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Mycotoxin genetics and gene clusters

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Abstract

Fungi produce low molecular weight secondary metabolites such as antibiotics and mycotoxins. Antibiotics cure diseases whereas mycotoxins cause diseases in plants, animals and human beings. Species such as *Aspergillus*, *Fusarium*, *Penicillium* and *Stachybotrys* are known to produce mycotoxins that accumulate in processed foods and feeds, although the incidence of infection occurs before processing, during the active growth of the organism. Among the mycotoxins, aflatoxins produced by *Aspergillus flavus* and *A. parasiticus* have been extensively studied at the molecular level. A complex biosynthetic pathway involving sixteen steps is mediated by individual major genes. These fungi have eight linkage groups, but the aflatoxin/sterigmatocystin (AF/ST) metabolic pathway genes have been mapped to only three linkage groups; ten of them belong to linkage group VII, and one of each to linkage group II and VIII. These genes are involved in both the regulatory and biosynthetic pathways and are clustered on the respective chromosomes. Clustering of genes in fungi indicates an evolutionary trend among genes that orchestrate gene function. Being linked together they segregate 'as a unit', thereby conferring a selective advantage to the organism. The evolution of gene clusters takes place through vertical or horizontal gene transfer. In fungi, horizontal gene transfer is most effective. Functionally, the mechanism of evolution of mycotoxin gene clusters in fungi seems to be similar to the evolution of a super-gene. The possible implications of evolutionary parallelism of gene clusters and super-genes is briefly explored.

Introduction

Many fungal species produce low molecular weight secondary metabolites. Some of them are economically important, either being useful or harmful to humans. Among the useful ones are the antibiotics, while the harmful ones include the mycotoxins. One of the most significant effects of these toxins is the induction of mycotoxicosis i.e. production of diseases in plants, animals and humans. An early record of mycotoxicosis is the occurrence of 'Turkey X' diseases in the 1960s (Lancaster et al., 1961). In cattle, it causes vomiting and sterility and in humans it induces vomiting, stomach ache and muscle pains. Usually, mycotoxicosis occurs as a result of eating crop products such as fodder, feed or food. Among the fungal genera that are known to produce mycotoxins, *Penicillium, Fusarium, Stachybotrys*, and *Aspergillus* are best known *Aspergillus, Penicillium*, and *Fusarium* often produce toxins in stored grains and hay, and in processed foods and feeds, although the incidence of infection takes place in the field. *Stachybotrys* species produce toxins by colonizing straw, hay or other cellulose rich products such as fodder and animal bedding. *Fusarium*-related toxins are primarily produced on maize and other cereal grains. *Aspergillus* species are notorious for producing aflatoxins in infected cereal grains and legumes, usually at low concentrations. However, in peanuts, cotton seed and fishmeal, they can produce high concentrations of toxins. Disease symptoms caused by aflatoxins in humans and animals are related to the type of toxins produced and to the age of the animal. Low doses of aflatoxins fed over a long period of time to pregnant sows, calves, fattening pigs, sheep, and mature cattle can led to abortion, reduced growth, nausea, feed refusal, and eventually

predisposition to other serious diseases such as liver and kidney cancer. Among the mycotoxins, aflatoxins produced by *Aspergillus* are the more important because of their economic importance.

Aspergillus species

Aflatoxins are highly toxic secondary metabolites produced by *Aspergillus* species such as *A. flavus* and *A. parasiticus.* Their attributes include antibiotic activity, carcinogenicity and toxicity in humans. Betalactam can be used to treat human diseases, but aflatoxin B_1 (AFB_1) causes liver cancer. Many aflatoxins, when present in foods, cause chronic aflatoxicosis in animals leading to haemorrhaging, suppression of immunity and loss in weight-gain. The importance of aflatoxins in human and animal diseases warrants an understanding of the genetic basis of their biosynthetic pathways.

