucose in the medium, whereas benzo(a)pyrene addition alone was able to increase the cell viability at 2 days post

exposure (**Figure 2.2B**). This suggests that benzo(a)pyrene can be used as carbon source, and such effects can be enhanced with the supplementation of a low amount of glucose, which may provide additional resources for energy and enzyme production.

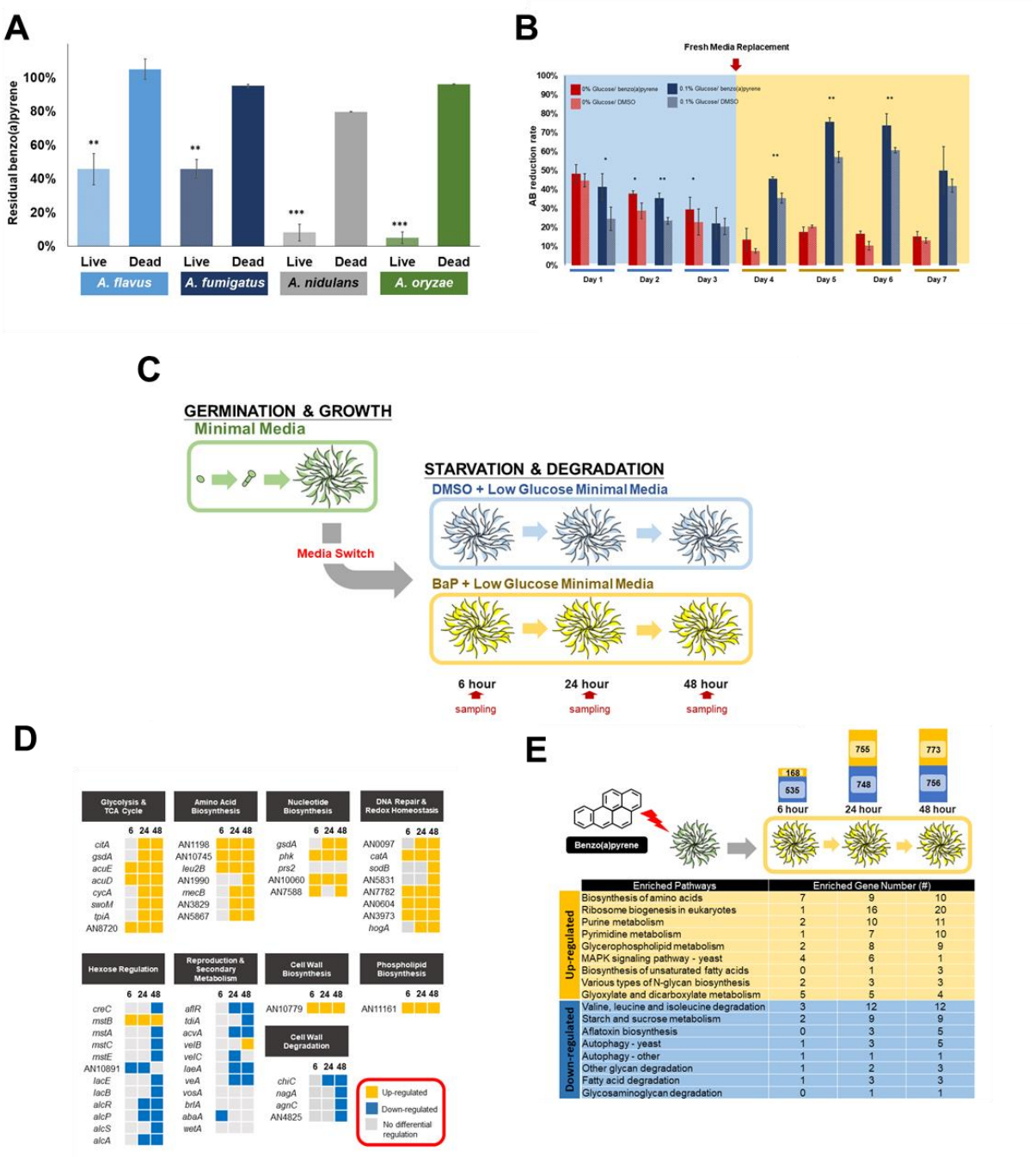


Figure 2.2: *Aspergillus nidulans* can efficiently degrade benzo(a)pyrene and utilize it as a source of energy. **A**. Percent benzo(a)pyrene remaining after culturing with each

indicated strain. All conditions were performed in triplicate and in three separate experiments. Mean values are plotted with a bar, and error bars represent standard error. Significance between Live and Dead was determined using student's t-test, and indicated as p<.01 (**) and p<.001 (***). **B**. AlamarBlue® reduction (%) was determined in cells treated with benzo(a)pyrene and solvent only (DMSO) in 0.1% glucose MM (red) and 0% glucose MM (blue). Timepoints (days) represent the number of days cells were incubated with benzo(a)pyrene or DMSO. Fresh media replacement indicates removal and washing of cells and addition of fresh MM media (0% or 0.1% glucose as indicated). All treatments were performed in triplicate. Mean values are plotted with a bar, and error bars represent standard error. Significance between benzo(a)pyrene and DMSO was determined at each timepoint using student's t-test, and indicated as p<.05 (*) and p<.01 (**). **C**. Experimental overview depicting preparation of cells for RNA sequencing. 10^6 spores/mL were inoculated in MM (1% glucose), then switched to 0.1% glucose media and either treated with 200 µM benzo(a)pyrene (BaP) or solvent only (DMSO). Samples were collected in triplicate at timepoints indicated. **D.** KEGG pathways which include differentially expressed genes at each timepoint. Upregulated pathways (yellow) and downregulated pathways (blue) represent comparison of benzo(a)pyrene to DMSO treatment at each timepoint. Bar chart above timepoints indicate total number of genes which were differentially expressed (log_2 fold change > 1 or <-1 and p-value < .05) at each indicated timepoint. **E**. Categories of responses seen in carbon starved cells and their expression level in benzo(a)pyrene treatment relative to DMSO. Upregulated (yellow) and downregulated (blue) indicate log_2 fold change > 1 or <-1 and p-value < .05.

2.4.3 Benzo(a)pyrene leads to upregulation of cell growth associated genes

To further test the hypothesis that the fungus uses benzo(a)pyrene as a food, we investigated the genome-wide expression responses of *A. nidulans* to benzo(a)pyrene treatment via RNA-seq. Transcript levels were measured in benzo(a)pyrene treated cells relative to controls (DMSO at 6, 24, and 48 h post-exposure; **Figure 2.2C**). We expected that transcriptomes at 6 hours would represent the recognition and uptake mechanisms, whereas the expression responses at 24 and 48 h may indicate metabolic and growth responses. The number of differentially accumulated transcripts were low in magnitude at 6 h, where 703 genes showed significant differential expression; this increased to 1,503 and 1,529 genes at 24 and 48 h, respectively. KEGG pathway analysis was used to determine the enriched metabolic pathways in benzo(a)pyrene-treated cells. The same pathways are enriched at each time point, but the number

increases as time progresses, indicating the time-dependent utilization of benzo(a)pyrene. Genes categorized into the pathways associated with cell growth such as ribosome biogenesis, biosynthesis of amino acids, nucleotide metabolism, biosynthesis of unsaturated fatty acids, N-glycan biosynthesis (cell wall), and glycerphospholipid (membrane) were upregulated as time progressed post benzo(a)pyrene treatment compared to controls **(Figure 2.2D)**. Conversely, genes categorized into the pathways indicative of cell starvation and stress, including amino acid degradation, autophagy, aflatoxin (sterigmatocystin) biosynthesis, and starch metabolism, were down-regulated in benzo(a)pyrene-treated cells (**Figure 2.2D**). These results indicate that benzo(a)pyrene-treated cells are actively growing compared to controls, and that the fungus is using benzo(a)pyrene as an energy source.

The transcriptomic response to carbon starvation in *A. nidulans* shows upregulation of genes involved in programmed cell death, macroautophagy, cell wall component degradation, asexual reproduction, and secondary metabolite production (Szilágyi *et al.*, 2013). Down regulation of genes involved in glycolysis and oxidative phosphorylation, cell wall component synthesis, and nitrogen and lipid anabolic pathways was also seen in the starving cells (Szilágyi *et al.*, 2013). We carried out an integrated analysis of the differentially expressed genes by benzo(a)pyrene treatment with the carbon starvation stress response and found that the benzo(a)pyrene treated cells showed upregulation of the following genes: 1) *citA*, *gsdA*, *acuE*, *acuD*, and *cycA* involved in the TCA cycle and oxidative phosphorylation; 2) AN11161 involved in phospholipid biosynthesis; 3) AN10779 involved in β-1,6-glucan biosynthesis; and 4) several genes involved in amino

acid biosynthesis pathways **(Figure 2.2E**). Benzo(a)pyrene-treated cells show down regulation of *aflR* and other sterigmatocystin biosynthesis genes, *abaA* involved in conidiation, *agnC*, *nagA*, *chiC*, and AN4825 involved in cell wall component hydrolase enzymes **(Figure 2.2E**). Taken together, the data demonstrate that benzo(a)pyrene enables the cells to grow more actively than control cells, and indicate that benzo(a)pyrene is metabolized and shuttled into carbon utilization pathways.

Finally, in an attempt to address whether benzo(a)pyrene metabolism causes oxidative stress and/or DNA damage responses in *A. nidulans* as in mammalian cells, we compared our RNA-seq data with those representing responses to cells treated with other known oxidizing compounds. Some redox balancing genes were upregulated in benzo(a)pyrene treated cells including *catA* and *sodB* **(Figure 2.2E)**. Expression of some DNA repair genes was also induced by benzo(a)pyrene including AN0604 and AN0097 **(Figure 2.2E)**. These results suggest that benzo(a)pyrene may cause DNA damage in *A. nidulans* as in mammalian cells, although it is likely that fungi have an additional capacity to prevent extensive DNA damage, as they also prevent self-poisoning during secondary metabolite production (Keller, 2015).

2.4.4 Identification and verification of the benzo(a)pyrene metabolizing CYP *bapA*

The rate-limiting step of benzo(a)pyrene metabolic degradation is typically the initial oxidation leading to an extremely reactive metabolite, which can then be metabolized

further. In mammalian cells, this step is mediated by a CYP, that adds a single molecular oxygen, leading to the formation of benzo(a)pyrene epoxide intermediates, which can be further converted into hydroxylated products (Bauer *et al.*, 1995; Gautier *et al.*, 1996; Shimada *et al.*, 1997; Kim *et al.*, 1998).

With the hypothesis that *A. nidulans* employs a CYP to degrade benzo(a)pyrene, we first examined our RNA-seq data to search for the CYP genes upregulated by benzo(a)pyrene treatment, and found that no specific CYPs were clearly induced by benzo(a)pyrene. We then used the CYPs of *P. chrysosporium*, Pc-PAH1 and Pc-PAH3, which, when expressed in the yeast, converted benzo(a)pyrene to 3-hydroxy benzo(a)pyrene (Syed *et al.*, 2010) to search for closely related CYPs in the *A. nidulans* genome. Despite the 723 million years of divergence between two fungi (Kumar *et al.*, 2017), AN1884 and AN11142 showed a very high similarity to Pc-PAH1, and AN1601 and AN7399 to Pc-PAH3 of the 119 predicted CYPs (**Figure 2.3A and Table 2.S1)**. The findings that Pc-PAH1 showed more benzo(a)pyrene metabolizing activity *in vitro* than Pc-PAH3 (Syed *et al.*, 2010) and AN1601 is likely involved in secondary metabolite biosynthesis (Cerquiera *et al,* 2014) led us to eliminate AN1601 and AN7399 from potential candidates involved in benzo(a)pyrene metabolism. Of the remaining candidates, progression of benzo(a)pyrene degradation and AN1884 mRNA levels were closely aligned, indicating that this CYP may be associated with metabolism of alternative carbon sources like benzo(a)pyrene (**Figure 2.3B**).

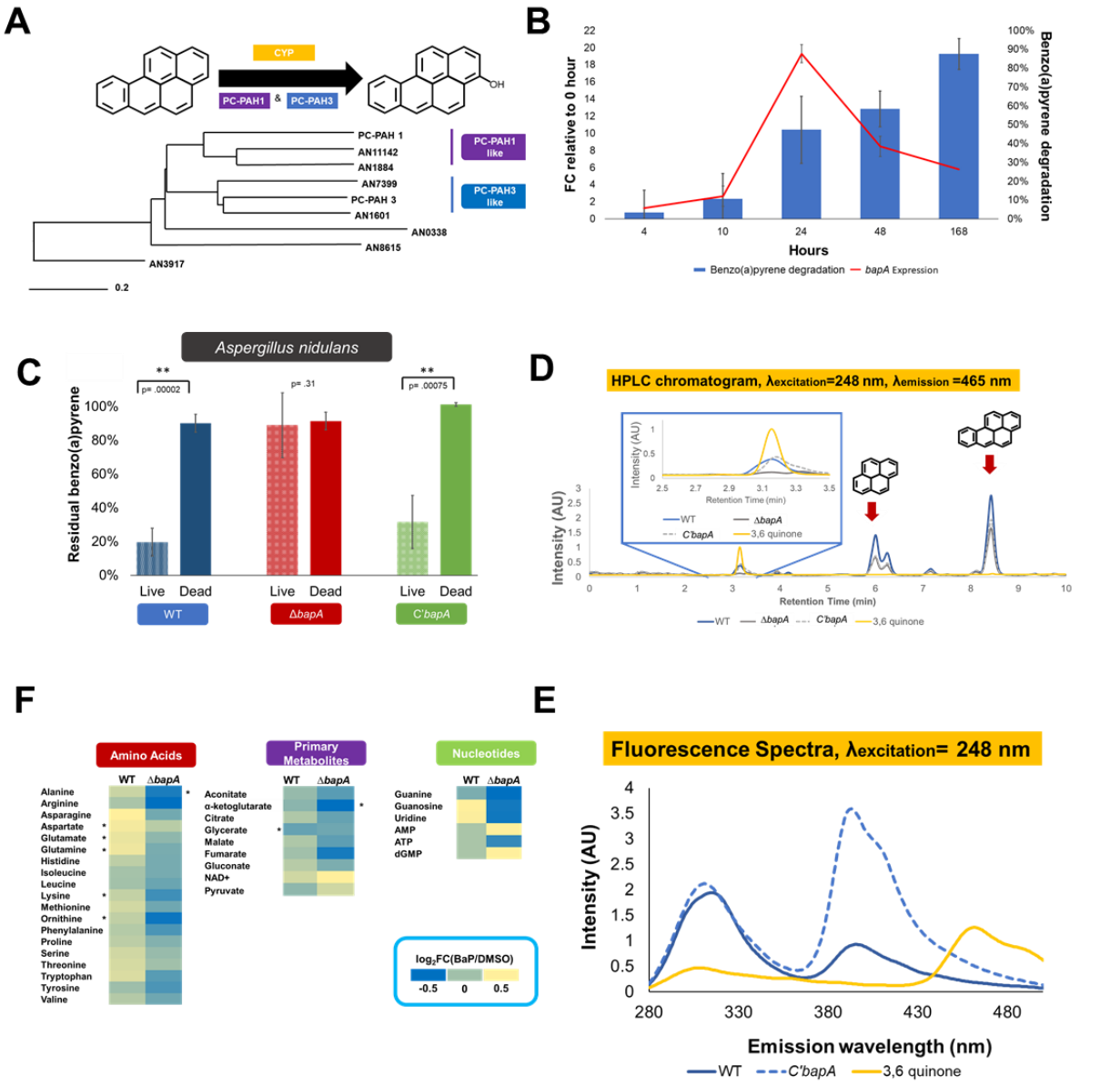


Figure 2.3: BapA initiates benzo(a)pyrene degradation by catalyzing the oxidation of benzo(a)pyrene. A. Phylogenetic tree representing candidate cytochrome P450 monooxygenases (CYPs) in *Aspergillus nidulans* and benzo(a)pyrene oxidizing CYPs in *Phanerochaete chrysosporium* (PC-PAH1 and PC-PAH3). **B**. Expression of *bapA* (AN1884, red line) was measured using $2^{-\Delta\Delta ct}$ in which *bapA* expression (c_t) was found relative to the reference gene *actA* (γ-actin) (Δc_t) and then relative to timepoint 0 ($\Delta\Delta c_t$) . Timepoint 0 indicates exponential growth (18 hours post germination) in MMG, and each timepoint represents hours after switch to 0.1% glucose MM. Each sample was performed using technical triplicate for RT-qPCR accuracy and three biological triplicates were used for each timepoint. The mean is plotted as a bar and error bars represent standard error. Degradation (blue bar) was found by measuring residual benzo(a)pyrene and subtracting from residual at timepoint 0. Recovery at timepoint 0 is cells grown for 18 hours in MMG then switched to MM 0.1% glucose and treated with 200 µM benzo(a)pyrene then immediately extracted as described. All timepoints were performed in triplicate and mean values were plotted as a bar and error bars represent standard error. **C**. Cells from each indicated

strain were incubated with benzo(a)pyrene for 7 days in 0.1% glucose MM and 200 µM benzo(a)pyrene. All conditions were performed in triplicate and in three separate experiments. Mean values are plotted with a bar, and error bars represent standard error. Significance between Live and Dead was determined using student's t-test, and indicated as p<.001 (***). **D**. Entire chromatogram showing injection of extract from benzo(a)pyrene incubation with microsome fraction of each indicated strain or authentic standard (of benzo(a)pyrene-3,6-dione), with enlarged portion of peaks with retention time 3.2 min (top). **E**. Fluorescence spectra collected at apex of peak with retention time 3.2 min and authentic standard of benzo(a)pyrene-3,6-dione as indicated. **F**. Relative quantification of cellular components indicated in wild-type and Δ*bapA* strains. Colored square represents $\log_2$FC of mean benzo(a)pyrene (BaP) quantity relative to mean control (DMSO). Each sample was prepared using biological triplicates. Significance between benzo(a)pyrene and control was determined using student's t-test where p<.05 (*).

To demonstrate its function in benzo(a)pyrene metabolism *in vivo* (endogenous fungus), we generated multiple deletion (Δ) strains in *A. nidulans*; all null mutant strains lost the ability to degrade benzo(a)pyrene (**Figure 2.3C**) with no distinct growth and developmental phenotypic changes (**Figure 2.S3).** To further verify that the lack of benzo(a)pyrene degradation ability is solely caused by the absence of AN1884, we re-introduced the AN1884 coding region, and found that all complementation strains restored the benzo(a)pyrene degradation ability to that of wild-type (WT; **Figure 2.3C**). These results indicate that the CYP encoded by AN1884 is responsible for the metabolic breakdown of benzo(a)pyrene under these conditions, and the locus AN1884 is named as *bapA* (**b**enzo**(a)p**yrene metabolism associated locus **A**). We further validated the essential role of BapA in benzo(a)pyrene utilization by measuring cell viability of the Δ*bapA* mutant in benzo(a)pyrene containing media relative to control media (DMSO). We found cells were significantly less viable in benzo(a)pyrene containing media at almost every time point tested, whereas WT and complemented (*C'bapA*) strains showed significantly higher cell viability in benzo(a)pyrene containing media (**Figure 2.S5**). This indicates lack of benzo(a)pyrene metabolism not only results in loss of carbon source utilization but also potentially causes toxic effect upon exposure

to benzo(a)pyrene. To our knowledge, this is the first report clearly providing the evidence of *in vivo* function of a CYP in biological degradation of benzo(a)pyrene in microbes.

