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CLONING AND CHARACTERIZATION OF GENETIC MARKERS FROM THE AFLATOXIN-PRODUCING FUNGUS ASPERGILLUS PARASITICUS

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Jyh-Song Horng

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James J Pestka
Major professor

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CLONING AND CHARACTERIZATION OF GENETIC MARKERS FROM THE AFLATOXIN-PRODUCING FUNGUS *ASPERGILLUS PARASITICUS*

By

Jyh-Song Horng

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ABSTRACT

CLONING AND CHARACTERIZATION OF GENETIC MARKERS FROM THE AFLATOXIN-PRODUCING FUNGUS *ASPERGILLUS PARASITICUS*

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Three selectable markers have been isolated from *Aspergillus parasiticus* as the initial step in developing genetic transformation systems for cloning aflatoxin biosynthetic genes. First, the *trpC*$^+$ gene in the tryptophan biosynthetic pathway was isolated by complementation of an *Escherichia coli trpC* mutant lacking phospho-ribosylanthranilate isomerase (PRAI) activity. The cloned gene complemented an *E. coli trpC* mutant deficient in indoleglycerolphosphate synthase (IGPS) activity and an *Aspergillus nidulans* mutant strain that was defective in all three enzymatic activities of the *trpC*$^+$ gene (glutamine amidotransferase, IGPS and PRAI), thus indicating the presence of a complete and functional *trpC*$^+$ gene. The location and organization of the *A. parasiticus trpC*$^+$ gene on the cloned DNA fragment were determined by deletion mapping and by hybridization to heterologous DNA probes prepared from cloned *trpC*$^+$ genes of *A. nidulans* and *Aspergillus niger*. These experiments suggested that the *A. parasiticus trpC*$^+$ gene encoded a trifunctional polypeptide with functional domains organized identically to those of analogous genes from other filamentous fungi. The *A. parasiticus trpC*$^+$ gene was expressed constitutively regardless of the nutritional status of the culture medium. Second, the nitrate reductase structural gene (*niaD*$^+$) was isolated by screening an *A. parasiticus*

genomic DNA library with DNA probes prepared from cloned *niaD*$^+$ genes of *A. niger* and *Aspergillus oryzae*. Three types of nitrate-nonutilizing mutants deficient in *niaD*$^+$, genes coding for a molybdenum cofactor (*cnx*$^+$), and a regulatory element in nitrogen assimilation (*nirA*$^+$), respectively, were also isolated based on their resistance to chlorate, a toxic analogue to nitrate. A homologous transformation system has been developed with *niaD*$^+$ as the selectable marker. Third, a gene with homologies to the *Neurospora crassa* β-tubulin gene was isolated from a genomic DNA library of a benomyl-resistant mutant of *A. parasiticus*. The possibility that this gene represents a mutated version of the β-tubulin gene and confers resistance to benomyl remains to be determined. The cloned *trpC*$^+$, *niaD*$^+$, and β-tubulin genes should be useful as general markers for fundamental genetic analyses, and as selectable markers in developing homologous transformation systems to analyze the aflatoxin biosynthetic pathway of *A. parasiticus*.

Dedicated to my parents, for their love and encouragement; to my wife Kitty, for her understanding and sharing excitement and frustration with me throughout my graduate career, and to our two clones, Cindy and Ivy, obtained by old-fashioned recombinant DNA.

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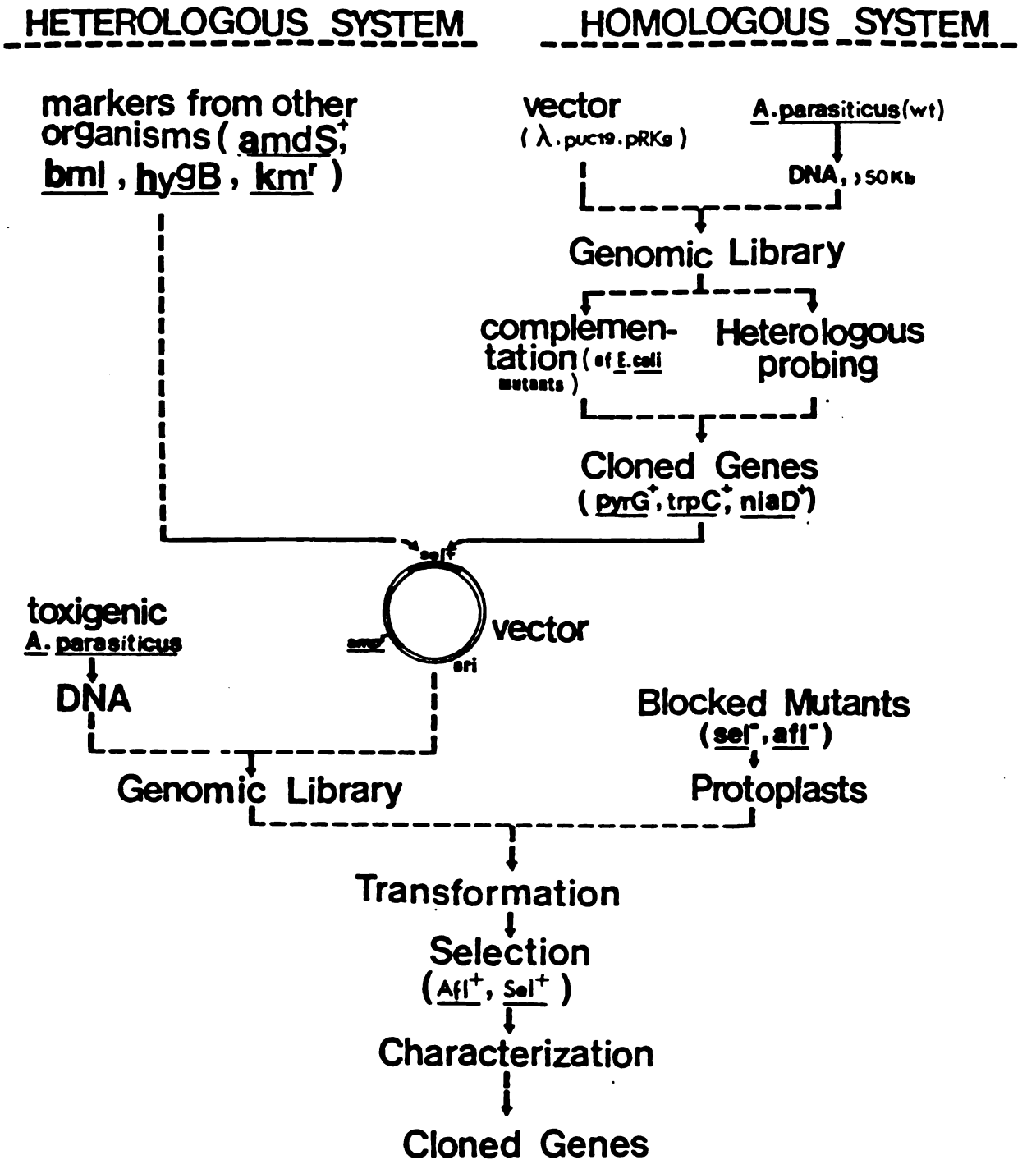
RATIONALE

Aspergillus parasiticus is an imperfect fungus which produces aflatoxins. The ubiquitous existence of this mold and the potent toxicity and carcinogenicity of aflatoxins represent a serious threat to food safety and public health. To date, protection against contamination of the food supply with these toxins is limited to preliminary detection, followed by elimination of contaminated items.

By investigating expression and regulation of genes involved in aflatoxin biosynthesis it should be possible to design strategies to efficiently control formation of these toxins. It is thus desirable to develop efficient DNA-mediated transformation systems for *A. parasiticus* to provide a mechanism to clone the genes involved in the aflatoxin biosynthetic pathway in this organism. However, the major obstacle in developing such a transformation system for *A. parasiticus* has been the lack of suitable selectable markers. Auxotrophic mutants of this imperfect fungus are not easily obtainable, presumably due to the presence of multiple nuclei in the conidia (Yuill, 1950) and the coenocytic nature of the fungal cytoplasm. Moreover, preliminary data indicate that *A. parasiticus* is highly resistant to various antibiotics such as hygromycin B and kanamycin, which are commonly used in transformation of eukaryotic cells. Utilization of cloned genes from other organisms as selectable markers to develop a heterologous transformation system for *A. parasiticus* is a feasible approach. However, available evidence suggests that heterologous genes will not always be expressed (Woloshuk et al., 1989), or expressed less efficiently in *A. parasiticus*, resulting in a lower transformation frequency. Together, these factors significantly impede development of homologous or heterologous transformation systems based on auxotrophic or dominant resistance genes as selectable markers.

A general approach to developing transformation systems for cloning genes in the aflatoxin biosynthetic pathway of *A. parasiticus* is depicted in Figure 1. The immediate goal of this investigation was to isolate and characterize selectable markers for use in developing transformation systems for *A. parasiticus* in suitable recipient strains. Three selectable markers from *A. parasiticus* were isolated and characterized. These three markers include the trifunctional *trpC*$^{+}$ gene in the tryptophan biosynthetic pathway, which has been widely used in transformation of filamentous fungi; the nitrate reductase structural gene (*niaD*$^{+}$) for which the corresponding mutants can be readily induced on the basis of a positive selection protocol (chlorate resistance), and a mutant allele of the β-tubulin gene which confers resistance to the fungicide benomyl, and is generally used as a dominant selectable marker in transformation of filamentous fungi.

Figure 1. A basic scheme for cloning genes involved in aflatoxin biosynthesis by complementation of blocked mutants. A vector containing a suitable selectable marker (*sel*$^+$) from heterologous or homologous sources is used to construct a genomic DNA library from an aflatoxigenic strain of *A. parasiticus*. Homologous selectable markers to be incorporated into the cloning vector are obtained by complementation of suitable *E. coli* mutant strains, or by screening an *A. parasiticus* wild-type (wt) genomic DNA library by hybridization with heterologous genes with sufficient nucleotide sequence similarity to the marker of interest. DNA purified from the library is used to transform protoplasts of appropriate blocked mutants that are defective in the aflatoxin biosynthesis. Transformants are first selected on the basis of selectable markers (*sel*$^+$) and then screened for their ability to produce aflatoxin (*afl*$^+$). Genetic markers are: *amdS*$^+$, coding for acetamidase; *bml*, conferring resistance to the fungicide benomyl; *hygB*, conferring resistance to the antibiotic hygromycin B; *km*^{r}, conferring resistance to the antibiotic kanamycin; *pyrG*$^+$, coding for orotidine 5'-phosphate decarboxylase; *trpC*$^+$, a trifunctional gene in the tryptophan biosynthetic pathway of filamentous fungi; *niaD*$^+$, the structural gene for nitrate reductase.


HETEROLOGOUS SYSTEM
HOMOLOGOUS SYSTEM
markers from other organisms (amdS+, bml , hygB , kmr)
vector
(λ . puc19 . pRK9)
A. parasiticus (wt)
DNA, > 50Kb
Genomic Library
complementation (of E. coli mutants)
Heterologous probing
Cloned Genes
(pyrG+, trpC+, niaD+)
sel+
ori
vector
toxigenic
A. parasiticus
DNA
Genomic Library
Blocked Mutants
(sel-, afl-)
Protoplasts
Transformation
Selection
(Afl+, Sel+)
Characterization
Cloned Genes

CHAPTER I

LITERATURE REVIEW

I. Genetics of Aflatoxin Biosynthesis

The aflatoxins are a group of carcinogenic, toxigenic, mutagenic, and teratogenic compounds of considerable health and economic importance. They are produced by certain strains of the closely related imperfect fungi *Aspergillus flavus*, *A. parasiticus*, and *A. nomius*, a strain which has recently evolved from *A. flavus* (Kurtzman et al., 1987). Aflatoxins are the most potent naturally occurring carcinogens known and are frequently found in agricultural commodities and dietary staples such as corn, peanuts, treenuts and cottonseed (Jelinek et al., 1989). The potential hazard of aflatoxins to human health was initially realized following their association with acute hepatotoxicity in poultry (Turkey X disease) in 1960 (Blount, 1961; Goldblatt, 1969) and subsequently with fatal toxicoses in India and West Africa (Ngindu et al., 1982). Aflatoxins in association with hepatitis B have been implicated as contributory epidemiological factors for primary human liver cancer in areas of Africa, China, and Southeast Asia where there is an extremely high incidence of this disease (Hsieh, 1986, 1989; Groopman et al., 1988; Yeh et al., 1989). The mode of action of this class of mycotoxins involves metabolic activation to a reactive epoxide and its subsequent covalent binding to cellular macromolecules such as DNA and proteins (Busby and Wogan, 1981; Swenson, 1981; Kiessling, 1986). Since their discovery in 1960, aflatoxins have been the subject of intense research by agricultural scientists including mycologists, chemists, and toxicologists (Goldblatt, 1969; Heathcote and Hibbert, 1978; Betina, 1984; Smith and Moss, 1985).

A. Aflatoxins as Secondary Metabolites

Aflatoxins are secondary metabolites because they are not essential for growth. Several additional criteria, as summarized by Bennett and Christensen (1983), which distinguish aflatoxins and other secondary metabolites from the primary metabolites, which are ubiquitous and necessary for growth are described below.

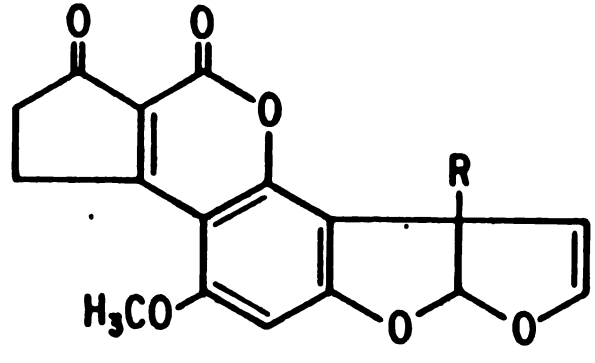
1. Limited taxonomic distribution

Aflatoxins is only produced by certain strains of a few fungal species: *A. flavus*, *A. nomius*, and *A. parasiticus* (Bennett, 1982). Moreover, the amount of aflatoxin produced by aflatoxigenic strains varies widely. In general, isolates of *A. parasiticus* consistently produce both B and G aflatoxins, while *A. flavus* isolates contain a greater proportion of nontoxigenic strains and tend to produce only B aflatoxins (Hesseltine et al., 1970; Klinch and Pitt, 1985).

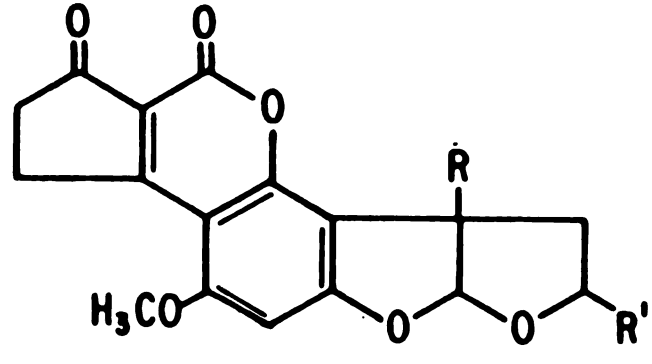
2. Produced in chemical families

The name "aflatoxins" describes a group of closely related compounds. The aflatoxins are substituted coumarins containing the reactive difuran moiety. The major aflatoxin families are called B_1, B_2, G_1, and G_2 (Figure 2) based on their respective blue and green fluorescence under long-wave ultraviolet (uv) light

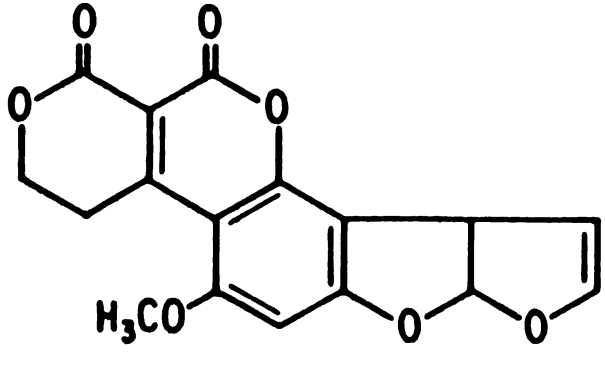
Figure 2. Structures of the major aflatoxins (adapted from Zaika and Buchanan, 1989).



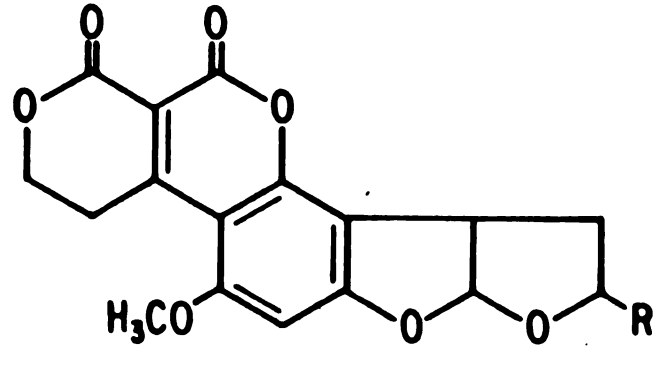
R = H, AFLATOXIN B_1
R = OH, AFLATOXIN M_1



R = R' = H, AFLATOXIN B_2
R = OH, R' = H, AFLATOXIN M_2
R = H, R' = OH, AFLATOXIN B_{2a}



AFLATOXIN G_1



R = H, AFLATOXIN G_2
R = OH, AFLATOXIN G_{2a}

and their relative chromatographic mobilities. Typically, aflatoxin B_1 is the major metabolite produced by all aflatoxigenic strains, with the other aflatoxins produced to a lesser extent. The hydroxylated aflatoxins (aflatoxin B_{2a} and G_{2a}) (Figure 2) occasionally occur as minor metabolites (Heathcote and Hibbert, 1978).

3. Produced after active cellular growth

Like other secondary metabolites, aflatoxins are synthesized after active growth of the mycelia has stopped. Morphological differentiation frequently occurs during this synthesis period (Turner, 1971).

4. Synthesized from simple building blocks

Aflatoxins are synthesized from a few simple precursors that are derived from primary metabolism, e.g., acetate, malonate, and the methyl group of methionine (Steyn et al., 1980; Applebaum and Marth, 1981; Bennett and Christensen, 1983).

B. The Biosynthesis of Aflatoxins

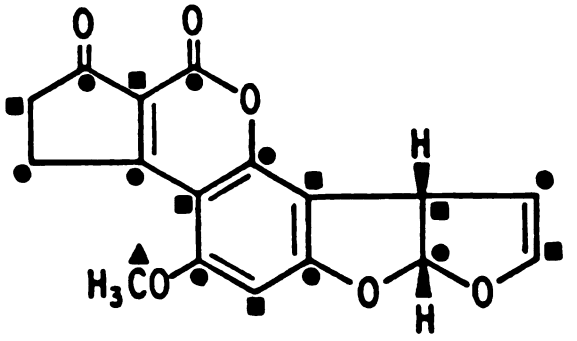
While great strides have been made in elucidating the chemistry, toxicity and biological activities of aflatoxins, information on their biochemistry, specifically their biosynthesis, has accumulated more slowly. However, due to improved experimental techniques and analytical instrumentation (as described below), aflatoxin biosynthesis

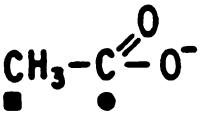
has attracted considerable research attention in recent years. The subject of aflatoxin biosynthesis has been extensively reviewed (Maggon et al., 1977; Bennett and Lee, 1979; Steyn, 1980; Bennett and Christensen, 1983; Turner and Aldridge, 1983; McCormick et al., 1986).

