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MINI-REVIEW

Understanding nonaflatoxigenicity of *Aspergillus sojae*: a windfall of aflatoxin biosynthesis research

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Abstract *Aspergillus* section *Flavi* includes aflatoxin-producing and nonproducing fungi. *Aspergillus sojae* is unable to produce aflatoxins and is generally recognized as safe for food fermentation. However, because of its taxonomical relatedness to aflatoxin-producing *Aspergillus parasiticus* and *A. flavus*, it is necessary to decipher the underlying mechanisms for its inability to produce aflatoxins. This review addresses the relationship between *A. sojae* and *A. parasiticus* and the advances that have been made in aflatoxin biosynthesis research, especially with regard to gene structure, genome organization, and gene regulation in *A. parasiticus* and *A. flavus* and how this has been used to assure the safety of *A. sojae* as an organism for food fermentation. The lack of aflatoxin-producing ability of *A. sojae* results primarily from an early termination point mutation in the pathway-specific *aflR* regulatory gene, which causes the truncation of the transcriptional activation domain of AflR and the abolishment of interaction between AflR and the AflJ co-activator. Both are required for gene expression. In addition, a defect in the polyketide synthase gene also contributes to its nonaflatoxigenicity.

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Introduction

Aspergillus sojae, like *Aspergillus oryzae*, is a koji (starter) mold for food fermentation. *A. sojae* is mainly used for the production of soy sauce, a condiment of global appeal with a multi-billion dollar market worldwide. *A. sojae* and *A. oryzae* are non-toxigenic variants of *Aspergillus flavus* and *A. parasiticus* and are grouped in section *Flavi* along with other toxigenic variants, such as *A. nomius*, *A. tamarii*, *A. pseudotamarii*, and *A. bombycis* (Klich and Pitt 1988). In agricultural fields, *Aspergillus flavus* is the main producer of the carcinogenic aflatoxins. (Frisvad et al. 2005; Goto et al. 1996; Ito et al. 2001). *Aspergillus* species classified outside of section *Flavi* also can produce aflatoxins. For example, *Aspergillus ochraceoroseus* from section *Ochraceorosei*, an *Aspergillus* species, SRRC 1468, morphologically resembling members of section *Circumdati*, and the ascomycete *Emericella astellata* and *E. venezuelensis* (*Aspergillus* section *Nidulantes*; Cary et al. 2005) also produce aflatoxins. Levels of aflatoxins in food and agricultural commodities are stringently regulated in most countries (Otsuki et al. 2001).

Consumer safety of fermented foods is determined by the type of organisms used, the quality of raw materials, and the production process (Blumenthal 2004; van Dijck et al.

2003). The production process is governed by Good Manufacturing Practice and Hazard Analysis of Critical Control Points. The safety focus is thus on the organisms and the raw materials used in the process. The primary safety evaluation of a production microorganism is its "toxigenic potential" (Pariza and Johnson 2001). A legal concept central to the US food law is Generally Recognized As Safe (GRAS). It refers to whole foods or food ingredients (including microorganisms for special uses), whose safety has been established based on either (1) a safe history of common use before 1958 or (2) publicly available scientific information (Schechtman 1992). No isolates of *A. sojae* or *A. oryzae* are known to produce aflatoxins (Wei and Jong 1986). Decades of use of *A. sojae* in food fermentation has suggested its biosafety. However, it is imperative to have a better understanding of why this organism lacks the ability to produce aflatoxins.

Relationship of *A. sojae* to *A. parasiticus*

A. sojae is morphologically similar to *A. parasiticus*, and it is generally agreed that *A. sojae* is a domesticated strain of *A. parasiticus*. Unlike *A. oryzae*, *A. sojae* has never been isolated from the agricultural soil (Wicklow 1984). *A. sojae* is separated from *A. parasiticus* by morphological characteristics, such as color of colony, texture, and conidial diameter (Klich 2002), but differences are not always obvious and intergradations are common. Culture characteristics can be affected by environment and nutritional conditions, which make it difficult to accurately score morphological data. Special skill and experience thus are required to differentiate the two species. In general, colonies of *A. sojae* are brownish olive, whereas those of *A. parasiticus* tend to be dark olive green. Conidial diameters of *A. sojae* isolates are larger (4–8 μm) than those of *A. parasiticus* (3–6 μm) (Matsushima et al. 2001b; Yuan et al. 1995). Sensitivity to bleomycin-modified minimal agar (MMA) provided a reliable method to differentiate between *A. sojae* and *A parasiticus* (Klich and Mullaney 1989). Although growth of both is reduced by bleomycin, *A. sojae* isolates are much more restricted in growth than *A. parasiticus* isolates. One study reported that colony diameters of *A. parasiticus* on bleomycin-containing MMA were larger than 16 mm, while those of *A. sojae* were smaller than 5 mm (Yuan et al. 1995). In contrast, colony diameters of both *A. parasiticus* and *A. sojae* were larger than 20 mm on MMA without bleomycin. Another study also reported that on bleomycin-containing MMA, colonies of *A. parasiticus* strains grew larger than 16 mm in diameter, whereas *A. sojae* strains either did not grow or formed microcolonies (<2 mm) that did not sporulate (Matsushima et al. 2001b).