In order to identify the benzo(a)pyrene metabolite(s) formed by BapA microsome containing fractions were isolated from the cells of WT, Δ*bapA*, and C'*bapA*, and incubated with benzo(a)pyrene. The WT and C'*bapA*, but not Δ*bapA*, chromatograms showed a small fluorescent peak with a shorter retention time than benzo(a)pyrene (**Figure 2.3D**). The retention time of this unknown metabolite was compared to known benzo(a)pyrene metabolites (i.e., benzo(a)pyrene-7,8-diol, benzo(a)pyrene-3,6-dione, and benzo(a)pyrene-7,8-dione) and matched that of benzo(a)pyrene-3,6-dione (**Figure 2.3D**). However, the fluorescence spectra revealed that sample peaks do not show the same fluorescing patterns to that of benzo(a)pyrene-3,6-dione (**Figure 2.3E**). Other known metabolite standards did not match the retention time of this peak (**Figure 2.S4**). We were unable to identify this metabolite since it is beyond the scope of this study. However, given that the polarity of the metabolite is similar to that of benzo(a)pyrene-3,6-dione, BapA may contribute to the formation an isomer with quinone groups in other positions.

Availability of the Δ*bapA* mutant for the first time allows us to further analyze the downstream metabolomics outcomes of benzo(a)pyrene. Since benzo(a)pyrene treatment caused upregulation of genes associated with amino acid biosynthesis, which

is an easily measured end point for carbon utilization, we quantified free amino acids in WT and Δ*bapA* cells treated with benzo(a)pyrene relative to the control (DMSO), and patterns of cytosolic amino acid accumulations in WT vs Δ*bapA* cells. Only benzo(a)pyrene-treated WT cells showed significant accumulation of glutamate, aspartate, glutamine, lysine, and the intermediate amino acid ornithine (**Figure 2.3F**). In agreement with AlamarBlue® data, Δ*bapA* cells showed significant accumulation of alanine, α-ketoglutarate and glutathione disulfide in control (DMSO) treatment (**Figure 2.3F**). This provides additional evidence that loss of benzo(a)pyrene metabolism causes lack of cell growth upon exposure to benzo(a)pyrene. While insignificant due to large deviations among samples, accumulation of several primary metabolites involved in energy metabolism such as citrate and malate, and nucleotides involved DNA/RNA synthesis, or signaling such as AMP appeared to be affected by benzo(a)pyrene (**Figure 2.3F**).

2.4.5 BapA is widely distributed in ascomycota and is functionally conserved in *Aspergillus flavus*

We performed a phylogenic analysis to determine how widely distributed BapA is in fungi. CYP617 members are limited to ascomycetes. The BapA subfamily CYP617D1 was mostly distributed in the genus *Aspergillus* (**Figure 2.4A**), suggesting the conserved functional role of BapA in the genus *Aspergillus*.

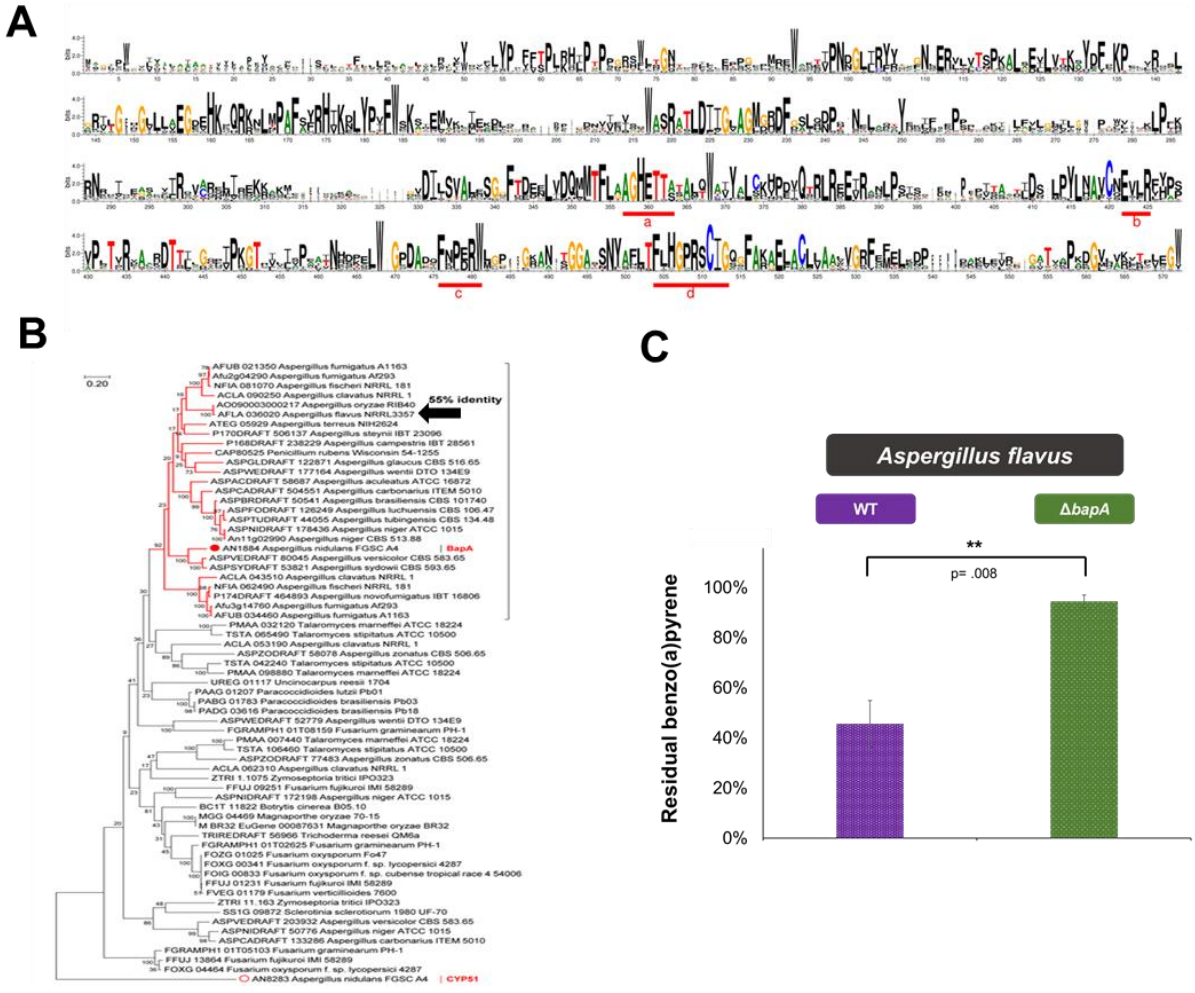


Figure 2.4: BapA is conserved in ascomycota and functionally conserved in *Aspergillus flavus* **A.** Phylogenetic tree showing all proteins in the fungal kingdom with greater than 40% sequence identity to *A. nidulans* BapA. **B**. Consensus logos which contribute to CYP functions (a) oxygen binding and activation, (b) and (c) assist in heme pocket positioning and core structure stabilization (d) heme binding. **C**. Cells from each indicated strain were incubated with benzo(a)pyrene for 7 days in 0.1% glucose MM and 200 µM benzo(a)pyrene. All conditions were performed in triplicate, with Δ*bapA* triplicates representing three separate mutants. Mean values are plotted with a bar, and error bars represent standard error. Significance between wild-type and Δ*bapA* was determined using student's t-test, and indicated as p<.01 (**).

The structural analysis showed that the four widely recognized consensus regions (a-d) (**Figure 2.4B**), contributing to the core function of P450s (Chen *et al.*, 2014), are highly

conserved in CYP617 family. Interestingly, the conserved motif a (AGHETT) of CYP617 family is very specific, and is highly similar to those of archaea, and bacteria (Chen *et al.*, 2014).

To examine a potential conservation of its function in other fungi, we identified a likely orthologue of BapA in *A. flavus* (*AFLA_036020*) and generated three individual null mutant strains. Δ*AFLA_036020* stains show no ability to degrade benzo(a)pyrene, corroborating the conserved and essential role of BapA in metabolic degradation of benzo(a)pyrene in *Aspergillus* fungi (**Figure 2.4C**) under glucose limiting conditions.

2.4.6 Requirement of fungal NF-kb-type regulators in benzo(a)pyrene degradation

Identification of this benzo(a)pyrene-metabolizing CYP allowed us to further investigate upstream regulatory components controlling its expression. This might be used to enhance production of BapA *in situ* and provides novel understanding of evolutionary conservation of benzo(a)pyrene responses. Velvet family proteins are a family of global transcription factors which are involved in many aspects of growth, reproduction, secondary metabolism, and energy allocation in many fungi (Bayram and Braus, 2012). They also contain a DNA binding domain that is structurally similar to the human transcription factor complex, which plays a role in cell survival Nf-κB (Ahmed *et al.*, 2014), including after exposure to benzo(a)pyrene (Pahl, 1999; Weng *et al.*, 2004; Bolotina *et al.*, 2007; Ji *et al.*, 2013).

With the hypothesis that the velvet family proteins play a role benzo(a)pyrene degradation by governing expression of carbon utilizing CYPs, we first tested mRNA levels of *bapA* in each *velvet* deletion mutant (Δ*veA,* Δ*velB,* Δ*velC, and* Δ*vosA*) and found that the two regulators VeA and VelB were necessary for proper expression of *bapA* (**Figure 2.5A**). Interestingly, the other two regulators VosA and VelC seem to play a repressive role at 10 hours post glucose starvation, and an activating role at later time points (**Figure 2.5A**). Timing is a critical element of fungal growth and development because it allows for proper allocation of energy, so low glucose conditions may trigger cellular responses which are very sensitive to timing, as also described by others (Kim *et al.*, 2011; Szilágyi *et al.*, 2013). To verify that the expression of *bapA* translates into benzo(a)pyrene degradation levels, we further tested the degradation ability of each deletion mutant and found that the Δ*veA* and Δ*velB* mutants were unable to degrade benzo(a)pyrene. On the contrary, the Δ*vosA* and Δ*velC* mutants were able to degrade the same amount of benzo(a)pyrene yet faster than WT (**Figure 2.5B**). These results indicate that VeA and VelB of the major *velvet* complex (Bayram *et al.*, 2008) play a key role in metabolic utilization of benzo(a)pyrene likely via controlling proper expression of *bapA*.

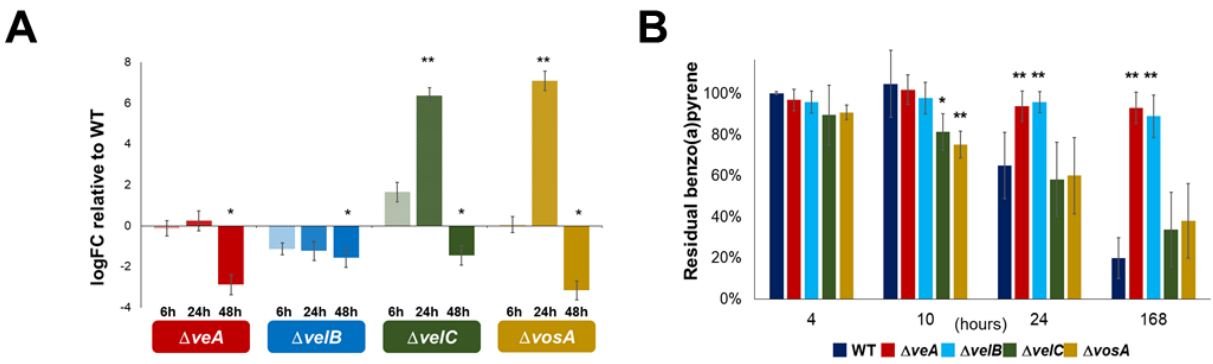


Figure 5: NF-kb-type regulators are required for *bapA* expression and benzo(a)pyrene degradation. A. Expression of *bapA* was measured using $2^{-\Delta\Delta ct}$ in which *bapA* expression (c_t) was found in each indicated deletion mutant background relative to the reference gene *actA* (ɣ-actin) (Δc_t) and then relative to wild-type ($\Delta\Delta c_t$). Timepoints indicate hours after switch to 0.1% glucose MM. Each sample was performed using technical triplicate for RT-qPCR accuracy and three biological triplicates were used for each timepoint and treatment. The mean is plotted as a bar and error bars represent standard error. Significance between wild-type and each deletion mutant was determined using student's t-test, and indicated as p<.05 (*) and p<.01 (**) **B.** Cells from each indicated deletion mutant strain were incubated with benzo(a)pyrene for 7 days in 0.1% glucose MM and 200 µM benzo(a)pyrene. All conditions were performed in triplicate and in three separate experiments. Mean values are plotted with a bar, and error bars represent standard error. Significance between wild-type (WT) and deletion mutant was determined using student's t-test, and indicated as p<.05 (*) and p<.01 (**).

2.5 Discussion

Benzo(a)pyrene is a contaminant of significant concern because of its ubiquity and toxicity. Humans are exposed to benzo(a)pyrene through air pollution and contaminated food and soil. Mammalian metabolism of benzo(a)pyrene results in reactive species, which cause damage to cells and form DNA adducts that can lead to cancer. Because the parent compound is so stable, biologically driven degradation remains the

predominant form of removal from the environment (Haritash and Kaushik, 2009). This means understanding how saprophytic bacteria and fungi effectively metabolize benzo(a)pyrene is critical to effective removal.

Our report is the first comprehensive study of fungal metabolism of organic contaminants, providing the data that infers *Aspergillus* fungi degrade benzo(a)pyrene and incorporate it into energy generation and amino acid biosynthesis (**Figure 2.6A**). This not only provides us with information that can help effectively implement bioremediation strategies, but also gives us a unique insight into evolution of the fungal CYPs and their biocatalytic activity. We propose a model in which VeA and VelB activate expression of *bapA* in response to nutrient limitation and BapA catalyzes the first step in benzo(a)pyrene degradation (**Figure 2.6B**). Benzo(a)pyrene is likely further enzymatically fragmented and the carbon is shuttled into energy generating pathways, which in turn represses further expression of *bapA* (**Figure 2.6B**). Degradation of benzo(a)pyrene most effectively occurs in *Aspergillus* sp. with the addition of a low concentration of glucose to support growth and fungal biosynthesis of reducing enzymes. Most importantly, we have discovered a specific cytochrome P450 enzyme, BapA, which is necessary for the rate limiting metabolic step.

Filamentous fungi harbor many more CYPs relative to their genome size than animals and bacteria, yet the function of many still remain unknown. The diversity of CYPs in fungi could be due to their need to metabolize many different carbon sources, including large cyclic compounds like lignin and plant polymers. It is also feasible that fungi, like animals, need detoxification systems reliant on CYP activity to avoid toxic compounds produced by competing microbes and plants. Our results demonstrate that the

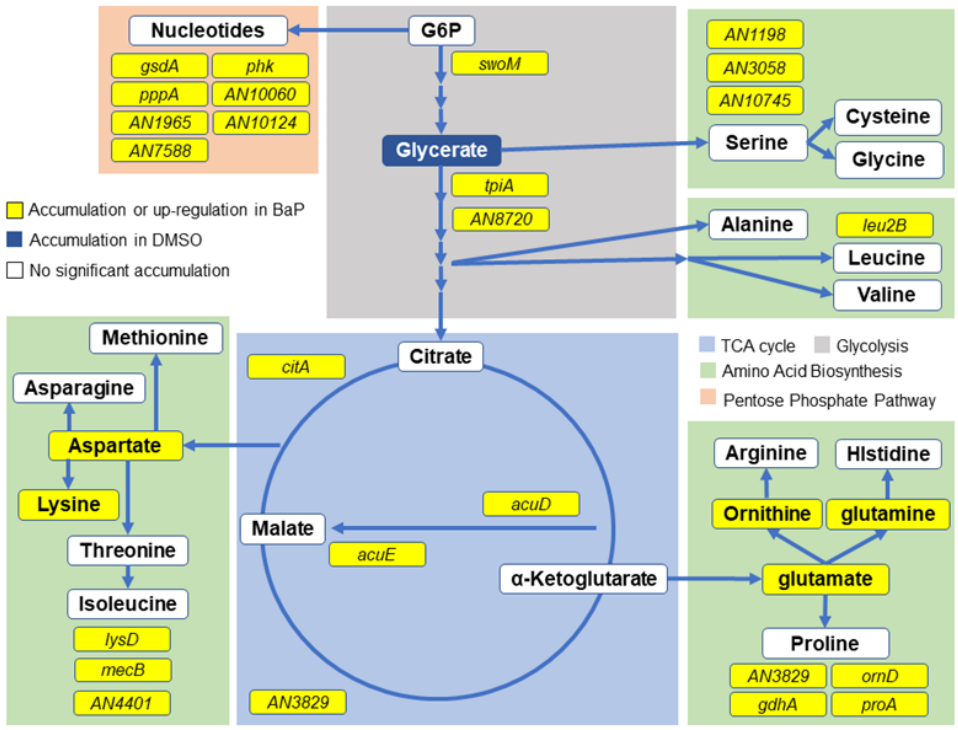
regulation of *bapA* is governed by response to carbon starvation, rather than exposure to a toxicant (benzo(a)pyrene).

The *A. nidulans*, *A. flavus*, and *A. fumigatus* genome each contains over 100 encoded CYPs, with 90, 93, and 57 family types respectively, yet only 45 types are shared(Chen *et al.*, 2014). Despite this diversity, BapA (CYP617D1) is found in all three distantly related *Aspergillus* and *Penicillium* sp., and in *A. nidulans* and *A. flavus* it plays the same functional role of metabolically transforming benzo(a)pyrene. Because CYPs demonstrate substrate promiscuity, it is likely that BapA oxidizes other compounds, such as other PAHs and/or large planar endogenous compounds. Deletion of *bapA* showed no obvious phenotypic differences from WT, revealing that BapA does not likely play a major housekeeping role during growth and development. The distantly related wood rotting fungus *P. chrysosporium* also contains two benzo(a)pyrene metabolizing CYPs which show high sequence similarity to BapA, although not enough to place them within the same CYP family. This indicates that benzo(a)pyrene metabolism conferred fitness multiple times in soil dwelling fungi.

Regulation of *bapA* also demonstrates a novel understanding of how *Aspergillus* sp. respond to organic contaminants like benzo(a)pyrene. Humans and fungi have evolved different strategies for dealing with exposure to benzo(a)pyrene, yet both employ the use of CYPs to transform this compound. Humans do not invest energy into utilizing carbon sources more complex than hexose and its polymers, so CYP transformation of benzo(a)pyrene yields more polar metabolites which can then be excreted. Regulation of encoded benzo(a)pyrene metabolizing CYPs is predominantly governed by the aryl hydrocarbon receptor (Shimada and Fujii-Kuriyama, 2004), yet benzo(a)pyrene and its

metabolites also activate the transcription factor NF-κB (Weng *et al.*, 2004; Bolotina *et al.*, 2007; Ji *et al.*, 2013). NF-κB is a protein heterodimer consisting of p50 and RelA which upon activation by many types of cellular stress, from microbial and viral proteins to ionizing radiation, initiates proper immune response, blocks apoptosis, and promotes cell survival (Pahl, 1999).