1. Overview of aflatoxin biosynthesis

The basic skeleton of aflatoxin B_1 [a C_{20} polyketide (decaketide)] is derived entirely from acetate units via the polyketide pathway, and methionine contributes the methoxy-methyl group (Figure 3) (Donkersloot et al., 1968; Biollaz et al., 1970; Hsieh and Mateles, 1970, 1971). The polyketide pathway can be divided into two distinct phases. "Phase one" is common to all compounds of polyacetate origin, while "phase two" is unique to the particular polyketide being synthesized (Bu'Lock, 1961). During "phase one" ten acetate units are assembled into a polyketide chain, i.e., anthrone (Figure 4). This assembly reaction is presumably catalyzed by the "polyketide synthase," an enzyme complex very similar to the eukaryotic fatty acid synthase in structural organization, function and mechanism of catalysis (Bennett and Christensen, 1983). The anthrone intermediate thus formed is further converted into aflatoxin B_1 in "phase two" by a series of steps involving several precursors and reactions including oxidation, reduction, condensation and molecular rearrangements (Figure 5). To date the known precursors to aflatoxin B_1 include seven anthraquinones (norsolorinic acid, averantin, averufanin, averufin, hydroxyversicolorone, versiconal hemiacetal acetate, and versicolorin A) and two xanthones [sterigmatocystin (ST), and O-methylsterigmatocystin (OMST)].

Figure 3. The origin of the carbon atoms and the arrangement of intact acetate units in aflatoxin B_1 (adapted from Biollaz et al., 1968, 1970).





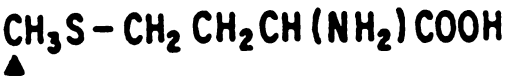


Figure 4. The hypothetical scheme for the assembly of anthraquinones by a "polyketide synthase" enzyme complex in *Aspergillus* species (adapted from Bennett and Christensen, 1983).

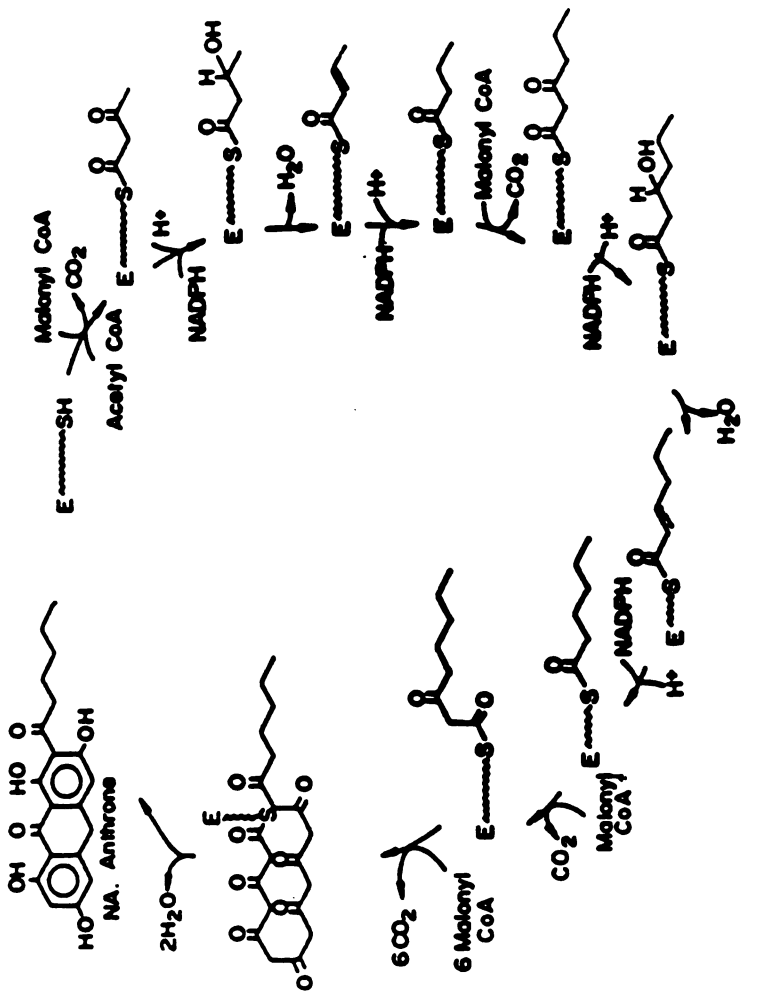

Malonyl CoA
Acetyl CoA
NADPH
NADPH
NADPH
Malonyl CoA
6 Malonyl CoA
Malonyl CoA
NADPH
NA. Anthrone

Figure 5. The proposed pathway for the biosynthesis of aflatoxin B_1. Arrows interrupted by a short double line indicate steps that are blocked in the pathway. The corresponding blocked mutants of *A. parasiticus* are shown. An arrow interrupted by a single dash line indicates the conversion step which is inhibited by the insecticide dichlorvos. Arrows may indicate more than one step.

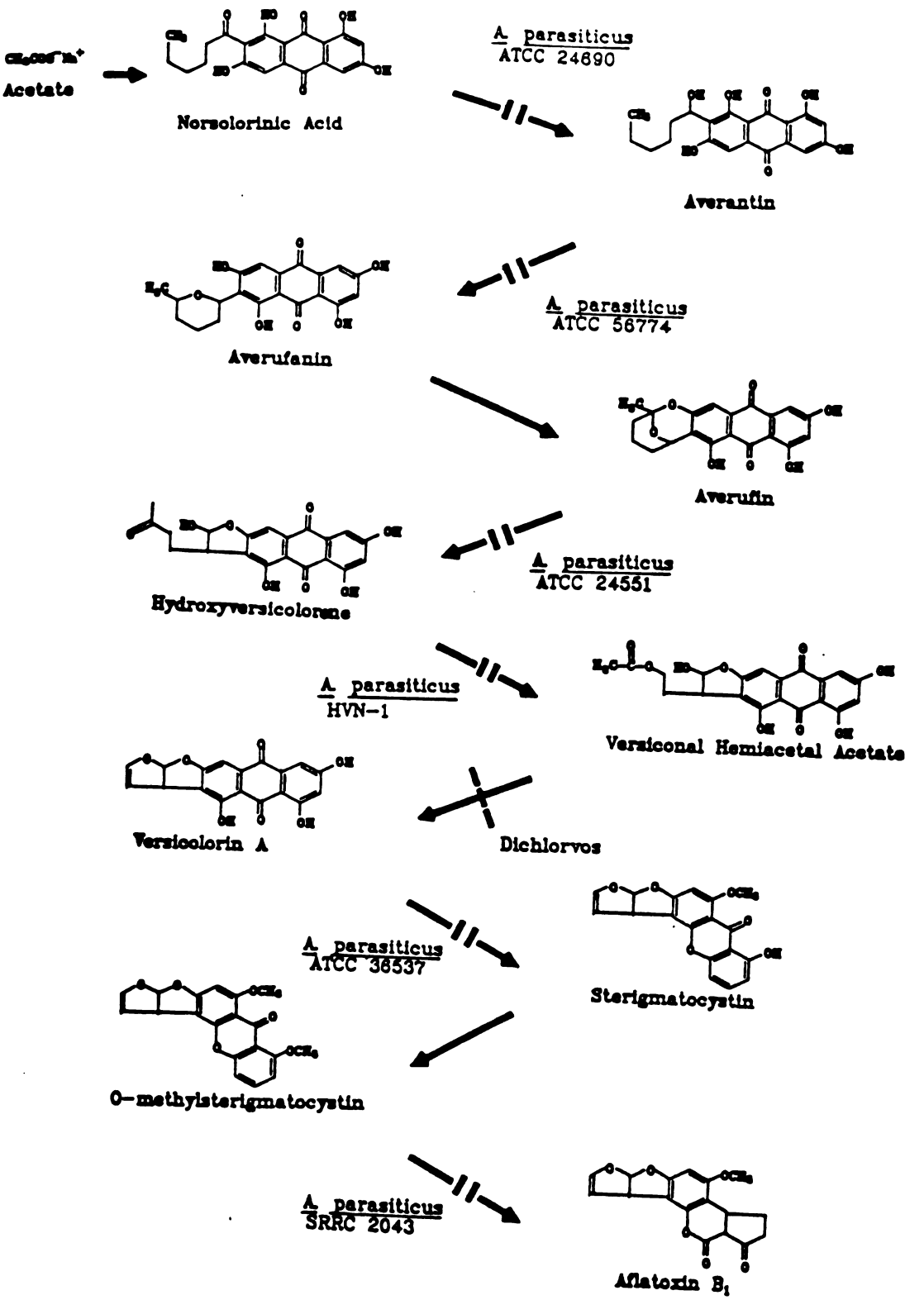
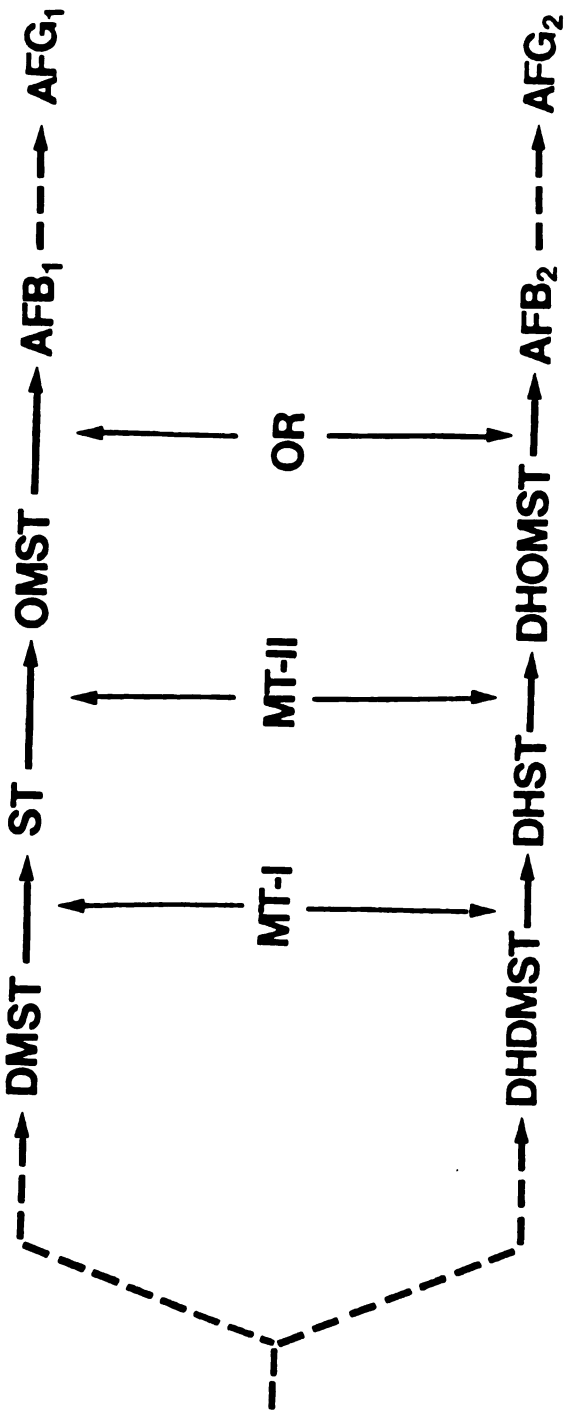

Acetate
Norsolorinic Acid
A. parasiticus ATCC 24690
Averantin
A. parasiticus ATCC 56774
Averufanin
Averufin
A. parasiticus ATCC 24551
Hydroxyversicolorene
A. parasiticus HVN-1
Versiconal Hemiacetal Acetate
Versicolorin A
Dichlorvos
A. parasiticus ATCC 38537
Sterigmatocystin
O-methylsterigmatocystin
A. parasiticus SRRC 2043
Aflatoxin B1

Figure 6. The metabolic scheme proposed for the late stages of aflatoxin biosynthesis (modified from Yabe et al., 1988, 1989). ____ , confirmed reactions; -----, hypothetical reactions. Abbreviations: AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; DMST, dimethyl-sterigmatocystin; DHDMST, dihydro-dimethyl-sterigmatocystin; ST, sterigmatocystin; DHST, dihydro-sterigmatocystin; OMST, O-methyl-sterigmatocystin; DHOMST, dihydro-O-methylsterigmatocystin; MT-I, O-methyltransferase I; MT-II, O-methyltransferase II; OR, oxidoreductase.


DMST
ST
OMST
AFB1
AFG1
MT-I
OR
MT-II
DHDMST
DHST
DHOMST
AFB2
AFG2

The biosynthetic pathway of the other aflatoxin families has not been well understood. Generally it has been assumed that aflatoxins B_2, G_1, and G_2 are synthesized by direct interconversions from aflatoxin B_1 (Bennett and Christensen, 1983). However, studies of Floyd and Bennett (1981), and Dutton et al. (1985) provided evidence that aflatoxin B_1 and B_2 could arise independently via a branched pathway. Recent findings (Cleveland et al., 1987; Floyd et al., 1987; Henderberg et al., 1988; Yabe et al., 1988, 1989; Cleveland, 1989) provide further support for this branched pathway hypothesis for the late stages of aflatoxin biogenesis. The currently-favored scheme is depicted in Figure 6.

2. Approaches to elucidating the aflatoxin biosynthetic pathway

Three approaches have been utilized for the elucidation of the aflatoxin biosynthetic pathway. In addition to the genetic approach, which is the major focus of this review, chemical and biochemical approaches have contributed tremendously to our understanding of this pathway.

a. Chemical approach. By marking aflatoxin B_1 with differentially labeled radioactive acetates and subjecting the compounds to selective degradation, Biollaz et al. (1968, 1970) established unambiguously the polyketide origins of aflatoxin B_1. The origin of the 16 skeletal carbon atoms was determined: 9 carbon atoms were derived from C-1 of acetate and 7 carbon atoms were derived from C-2 of acetate (Figure 3). This powerful technique, however, is tedious to perform, and therefore is no longer widely applied to the aflatoxin biosynthesis. Nuclear magnetic resonance

(NMR), in particular ^{13}C-NMR (Sequin and Scott, 1974), represents another powerful technique that has been widely used for exploring biosynthetic pathways in recent years. Studies using this technique have provided information concerning bonds broken and formed during the aflatoxin biosynthetic process, and have revealed some mechanistic details concerning formation and subsequent conversion of each individual aflatoxin precursors (Steyn et al., 1980; Townsend, 1986).

b. Biochemical approach. Our current knowledge about aflatoxin biosynthesis was derived mostly from the utilization of versatile biochemical techniques. Several techniques have contributed significantly to the elucidation of the aflatoxin biosynthetic pathway.

1) Bioconversion with blocked mutants. Aflatoxigenic *Aspergillus* spp. can be mutagenized to yield mutants that do not produce or produce a limited amount of aflatoxins. These blocked mutants (Figure 5 and Table 2) are presumably impaired in one or more enzymatic steps involved in the aflatoxin biosynthesis. Singh and Hsieh (1977) pioneered the use of biotransformations by blocked mutants to establish a tentative sequence for the intermediates of the aflatoxin pathway. When a mutant blocked early in the pathway is incubated with a putative late intermediate, the recovery of aflatoxins simultaneously supports the role of the putative intermediate as an aflatoxin precursor and places the compound past the site of the mutant block. Radioactive aflatoxin precursors used in biofeeding studies are normally prepared by feeding a radioactive acetate to the blocked mutant that has accumulated the desired precursor.

2) Radiolabeling. As summarized in Table 1, metabolism of ^{14}C-labeled precursor molecules by blocked mutants has been routinely used in radiotracing experiments to confirm and place suspect precursors in the aflatoxin biosynthetic pathway. Radioactive compounds can also be used in kinetic pulse-labeling experiments to follow the metabolism of radioactive precursors as a function of time, thus revealing an ordered sequence of metabolite appearance (Zamir and Ginsburg, 1979; Zamir and Hufford, 1981). This technique is also advantageous because transient aflatoxin precursors can be distinguished from "dead-end" metabolites by the rapid formation and disappearance of the former.

3) Metabolic inhibitors. A number of chemicals are able to inhibit aflatoxin production (Zaika and Buchanan, 1987), but only dichlorvos (dimethyl-2,2-dichlorovinyl phosphate, an insecticide) has been frequently used for the elucidation of the aflatoxin biosynthetic pathway. Dichlorvos specifically inhibits the enzymatic conversion of versiconal hemiacetal acetate to versicolorin A in the aflatoxin biosynthetic pathway (Rao and Harein, 1972; Hsieh, 1973; Bennett et al., 1976; Singh and Hsieh, 1977). The effect of dichlorvos on intermediate accumulation by *A. parasiticus* blocked mutants is summarized in Table 1.

4) Enzymology. Only limited information is available with respect to the enzymes associated with the aflatoxin biosynthesis (Dutton, 1988). A few cell-free systems have been developed for conversion of certain precursors into aflatoxin B_1 or other

Table 1. Incorporation of labeled precursors into aflatoxin B_1.

Labeled compound	Blocked mutant	^{14}C incorporation (%)[1]	Dichlorvos inhibition of label into aflatoxin B_1
Norsolorinic Acid	NOR-1	2.2	n.a.[2]
Averantin	AVN-1	10.2-28.5	Yes
Averufanin	n.a.	23.0	n.a.
Averufin	AVR-1	7.4-49.4	Yes
Hydroxyversicolorone	HVN-1	n.a.	n.a.
Versiconal hemiacetal acetate	n.a.	8.0-13.7	Yes
Versicolorin A	VER-1	34.5-50.5	No
Sterigmatocystin	n.a.	17.0-65.0	No
O-Methyl-sterigmatocystin	SRRC 2043	72.5	No

[1]: Data are adapted from Bennett and Lee (1979), and Bennett and Christensen (1983) except for averufanin (McCormick et al., 1987), hydroxyversicolorone (Townsend et al., 1988) and O-methylsterigmatocystin (Bhatnagar et al., 1987).

[2]: Data not available.

Table 2. Aflatoxin intermediates accumulated by blocked mutants of *A. parasiticus*

Blocked mutant	Genotype	Intermediate accumulated
ATCC 24690 (NOR-1)	*nor-1*	Norsolorinic Acid
ATCC 56774 (AVN-1)	*avn-1, nor-1*	Averantin, Versicolorin A
ATCC 24551 (AVR-1)	*avr-1*	Averufin
HVN-1	*hvn-1*	Hydroxyversicolorone
ATCC 36537 (VER-1)	*ver-1*	Versicolorin A
SRRC 2043	n.a.[2]	O-Methyl-sterigmatocystin

[1]: Mutants NOR-1 (Lee et al., 1970), AVN-1 (Bennett et al., 1980a), AVR-1 (Donkersloot et al., 1972) and VER-1 (Lee et al., 1975) were derived from the same wild-type strain *A. parasiticus* NRRL 5862 (SU-1 or ATCC 56775) by mutagenesis with UV-light or nitrosoguanidine. Mutant HVN-1 (Townsend et al., 1988) was derived from *A. parasiticus* SU-7. SRRC 2043 (Bhatnagar et al., 1987) was originated from an *A. parasiticus* isolate CP461 (Dorner et al., 1984).

[2]: Data not available.

intermediates in the pathway (Singh and Hsieh, 1976; Anderson and Dutton, 1979; Anderson and Dutton, 1980; Wan and Hsieh, 1980; Jeenah and Dutton, 1983; Bhatnagar et al., 1989), but most of the nature of the enzymatic activities involved remain unclear to date. Purification and characterization of some of the relevant enzymes has been reported recently (Cleveland and Bhatnagar, 1987; Cleveland et al., 1987; Bhatnagar et al., 1988, Yabe et al., 1989). For example, Yabe et al. (1989) have identified two distinct O-methyltransferase activities in the aflatoxin biosynthetic pathway (Figure 6). One of these is responsible for the conversion of dimethyl sterigmatocystin (DMST) and dihydro-DMST to ST and dihydro-ST, respectively. The other is involved in the conversion of ST and dihydro-ST to OMST and dihydro-OMST, respectively. The latter enzyme has been purified to homogeneity by Bhatnagar et al. (1988). An attempt to clone the gene coding for this enzyme using a cDNA expression library was also initiated by Cleveland and Bhatnagar (1988).

