Chapter 2
Aflatoxin Biosynthesis

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Abstract Aflatoxins are highly toxic contaminants synthesized by several toxigenic strains of *Aspergillus* as secondary metabolites. The biosynthesis of aflatoxins is a complicated process involving a chain of reactions which are catalyzed by various enzymes encoded by genes present on aflatoxin cluster. The genetic variations among different fungal strains can impact the final compound being produced. This chapter focuses on the biosynthetic pathway for aflatoxin production starting from acetate and finishing with the production of aflatoxin. The role of various genes and their encoded enzymes at every reaction has been described. Furthermore, the impact of different factors such as light, temperature, water activity, oxidative stress, carbon sources, nitrogen sources, and pH on aflatoxin biosynthesis has also been described. AFB_1 is the most common and toxic aflatoxin being consumed through various sources. Inside the human or animal body, AFB_1 is metabolized to different forms, making it either highly toxic or less toxic depending on the metabolism channel. The metabolism of AFB_1 has also been covered in this chapter.

Keywords Aflatoxins · Synthesized · Process · Reactions · Biosynthetic · Genes · Factors · Metabolism

2.1 Biosynthesis

Biosynthesis is an enzyme-catalyzed, multistep process involving the conversion of substrates into highly complex compounds within living organisms. During this process, the simpler compounds are modified either through conversion into other

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compounds or through joining together in order to form macromolecules. The biosynthesis of metabolites is carried out through certain metabolic pathways. Aflatoxins are synthesized by certain fungal species as secondary metabolites through a chain of reactions. The study regarding the biosynthesis of secondary metabolites generally involves the identification of reactions and their sequence through which the cells convert the primary metabolites into the final molecule. Additionally, the features regulating these processes are also identified. Even though a great variation may exist in chemical structures of metabolites, the biosynthesis of most secondary microbial metabolites may occur only through a certain number of biosynthetic pathways.

The biosynthetic pathway elucidation is a multistep procedure which may involve the identification of the primary metabolite from which the final molecule is being made and isolation of intermediate compounds formed along the pathway in order to hypothesize the sequence of reactions based on their chemical structures. The identification of enzymes involved at each point during the biosynthetic pathway and their isolation is also important in the understanding of this process. Furthermore, in order to completely understand the biosynthetic process, identification of regulatory factors and biosynthetic genes is also important.

2.2 Aflatoxin Biosynthesis

A complicated biosynthetic pathway consisting of at least 27 reactions catalyzed by different enzymes is involved in the production of aflatoxins (Roze et al. 2013; Yu 2012). The genes containing the codes for enzymes involved in the AF biosynthetic pathway are grouped in a cluster. The expression of these genes is controlled by two cluster-specific regulatory genes, namely, *aflR* and *aflS* (Chang 2003; Price et al. 2006). Additionally, the synthesis of AFs can also be influenced by environmental stimuli such as oxidative stress, nutrient sources, pH, and light which may initiate complex mechanisms through activation of various cell signaling pathways, hence modifying the expression of the genes playing a role in the production of toxin (Affeldt et al. 2014; Klich 2007; Montibus et al. 2015).

The DNA information in aspergilli is structured in eight chromosomes, and the genes resulting in AF synthesis are situated in the 54th cluster, 75-kb region of the fungal genome on chromosome III, 80 kb from the telomere of chromosome 3 (Georgianna and Payne 2009). This cluster consists of 30 genes (Fig. 2.1) and is mainly controlled by the regulatory genes *aflR* and *aflS* (Chang 2003; Price et al. 2006). The gene cluster involved in AF production has been extensively analyzed in *A. flavus* as well as in *A. parasiticus*. The studies have revealed the homology of the clustered genes among these species to be between 90 and 99% (Yu et al. 2000b). Due to the presence of these changes, one of the major differences existing among *A. flavus* and *A. parasiticus* is their ability to synthesize B- and G-type aflatoxins. The *A. flavus* majorly synthesizes aflatoxins B_1 and B_2, while *A. parasiticus* possesses the ability to produce both B and G types of aflatoxin. The functional genes

Fig. 2.1 Gene cluster for aflatoxin biosynthesis. This figure is adopted from the work of Yu (2012)

responsible for the synthesis of G-type aflatoxin include *aflF*, *aflU*, and *nadA* which, respectively, encode for an aryl alcohol dehydrogenase, a cytochrome P450 monooxygenase, and an oxidase (Ehrlich et al. 2004; Ehrlich et al. 2008). Furthermore, the elucidation of the aflatoxin-producing cluster has also been aided by studies on *A. nidulans* which has the capability to produce sterigmatocystin, an intermediate metabolite produced along the AF biosynthetic pathway. The *A. parasiticus* and *A. nidulans* exhibit the homology between 55 and 75%.

Birch (1967) first proposed the formation of aflatoxins through a polyketide pathway, and extensive studies have shown the involvement of at least 27 enzymatic reactions till now. Moreover, extensive studies have shown that along with other enzymes, cytochrome P450 also exhibits an important part in the production of aflatoxin. These enzymes are responsible for the attachment of functional groups (i.e., acetyl, methyl) during AF biosynthesis (Nelson 2011). Among the biosynthetic pathways for the production of mycotoxins known to date, the aflatoxin gene cluster has the highest number of cytochrome P450 (Roze et al. 2015). Thus owing to the number of oxidative changes involved, AF biosynthesis is among the longest and most complex processes (Minto and Townsend 1997).

2.3 Aflatoxin Biosynthetic Pathway

Aflatoxin biosynthetic pathway comprises of cascade of reactions catalyzed by various enzymes (Fig. 2.2).