A


Nucleotides
gsdA
phk
pppA
AN10060
AN1965
AN10124
AN7588
G6P
swoM
Glycerate
tpiA
AN8720
AN1198
AN3058
AN10745
Serine
Cysteine
Glycine
Alanine
leu2B
Leucine
Valine
Accumulation or up-regulation in BaP
Accumulation in DMSO
No significant accumulation
TCA cycle
Glycolysis
Amino Acid Biosynthesis
Pentose Phosphate Pathway
Methionine
Asparagine
Aspartate
Lysine
Threonine
Isoleucine
lysD
mecB
AN4401
citA
Citrate
acuD
Malate
acuE
α-Ketoglutarate
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ornD
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proA

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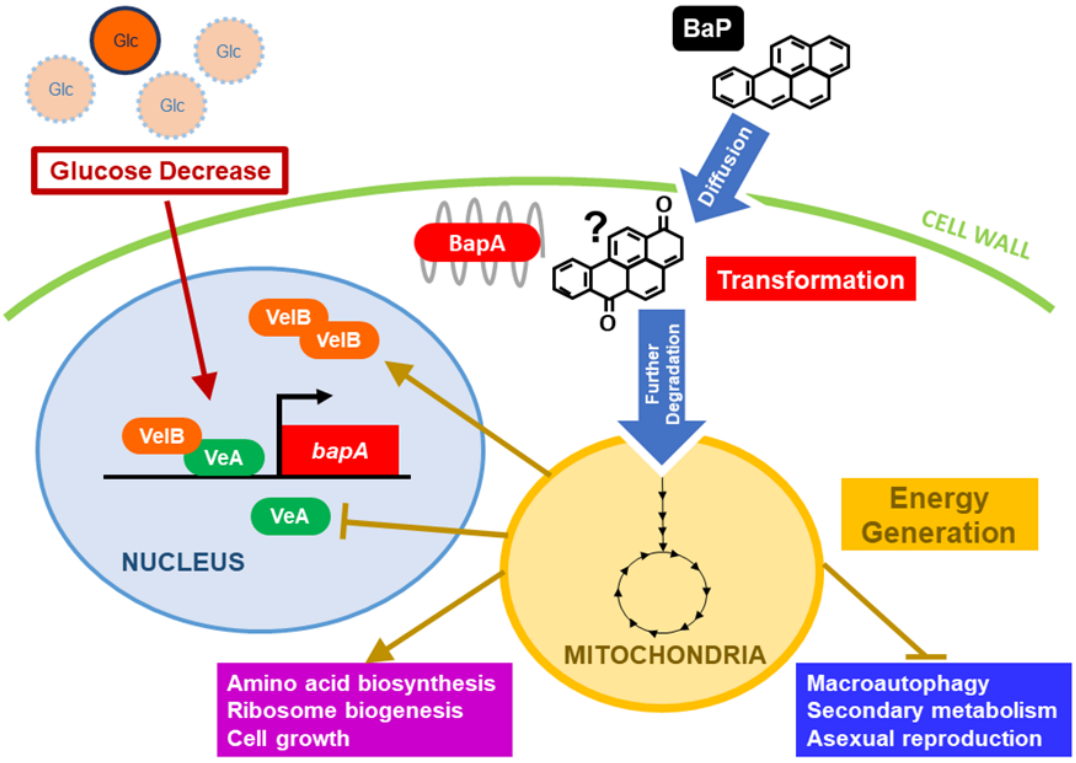
Glc
Glc
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Glucose Decrease
BaP
Diffusion
CELL WALL
BapA
?
Transformation
VelB
VelB
VelB
VeA
bapA
VeA
NUCLEUS
Further Degradation
Energy Generation
MITOCHONDRIA
Amino acid biosynthesis
Ribosome biogenesis
Cell growth
Macroautophagy
Secondary metabolism
Asexual reproduction

Figure 2.6: Proposed pathway of benzo(a)pyrene degradation and metabolic utilization. A. Benzo(a)pyrene treatment affects changes in energy generation pathways indicated by colored blocks. In each block genes which are associated with this pathway that were differentially upregulated in benzo(a)pyrene (BaP) vs DMSO treatment (log_2FC >1 and p-value < .05) in RNA seq experiment are shown in yellow. Amino acids and other compounds which are found in greater quantity as a result of benzo(a)pyrene treatment in wild-type but not Δ*bapA* cells are shown in yellow and white boxes, where yellow indicates statistical significance (p< .05). Blue boxes represent cellular components which significantly decrease as a result of benzo(a)pyrene treatment in wild-type but not Δ*bapA* cells (p< .05). **B**. Proposed molecular pathway resulting in expression of *bapA*, degradation of benzo(a)pyrene and cellular responses to energy generated from benzo(a)pyrene carbon.

Filamentous fungi, on the other hand, act more as ecological scavengers and are capable of utilizing large carbon containing compounds such as plant cell wall polymers. These fungi have evolved with the global regulators called the *velvet* family proteins, which contain a DNA binding domain structurally similar to that of NF-kb p50 (Ahmed *et al.*, 2014). The *velvet* regulators in *Aspergillus* sp. govern environmental sensing, orchestration of cell growth, reproduction, stress response, spore viability, and biosynthesis of various secondary metabolites which similarly helps the fungus survive environmental stressors (Bayram and Braus, 2012; Park and Yu, 2012).

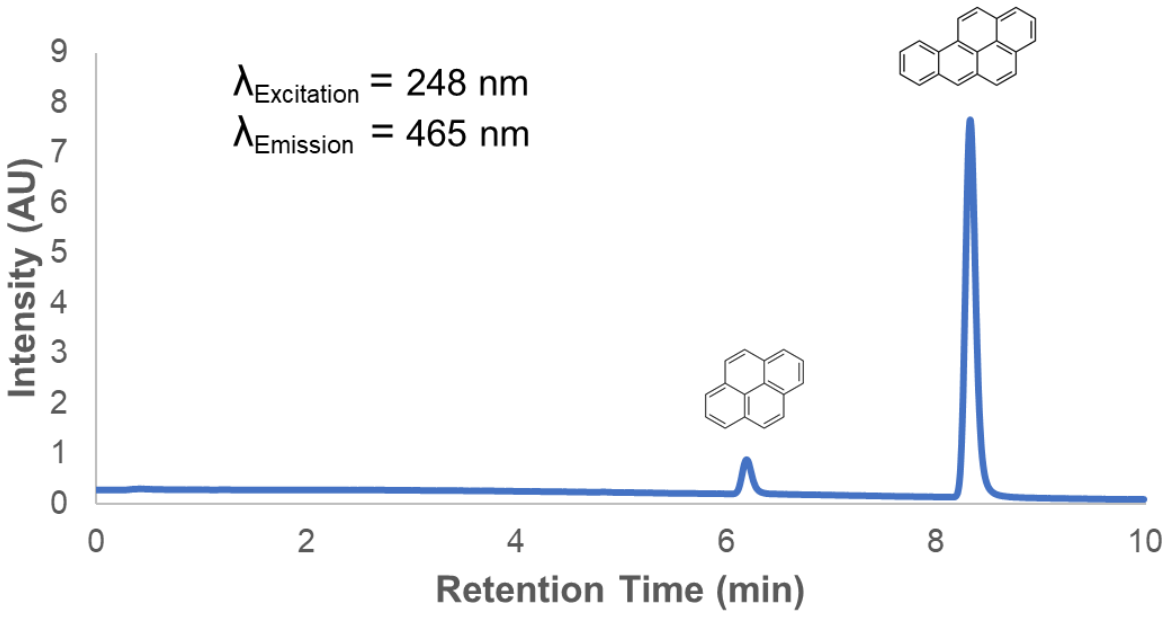
In this study, we have shown that CYP mediated degradation of benzo(a)pyrene requires functions of the velvet family proteins VeA and VelB. These regulatory proteins control the transcript *bapA* in response to stress resulting from carbon insufficiency, as opposed to exposure to xenobiotics. As this CYP is functionally conserved across distantly related fungi, it is likely to play the same role in many ascomycete fungi. Further investigation of substrates metabolized by BapA would reveal its activity on

other environmental contaminants as well as give insight into a possible endogenous function.

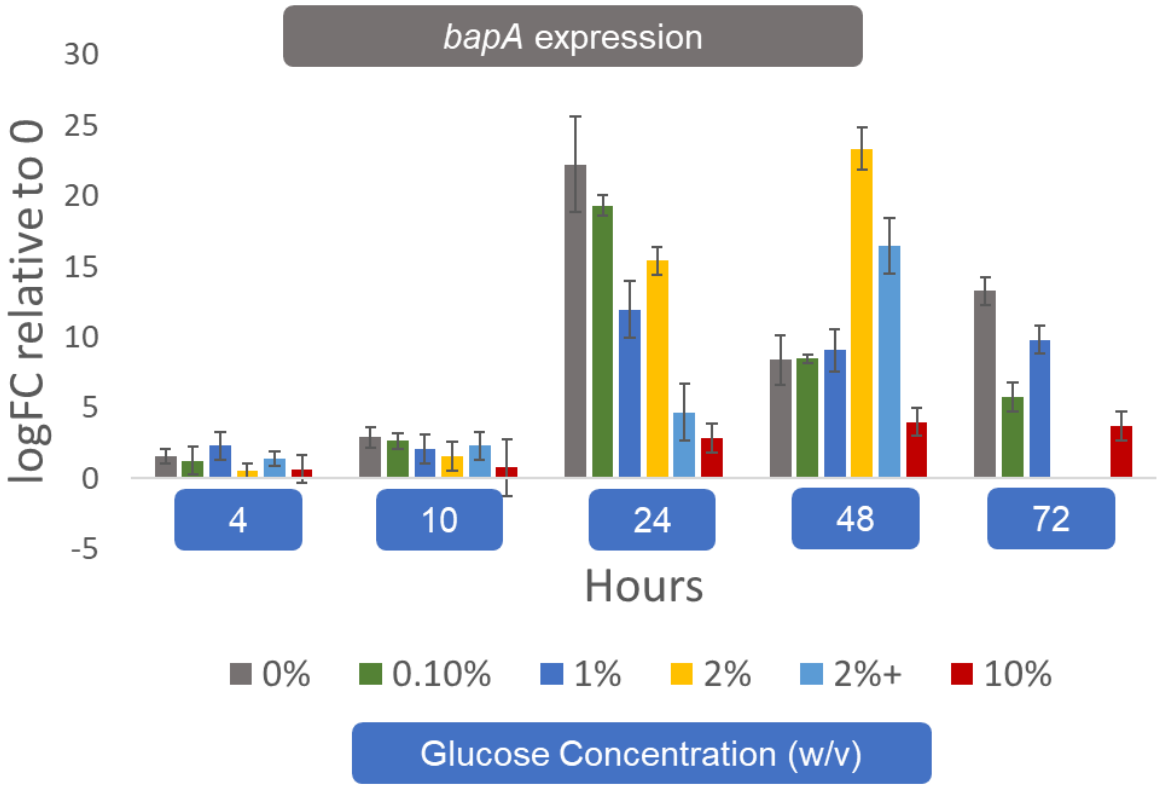
2.6 Acknowledgements

The authors thank U.S. EPA STAR Fellowship for the support of the research and the University of Wisconsin Biotechnology Center DNA Sequencing Facility for providing Illumina sequencing facilities and services. We would also like to thank Nancy Keller, Philipp Wiemann and Mengyao Niu for their insights and suggestions regarding biology and metabolism of *Aspergillus* sp.; Andrew Maizel for his proficient implementation of HPLC method; Christopher Bradfield, Anna Shen, and Susan Moran for their vast knowledge of cytochrome P450 methodology and technical help; and Trevor Penning for his assistance with benzo(a)pyrene metabolites. Without their help, this research would not be possible, however, they may not agree with all of the conclusions of this paper.

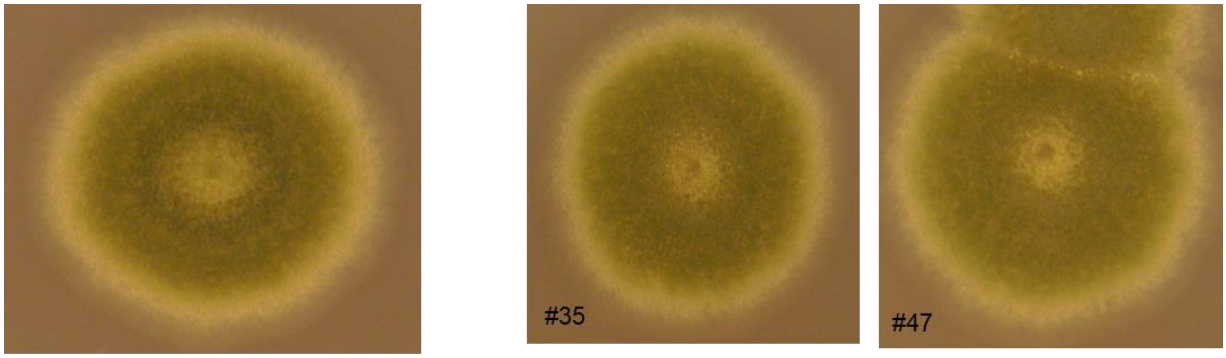
2.7 Supplementary Figures and Tables



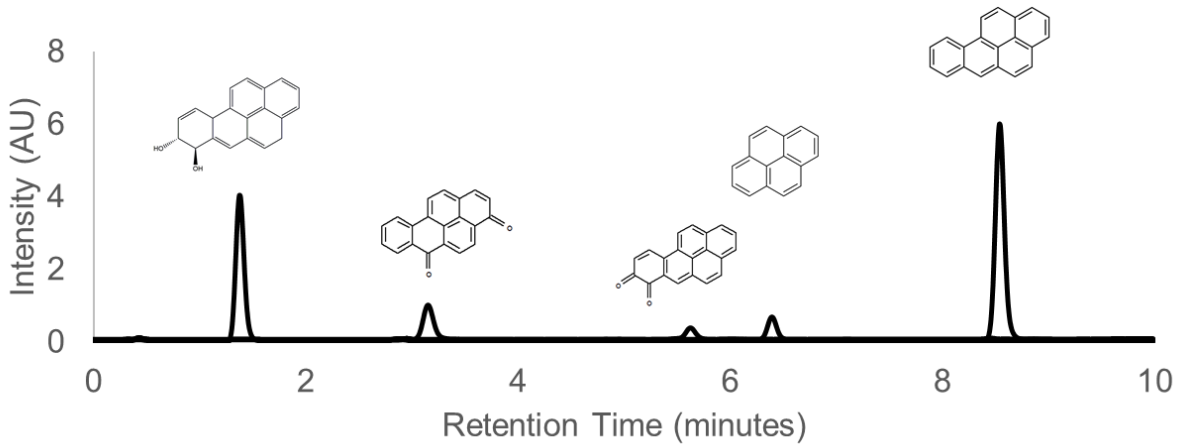
S2.1: **Chromatogram showing compounds extracted from whole cell cultures**.
Extract from *Aspergillus nidulans* cultures after 7 days incubation with benzo(a)pyrene was prepared and analyzed by HPLC as described in methods. Pyrene and benzo(a)pyrene peak retention times matched those of authentic standards as indicated by chemical structure.



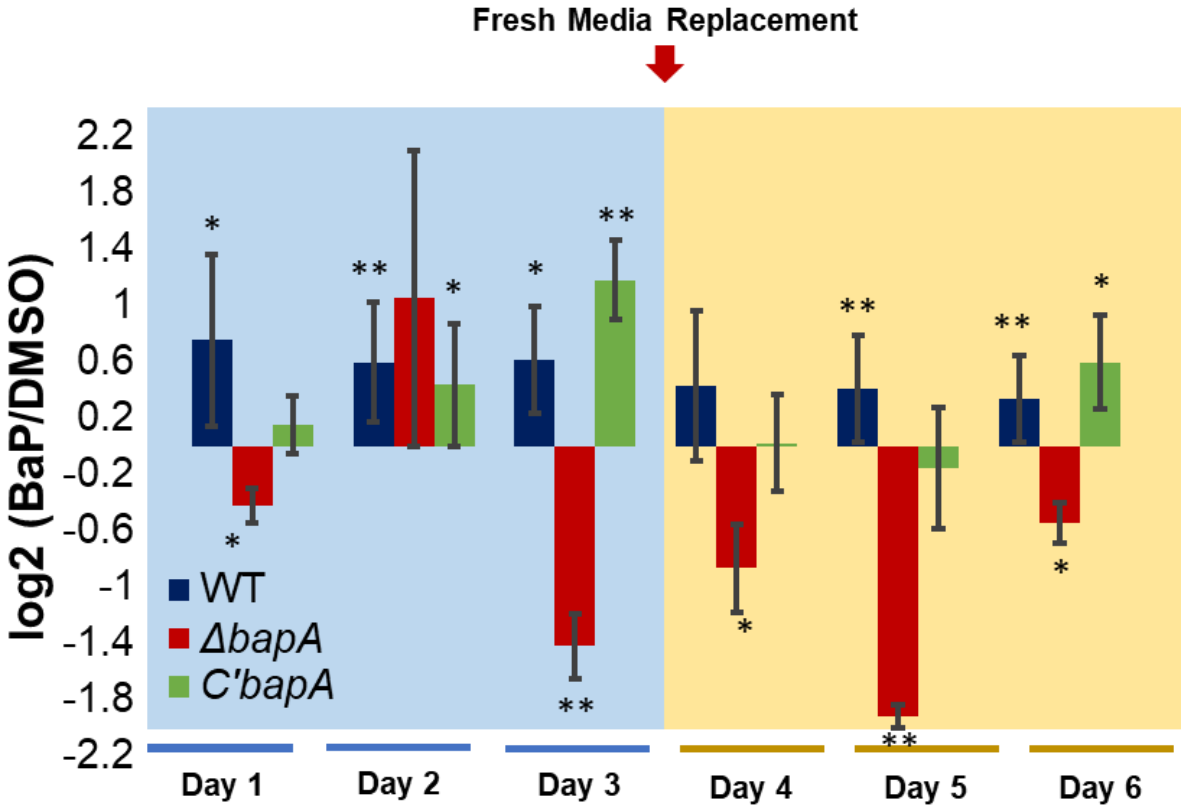
S2.2: Expression of *bapA* increases as glucose becomes scarce. Expression of *AnibapA* (AN1884) was measured using $2^{-\Delta\Delta ct}$ in which *bapA* expression (c_t) was found relative to the reference gene *actA* (ɣ-actin) (Δc_t) and then relative to timepoint 0 ($\Delta\Delta c_t$). Timepoint 0 indicates exponential growth (18 hours post germination) in MMG, and each timepoint represents hours after switch to minimal media indicated. Complete media is indicated by 2%+ Each sample was performed using technical triplicate for RT-qPCR accuracy and three biological triplicates were used for each timepoint. The mean is plotted as a bar and error bars represent standard error.