C. Intraspecific Variation in Toxin Production

The aflatoxin-producing ability is strain-specific among natural populations of *A. flavus* and *A. parasiticus*. Not all wild-type strains of these fungi produce aflatoxins. For example, Diener and Davis (1969) screened more than one thousand natural isolates of *A. flavus* collected from six countries and found that only approximately 60% of them produced detectable aflatoxins. Bennett (1982) surveyed published literature studies encompassing 3343 isolates and found that a total of 1847 (56%) were aflatoxigenic. Recently Wei and Jong (1986) surveyed

the aflatoxin-producing ability of 169 strains of the *Aspergillus* reference cultures in the *A. flavus* group maintained in the American Type Culture Collection (ATCC). A relatively low percentage (33%) of *A. flavus* strains were found aflatoxigenic, when compared to *A. parasiticus* strains (85%).

Aflatoxin-producing ability can be lost or decreased after serial transfer in the laboratory (Mayne et al., 1971). The basis of this phenomenon is presumably genetic, but little is known about inheritance of aflatoxin-producing ability.

D. Interspecific Variation in Toxin Production

Aflatoxin production has been reported only from the fungal species *A. flavus*, *A. parasiticus* and *A. nomius*. Early reports that other fungal species also produced aflatoxin have never been confirmed (Detroy et al., 1971; Bennett and Papa, 1988). *A. flavus* and *A. parasiticus* are classified in the *A. flavus* group by Raper and Fennell (1965) along with the koji molds (*A. oryzae* and *A. sojae*) which have been used extensively in Asia for the production of various types of fermented foods. The *A. flavus* group is taxonomically complex. The morphological characteristics of strains within this group are not distinct enough to allow unambiguous differentiation among strains. Because of the possibility that koji molds might be aflatoxigenic, hundreds of Japanese industrial and domestic strains of *A. oryzae* and *A. sojae* have been screened. No aflatoxin has been detected in early studies (Murakami et al., 1967, 1968a, 1968b; Murakami, 1971), or in a more recent study by Wei and Jong (1986). Wicklow (1983) has recently reexamined various koji molds, and, based on their morphological and cultural characteristics, has concluded

that *A. oryzae* has originated as a "domesticated" strain of *A. flavus*, with a similar relationship existing between *A. sojae* and *A. parasiticus*. In support of this theory, homology studies based on DNA hybridization (Kurtzman et al., 1986) showed no detectable differences between *A. flavus* and *A. oryzae*, and 91% relatedness between *A. parasiticus* and *A. sojae*.

Several other fungal species have been reported to contain parts of the aflatoxin biosynthetic pathway (Moss, 1977; Steyn et al., 1980). Moreover, as summarized by Bennett and Deutsch (1985), a number of mold species also produce sterigmatocystin, one of the known intermediates in the aflatoxin pathway (Davies et al., 1960; Dean, 1963; Holzapfel et al., 1966; Mislivec et al., 1975; Schroeder and Kelton, 1975; Hamasaki et al., 1977; Rabie et al., 1977; Udagawa et al., 1979; Ayer et al., 1981; Davis, 1981; Sekita et al., 1981). These data suggest that genes encoding enzymes in the aflatoxin biosynthetic pathway up to sterigmatocystin are widely distributed in nature, while genes encoding enzymes for the last few steps are limited to *A. flavus* and *A. parasiticus*.

From a genetic point of view, the reason that certain isolates of *A. flavus*, *A. parasiticus*, or the koji molds do not produce detectable aflatoxins could be that they lack of genes for the aflatoxin pathway, or that they are simply not expressing these genes. Similar reasoning could be used to explain fungal strains which share part of the aflatoxin pathway but produce no aflatoxins. To date, there are not sufficient data to distinguish between these possibilities. Attempts have been inititated in this lab and others (e.g., Woloshuk et al., 1989) to clone the structural genes of the aflatoxin pathway. These cloned genes can then be used as probes to unambiguously answer these questions.

E. Mutational Analysis of Aflatoxigenic Fungi

A number of mutants have been isolated from *A. flavus* and *A. parasiticus*, and are summarized in a recent review by Bennett and Papa (1988). Some of these have been useful as markers for the elucidation of the parasexual cycle (see below); others have been used to shed light on aflatoxin biosynthesis (Steyn, 1980; Bennett and Christensen, 1983).

1. *A. parasiticus*

Three types of mutants have been described in *A. parasiticus*: (1) mutants blocked in the aflatoxin pathway and accumulate aflatoxin intermediates; (2) spore color and auxotrophic mutants and (3) the so-called *fan* and *fluff* variants which have lost or reduced aflatoxigenicity.

Intermediate-accumulating mutants (Table 2) have brightly-colored mycelia which have been used as a visual screen in parasexual studies. Several rapid plate assays for the aflatoxin-producing ability have been developed to facilitate isolation of blocked mutants (Table 3). In these assays, fluorescence of the aflatoxin-producing colonies under the long-wave uv light enables one to differentiate between aflatoxin-producing and -nonproducing isolates. Spore color and auxotrophic mutants are generally used as markers in elucidating the parasexual cycle and generating linkage maps.

Table 3. Quick screening media for analysis of aflatoxin-producing ability*.

Name of medium	Reference
Aspergillus Differential Medium (ADM)	Bothast and Fennell (1974)
Hyflo-Supercel Peanut Agar (HSPA)	deVogel et al. (1965)
Aflatoxin Producing Ability Medium (APA)	Hara et al. (1974)
Coconut Agar Medium (CAM)	Lin and Dianese (1976)
	Davis et al. (1987)
	Lemke et al. (1988, 1989)
Silica Gel Medium (SGM)	Torrey and Marth (1976)
Fluorescence Agar Medium (FAM)	Lennox and Davis (1983)

* Modified from Bennett and Deutsch (1985). After incubation at 30°C for 2 to 4 days, cultures in Petri plates were inverted and viewed under illumination with a long-wave ultraviolet light. Aflatoxin-producing ability was indicated by a bright blue fluorescence zone around the producing colonies in all cases except for ADM in which formation of a yellow-orange pigment was a positive evidence for aflatoxigenesis.

Strains of *A. parasiticus* which produce no detectable or less aflatoxins are of particular interest. They can be induced by successive transfers of mycelial macerates in defined media. Two such variants (*fan* and *fluff*) were isolated by Bennett et al. (1981a). These strains share the following common features: (1) Both arose spontaneously by positive selection under conditions that favor rapid vegetative growth; (2) Attenuation or loss of the aflatoxin-producing ability is accompanied by loss of non-vegetative functions. For example, haploid and diploid isolates of the *fan* and *fluff* phenotype exhibited less sporulation and altered colony characteristics. The *fan* variants yielded flat growth with heavy sporulation in the center of the colony but only scant sporulation at the edges. The *fluff* variants produced abundant fluffy, aerial mycelium, and few spores. The *fan* variants produced no detectable aflatoxins, but the *fluff* variants were unstable with respect to aflatoxigenicity (Bennett et al., 1986); (3) Both *fan* and *fluff* were recessive traits. Complementation occurred resulting in production of aflatoxin in heterozygous diploids derived from blocked mutants and the *fan* variants (Bennett et al., 1981b, 1986); (4) Both are chromosomal mutations but are not linked to any structural genes for aflatoxin synthesis (Bennett et al., 1981b).

It is interesting to note that the "*lazy*" mutants of *Fusarium graminearum* isolated by Duncan and Bu'Lock (1985) showed the similar phenotypic and genotypic characteristics to the *fan* and *fluff* variants of *Aspergillus* (Bu'Lock, 1986; Bu'Lock et al., 1986a, 1986b). These mutants arose by a chromosomal change and were most readily obtained through repeated serial subculture under non-limiting conditions on rich media. They were characterized by a radically different colony morphology and pigmentation as well as by the substantial or complete loss of the

zearalenone-producing ability. Bu'Lock (1986) and Bu'Lock et al. (1986a, 1986b) suggested that the *lazy* strains contained the full complement of structural genes for zearalenone production but were unable to express them because the *lazy* mutation affected a regulatory function that governed the expression of the structural genes for zearalenone synthesis. Whether or not this is also the case for the *fan* and *fluff* variants remains unclear.

As suggested by Bennett (1982), the high frequency with which *fan* and *fluff* variants are isolated, the pleiotropic nature of their respective phenotypes, and the anomalous behavior of genes *nor-1* and *ver-1* in crosses involving *fan* and *fluff* imply that genetic transpositions are involved. However, direct evidence for this proposition is lacking.

2. *A. flavus*

Three major classes of mutants have been isolated in *A. flavus*, including (1) high aflatoxin B_2-accumulating strains; (2) spore color and auxotrophic mutants, and (3) aflatoxin-negative mutants. Among these mutants, of particular interest are those which accumulate a high level of aflatoxin B_2. A number of these mutants have been isolated from natural environments (Van Walbeek et al., 1968; Schroeder and Carlton, 1973; Gunasekaran, 1981). The strain isolated by Schroeder and Carlton has been used to elucidate the late stages of aflatoxin biosynthesis (Dutton et al., 1985). A mutant producing a high level of aflatoxin B_2 over B_1 was induced by nitrosoguanidine treatment (Papa, 1977a). This mutant (*afl-B2*) has been linked to the histidine locus on linkage group VIII (Papa, 1977a, 1977b, 1977c, 1981).

Other classes of mutants were isolated and characterized by Bennett and Papa (1988). These mutants have been used in parasexual analysis and genetic mapping.

F. Viral Association with Aflatoxin Production

The possibility that aflatoxin production is influenced by extrachromosomal genetic elements in a non-toxigenic isolate of *A. flavus* (NRRL 5565) was investigated by Schmidt et al., (1983). This strain harbored unusual proteinaceous virus-like particles (Wood et al., 1974) consisting of double-stranded RNA (ds-RNA) components that shared characteristic features with a virus that is associated with *Penicillium* strains, particularly *P. chrysogenum*. (Schmidt et al., 1986). Since the viral particles in *A. flavus* NRRL 5565 showed unusual properties, such as an extremely low replication rate and a high proportion of smaller, empty capsids (Wood et al., 1974; Schmidt et al., 1986) that could be attributed only to host influences, Schmidt et al., (1986) suggested that the virus in *A. flavus* NRRL 5565 normally resides in a foreign host.

Elimination of this virus by viral antimetabolites (cycloheximide and 5-fluorouracil) resulted in the simultaneous losses of its ds-RNA trait and initiation of stable aflatoxin formation by *A. flavus* (Schmidt et al., 1983), which, in turn could be switched off by artificial infection with a ds-RNA virus from *P. chrysogenum* (Schmidt and Esser, 1985; Schmidt et al., 1986). This finding suggested that the virus from *P. chrysogenum* was able to repress aflatoxin production of *A. flavus* and supported the hypothesis that this virus may have been transmitted from a foreign host to the originally toxigenic strain of *A. flavus* NRRL 5565. These data also

suggested that the viral ds-RNA present in NRRL 5565 somehow repressed aflatoxin synthesis. When removed or reduced in titer by treatment with antiviral drugs, the fungus regained a native ability to produce aflatoxin. Thus, the nonproduction of aflatoxins was due, in this case, to the failure of gene expression rather than the absence of structural genes of aflatoxin biosynthesis. The mechanism of this viral control of aflatoxin formation remains unknown at this moment. However, it should be noted that a yellow-spored mutant of *A. flavus* NRRL 5565 consistently produced no detectable aflatoxin even after repeated exposure to cycloheximide (Lemke et al., 1989), suggesting that previous results of aflatoxin production in this strain following cycloheximide treatment could be due to contamination by a toxigenic strain. Further investigation on this phenomenon may shed light on genetic regulation of aflatoxin biosynthesis in this particular *A. flavus* strain.

G. The Parasexual Analysis

Although neither *A. flavus* nor *A. parasiticus* possess a sexual stage (teleomorph), there is evidence that a parasexual cycle functions in both species (Bennett, 1979; Gussack et al., 1977; Papa, 1973, 1978). Parasexual analysis involves formation of heterokaryons followed by isolation of heterozygous diploids from these heterokaryons, and the subsequent recovery of recombinant cells from the heterozygous diploid (Roper, 1966). Unlinked genes segregate independently during haploidization, and recombination between linked genes occurs during mitotic crossing over. This process provides an "alternative sex" for many imperfect

(anamorphic) fungi and has made formal genetic analysis possible for the aflatoxigenic molds.

The genetics of *A. flavus* is more developed than that of *A. parasiticus*. Over Thirty genes have been mapped to 8 linkage groups via parasexual analysis (Papa, 1976, 1977a, 1977b, 1977c, 1979, 1980, 1982, 1984). A complete list of these genetic loci of *A. flavus*, including known linkage assignments, has been compiled by Bennett and Papa (1988). It appears that the genes encoding proteins responsible for aflatoxin synthesis and regulation of this metabolic pathway have been mapped to different chromosomes with some clustering of loci on particular chromosomes.

Fewer genetic loci have been studied in *A. parasiticus*. Papa (1978) assigned seven loci to four linkage groups (I through IV), and ten loci were assigned to six linkage groups by Bradshaw et al. (1983).

As pointed out by Bennett and Papa (1988), different laboratories used different parent strains, different genetic markers and nomenclatures, as well as different blocked aflatoxin mutants because virtually no collaborative studies have been initiated among laboratories. Therefore, until genetic nomenclature and linkage groups are standardized, the mapping data from each laboratory must be evaluated with caution. The conventional designations for fungal gene symbols and phenotypes as described by Bennett and Lasure (1985b) and Yoder et al. (1986) are followed throughout this thesis.

Although parasexual analysis allows genetic analysis of aflatoxigenic molds, it is somewhat unreliable and laborious to perform due to the following reasons as summarized by Bennett (1982), which significantly impede the development of the genetic maps for the aflatoxigenic molds.

a. Cell ploidy. In parasexual analysis, a haploidization agent such as *p*-fluorophenylalanine (FPA) or benlate (containing 50% benomyl) is usually used to increase the number of haploid segregants from heterozygous diploids. In species with uninucleate conidia (e.g., *A. nidulans*), ploidy is readily determined by conidial diameter, with diploid spores derived from nuclear fusions being larger than haploid spores. However, determination of cell ploidy is nearly impossible in wild-type *A. flavus* and *A. parasiticus* which have multinucleate conidia (Yuill, 1950). First, microscopical examinations are not able to detect differences in conidial diameters in haploid, heterokaryotic and diploid spores of these fungi. Second, total DNA content is nearly equal in these spores because spores in diploids have fewer nuclei than do haploids or heterokaryons (Leaich and Papa, 1975; Bennett et al., 1980b). The inability to directly detect cell ploidy by spore diameter entails the utilization of indirect methods which are laborious, and often can not be used to distinguish from strain instability arising from aneuploidy such as has been detected in *A. nidulans* (Birkett and Roper, 1977).

b. Genetic segregation. During parasexual cycle, a number of auxotrophic markers are recovered in extremely low yields on media containing haploidization agents (FPA and benlate). Furthermore, spore and mycelial color markers have been found to segregate nonrandomly (Bennett, 1979; Bennett et al., 1980b).

c. Heterokaryon incompatibility. Strains belonging to the same heterokaryon (vegetative) incompatibility (or compatibility) group generally do not mate or allow

hyphal anastomosis to form a heterokaryon. Th phenomenon is widespread in the *A. flavus* group. Formation of heterokaryons has been reported only among complementing auxotrophs derived from the same ancestral wild-type strain (Gussack et al., 1977; Bennett, 1982). Papa (1986) detected 22 different heterokaryon compatibility groups among 32 *A. flavus* strains collected from 15 Georgia counties. Strains within the same heterokaryon compatibility group were not restricted to the same geographical area. Six intraspecific crossing of auxotrophs from *A. flavus* PC-7 and *A. flavus* NRRL 5565 failed to grow, as did 12 interspecific combinations of auxotrophs of *A. flavus* and *A. parasiticus* (Gussack et al., 1977). Attempted protoplast fusions between *A. flavus* and *A. parasiticus* auxotrophs were also unsuccessful (Bennett, 1982).

H. Genetic Transformation in Aflatoxigenic Fungi

As discussed above, the development of aflatoxin genetics is still at a very early stage. The difficulties arising from the lack of a sexual reproduction cycle of the aflatoxigenic molds make traditional Mendelian genetic analysis of aflatoxin biosynthesis nearly impossible. In addition, as summarized by Bennett (1982), a number of technical problems impede research on the genetic basis of biosynthesis of other fungal secondary metabolites as well. First, aflatoxins are not direct gene products. They are produced by the enzymatic conversion of small precursor molecules. Second, cell-free systems are poorly developed and only a few of the enzymes involved in aflatoxin biosynthesis have been isolated (as discussed in section B). Third, strain instability in originally high aflatoxin-producing strains

occurs at a higher frequency than background mutation rates. Fourth, mutants affecting the aflatoxin biosynthetic pathway are difficult to obtain because conditional lethal protocols cannot be employed, because with all secondary metabolites, aflatoxins are not essential for growth of the producing organism.

Recent advancements in the molecular biology of filamentous fungi (as reviewed below) offer innovative and more effective approaches for studying aflatoxin biosynthesis. Transformation is a gene transfer process in which a naked DNA molecule is introduced into cells. It is generally considered an essential step in modern genetic engineering techniques. Transformation systems developed for a variety of filamentous fungi have resulted in cloning of a growing number of genes by functional complementation of mutations (Fincham, 1989). Such a transformation system has recently been developed for *A. flavus* (Woloshuk et al., 1989). This system is based on complementation of a pyrimidine auxotrophic mutation with vectors containing the heterologous *pyr-4*$^{+}$ gene of *N. crassa* as a selectable marker. The pyrimidine mutation in the recipient strain was obtained by screening mutagenized cells for resistance to the toxic metabolite 5-fluoro-orotic acid (5-FOA) (Boeke et al., 1984). The pyrimidine mutation in one strain could be transferred into different strains through parasexual recombination, thus making mutant isolation somewhat easier to perform. The transformation frequency of *A. flavus* with this system is generally low (about 20 transformants/μg DNA) but, in theory, is sufficient to screen a cosmid genomic DNA library. Coupled with aflatoxin-nonproducing mutants, this system may allow one to clone genes involved in aflatoxin synthesis in *A. flavus*. A more versatile transformation system based on the homologous benomyl resistance gene (a mutated version of the β-tubulin gene) as a

selectable marker is also being developed for *A. flavus* (Seip et al., 1988, 1989). This type of dominant selectable marker is highly desirable because any fungal strain sensitive to the inhibitors (benomyl) can be used as a potential recipient in transformation.

A. parasiticus offers a distinct advantage over *A. flavus* in studying aflatoxin biosynthesis at the molecular level since there exist several blocked mutants of this fungus which will allow cloning of genes involved in the aflatoxin biosynthetic pathway by functional complementation. Development and characterization of selectable markers for genetic transformation systems in the aflatoxigenic *A. parasiticus* is the major goal of this investigation.

II. Genetic Transformation in Filamentous Fungi

The importance of filamentous fungi in medicine, industry, and agriculture has led to intensive investigations into their physiology and metabolic regulation (Bennett and Lasure, 1985a; Timberlake, 1985). However, due to the lack of a sexual stage, fundamental genetic analysis in many of these important groups of fungi has been impossible or difficult to perform. Therefore, it was desirable to develop a deoxyribonucleic acid (DNA)-mediated transformation systems to conduct genetic studies.

The first confirmed DNA-mediated transformation in filamentous fungi was reported by Case et al. (1979) who used the cloned *N. crassa qa-2*$^+$ (catabolic dehydroquinase) gene as a selectable marker. Transformants were shown by Southern hybridization analysis to contain chromosomally integrated plasmid molecules. Subsequently, many *Neurospora* genes have been used as selectable markers for transformation, including *pyr-4*$^+$ (Ballance et al., 1983), *trp-1*$^+$ (Schechtman and Yanofsky, 1983), *am*$^+$ (Kinnaird et al., 1982), and *bml* (benomyl resistance) (Orbach et al., 1986). Transformation of *A. nidulans*, another well-studied filamentous fungus, was first reported by Ballance et al. (1983), who used the *N. crassa pyr-4*$^+$ gene to complement an *A. nidulans pyrG* mutation. Shortly after, Tilburn et al. (1983) used the *A. nidulans amdS*$^+$ gene, encoding acetamidase (Hynes et al., 1983), and Yelton et al. (1984) used the *A. nidulans trpC*$^+$ gene for transformation. In each case, transforming DNA became integrated into the genome. Subsequently, other *A. nidulans* genes were used as transformation markers, including *argB*$^+$ (Berse et al., 1983; John and Peberdy, 1984), *prn*$^+$ (Arst et al.,

Table 4. Filamentous fungi in which transformation has been achieved*.