Aflatoxins are synthesized through a complex pathway involving at least sixteen steps following the synthesis of the first stable intermediate, norsolorinic acid (NOR) (Bhatnagar et al., 1992). A proposed biosynthetic pathway scheme involved in aflatoxin/sterigmatocystin (AF/ST) is shown in Figure 1. This review will summarize the genetic steps involved in the pathway with emphasis on the mechanism of clustering of genes and the possibility of evolution of a 'super-gene'.

Genetics of aflatoxin production

The genetic basis of aflatoxin production in fungi has been extensively studied in *A. flavus, A. parasiticus*, and *A. nidulans*. These fungi lack a regular sexual cycle, but have a well-defined parasexual cycle as an alternative to sexual reproduction (Papa, 1973). Briefly, the parasexual cycle involves fusion between unlike haploid nuclei leading to the formation of a heterokaryon in which crossing over occurs. Crossing over assures a reassortment of the genes on the chromosomes and eventually the chromosomes reshuffle through haploidization. The sequence of events of a parasexual cycle mimics the effects of a regular sexual cycle. Parasexuality is usually limited to intra-strain fusion. In inter-strain fusion heterokaryon incompatibility ensues and therefore inter-strain or inter-specific crosses are limited. Heterokaryon incompatibility is used as an experimental tool to establish vegetative compatibility groups (VCG) in facultative fungi.

In *A. flavus* from maize, thirty-two strains were used to generate heterokaryons by mating them in all possible combinations. Twenty-two VCGs were detected (Bennett and Papa, 1988). Fundamental genetic work of Papa and his associates identified eight linkage groups (= 8 chromosomes) in *A. flavus* using both aflatoxin and morphological auxotrophic mutants (see Bennett and Papa, 1988). Six genes controlling auxotrophy and conidial mutants have been identified in *A. flavus*. Additionally, twenty-six strains were mutated for aflatoxin biosynthesis. All auxotrophic, conidial and aflatoxin mutants were mapped to eight linkage groups. Aflatoxin genes were mapped to only three linkage groups; ten of them belong to linkage group VII, and one of each to linkage group II and linkage group VIII. The aflatoxin alleles were recessive to the dominant alleles. The only dominant allele controlling aflatoxin was due to a deletion of over 120 kb (Woloshuk et al., 1995).

Originally eight linkage groups were identified on the basis of genetic analysis of morphological and aflatoxin mutants (Bennett and Papa, 1988). However, recent studies indicate that there may only be seven chromosomes in *A. flavus* and *A. parasiticus*. Payne has suggested (Personal Communications) that the heavier band (7.0 Mb) may in fact be a composite of two separate bands having approximately the same molecular weight.

Biosynthetic pathways

The biochemistry of aflatoxin biosynthesis has been reviewed recently (Payne and Brown, 1998; Hicks et al., 2001). Briefly, there are sixteen enzymatic steps starting from a polyketide precursor to AF/ST biosynthesis (Figure 1). The first step involves a fatty acid synthase (FAS) enzyme that turns acetate units into a fatty acid precursor molecule (Brown et al., 1996a; Watanabe et al., 1996). Genetic evidence indicates that a single gene is involved in this first stage (Mahanti et al., 1996). The precursor molecule (a polyketide) is converted by polyketide synthase (PKS) to an unstable intermediate, noranthrone, which is immediately converted to a stable molecule NOR. However, the conversion of an intermediate molecule to NOR has been proposed (see Hicks et al., 2001). In *A. nidulans,* the FAS is composed of two distinctive FAS complexes, one is needed for primary fatty acid metabolism and the other for sterigmatocystin (ST) biosynthesis. Four genes are involved and two of them, *stc J* and *stc K*, are