2.3.1 Acetate to Norsolorinic Acid

Aflatoxins are the polyketide derivative furanocoumarin compounds. The biosynthesis of these substances require the hexanoate starter components (from acetyl-CoA and malonyl-CoA) which act as an initial substrate in a series of reactions ultimately leading to the formation of aflatoxins (Roze et al. 2013). The first step in the biosynthetic pathway for aflatoxins involves the formation of norsolorinic acid (NOR). The NOR is the first stable compound among the aflatoxin precursors

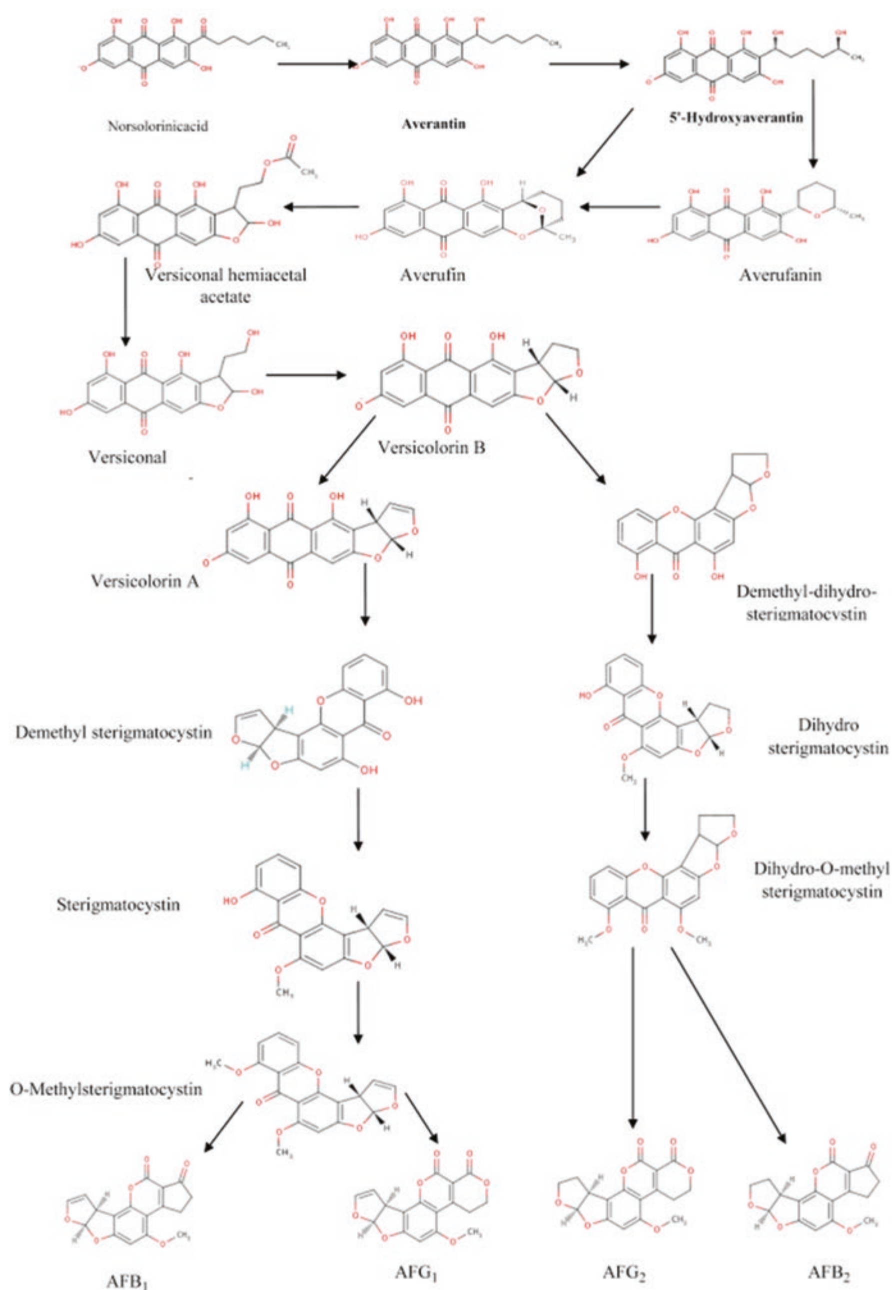

Fig. 2.2 Aflatoxin biosynthesis pathway. This figure is adopted from work of Gacem and El Hadj-Khelil (2016)

(Bennett et al. 1971). The formation of polyketide from a hexanoyl unit is assisted by a couple of fatty acid synthase enzymes and a polyketide synthase enzyme.

The enzymes catalyzing these changes are encoded majorly by four genes. The *afl*A and *afl*B were formerly known as *fas-2* and *fas-1*, respectively, because of their involvement in encoding fatty acid synthases. The *afl*A and *afl*B synthesize α and β protein subunits which are considered to play an important role in the formation of a polyketide structure from hexanoate units (Roze et al. 2013; Yu et al. 2004a). The gene *afl*C, also known as *pks*A, contains the coding regarding the synthesis of polyketide skeletons. The polyketide synthase is involved in the chain elongation that occurs in most of the secondary metabolites that are acetate derivatives. This enzyme has been found to further convert the polyketide structure into norsolorinic acid anthrone (NAA) (Roze et al. 2013). Furthermore, another gene, *hyp*C, is known to encode noranthrone oxidase, a 17-kDa enzyme that has been demonstrated to be involved in the catalytic transition of NAA to NOR (Ehrlich and Yu 2009). The *hyp*C is located in the region between *afl*C and *nor*-1. The *nor*-1 gene, due to its participation in aflatoxin biosynthesis, is also known as *afl*D.