Molecular methods to differentiate *A. sojae* from others in section *Flavi*

On the basis of DNA complementarity, *A. sojae*, *A. oryzae*, *A. parasiticus*, and *A. flavus* were found to be genetically similar (Kurtzman et al. 1986). Phylogenetic studies have indicated that *A. oryzae* may have originated from an ancestral non-aflatoxigenic *A. flavus* (Chang et al. 2006; Geiser et al. 1998, 2000). Whole genome comparison of *A. flavus* NRRL3357 and *A. oryzae* RIB40 shows that these two fungi are very similar in genome size and number of predicted genes, although each also has unique genes (Payne et al. 2006). Genome sequence information about *A. sojae* and *A. parasiticus* is not available. Published data showed that genes of *A. parasiticus* and *A. flavus* commonly share ~97–99% nucleotide identity. The close relatedness among the four aspergilli indeed is a challenge to the accurate classification of each species without error. Misclassification of *A. sojae*, *A. oryzae*, *A. parasiticus*, or *A. flavus* isolates based on morphological characteristics is therefore not uncommon (Wang et al. 2001). A variety of methods have been attempted to differentiate these aflatoxin-producing and non-producing aspergilli. Mitochondrial DNA restriction fragment length polymorphism (RFLP) can distinguish *A. flavus*, *A. parasiticus*, and *A. nomius* (Moody and Tyler 1990a, b). Single-strand conformation polymorphism and heteroduplex panel analysis, both examining polymerase chain reaction (PCR) fragments derived from the internal transcribed spacer (ITS) regions (ITS1-5.8S-ITS2) of the rRNA gene, separate the *A. parasiticus*/*sojae* group from the *A. flavus*/*A. oryzae* group but cannot distinguish individual species (Kumeda and Asao 1996, 2001). Amplified fragment length polymorphism also can separate the *A. parasiticus*/*A. sojae* group from the *A. flavus*/*A. oryzae* group but is unable to distinguish *A. sojae* from *A. parasiticus* (Montiel et al. 2003). Nonetheless, some *A. oryzae* isolates can be separated from *A. flavus* by *Sma*I RFLP patterns (Klich and Mullaney 1987). Random amplified polymorphic DNA (RAPD) markers can distinguish *A. sojae* from *A. parasiticus*, and the *A. sojae* strains were further separated into two groups by their RAPD patterns (Yuan et al. 1995). Single nucleotide polymorphism (SNP) seems to be the most unambiguous way to differentiate *A. parasiticus* from *A. sojae*. The SNPs in the 5′-untranslated region and in the complete coding region of the aflatoxin biosynthesis regulatory gene, *aflR* (Chang et al. 1995a; Lee et al. 2006a) and the partial mitochondrial cytochrome b gene can separate *A. sojae* from *A. parasiticus* as well as from *A. oryzae* and *A. flavus* (Wang et al. 2001). The successful separation of *A. sojae* from *A. parasiticus* suggests subtle differences present in the *A. sojae* genome sequence, possibly introduced after long periods of domestication.