S2.3: Growth and sporulation of Δ*bapA* does not differ from wild-type (WT). Colony photos of each indicated strain showing growth and sporulation 3 days post inoculation on MMG.



S2.4: **Chromatogram of authentic standards of known benzo(a)pyene metabolites.** Metabolite standards benzo(a)pyrene-7,8-diol, benzo(a)pyrene-3,6-dione, and benzo(a)pyrene-7,8-dione, pyrene and benzo(a)pyrene indicated by chemical structure were diluted to 1 uM in 1:1 solvent A: solvent B and run by HPLC method described in methods with FLD $\lambda_{excitation}$ = 248 nm, $\lambda_{emission}$ = 465 nm.



S2.5: *AnibapA* shows reduced cell viability relative to control treatment. Relative AlamarBlue® reduction (%) was determined in cells treated with benzo(a)pyrene and solvent only (DMSO) in 0.1% glucose MM in each indicated strain. Timepoints (days) represent the number of days cells were incubated with benzo(a)pyrene or DMSO. Fresh media replacement indicates removal and washing of cells and addition of fresh media. All treatments were performed in triplicate. Mean values are plotted with a bar, and error bars represent standard error. Significance between benzo(a)pyrene and DMSO was determined at each timepoint using student's t-test, and indicated as $p<.05$ (*) and $p<.01$ (**).

	CYP name (aa#)	A. nidulans locus	identities	E-value	A. flavus locus	E-value	A. fumigatus locus	E-value
Pc-Pah-1	CYP617 D1 (544)	AN1884	32%	3e-66	AFL2G_ 02761	4.00E-99	Afu2g04 290	0.00E+0 0
	CYP547 C1 (549)	AN11142	27%	4E-23	AFL2G_ 11890	0.00E+0 0	Afu8g02 610	0.00E+0 0
	CYP539 D1 (538)	AN3917	24%	8E-21	AFL2G_ 09153	0.00E+0 0	Afu6g08 460	0.00E+0 0
	CYP680 A1 (515)	AN0338	25%	4e-12	AFL2G_ 11819	5.00E-75	Afu5g01 360	6.00E-73
Pc-Pah-3	CYP620 E1 (524)	AN1601	36%	1e-99	AFL2G_ 00089	0.00E+0 0	Afu8g00 510	7.00E-124
	CYP663 A1 (543)	AN7399	31%	2e-59	AFL2G_ 02173	2.00E-64	Afu1g11 390	4.00E-69
	CYP677 A1 (502)	AN8615	31%	2e-08	AFL2G_ 06876	2.00E-76	Afu5g01 360	3.00E-50

Table S2.1: Candidate CYPs with high sequence similarity to PAH metabolizing CYPs in wood rotting fungus. Identities and e-value of CYPs in *Aspergillus nidulans* identified by alignment using BLASTp of benzo(a)pyrene metabolizing CYPs in *Phanerochaete chrysosporium.* Orthologs in *A. flavus* and *A. fumigatus* show e-value of alignment to CYP in *A. nidulans*.

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Chapter 3:

Dual role of a cytochrome p450 monooxygenase in *Aspergillus flavus* contributes to biosynthesis of a secondary metabolite and degradation of benzo(a)pyrene

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EOL and J-HY contributed to designing and performing the experiments, and analyzing the data. EOL, CR and J-HY contributed to writing and editing the chapter.

3.1 Abstract

Cytochrome P450 monooxygenases (CYPs) play important physiological roles in organisms and are encoded in great abundance in soil-dwelling filamentous fungi. *Aspergillus flavus* contains 153 CYPs, yet the function of many are not known. Two major roles played by CYPs that are of great importance to humans are the biosynthesis of secondary metabolites and the degradation of toxic environmental contaminants. Because CYPs demonstrate substrate promiscuity, it is possible a single CYP is capable fulfilling both roles. Here we demonstrate a CYP, AFLA_135430, which is positioned within a secondary metabolite cluster may play a dual role in the degradation of the problematic soil contaminant benzo(a)pyrene. Deletion of this CYP results in morphological changes including aberrations in growth, improper reproduction and imbalance of secondary metabolite production. This null mutant also shows significantly less degradation of benzo(a)pyrene compared to wild-type. This study unravels novel findings which could be used to understand evolutionary events that led to CYP diversity and function in fungi, as well as assist in the engineering of bioremediation strategies.

3.2 Introduction

Fungi have evolved numerous methods for surviving in hostile and nutrient limited environments, including a vast array of cytochrome P450 monooxygenases (CYPs; (Chen *et al.*, 2014). CYPs are a superfamily of enzymes which are found in every kingdom of life, even viruses (Lamb *et al.*, 2009). In fact, soil-dwelling filamentous fungi encode more numerous CYPs relative to their genome size than bacteria, animals and fungi that reside in other environments (Cresnar and Petric, 2011). CYPs play both anabolic (e.g., membrane and hormone synthesis) and catabolic functions (for toxin elimination or energy generation). CYP families CYP51 and CYP61 play housekeeping roles in fungi, yet the majority of the remaining CYPs have not been described (Chen *et al.*, 2014). Some CYPs in fungi are upregulated after exposure to contaminants like polycyclic aromatic hydrocarbons (PAHs), and may play a role similar to animals' detoxification system (Syed *et al.*, 2010).

Polycyclic aromatic hydrocarbons (PAHs) are by-products of the partial combustion of organic matter and have many sources in the environment. Their ubiquity has increased due to growing use of coal and fossil fuels for energy that consequently contributes to their formation (U.S. Centers for Disease Control Agency for Toxic Substances and Disease Registry (ATSDR), 1995). Additionally, high molecular weight PAHs are extremely stable in the environment and are not susceptible to abiotic degradation (Abdel-Shafy and Mansour, 2016). Many organisms, however, are equipped with enzymes to transform compounds like PAHs, including microorganisms which dwell in PAH contaminated soils (**Chapter 1**). Some CYPs are capable of metabolizing PAHs,

making them more water soluble and excretable. CYP activation of the PAH benzo(a)pyrene in humans, however, results in the formation of reactive metabolites which causes DNA adducts and other oxidative damage in cells (Ortiz de Montellano, 2005). Microbial metabolism of PAHs is critical to the removal of PAHs to prevent human exposure.

Aspergillus flavus is a common soil dwelling fungus, and opportunistic plant pathogen which infects oilseed crops such as peanuts and maize (Klich, 2007). The genome of *A. flavus* encodes an impressive 153 CYPs (Khaldi *et al.*, 2010; Cerqueira, Arnaud, Inglis, Skrzypek, Binkley, Simison, Miyasato, *et al.*, 2014). Although the function of many are not yet know, secondary metabolite gene clusters are known to contain one or more CYPs (Khaldi *et al.*, 2010). These CYPs act as tailoring enzymes which modify the secondary metabolite assembled by the rest of the proteins in the cluster (Keller, 2015). Fungal secondary metabolite clusters encode the enzymatic machinery to produce clinically important compounds that serve as antimicrobials, cholesterol lowering drugs, and anti-cancer medications (Keller, 2015). They also encode toxins like the most potent naturally produced carcinogen, aflatoxin B1 (Klich, 2007). Some secondary metabolite gene clusters contain enzymes, possibly CYPs, to protect the fungus from self-harm (Keller, 2015). For instance, *gliT* encodes an oxidoreductase found in the gliotoxin gene cluster in *Aspergillus fumigatus* which can metabolize gliotoxin into a less toxic compound (Scharf *et al.*, 2010). CYPs demonstrate substrate promiscuity, resulting in the ability to metabolize many different compounds including those which are not endogenous. This enables the fungus to adapt during changing selective pressure (Nath and Atkins, 2008), and explains how fungi are able to metabolize so

many different compounds, including man-made chemicals which end up in the environment (Durairaj, Hur and Yun, 2016).

In this study we show a CYP belonging to a predicted secondary metabolite cluster may fortuitously play a dual role in the degradation of benzo(a)pyrene in *Aspergillus flavus*. We also characterize a previously unstudied secondary metabolite gene cluster revealing the metabolite's function in development and production of other secondary metabolites. This information is important for our understanding of the endogenous function of contaminant degrading CYPs, which we can use to engineer strategies to assist with bioremediation techniques. This study also opens the possibility to discover a new fungal secondary metabolite which may have a useful role to humans.

3.3 Materials and Methods

Strains, media, and culture conditions

Aspergillus strains used in this study are listed in **Table 3.1**. All media used in this study were prepared as previously described (Kafer, 1977). Briefly, for benzo(a)pyrene degradation, 10^6 spores/mL were added to 400 mL minimal media (MM) with 1% glucose (GMM) in 2 L flasks and incubated 18 hours in 220 rpm at 37°C. The mycelial aggregates were then collected on sterile Miracloth, rinsed, and transferred to 100 mL MM with 0.1% glucose in 250 mL Erlenmeyer flasks. Heat killed controls were autoclaved on liquid cycle for 20 minutes at 121°C. Stock solution benzo(a)pyrene (Sigma-Aldrich) (100 mM) in dimethyl sulfoxide (DMSO) was added to the cultures to a final concentration of 200 μM; the same volume DMSO was added to controls. All flasks

were further incubated at 220 rpm, at 37°C for 7 days. For aflatoxin B1 quantification, 10^6 spores/mL were added to 100 mL of potato dextrose broth and either incubated in 250 mL Erlenmeyer flasks at 220 rpm and 37°C (shaking cultures) or 2 mL were pipetted into a 50 mL sterile culturing tube and positioned at a slant at 30°C for 5 days. Peanuts were inoculated as described previously (Kale *et al.*, 2008). Briefly, sterilized peanut cotyledons were mixed with 10 mL sterilized water containing 10^5 spores or as a control water alone. The cotyledons were then plated on wet filter paper lined petri dishes to retain moisture.

Physiological experiments

Spore and sclerotia production were performed as previously described (Amaike and Keller, 2009). Briefly, 200 spores were spread on GMM plates and sealed and placed in complete darkness to induce sclerotia formation or left exposed to air in continuous light to induce conidiation, at 30°C for 5 days. Spores were counted by washing plates with 10 mL 0.01% Tween 80 buffer (v/v) in water, diluted 10x and counted using a hematocytometer. Sclerotia were exposed by spraying plates with 70% ethanol to remove spores and counted manually.

Table 3.1. *Aspergillus* strains used in this study.

Strain	Species/Genotype	Source/Reference
NRRL 3357	*Aspergillus flavus* Wild Type	FGSC [a]
3357.5	*pyrG−*	(Szewczyk *et al.*, 2006)
TEO3	Δ*AFLA_135430*::*AfupyrG+* ; *pyrG−*	This study

[a] Fungal Genetic Stock Center

Generation of *AFLA_135430* deletion

The oligonucleotides used in this study are listed **Table 3.2**. Double-joint PCR was used to generate the deletion constructs of *AFLA_135430* (Yu *et al.*, 2004). Briefly, the deletion construct containing A. fumigatus pyrG marker with 5' and 3' flanking regions of *AFLA_135430* was introduced into the recipient strain NRRL3357.5 (Szewczyk *et al.*, 2006). Multiple deletion mutants (Δ*AFLA_135430*) in A. flavus were generated and confirmed using PCR which all behaved the same in physiological experiments and aflatoxin B1 quantification. We randomly chose TEO3 (Δ*AFLA_135430*) as the testing strains for further experiments.

Table 3.2: Primers used in this study

Name	Sequence (5' - 3') [a]	Purpose
oEO-8	AACACAAACCCGGCCATATCG	AFLA_135430.2 flanking region 5'
Oeo-9	*TTTGGCCTGTATCATGACTTCAGC*AGATAGTATTATAGTAATTATAG	AFLA_135430 3' with *AfupyrG* tail
oEO-10	*GTGACGACAATACCTCCCGACGAT*GATAACCGTGAGGCATGAATG	AFLA_135430 5' with *AfupyrG* tail
oEO-11	TTAGGAATGCCCCTCCGCAC	AFLA_135430 flanking region 3'
oEO-12	GCTATTGCCATGATTGAGAT	AFLA_135430 flanking region 5' nested
oEO-13	AGTACAGCGGCTGAAAACAGTCT	AFLA_135430 flanking region 3' nested
oMK-371	GAGGGATTCAGCAACCTGAT	AFLA_135430.2 qPCR 5'
oMK-372	TAGGATGCCGCTTAGACCTT	AFLA_135430.2 qPCR 3'
oEO-82	GAGGTTGTATGTGCATTCCG	AFLA_135490 qPCR 5'
oEO-83	TGTCTGAGTGATGGGCTCTC	AFLA_135490 qPCR 3'
oEO-84	CCAAATCTCCCAGAAAGTGACA	AFLA_135500 qPCR 5'

oEO-85	TGTCGACATTCCCAGGTAAGG	AFLA_135500 qPCR 3’
oMK-379	TCGTTACCTCACCTGCTCTG	Afl-qPCR tubulin F1
oMK-380	TGGAGGACATCTTGAGACCA	Afl-qPCR tubulin R1
	[a] Tail sequences are shown in italics. Restriction enzyme sites are in bold	

Extraction and HPLC quantification

The extraction and quantification of benzo(a)pyrene was previously described (**Chapter 2**). Briefly, cell cultures were extracted using pyrene (Sigma-Aldrich; final concentration = 200 µM) as an internal standard to correct for extraction efficiency. The entire mixture was sonicated using Sonic Dismembrator model 100 (Fisher Scientific), centrifuged to remove particulate matter, and diluted 100x in 1:1 solvent A (30 mM acetate buffer pH 4.7, 10% acetonitrile): solvent B (acetonitrile). Benzo(a)pyrene and pyrene were quantified by high-performance liquid chromatograph (HPLC) using method previously described (Chapter 2).

RNA preparation and qRT-PCR

RNA prep and qRT-PCR were performed as described in chapter 2. Briefly, Fungal cells from the submerged cultures were collected at designated time points, squeeze-dried, flash frozen in liquid nitrogen, and stored at -80°C until subject to RNA preparation. Development in light and dark was performed as follows: 10^6 spores/mL were used to inoculated 200 mL GMM and grown at 30°C for 18 hours. Mycelium pellets were filtered and washed, then thinly spread on GMM agar plates, and sealed and placed in

complete darkness (sclerotia development) or left unsealed and exposed to continuous light (conidiation). At designated timepoints, a 1x1 inch square was cut from the center of the plate, the mycelium squeeze-dried, flash frozen in liquid nitrogen, and stored at -80°C until subject to RNA preparation .

Total RNA isolation was done using Trizol as described previously (Yu *et al.*, 2004). cDNA was prepared using AMV reverse transcriptase kit (NewEngland Biolabs) with oligo-dT primer. Reverse transcriptase quantitative PCR (RT-qPCR) was performed with iTaq universal SYBR green supermix (Bio-Rad, Hercules, CA) on a Bio-Rad CFX96 real-time PCR detection system. mRNA was normalized using $2^{\Delta\Delta C}$t method (Livak and Schmittgen, 2001) Primers used are listed in **Table 3.2**.

Microscopy

Sony digital (DSC-F828) camera was used to take colony photos of growth and reproduction. Micrographs showing peanut colonization were taken using a Zeiss M2Bio microscope equipped with AxioCam and AxioVision digital imaging software.

Aflatoxin B1 quantification

Aflatoxin (AFB1) was extracted and quantified as previously described (Wu *et al.*, 2017). Briefly, two mL of the culture (without filtration to account for AFB1 in the hyphae), or the entire stationary culture tube were mixed with an equal volume of chloroform, vigorously vortexed, and centrifuged. The chloroform (bottom) layer of each sample was

transferred and evaporated in a glass tube overnight. Peanut extraction was done as described previously (Kale *et al.*, 2008) by mixing peanut cotyledons with 5 mL chloroform and left at 150 rpm for 10 minutes in a rotary shaker. The chloroform layer was removed and dried in a glass tube. For HPLC analysis, each dried sample was dissolved in 500 µL methanol and filtered through a 0.45 µm filter into HPLC vials. Each sample was injected into the HPLC (Agilent 1260 series) at a flow rate of 0.8 mL/min with water: acetonitrile: methanol (20:40:40, v/v). AFB1 was detected using a diode array detector at a wavelength of 365 nm (Lillehoj, Ciegler and Hall, 1967). The injected volume was 10 µL and the separation was performed via Agilent HPLC column Zorbax Eclipse XDB-C18 (5 µm, 4.6 x 250 mm). For TLC analysis, dried samples were reconstituted in 100 µL chloroform and separated silica gel TLC plate using a chloroform: acetone (95:5 (vol/vol)) solvent system (Kale *et al.*, 2008).

3.4 Results

3.4.1 *AFLA_135430* belongs to a predicted secondary metabolite cluster

Aspergillus spp. are able to degrade the PAH benzo(a)pyrene, including *A. flavus* (**Chapter 2**). Although we were able to identify one CYP (BapA) which is responsible for benzo(a)pyrene degradation in both *A. nidulans* and *A. flavus*, we hypothesized that there are more than one benzo(a)pyrene degrading CYP in *A. flavus,* considering mammals encode several benzo(a)pyrene metabolizing CYPs and given that *A. flavus*

contains more encoded CYPs (153) than *A. nidulans* (119; (Chen *et al.*, 2014) and humans (57; (Nelson, 2009).

In order to identify additional CYPs in *A. flavus* which may be responsible for PAH degradation, we used blastp to look for proteins with high homology to the PAH degradation CYPs in *Phanerochaete chrysosporium* (Syed *et al.*, 2010) and *A. nidulans* (Chapter 2). *AFLA_135430* showed high similarity to PC-PAH 5 and PC-PAH 1 in *P. chrysosporium*, which were shown to metabolize pyrene and benzo(a)pyrene (**Table 3.3**; Syed *et al.*, 2010). *AFLA_135430* was also found encoded near a non-ribosomal protein synthase (NRPS), and is predicted to be part of a secondary metabolite gene cluster (Khaldi *et al.*, 2010). We hypothesized that CYPs involved in secondary metabolite biosynthesis could also be involved in contaminant degradation and tested this by analyzing the role AFLA_135430 plays in both secondary metabolite biosynthesis, and benzo(a)pyrene degradation.