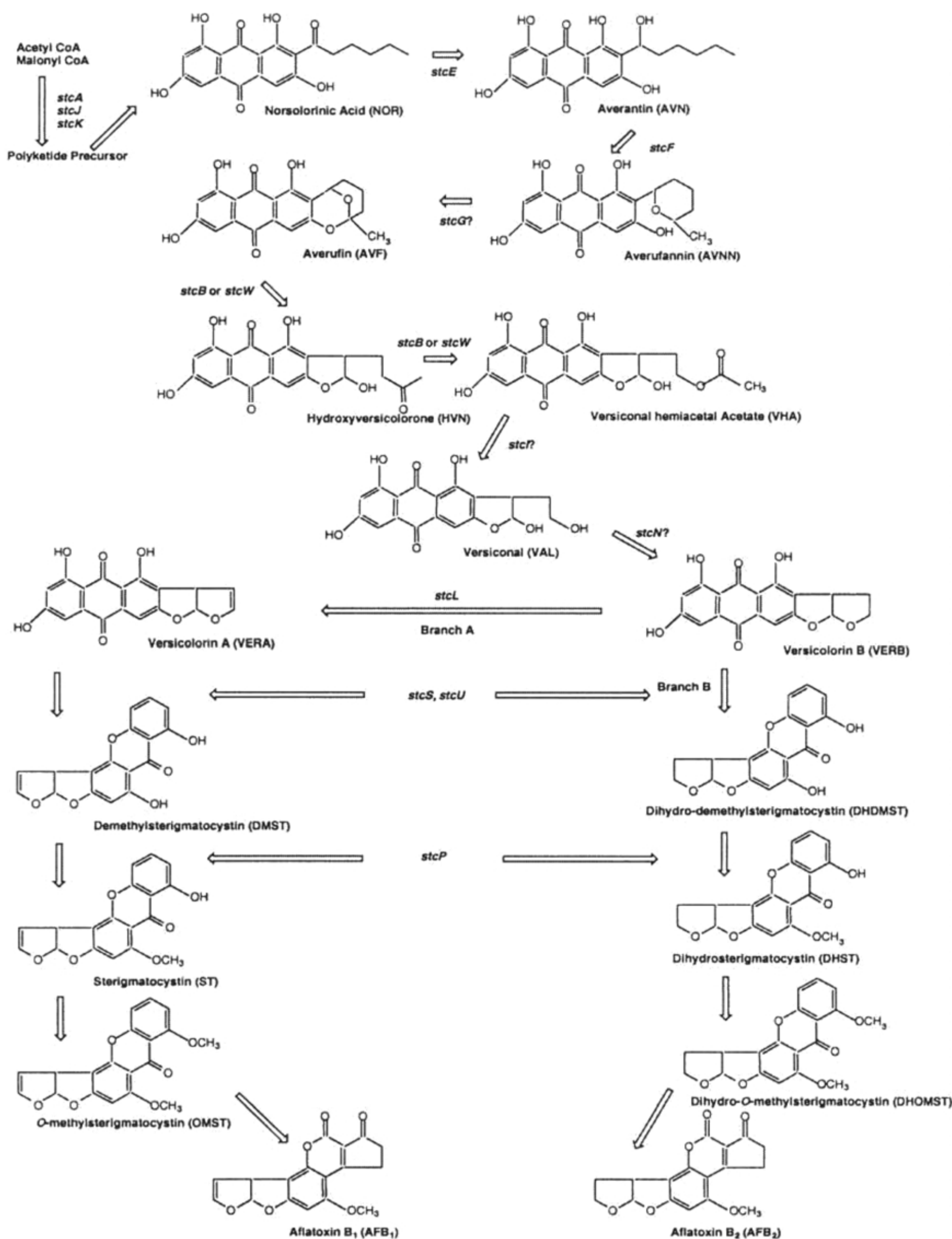

Figure 1. The AF/ST biosynthetic pathway. Malonyl CoA and acetyl CoA are converted to a polyketide precursor by the combined activities of a FAS and PKS. The polyketide precursor undergoes a series of conversions which results in formation of ST (*A. nidulans*), AFB_1/AFB_2 (*A. flavus*) or $AFB_1/AFB_2/ AFG_1/AFG_2$ (*A. parasiticus*). The *A. nidulans stc* (sterigmatocystin cluster) genes responsible for producing various enzymes in the pathway are indicated. A question mark following the gene indicates that function has not been demonstrated and activity is only postulated (reproduced from Julie K. Hicks, Kiminori Shimizu and Nancy P. Keller (2001)).

part of a ST gene cluster. These genes are part of the FAS-α and FAS-β subunit domains.