Aflatoxin biosynthesis gene cluster

The biosynthesis of the potent carcinogen, aflatoxin B_1, has been studied extensively (Brown et al. 1999; Minto and Townsend 1997; Trail et al. 1995; Yabe and Nakajima 2004; Yu et al. 2004a). There are more than 25 enzymatic steps required for aflatoxin biosynthesis. Earlier genetic complementation experiments and restriction mapping of cosmid and lambda library clones of *A. parasiticus* and *A. flavus* revealed that genes possibly associated with aflatoxin biosynthesis are clustered in the fungal genome (Chang et al. 1993; Skory et al. 1993; Trail et al. 1994; Yu et al. 1995). Chromosomal walking of *A. parasiticus* and compilation of all published sequences showed that genes proven to be involved in aflatoxin biosynthesis are indeed clustered in a 70-kb region in the *A. parasiticus* genome (Yu et al. 2004a; Fig. 1). Since then, three more genes (*hypB1*, *hypB2*, and *nadA*) have been shown to belong to the aflatoxin gene cluster. The functions of most of the clustered genes also have been elucidated (Yabe and Nakajima 2004; Yu et al. 2004a). Recent additions to the previous genes whose functions in the biosynthesis were elucidated are *cypA*, required for G aflatoxin formation (Ehrlich et al. 2004), *aflY* (*hypA*), which encodes a Baeyer–Villiger oxidase necessary for formation of the xanthone ring of demethylsterigmatocystin (Ehrlich et al. 2005a), *cypX*, which encodes a monooxygenase that converts averufin to hydroxyversicolorone (HVN; Wen et al. 2005), *moxY*, which encodes a monooxygenase that catalyzes Baeyer–Villiger oxidation of HVN, and versicolorone to versiconal hemiacetal acetate and versiconol acetate, *aflX* (*ordB*), which encodes an oxidoreductase that catalyzes one step in the conversion of versicolorin A to demethylsterigmatocystin (Cary et al. 2006), *vbs*, which encodes a cyclase that catalyzes oxidation of versiconal to versicolorin B and 5′-oxoaverantin to averufin (Sakuno et al. 2005). Although *aflT* encodes a MFS transporter, its role in aflatoxin secretion could not be demonstrated (Chang et al. 2004). The functions of *norA*, *norB*, *hypB1*, *hypB2*, and *nadA* have not yet been resolved.

Sequencing of the aflatoxin gene clusters of *A. flavus* isolates that produce large (L) or small (S) sclerotia, an *A. nomius* isolate, and an unnamed taxon closely related to *A. flavus* and *A. parasiticus* (Ehrlich et al. 2005b) has confirmed that the order of biosynthetic genes is the same as that of *A. parasiticus*. Most recently, whole genome sequences of *A. flavus* NRRL 3357 and *A. oryzae* RIB40 showed that their aflatoxin gene clusters are located near a telomere on chromosome 3 (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao). Compared to *A. flavus*, *A. oryzae* RIB40 contains a deletion in *aflT*, a frameshift mutation in *norA*, and a nucleotide substitution in *verA* (Tominaga et al. 2006). These defects certainly attribute to the non-aflatoxigenicity of *A. oryzae*, but they appear not to be the primary reason. About 10 to 40% of L type *A. flavus* isolates in the field do not produce aflatoxins. In contrast, field isolates of S type *A. flavus* and *A. parasiticus* and *A. nomius* isolates are usually aflatoxigenic. Why the aflatoxin producing ability of *A. parasiticus*, the putative progenitor of *A. sojae*, is so stable is not clear.

Transcriptional regulation of aflatoxin biosynthesis

Of all the genes involved in aflatoxin biosynthesis identified so far, *aflR* is the only one that encodes a recognized transcription factor. It was first cloned from an *A. flavus* by its ability to restore aflatoxin production of a blocked mutant (Payne et al. 1993). An ortholog was independently isolated from *A. parasiticus* based on its ability to cause transformants to overproduce aflatoxin pathway intermediates in an O-methylsterigmatocystin-accumulating mutant (Chang et al. 1993, 1995b). Overexpression of *aflR* in *A. flavus* upregulates aflatoxin pathway gene transcription and aflatoxin accumulation (Flaherty and Payne 1997) in a fashion similar to that reported for *A. parasiticus* (Chang et al. 1995b). Deletion of *aflR* in *A. parasiticus* abolished the expression of other pathway genes, such as *ver1* and *omtA* (Cary et al. 2000). The *aflR* gene encodes a Cys6-type binuclear zinc finger protein, which binds to the sequence 5′-TCGN5CGR-3′. This consensus motif is found in most of the promoter regions of *A. parasiticus* aflatoxin biosynthesis genes (Ehrlich et al. 1999). Indeed, transcription profiling by microarray assays comparing wild type and *aflR*-deleted *A. parasiticus* strains identified 23 upregulated genes in the wild type under conditions conducive to aflatoxin production (Price et al. 2006). These include 20 genes in the aflatoxin biosynthesis gene cluster and three genes outside the cluster (*nadA*, *hlyC*, and *niiA*) all having AflR binding sites. Thus, the *aflR* gene appears to be specifically involved in the regulation of aflatoxin biosynthesis.