2.3.2 Norsolorinic Acid to Averantin

The norsolorinic acid is further converted into averantin (AVN). The conversion of NOR into AVN is directed by the *afl*D (*nor-1*) gene. The cloning of the *afl*D gene was achieved through genetic complementation. The *afl*D gene encodes a ketoreductase that is required in order to convert the 1′-keto group of NOR to the 1′-hydroxyl group of AVN (Zhou and Linz 1999). Earlier it was predicted that the *aflE* (*norA*) and *aflF* (*norB*) genes present on the AF cluster were associated with this step. The short-chain aryl alcohol dehydrogenases, which may have the tendency to further assist the conversion of NOR to AVN, are encoded by these genes (Cary et al. 1996). However, they have also been shown to take part in other steps involved in the series of reactions catalyzed by enzymes during AFB_1 biosynthesis.

2.3.3 Averantin to 5′-Hydroxyaverantin

The earliest proof revealing the conversion of *Averantin* (AVN) to 5′-hydroxyaverantin (HAVN) was established through the experiments involving radioisotope incorporation (Bennett et al. 1980). The studies showed that in *A.* parasiticus, the transformation of AVN to averufin (AVF) is accomplished by two enzymatic reactions among which the first reaction involves the conversion of polyketide anthraquinone averantin into HAVN through hydroxylation. This reaction is catalyzed by P450 monooxygenase enzymes (Yabe et al. 1991). The gene *ord-1*, encoding the P450 monooxygenase, was cloned and disrupted by Yu et al. (1997). The studies of

ord-1 mutant in the presence of a substrate proved that HAVN is an intermediate compound formed during the transformation of AVN to AVF. The *ord-1* gene that exhibits a high degree of similarity in sequence to *A. nidulans stcf* (Brown et al. 1996) was renamed as *aflG* (*avnA*).

2.3.4 5′-Hydroxyaverantin to Averufin

Averufin (AVF) is among key intermediary compounds formed during the biosynthetic formation of aflatoxins through many studies (Keller et al. 2000). Furthermore, initially the involvement of several metabolites were reported during the transformation of AVN to AVF (Bhatnagar et al. 1992). However, the later studies negated the involvement of some of these metabolites as an intermediate in aflatoxin formation. One of such metabolites was averufanin (AVNN) which proved to be a shunt metabolite instead of being an intermediate in aflatoxin biosynthetic pathway (Sakuno et al. 2003; Yabe and Nakajima 2004).

An alcohol dehydrogenase-encoding gene cluster, *aflH* (*adhA*), was characterized by Chang et al. (2000) in *A. parasiticus*. The experiments showed that *adhA* deletion mutants resulted in predominant accumulation of HAVN. However, after prolonged growth periods, the mutant strains showed production of AVNN in minor quantities which was observed to be a shunt metabolite. Hence, the transformation of HAVN might be directly into the AVF or indirectly, as a result of the actions of additional cytosolic enzymes. Two cytosolic enzymes along with 5′-oxoaverantin (OAVN), a novel AF intermediate, were described during further studies by Sakuno et al. (2003). Among the series of intermediates formed during aflatoxin biosynthesis, OAVN was observed to be formed during the transformation of HAVN to AVF. The *aflH* (*adhA*) gene is responsible for encoding alcohol dehydrogenase enzyme that is involved in catalytic conversion of HAVN to OAVN. However, it was observed that the *adhA* deletion mutant was leaky, revealing the possible involvement of other genes or enzymes during transformation of OAVN to AVF. The study by Sakuno et al. (2005) showed the association of *aflK* (*vbs*) gene with the transformation of OAVN to AVF. Although initially the *aflK* gene was linked with the transformation of a versiconal compound into versicolorin B only, this was the first time described that the same enzyme can be involved in the catalysis of two reactions during AFB_1 biosynthesis. It was further hypothesized that this might have happened as a result of evolution in the gene cluster of AFB_1.

2.3.5 Averufin to Versiconal Hemiacetal Acetate

The oxidation of averufin (AVF) transforms it into versiconal hemiacetal acetate (VHA). VHA is known as a precursor to aflatoxin (Fitzell et al. 1977). The gene *aflI* (*avfA*) has been shown to take part in the biotransformation of AVF to VHA. Yu

et al. (2000b) revealed that when AVF-accumulating mutant, *A. parasiticus* SRRC 165, was complemented with *aflI* (*avfA*) gene from *A. flavus*, it restored the ability of strain transform AVF to VHA consequently producing aflatoxins, hence confirming the role of *aflI* (*avfA*) in this process. However, the precise role of *aflI* in the AVF oxidation has still not been completely clarified, though it has been commonly projected that the *aflI* encoded enzyme is involved in the catalysis of the ring closure step during the production of hydroxyversicolorone (HVN).

Furthermore, the gene *aflV*-encoded enzyme has been shown to be responsible for catalyzing the process of AVF transformation to HVN and the *aflW* gene product to be involved in conversion of HVN to VHA through a Baeyer-Villiger reaction. The gene *aflV* (*cypX*) is involved in encoding P450 microsomal monooxygenase, and *aflW* (*moxY*) has been found to encode a cytosolic monooxygenase (Wen et al. 2005).

2.3.6 Versiconal Hemiacetal Acetate to Versiconal

The subsequent phase after the formation of VHA includes its transformation into a versiconal (VAL). Various studies have revealed the involvement of an esterase in this conversion (Kusumoto and Hsieh 1996). The esterase enzyme was identified to have been encoded by *aflJ* (*estA*) (Yu et al. 2003). Furthermore, the esterase enzyme has also been isolated from *A. parasiticus* (Hsieh 1989; Kusumoto and Hsieh 1996). Additionally, the *aflJ* deletion mutants of *A. parasiticus* showed the accumulation of some metabolites such as versicolorin A (VERA) and VHA (Chang et al. 2004). In addition, trace amounts of versiconoc acetate (VOAc) along with other downstream metabolites in aflatoxin biosynthetic pathway including VAL and versicolorin B were also accumulated. The enzyme esterase is also known to exhibit involvement in the reversible transformation of VHA to VOAc. Furthermore, a study by Chang et al. (2004) confirmed the participation of esterase encoded by *aflJ* in transformation of VHA to VAL and VOAc to VOH during the biosynthesis of aflatoxins.