Type of fungus	Reference
Oomycetes	
Achlya ambisexualis	Manavathu et al. (1987)
Ascomycetes	
Ascobolus immersus	Faugeron et al. (1989)
Aspergillus nidulans	Tilburn et al. (1983)
Claviceps purpurea	van Engelenburg et al. (1989)
Cochliobolus heterostrophus[1]	Turgeon et al. (1985)
Endothia parasitica[2]	Van Alfen et al. (1988)
Gaeumannomyces graminis	Hensen et al. (1988)
Gibberella pulicaris[3]	Salch & Beremand (1989)
Glomerella cingulata[4]	Rodriguez & Yoder (1987)
Hypoxylon mammatum	Griffin et al. (1989)
Leptosphaeria maculans[5]	Farman & Oliver (1988)
Magnaporthe grisea[6]	Parsons et al. (1987)
Nectria haematococca[7]	Van Etten & Kistler (1988)
Neurospora crassa	Case et al. (1979)
Podospora anserina	Brygoo & Debuchy (1985)
Sordaria macrospora	Le Chevanton et al. (1989)
Related Fungi Imperfecti	
Aspergillus awamori	Cullen & Leong (1986)
Aspergillus flavus	Woloshuk et al. (1989)
Aspergillus ficuum	Mullaney et al. (1988)
Aspergillus niger	Goosen et al. (1987)
Aspergillus oryzae	Mattern et al. (1987)
Aspergillus terreus	Katz & Hynes (1989b)
Botrytis squamosa	Huang et al. (1989)
Cephalosporium acremonium	Skatrud & Queener (1984)
Colletotrichum capsici	Soliday et al. (1989)
Colletotrichum graminicola	Panaccione et al. (1988)
Colletotrichum gloeosporioides	TeBeest & Dickman (1989)
Colletotrichum lindemuthianum	Daboussi et al. (1989)
Colletotrichum trifolii	Dickman (1988)
Fulvia fulva[8]	Oliver et al. (1987)
Penicillium caseicolum	Daboussi et al. (1989)
Penicillium chrysogenum	Díez et al. (1987)
Penicillium nalgiovense	Geisen & Leistner (1989)

Table 4 (cont'd.).

Basidiomycetes	
Coprinus cinereus	Binninger et al. (1987)
Phanerochaete chrysosporium	Alic et al. (1989)
Schizophyllum commune	Muñoz-Rivas et al. (1986b)
Ustilago maydis	Banks (1983a)
Ustilago hordei	Holden et al. (1988)
Ustilago nigra	Holden et al. (1988)
Ustilago violacea	Bej & Perlin (1989)
Zygomycetes	
Absidia glauca	Wöstemeyer et al. (1987)
Mucor circinelloides	Van Heeswijck & Roncero (1984)
Phycomyces blakesleeanus	Revuelta & Jayaram (1986)
Other Fungi Imperfecti	
Aphanocladium album	Daboussi et al. (1989)
Beauveria bassiana	Daboussi et al. (1989)
Curvularia lunata	Osiewacz & Weber (1989)
Fusarium culmorum	Madhosingh & Orr (1985)
Fusarium graminearum	Dickman & Leslie (1989)
Fusarium moniliforme	Leslie & Dickman (1989)
Fusarium oxysporium	Kistler & Benny (1988)
Gliocladium virens	Thomas & Kenerley (1989)
Gliocladium roseum	Thomas & Kenerley (1989)
Metarhizium anisopliae	Bernier et al. (1989)
Pseudocercosporella herpotrichoides	Blakemore et al. (1989)
Scytalidium flavo-brunneum	Caprioglio & Parks (1989)
Septoria nodorum[9]	Cooley et al. (1988)
Trichoderma reesei	Penttilä et al. (1987)

*: Adapted from Fincham (1989).
[1]: Anamorph: *Helminthosporium maydis* = *Bipolaris maydis* = *Dreschlera maydis*.
[2]: Synonym: *Cryphonectria parasitica*, the causal agent of chestnut blight.
[3]: Anamorph: *Fusarium sambucinum*, causing dry rot on potato tubers.
[4]: Anamorph: *Colletotrichum lindemuthianum*, a pathogen of bean.
[5]: Synonym: *Phoma lingam*, causing the blackleg disease of brassicas.
[6]: Anamorph: *Pyricularia oryzae* or *P. grisea*, the causal agent of rice blast disease.
[7]: Anamorph: *Fusarium solani* f. sp. *pisi*, a pathogen of pea.
[8]: Synonym: *Cladosporium fulvum*, a major foliar pathogen of tomato.
[9]: Teleomorph: *Leptosphaeria nodorum*, causing a leaf spot disease of wheat.

1985), *pyrG*$^{+}$ (Oakley et al., 1987), *pabaA*$^{+}$ (Timberlake and Marshall, 1989), and *BenA*^{R} (Dunne and Oakley, 1988).

Following the success with *N. crassa* and *A. nidulans*, transformation systems were rapidly developed in many other fungal species of medical, industrial, and agricultural significance (Fincham, 1989; Table 4). Transformation of filamentous fungi has been extensively reviewed (Bennett and Lasure, 1985a; Johnstone, 1985; Mishra, 1985; Timberlake, 1985; Cullen and Leong, 1986; Esser and Mohr, 1986; Hynes, 1986; Saunders et al., 1986; Rambosek and Leach, 1987; Leong, 1988; Schwab, 1988; Fincham, 1989; Timberlake and Marshall, 1989).

A. Transformation Techniques

Although the original transformation protocols developed for *N. crassa* and *A. nidulans* have been modified and improved, they remain fundamentally unchanged. These include preparation of competent cells and DNA uptake.

1. Preparation of competent cells

DNA-mediated transformation requires that an organism take up and express exogenous DNA molecules that can confer a selectable advantage on those cells which receive them. Cells that are capable of taking up exogenous DNA are referred to as "competent." In filamentous fungi, competence can be achieved in the following ways:

a. Protoplast. The fungal cell wall is a multilayer network structure of complicated organization which serves as an efficient barrier to DNA entry (Bartnicki-Garcia, 1968; Rosenberger, 1976; Farkaš, 1985; Wessels, 1986; Cabib et al., 1988). Protoplasts are typically generated by digesting away the fungal cell walls with cell wall-degrading enzymes. Novozym 234, a commercially available hydrolytic enzyme mixture (notably 1,3-glucanases and chitinase) secreted by the filamentous fungus *Trichoderma harzianum*, has been commonly used for this purpose (Hamlyn et al., 1981). In some fungal species (Turgeon et al., 1985; Binninger et al., 1987), it is necessary to use other enzymes in conjunction with Novozym 234. These include enzymes from snail gut (Glusulase, helicase and β-glucuronidase), Zymolyase, cellulase, chitinase, driselase or others (Peberdy, 1985).

Experimental data suggests that the particular enzyme used for digestion of the fungal cell wall was often of great importance. For example, using Glusulase Kinsey et al. (1984) obtain a high yield of transformants (10^4/μg DNA) when selecting for the *am*$^+$ (glutamate dehydrogenase) gene. With different lot of Glusulase, however, the frequency decreased 100-fold. Akins and Lambowitz (1985) similarly found that one particular lot of Novozym 234 was most suitable for preparation of competent *Neurospora* protoplasts. Because empirical observation suggests that there is significant variation in efficiency of transformation between batches of enzymes, Rambosek and Leach (1987) suggested "When in doubt, try another batch of enzymes to generate protoplasts."

Protoplasts can be prepared from various cell types of filamentous fungi. In For example, young mycelium has been a major source of protoplasts for *Neurospora* (Buxton and Radford, 1984), *Aspergillus* (Yelton et al., 1984), and *Penicillium*

species (Sánchez et al., 1987), as well as for *Podospora anserina* (Brygoo and Debuchy, 1985) and *Ascobolus immersus* (Faugeron et al., 1989) which do not produce conidia. Protoplasts released can be easily separated from the hyphal debris by filtration. For *Basidiomycetes*, the sexual basidiospores (Muñoz-Rivas et al., 1986b), the dikaryotic mycelium, or the asexual oidia (Binninger et al., 1987) has been used for protoplast production.

One of the main advantages of preparing protoplasts from spores is obtaining a homogeneous suspension of protoplasts with only one nucleus. Mononucleate protoplasts are most suitable for recombination and transformation experiments (Bos et al., 1983). Moreover, protoplasts from fungal spores are homogeneous in terms of size distribution and physiological condition, whereas protoplasts derived from mycelium are heterogeneous because of age differences of the hyphal cells from which they are originated (Peberdy and Gibson, 1971; Anné et al., 1974). Thus, protoplasts from fungal spores offer certain advantages over those from mycelium for genetic and physiological experiments. Several reports describe production of protoplasts from fungal spores (Chu and Alexander, 1972; Emerson and Emerson, 1958; Bachmann and Bonner, 1959; Weiss, 1965; García Acha and Villanueva, 1963, 1964; García Acha et al., 1966; Laborda et al., 1974; Moore and Peberdy, 1976; Bos and Slakhorst, 1981; Bos, 1985).

Freezing has been used to preserve fungal protoplasts for successive transformation experiments. However, the effect(s) of freezing on competence and survival of protoplasts has not been fully investigated. Competent *Neurospora* protoplasts could be stored frozen in the transformation buffer at -70°C for more than one month without loss in transformation frequency (Akins and Lambowitz, 1985).

Neurospora protoplasts stabilized with sorbitol remained viable indefinitely at -70°C (Vollmer and Yanofsky, 1986). Frozen protoplasts of *A. nidulans* prepared according to this procedure yielded more reproducible transformation results than freshly prepared protoplasts (Cullen et al., 1987). However, storage of protoplasts of *Trichoderma* at -70°C resulted in 50% reduction in transformation frequency (Penttilä et al., 1987). A similar result was observed for protoplasts of *Schizophyllum* (Specht et al., 1988). However, they could be stored on ice for 20h before use without affecting the transformation frequency. Addition of glycerol to 15% (v/v) had no effect on protoplast viability, but completely inhibited transformation in *Schizophyllum.*

Protoplasts are fragile and must be protected by osmotic stabilizers in the suspending medium. The type and concentration of stabilizer may influence the yield and the stability of protoplasts, and depending on the organism used, a wide range of osmotic stabilizers including inorganic salts, sugars and sugar alcohols have been used to stabilize protoplasts released from fungi (Davis, 1985). There is no single stabilizer suitable for all fungi. Some osmotic stabilizers such as KCl, $MgSO_4$, and the sugar alcohol sorbitol and mannitol are used more often than others (Musílková and Fencl, 1968; Sietsma and de Boer, 1973; Peberdy et al., 1976). Magnesium sulfate (1.2 M) has promoted the release of highly vacuolated protoplasts from *S. commune* (de Vries and Wessels, 1972) and *A. nidulans* (Peberdy and Isaac, 1976; Tilburn et al. 1983; Yelton et al., 1984) which could be easily separated from mycelial debris because they floated on the supernatant after centrifugation. However, protoplasts isolated from *A. fumigatus* in the presence of magnesium sulfate are osmotically very fragile (Hearn et al., 1980).

An essential requirement for obtaining growing colonies from protoplasts is the maintenance of the osmotic stabilizer in the growth medium until the cell wall has been regenerated. The same stabilizer is generally used for regeneration as well as for protoplast preparation, but the choice of plating conditions may be determined by the selectable marker in use. For example, cesium chloride is commonly added into media to reduce background nitrogen utilization by nontransformed cells. In this case, sucrose is the stabilizer of choice instead of potassium chloride (Tilburn et al., 1983). In contrast, sucrose or sorbitol prevents direct selection for carbon utilization genes. In general, spreading of the transformed cells embedded in an agar overlay of selective medium tends to improve the transformation yield by increasing the regeneration rate of protoplasts (Ballance and Turner, 1985; Bergès and Barreau, 1989). However, there were reported cases in which directly spreading the transformed cells or embedding them in the agar overlay were indifferent with respect to transformants recovered. Oakley et al. (1987) were able to achieve a high transformation frequency (2400/μg DNA) in *A. nidulans* by eliminating top agar and plating transformed cells directly on the selective medium.

b. Alternatives to protoplast. Several alternative methods have been developed that allow transformation of fungal cells without the need for tedious protoplast preparation.

One approach is to use a mutant recipient strain that may have more permeable membranes or cell walls. For example, the *inl* mutant of *N. crassa* was thought to be particularly competent to take up DNA because of the greater membrane porosity when it was starved for inositol (Mishra and Tatum, 1973; Mishra et al.,

1973). Germlings of this strain were transformable, although the frequency was low when compared to methods involving protoplasts (Mishra, 1977, 1979; Wootton et al., 1980). Cell wall-deficient mutants of *Neurospora* have also been used to facilitate the uptake of DNA without protoplast formation (Mishra et al., 1973). A cell-wall-less strain of *Neurospora* called *slime* could be used without glusulase treatment. However, genetic analysis of the *slime* strain of *Neurospora* is difficult, making the use of this strain less attractive in transformation experiments (Mishra, 1985).

The use of high concentrations of alkali metal ions (Iimura et al., 1983; Ito et al., 1983) represents another effective way to induce permeability of DNA into intact fungal cells. Lithium acetate at 0.1 M has been widely used for this purpose (Ito et al., 1983). This procedure has been successfully applied to *N. crassa* (Dhawale et al., 1984), *Coprinus cinereus* (Binninger et al., 1987), *Colletotrichum trifolii* (Dickman, 1988) and *Fusarium solani* f. sp. *pisi* (*Nectria hematococa*) (Soliday et al., 1989, Marek et al., 1989). Frequencies of transformation are generally lower than those obtained using protoplast methods but considerable time can be saved since generation of protoplasts is not necessary. This method, however, could not be applied in *Ustilago maydis* due to the toxic effect of the lithium ion to this fungus (Leong, 1988). In contrast, whole sporidial cells from *U. violacea* could be transformed at a high frequency when treated with lithium acetate and polyethylene glycol (PEG) (Bej and Perlin, 1989). How alkali metal cations assist the passage of DNA into cells is not well understood.

2. Uptake of DNA

How cells become competent to take up exogenous DNA molecules remains unclear, and is a subject being intensively investigated. Nevertheless, several procedures are available to enhance entry of DNA into fungal cells.

a. Calcium and PEG. Calcium chloride (10 or 50 mM) is typically used in transformation protocols for *Aspergillus* species and other filamentous fungi. A treating time of 15 to 30 min at room temperature or on ice is generally sufficient for DNA uptake.

Dimethyl sulfoxide (1%) has been added to the transformation mixtures for *Neurospora*, and some protocols have included heparin (Kinsey and Rambosek, 1984) and spermidine as well (Vollmer and Yanofsky, 1986). However, these additives is not commonly used in transformation of fungi other than *Neurospora* species.

PEG is one of the key components in virtually all fungal transformation protocols. It brings the treated cells together and thus allows fusion to occur. DNA molecules are presumably internalized during this process because no transformation occurs when PEG is omitted (Timberlake and Marshall, 1989). The effect(s) on the transformation frequency of varying the molecular weight and concentration of PEG has not been carefully investigated. Concentrations of PEG commonly used range from 20% to 70% with PEG 4000 (from various sources). PEG 6000 and PEG 8000 at 25% or 50% are also common. Tilburn et al. (1983) found that increasing the concentration of PEG from 25 to 60% increased the frequency of

transformation in *A. nidulans*, but Turner and Ballance (1985) did not find the same effect. Increasing the concentration of PEG 8000 from 25% to 50% also increased the transformation frequency 3 to 4-fold in *Penicillium* (Cantoral et al., 1987).

b. Liposome-mediated uptake. An alternative way of introducing DNA into cells that could in principle be more efficient was devised by Fraley and coworkers (Fraley and Papahadjopoulos, 1981). They showed that DNA molecules can be encapsulated within the aqueous interior of phospholipid vesicles (liposomes). Subsequent fusion of liposomes with the plasma membrane results in intracellular delivery of the encapsulated nucleic acid. Whereas transformation by naked DNA is DNase-sensitive, that by liposomes is presumably not. Radford et al. (1981) first applied this technique in transformation of *Neurospora*. This procedure is effective in protecting transforming DNA but is not widely adopted because it appears to offer no advantages over the routine procedures with naked DNA to justify the extra work of liposome preparation.

c. Heat shock treatment. During the transformation protocol, bacterial cells are generally treated with a short pulse of heat to enhance their competence in taking up DNA (Hanahan, 1983). However, the effect of heat shock treatment on the transformation frequency of filamentous fungi has not been well documented except in *Podospora*. Bergès and Barreau (1989) observed that heat shock at an elevated temperature (48°C) for 5 min improved the transformation efficiency 5- to 10-fold in *P. anserina* protoplasts only if the heat shock was applied before the addition of transforming DNA. An increase in competence was observed immediately after the

heat shock. Heat-shocked cells remained competent for 20 to 30 min. The improvement in transformation efficiency after heat shock did not depend on the selectable markers used for transformation. The mechanism by which heat shock improves competence remains unclear, perhaps explaining why heat shock has not been widely used in fungal transformation.

d. Electroporation. DNA can also be introduced into protoplasts by exposing cells to a brief electrical pulse or pulses of high field strength (Zimmermann, 1982; Zimmermann and Vienken, 1982). This technique, called electroporation, renders cell membranes temporarily permeable to macromolecules such as DNA. Electroporation has been used successfully to study the transformation and expression of gene products in various cell types where other methods failed (Fromm et al., 1987; Knight and Scrutton, 1986; Neumann and Bierth, 1986).

Ward et al. (1988) first transformed protoplasts of *A. niger* to oligomycin resistance by electroporation. Subsequently protoplasts from several other filamentous fungi have been successfully transformed by this technique, including *A. nidulans*, *F. solani* (Richey et al. 1989), *A. awamori*, *A. niger* (Ward et al., 1989), and *Gliocladium* spp. (Thomas and Kenerley, 1989). In certain cases, transformation frequencies were comparable to those obtained by the traditional protocol involving treatment of protoplasts with PEG and calcium ion. Electroporation may not be an appropriate method of transformation for species such as *A. nidulans* or *N. crassa* for which highly efficient PEG-mediated transformation protocols are already available. However, this method may provide an alternative of introducing foreign DNA into fungi that cannot be transformed by traditional methods.

e. Other procedures. In addition to procedures described above, transformation protocols involving particle bombardment (Klein et al., 1987; Fox et al., 1988; Johnston et al., 1988) or partial cell breakage by blending with glass beads (Costanzo and Fox, 1988a, 1988b) have been proven feasible in yeasts, and could also be useful with filamentous fungi.

B. Selection of Transformants

Transformation systems require the presence of a marker on the transforming vector which allows growth of transformed colonies under selective conditions.

1. Selectable markers

A wide variety of selection systems are now available in the filamentous fungi (Schwab, 1988) which fall into two broad classes: auxotrophic markers and dominant resistance markers. Occasionally certain specific selectable (or screenable) markers such as those based on spore color or colonial morphology are also useful.

a. Auxotrophic selectable markers

The use of auxotrophic selectable markers involves complementation of an auxotrophic mutation with the wild-type gene. Selection of transformants with this

system is usually straightforward. Several wild-type genes from filamentous fungi (Schwab, 1988; Fincham, 1989) have been particularly useful as general-purpose selectable markers and have frequently been incorporated into cloning vectors. A number of them are functional in several different fungal species (Table 5).

In order for this type of selection system to work, it is necessary to obtain both a cloned wild-type gene (either from the same or different fungal strains) as well as the corresponding mutation in the recipient strain. This latter requirement is not always easy to meet, especially for genetically undeveloped fungi. One way to overcome these limitations is to utilize positive selection protocols which have been successful in generating a number of mutants in filamentous fungi.