Regions of *A. parasiticus* AflR associated with its ability to activate transcription have been identified by *Saccharo-*

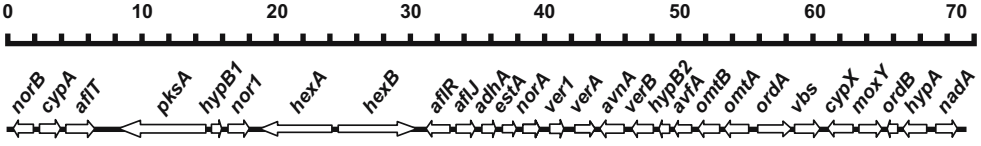

Fig. 1 The organization of the aflatoxin biosynthesis gene cluster in the genome of *A. parasiticus*

myces cerevisiae one-hybrid analyses (Chang et al. 1999). The fusion construct that encodes both the yeast Gal4p and AflR DNA-binding domains is not able to activate transcription, whereas the fusion construct that encodes the Gal4p DNA-binding domain and the full-length AflR has only 15% activity of the *aflRC* construct that encodes the Gal4p DNA-binding domain and the AflR carboxy-terminal half. Deletion analysis of *aflRC* shows that the 23 amino acids (422 to 444) at the carboxyl terminus are important for activating the *GAL1::lacZ* gene expression. Simultaneous substitutions of Arg427, Arg429, and Arg431 with Leu also decrease by 50-fold the ability to activate transcription. These results suggest that the transcriptional activation domain is located near the carboxyl terminus of AflR.

The *aflJ* gene is divergently transcribed from *aflR*. The predicted AflJ protein has no known sequence homologies to proteins deposited in the databases. Compared to *aflR* transformants, the production of aflatoxin pathway intermediates was enhanced in *A. parasiticus* transformants containing an additional *aflR* plus *aflJ* region (Chang et al. 1995b, 2002). *A. flavus aflJ* deletion mutants fail to produce aflatoxins although transcripts of the aflatoxin pathway genes examined were still made (Meyers et al. 1998). Like *A. flavus*, disruption of *aflJ* in *A. parasiticus* resulted in mutants that were unable to produce aflatoxin intermediates. Quantitative PCR showed that *A. parasiticus aflJ* deletion mutants had significantly decreased transcript levels of the aflatoxin biosynthesis genes for early (*pksA* and *nor1*), middle (*ver1*), and late (*omtA*) steps of the aflatoxin pathway (Chang 2003). Deletion of *aflJ*, however, did not have any effect on *aflR* transcript levels, and vice versa. Yeast two-hybrid assays showed that AflJ only bound to the carboxy-terminal region of AflR (Chang et al. 1999). Asp436 of AflR was crucial for AflR's ability to activate transcription but replacing it with His had little effect on the interaction of AflR with AflJ. These data conclude that AflJ is a transcriptional co-activator and suggest that the AflR–AflJ interacting region does not overlap with the AflR transcription activation region.

Aflatoxin gene orthologs in *A. sojae*

A. sojae strains have been proven to have orthologs of the aflatoxin biosynthesis genes, *aflR*, *omt-1* (=*omtA*), *avfA*, and *omtB* (Chang et al. 1995a; Klich et al. 1995; Yu et al. 2000). To date, sequences of less than ten aflatoxin gene orthologs have been reported for *A. sojae*. Complete gene sequences available in the GenBank database (*pksA*, *aflR*, and *aflJ*) and partial sequences such as *nor1*, *norA*, and *omtA* (Yu, personal communication) suggest that the aflatoxin genes in *A. sojae* are most similar to those of *A. parasiticus* (identity ~98–99%). The intergenic and coding regions of *A. parasiticus cypA* and *norB* also appear to be highly conserved in *A. sojae* isolates (Chang, unpublished results). In contrast, a deletion of either 1.0 or 1.5 kb was found in the same region in *A. flavus* or *A. oryzae* (Chang et al. 2006; Ehrlich et al. 2004; Tominaga et al. 2006). These results suggest that *A. sojae* possesses an orthologous aflatoxin gene cluster, and gene organization is identical to that of *A. parasiticus*.