2.3.7 Versiconal to Versicolorin B

The conversion of VAL/VHOH to VERB was identified to have been catalyzed by a cyclase enzyme, named versicolorin B synthase (McGuire et al. 1996; Silva and Townsend 1997). This gene was cloned and named *vbs* (Silva et al. 1996). It was also observed during these studies that the recombinant proteins of the vbs gene exhibited the cyclase activity. The *vbs* gene was renamed as *aflK* due to its involvement in aflatoxin biosynthesis reaction cascade (Yu et al. 2004b). This enzyme is also involved in the conversion of OAVN into AVF as mentioned earlier. Furthermore, the closure of the bisfuran ring is also catalyzed by the *aflK*-encoded enzyme. The bisfuran ring is known to be responsible for the toxic character of aflatoxins as it binds with DNA after metabolization (Yu et al. 2004b).

2.3.8 Versicolorin B to Versicolorin a

The subsequent transformations of VERB are critical in determining the type of aflatoxin going to be synthesized. The VERB structure contains the tetrahydrofuran ring that is similar to the one present in AFB2/AFG2, hence forming the AFB2/AFG2 as a final product of aflatoxin biosynthetic pathway. Contrarily, the transformation of VERB to versicolorin A (VERA) leads to the formation of AFB1 or AFG1 eventually, as these toxins contain a dihydrobisfuran ring like VERA. The transformation of VERB to VERA involves the desaturation of the bisfuran ring (Yabe et al. 1993). It was identified that the *stcL*-disrupted *A. nidulans* did not synthesize sterigmatocystin (ST) compounds, consequently resulting in VERB accumulation (Kelkar et al. 1997). Furthermore, *aflL* (*verB*), a homologue of *stcL* in *A. parasiticus* and *A. flavus*, is considered to be involved in the conversion of VERB to VERA as it encodes a cytochrome P450 monooxygenase/desaturase (Kelkar et al. 1997).

2.3.9 Versicolorin a to Demethylsterigmatocystin and Versicolorin B to Dihydrodemethylsterigmatocystin

The biosynthetic pathway of aflatoxins involves the conversion of VERA to demethylsterigmatocystin (DMST) resulting in the formation of AFB_1 or AFG_1. Additionally, the transformation of VERB to dihydrodemethylsterigmatocystin (DHDMST) eventually leads to formation of AFB_2 or AFG_2. Henry and Townsend (2005) have described the changes occurring during this chain of reactions in detail.

Four genes have been observed to take part in the VERA to DMST conversion. The *ver-1* also known as *aflM* is a ketoreductase encoding gene that is similar to *nor-1*. The gene *aflM* was observed to be involved in the transformation of VERA to an intermediate compound which has still not been isolated. The homologous gene for *aflM* was also identified in *A. nidulans* as *stcU*. Furthermore, another gene, *aflN* (*verA*) responsible for coding a cytochrome P450-type monooxygenase, was shown to catalyze the transformation of VERA to another intermediate before converting into DMST. The *aflN* homologue in *A. nidulans* has also been identified as *stcS* (Yu et al. 2004a, b). Additionally, the disruption of *stcU* and *stcS* resulted in accumulation of VERA, hence confirming their requirement in the transformation of VERA to DMST ultimately (Keller et al. 1995). However, their exact function remains to be identified. The third gene involved in the transformation of VERA, *aflY* (*hypA*), is considered to encode a Baeyer-Villiger monooxygenase which appears to act as a mediator between two hypothetical structures during the transformation of VERA to DMST. The disruption of *aflY* in *A. parasiticus* resulted in accumulation of VERA which may suggest that it is involved as a part of the enzyme complex without permitting the development of intermediate compounds. The gene *aflX* (*ordB*) is further responsible for coding an oxidoreductase that is involved in the catalysis of oxidative decarboxylation and ring closure of the intermediate formed after *aflY*-catalyzed oxidation.

2.3.10 Demethylsterigmatocystin to Sterigmatocystin and Dihydrodemethylsterigmatocystin to Dihydrosterigmatocystin

The involvement of *O*-methyltransferases in aflatoxin biosynthesis was confirmed after studies on purified enzymes. It was revealed that *O*-methyltransferase I is involved in the catalysis of methyl transfer from S-adenosylmethionine (SAM) to the hydroxyls of DHDMST as well as DMST in order to produce DHST and ST, respectively. The *O*-methyltransferase I is a 43-kDa enzyme that has been isolated from *A. parasiticus*. The gene corresponding to this enzyme, *dmtA*, was isolated from *A. flavus*, *A. parasiticus*, and *A. sojae* and was later named *aflO* (*omtB*) (for O-methyltransferase B) (Yu et al. 2000b). The *stcP* gene in *A. nidulans* was identified to be homologous to the *aflO* gene. Furthermore, the disruption of *aflO* caused failure of DMST transformation to ST.