1) Mutants defective in orotidine 5'-monophosphate (OMP) pyrophosphorylase and/or OMP decarboxylase. The biosynthesis of uridine monophosphate (UMP) in filamentous fungi proceeds from aspartate and carbamoyl phosphate through the intermediate orotic acid, to OMP which is finally decarboxylated to UMP (Radford et al., 1985). The genes involved in the conversion of orotic acid to UMP have been named *pyrF*$^+$ (OMP pyrophosphorylase) and *pyrG*$^+$ (OMP decarboxylase) in *Aspergillus* (Palmer and Cove, 1975), whereas the same genes in *Neurospora* are known as *pyr-2*$^+$ and *pyr-4*$^+$ respectively (Caroline, 1969; Perkins et al., 1982). Boeke et al. (1984) found that wild-type strains of *S. cerevisiae* were sensitive to the pyrimidine analog 5-fluoroorotic acid (5-FOA), presumably due to its conversion into the toxic 5-fluoro-UMP. Mutants resistant to 5-FOA were frequently uracil auxotrophs. Thus, this toxic pyrimidine analog is potentially useful in isolating mutants defective in genes encoding enzymes responsible for conversion of orotic

Table 5. Selectable markers used across species lines in filamentous fungi[a].

Marker	Phenotype	Species of origin	Species in which marker was used	Reference
acu-7[+]	Isocitrate lyase	*Coprinus cinereus*	*Aspergillus nidulans*	Hynes (1989)
ade5[+]	Adenine synthesis	*Schizophyllum commune*	*Phanerochaete chrysosporium*	Alic et al. (1989)
amdS[+]	Acetamide utilization	*A. nidulans*	*Aspergillus ficuum*	Mullaney et al. (1988)
			Aspergillus niger	Kelly & Hynes (1985)
			Aspergillus oryzae	Christensen et al. (1988)
			Cochliobolus heterostrophus	Turgeon et al. (1985)
			Glomerella cingulata	Rodriguez & Yoder (1987)
			Penicillium chrysogenum	Beri & Turner (1987)
			Penicillium nalgiovense	Geisen & Leistner (1989)
			Septoria nodorum	Cooley et al. (1988)
			Trichoderma reesei	Penttilä et al. (1987)
argB[+]	Arginine synthesis	*A. nidulans*	*A. niger*	Buxton et al. (1985)
			A. oryzae	Gomi et al. (1987)
			Magnaporthe grisea	Parsons et al. (1987)
			Nectria haematococca	Van Etten & Kistler (1988)
			Trichoderma reesei	Penttilä et al. (1987)
		A. niger	*A. nidulans*	Buxton et al. (1987)
bml	Benomyl resistance	*Neurospora crassa*	*A. nidulans*	Orbach et al. (1986)
			A. niger	Rambosek & Leach (1987)
			A. oryzae	Unkles et al. (1989a)
			Colletotrichum gloeosporioides	TeBeest & Dickman (1989)

Table 5 (cont'd.).

			Colletotrichum graminicola	Panaccione et al. (1988)
			Colletotrichum trifolii	Dickman (1988)
			Endothia parasitica	Van Alfen et al. (1988)
			Gaeumannomyces graminis	Henson et al. (1988)
			Hypoxylon mammatum	Griffin et al. (1989)
			Metarhizium anisopliae	Bernier et al. (1989)
			P. chrysogenum	Picknett & Saunders (1989)
			Podospora anserina	Berges & Barreau (1989)
				Fernandez-Larrea & Stahl (1989)
			Pseudocercosporella herpotrichoides	Blakemore et al. (1989)
ble	Bleomycin resistance	*Escherichia coli*	*A. nidulans*	Mattern et al. (1988)
			A. niger	Katz & Hynes (1989b).
			A. oryzae	Mattern et al. (1987)
			Aspergillus terreus	Katz & Hynes (1989b)
			Claviceps purpurea	van Engelenburg et al. (1989)
			P. chrysogenum	Kolar et al. (1988)
hygB	Hygromycin B resistance	*E. coli*	*A. nidulans*	Cullen et al. (1987)
			A. niger	Punt et al. (1987)
			A. ficuum	Mullaney et al. (1988)
			Botrytis squamosa	Huang et al. (1989)
			Cephalosporium acremonium	Skatrud et al. (1987)
			C. heterostrophus	Turgeon et al. (1987)
			C. trifolii	Dickman (1988)
			Colletotrichum capsici	Soliday et al. (1989)
			Curvularia lunata	Osiewacz & Weber (1989)
			E. parasitica	Van Alfen et al. (1988)
			Fulvia fulva	Oliver et al. (1988)
			Fusarium moniliforme	Leslie & Dickman (1989)
			Fusarium oxysporum	Kistler & Benny (1988)
			Fusarium solani f. sp. *pisi*	Soliday et al. (1989)
			Gibberella pulicaris	Salch & Beremand (1989)

Table 5 (cont'd.).

			Gliocladium virens	Thomas & Kenerley (1989)
			G. cingulata	Rodriguez & Yoder (1987)
			Leptosphaeria maculans	Farman & Oliver (1988)
			P. herpotrichoides	Blakemore et al. (1989)
			Septoria nodorum	Cooley et al. (1988)
			Trichoderma spp.	Siyan et al. (1989)
			Ustilago maydis	Wang et al. (1988)
			Ustilago hordei	Holden et al. (1988)
			Ustilago nigra	Holden et al. (1988)
			Ustilago violacea	Bej & Perlin (1989)
lacZ⁺	β-Galacto-sidase	*E. coli*	*A. nidulans*	Roberts et al. (1989)
			A. niger	Davis et al. (1988)
			A. oryzae	de Ruiter-Jacobs et al. (1989)
			P. chrysogenum	Kolar et al. (1988)
			T. reesei	Penttilä et al. (1987)
*neo*ʳ	Neomycin resistance	*E. coli*	*Absidia glauca*	Wöstemeyer et al. (1987)
			Achlya ambisexualis	Manavathu et al. (1987)
			A. niger	Rambosek & Leach (1987)
			C. acremonium	Peñalva (1985)
			F. solani	Marek et al. (1989)
			N. crassa	Bull & Wootton (1984)
			P. chrysogenum	Rambosek & Leach (1987)
			P. chrysosporium	Randall et al. (1987)
			Phycomyces blakesleeanus	Revuelta & Jayaram (1986)
			S. commune	Ullrich et al. (1985)
			U. maydis	Banks (1983)
niaD⁺	Nitrate reductase	*A. nidulans*	*Aphanocladium album*	Daboussi et al. (1989)
			Beauveria bassiana	Daboussi et al. (1989)
			Colletotrichum lindemuthianum	Daboussi et al. (1989)
			F. oxysporum	Malardier et al. (1989)

Table 5 (cont'd.).

			N. haematococca	Daboussi et al. (1989)
			Penicillium caseicolum	Daboussi et al. (1989)
			P. chrysogenum	Whitehead et al. (1989)
			Pyricularia oryzae	Daboussi et al. (1989)
		A. oryzae	*A. nidulans*	Unkles et al. (1989a)
			A. niger	Unkles et al. (1989a)
			P. chrysogenum	Unkles et al. (1989a)
		A. niger	*A. nidulans*	Unkles et al. (1989b)
			A. oryzae	Unkles et al. (1989b)
			P. chrysogenum	Unkles et al. (1989b)
		F. solani	*A. nidulans*	Richey et al. (1989)
nit-2$^+$	Nitrogen utilization	*N. crassa*	*A. nidulans*	Davis & Hynes (1987)
			Fusarium graminearum	Dickman & Leslie (1989)
pyr-4$^+$	Pyrimidine synthesis	*N. crassa*	*Aspergillus awamori*	Cullen & Leong (1986)
			Aspergillus flavus	Woloshuk et al. (1989)
			A. niger	van Hartingsveldt et al (1987)
			A. nidulans	Ballance et al. (1983)
			P. chrysogenum	Díez et al. (1987)
			T. reesei	Cullen & Leong (1986)
pyrG$^+$	Pyrimidine synthesis	*A. niger*	*A. awamori*	Ward et al. (1989)
			A. nidulans	Goosen et al. (1987)
			A. oryzae	Mattern et al. (1987)
		A. oryzae	*A. niger*	de Ruiter-Jacobs et al. (1989)
trp1$^+$	tryptophan synthesis	*S. commune*	*C. cinereus*	Casselton & Fuente Herce (1989)

Table 5 (Cont'd.).

trpC•	tryptophan synthesis	*A. niger*	*A. nidulans*	Kos et al. (1985)
		A. parasiticus	*A. nidulans*	Horng et al. (1989)
		C. heterostrophus	*A. nidulans*	Turgeon et al. (1986)
		P. chrysosporium	*C. cinereus*	Casselton & Fuente Herce (1989)
uidA•	β-Glucuro-nidase	*E. coli*	*A. nidulans*	Roberts et al. (1989)
			A. niger	Roberts et al. (1989)
			A. oryzae	Unkles et al. (1989a)
			F. fulva	Roberts et al. (1989)

•: Modified from Fincham (1989).
*: Including varieties *graminis* and *tritici*.

acid to UMP. Using this protocol, pyrimidine auxotrophs have been readily isolated and used as recipients in transformation of a variety of filamentous fungi including *A. flavus* (Woloshuk et al., 1989), *A. nidulans* (Dunne and Oakley, 1988), *A. niger* (van Hartingsveldt et al., 1987; Goosen et al., 1987), *A. oryzae* (Mattern et al., 1987), *C. graminicola* (Rasmussen et al., 1989), *P. anserina* (Razanamparany and Bégueret, 1986), *P. chrysogenum* (Díez et al., 1987), and *U. maydis* (Kronstad et al., 1989).

2) Mutants defective in nitrate reductase. Mutants in the nitrate reductase structural gene of filamentous fungi [*niaD*$^+$ in *Aspergillus* (Cove, 1976a, 1979); *nit-3*$^+$ in *Neurospora* (Tomsett and Garrett, 1980)] can be isolated readily by selection for chlorate resistance. Chlorate toxicity arises presumably from its mimicking nitrate's effect on the control of nitrogen catabolism (Cove, 1979). Although mutation in several genes could result in chlorate resistance in filamentous fungi (Cove, 1976b), the *niaD/nit-3* mutants could be easily distinguished from other chlorate resistant mutants by a series of simple phenotypic tests (Cove, 1979; Birkett and Rowlands, 1981; Correll et al., 1987; Klittich and Leslie, 1988; Newton and Caten, 1988). Nitrate reductase genes have recently been cloned from *A. nidulans* (Malardier et al., 1989), *N. crassa* (Fu and Marzluf, 1987), *A. oryzae* (Unkles et al., 1989a) as well as *A. niger* (Unkles et al., 1989b), which should facilitate usage of these genes as selectable markers in transformation of other filamentous fungi. Whitehead et al. (1989) has successfully transformed a *niaD* mutant of *Penicillium* with the cloned *niaD*$^+$ genes from *A. nidulans* and *A. niger*. A *niaD* mutant of *F. oxysporum* has also been transformed successfully with the cloned *niaD*$^+$ gene of *A. nidulans*

(Malardier et al., 1989). Furthermore, Unkles et al. (1989a) has successfully expressed the *A. oryzae niaD*$^+$ gene in *A. nidulans*, *A. niger* and *P. chrysogenum*. The versatility of this transformation system was further demonstrated by Daboussi et al. (1989) who transformed seven filamentous fungi with the *A. nidulans niaD*$^+$ gene (Table 5). Thus, the nitrate system can potentially be used as a universal selection system for transformation of nitrate-assimilating fungi.

In addition to 5-FOA and chlorate, other toxic metabolic analogs are available for selection of mutations in specific nutritional genes, particularly those associated with amino acid metabolism. For example, in *S. cerevisiae*, α-amino-adipate can be used to select mutations at the *LYS2*$^+$ and *LYS5*$^+$ loci (Chattoo et al., 1979) and methyl mercury selects for *met2* and *met15* mutations (Singh and Sherman, 1974, 1975). In *E. coli* (Zurawski et al., 1978) and plants (Last and Fink, 1988), tryptophan-requiring mutants could be isolated by selecting for resistance to 5-methylanthranilic acid. Selection of the corresponding mutants in filamentous fungi with these chemicals may also be feasible.

b. Dominant selectable markers

Dominant selectable markers (e.g., those encoding resistance to antibiotics) are routinely used in transformation of prokaryotic cells. Any fungal species should be transformable with this type of selectable markers as long as it is sensitive to the applied selective pressure, and that the cloned gene encoding the resistance phenotype can be expressed in the recipient under selection. Therefore, dominant selec-

tion of transformants offers the distinct advantage of not requiring the presence of a particular mutation in the recipient. A number of mutations conferring drug resistance have been proven to be suitable for transformation in a variety of filamentous fungi. Some of these dominant resistance markers are also listed in Table 5 (Fincham, 1989).

1) Benomyl resistance. Most mutations conferring benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate], a fungicide) resistance in fungi are due to an alternation in the β-tubulin structural gene which prevents binding of benomyl to the β-tubulin molecules (Davidse, 1986; Morris, 1986). A cloned β-tubulin gene from a benomyl-resistant mutant, therefore, should be usable as a dominant selectable marker in transformation experiments. A mutated β-tubulin gene has been cloned from *N. crassa* (Orbach et al., 1986) and successfully used as a dominant selectable marker in transforming this fungi. Subsequently several fungal species have also been transformed successfully using this marker (Table 5). Fungal species in which a transformation system is being developed using a homologous β-tubulin gene as a dominant selectable marker include *A. flavus* (Seip et al., 1988, 1989) and *Erysiphe graminis* (Sherwood and Sommerville, 1988).

2) Hygromycin B resistance. The aminocyclitol antibiotic hygromycin B binds to 70S or 80S ribosomes, inhibiting protein synthesis and growth of prokaryotic or eukaryotic organisms. Hygromycin B is produced by the prokaryote *Streptomyces hygroscopicus*, which protects its own ribosome formation by synthesizing the enzyme hygromycin B phosphotransferase. This enzyme phosphorylates the drug

making it inactive in the host (Pardo et al., 1985). Genes (*hygB* or *hph*) encoding hygromycin B phosphotransferase have been cloned from both *S. hygroscopius* (Malpartida et al., 1983) and *E. coli* (Rao et al., 1983; Kaster et al., 1983). Resistance to hygromycin B has been used as the basis of transformation systems for a number of eukaryotes, including mammalian cells (Santerre et al., 1984), plant cells (Waldron et al., 1985), and *S. cerevisiae* (Kaster et al., 1984). In filamentous fungi, the *hygB* gene is the most widely utilized selectable marker in transformation of genetically undeveloped strains which are sensitive to this drug (Table 5). The list will most likely be extended as the hygromycin B resistance marker has been tried on more and more fungal species. It is important to note that in all these cases, a eukaryotic promoter has been required to obtain adequate levels of expression of the prokaryotic *hygB* or *hph* genes.

3) Oligomycin resistance. Oligomycin is a hydrophobic antibiotic which inhibits the subunit 9 oligomer in the membrane-bound (Fo) portion of the mitochondrial ATP synthase complex (Enns and Criddle, 1977). Mutants resistant to this antibiotic can be readily obtained. In *A. nidulans* nuclear and extranuclear mutations have been isolated which confer oligomycin resistance (Rowlands and Turner, 1973). Despite a variety of mutant phenotypes, all nuclear resistance mutants mapped to a single locus (*oliC*) (Rowlands and Turner, 1977) which encoded an altered allele of the subunit 9 structural gene (Sebald and Hoppe, 1981). The nuclear resistance mutants showed incomplete dominance in heterozygous diploids and were hypersensitive to triethyltin. The semi-dominant nature of the *oliC* gene presumably resulted from a mixture of resistant and sensitive subunits in the subunit 9 oligomer. The

extranuclear mutation possibly resides in the mitochondrial gene for subunit 6 (Sebald and Hoppe, 1981).

A cloned oligomycin-resistance allele (*oliC31*) of *A. nidulans* was shown to be useful as a semi-dominant selectable marker for transformation of this fungus (Ward et al., 1986). However, the *A. nidulans oliC31* gene was not able to act as a marker in *A. niger* (Ward et al., 1988). Oligomycin-resistance alleles isolated from resistant mutants of *A. niger* (Ward et al., 1988) and *Penicillium* (Bull et al., 1988) have also been used successfully in transformation of these fungi. Particular features of this system include the fact that homologous integration of the plasmid at the *oliC* locus can be phenotypically distinguished from non-homologous integration. Transformants having a plasmid integrated at the *oliC* locus are fully oligomycin resistant or initially semi-resistant but frequently give rise to fully resistant sectors on oligomycin media. Transformants having a plasmid integrated elsewhere in the genome show stable semiresistant phenotype (Ward et al., 1986, 1988). In addition, triethyltin could be used for selection against the *oliC* allele which may find use in gene replacement experiments.

4) Kanamycin resistance. The gene encoding the aminoglycoside 3'-phosphotransferase is responsible for resistance to antibiotics neomycin, kanamycin and its analog G-418. The enzyme phosphorylates these antibiotics and renders them inactive. The kanamycin-resistance gene, derived from the bacterial transposon Tn5 (Jorgensen et al., 1979) or Tn903 (Oka et al., 1981), has been successfully expressed in a slime mold (Hirth et al., 1982), in yeasts (Jiménez and Davies, 1980; Webster and Dickson, 1983; Das et al., 1984; Sakai et al., 1984; Sakai and

Yamamoto, 1986) and in higher eukaryotes (Colbére-Garapin et al., 1981). Filamentous fungi which have been successfully transformed to G-418 resistance are listed in Table 5. In a few cases, the kanamycin-resistance gene could be driven by its native bacterial promoter. However, in the majority of cases, expression of this resistance gene has been placed under control of the promoter from the recipient fungus itself or from the SV 40 virus.

5) Phleomycin resistance. Phleomycin belongs to the metallo-glycopeptide group of antibiotics of the bleomycin family (Berdy, 1980). Phleomycin and bleomycin are very closely related classes of compounds produced by different strains of *Streptomyces verticillus* (Umezawa, 1974). Each class of these water-soluble antibiotics is composed of a mixture of several components differing in the structure of the basic group (Grigg et al., 1985). At low concentrations the phleomycins are effective in killing prokaryotic or eukaryotic organisms. Phleomycin and bleomycin cause scission of DNA *in vivo* and *in vitro* (Kross et al., 1982) preferentially at inverted repeat sequences in single stranded DNA (Ueda et al., 1985) and at various unmethylated sites in double stranded DNA (Hertzberg et al., 1985). This reaction is mediated by oxygen in the presence of iron salts (Pratviel et al., 1986). Two bleomycin resistance genes (*ble*) have been characterized from Tn5 (Collis and Hall, 1985) and from *Staphylococcus aureus* (Semon et al., 1987). Driven by the promoter from the *S. cerevisiae CYC1*$^+$ (iso-1 cytochrome C) gene, the *ble* gene from Tn5 has been used as a dominant selectable marker in transformation of this fungi (Gatignol et al., 1987). Filamentous fungi which have been transformed by the *ble*

gene into phleomycin resistance are listed in Table 5. In each of these cases, expression of the *ble* gene was driven by a fungal promoter.

6) Utilization of acetamide. In filamentous fungi, acetamidase (encoded by the *amdS*$^+$ gene) converts acetamide into acetate and ammonium, thereby allowing growth on acetamide as a sole nitrogen or carbon source (Hynes and Davis, 1986). The *amdS*$^+$ gene has been cloned from *A. nidulans* (Hynes et al., 1983) and subsequently used in development of a transformation system for this fungus (Tilburn et al., 1983). This cloned gene has been proven a valuable heterologous selectable marker for the transformation of several commercially important fungal species (Table 5). Selection depends on poor utilization of acetamide as a sole nitrogen source by the recipient strain. It therefore has the potential for use with any fungal species which can be grown on nitrogen-limiting defined medium and which has low levels of acetamidase activity. Moreover, in fungal species which are capable of utilizing acetamide, mutant strains lacking acetamidase activity can be isolated by treatment with fluoro-acetamide, an amide analogue (Hynes, 1979). This compound is converted to the toxic fluoro-acetate by the acetamidase. Mutations preventing or reducing *amdS*$^+$ expression cause resistance to the analogue. An acetamide uptake system has not been found in organisms studied to date. Therefore, selection for mutations affecting utilization of acetamide will most likely isolate mutants altered in the expression of the *amdS*$^+$ structural gene (Hynes and Davis, 1986).