Expression of *aflR* in *A. sojae* strains

Transcription of only some aflatoxin biosynthesis genes has been demonstrated in *A. sojae*. Northern (RNA) analysis showed that *A. sojae* strains lack transcripts of *nor1*, *ver1*, and *omtA*, but transcripts of *aflR* and *uvm8* (=*fas1, hexA*) were found (Klich et al. 1997). The *A. parasiticus aflR* probe hybridized weakly to the total RNA of *A. sojae* SRRC 299 and SRRC 1123 and barely hybridized to the total RNA of SRRC 1126. However, another study showed that *A. sojae* strain 477, a soy-sauce-producing strain, lacks *aflR*, *pksA*, and *nor1* transcripts even under aflatoxin-conducive growth conditions (Matsushima et al. 2001b). Enzymatic activities associated with aflatoxin biosynthesis also were not detected. In the same study, all other industrial *A. sojae* strains examined lack the *aflR* transcript. Quantitative PCR analyses showed that the *aflR* transcript level in *A. sojae* at different growth stages, compared to that of a wild-type *A. parasiticus* strain, range from ~28–33% for SRRC 299, ~60–90% for SRRC 1123, and ~9–17% for SRRC 1126 (Chang 2004). These varied reports on levels of *aflR* mRNA may reflect the history of each *A. sojae* strain because industrial strains usually have undergone mutagenic treatments to obtain desired traits, such as fast growth or elevated protease activities (Sekine et al. 1969). The steady-state levels of aflatoxin gene transcripts of *pksA*, *nor1*, *ver1*, and *omtA* in *A. sojae* strains are less than 5% of *A. parasiticus* (Chang 2004). Therefore, defects in *aflR* expression likely cause the lack of expression of other aflatoxin biosynthesis genes and results in a loss of aflatoxin production in *A. sojae* strains.

The *aflR* gene ortholog of *A. sojae*

A. sojae aflR shares 99% nucleotide identity to *A. parasiticus aflR*. *A. sojae aflR* has two distinct characteristics in its sequence (Lee et al. 2006a; Matsushima et al. 2001a; Watson et al. 1999). One is an inserted six-base repeat, CTCATG, in the amino-terminal coding region at nucleotides 335–340, and another is a C to T transition at nucleotide 1153, which results in introduction of a

premature stop codon. The sequence repeat generates a HisAlaHisAla motif, instead of the single HisAla found in *A. parasticus* AflR. The stop codon deletes part of the carboxy-terminal coding region giving a predicted protein 62 amino acids shorter than *A. parasiticus* AflR. Yeast GAL4 one-hybrid analyses showed that the transcription-activating ability of *A. sojae* AflR is only about 15% of *A. parasiticus* AflR (Matsushima et al. 2001a). Introducing a copy of *A. sojae aflR* into an *A. parasiticus* strain also did not increase aflatoxin production as would be expected with intact *aflR*. In contrast, introducing a chimeric *aflR* construct comprised of the *A. sojae aflR* (5′ region) and the *A. parasiticus aflR* (3′ region) into the *A. parasiticus* strain increased aflatoxin productivity. Consistent results were obtained by the introduction of a full-length *A. sojae aflR* gene into an *A. parasiticus aflR* deletion mutant, which was derived from a strain accumulating the aflatoxin pathway intermediate, versicolorin A (VERA; Takahashi et al. 2002). The *aflR*-deleted *A. parasiticus* mutant is incapable of making aflatoxin metabolites. Although the *A. sojae aflR* gene was expressed in the *A. parasiticus* mutant at about 50% the level of the control strain, such expression was unable to restore VERA production. Even when the *A. sojae aflR* was placed under the control of the strong promoter of an *A. oryzae* amylase gene (*amyB*), it still could not restore VERA production. The chimeric construct mentioned above restores VERA production, but another chimeric construct comprising half of *A. parasticus aflR* (5′ region) and half of *A. sojae aflR* (3′ region) cannot. Thus, the *A. sojae aflR* promoter and 5′ coding region are functional in *A. parasiticus*. The premature termination defect in the 3′ coding region of *A. sojae aflR* must result in loss of the domain necessary for AflR's ability to activate transcription (Chang et al. 1999).