2.3.11 Sterigmatocystin to O-Methylsterigmatocystin and DHST to Dihydro-O-Methylsterigmatocystin

The *O*-methyltransferase catalyzes the transformation of ST to OMST and DHST to DHOMST. The gene containing the code for *O*-methyltransferase was first cloned using *A. parasiticus* through reverse genetics by producing antibodies against the *O*-methyltransferase isolated from *A. parasiticus* (Keller et al. 1993). The gene responsible for these transformations, *aflP* (*omtA*), was formerly named as *omt-1* and then *omtA* (Yu et al. 1993). The enzyme *O*-methyltransferase A is substrate specific; hence it cannot methylate DMST or DHDMST. In addition to *A.* parasiticus, the genomic sequence of *aflP* was cloned from *A. flavus* as well (Yu et al. 1995). Furthermore, the homologue for *aflP* was also identified in different other species of *Aspergillus*, either aflatoxigenic or non-aflatoxigenic (Klich et al. 1995). However, the orthologue of *aflP* is absent in *A. nidulans* which can explain the absence of aflatoxin as an end product and the presence of ST in this particular specie.

2.3.12 Formation of Aflatoxin B and Aflatoxin G

During the final conversions of OMST into AFB_1, various genes are involved including *aflQ*, *hypB*, *aflE*, and *hypE*. The roles of *aflQ* and *hypB* have been accurately defined in the final transformations; however partial roles have been reported for various other genes involved in AF biosynthesis. The gene responsible for encoding a P450 monooxygenase, *aflQ* (ordA), is present adjacent to *aflP* in the aflatoxin cluster. The expression of this gene ultimately leads to the transformation of OMST

into aflatoxin B_1 or G_1 and DHOMST into aflatoxin B_2 or G_2 (Ehrlich 2009). The *aflQ* transforms OMST into 11-hydroxy-O-methylsterigmatocystin (HOMST), a precursor of AFB_1 (Zeng et al. 2011). Nevertheless, it is still not clear whether two successive reactions involving monooxygenase are catalyzed by *aflQ* (*ordA*) gene product, OrdA, during the later steps of aflatoxin biosynthesis. The *hypB* gene encodes an oxidase reported to take part in conversion of HOMST into a seven-ring lactone (MW, 370 Da) and is expressed under conditions suitable for aflatoxin production (Ehrlich et al. 2010). This compound is converted into another unknown intermediary product through hydrolytic enzymes which do not belong to the aflatoxin cluster (Ehrlich 2009). The synthesis of G-group aflatoxins has been proposed to involve additional enzyme(s) (Yu et al. 1998). Further studies showed that cytochrome P450 monooxygenase encoded by *cypA* gene is involved in the formation of G-type aflatoxins (Ehrlich et al. 2004). Furthermore, the *nadA* gene that was earlier considered as a member of sugar utilization cluster (Yu et al. 2000a) was also shown to be a member of the adjoining aflatoxin cluster through microarray studies and participated in the formation of AFG_1/AFG_2 (Yu et al. 2011). The disruption of *nadA* gene showed that a recently observed AF intermediate, NADA (formed after OMST), is converted into G_1 type of aflatoxin through a cytosolic enzyme named NadA. Initially, the gene *aflE* (*norA*), homologous to *aflD* in the aflatoxin cluster, was believed to be involved in the conversion of NOR to AVN; however, further studies depicted the involvement of *aflE* in mainly final two transformations during formation of AFB_1. Although the role of *aflE* in AF biosynthetic pathway has been confirmed as its absence resulted in accumulation of deoxyaflatoxin, its exact position on AF gene cluster is still not known. Furthermore, the *hypB* was also predicted for its involvement in one of the steps exhibiting oxidation during the transformation of OMST to AFs. The involvement of *hypE* (*aflLa*) during the last steps of AFB_1 biosynthesis was also proposed because the disruption of *hypE* led to the production of an intermediary compound prior to the formation of deoxyAFB_1 synthesis. The *hypE* depicts homologies with several bacterial enzymes, and its participation along AF enzymatic pathway was suggested in combination with P450 monooxygenase (Ehrlich 2009). *A. flavus* is involved in the production of only B_1 and B_2 aflatoxins, while *A. parasiticus* is capable of producing aflatoxins B_1, B_2, G_1, and G_2. The presence of the intact *nadA* and *norB* genes has only been shown in *A. parasiticus* which is the G-group producer. The data proposes that *norB* is responsible for encoding another enzyme which is predominantly involved in the formation of AFG_1 and AFG_2 (Ehrlich et al. 2008).

2.3.13 Formation of Aflatoxin M

The aflatoxins M_1 and M_2 are the products of AFB_1 and AFB_2 biologically converted within mammals. These products were initially separated from bovine milk (Garrido et al. 2003). After aflatoxins enter a mammalian (humans or animals) body, the liver cytochrome P450 enzymes metabolize them, consequently converting them to a

reactive epoxide intermediate or hydroxylated aflatoxins M_1 and M_2. The epoxide intermediate is a more toxic compound exhibiting higher carcinogenicity, while aflatoxins M_1 and AFM_2 are less harmful metabolites of aflatoxins B_1 and AFB_2, respectively. Some recent studies involving feeding of aspertoxin (12c-hydroxy-OMST) (Yabe et al. 2012) revealed that *A. parasiticus* also exhibits production of some minor aflatoxins including M_1, M_2, GM_1, and GM_2. Furthermore, feeding of *O*-methylsterigmatocystin (OMST) to *A. parasiticus* resulted in production of AFM_1 and $AFGM_1$ along with AFB_1 and AFG_1, while feeding with DHOMST resulted in the production of aflatoxins M_2 and GM_2 in addition to B_2 and G_2. This revealed that *OrdA* is responsible for catalyzing the reaction involving 12c-hydroxylation resulting in transformation of OMST to aspertoxin and also the subsequent transformation from aspertoxin to AFM_1. In this scenario, the AFB_1 is not a precursor of AFM_1.

2.4 Factors Affecting Aflatoxin Biosynthesis

The aflatoxin biosynthesis is a complicated process based on a chain of reactions catalyzed by various enzymes coded by different genes. The synthesis of aflatoxins is affected by different nutritional and environmental factors which are discussed below.