7) Sulfonamide. Sulfonamides are structural analogues of para-aminobenzoic acid. They act as competitive growth inhibitors by reducing the amount of synthesis of

functional dihydrofolate by dihydropteroate synthetase (DHPS). Recently, a sulfonamide resistance gene encoding DHPS has been used as a dominant selectable marker in transformation of *P. chrysogenum* (Carramolino et al., 1989). Expression of the sulfonamide resistance marker in this fungus was made possible by replacing the native bacterial promoter with the *trpC*$^+$ promoter from *P. chrysogenum.*

8) Others. A gene conferring resistance to the herbicide glyphosate [N-(phosphonomethyl)-glycine] has also been used as a dominant selectable marker in transformation of yeast (Kunze et al., 1989). Glyphosate inhibits EPSP synthase, an enzyme which catalyzes the conversion of shikimate-3-phosphate into 5-enol-shikimate-3-phosphate. The gene encoding EPSP synthase has been cloned (as part of the pentafunctional *arom*$^+$ polypeptide) from *S. cerevisiae* (Larimer et al., 1983) and from *E. coli (aroA*$^+$) (Duncan and Coggins, 1984). Application of these markers in the selection for transformants of filamentous fungi remains to be explored.

c. Visual screening of transformants

Expression of transforming genes can be directly visualized in certain cases on plates with or without a visual indicator.

1) Reporter genes. Vectors containing a fusion of the *E. coli lacZ*$^+$ (β-galactosidase) gene inserted in frame into the coding region of a fungal structural gene allow visual detection of the transformants. Fungal colonies transformed with this construction turn blue on media containing the chromogenic β-galactoside derivative

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). In filamentous fungi, structural genes of *A. nidulans* are commonly used for construction of such translational fusions. These include *trpC*$^+$ (van Gorcom et al., 1985, 1986; Wernars et al., 1987), *gpd*$^+$ (van Gorcom et al., 1986), and *amdS*$^+$ (Davis et al., 1988). In addition to *A. nidulans*, similar gene fusions have also been shown to function in *A. niger* (van Gorcom et al., 1986; Davis et al., 1988) and *P. chrysogenum* (Kolar et al., 1988). The level of expression of the *lacZ*$^+$ gene under control of the *gpd*$^+$ gene promoter was approximately tenfold higher than that under control of the *trpC*$^+$ promoter (van Gorcom et al., 1986).

The *E. coli uidA*$^+$ gene, encoding β-glucuronidase, has recently been developed (Roberts et al., 1989) as an alternative reporter gene to the *lacZ*$^+$. A chimeric β-glucuronidase gene was created by ligating the *A. nidulans gpd*$^+$ gene (encoding glyceraldehyde 3-phosphate dehydrogenase) promoter to the coding sequence of the *E. coli uidA*$^+$ gene. Cotransformation of this vector into *A. nidulans*, *A. niger* and a tomato pathogen *Fulvia fulva* (syn. *Cladosporium fulvum*) resulted in the expression of β-glucuronidase. Transformants turned blue on plates containing the enzyme substrate X-gluc (5-bromo-4-chloro-3-indolyl glucuronide). *A. oryzae* was able to express the *E. coli uidA*$^+$ gene when cotransformed with the homologous *niaD*$^+$ gene (Unkles et al., 1989a). This system is suitable for those fungi (e.g., wood rot fungi and some plant pathogens) in which a gene expression system based on the *lacZ*$^+$ gene is not feasible (Roberts et al., 1989). These fungi normally produce abundant endogenous β-galactosidase even in the presence of glucose as the carbon source to repress production of this enzyme. In order to apply this system, the endogenous β-glucuronidase activity in these fungi should not be detectable.

2) Spore color. Genes responsible for the formation of fungal spore color can be used as visual screenable markers. In *Aspergillus*, the *yA*$^+$ gene encodes a *p*-diphenol oxidase, or laccase, which is needed to convert the yellow pigment intermediate to the mature green pigment of the conidia (Clutterbuck, 1972). *yA*$^+$ is one of only a few genes selectively activated during asexual reproduction in *A. nidulans* that has a well defined physiological function in conidiophore development or spore differentiation (Glutterbuck, 1977; Timberlake, 1987, Timberlake and Marshall, 1988). A mutant defective in the *yA*$^+$ locus produced yellow spores, and when successfully transformed with the wild-type *yA*$^+$ gene, was capable of producing green spores (Yelton et al., 1985; O'Hara and Timberlake, 1989). Thus, in this particular case the transformants could be easily identified by the change in spore color.

3) Pigment production. The imperfect filamentous fungus *Scytalidium flavobrunneum* produces a potent antifungal metabolite, 15-aza-sterol, and a reddish brown pigment at the end of the exponential phase of growth (Rodriguez and Parks, 1980). A plasmid (pSFB-1) was found (Caprioglio and Parks, 1988) in the wild-type strain which conferred the ability to synthesize both the pigment and the aza-sterol simultaneously when transformed into a mutant strain lacking this plasmid (Caprioglio and Parks, 1989). In this case, successful transformants were identified visually by production of the reddish brown pigment that is characteristic of the plasmid-containing wild type.

4) Morphological changes. Changes in size, shape and other morphological features resulting from genetic transformation represent another type of visual selection. For example, in the ascomycete *P. anserina*, senescence (vegetative death) through premature strain aging is under nucleo-cytoplasmic control and is inducible in juvenile mycelia by a movable, circular mitochondrial plasmid DNA (plDNA or α senDNA), which originates from an intron of the subunit 1 gene for the mitochondrial cytochrome oxidase and is highly amplified in aging mycelia (Kück, 1989). Juvenile protoplasts could be transformed into senescence by using purified plDNA (Tudzynski and Esser, 1980). Unequivocal selection of aging transformants was made possible by using a long-living mutant for which spontaneous occurrence of aging was not observed (Tudzynski et al., 1982).

2. Cotransformation

Cotransformation is a technique in which a transforming gene that cannot be directly selected is assimilated along with a more readily selectable marker. It appears that when recipient cells are simultaneously exposed to different transforming DNAs, the most competent cells tend to take up more than one type of DNA molecules (Fincham, 1989).

The efficiency of cotransformation depends somewhat on the fungal species, vectors and selectable markers used in transformation. In the majority of cases, it has been proven to be a high-efficiency process in filamentous fungi. For example, *A. niger* was transformed with two plasmids containing, respectively, the *amdS*$^+$ and the *aciA*$^+$ genes of *A. nidulans*. The seven transformants selected for acetamide

utilization also contained *aciA*$^+$ sequences derived from the unselected plasmid (Kelly and Hynes, 1985). In *A. nidulans*, Wernars et al. (1987) has shown that approximately 85% of the AmdS$^+$ transformants, obtained with the cloned *amdS*$^+$ gene and the *trpC-lacZ* hybird gene on separate vectors, exhibited expression of a functional β-galactosidase fusion protein. This percentage of β-galactosidase-expressing transformants was as high as that obtained with both genes on one composite vector. About 80% and 60% of the hygromycin B resistant transformants of *A. niger* and *A. nidulans*, respectively, were cotransformed with the second marker (*amdS*$^+$ or *pyrG*$^+$) (Punt et al., 1987). When *P. chrysogenum* was cotransformed with *amdS*$^+$ and the *gpd-lacZ* hybrid gene, 90% of the AmdS$^+$ transformants formed blue colonies when transferred to XGal plates (Kolar et al., 1988). In *Trichoderma*, 86% of the Arg$^+$ transformants were also *amdS*$^+$ when cotransformed with *argB*$^+$ and *amdS*$^+$ of *A. nidulans* (Penttilä et al., 1987). Up to 47.5% of the Trp$^+$ transformants were also *acu*$^+$ when a double mutant of *Coprinus cinereus* was transformed with a mixture of two plasmids carrying, respectively, *trp-1*$^+$ and the putative *acu-7*$^+$ gene (Mellon et al., 1987). Cotransformation of *A. nidulans*, *A. niger* and *F. fulva* (Roberts et al., 1989) using selected and unselected DNAs resulted in efficiencies varying from 20% with a phleomycin vector to 44% with the β-glucuronidase vector. When transformed with a mixture of plasmids carrying the hygromycin B resistance marker and the *A. nidulans gpd-E. coli lacZ* hybrid gene, 10 of the 13 hygromycin B-resistant transformants of *A. ficuum* produced a blue color in media containing X-Gal (Mullaney et al., 1988). In all these cases, approximately equal concentrations of the cotransforming DNAs were used.

There are also cases in which cotransformation is less efficient. For example, when *A. nidulans* was cotransformed with a mixture of *pyr-4*$^+$ (carried on a plasmid) and *oliC31* (carried on a lambda), selection for *pyr-4*$^+$ resulted in *oliC31* cotransformation at a frequency of only 4% (Turner and Ballance, 1985). A *pyrG* mutant of *A. niger* was transformed with a 1:1 mixture of plasmids containing the *A. niger pyrG*$^+$ and the *A. nidulans gpd-E. coli lacZ* fusion. About 10% of the Pyr$^+$ transformants showed a blue color on X-gal medium (van Hartingsveldt et al., 1987). Cotransformation of an *A. oryzae niaD* mutant with the native *pyrG*$^+$, the *E. coli uidA*$^+$, as well as a mutant allele of the *N. crassa tub-2*$^+$ gene (conferring benomyl resistance) resulted in cotransformation frequencies of only 4, 9, and 6%, respectively (Unkles et al., 1989). It appears that the maximum cotransformation frequency which can be achieved depends on the nature of the cotransforming DNA and the order of selection for marker genes. For example, in one cotransformation experiment conducted by Wernars et al. (1987), an *amdS*, *trpC* double mutant of *A. nidulans* was transformed with a mixture of both plasmids containing the corresponding wild-type genes, and either AmdS$^+$ or Trp$^+$ transformants were selected. When the AmdS$^+$ transformants were selected first, 95% of them were also *trpC*$^+$. On the other hand, if Trp$^+$ transformants were selected first, only 50-60% of the colonies also possessed the AmdS$^+$ phenotypes.

Cotransformation is a useful technique for testing expression of unselected DNA sequences. It avoids the need to make technically difficult and often complex plasmid constructs containing more than one gene. Genes encoding the isocitrate lyase of *A. nidulans* (*acuD*$^+$) (Ballance and Turner, 1986) and *C. cinereus* (*acu-7*$^+$) (Mellon et al., 1987) as well as acetate utilization genes of *N. crassa* (Thomas et

al., 1988) and *A. nidulans* (Sandeman and Hynes, 1989; Katz and Hynes, 1989c) have been cloned and tested for their functions by this technique. A test for the heterologous expression of the *C. cinereus acu-7*$^{+}$ gene in an *acuD* mutant of *A. nidulans* was also made possible by cotransformation (Hynes, 1989).

It is important to note that the unselected gene could be carried in a lambda vector, although the cotransformation was less efficient in this case (Turner and Ballance, 1985; Mellon et al., 1987). Thus, genes carried on lambda or cosmid vectors could be tested directly for function without the need to subclone them (Timberlake et al., 1985).

C. Fates of Transforming DNA

Without a fungal replication origin, transforming DNA is frequently integrated into the host chromosome. Alternatively, transforming DNA can replicate autonomously in the recipient species.

1. Integration into chromosomes

The data presently available (Fincham, 1989; Timberlake and Marshall, 1989; Rambosek and Leach, 1987; Hynes, 1986) clearly indicate that incorporation of the transforming DNA into the genome of the recipient cells is the most frequent events in transformation of filamentous fungi.

a. Modes of integration. Three types of integration (Figure 7), originally proposed by Hinnen et al. (1978) for transformation of *S. cerevisiae*, also apply to filamentous fungi. The three types of integration are thought to differ in the nature of the recombination event(s) leading to integration. The frequency with which the various types of integration events occur varies with the plasmid and the recipient strain or species used.

1) Type I (Homologous integration). Transformants of this type result from a single cross-over between cloned and chromosomal fungal sequences resulting in the integration of vector sequences, which are flanked by the cloned (transforming) and the chromosomal fungal sequences. Homologous integration is a common event in many fungal transformation systems (Case et al., 1979; Tilburn et al., 1983; Yelton et al., 1984, Upshall, 1986). Several copies of transforming DNA have been detected in transformants in *A. nidulans* (Kelly and Hynes, 1985).

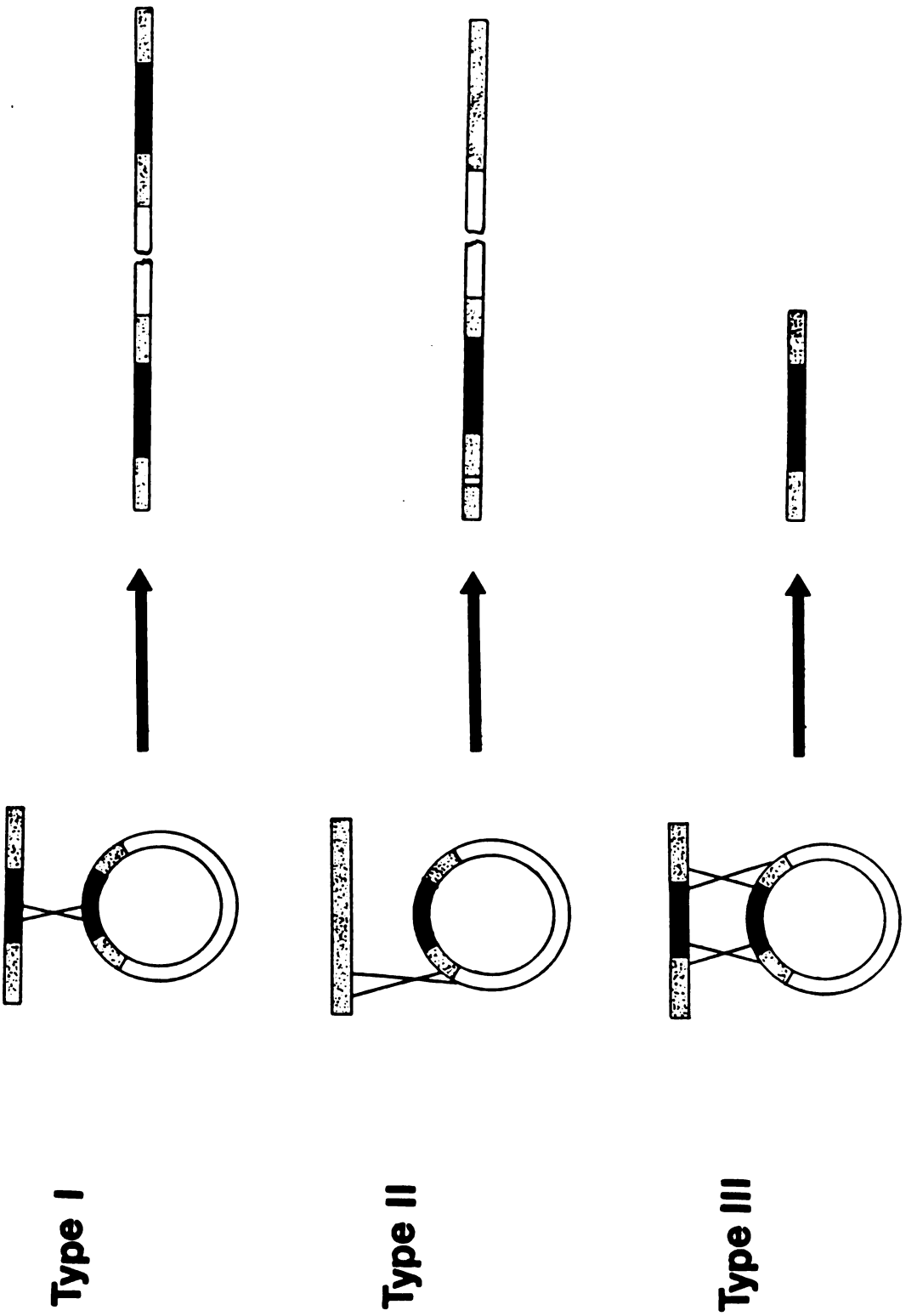
2) Type II (Nonhomologous integration). Transformants of this type arise from integration at sites other than the resident chromosomal locus. It occurs frequently in heterologous transformation systems where there is no detectable homology between introduced sequences and the recipient genome. For example, the *N. crassa* *pyr-4*$^{+}$ gene has low homology with *A. nidulans* genome, however, integrative transformants were readily obtained (Ballance et al., 1983; Ballance and Turner, 1985). Integration at nonhomologous sites occurs at a reasonable frequency even where a homologous sequence exists (Case et al., 1979; Tilburn et al., 1983; Yelton et al., 1984).

3) Type III (Gene conversion or replacement). Transformants of this type are attributed to either gene conversion or a double cross-over event in which the cloned fungal sequences replace their chromosomal counterparts with no integration of the vector sequences (Case et al., 1979; Yelton et al., 1984). Apparent gene conversion events have been observed between *oliC*^{R} and *oliC* genes located in different parts of the *A. nidulans* genome (Ward et al., 1986).

b. Analysis of integration events. Traditional genetic linkage analysis as well as modern molecular probing techniques are usually employed to distinguish among the three integration events described above. Whether or not the integrated transforming DNA is linked to the homologous chromosomal locus can be determined by analyzing progenies of a cross between the transformant and the wild type. Absence or extremely low frequency of the untransformed phenotype among the products of the cross indicates close linkage of the integrated DNA to the resident locus. An unlinked transformant is expected to yield 25% of progeny with the untransformed phenotype from such a cross (Case et al., 1979; Kinsey and Rambosek, 1984; Brygoo and Debuchy, 1985; Dhawale and Marzluf, 1985; Wernars et al., 1985; Picard et al., 1987; Kim and Marzluf, 1988).

More conclusive results can be obtained by Southern hybridization analysis of restriction fragments of genomic DNA from transformants. Molecular analysis of transformants has led to the identification of all three types of integration events in virtually all filamentous fungi investigated (Kim and Marzluf, 1988; Grant et al., 1984; de Graaff et al., 1988; Faugeron et al., 1989; Tilburn et al., 1983; Binninger

Figure 7. Three patterns of integration of transforming DNA into the fungal genome (adapted from Peberdy, 1989). **Type I**: integration at a homologous locus by a single recombination event. **Type II**: integration at random sites of weaker homology by a single recombination event. **Type III**: integration at a homologous locus involving a double recombination resulting in a gene conversion. DNA sequences: solid bars, fungal genes used for selection; striped bars, flanking regions of fungal DNA; open bars, bacterial plasmid DNA.


Type I
Type II
Type III

et al., 1987; Mellon et al., 1987; Alic et al., 1989; Wang et al., 1989; Bull et al., 1988; Van Hartingsveldt et al., 1987). However, the distribution of these three events varies from species to species. There is not sufficient data to accurately estimate relative frequencies of the three types of integration events in filamentous fungi. The mechanism of integration remains virtually unknown (Razanamparany and Bégueret, 1988; Fincham, 1989).

There are several examples of integration of transforming DNA sequences in tandemly repeated copies in filamentous fungi (Yelton et al., 1984; Wernars et al., 1985, 1987; Goyon and Faugeron, 1989).

c. Stability of integrated sequences. In general, integrated transforming DNA sequences are mitotically stable (Boylan et al., 1986). This high degree of mitotic stability is most desirable for genetic engineering of industrial strains. In contrast, integrated DNA sequences are often meiotically unstable (Tilburn et al., 1983; Yelton et al., 1985). In *N. crassa* (Selker et al., 1987) and *A. immersus* (Goyon and Faugeron, 1989; Faugeron et al., 1989), integrated DNA sequences are often eliminated at a high frequency during the sexual phase by a process called repeat-induced point mutation (RIP) (see below).