The first stable compound, NOR, is converted to averantin (AVN) with the help of two enzymes, though their nature is obscure (Bhatnagar et al. 1992). In *A. parasiticus*, Chang et al. (1992) isolated a gene (*nor-1*) that complemented a NOR accumulation defect. Another gene *norA*, with a similar function, has been shown to convert NOR to AVN (Cary et al., 1996). In *A. nidulans*, a single gene (*stc E*) is involved in conversion of NOR to AVN (Brown et al., 1996a,b; Butchko et al., 1999). The AVN conversion to averufin (AVF) is carried out by at least two enzymes. The first reaction yields an intermediate compound, 5′ hydroxyaverantin (HAVN), and the second converts HAVN to AVF (Yabe et al., 1991; Yu et al., 1997; Chang et al., 2000). AVF is first converted to hydroxyversicolorone (HVN) and then to versiconal hemiacetal acetate (VHA). VHA is converted to versiconal (VAL) and VAL to versicolorin B (VERB). VERB is oxidized to versicolorin (VERA). VERA and VERB initiate pathway A and B, respectively, using the same enzymes (Figure 1). In pathway A, VERA is converted to demethylsterigmatocystin (DMST) followed by the production of sterigmatocystin (ST) in *A. nidulans* (Kelkar et al., 1996; 1997). In *A. flavus*, both pathways produce (AFB_1) and aflatoxin B_2 (AFB_2), whereas *A. parasiticus* produces AFB_1/AFG_1 and AFB_2/AFG_2 as their end products (Yabe et al., 1999).

Genes involved in pathways

Regulatory and biosynthetic genes have been categorized in the AF/ST pathway in *Aspergillus*. Both types of genes are clustered in a 60–75 kb region of the DNA (Brown et al., 1996b; Trail et al., 1995; Yu et al., 1995). Analysis of gene clusters revealed that *A. flavus, A. parasiticus* and *A. nidulans* contain the same genes in their AF/ST pathway, whereas the order and direction of ST gene expression in *A. nidulans* are rearranged (Cary et al., 2000).

Gene clusters and super-genes

A successful adaptation of an organism to its environment requires the cooperation of many genes that contribute to its survival. Genes assort independently when present on separate chromosomes or are separated by crossing over when present on the same chromosome. If the genes are tightly linked, they will rarely be broken apart by chiasmata and thus segregate together as a unit. Clustering of genes in fungi indicates tight linkage among the genes that orchestrate gene function and that segregate as a unit.

Functionally, the evolution of gene clusters in fungi and bacteria is identical to super-gene formation (Darlington and Mather, 1949). However, super-gene formation in diploid organisms has its basis in polymorphic adaptation, which in turn relies on heterozygous advantage. Heterozygous advantage is established in sexually reproducing organisms, but not in haploid organisms. However, the occurrence of the parasexual cycle in some fungi may substitute for heterozygous advantage.

The control of polymorphic adaptations is different in diploid organisms in the sense that genes must be held together so that their alleles can be segregated from one another in a block called a *super-gene*. The loci of a super-gene may become separated by rare chiasmata formation, which produces an ill-adapted group that will be eliminated like a disadvantageous mutant. If the linked genes interact usefully, structural changes to bring them on the same chromosome will be favoured.