2.4.1 Oxidative Stress

The aflatoxins are considered to be biosynthesized by *A. parasiticus* and *A. flavus* as a response by their cells against oxidative stress. A study showed that the aflatoxigenic strains of *A. parasiticus* require more oxygen as compared to non-aflatoxigenic strains during the phase of their active growth (Jayashree and Subramanyam 2000). It was further shown that aflatoxin synthesis in *A. parasiticus* is prompted by an increase in oxidative stress. The higher oxygen demand may consequently cause an increase in reactive oxygen species (ROS), hence increasing oxidative stress (Walsh et al. 2011). The relation of oxidative stress with aflatoxin synthesis was further confirmed as its alleviation resulted in a decrease in aflatoxin production (Huang et al. 2009). The microarray analysis revealed the downregulation of all the genes of *A. flavus* involved in AF biosynthesis after treatment with an antioxidant caffeic acid (Kim et al. 2008). Additionally, the toxigenic *A. parasiticus* possesses higher antioxidant activities by enzymes like superoxide dismutase in comparison to its nontoxigenic forms (Narasaiah et al. 2006). Catalases (CAT), superoxide dismutases (SOD), and glutathione peroxidase (GPX) play critical roles in the defense system of fungal strains against reactive oxygen species (ROS), hence facilitating the cellular defense to cope with oxidative stress. Among these, SOD provides frontline defense through conversion of the free radicals into H_2O_2 and O_2. Afterward,

peroxidases and catalases assist the transformation of H_2O_2 into H_2O and/or O_2 in to H_2O, respectively (Weydert and Cullen 2010). Aflatoxin production is considered to perform as a secondary defense mechanism after the primary mechanism that involves antioxidant enzymes (Hong et al. 2013). Furthermore, the AFB_1 biosynthesis is promoted in the availability of acetate units, and the beta-oxidation of fatty acids results in their degradation into acetate units among fungal strains (Maggio-Hall and Keller 2004), hence linking this pathway indirectly to AFB_1 production.

2.4.2 Carbon

The synthesis of secondary metabolites is well known for being dependent upon the availability of carbon and its source. Sugars are considered to be a favorable source of carbon aflatoxin biosynthesis as they produce the polyketide starter units (Davis and Diener 1968; Maggio-Hall et al. 2005). Various studies have shown the presence of simple sugars to be related to higher levels of aflatoxin synthesis in *A. parasiticus*, *A. flavus*, and *A. nidulans* (Bhatnagar et al. 2006; Calvo et al. 2002; Liu et al. 2016). In contrast, D-glucal (a glucose derivative), when used as the principle sugar source in the medium, was able to inhibit the aflatoxin production because D-glucal is not metabolized by fungi (Zhang et al. 2014). The carbon source utilization among *Aspergillus* is mediated by a sugar cluster which contains four genes grouped in a 7.5-kB cluster. This cluster of genes is positioned next to aflatoxin gene cluster in *A. flavus* and *A. parasiticus* (Bhatnagar et al. 2006; Yu et al. 2000a). Many genes present in the aflatoxin cluster have CreA-binding sites close to their promoter regions, which may be a basis of forming its relation with regulation of AF production (Georgianna and Payne 2009). CreA is a transcription factor that in combination with genes is involved in the process of carbon catabolic repression (CCR). The aspergilli use CCR as a strategic mechanism in order to preserve energy and regulate the carbon catabolism for using the most favorable carbon source (Deepika et al. 2016; Ruijter and Visser 1997). Furthermore, CreA also plays an important role in several other functions in addition to AFB_1 production in *A. flavus* (Fasoyin et al. 2018).

2.4.3 Nitrogen

Among *Aspergillus* species, the nitrogen sources are regulated by nitrogen metabolite repression mechanism. The nitrogen sources have been found to affect the synthesis of aflatoxin and ST in various ways (Calvo et al. 2002). In this perspective, AreA plays a critical role as it modulates the genes responsible for utilization of substitute nitrogen sources. Different nitrogen sources may affect the AF production differently as some substrates, such as asparagine, glutamate, and ammonium salts,

support the AF synthesis, while some substrates like sodium nitrate and tryptophan do not favor AF production (Yu 2012). It is because the nitrogen source media govern the under- and over-expression of the *areA* gene, consequently resulting in a higher or lower production levels of aflatoxin (Fasoyin et al. 2019).

2.4.4 pH

The lower pH levels are generally associated with high contents of aflatoxin production in *A. flavus*. A study showed a tenfold increase in AF production when the pH was reduced to 4.0, while an increase in pH caused a decrease in AF synthesis (Cotty 1988). For *A. parasiticus*, a decrease in pH level to <6.0 was found to stimulate the AFB synthesis, whereas the higher pH level favored the synthesis of G-type aflatoxin (Buchanan and Ayres 1975). The *aflM* expression is higher in acidic media as compared to that in neutral or alkaline media. Additionally, the fungal growth results in pH reduction, consequently enhancing AF production with time (Keller et al. 1997).

2.4.5 Light

The stimulus of light exerts a high impact on adaptation as well as survival of fungal strains. Light may have an impact on growth and morphological features of the fungus, consequently affecting the production of secondary metabolites as well (Rangel et al. 2015). The light induces a strong "velvet comlex" in aspergilli that is governed by a global regulator *veA* that controls a number of genetic elements including photoreceptors (Purschwitz et al. 2008). VeA regulates half of the gene clusters involved in secondary metabolite formation (28 out of the 56), including the AF gene cluster (Cary et al. 2015). The *veA* is an essential gene for the production of AFB_1 in *A. flavus* (Duran et al. 2009). The null mutants of *veA* and *IaeA* did not exhibit *aflR* expression (Amaike and Keller 2009). Deletion of *veA* in *A. parasiticus* resulted in the absence of versicolorin A, an aflatoxin intermediate, hence confirming its role in *aflR*/*aflS* expression (Calvo et al. 2004).