Lack of AflR–AflJ interactions in *A. sojae*

In *A. parasiticus*, both *aflR*, the aflatoxin pathway-specific regulatory gene, and *aflJ*, a co-activator gene, are necessary for transcription of other aflatoxin biosynthesis genes. *A. sojae aflJ* differs from *A. parasiticus aflJ* in that it encodes a predicted protein with Ser39 replaced by Ala and Ser283 replaced by Pro. Two-hybrid assays performed in *S. cerevisiae* showed that truncated *A. sojae* AflR does not bind to either *A. sojae* AflJ or *A. parasiticus* AflJ. In contrast, if the "T" in the stop codon of *A. sojae aflR* is replaced with a "C" binding is normal with either AflJ of *A. sojae* or *A. parasiticus* (Chang 2004). Deletion analyses showed that both amino- and carboxy-terminal regions of the *A. sojae* AflJ are necessary for the AflR–AflJ interaction. Deletion of the first nine amino acids of *A. sojae* AflJ decreased interaction about tenfold, and deletion of the last 11 amino acids completely abolished the interaction. Therefore, the truncated *A. sojae* AflR not only is impaired in its ability to activate transcription of aflatoxin biosynthesis genes but also is unable to interact with AflJ if it were made by *A. sojae*. Consequently, the lack of aflatoxin-producing ability of *A. sojae* results from at least two defects in these genes necessary for the expression of other aflatoxin genes.

Aspergillus sojae pksA

The *A. sojae* aflatoxin gene ortholog of the polyketide synthase gene, *pksA*, is also defective. It contains a pretermination stop codon in its sequence and encodes a truncated product of 1,847 amino acids. The nucleotide in position 6133 in *pksA* of *A. sojae* isolates (GenBank Accession numbers: AY607768, AY607769, and AY607770) is a T, but *A. parasiticus*, *A. oryzae*, *A. flavus* (S type, L type, and S_{BG} type), and *A. nomius* all have a C at the same position (Fig. 2). This suggests that *A. sojae* was selected from a variant of *A. parasiticus*, or the nucleotide change was introduced during domestication. The intact *A. parasiticus* polyketide synthase (PksA) consists of 2,109 amino acids. PksA contains functional domains for acyl carrier protein (ACP), β-ketoacyl-ACP synthase, acyltransferase, and thioesterase. In the synthesis of aflatoxin, PksA must accept a C6 fatty acid starter unit made by fatty acid synthases, HexA and HexB, before

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As(3)   GACCACGATCAT...GGCGCCGACACCGGAAGTCCGCCTGCACTTGATCTGAAACCCTAATGC
Ap      GACCACGGTCAT...GGCGCCGACACCGGAAGTCCGCCTGCACTTGATCTGAAGCCCTACTGC
Ao      GATCACGATCAT...GGCGCCGACACCGGAAGTCCGCCTGCACTTGACCTGAAGCCCTACTGC
Af(L)   GATCACGGTCAT...GGCGCCGACACCGGAAGTCCGCCTGCACTTGACCTGAAGCCCTACTGC
Af(S)   GATCACGGTCAT...GGCGCCGACACCGGAAGTCCGCCTGCACTTGACCTGAAGCCCTACTGC
Af(B/G) GATCACGGTCAT...GGCGCCGACACTGGAAGTCCGCCTGCACTTGATCTGAAGCCCTACTGC
An      GATCACGGTCAT...GGTGCCGACACTGGAAGTCCGCCTTCACTTGATCTGAAGCCCTACTGC
1826 → D  H  D  H  ...G  A  D  T  G  S  P  P  A  L  D  L  K  P  *  C ← 1849
          (G)                                 (S)                 (Y)
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Fig. 2 Comparison of a portion of the *pksA* sequence of different species in *Aspergillus* section *Flavi*. *As* (3), three *A. sojae* strains of SRRC 299, SRRC 1123, and SRRC 1126; *Ap*, *A. parasiticus* ATCC 56775; *Ao*, *A. oryzae* RIB40; *Af*(L), *A. flavus* AF13; *Af*(S), *A. flavus* AF 70; *Af* (B/G), *A. flavus* BN008; *An*, *A. nomius* NRRL 13137