Table 3.3: **Blastp of PAH degrading cytochrome p450s in *Phanerochaete chrysosporium***

	CYP name (aa#)	***A. nidulans* locus**	***A. flavus* Locus** (Broad Institute* and NCBI)	**E-value (to Ani)**	**E-value (to PC)**
Pc-Pah-1 **Benzo(a)pyrene and Pyrene**	**CYP617D1 (544)**	AN1884	AFL2G_02761 AFLA_036020	4.00E-99	7e-41
	CYP547C1 (549)	AN11142	AFL2G_11890 AFLA_122460	0.00E+00	2e-47
	CYP539D1 (538)	AN3917	AFL2G_09153 AFLA_128090	0.00E+00	2e-79
	CYP677A1 (502)	AN8615	AFL2G_06876 AFLA_135430	2.00E-76	8e-08

Pc-Pah-2 **Pyrene**	**CYP620D1 (529)**	AN3394	AFL2G_00089 AFLA_058760	6.00E-125	2e-114
	CYP530A3 (525)	AN8358	AFL2G_02173 AFLA_029720	6.00E-70	1e-67
	CYP619B1 (554)	AN10613	AFL2G_03370 AFLA_017300 **Identities 41%[1]**	2.00E-48	7e-113
Pc-Pah-3 **Benzo(a)pyrene and Pyrene**	**CYP663A1 (543)**	AN7399	AFL2G_02173 AFLA_029720	2.00E-64	4e-55
Pc-Pah-5 **Pyrene**	**CYP677A1 (502)**	AN8615	AFL2G_06876 AFLA_135430	2.00E-76	3e-101
	CYP680A1 (515)	AN0338	AFL2G_11819 AFLA_121680	5.00E-75	5e-74
	CYP682A1 (511)	AN7932	AFL2G_00090 AFLA_073140	2.00E-105	4e-37
	CYP58C1 (533)	AN5360	AFL2G_07403 AFLA_065760	2.00E-125	5e-70
	CYP58D1 (519)	AN9313	AFL2G_03972 AFLA_105070	0.00E+00	2e-64
	IvoC (512)	AN10573	AFL2G_00090 AFLA_073140	4.00E-97	4e-37
	CYP682D1 (531)	AN1794	AFL2G_00090 AFLA_073140	0.00E+00	4e-37
	CYP681A1 (517)	AN3609	AFL2G_00090 AFLA_073140	4.00E-83	4e-37

[1] Greater than 40% identities indicates same CYP family (Nelson, 2006)

The predicted cluster encodes two CYPs, AFLA_135430 and AFLA_135440, a trichodiene synthase (AFLA_135450), an ornithine decarboxylase (AFLA_135470), a

galactose-proton symport (AFLA_135480), an NRPS (AFLA_135490), and two proteins of unknown function (AFLA_135460 and AFLA_135500; **Figure 3.1A**; (Khaldi *et al.*, 2010). For the proteins with unknown functions, we used blastp in order to reveal similar proteins which may have an annotated function. AFLA_135460 contained a region belonging to the isoprenoid C1 biosynthetic superfamily and showed sequence similarity to a fusicoccadiene synthase (e-value: 1e-144). AFLA_135500 showed high sequence similarity to ZnII(2)Cys6 transcription factor in *Aspergillus kawachii*, although the sequence was less conserved in the DNA binding domain (**Figure 3.1A**). Regulation of secondary metabolite gene clusters may or may not contain a pathway specific regulatory protein, as some cluster regulation is intertwined into larger superclusters (Wiemann *et al.*, 2013). Regardless, we considered AFLA_135490 (NRPS) and AFLA_135500 (a possible transcription factor) to be the drivers of metabolite synthesis, whereas the AFLA_135430 (CYP) to play a modifying role.

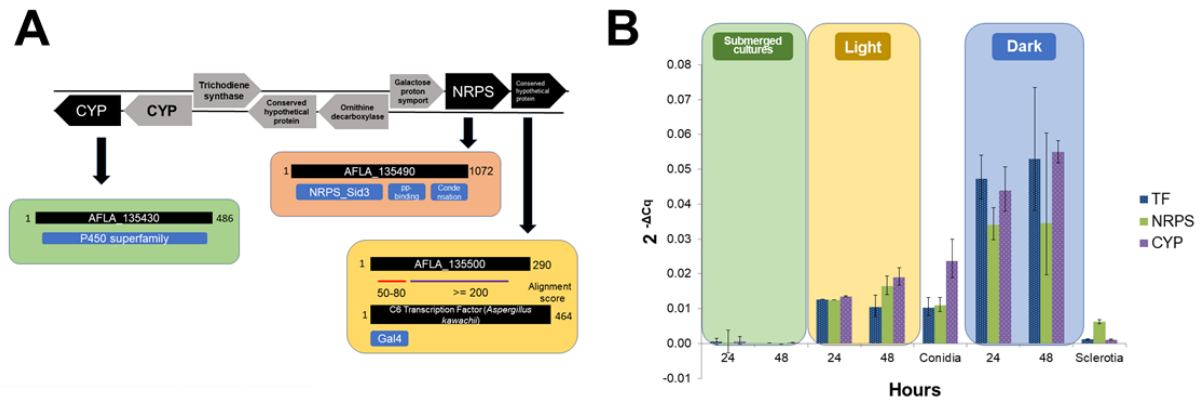

Figure 3.1: AFLA_135430 is encoded in a predicted secondary metabolite gene cluster. A. Examination of genes within the predicted cluster. AFLA_135490 is a non-ribosomal peptide synthase containing a siderophore like domain (NRPS_sid3), and AFLA_135500 shows homology to a $Cys_6Zn(II)_2$ transcription factor, although homology is not conserved the DNA binding domain (Gal4). **B.** Relative expression of AFLA_135430 (CYP) and biosynthetic backbone AFLA_135490 (NRPS) and transcription factor AFLA_135500 (TF) in various stages of *Aspergillus flavus* life cycle. Expression of each target gene (c_t) was normalized to the reference gene β-tubulin (Δc_t).

Because this was only a prediction of members in the secondary metabolite cluster, we wanted to see if expression of these key cluster genes and our CYP of interest are coordinated. By inducing different developmental stages in the *Aspergillus* life cycle, we can not only determine if gene expression is coordinated but predict the regulatory elements which orchestrate its expression given the developmental stage which it is being expressed. All three genes showed the same pattern of expression in each growth condition indicating that they are indeed an integrated cluster (**Figure 3.1B**). Relative to the house keeping gene, β-tubulin, the three members of this cluster are upregulated during developmental stages, especially in dark conditions (**Figure 3.1B**). This indicates this cluster may be under the control of the velvet family complex which coordinates development and secondary metabolite synthesis in response to light (Bayram and Braus, 2012).

3.4.2 Deletion of AFLA_135430 reduces the ability of cells to degrade benzo(a)pyrene

Benzo(a)pyrene is the PAH of most concern because of its toxicity to humans (Kim *et al.*, 1998; Tsuji *et al.*, 2011), making it important to understand if and how fungal CYPs can metabolize it. By deleting *AFLA_135430* we were able to determine if this CYP plays a role in metabolizing benzo(a)pyrene in order to degrade it. We found Δ*AFLA_135430* was able to degrade benzo(a)pyrene, but significantly less than wild-type (**Figure 3.2A**). There are two possible reasons for reduced degradation: 1) this CYP can metabolize benzo(a)pyrene by initiating the first enzymatic step, though it is

not the only CYP which is able to under these conditions, or 2) by deleting this gene, the viability and fecundity of the strain was reduced which leads to general reduced biocatalytic activity.

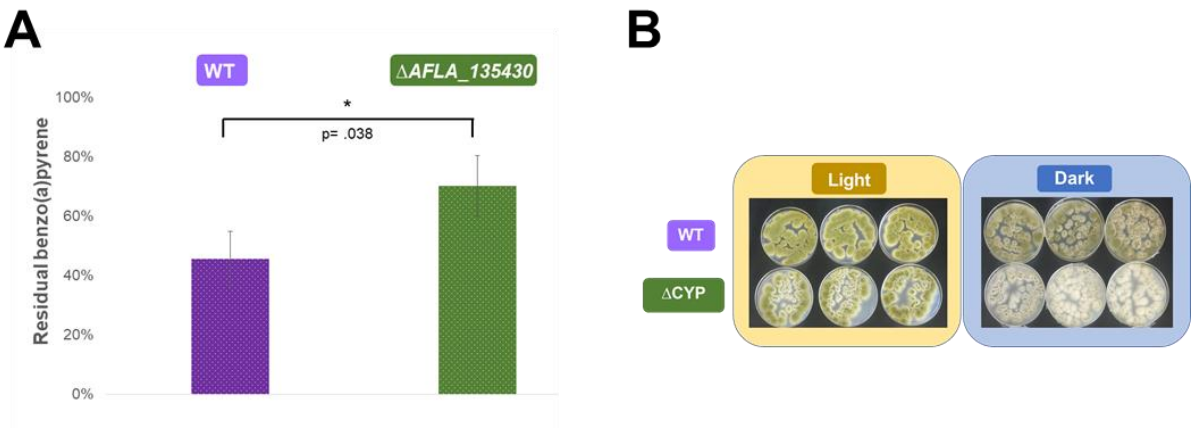

Figure 3.2: **ΔAFLA_135430 shows reduced benzo(a)pyrene degradation and abnormal physiology compared to wild-type**. **A**. Residual benzo(a)pyrene remaining after 7 days growth with each indicated strain. Each experiment was performed in triplicate, with null mutant ΔAFLA_135430 represented by three independent null mutants. Mean is plotted as a bar and error bars representing standard error. Significance was determined using student's t test with p< .05 (*). **B**. Abnormal appearance of AFLA_135430 (CYP) compared to wildtype.

Because we noticed decreased conidiation and increased sclerotia formation in ΔAFLA_135430 colonies compared to wild-type (**Figure 3.2B**), we decided to further investigate the phenotypic differences in growth and development. These apparent differences may mean either imbalance of the secondary metabolite produced by this cluster causes disruption of normal development, or that there is an epigenetic effect resulting from disruption of this locus. This is not enough evidence, however, to conclusively say whether or not the general catalytic ability of this strain is negatively affected by the imbalance of secondary metabolite synthesis.

3.4.3 AFLA_135430 positively influences sporulation and negatively affects aflatoxin accumulation

In order to get a better idea of the fecundity of this strain, as well as investigate the endogenous role of the secondary metabolite, we measured the specific differences in growth, reproduction and AFB1 (a key secondary metabolite) production of Δ*AFLA_135430*. Growth and development are tightly regulated in fungi in response to environmental stimuli in order to utilize available energy appropriately. Secondary metabolites serve many purposes, including signaling differentiation and reproduction (Demain and Fang, 2000). By examining Δ*AFLA_135430*, which we posit is unable to produce the active secondary metabolite, we can determine if this secondary metabolite is indeed involved in the necessary regulation of development. Δ*AFLA_135430* produced significantly fewer spores in both light and dark reproduction conditions (**Figure 3.3A**). Sclerotia, which are the overwintering bodies of *Aspergillus flavus*, were produced in light conditions in Δ*AFLA_135430* but not in wild-type. Interestingly, Δ*AFLA_135430* produced significantly fewer sclerotia in dark conditions compared to wildtype, even accounting for the reduced colony growth (**Figure 3.3A**). The exact effect of AFLA_135430 on growth seems to also be dependent on the environment. In dark conditions with multiple separate growing colonies, growth seems to be stunted by deletion of *AFLA_135430* (**Figure 3.3A**). In contrast, Δ*AFLA_135430* grew faster and more than wildtype when exposed to light and from a single inoculation point in race tubes (**Figure 3.3B**). This is not enough evidence, however, to conclusively say whether

or not the general catalytic ability of this strain is negatively affected by the imbalance of secondary metabolite synthesis.

Because the NRPS contains a siderophore-like domain (**Figure 3.1C**) and *A. flavus* is a problematic plant pathogen which produces secondary metabolites to assist in the colonization of plants, we attempted to discover whether the secondary metabolite produced by this cluster has any effect on peanut colonization. We found a visibly smaller dark band on the exterior of peanuts infected with Δ*AFLA_135430* compared to wildtype, which may suggest reduced hyphal penetration (**Figure 3.3C**). The number of spores produced on during peanut colonization in Δ*AFLA_135430* was fewer than in wild-type, which suggests decreased virulence as well (**Figure 3.3D**). However, reduced growth and decreased sporulation was also seen on agar media, indicating that these data are general defects in Δ*AFLA_135430* and not directly related to colonization of plant tissue.

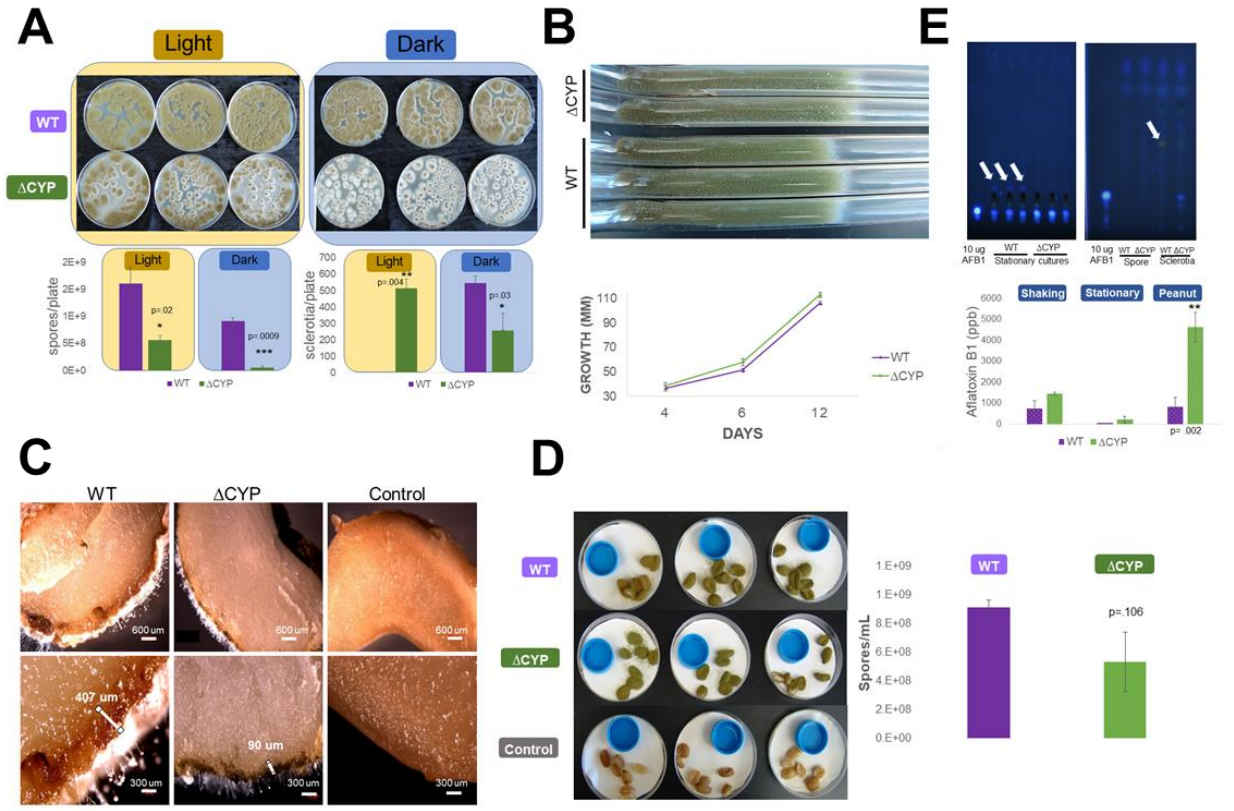

Figure 3.3: **ΔAFLA_135430 demonstrates reduced conidiation, increased sclerotia production, imbalance of secondary metabolite production, and aberrant growth compared to wild-type.** **A.** Quantification of spores and sclerotia in dark and light inducing conditions in wild-type (WT) and Δ*AFLA_135430* (CYP). All experiments were performed in triplicate with Δ*AFLA_135430* represented by three independent null mutants. The mean is plotted as a bar and error bars represent standard error. Significance was determined using student's t test with p<.05 (*) and p<.01 (**). **B**. Growth rate in days of wild-type (WT) and Δ*AFLA_135430* (CYP). Wild-type samples were performed in triplicate and Δ*AFLA_135430* represented by two independent null mutants. Mean was plotted as a line, with error bars representing the standard error. No significance was found due to lack of triplicates. **C**. Quantification of spores produced when grown on peanut cultures. Bars represent mean spore number in wild-type (WT) and Δ*AFLA_135430* (CYP) and error bars represent standard error. No spores were found on water (control) peanuts. No significance was seen due to large deviation. **D**. Microscopy images showing peanut colonization by wild-type (WT) and Δ*AFLA_135430* (CYP) and water (control). Measurement of dark band represented by white bar on peanut edge. No dark band was found on peanuts colonized with water only. **E**. Aflatoxin B1 (AFB1) and other secondary metabolite separation on TLC plates (top) and quantification of AFB1 using HPLC (bottom) in wild-type (WT) and Δ*AFLA_135430* (CYP) strains. Experiments were performed in triplicate with Δ*AFLA_135430* represented by three independent null strains except for spore and sclerotia samples which represent only one wild-type sample and one null mutant strain. Mean AFB1 production in each strain is plotted a bar with error bars representing standard error. Significance was determined using student's t test with p< .01 (**).

The amount of AFB1 produced by Δ*AFLA_135430* was greater than wildtype in all growth conditions, yet this difference was only significant on peanut cultures. This was quantified by HPLC, but we can also see qualitative evidence via TLC plates that AFB1 is more abundant, yet several other unknown metabolites are depleted (**Figure 3.3E**). This indicates global imbalance of the secondary metabolite profile.

3.5 Discussion

Evidence of CYP mediated metabolism of PAHs has been demonstrated in fungi (Cerniglia and Sutherland, 2010), yet the specific CYPs involved have not been studied in great detail. This has left many questions regarding their regulation, substrate promiscuity, and potential endogenous function. We were able to partially demonstrate the function of a predicted PAH metabolizing CYP in *A. flavus*, which shows sequence similarity to a PAH metabolizing CYP in the distantly related wood rotting fungus *P. chrysosporium*. We hypothesized the endogenous function of this CYP, *AFLA_135430*, is secondary metabolite modification during biosynthesis because of its position in a predicted secondary metabolite gene cluster (Khaldi *et al.*, 2010), and its coordinated regulation with other encoded cluster member. Although AFLA_036020 plays the dominant role in benzo(a)pyrene degradation in carbon limited media (**Chapter 2**), Δ*AFLA_135430* also demonstrated reduced benzo(a)pyrene degradation *in vivo* compared to wild-type in carbon limited media indicating this monooxygenase may coincidentally contribute to PAH degradation as well. Because *A. flavus* encodes more numerous CYPs than several other *Aspergillus sp*.(Chen *et al.*, 2014), it is possible that

more than one CYP is utilized depending on the culturing conditions. *P. chrysosporium* utilizes two different degradation mechanisms depending on the culturing conditions: CYP mediated intracellular degradation in nutrient sufficient media and peroxidase mediated extracellular degradation in nutrient limited media (Syed *et al.*, 2010). Thorough investigation of Δ*AFLA_135430* expression in various media types, including PAH containing media, would give insight into its response to nutrient stimuli and xenobiotic exposure. Conditions which stimulate the expression of this gene cluster may yield a clearer phenotype of defective benzo(a)pyrene degradation. For instance, expression of *AFLA_135430* is relatively high in dark, sclerotia inducing conditions, meaning it may play the dominant benzo(a)pyrene degrading role. Testing degradation in Δ*AFLA_135430* under these conditions may yield a more debilitated degradation phenotype.

Δ*AFLA_135430* also showed defects in growth, development, and secondary metabolite production, which may have contributed to reduced benzo(a)pyrene degradation. Disruption of programmed growth and reproduction may result in energy expenditure which cannot then be used for toxin metabolism. It is known that deletion of secondary metabolite biosynthetic genes cause many physiological responses such as defective growth and reproduction, reduced stress tolerance, diminished virulence, and uncoordinated production of other secondary metabolites (Bok and Keller, 2004; Forseth *et al.*, 2012; O'Hanlon *et al.*, 2012; Cary *et al.*, 2015). However, to our knowledge there is no evidence to show deletion of only one tailoring enzyme in a secondary metabolite gene cluster, like a CYP, causes such a profound phenotype.