2.4.6 Temperature

Temperature is among the major influencing factors affecting aflatoxin synthesis. It is associated with promoting the expression of the structural biosynthesis genes (*aflD* and *aflO*). However, it does not induce expression of regulatory genes (*aflR* and *aflS*) (Gallo et al. 2016). A study by OBrian et al. (2007) showed that AF

biosynthetic genes were expressed more under the temperature of 28 °C as compared to higher temperature of 37 °C, but the aflatoxin pathway regulatory genes, *aflR* and *aflS*, did not show difference at these two temperatures. Furthermore, the genes involved in aflatoxin biosynthesis were found to be downregulated when exposed to elevated temperatures of 42 °C (Liu et al. 2017).

2.4.7 Water Activity

The water activity (a_w) may also affect aflatoxin biosynthesis. All the genes involved in AFB_1 biosynthesis and *laeA* were found to be inhibited under the a_w of 0.99 as compared to the a_w of 0.96, while highest expression was observed at a_w 0.92 (Liu et al. 2017).

2.5 Metabolism of Aflatoxin B_1

The metabolism of aflatoxin B_1 occurs through oxidative reactions catalyzed by the members of cytochrome P450 (CYP450) supergene family of isoenzymes. The CYP450 enzyme family is composed of hemoproteins and electron carriers that, during the cellular respiration, catalyze or enhance the oxidation-reduction reactions (Lamb et al. 2009). Earlier, it had been considered that CYP450 specifically originated from the liver, but later studies showed that they are distributed throughout the body (Ding and Kaminsky 2003). However, xenobiotics are mainly metabolized in the liver (Shimada et al. 1994). The isoforms of CYP450 involved in AFB_1 metabolism in the body include CYP1A1, CYP1A2, CYP3A4, CYP2Cs, CYP3A5, and CYP3A7 (Shimada et al. 1994). The glutathione S-transferase (GST) and AFB1-aldehyde reductase also catalyze the AFB_1 metabolism resulting in the formation of reactive metabolites, among which some can be used as the biomarkers for AF exposure (Bbosa et al. 2013). The metabolites of AFB_1 formed by actions of different CYP450 isoenzymes vary in their carcinogenic potential. The toxic impacts of AFB_1 are associated with activation as well as detoxification rate at the primary and secondary levels of metabolism (Neal et al. 1987). Furthermore, the fate of aflatoxin B_1 metabolism varies among and within humans and animals. In addition, the activation rate of aflatoxins varies among children and adults belonging to the same species as well, consequently affecting their resistance toward AFB_1 toxicity (Ramsdell and Eaton 1990). Additionally, AFB_1 metabolism varies among humans belonging to different regions of the world. The main pathways involved in AFB_1 metabolism include O-dealkylation, ketoreduction, epoxidation, and hydroxylation (Fig. 2.3). These reactions can result in formation of either highly toxic metabolite (AFBO and AFM1) or relatively nontoxic compounds (AFP_1, AFQ_1, or AFB_{2a}) (Wu et al. 2009).

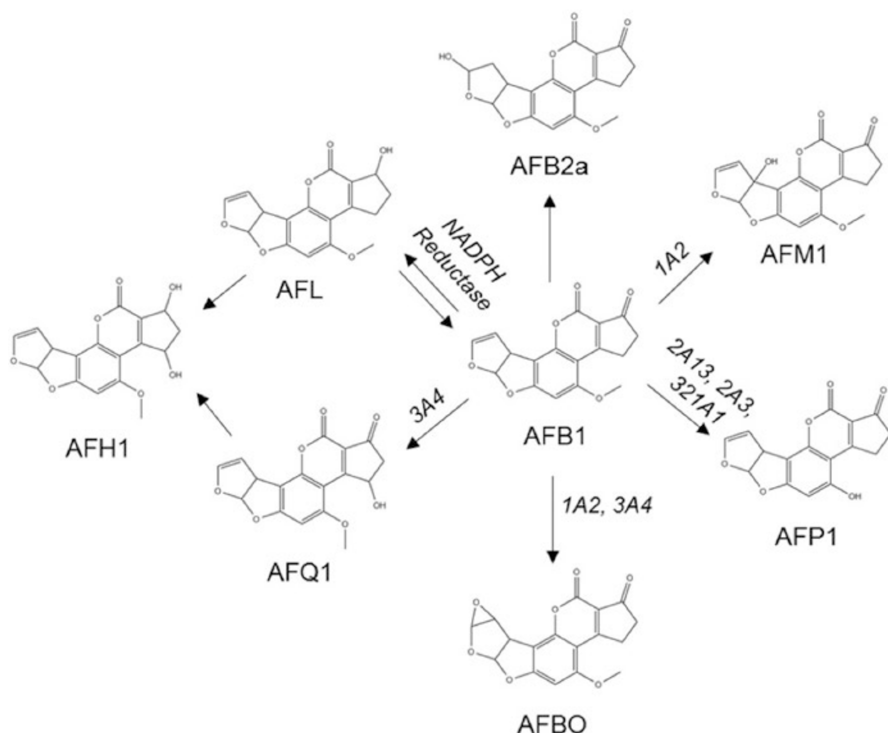

Fig. 2.3 AFB_1 metabolic pathway mediated by CYP450. This figure is adopted from the work of Rushing and Selim (2019)

2.5.1 Aflatoxin B1–8,9-Epoxide

The AFB_1 is metabolized to B1–8,9-epoxide (AFBO) through the help of enzyme system P450 in the liver. The AFBO has two isomers, *endo*-8,9-epoxide and *exo*-8,9-epoxide (Raney et al. 1992a). The isoenzymes, CYP3A4 and CYP1A2, are primarily responsible for this conversion. The CYP1A2 acts as a primary producer of AFBO when AFB_1 is in lower concentrations. In contrast, at higher AFB_1 concentrations, CYP3A4 majorly produces AFBO resulting in the formation of *exo* AFBO isomers only (Ueng et al. 1995). Also, the CYP1A2 produces more *exo* isomers as compared to CYP3A4 at lower AFB_1 levels (Gallagher et al. 1996). This intermediate exhibits extremely electrophilic character, allowing it to instantly react with amines of proteins as well as of nucleic acids. It reacts with DNA and attaches with N_7 position of guanine, consequently forming AFB_1-N_7-guanine adduct imparting AFB1-*exo*-8,9-epoxide highly carcinogenic character (Johnson and Guengerich 1997).