2. Autonomous replication

Transformation vectors capable of autonomous replication have been reported in several filamentous fungi including *Mucor* (van Heeswijck and Roncero, 1984; van Heeswijck, 1986), *Phycomyces* (Revuelta and Jayaram, 1986; Suárez and Eslava,

1988), *Absidia* (Wöstemeyer et al., 1987), *Podospora* (Tudzynski et al., 1980; Stahl et al., 1982), *Ustilago* (Tsukuda et al., 1988) and *Neurospora* (Stohl and Lambowitz, 1983; Stohl et al., 1984; Grant et al., 1984). There is also limited evidence to suggest their existence in *A. nidulans* (Johnstone, 1985), *A. niger* (Rambosek and Leach, 1987) and the koji mold *A. oryzae* (Iimura et al., 1987).

a. Features of autonomously replicating plasmids. In general, autonomously replicating plasmids (ARPs) can be distinguished from integrating vectors by the following criteria (Rambosek and Leach, 1987): (1) ARPs normally give a high frequency of transformation; (2) Transformants containing ARP are mitotically and meiotically unstable under nonselective growth conditions; (3) DNA isolated from transformants with ARP readily transforms *E. coli*. However, genomic DNA from integrated transformants doesn't; (4) Southern analysis of undigested DNA from transformants with ARPs reveals the presence of monomer-sized ARPs, while the integrated plasmid DNA comigrates with undigested chromosomal DNA in a similar analysis.

b. Origins of replication. In filamentous fungi, the origin that allows autonomous DNA replication is generally derived from mitochondrial plasmids or chromosomal sequences capable of autonomous replication in yeast cells.

1) Mitochondrial plasmids. Although nuclear plasmids are not known in filamentous fungi, several mitochondrial plasmids have been discovered in various species (Tudzynski and Esser, 1985; Garber et al., 1986).

It is interesting to note that a restriction DNA fragment containing the terminal inverted repeat of a linear plasmid isolated from the mitochondria of *N. haematococca* (anamorph *F. solani*) (Samac and Leong, 1988) conferred autonomous replication when incorporated into an integrative transformation vector of *U. maydis* (Samac and Leong, 1989). Whether this plasmid DNA fragment will also support autonomous replication of vectors in other fungi remains to be investigated.

2) Autonomous replication sequences in filamentous fungi. Autonomous replication sequences (ARSs) refer to DNA sequences isolated from other organisms that promote autonomous replication in yeast. They often do not represent authentic origins of DNA replication in the homologous system. ARSs were first identified as sequences linked to genetic markers that transformed yeast cells at high frequencies (Hsiao and Carbon, 1979; Struhl et al., 1979). Plasmid DNA lacking an ARS can transform yeast cells only by rare integrative recombination into the cellular genome (Hinnen et al., 1978). In contrast, the transformation frequency may be 10^3 to 10^4 times higher when the plasmid carrys an ARS. This differential transformation frequency has been useful for defining ARS sequences operationally (Stinchcomb et al., 1980). Putative ARSs have been isolated from a variety of filamentous fungi, including *U. maydis* (Banks, 1983b), *C. acremonium* (Skatrud and Queener, 1984), *N. crassa* (Suzci and Radford, 1983; Buxton and Radford, 1984), *Phycomyces* (Revuelta and Jayaram, 1986), *P. anserina* (Lazdins and Cummings, 1982), and *C. heterostrophus* (Yoder and Turgeon, 1985).

ARS sequences exhibit only a limited function across species lines of filamentous fungi. For example, the *A. nidulans ansl* sequence identified by Ballance

and Turner (1985) is capable of increasing the transformation frequencies of vectors containing the *N. crassa pyr-4*$^+$, and the *A. nidulans argB*$^+$ as well as *trpC*$^+$ genes (Cullen et al., 1987). The efficiency of *P. chrysogenum* transformation was also improved by employing *ans1*-bearing plasmids (Cantoral et al., 1987). However, vector constructs carrying the *ans1* sequence and the *A. niger niaD*$^+$ gene did not show increased transformation frequencies (Whitehead et al., 1989). Genetic mapping and DNA sequencing data (Cullen et al., 1987) suggested that *ans1* may be related to the centromere sequence. Whether this is the basis of the effect of *ans1* on the efficiency of transformation needs further investigation.

An *A. nidulans* genomic DNA sequence of unknown biological function (designated *unt*) has been cloned by Johnstone (1985). Like *ans1*, *unt* is reiterated several times in the *Aspergillus* genome, and is capable of enhancing the frequency of transformation, but its mechanism of activity appears to be different. *Aspergillus*, transformed with a vector containing *unt*, is very unstable and usually contains considerable amounts of free circular plasmid molecules as well as multiple integrated copies (Johnstone and Clutterbuck, 1986). The nature of this sequence remains unknown to date.

Despite failures in the search for an ARP from the *Ascomycetes*, evidence for ARP was obtained from the *Zygomycetes* and *Basidiomycetes*. In the *Zygomycetes* such as *Mucor* (van Heeswijck and Roncero, 1984; van Heeswijck, 1986), *Phycomyces* (Suárez and Eslava, 1988; Revuelta and Jayaram, 1986), and *Absidia* (Wöstemeyer et al., 1987), Southern analysis has shown the presence of plasmids capable of replicating indefinitely in the transformants under selective conditions.

An ARS sequence was presumably responsible for the autonomous replication in the cases of *Phycomyces* and *Mucor*.

Recently, a more detailed study on ARS elements in a basidiomycete, *U. maydis*, was reported by Tsukuda et al. (1988). A selection scheme similar to that used in yeast which exploited the high transformation frequency of ARP was adopted to isolate DNA segments from *U. maydis* capable of autonomous replication. DNA fragments 2-10 kb in size were cloned into an integrative transforming vector (pHL1) containing the hygromycin B resistance marker fused to heat shock gene promoter of *Ustilago*. DNA enriched in plasmid molecules was extracted from the pooled hygromycin-resistant transformants and used to transform competent *E. coli* cells. A high percentage of plasmids recovered from *E. coli* transformants was found to contain *U. maydis* DNA inserts which increased the frequency of *U. maydis* transformation several-thousandfold. Evidence of transmission of these ARS-containing plasmids as extrachromosomal elements through replication was obtained by monitoring the sensitivity of methylated plasmid DNA to restriction endonuclease digestion after transformation. ARS-containing plasmid prepared from the *dam*$^+$ *E. coli* strain DH5, which methylates adenine in the sequence GATC, was cut by *Dpn*I but not by its isoschizomer *Mbo*I. However, after transformation of *U. maydis* and 20 generations of growth under antibiotic selection, plasmid DNA extracted from the transformants had become resistant to digestion by *Dpn*I but sensitive to *Mbo*I, indicating a loss of methylation. This loss in the pattern of DNA methylation is in agreement with a mode of transmission in which the unmethylated sites arise from new synthesis of plasmid DNA during autonomous replication. These plasmids were maintained at a level of 25 copies per cell but were mitotically

unstable as expected. One 1.7-kb ARS (designated UARS1) could be reduced further into three separate fragments, each of which retained ARS activity. The smallest fragment was 383 base pairs long, which, although not active itself in yeast, contained seven 8-bp direct repeats, two contiguous 30-bp direct repeats, and five 11-bp units in both orientations with sequences similar but not identical to the consensus sequence found to be crucial for ARS activity in *S. cerevisiae*. Whether any of these sequences plays a functional role in conferring autonomous replication activity remains to be determined.

c. ARP and abortive transformants. A common observation in fungal transformation systems is the presence of "abortive" transformants (Buxton and Radford, 1984; Kinnaird et al., 1982), that is, colonies that initially grow on selective media but not after subculturing. One theory suggests that in abortive colonies the transforming DNA has not been stabilized by integration or by autonomous replication and is only transiently expressed. The transformant grows initially, but as the gene product that relieves the selective pressure is diluted out, growth is halted. In support of this theory, Tilburn et al. (1983) found that transforming DNA is present in abortive transformants after cessation of growth and noted that sectors of vigorous growth could develop from abortive colonies. Southern analysis was used to show that these sectors contained transforming DNA that was stabilized by integration into the recipient genome.

In summary, autonomously replicating plasmids have been reported but have not been widely used as molecular cloning vectors in filamentous fungi. Construc-

tion of vectors lacking genomic homology is not useful in preventing integration since transforming plasmids readily integrate at either homologous or heterologous sites regardless of the homology (Tilburn et al., 1983; Russell et al., 1989). The development of mutant strains completely defective in integrative recombination represents a feasible approach. On the other hand, centromeric sequences may be used to develop autonomously replicating vectors with increased mitotic stability during segregation. However, such sequences are not yet available from filamentous fungi. The cloned *S. cerevisiae* centromeres provide an alternative source, but they do not display detectable stabilizing activity in *A. nidulans* (Boylan et al., 1986). Telomeric sequences from *Tetrahymena thermophila* are able to maintain an integrative linear plasmid of *P. anserina* extrachromosomally (Perrot et al., 1987). A telomere DNA has been isolated from *N. crassa* (Schechtman, 1987). Vectors constructed based on this telomeric sequence may allow development of autonomous replicating linear plasmids in filamentous fungi.

D. Recovery of Transforming DNA

Transformation is frequently used to directly clone genes by selection for function. The ability to recover the cloned sequences from the transformants is one of the most desirable features of a transformation system which has led to efforts to isolate the autonomously replicating shuttle vectors as discussed above. However, the frequencies of integrative fungal transformation can be sufficiently high for direct cloning and the integrated transforming DNA can be recovered in several ways.

1. Recovery of plasmids

Although transforming plasmids frequently integrated into the host chromosome, they can be recovered intact by direct transformation of *E. coli* with undigested genomic DNA from transformants (Stohl and Lambowitz, 1983; Ballance and Turner, 1985; Johnstone et al., 1985). It appears that excision of an integrated plasmid can occur, resulting in free plasmid molecules in transformants (Johnstone et al., 1985; Diallinas and Scazzocchio, 1989). Rearrangement or partial deletion of transforming plasmid DNA can occur during the recovery process (Paietta and Marzluf, 1985; Barnes and MacDonald, 1986). However, they often still carry the selectable marker.

Alternatively, an integrated transforming DNA sequence can be recovered by cleaving the transformant genomic DNA with a restriction enzyme that cuts once within the transforming sequence. The resulting DNA fragments are circularized and used to transform *E. coli* (Yelton et al., 1984; Ballance and Turner, 1985). For example, the genes encoding isocitrate lyase (Turner and Ballance, 1985) and orotidine 5'-phosphate decarboxylase (Oakley et al., 1987) have been cloned in this way.

2. Recovery of cosmids

Cosmids are plasmids with bacteriophage lambda *cos* (packaging) sequences. The genomic DNA isolated from transformants carrying cosmid vectors can be sub-

jected to an in vitro lambda packaging system to recover an intact copy of the transforming DNA. This cosmid rescue procedure has been successfully used to clone *yA*$^+$ (encoding laccase) and other developmentally regulated *Aspergillus* genes (Yelton et al., 1985).

3. Sib selection

This strategy is originally developed by Akins and Lambowitz (1985) for *N. crassa*, and represents a very efficient procedure for marker rescue especially when coupled with cosmid genomic banks (Vollmer and Yanofsky, 1986). In this approach, a transforming marker is recovered by successive rounds of transformation of a suitable recipient strain, using pools of DNAs from a genomic library which are progressively reduced in complexity until a single candidate clone is identified. A similar procedure was used to identify plasmid clones containing the *A. nidulans pyrG*$^+$ gene (Oakley et al., 1987), and to identify the mating-type *b1* allele from a cosmid library of *U. maydis* (Kronstad and Leong, 1989).

E. Applications of Transformation

Transformation represents a new approach to altering the biological characteristics of filamentous fungi. It has been used extensively in studying control mechanisms for growth, metabolism, and development as well as for isolation and manipulation of genes of potential application in medicine, industry and agriculture.

1. Cloning genes by complementation

Techniques used for isolating genes from other organisms have been used in filamentous fungi with varying degrees of success. Basic cloning strategies such as differential hybridization (Hynes et al., 1983), hybridization with synthetic DNA probes (Kinnaird et al., 1982), and cDNAs prepared from abundant messenger RNAs (Nunberg et al., 1984) have been employed successfully.

Cloning genes by functional complementation of mutation can be heterologous or homologous in nature. In heterologous systems, appropriate mutant strains of *E. coli*, yeast or other fungal species than the recipient strain are often used as recipients, whereas for homologous systems mutants of filamentous fungi from which the desired wild-type gene will be isolated from is the recipient.

a. Heterologous complementation. Several fungal genes, for example, the *N. crassa qa-2*$^+$ gene (Vapnek et al., 1977) and the *A. nidulans trpC*$^+$ gene (Yelton et al., 1984) have been successfully cloned by complementation of *E. coli* auxotrophic mutants. However, this approach is not generally applicable since regulation and expression signals of fungal genes may not be recognized in *E. coli*. Genes isolated by complementation of *E. coli* mutants are presumed not to have introns in the functional domain.

Yeast cells, in general, are not suitable hosts for heterologous complementation since they can not efficiently process introns of genes from filamentous fungi (Penttilä et al., 1984; McKnight et al., 1985; Woudt et al., 1985; Innis et al., 1985). However, cDNA libraries constructed on a yeast promoter have been successfully

used to isolate several genes from filamentous fungi (McKnight et al., 1985; Kronstad et al., 1989).

The close relatedness of certain fungi to the well-characterized perfect fungus *A. nidulans* has made cloning genes by complementation of the latter feasible, in particular for those genes that are reasonably conserved through evolution. For example, the *A. niger argB*$^+$ gene (encoding ornithine carbamoyl transferase) has been cloned by transforming an *argB* mutant of *A. nidulans* with a genomic DNA library of *A. niger* (Buxton et al., 1987). Alternatively, a gene can be cloned from one species by detecting its expression in a different species. For example, a gene encoding pisatin demethylase from the plant pathogen *N. haematacocca* was cloned by screening its enzymatic activity in *A. nidulans* transformed with DNAs from a cosmid library (Weltring et al., 1988).

b. Homologous complementation. Virtually any gene can be cloned by homologous complementation providing that an appropriate selection protocol and a mutant recipient with a low reversion frequency are available.

The rapid advancement in molecular genetics of *A. nidulans* and *N. crassa* has allowed a number of structural genes to be cloned (Rambosek and Leach, 1987; Schwab, 1988). In addition, a strategy to cloning promoter sequences has been developed in *Cochliobolus* (Turgeon et al., 1987). This technique should be of general applicability for cloning regulatory sequences in other less well characterized fungal species.

2. Gene disruption/replacement

Gene disruption or replacement refers to a procedure whereby an altered cloned gene is used to disrupt or replace its chromosomal counterpart by homologous recombination after transformation. Insertion of plasmids at specific chromosomal sites is important for analysis of mutations generated *in vitro* and for making targeted *in vivo* mutations.

a. Approaches to gene disruption

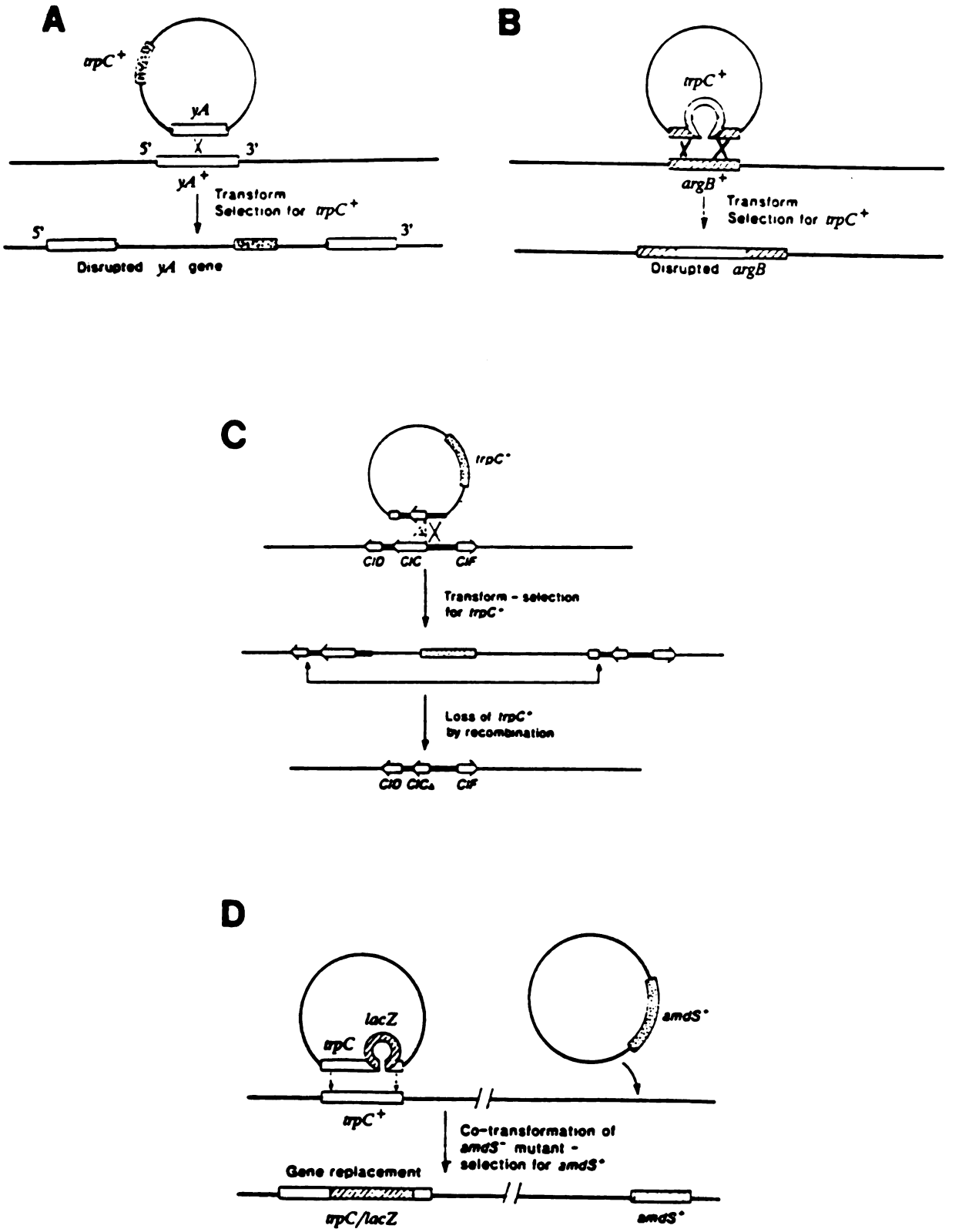
Several transformation techniques are available for selection of plasmid integration events at specific sites (Fincham, 1989; Leong, 1988) (Figure 8).

1) Transformation with a truncated cloned gene. In this approach (Figure 8A) a homologous recombination by a single crossover between the transforming plasmid , which carries an internal fragment of the target gene, and the resident locus of the recipient chromosome results in two incomplete genes, one lacking sequences at the 5' end of the gene and the other lacking sequences at the 3' end. For example, mutants of the *A. nidulans* *yA*$^+$ gene produce yellow instead of the normal green spores (Pontecorvo, 1953). Homologous integration with transforming plasmids carrying a truncated *yA*$^+$ gene results in formation of readily selectable yellow-spored mutants (O'Hara and Timberlake, 1989).

2) One-step gene replacement. In this approach (Figure 8B) a plasmid (linear or circular) containing a selectable marker inserted into (and thus disrupting) the cloned gene of interest is used to transform a mutant defective in the selectable gene. Selection is made for the disrupting marker. Type III integration will result in replacement of the target gene by the nonfunctional disrupted allele. Undesirable transformants arising from type I integration of the disrupting marker into the chromosomal locus can be largely avoided by using a deletion mutant as the recipient. Alternatively, transformants derived from type III integration should contain no vector DNA sequences, and thus can be distinguished from those derived from type I event by Southern analysis of the chromosomal DNA.