It can also be hypothesized that *AFLA_135430* plays a role in degradation of AFB1 and/or other secondary metabolites. This could explain why we find more AFB1 in Δ*AFLA_135430* cultures. Because secondary metabolites are toxic to the fungus itself, biosynthetic gene clusters often contain genes which protect the fungus from self-harm (Keller, 2015). CYPs play a detoxification role in mammals and (likely) fungi, so promiscuity in substrate metabolism allows the fungus to adapt to changing environmental toxin exposure . Expression of *AFLA_135430* in a heterologous system would give insight into the diversity of compounds this enzyme is capable of metabolizing, including both environmental contaminants and secondary metabolites.

It is also important to identify the specific secondary metabolite produced by this cluster. Conversations with the Keller lab indicated overexpression of the NRPS in this CYP containing cluster (*AFLA_135490*) results in production of a large hydrophobic compound (Brandon Pfannenstiel, personal communication, May 23, 2018) which fits the description of high molecular weight hydrocarbons like benzo(a)pyrene. By examining the substrate diversity of this CYP we may be able to not only utilize this knowledge for bioremediation purposes, but also gain insight into the endogenous function of CYPs and reveal the evolutionary ancestry of PAH degrading CYPs in fungi.

3.6 Conclusions

A. flavus encodes 153 CYPs which exceeds the number found in many other fungal species (Chen *et al.*, 2014). The function of the majority are not yet known, with the exception of housekeeping CYPs belonging to CYP51 and CYP61 families, and several

which have been experimentally shown to be involved in secondary metabolite biosynthesis (Georgianna *et al.*, 2010). Since many other predicted secondary metabolite gene clusters contain CYPs, it is likely these also play a role in metabolite biosynthesis. What is less well understood, is whether CYPs within these biosynthetic clusters are able to metabolize either endogenous or exogenous toxic compounds in order to protect the fungus from harm. Other oxidizing enzymes are found in secondary metabolite gene clusters, like GliT within the gliotoxin biosynthetic cluster which transforms gliotoxin into a less toxic compound. We hypothesized that AFLA_135430 which is found in a predicted secondary metabolite cluster, transforms this secondary metabolite either during biosynthesis, or as a means of protecting fungal cells from damage incurred by the metabolite itself. We additionally hypothesized that AFLA_135430 is able to transform benzo(a)pyrene as a side effect due to the structural similarity of this five ringed PAH to the secondary metabolite.

We were first able to show this CYP shows coordinated regulation with the NRPS and possible transcription factor encoded in the predicted biosynthetic cluster in dark, sclerotia inducing conditions. This indicates these genes do behave like they are in a coordinated cluster which is regulated by the well know velvet complex, VelB, VeA, and LaeA that is responsible for secondary metabolite production in response to light (Bayram *et al.*, 2008). We also showed that deletion of *AFLA_135430* results in decreased conidiation, increased sclerotia formation in light, aberrations in growth, and imbalance of secondary metabolite production.

Lastly, we showed reduced degradation of benzo(a)pyrene by Δ*AFLA_135430* under glucose limiting conditions. We were not able to conclude if this were due to the specific

result of the CYP deletion, or if the growth and developmental defects caused general loss of catalytic activity in this strain. Further experiments, such as expression of *AFLA_135430* in a heterologous system could more directly answer the specific substrates this CYP is capable of metabolizing. Using various culturing methods i.e. high glucose and constant dark to optimize expression of this cluster may also reveal if this CYP is the predominant mechanism for benzo(a)pyrene degradation under these conditions.

Overall, these results give insight into conditions which may be used to optimize PAH degradation in contaminated sites. A closer examination of metabolism of endogenous compounds by this CYP including identification of the metabolite produced by this biosynthetic cluster, would give insight for the evolutionary role of PAH degrading CYPs in fungi.

3.7 Acknowledgements

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Chapter 4:

Accumulation of the environmental contaminant benzo(a)pyrene in spores of the pathogenic fungus *Aspergillus fumigatus*

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EOL, NR, AH, CR, and J-H Y contributed to designing and performing the experiments, and analyzing the data. EOL, CR, and J-HY contributed to writing and editing the chapter.

4.1 Abstract

Fungal spores represent a major health concern, rivaling man-made air pollution for negative respiratory health consequences in humans. Although there is evidence that the unique metabolic capabilities of fungi allow them to metabolize organic compounds which are toxic to animals, there has been little investigation into the transport of environmental contaminants to fungal spores. Benzo(a)pyrene is a contaminant of concern due to its ubiquity in the environment, and its immunotoxic effects which result in reduced defense against microbial invaders. In this study we find that the common environmental fungus *Aspergillus fumigatus* accumulates benzo(a)pyrene in its asexual spores in a dose responsive manner when cultured in nutrient-poor media. Benzo(a)pyrene was visualized in non-lipid body vesicle-like structures in developing conidiophores, and quantification techniques show the amount in spores exceeds the amount in growth media. Further cellular analyses of spores show benzo(a)pyrene-containing spores may interfere with trehalose and β-glucan biogenesis in spores, thereby affecting the overall host-pathogen interaction. Although we were unable to show significant data to demonstrate increased survival of benzo(a)pyrene containing spores against innate immune system responses in zebrafish, there is literature to suggest benzo(a)pyrene instead inhibits components of the adaptive immune response to spores. Our study implies that other respiratory effects caused by inhalation of benzo(a)pyrene-containing fungal spores need to be investigated further.

4.2 Introduction

Filamentous fungi fill a valuable ecological niche as saprophytes by degrading organic compounds which are not easily degraded by many other organisms. This has made them potential solutions for the accumulation of recalcitrant man-made pollutants in the environment, and have been studied in the context of bioremediation in recent years (Harms, Schlosser and Wick, 2011). Bioremediation strategies including biosorption and bioaccumulation are well studied phenomena in fungi. Biosorption is the chemically favorable partitioning of contaminants to cell wall components and has received positive attention for its ability to remove inorganic contaminants from soil (Dhankhar and Hooda, 2011). Bioaccumulation is the active uptake and storage of environmental contaminants in living tissue which has generally received negative attention because it causes contamination of edible mushrooms (Isildak *et al.*, 2007; Carvalho *et al.*, 2014). Accumulation of environmental contaminants within spores in non-mushroom forming filamentous fungi (molds), however, has not been studied.

Fungi receive considerable attention as indoor air pollutants themselves because they produce an abundance of reproductive spores, of which humans inhale 10^3 - 10^{10} daily (Brown *et al.*, 2012). Inhaled spores cause negative health effects ranging from allergic sensitization to invasive colonization of lung tissue (Latge, 1999). Treatment of fungal infections is challenging because many of the basic mechanisms necessary for human cell survival are shared by closely related eukaryotic fungi. *Aspergillus fumigatus* is an extremely effective opportunistic human pathogen that causes many negative health

effects especially in immunocompromised populations. Invasive aspergillosis (IA) for instance has fatality rates in some clinical groups as high as 90% (Lin, Schranz and Teutsch, 2001). The qualities that make *A. fumigatus* particularly good at invading lung tissue are its ability to grow in a wide range of environmental conditions, the enormous number and efficient dispersion of spores it produces (Taha *et al.*, 2005), small spore size (2-3 µm) which can reach lower airways, and the robust stress response to evade human immune responses (Kwon-Chung and Sugui, 2013). Immunocompetent population do not usually succumb to IA, however. Besides epithelial recognition and mucosal clearance (Bals and Hiemstra, 2004), phagosomes, especially alveolar macrophages represent the first line of innate immune mediated defense against *A. fumigatus* spores (Gerson *et al.*, 1984; Segal, 2009; Espinosa *et al.*, 2014). Macrophages alone rapidly phagocytose spores in the zebrafish model of IA, which contributes to overall host survival during the fungal burden (Knox *et al.*, 2014). Macrophages and neutrophils together contribute to host clearance in the less common event in which spores germinate within tissue (Knox *et al.*, 2014).

A. fumigatus spores have several endogenous mechanisms to avoid macrophage killing, such as pigments and toxic secondary metabolites which provide resistance to oxidative stress and act as virulence factors (Dagenais and Keller, 2009). There is also a link between exposure to environmental contaminants like the polycyclic aromatic hydrocarbon (PAH) benzo(a)pyrene and increased incidence of microbial infections (Clark *et al.*, 2016). Benzo(a)pyrene is a particularly harmful PAH because cellular metabolism by cytochrome p450 monooxygenases forms reactive metabolites that cause DNA adducts and cytotoxic effects. Benzo(a)pyrene is metabolized by CYPs

expressed in alveolar macrophages (Dehnen, 1975) and impairs both cellular differentiation from monocytes (van Grevenynghe *et al.*, 2003) and macrophage function (Braun *et al.*, 1998). This might make animals more susceptible to infection from inhaled fungal spores.

Although one study has shown the environmental contaminant benzo(a)pyrene accumulates in vegetative cells of the filamentous fungus *Fusarium solani* (Fayeulle *et al.*, 2014), no studies have investigated whether benzo(a)pyrene or any environmental contaminant is transported into fungal spores. There has also been no investigation into the immunotoxic effects of benzo(a)pyrene on the innate immune response against fungal spores. Here we show *Aspergillus fumigatus* grown on benzo(a)pyrene containing nutrient poor media produces spores which contain high amounts of benzo(a)pyrene. The amount of benzo(a)pyrene in spores positively correlates with the concentration of benzo(a)pyrene in the media. We did not, however, find that benzo(a)pyrene containing spores more effectively evade the innate immune response in the zebrafish model. We discuss possible reasons for this finding and propose additional research questions that are important to address.

4.3 Materials and Methods

Strains, media and culture conditions

Aspergillus fumigatus strain used in this study is AF293 (Brookman and Denning, 2000). All media used in this study were prepared as previously described (Kafer, 1977). Briefly, spores were grown to avoid direct contact with media containing benzo(a)pyrene

(**Figure 4.1A**)**.** 10^6 spores were spread on petri dishes containing 10 mL 0.1% glucose MM, 1% glucose MM (GMM) or complete media (CM) to which either a 100 mM stock solution benzo(a)pyrene (Sigma-Aldrich) in DMSO was added to the final designated concentration or equal volume of DMSO as a control. 5 mL of the corresponding media type containing only DMSO was poured on top and allowed to solidify. Plates were incubated for 3 days at 37°C and spores were quantified by washing plates with 10 mL 0.01% tween80 buffer (v/v) in water, diluted 10x and counted using a hematocytometer.

Benzo(a)pyrene extraction and HPLC quantification

10^7 spores in water suspension were collected in 2 mL tubes (BioExpress) and centrifuged at 13000 g for 10 minutes. Water was removed using a vacuum suction and spores were dried briefly in drying oven (70°C) and weighed. 1.5 mL 1:1 ethyl acetate: hexane was added with 200 µL 0.5 mm zirconia/silica beads (ThermoFisher) and shaken in mini bead beater (Biospec products) for 2 minutes. Cell debris was removed by centrifugation at 13000g for 10 minutes and solvent was removed and dried under N_2. Extract was resuspended in 50 µL 1 solvent A: 1 solvent B HPLC running buffer (**Chapter 2**). Benzo(a)pyrene was quantified by high-performance liquid chromatography using an Agilent 1260 HPLC system as described previously (**Chapter 2).**

Trehalose and β-glucan Analysis

Spore trehalose content was determined as previously described (Ni and Yu, 2007). Briefly, 3-day old conidia from benzo(a)pyrene or control (DMSO) plates were collected,

quantified and diluted to 10^8 spores/mL. The amount of glucose liberated from treatment with trehalase (Sigma, St Louis, MO, USA) was quantified using glucose assay kit (Sigma, St Louis, MO, USA) and represented as µg glucose per mL of spore suspension (10^8 spores). This assay was performed in triplicate.

1,3-β-glucan quantification was performed as previously described (Park *et al.*, 2015), using Glucatell® assay (Associates of Cape Cod, USA) following the manufacturer's instructions (Odabasi *et al.*, 2004). Briefly, 3-day old conidia on benzo(a)pyrene or control (DMSO) plates were harvested and quantified. 10^5 spores were centrifuged and resuspended in 25 µL sterile deionized water. Glucatell® reagent was added and the mixture was incubated at 37°C for 30 minutes, followed by termination with diazo-reagent, and absorbance was measured at 540 nm. This assay was performed in triplicate.

Microscopy

Fluorescence microscopy

Microscopy of benzo(a)pyrene and lipid body localization was carried out on a Nikon Eclipse Ti fluorescence microscope using a 60x Nikon Plan Apo VC Oil DIC objective. For condiophore development, 5x 10^4 spores were added to sterilized coverslips immersed in 3 mL 1 % glucose MM in a 6-well plate (Corning Incorporated) and grown for 12 hours at 30°C. Media was then removed, coverslips washed briefly with sterile deionized water, and replaced with 3 mL 0.1% glucose containing 200 µM benzo(a)pyrene or equal volume DMSO (control). Coniophores were allowed to develop

by an additional incubation at 37°C for 24 hours. Cells were then stained by adding 24 µL 0.1% (w/v) Nile Red (ThermoFischer) in 100% ethanol to coverslips immersed in media, then removed and washed with PBS three times. DAPI filter (λ_{ex}= 400 nm, λ_{em}= 430-480 nm) was used to visualize benzo(a)pyrene and RFP filter (λ_{ex}= 488 nm, λ_{em}= 570-610 nm) were used to visualize Nile Red. Images were process using NIS elements advanced research analysis software.

Spore persistence assay

A vertebrate model of invasive aspergillosis using zebrafish larvae was used to determine virulence of benzo(a)pyrene containing spores as previously described (Knox *et al.*, 2014), with the following exceptions. Spores were grown as described above, in media containing 0.1% glucose and 200 µM benzo(a)pyrene or an equal volume of DMSO (control) and injected into the hindbrain of immunocompetent zebrafish larvae. Larvae were homogenized and spread evenly on GMM containing plates at designated timepoints, and colony forming units (CFU) were counted manually.

4.4 Results

4.4.1 Spores from *Aspergillus fumigatus* contain benzo(a)pyrene from growth media

Although it has been shown *Aspergillus spp*. including *Aspergillus fumigatus* are capable of degrading benzo(a)pyrene (Chapter 2), we wanted to test the hypothesis that unmetabolized benzo(a)pyrene can be transported to reproductive bodies during sporulation. To address this we grew *Aspergillus fumigatus* on agar media to promote sporulation. By adding a layer of media on top of spores that does not contain benzo(a)pyrene we were able to detect only benzo(a)pyrene which was transported to spores, not which has adhered to the spore surface (**Figure 4.1A**). The identity of benzo(a)pyrene in the spores was confirmed by comparing the retention time and fluorescence spectra with an authentic standard. We found a large peak in cells grown in benzo(a)pyrene containing media, but not control media that shares the same retention time (**Figure 4.1C**) and fluorescence spectra (**Figure 4.1D**) as benzo(a)pyrene. This provided conclusive evidence supporting the hypothesis that spores contain benzo(a)pyrene which has been uptaken by fungal hyphae and transported during sporulation.

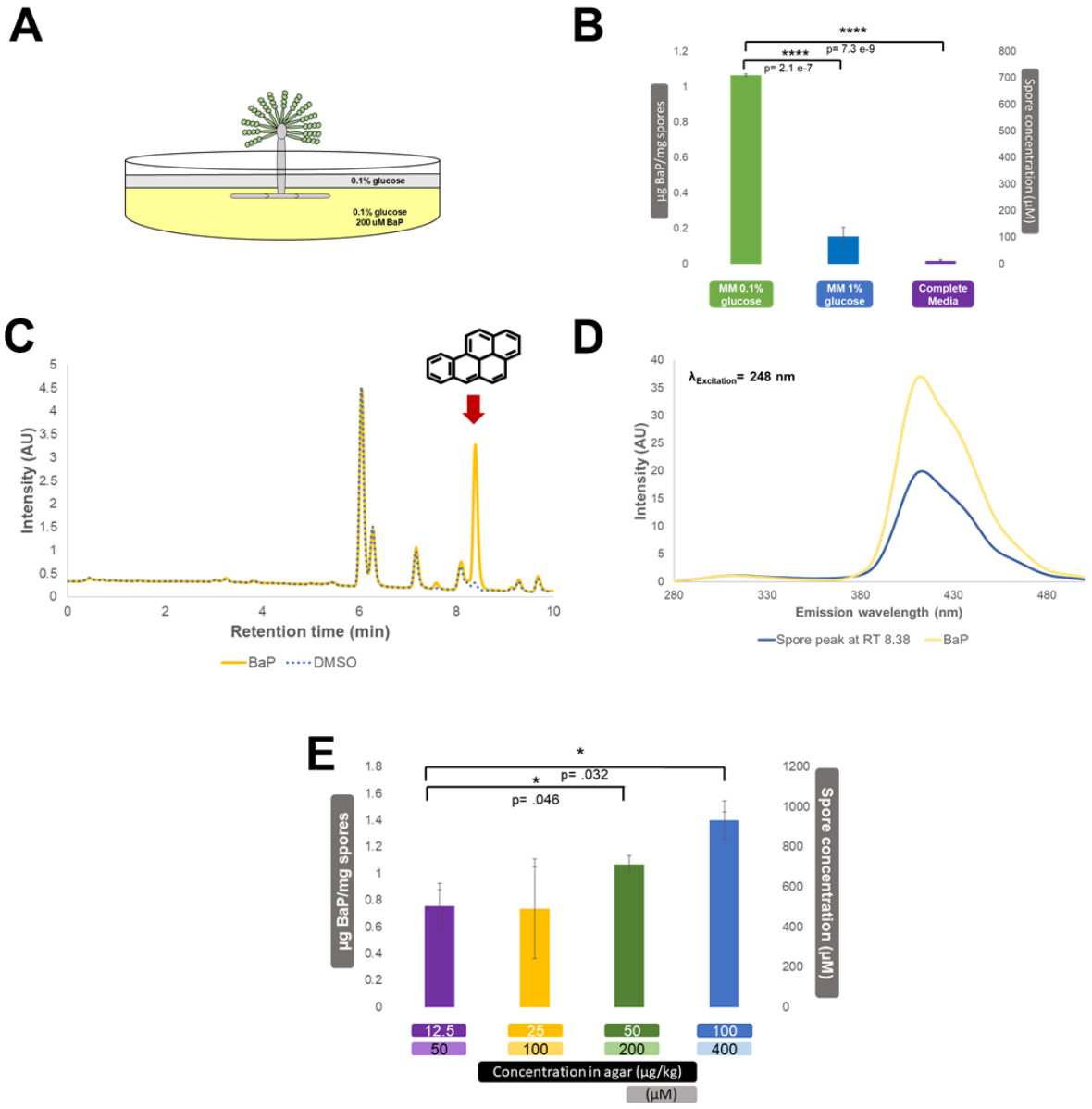

Figure 4.1: Aspergillus fumigatus produces spores that contain benzo(a)pyrene when hyphae are grown in benzo(a)pyrene containing media. **A**. Schematic showing inoculation technique and growth conditions to ensure spores do not contain benzo(a)pyrene which adheres to the spore surface. **B**. The nutritional components of media lead to variable concentrations of benzo(a)pyrene found in spores. Minimal media (MM) contains only glucose, trace elements, and nitrogen salts whereas complete media also contains peptides, and casein, and yeast extract. **C**. HPLC chromatogram showing extract of spores produced from benzo(a)pyrene containing media (BaP) and solvent only control (DMSO). Sample peak shares the same retention time as benzo(a)pyrene authentic standard (structure shown). **D**. Fluorescence spectra of sample peak which shares the same retention time as benzo(a)pyrene standard (retention time (RT)= 8.38 minutes), compared to the fluorescence

spectrum of 1 µM benzo(a)pyrene standard. **E**. Concentration of benzo(a)pyrene found in spores given in two different unit measurement (µg/mg spores and µM) at increasing concentrations of benzo(a)pyrene in the media given in two different unit measurements (µg/kg of media and µM).