In haploid organisms such as *Neurospora* and *Aspergillus*, polymorphism may be maintained through *frequency-dependent selection*. Frequency-dependent selection implies that an allele tend to be selected *for* when it is useful and *against* when it is not useful or is neutral to the organism. This type of selection is only plausible in haploid organisms through mutations. Useful mutations tend to accumulate on a single chromosome and this results in establishing stable coexistence of a variety of alleles in the populations. When the useful alleles form a cluster or a super-gene, heterokaryon incompatibility keeps them isolated in natural populations. In *A. nidulans,* Grindle (1963) found that independently isolated wild-type strains were often mutually incompatible. Compatibility often occurs between strains found in the same habitat. Widespread incompatibility in fungi suggests delineation of related species such as *A. flavus and A. parasiticus*. These fungi are mainly responsible for aflatoxin formation and possess a common aflatoxin pathway. The genes that control the enzymatic steps in the pathway are clustered and seem to be identical to the super-gene category of diploid organisms.

Early examples of clustering of genes are found in carbon-catabolic pathways in fungi. The utilization of galactose in yeast and quinate in *Neurospora crassa* are two examples of gene clusters in which functionally

related genes in contiguous groups are subject to common transcription control resulting in the release of common messenger RNA – a situation analogous to the 'operon system'. In *Aspergillus*, the clustering of genes which have a common regulatory function seems to be identical to quinate catabolism in *Neurospora* (Case and Giles, 1977), but may not be candidates for operon formation. Clustering of genes or super-gene formation in *Aspergillus* has an adaptive advantage under adverse ecological conditions. The aflatoxins are produced under the adverse conditions which occur prior to harvest – a measure facilitating survival under dry conditions.

Evolution of gene clusters

Genes that orchestrate secondary metabolites are usually clustered on chromosomes. Antibiotic and mycotoxin-producing genes are good examples of gene clusters in bacteria and fungi. For example, production of the antibiotic B-lactam and AF/ST in *Penicillium* and *Aspergillus* are under the control of gene clusters (Gutierrez et al., 1999; Brown et al., 1996a,b). Genes for penicillin and cephalosporin are also present in bacteria. The regulation of gene clusters in bacteria is usually under the control of an 'operon' system, whereas such control is lacking in fungi (Keller and Hohn, 1997). However, the carbon-catabolic pathway of utilization of galactose in yeast and quinate in *Neurospora crassa* may conform to operon organization of genes suggesting a common transcriptional control. Structural gene clusters in mycotoxin-producing fungi do not transcribe m-RNA in one piece, therefore, do not operate as an 'operon.'

The evolution of gene clusters has been suggested to take place through horizontal gene transfer among organisms (Rosewich and Kistler, 2000). As compared to vertical gene transfer from common ancestors, horizontal gene transfer takes place among parallel and related species. Clustering of genes in mycotoxin-producing fungi seems to evolve through horizontal gene transfer. In this regard, a 'selfish operon' hypothesis has been proposed (Lawrence and Roth, 1996). This hypothesis proposes the clustering of genes through genetic rearrangements that link together genes which operate in a single metabolic pathway such as the AF/ST pathway in *Aspergillus.*

If any set of linked genes interact usefully, structural interchanges bringing them on the same chromosome will be favoured and selected during natural selection. Inversions and translocations have been suggested as the most effective chromosomal changes in bringing together genes that have an adaptive positive value. In the case of mycotoxin-producing fungi, the cooperating genes have an adaptive value for the organism enabling them to colonize ecological niches that would otherwise pose adverse environmental conditions. Mycotoxin-producing fungi experience adverse niches such as dry grains or straw where the development of the organism is under test. Such development can be best served by those species that orchestrate their genes in a clustered form.

The AF/ST pathway shows common genes among *A. parasiticus, A. flavus*, and *A. nidulans*. These genes are clustered in all three species (Payne and Brown, 1998). Aflatoxin genes, which seem to have common ancestral origins, accumulated in these species through horizontal transfer. The gene clusters in these fungi are still evolving because introgressed DNA present in a 60–75 kb chromosomal region contains loosely linked genes. Such pieces of introgressed DNA are usually junk DNA and have no purpose. Junk DNA would be lost over time through deletions and the toxin genes would thus become more closely linked.