proceeding to condense seven malonates to generate the polyketide backbone. Therefore, PksA does not contain domains for β-ketoacyl-ACP reductase, dehydrase, and enoyl reductase, which are highly conserved in fatty acid synthases and necessary for the production of the non-reducing C6 starter unit. Most recently, the starter unit–ACP transacylase domain (amino acids 5–350) that selects the hexanoyl-CoA starter unit to load onto the ACP domain and initiates the polyketide biosynthesis has been identified in PkaA (Crawford et al. 2006). This domain is intact in the PksA of the *A. sojae* isolates examined (unpublished data). A thioesterase activity is needed to hydrolyze the completed polyketide chain from ACP. BLASTP analysis (Schaffer et al. 2001) indicates that a region at the carboxyl terminus (amino acids 1,850–2,100) of *A. parasiticus* PksA is homologous to the thioesterase domain of type I polyketide synthases (COG3319, clusters of orthologous groups of proteins, NCBI). This region corresponds to the truncated portion of the predicted *A. sojae* PksA. Thus, *A. sojae* PksA would be a defective polyketide synthase lacking the TE domain even if it were made.

Diversity of *A. sojae* population

In a recent study of the *aflR* gene sequences of 12 *A. sojae* strains, five (BCRC 30419, 30431, 31200, 32265, and 38021) do not contain the *aflR* gene despite the showing that the rDNA gene control yields positive PCR products (Lee et al. 2006a). Noticeably, BCRC 30419 (ATCC42249), 30431 (ATCC20235), 31200, and 38021 are the four strains, which form group II *A. sojae* in the RAPD study carried out earlier (Yuan et al. 1995). In that study, *A. parasiticus* strains also were divided into groups A and B, which suggests that *A. parasiticus* strains are not genetically homogeneous. Although *A. parasiticus* isolates are typically aflatoxigenic, the percent of naturally occurring *A. parasiticus* isolates not producing aflatoxins has been reported to range from 3 to 6% (Barros et al. 2006; Horn et al. 1996; Vaamonde et al. 2003). *A. sojae* strains, which have never been isolated from nature, may have been selected for food fermentation from genetically variable *A. parasiticus* progenitors that differ in the aflatoxin gene clusters. This is not unlikely. *A. flavus* populations are diverse. A significant portion of nonaflatoxigenic *A. flavus* isolates have been found to contain various deletions in the aflatoxin gene cluster (Chang et al. 2005). Studies have suggested that some isolates of *A. oryzae* may evolve by domestication from certain groups of nonaflatoxigenic *A. flavus* isolates (Geiser et al. 1998, 2000). This notion is supported by the finding that *A. oryzae* RIB strains categorized in group 2 (Lee et al. 2006b), *A. oryzae* SRRC 2103, and *A. oryzae* SRRC 2044 (Chang et al. 2006) have chromosomal breakpoints in the aflatoxin gene cluster identical to the *A. flavus* isolates having pattern E deletion (Chang et al. 2005). Other patterns of deletion are also common for *A. oryzae* and *A. flavus* isolates. *A. parasiticus* populations have lower genetic diversity compared to *A. flavus* populations (Horn et al. 1996; Vaamonde et al. 2003). This may explain why genetically diverse *A. parasiticus* or *A. sojae* isolates were infrequently found. As more *A. sojae* and *A. parasiticus* strains are examined, a clearer picture of the population diversity and the phylogenetic relationship may emerge.

Conclusion

Progress has been made in the understanding of what genetic changes contribute to the lack of aflatoxin production by *A. sojae*. In the past, functional analyses of *A. sojae* aflatoxin gene orthologs were carried out in *A. parasiticus* strains with genetic background conducive to aflatoxin biosynthesis or in yeast system. Future work likely would uncover defects in other structural or regulatory components of *A. sojae* associated with aflatoxin production, for example, those uncharacterized aflatoxin biosynthesis genes, genes involved in signal transduction pathways (Hicks et al. 1997; Roze et al. 2004), or genes controlling global expression of secondary metabolism or development (Bok et al. 2005; Calvo et al. 2004). Gene profiling using microarray is a powerful tool to detect differentially expressed genes in strains within a population, between populations, and of closely related species. The *A. flavus* expressed sequence tag sequence and the entire genome sequence of *A. flavus* are now available (Payne et al. 2006; Yu et al. 2004b). Microarray analysis has revealed that some genes are expressed significantly different between *A. flavus* and *A. parasiticus* despite their close relatedness at the DNA level (Wilkinson et al. 2007). The same technique may be used to identify other genes associated with loss of aflatoxin production in *A. sojae*, thus providing further insight into its biosafety.

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