We also wanted to address the specific environmental stimuli which might influence the quantity of benzo(a)pyrene translocated to spores. Since benzo(a)pyrene degradation and metabolic utilization is dependent on glucose concentration in *Aspergillus nidulans* (**Chapter 2**) we addressed whether carbon availability and nutritional supplementation has an impact on concentration of benzo(a)pyrene found in spores in *Aspergillus fumigatus*. We found media containing a low concentration of glucose (0.1%) accumulated almost 7 times the concentration of benzo(a)pyrene (1.07 µg/mg spores ± .01) in spores as media containing a higher concentration (1%) of glucose (0.16 µg/mg spores ± .05; **Figure 4.1B**). In media containing additional elements like peptides and yeast extract (complete media) to support robust fungal growth *A. fumigatus* produced spores with concentrations almost tenfold lower than that (.02 ± .01 µg/mg spores; **Figure 4.1B**). This suggests 1) that the robust growth resulting from carbon sufficiency of *A. fumigatus* leads to increased degradation of benzo(a)pyrene or that 2) robust growth of *A. fumigatus* reduces transport of benzo(a)pyrene to developing spores for another reason, i.e. reduced need for allocation of limited resources to developing spores. We also observed that in 0.1% glucose containing media, benzo(a)pyrene hyper-accumulated in spores relative to the amount found in the media (200 µM or 50 µg/mL; **Figure 4.1B**). This indicates either a physiological or chemical preference for the presence of benzo(a)pyrene in spores.

Benzo(a)pyrene concentration in the media is also a contributing factor to the concentration of benzo(a)pyrene found translocated to the spores. Concentrations of benzo(a)pyrene found in spores responded to concentration in media is a dose responsive manner in 0.1% glucose media (**Figure 4.1E**). We did not determine the maximum amount of benzo(a)pyrene transported to fungal spores, however we were interested in studying the environmentally relevant benzo(a)pyrene concentrations in soil. Concentrations used in these experiments do not exceed the U.S. Environmental Protection Agency's (EPA) dose for 1 x 10^{-6} cancer risk (1 in one million people will get cancer if exposed all day every day to this dose) of 110 µg/kg in residential soil (U.S. EPA, 2017).

Hyphae show benzo(a)pyrene accumulation in vesicle like structures

The auto-fluorescence of benzo(a)pyrene was used to visualize intracellular localization with fluorescence microscopy in order to evaluate how benzo(a)pyrene was being transported to spores during hyphal growth and development. We were able to detect a blue fluorescent signal ($\lambda_{excitation}$= 394-409 nm, $\lambda_{emission}$= 430-480 nm) in vesicle-like structures in benzo(a)pyrene treated cells but not the DMSO control (DAPI, **Figure 4.2**). This is consistent with fluorescence of benzo(a)pyrene (**Figure 4.1D**) and, since we did not see autofluorescence in the control fungal cells, we attributed this fluorescent signal to benzo(a)pyrene. We also attempted to localize benzo(a)pyrene within mature fungal spores to determine its distribution but were unable to visualize a clear fluorescent signal (data not shown). This may be due to the relatively low concentration of

benzo(a)pyrene in fungal spores, or lack of magnification, and resolution in the microscopy technique used.

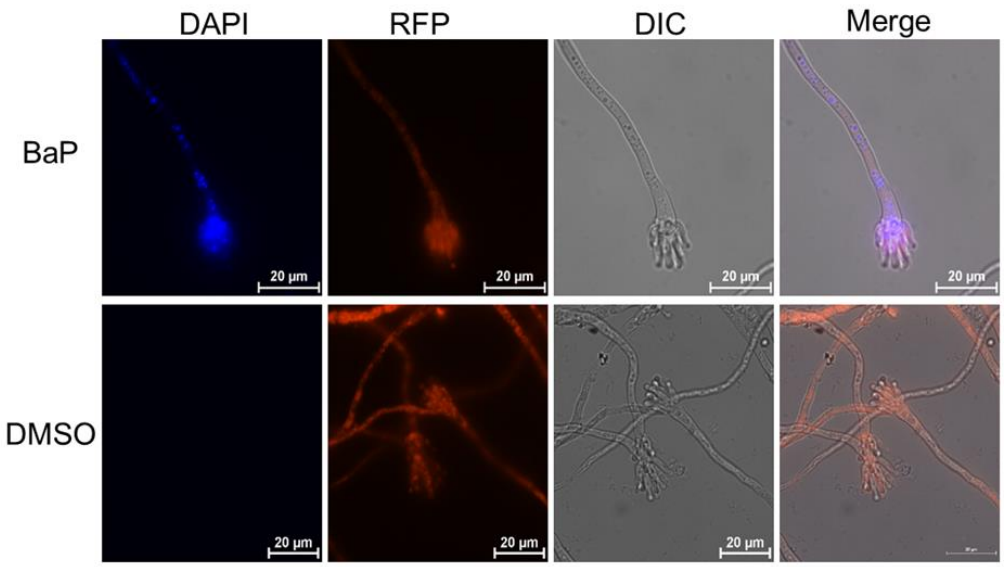

Figure 4.2: **Benzo(a)pyrene is found in vesicle-like structure in developing conidiophore which does not colocalize with lipid bodies**. Images showing fluorescent signals in benzo(a)pyrene treated hyphae (BaP) and vehicle only control (DMSO). Nile red signal shown using RFP filter and benzo(a)pyrene exclusive signal visible using DAPI filter.

We hypothesized that these vesicle-like structures are lipid bodies because these structures have been shown to sequester toxic compounds in fungal cells as a form of defense (Chang *et al.*, 2015), lipid bodies are shown to migrate to forming conidia in *Aspergillus nidulans* (Tsitsigiannis, Zarnowski and Keller, 2004), and the hydrophobic nature of benzo(a)pyrene would preferentially partition to nonpolar structures (Mackay and Paterson, 1991). We used Nile Red to specifically stain lipid bodies and detected a red fluorescent signal within metulae of developing conidiophores consistent with others

(Tsitsigiannis, Zarnowski and Keller, 2004; **Figure 4.2**). Although there was a small amount of colocalization of benzo(a)pyrene (DAPI) and lipid body (RFP) signals within metulae and the conidiphore head, we did not find colocalization within vesicle like structures in conidiophore stalk (**Figure 4.2**). This indicates some other type of vesicle plays the dominant role in transporting benzo(a)pyrene to the spores during development.

4.4.2 Benzo(a)pyrene containing spores do not cause increased persistence against innate immune response

The major concern resulting from a cytotoxic compound contained within an opportunistic pathogenic fungus is the potential damage to and reduced effectiveness of immune responses to protect the host from infection. We used a zebrafish model to investigate whether exposure to benzo(a)pyrene-containing spores causes increased spore persistence against the zebrafish innate immune response. Each timepoint showed no significant difference between persistence of benzo(a)pyrene containing spores and the DMSO only control (**Figure 4.3A**). This indicates benzo(a)pyrene-containing spores did not survive innate immune responses better than the control in immunocompetent zebrafish. However, every timepoint except zero days post spore injection showed higher average colony forming units (CFU) per zebrafish larva in benzo(a)pyrene containing spores than the control which supports our hypothesis (**Figure 4.3A**). The lack of statistical significance may be due to a true lack of any cytotoxic effect of benzo(a)pyrene on immune cells in the zebrafish model or could be

due to other variables such as a lack of cytotoxic effect of benzo(a)pyrene at the tested concentration.

4.4.3 Benzo(a)pyrene treated causes reduced spore components

In order to assess any additional variables within the physiology of the fungal spores that could affect virulence and confound our results, we addressed the impact benzo(a)pyrene has on endogenous components of the spore. Trehalose is a dissacharide absent in mammalians that protects fungal spores from desiccation, contributes to an effective stress response and influences virulence (Tournu, Fiori and Van Dijck, 2013). We measured trehalose content in benzo(a)pyrene and control (DMSO) spores and found a reduced concentration in the presence of benzo(a)pyrene, regardless of nutrient composition of the media (**Figure 4.3B**). We also measured 1,3-β-glucan content in spores, as it is unique to fungal cells and is recognized by alveolar macrophages (Brown *et al.*, 2002). We found a reduced amount of 1,3-β-glucan in benzo(a)pyrene containing spores than in the control in nutrient limited media (**Figure 4.3B**). The opposite trend was true in complete media, however the difference was not significant (**Figure 4.3B**). Both of these results together indicate that reduced decreased trehalose content and reduced 1,3-β-glucan content would result in further virulence of spores containing benzo(a)pyrene.

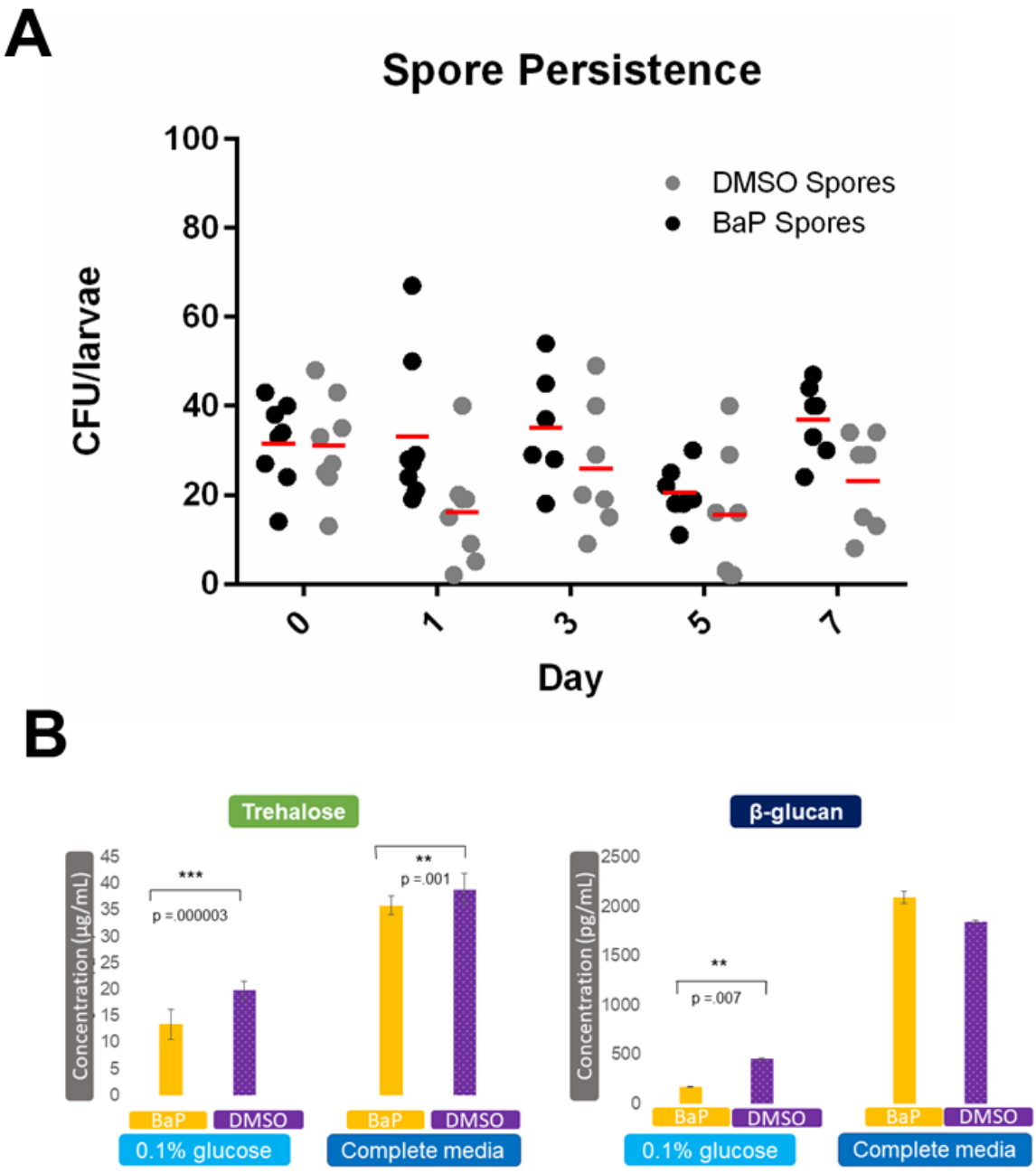

Figure 4.3: **Spores containing benzo(a)pyrene do not survive innate immune defense in zebrafish better than control spores.** **A**. Spore persistence assay showing number colony forming units (CFU) after plating homogenized zebrafish injected with benzo(a)pyrene (BaP) containing spores or vehicle only (DMSO) control. X-axis represents day post injection in zebrafish larvae. **B**. Quantification of compounds in trehalose and 1,3 β-glucan in spores produced in benzo(a)pyrene containing media (BaP) and vehicle only control media (DMSO).

4.5 Discussion

Inhalation of spores from *A. fumigatus* represents a major human health hazard, especially in populations with deficient or suppressed immune responses (Segal, 2009). One factor that makes *A. fumigatus* an effective pathogen is its ubiquity in the environment, as it can grow in almost any aerobic environment, including those contaminated with toxic PAHs (Ye *et al.*, 2010). Another factor is the production of easily dispersed spores which are inhaled by humans on a daily basis, and are able to reach lower airways because of their small size (Kwon-Chung and Sugui, 2013). Given these factors together, if *A. fumigatus* uptakes compounds from the environment and transports them to spores, humans living near contaminated sites may be exposed to more potentially toxic and virulent spores than previously considered.

Here we demonstrate that *A. fumigatus* accumulates benzo(a)pyrene within spores under specific nutrient limiting conditions. Since benzo(a)pyrene is an immunotoxin, and exposure increases the risk of bacterial infections (Clark *et al.*, 2016), we hypothesized *A. fumigatus* spores containing benzo(a)pyrene are better able to survive innate immune system defenses that could lead to higher incidence of invasive fungal growth. Using a zebrafish model of the innate immune response, however, we did not find significant increased survival of benzo(a)pyrene containing spores. The trend seen in the data, however, does show increased persistence of spores grown in benzo(a)pyrene containing media on average which supports our hypothesis (**Figure**

4.3A). The lack of statistical significance may be due to the specific conditions chosen for this experiment including low concentrations of benzo(a)pyrene contained in spores.

We also hypothesized that benzo(a)pyrene exposure during spore development may have led to biochemical differences in the spore physiology. Differences in spore contents could potentially confound immune responses specific to benzo(a)pyrene treatment. In *A. fumigatus*, a strain with deletions in two trehalose biosynthesis genes, *tpsA* and *tpsB*, produces spores which are hypervirulent in a murine mouse model despite defective germination (Al-Bader *et al.*, 2010). This indicates that although trehalose confers protection and growth support for spores, it inhibits spores from effectively evading host immune responses. Benzo(a)pyrene treatment during development of *A. fumigatus* spores showed decreased levels of trehalose which may contribute to hypervirulence (**Figure 4.3B**). According to our results, however, neither benzo(a)pyrene or decreased trehalose content impacted the survival of spores against innate immune responses (**Figure 4.3A**).

1,3-β-glucan is a major component of fungal cells walls and plays a major role in initiating proper immune response (Brown and Gordon, 2003). Alveolar macrophages recognize the 1,3-β-glucan fungal signature via the receptor dectin-1 which initiates proper signaling to the adaptive immune system to promote attack against fungal infection (Brown *et al.*, 2002; Steele *et al.*, 2005). We found less 1,3-β-glucan in benzo(a)pyrene containing spores than in control spores (**Figure 4.3B**), indicating again that this difference would favor higher survivability in benzo(a)pyrene containing spores. However, this response is more evident in swollen and germinating spores where β-glucans become increasingly exposed compared to resting spores (Steele *et al.*, 2005).

We are uncertain as to whether lower 1,3-β-glucan content persists after germination in our benzo(a)pyrene containing spores. Additionally, in this zebrafish model the adaptive immune response is not yet developed in fish larvae (Knox *et al.*, 2014), so any effect of benzo(a)pyrene on adaptive immunity is not seen. Since we did not measure any increase in survivability of benzo(a)pyrene containing spores, the contribution of 1,3-β-glucan content was not considered. Regardless, is interesting and somewhat unexpected to see such striking differences in spore components in response to benzo(a)pyrene exposure during spore formation.

Further investigation is needed to assess whether benzo(a)pyrene containing spores shows no effect on survival against innate immune response at any concentration. The low glucose media which shows the greatest benzo(a)pyrene accumulation accurately reflects most soil environments where free glucose is not readily available (Gieseking, 1975). Our study, however, focuses on benzo(a)pyrene concentrations that do not exceed levels that would warrant U.S. EPA intervention (U.S. EPA, 2017). But for populations living in close proximity to PAH contaminated sites, like U.S. EPA regulated Superfund sites, higher levels of benzo(a)pyrene in the soil may mean exposure to spores containing higher concentrations in spores which are inhaled. Since humans inhale a range of fungal spores which spans several orders of magnitude (Brown *et al.*, 2012), it is important to assess the increased exposure to benzo(a)pyrene in general from spores. Our results show the average amount of benzo(a)pyrene contained in the highest concentration of benzo(a)pyrene-containing media is 0.036 ng/10^6 spores (**Figure 4.1E**, conversion not shown). Humans on average are exposed to 2.2 µg of benzo(a)pyrene per day (Hattemer-Frey and Travis, 1991). If a given population were to

inhale the maximum number of predicted inhaled spores (10^{10}; Brown et al. 2012), this population could potentially be exposed to over 15% more benzo(a)pyrene than currently calculated. Besides immunosuppression, benzo(a)pyrene causes many other negative health effects, including lung cancer (U.S. EPA, 2017). A better understanding of the physiological interactions between fungal spores, and the human respiratory system would help mitigate this currently poorly understood health risk.