2.5.2 AFQ1

AFQ_1 is a relatively nontoxic metabolite of AFB_1 formed through hydroxylation mediated solely by CYP3A4 (Kamdem et al. 2006). It was first observed in monkey liver microsomal preparations exposed to AFB_1. Generally, AFQ_1 is produced in much higher amounts in comparison to AFM_1; however, rat microsomes were observed to produce lower amounts (Masri et al. 1974). It was shown that AFQ_1 occurred in humans frequently in amounts ranging from 1 to 11% of initial AFB_1 amounts (Yourtee et al. 1987). However, the potential of AFQ_1 to bind with DNA is significantly lower as compared to that of AFBO, hence making it a detoxification product of AFB_1 in comparison to AFM_1 which is toxic in nature (Raney et al. 1992b). Furthermore, another study showed the presence of AFQ_1 in levels higher than AFM_1 and AFB_1-N_7-guanine after monitoring the urinary and fecal samples. Moreover, the concentrations were found to be higher in fecal matter in comparison to urine, making it a potential biomarker source for evaluation of AFB_1 exposure (Mykkänen et al. 2005). Even though AFQ_1 is one of the most abundant metabolites of AFB_1, it is seldom used as a biomarker for AFB_1 exposure assessment.

2.5.3 AFP_1

AFP_1 is also a detoxification metabolite of AFB_1 produced by hydroxylation through P450 enzymes including CYP2A13, CYP2A3, and CYP321A1 (He et al. 2006; Niu et al. 2008). Studies have shown the presence of this metabolite in urine of individuals exposed to AFB_1 and those who had developed hepatocellular carcinoma (HCC) probably as a consequence of AFB_1 exposure (Ross et al. 1992).

2.5.4 Aflatoxicol

In contrast to other metabolites of AFB_1, aflatoxicol (AFL) is found in cytosolic fractions of liver preparations. The formation of AFL is mediated by NADPH reductase, typically in the cytosol (Partanen et al. 2010). Unlike AFP_1 and AFQ_1, AFL retains its DNA-binding capacity, consequently retaining its toxic nature. Therefore, it is not considered as a detoxification product of AFB_1. Furthermore, the AFL acts as a reservoir for AFB_1 as it has a tendency to be converted back to AFB_1 though enzyme actions, which further enhances the toxic effects (Partanen et al. 2010). The AFL is the only AFB_1 metabolite that can be transferred through the placenta of a human and can be formed from placenta as well. This metabolite has been observed in human urine as well as in breast milk of individuals exposed to AFB_1 (Kussak et al. 1998).

2.5.5 AFH$_1$

AFH$_1$ resembles AFL structurally with an additional hydroxyl group at the terminal cyclopentenone ring. Two enzyme systems are involved in the metabolic conversion of AFB$_1$ to AFH$_1$, namely, the microsomal hydroxylase and cytoplasmic reductase. However, it is not clear whether the AFH$_1$ is formed through hydroxylation of AFL or reduction of AFQ$_1$.

2.5.6 AFB2a

AFB2a was initially characterized as a product of AFB$_1$ formed as a result of acid catalysis. The mild acidic conditions promote the addition of waster across the 8,9-double bond to form the hemiacetal ring. This nonenzymatic transformation was observed in acidic media of molds that had been added with AFB$_1$ (Ciegler et al. 1966). It has been shown that AFB2a possesses lower toxicity as compared to AFB$_1$ due to lower DNA-binding capacity, hence making it a detoxification product of AFB$_1$. However, it has a unique tendency to bind with cellular proteins which can contribute to other cellular toxicities. In addition, this binding usually occurs with primary amines in alkaline conditions. The binding may take place on phosphoethanolamine head groups of phospholipids. It is one-of-a-kind structurally characterized aflatoxin-lipid adduct till now (Rushing and Selim 2017).

2.6 Conclusion

Aflatoxins are highly toxic compounds produced by various fungal strains belonging to *Aspergillus*. These toxins are produced as secondary metabolites through a complex biosynthetic pathway which has been under investigation for decades. The aflatoxin biosynthesis involves a number of reactions catalyzed by different enzymes which consequently produce intermediate compounds, ultimately forming aflatoxins. The aflatoxin formation is regulated by expression of genes at different steps in the chain of reactions. Studies on AF biosynthesis have helped reveal the type of enzymes and the genes responsible for encoding these enzymes taking part in the biosynthesis of aflatoxins. The production of aflatoxin is affected by several factors such as light, temperature, pH, water activity, and nutrient sources. The knowledge about the impact of these factors on gene expression can help propose effective strategies to prevent contamination of food as well as feed commodities with aflatoxins. Additionally, comprehensive understanding about AFB$_1$ metabolism can also help identify different biomarkers in order to assess the exposure of the population to aflatoxins. This can aid in determining an accurate estimate regarding the threat of aflatoxins being faced by populations of different regions around the world.Conflict of InterestThe authors declare no conflict of interest.

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