Horizontal gene transfer has also been demonstrated in the case of penicillin biosynthetic gene clusters in *P. chrysogenum*. DNA sequences of several naturally occurring mutants of *P. chrysogenum* show a single copy of the gene cluster, as well as multiple copies of biosynthetic gene clusters. The borders of a single copy or multiple copies of gene clusters are flanked by an identical hexanucleotide repeat (TTTACA). Single copy cluster or multiple copies of clusters is evident from the amount of penicillin produced by the respective strains. The occurrence of the hexanucleotide repeat border, which occurs in many penicillin-producing strains, suggests a common origin and transfer of gene clusters in Penicillium. Similar mechanisms seem to hold true in *Aspergillus*.

Function of gene clusters in *Aspergillus* species

Biosynthetic genes

Sixteen genes have been identified in the AF/ST biosynthetic pathway of *A. flavus and A. parasiticus* (see Hicks et al., 2001). These genes are clustered on a 60–75 kb section of DNA (Brown et al., 1996a,b). The gene clusters of the two species contain almost

the same genes (Cary et al., 2000; Keller and Hohn, 1997). However, the gene order and directions of their expression is different from *A. nidulans*. This may have occurred because *A. nidulans* has a different developmental life cycle. The gene clusters of the *Aspergillus* have a common regulatory control.

The aflR *gene*

The *aflR* (aflatoxin regulation) gene is essential for positive co-regulation of AF/ST gene clusters (Payne and Brown, 1998). This observation is supported by the fact that a deletion in or a mutation of the *aflR* gene in *A. flavus* and *A. parasiticus* impaired the AF biosynthetic ability of the biosynthetic genes. When the impaired regulatory gene was complemented with its wild-type allele, the function of the biosynthetic genes was regained (Chang et al., 1993; Yu et al., 1996a). Furthermore, the number of copies of the *aflR* gene in three *Aspergillus* species directly influenced the corresponding level of transcription of the AF/ST genes (Chang et al., 1995a).

The *aflR* genes from three species produced functionally homologous proteins, however, their DNA sequences varied greatly (Yu et al., 1996). Payne and Brown (1998) showed that *A. flavus* and *A. parasiticus* share 95% DNA sequence homology while *A. nidulans* only share 33% identity with the other two species. The *aflR* genes produce proteins that belong to a zinc-binuclear cluster (Zn(II)2 Cys6) family (Chang et al., 1993; Todd and Adrianopoulus, 1997). The base, encoding the zinc-binuclear cluster binding domain showed 71% identity with *aflR* genes of *A. flavus* and *A. nidulans*. The protein from the zinc-binuclear cluster domain binds to and recognizes a palindromic sequence in the promoters of biosynthetic genes. The first primary binding sequence, 5′-TCGN(5) CGA-3′, of *aflR* was identified in *A. nidulans* (Fernandes et al., 1998) and the second *aflR* binding sequence, 5′-TTAGGCCTAA-3′, was reported from *A. flavus* and *A. parasiticus* (Chang et al., 1995b; Payne and Brown, 1998). However, the first primary sequence remains the major binding site in *A. parasiticus* (Ehrlich et al., 1999). Interestingly, the primary sequence is conserved in the promoters of AF genes while it is repeated one or more times in the promoters of the ST cluster. Other aspects of the *aflR* gene are discussed by Hick et al. (2001). Importantly, *aflR* is not sufficient to activate AF/ST biosynthetic genes, particularly during primary growth. Several reports suggest that inhibitory factors may be operative during the early periods of fungal growth (Butchko et al., 1999). The data indicate that there may be post-transcriptional or post-translational regulation of aflR by other proteins (see Hicks et al., 2001).

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