4.6 References

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Chapter 5:
Conclusions and Future Directions

5.1 Introduction

Filamentous fungi have long been acknowledged as the planet's 'garbage men' as they fill a valuable saprophytic ecological niche by degrading complex organic plant polymers like cellulose and lignin, which other organisms lack the enzymes to metabolize. They have unique characteristics that make them especially well-suited to scavenge nutrient limited soils which are inhospitable to many other organisms (Harms, Schlosser and Wick, 2011). *Aspergillus* is a genus of ubiquitous fungus that can be found living in almost any aerobic environment, including those contaminated with toxic soil contaminants (Mukherjee, 2016). Their unique metabolic capabilities, including an impressive repertoire of cytochrome P450 monooxygenase enzymes (CYPs), allow them to be enormous contributors to the removal of toxic compounds from the environment which reduces exposure risks to humans. CYPs are a superfamily of enzymes found in every kingdom of life, including viruses, and play many roles, both anabolic and catabolic (Lamb *et al.*, 2009). One possible reason that filamentous fungi encode such an impressive number of CYPs relative to their genome size (**Table 1.1**) is that in order to survive hostile and nutrient limiting conditions, these fungi must have an extensive and adaptable toolbox in order to secure an ecological niche. *Aspergillus sp.* encode numerous CYPs for which the function of many have not yet been described. *A. nidulans*, *A. flavus,* and *A. fumigatus* encode 119, 153, and 75 CYPs respectively, yet there has been no study specifically identifying any of these to be involved in contaminant degradation (Chen *et al.*, 2014). Understanding the role and

function of CYPs in degrading environmental contaminants is extremely valuable and can contribute to the prevention of human exposure to toxicants.

Polycyclic aromatic hydrocarbons (PAH) are compounds consisting of fused benzene rings that are found in many environments. Among them, the five-ringed benzo(a)pyrene represents the most problematic PAH. Its toxicity in humans is not due to the parent compound itself, but reactive metabolites are formed when oxidized by CYPs, causing oxidative damage and DNA adducts. CYP metabolism of benzo(a)pyrene has been studied in humans and animal models for decades. Therefore, a lot is already known about how these enzymes interact with benzo(a)pyrene and many assays exist to study the genetics, biochemistry and analytical measurements of benzo(a)pyrene metabolism. This has made benzo(a)pyrene an important, and useful compound to study.

Given the promise of using fungi for bioremediation, the majority of the literature has neglected to assess any potential consequences of the interaction between toxic contaminants and fungi. *A. fumigatus* itself acts as an air pollutant because of the abundant and pathogenic spores it produces (Segal, 2009). Its ubiquity has made it an effective opportunistic pathogen (Kwon-Chung and Sugui, 2013), yet no study has looked at the potential of this fungus to disperse environmental contaminants by uptake into its spores.

Given that the interaction of *Aspergillus sp*. with benzo(a)pyrene is likely to cause beneficial and detrimental health effects, the purpose of this research was to explore both. Specifically, the goals of this research were three-fold: 1) Utilize the model organism *A. nidulans* to comprehensively investigate the CYP mediated metabolism of

the archetypal environmental contaminant benzo(a)pyrene, 2) Propose the endogenous function of a benzo(a)pyrene metabolizing CYP by elucidating its role within a secondary metabolite gene cluster in *A. flavus*, 3) Demonstrate the consequences of contaminant interaction with fungi by showing benzo(a)pyrene accumulation within fungal spores in *A. fumigatus* (**Figure 5.1**).

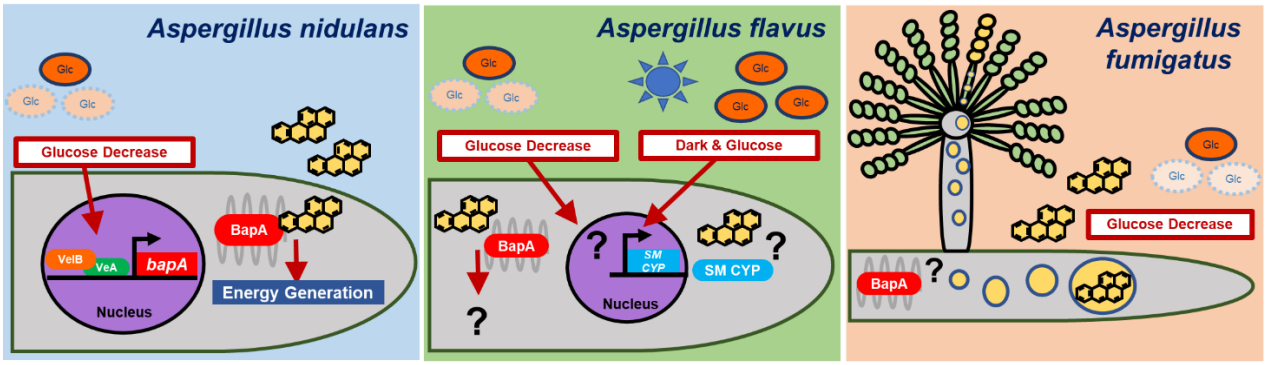

Figure 5.1: Graphical abstract showing topics related to the interaction between benzo(a)pyrene and fungi in three *Aspergillus sp*. and the conclusions drawn from this dissertation research.

5.2 Conclusions

We were able to investigate many aspects of benzo(a)pyrene metabolism in *A. nidulans.* Increased cell viability and transcriptional responses to benzo(a)pyrene treatment demonstrate that *A. nidulans* is capable of utilizing this organic contaminant as a substrate for growth. This implies that the transformation of benzo(a)pyrene yields compounds which are no longer toxic to animals, as both aerobic fungi and humans utilize the same biochemical energy generating pathways (Watson and Smith, 1967). In addition, we were able to show that this pathway is mediated by a benzo(a)pyrene

metabolizing CYP, BapA (AN1884; ***b***enzo(***a***)**p**yrene metabolism associated locus **A**). The null mutant Δ*bapA* was not able to degrade benzo(a)pyrene under nutrient limiting conditions, and no longer showed cell viability nor contained significantly more newly synthesized (cytosolic) amino acids when cultured with benzo(a)pyrene compared to the control. The BapA subfamily (CYP617D1) was shown to be conserved in *Aspergillus sp*., and deletion of the *bapA* ortholog in *A. flavus* (AFLA_036020) showed the same phenotypic lack of benzo(a)pyrene metabolizing ability. The Nf-κB-like regulators VeA and VelB were shown to be necessary for the upregulation of *bapA* under glucose limiting conditions in *A. nidulans.* This demonstrates interesting evolutionary similarities and differences in the responses of fungi and animals to toxic compounds.

Although we were able to thoroughly investigate the role of one benzo(a)pyrene degrading CYPs in *A. nidulans*, such a large repertoire of CYPs encoded in the genomes of *Aspergillus sp.* suggests more than one is capable of benzo(a)pyrene metabolizing capabilities. Using the hypothesis that substrate promiscuity in CYPs enables metabolism of more than one substrate, we sought to demonstrate the ability of a fungal CYP in *A. flavus* to metabolize both an endogenous and exogenous substrate. AFLA_135430 (*smcypA*) is contained within a predicted secondary metabolite gene cluster and shows sequence similarity to CYPs in the white rot fungus *Phanerochaete chrysosporium* that can oxidize the PAHs pyrene and benzo(a)pyrene (Syed *et al.*, 2010). Deletion *smcypA* provided evidence to support our hypothesis. The null mutant (Δ*AFLA_135430*) showed alterations in growth and development, and secondary metabolite production imbalance which is consistent with disruption of other secondary metabolite gene clusters (Bok and Keller, 2004; Forseth *et al.*, 2012; Cary *et al.*, 2015).

Δ*smcypA* also showed reduced, although not abolished, degradation of benzo(a)pyrene under glucose limiting conditions. This was not conclusive evidence that SmcypA is involved in benzo(a)pyrene metabolism, especially since deletion of the *bapA* orthologue in *A. flavus* (AFLA_036020) showed almost no benzo(a)pyrene metabolic activity, suggesting *bapA* plays the dominant role in metabolizing benzo(a)pyrene under carbon limitation.

Our most surprising finding was the accumulation of benzo(a)pyrene in spores during development in *Aspergillus fumigatus* (**Chapter 4**). Since there was no external contact between the spore surface, and benzo(a)pyrene containing media, this occurred via uptake and transport through fungal hyphae. Bioaccumulation of benzo(a)pyrene is generally not a concern because it is biotically degraded by many organisms, except in aquatic environments that harbor more abundant non-metabolizing organisms (Rust *et al.*, 2004). However, of all the *Aspergillus sp.* tested, *A. fumigatus* did not degrade benzo(a)pyrene as efficiently as other species in glucose limited media (**Figure 2.1A**). It was previously observed that benzo(a)pyrene accumulates in vesicle-like structure in *Fusarium solani*, however transport during sporulation was not addressed in this study (Fayeulle *et al.*, 2014). We were able to visualize similar benzo(a)pyrene containing vesicles in the developing conidiophore structure, as well and measure concentrations of benzo(a)pyrene in mature spores which was higher than that of the media (**Figure 4.1A and Figure 4.2**). This provides evidence that further investigation into the health concerns of fungal mediated sequestration of toxic environmental contaminants into spores is warranted.

5.2 Future Directions

5.2.1 Identification of additional PAH metabolizing CYPs

Since very little is known about CYP activity towards benzo(a)pyrene in fungi, especially in fungi that encode a plethora of CYPs, using examples from other organisms can help formulate hypotheses to explain our results. Humans, for instance, encode fewer total CYPs (57; D. Nelson 2009) than *A. flavus* (153; Chen et al. 2014), but at least 13 of these CYPs in humans show benzo(a)pyrene metabolizing activity, CYP1A1, 1A2, 2A6, 1B1, 2B6, 2C8, 2C9, 2C10, 2C18, 2C19 , 3A4, 3A5, and 2E1 (Bauer *et al.*, 1995; Šulc *et al.*, 2016). Benzo(a)pyrene metabolism *in vivo* consequently relies on several factors: amount of benzo(a)pyrene metabolic activity each CYP is capable of, the cell type, and the inducibility of CYPs within that cell type.

It is possible benzo(a)pyrene metabolism acts similarly in eukaryotic fungi. Fungi have less sophisticated cell differentiation than humans, however differences in reproductive cellular structures and vegetative hyphae demonstrate differentiation is apparent (Chi and Craven, 2016). Even within hyphae, more metabolic activity occurs at hyphal tips than in older, more vacuolized cells towards the hyphal base (Arman *et al.*, 2005). Therefore, environmental stimuli, cell type, and inducibility of CYPs within fungal cells all likely play a role in benzo(a)pyrene degradation in *Aspergillus sp*. Since the null mutant Δ*smcypA* (AFLA_135430) did not show abolishment of benzo(a)pyrene degradation ability in glucose limiting conditions, another set of parameters may show a more dramatic phenotype. This CYP is highly expressed in hyphae with 1% glucose in dark conditions (**Figure 3.1A**) relative to other conditions. Testing the degradation of

Δ*smcypA* under these conditions may yield greater reduction in benzo(a)pyrene metabolism than the glucose limiting conditions that seem to favor *bapA* expression. Utilizing the expression profiles of all encoded CYPs in the model system *Aspergillus nidulans* in response to media components, environmental stimuli which induce development, and various PAHs would give insight into the regulation of *bapA* and *smcypA,* as well as identify other potential PAH metabolizing candidates.

Using a heterologous system to express *anibapA*, *aflbapA,* and *aflsmcypA* would facilitate measurement of benzo(a)pyrene metabolizing activity. *A. nidulans* and *A. flavus* degrade different amounts of benzo(a)pyrene *in vivo*, 92% ± 4.9% and 54% ± 9.3% respectively in glucose limiting conditions (**Figure 2.2A**). The exact reason for this difference is not clear, but it is especially important for the design and implementation of effective bioremediation strategies. Heterologous expression would reveal if differences in metabolism of benzo(a)pyrene occurs as a result of CYP protein-substrate interactions. Other factors may which may be responsible for differences in degradation ability are uptake kinetics, or differences in cellular morphology (i.e. early reproductive induction) that would not affect *in vitro* metabolism. Elucidation of the effect of individual variables could provide some clarity as to the exact cause of increased benzo(a)pyrene degradation ability.

5.2.2 CYP activity towards other substrates

A heterologous expression system could also be used as a measure of substrate flexibility. In other organisms such as the wood rotting fungus *P. chrysosporium* CYPs metabolize more than one PAH. PC-PAH1 and PC-PAH3 in *P. chrysosporium* oxidize both pyrene and benzo(a)pyrene in the heterologous *Pichia pastoris* system, while other CYPs metabolized either phenanthrene and pyrene, or pyrene alone (Syed *et al.*, 2010). This indicates limitations on the size of PAH but does not demonstrate rigid structural specificity. Human encoded CYPs demonstrate an enormous range of metabolic activity. For example, the CYP1A1 that is responsible for benzo(a)pyrene activation (carcinogenic metabolite formation) also metabolizes many other large, planar molecules including endogenous substrates like arachidonic and eicosapentoic acid (Sridhar *et al.*, 2017). By building a profile of the metabolizing capabilities of several fungal CYPs, pairwise comparisons of amino acid sequences may help identify important sequence motifs which enable the protein to catalyze PAH specific reactions. This would assist in identifying PAH degrading CYPs in many other fungi, as well as give some insight into their evolution.

Identification of the secondary metabolite produced by the cluster encoding *AflsmcypA* is an important contributor to understanding the substrate promiscuity of PAH degrading CYPs. Overexpression of the backbone non-ribosomal peptide synthase gene in this cluster was constructed by the Keller lab, and identification of a large, hydrophobic compound was detected (Brandon Pfannenstiel, personal communication, May 23, 2018). It is reasonable to hypothesize that oxidation of this compound during biosynthesis is the endogenous role of AflSmcypA, whereas oxidation of

benzo(a)pyrene is a result of substrate promiscuity. Confirming the identity of this secondary metabolite, and the role of aflSmcypA in its oxidation could not only yield important insights into the function of this secondary metabolite, and its potentially useful properties, but also open avenues for exploring the evolution of CYP number and function in *Aspergillus sp*.

The evolution of mechanisms used to regulate CYP expression yields other interesting insights. The Nf-κB like velvet regulators VeA and VelB are necessary for the expression of *bapA* in *A. nidulans*. Nf-κB in humans plays a role in responding to cellular damage to promote cell survival (Pahl, 1999). Interestingly Nf-κB activity responds to both cellular damage caused by benzo(a)pyrene metabolites, and to benzo(a)pyrene itself (Weng *et al.*, 2004; Bolotina *et al.*, 2007; Ji *et al.*, 2013). VeA and VelB respond to nutrient limitation and upregulate *bapA* in order to potentially utilize alternative carbon containing molecules as a substrate for growth. These responses demonstrate vastly different strategies for dealing with exposure to potentially damaging, albeit, carbon containing molecules like benzo(a)pyrene.

Within fungi (maybe even within a particular fungal species), environmental stimuli induce variable responses. BapA shows sequence similarity to a CYP in the distantly related fungus *P. chrysosporium* (PC-PAH1). Although similar, these two CYPs do not share enough sequence identities to place them in the same CYP family (Nelson, 2006). This indicates during the evolution of filamentous fungi, benzo(a)pyrene metabolic capabilities conferred fitness on multiple separate occasions. Unlike *AnibapA* that is upregulated in response to carbon depletion, *PC-PAH1* expression is upregulated in response to PAH exposure, which is similar to the mammalian aryl

hydrocarbon receptor mediated response in animals (Pandini *et al.*, 2007; Syed *et al.*, 2010). The transcriptional regulatory mechanisms which guide CYP expression in response to PAHs has not been studied, though there is evidence to support the hypothesis that fungi upregulate CYPs as a xenobiotic detoxification defense strategy (Lah *et al.*, 2011).

5.2.3 Physiological role of benzo(a)pyrene sequestration in fungal spores and potential toxic consequences in humans

Although we were able to demonstrate the transport of benzo(a)pyrene into spores in *Aspergillus fumigatus*, the biological outcome and specific mechanisms used were not explored. We were not able to identify the benzo(a)pyrene containing vesicles within the developing conidiophore so it is not clear whether transport of benzo(a)pyrene is the result of chemically favorable partitioning, or whether this process in biologically driven. *Aspergillus fumigatus* produces spores that contain a hydrophobic rodlet layer on the surface in order to evade macrophage recognition, which is a chemically favorable environment for the lipophilic benzo(a)pyrene (Sims and Overcash, 1983; Paris *et al.*, 2003). Alternatively, other toxic compounds like aflatoxin B1 are compartmentalized, synthesized, and exported using vesicle-vacuole transport mechanisms in *Aspergillus parasiticus,* which highlights the sophisticated mechanisms used by fungi to protect cells from exposure to toxins (Chanda *et al.*, 2009). Although this example does not show vesicle transport to spores, other fungi have trafficking mechanisms which act specifically during sporulation (Morishita *et al.*, 2007). By using an inhibitor of spore-specific trafficking proteins it would be possible to see if benzo(a)pyrene accumulates in

spores yielding evidence of whether transport of benzo(a)pyrene is chemically or biologically driven. This may give insight into the potential for other compounds to be transported to spores, revealing the extent of potentially harmful consequences of this process.

We were also not able to show benzo(a)pyrene containing spores were better able to survive the innate immune response in zebrafish, despite evidence that benzo(a)pyrene causes increased incidence of bacterial infections in some populations (Clark *et al.*, 2016). It is not clear whether our results were due to assay flaws, or if other variables such as the low benzo(a)pyrene concentration in spores were the cause of no measurable effect. It is possible that although alveolar macrophages do contain benzo(a)pyrene activating CYPs (Dehnen, 1975) and alveolar macrophages in populations of smokers contain aromatic DNA adducts (Godschalk *et al.*, 1998) this damaging event is not enough to disrupt alveolar macrophage activity *in vivo* against *Aspergillus* spores. There is evidence to show benzo(a)pyrene causes reduced differentiation of macrophages from monocytes (van Grevenynghe *et al.*, 2003), so perhaps benzo(a)pyrene containing spores are more virulent following longer periods of exposure. Benzo(a)pyrene additionally affects macrophage cytokine production to direct adaptive immune recruitment (Lyte and Bick, 1986; Braun *et al.*, 1998; Tsuji *et al.*, 2011), so phenotypic differences in benzo(a)pyrene containing spores could be more evident in models which include the roles of the adaptive immune system.

5.3 Final Conclusion

Fungi in general greatly impact the lives of humans in many different ways. Beneficial characteristics of *Aspergillus spp.* include production of pharmaceutically and industrially important compounds, degradation of complex plant polymers which contributes to the global carbon cycle, and elimination of toxic organic compounds from the environment. *Aspergillus spp.* also cause potential harm to humans because of their ability to produce toxic secondary metabolites, are pathogenic towards agriculturally important plants and cause difficult to treat infections in vulnerable populations.

Through our research we have found that the interaction of *Aspergillus spp*. with the prototypical environmental contaminant benzo(a)pyrene falls into both beneficial and potentially harmful categories. The metabolic degradation of benzo(a)pyrene by *Aspergillus spp.* reduces human exposure to the compound. The utilization of benzo(a)pyrene as a substrate for growth indicates non-toxic metabolites are left in the environment, alleviating exposure risks to humans. Accumulation of benzo(a)pyrene in fungal spores, however, is a concerning phenomenon that is currently not addressed by environmental regulations. Additional research needs to be undertaken in order to more appropriately utilize and respond to the trade-offs of these fungal interactions with environmental contaminants.

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