Gene disruption seems to be a low-frequency event in *A. nidulans* and *N. crassa* (Paietta and Marzluf, 1985; Miller et al., 1985). However, it may occur at a higher frequency in *U. maydis*. To disrupt the genomic copy of the *U. maydis* *pyr6*$^+$ gene, Kronstad et al. (1989) inserted the hygromycin B resistance marker into a DNA fragment containing the cloned *pyr6*$^+$. The resulting linear construct was used to transform *U. maydis* to hygromycin B resistance. Approximate 70% of the transformants required uracil for growth. Southern blot analysis revealed that allthese transformants resulted from direct gene replacement by homologous recombination. Similar results were also obtained in a gene replacement experiment using a DNA fragment carrying an allele of the mating-type *b* locus of *U. maydis* (Kronstad and Leong, 1989). By inserting the hygromycin B resistance marker into the cloned *leu*$^+$ gene, Fotheringham and Holloman (1989) have shown that the one-step disruption procedure in *U. maydis* could be as effective as in yeast. Among

Figure 8. Examples of gene disruption/replacement (modified from Fincham, 1989). (A) Transformation with a truncated cloned gene. Integration of the transforming plasmid carrying a deleted copy of the *yA*$^+$ gene by homologous recombination splits the chromosomal *yA*$^+$ gene into two nonfunctional pieces. (B) One-step gene disruption (Miller et al., 1985). The transforming plasmid carries a copy of *argB*$^+$ disrupted by the selectable marker *trpC*$^+$. Type III integration of the *trpC*$^+$ selectable marker at the chromosomal *argB*$^+$ locus results in its disruption. (C) Two-step gene replacement. A plasmid containing a partially deleted *spoC1-C*$^+$ gene of the *A. nidulans spoC1*$^+$ gene cluster (thicker lines with open arrows) and the *trpC*$^+$ gene is used to transform a *trpC* mutant (Miller et al., 1985). A Trp$^+$ transformant containing a tandem duplication of the *spoC1-C*$^+$ region is self-fertilized. *TrpC* mutants are obtained which have lost *trpC*$^+$ but retained the partially deleted *spoC1-C*$^+$ gene by intrachromosomal recombination. (D) Gene replacement by cotransformation (Wernars et al., 1987). Two plasmids, one carrying the *A. nidulans trpC/lacZ* fusion gene and one carrying *amdS*$^+$, are used in co-transformation. *LacZ*$^+$ gene activity is detected among a large number of AmdS$^+$ transformants screened.


A
trpC+
yA
5'
3'
yA+
Transform
Selection for trpC+
5'
3'
Disrupted yA gene
B
trpC+
argB+
Transform
Selection for trpC+
Disrupted argB
C
trpC+
ClD ClC ClF
Transform - selection
for trpC+
Loss of trpC+
by recombination
ClD ClC∆ ClF
D
lacZ
trpC
trpC+
amdS+
Co-transformation of
amdS- mutant -
selection for amdS+
Gene replacement
trpC/lacZ
amdS+

hygromycin B resistant transformants, one quarter to one half were simultaneously Leu⁻.

3) Two-step gene replacement. In this approach (Figure 8C) type I integrative transformants are isolated which contain tandem copies of the target gene under investigation and the modified replacement version. Then segregants are identified which have lost sequences associated with the selectable marker through differential double reciprocal recombinations, resulting in restoration of the native gene or its replacement. In general, these two recombination events can be distinguished by Southern hybridization analysis. Alternatively, they can be readily differentiated without resort to Southern analysis in cases where a simple assay is available to detect the replacement gene. For example, a simple color assay for β-galactosidase was used to detect meiotic progenies of *A. nidulans* expressing the *amdS-lacZ* fusion which replaced the resident *amdS*⁺ gene during a two-step gene replacement experiment (Davis et al., 1988).

Selectable markers which can be selected (positively selective) and counter-selected (negatively selective) are extremely useful in gene replacement experiments. For example, in filamentous fungi, mutations resulting in loss of orotidine 5'-phosphate decarboxylase (encoded by *pyrG*⁺, *pyr-4*⁺ or *pyr6*⁺, depending on fungal species) confer resistance to the normally inhibitory analog 5-FOA (Diéz et al., 1987; Dunne and Oakley, 1988; Kronstad et al., 1989). This two-way selection has been used in *A. nidulans* for a two-step gene replacement of a mutant *benA* allele (β-tubulin) with the wild-type allele (Dunne and Oakley, 1988). A benomyl-resistant (*benA*), pyrimidine-auxotrophic (*pyrG*) strain of *A. nidulans* was transformed with a

plasmid carrying the wild-type *benA* allele and the *pyr-4*$^+$ gene of *N. crassa*. The *pyr-4*$^+$ gene complemented *pyrG* but had insufficient homology to direct integration at the *pyrG*$^+$ locus. Consequently, the majority of the primary transformants carried a duplication of *benA* alleles (one wild type and one benomyl resistant) interrupted by plasmid sequences due to integration at the *benA* locus. Such transformants (Pyr$^+$, Ben^i) showed intermediate degree of benomyl resistance and were not able to grow in the presence of high levels of benomyl. The cells from the transformant colony were then incubated on medium containing 5-FOA which killed cells carrying a functional *pyr-4*$^+$ gene and thus selected for cells that had lost the functional *pyr-4*$^+$ by reciprocal recombination. Of the twenty-two such Pyr$^-$ colonies isolated, 7 were benomyl sensitive (Ben^s) and 15 were Ben^r. Southern hybridization of DNA from 3 of the Ben^s, Pyr$^-$ colonies revealed that the plasmid sequences had been lost and only a single copy of *benA* was present in each case, demonstrating the replacement of the resident mutant *benA* allele by the transforming wild-type allele.

Gene replacement can also be achieved in transformants of *A. immersus* by a two-step procedure (Goyon and Faugeron, 1989) similar to that described for *A. nidulans* and other filamentous fungi.

4) Gene replacement by cotransformation. Cotransformation can be used in gene replacement (Figure 8D). For example, about 75% of the *A. nidulans* AmdS$^+$ transformants express β-galactosidase activity when cotransformed with the homologous *amdS*$^+$ and the *trpC-lacZ* hybrid gene. Some of these transformants have *trpC*$^+$ replaced by the *trpC-lacZ* fusion (Wernars et al., 1987).

In summary, direct and indirect gene disruption and replacement have been demonstrated in several filamentous fungi. The efficiency with which these events occur is sufficiently high that the desired transformants can readily be found among the background of nonspecific integrations. These powerful techniques can be used (i) to return an *in vitro*-altered gene to its resident site, and thus create a mutant which could otherwise difficult to obtain (Wernars et al., 1987); (ii) to prove the identity of newly cloned genes (Osmani et al., 1988a; Boylan et al., 1987; Turner et al., 1985), and map their chromosomal location (May et al., 1985); (iii) to demonstrate the physiological functions of cloned genes (Aramayo et al., 1989), and (iv) to analyze regulatory genetic elements (Frederick et al., 1989).

b. The RIP effect: a unique gene disruption

Any cloned *N. crassa* gene can be potentially disrupted by the premeiotic disruption procedure discovered by Selker et al. (1987). They found that crossing a strain containing duplicated DNA sequences generated by transformation with any other strain resulted in heavy methylations and numerous nucleotide sequence changes in both duplicated copies in part of the meiotic products (ascospores). The effect presumably will destroy the function of any gene present within the duplicated region. The phenomenon was initially called the RIP (rearrangement induced premeiotically) effect because it occurs immediately before karyogamy and meiosis but later renamed to "repeat-induced point mutation" because the changes were found usually within the duplicated sequences without rearrangement of their positions in the genome (Fincham et al., 1989; Cambareri et al., 1989). A detailed

characterization of this phenomenon by DNA sequencing and heteroduplex analyses (Cambareri et al., 1989) indicated that the process produces an extremely high frequency of G-C to AT transition point mutations (of the order of 50% of G-C base pairs may be affected). The changes occur principally at sites where adenine is 3' of the changed cytosine. The mechanism and function of the RIP effect remain unclear.

The RIP effect also exists in *Ascobolus* (Goyon and Faugeron, 1989). As pointed out by Fincham (1988, 1989), the RIP effect probably accounts for (1) a reported case of two-copy lethality in *P. anserina* (Debuchy et al., 1988); (2) the fact that many transformants fail to transmit the transformed character through crosses in *A. nidulans* (Tilburn et al., 1983; Yelton et al., 1984), *P. anserina* (Picard et al., 1987; Razanamparany and Bégueret, 1986), as well as in *N. crassa* (Szabo and Schablik, 1982), and (3) the observation of Case (1986) that transforming *qa-2*$^+$ sequences whose activity had been lost during outcrossing appeared to remain present (but nonfunctional) in the genome. The RIP effect may explain the meiotic instability of selectable markers in transformants with closely linked duplicated genes. Unlinked duplicate gene copies of the selectable marker would show less meiotic instability. Furthermore, the RIP effect may play a role in limiting the distribution of the Tad transposon in strains of *Neurospora*. The Tad transposable element was detected as a 7-kb insertion in two independently isolated spontaneous forward mutants of the *N. crassa am*$^+$ gene (Kinsey and Helber, 1989). DNA hybridization analysis (Kinsey, 1989) indicated that Tad was present only in the *Neurospora* strain of origin from Adiopodumé, Ivory Coast; laboratory strains do not contain this element. This has led Kinsey (1989) to suggest that the RIP process

might play a role in protecting *Neurospora* against transposable elements. Tad DNA sequences would become extensively altered and failed to hybridize the cloned Tad probe if the RIP process occurred during repeated crosses.

Not all *Ascomycetes* with a dikaryotic phase in their life history are subject to the RIP effect. For example, duplications created by transformation in *Sordaria macrospora*, an ascomycete closely related to *N. crassa*, are not inactivated during meiosis (Le Chevanton et al., 1989). As the inactivation of duplicated sequences takes place just before meiosis, it was suggested that such inactivation may act in heterothallic species (e.g., *N. crassa*) as a regulatory mechanism associated with the coordinated expression of the two nuclei of different mating type. Such a mechanism would not be required in a homothallic species such as *S. macrospora* in which the two nuclei are most likely derived from the same parental nucleus.

3. Cloning genes by insertional inactivation

As discussed previously, plasmids lacking homology with the host genome frequently integrate at random chromosomal sites in transformation. This feature is similar to a transposable element, and offers a convenient method for cloning genes by selection for loss of function due to insertional inactivation (Timberlake and Marshall, 1989). This approach does not require previous isolation of mutants as does the functional complementation technique.

Using this inactivational cloning technique, Diallinas and Scazzocchio (1989) were able to clone the *A. nidulans uapA*$^+$ gene encoding the uric acid/xanthine permease. Cloning *uapA*$^+$ by direct complementation is laborious because *uapA*

mutants are leaky, due to the presence of other permeases that transport uric acid into the cell (Arst and Scazzocchio, 1975). In their procedure, a yellow-spored strain of *A. nidulans* carrying a complete deletion of the *amdS*$^+$ gene was transformed with a plasmid carrying an intact *amdS*$^+$ gene and bearing no sequences homologous to the genomic DNA of the recipient strain. Selection of the *uapA* mutants resulting from inactivational integration at the *uapA*$^+$ locus was made simple by an observation by Alderson and Scazzocchio (1967): in the presence of the xanthine analog 2-thioxanthine, wild-type strains of *A. nidulans* produce yellow rather than normal green conidia. This effect requires uptake of the analog and its subsequent oxidation to 2-thiouric acid which presumably acts by chelating the copper of laccase (polyphenol oxidase) which is involved in the conversion of yellow to green pigment (Alderson and Scazzocchio, 1967). Mutations at any locus necessary for this process result in resistance to 2-thioxanthine and produce green conidia even in the presence of 2-thioxanthine. Thus, transformants were selected on acetamide as nitrogen source in the presence of 2-thioxanthine. Among approximately 12,000 *A. nidulans* AmdS$^+$ transformants, 34 were 2-thioxanthine-resistant, i.e., showed green conidia. In theory, integration events at nine genes could result in resistance to 2-thioxanthine. However, these possibilities can be easily distinguished by growth and complementation tests (Darlington and Scazzocchio, 1967; Alderson and Scazzocchio, 1967; Scazzocchio and Arst, 1978). Genetic mapping indicated that all 34 green-spored transformants were *uapA* mutants due to integration of the *amdS*$^+$ gene at the *uapA*$^+$ locus. A plasmid containing an intact *amdS*$^+$ gene but an incomplete *uapA*$^+$ gene was recovered by transforming *E. coli* to ampicillin resistance with genomic DNA prepared from transformants. A functional

copy of the *uapA*$^+$ gene was finally isolated from a gene library by using this plasmid as a probe.

4. Gene fusions

The *E. coli* β-galactosidase is easily detected in plates or protein extracts, and therefore its structural gene (*lacZ*$^+$) is frequently used for transcriptional or translational fusions to measure the activity of a regulatory DNA sequence from filamentous fungi (Davis et al., 1988; Hamer and Timberlake, 1987; van den Hondel et al., 1985, 1986; van Gorcom et al., 1985). Gene replacement can be used to target the fused hybrid gene at specific chromosomal sites (see above) to avoid position effects on gene expression (Miller et al., 1987).

Similarly, regulatable promoters can be used to study expression patterns of structural genes. For example, several *A. nidulans* structural genes have been fused to the *A. nidulans alcA*$^+$ promoter to investigate the functions of their products (Osmani et al., 1988b; Adams et al., 1988; Mirabito et al., 1989; Waring et al., 1989). The *alcA*$^+$ gene encodes catabolic alcohol dehydrogenase and the promoter is subject to substrate induction and carbon catabolite repression (Gwynne et al., 1987). Expression of genes fused to this promoter can be manipulated by growth conditions.

5. Titration of regulatory gene products

Expression of the *A. nidulans amdS*$^+$ gene is controlled by the *trans*-acting gene products of *areA*$^+$ and *amdR*$^+$ under different growth conditions (Hynes and Davis, 1986). Transformants with multiple copies of *amdS*$^+$ grow poorly on nitrogen sources due to titration of the *areA*$^+$ and *amdR*$^+$ gene products by binding sites upstream of each of the multiple copies of *amdS*$^+$ (Kelly and Hynes, 1987). The activities regulated by *amdR*$^+$ were depressed upon introduction of multiple copies of an *amdS*$^+$ upstream DNA fragment, but could be restored by transformation with multiple copies of *amdR*$^+$ (Andrianopoulos and Hynes, 1988).

This titration approach has allowed identification and mapping of *cis*-acting regulatory sequences (Hynes et al., 1988) as well as cloning of genes that are controlled by common *trans*-acting factors (Katz and Hynes, 1989a; Richardson et al., 1989).

6. Biotechnology

Filamentous fungi have a long history of use in fermentations for production of a variety of industrial enzymes and pharmaceuticals. In general, these organisms are more versatile hosts for the development of heterologous gene expression systems than other organisms such as *E. coli* and *S. cerevisiae* (van Brunt, 1986; Cullen and Leong, 1986; Upshall, 1986) because many of them have the ability to properly process and secrete high level of heterologous proteins of eukaryotic origin. For example, *A. niger* has been reported to secrete up to 20 grams per liter of the

glycoprotein amyloglucosidase (van Brunt, 1986). An additional advantage is the ability to generate transformants containing multiple copies of integrated plasmid sequences of sufficient mitotic stability.

With the development of molecular methods for manipulating filamentous fungi, the exploitation of their secretion and processing capability to produce heterologous proteins, especially those with therapeutic and commercial value is being actively investigated (Saunders et al., 1989). Results from such attempts are summarized in Table 6. In general, the rapid developments in the molecular biology of *A. nidulans* make this fungus suitable for initial development of a secretion system, from which technology may be transferred to the important industrial species such as *A. niger*, *A. awamori*, and *A. oryzae*. Cotransformation, which is a very efficient procedure in *Aspergillus* (see previous discussion), is usually used to introduce the desired gene into the expression host. Both constitutive (Upshall et al., 1987) and controllable (Gwynne et al., 1987a, 1989; Christensen et al., 1988; Turnbull et al., 1989; Harkki et al., 1989; Cullen et al., 1987) promoter elements have been used in construction of heterologous expression systems, but the latter is most desirable. Being able to control production of the desired protein allows one to direct product synthesis at particular times during the fermentation process. Thus, the secreted products are only exposed briefly to the extracellular environment prior to harvest. For example, the expression of the *A. nidulans alcA*$^+$ is normally repressed by glucose but can be induced in the absence of it (Sealy-Lewis and Lockington, 1984; Lockington et al., 1985). This allows the construction of strains which can be rapidly grown in the presence of glucose to accumulate biomass and which can then be induced to initiate the production (and

Table 6. Filamentous fungi as hosts for heterologous gene expression and product secretion.

Host	Vector	Origins of Control sequences	Product secreted	Conc. secreted[1]	Reference
A. nidulans	pGL2B	*A. niger* glucoamylase	human α_2-interferon	2-3 µg/l	Gwynne et al. (1987b)
A. nidulans	pALCA1S	*A. nidulans alcA*[*2]	human α_2-interferon	1 mg/l	Gwynne et al. (1987b)
A. nidulans	pGL2C	*A. niger* glucoamylase	*Cellulomonas fimi* endoglucanase	n. a.	Gwynne et al. (1987b)
A. nidulans	pALCA1S	*A. nidulans alcA*[*]	*Cellulomonas fimi* endoglucanase	n. a.	Gwynne et al. (1987b)
A. nidulans	pALCA1S	*A. nidulans alcA*[*]	human epidermal growth factor	1-2 mg/l	Gwynne et al. (1989)
A. nidulans	pALCA1S	*A. nidulans alcA*[*]	*A. niger* glucoamylase	1.2 g/l	Gwynne et al. (1989)
A. nidulans	pL_2	*A. nidulans amdS*[*3]	*E. coli* heat labile enterotoxin subunit B	24 µg/g	Turnbull et al. (1989)
A. nidulans	pGRG	*A. niger* glucoamylase	bovine chymosin	23-146 µg/g	Cullen et al. (1987)
A. nidulans	pM159	*A. nidulans tpiA*[*4]	human tissue plasminogen acitvator	100 µg/l	Upshall et al. (1987)
A. nidulans	pMMAN2	*M. miehei* aspartic protease	*M. miehei* aspartic protease	n. a.	Gray et al. (1986)
A. oryzae	pBoel777	*A. oryzae* α-amylase[5] *A. niger* glucoamylase[6]	*Rhizomucor miehei* aspartic protease	0.5-3.3 mg/ml	Christensen et al. (1988)
	pRML-787	*A. oryzae* α-amylase[5] *A. niger* glucoamylase[6]	*R. miehei* lipase	n. a.	Huge-Jensen et al. (1989)
M. circinelloides	pMA67	*M. miehei* aspartic protease	*M. miehei* aspartic protease	1-12 µg/ml	Dickinson et al. (1987)
T. reesei	pAMH	*T. reesei cbh1*[*7]	bovine chymosin	17-40 mg/l	Harkki et al. (1989)

[1]: The units of concentration represent the weight of secreted product per unit volume of fermentation broth (w/v), or per unit weight of fungal mycelia (w/w); n. a.: data not available.

[2]: Encoding alcohol dehydrogenase; [3]: encoding acetamidase; [4]: encoding triose phosphate isomerase; [5] supplies the regulatory sequences at the 5' end. [6]: Supplies the regulatory sequences at the 3' end; [7]: encoding cellobiohydrolase I.

secretion) of the recombinant protein (Gwynne et al., 1987b, 1989). By using a recombinant *A. nidulans* host with multiple integrated copies of the *alcR*$^+$ regulatory gene whose gene product is a positive transcriptional regulatory factor for the *alcA*$^+$ gene (Doy et al., 1985; Gwynne et al., 1987a; Felenbok et al., 1988), Gwynne et al. (1989) was able to increase the secretion efficiency of *A. niger* glucoamylase up to 1.2 g/liter complex medium. Sufficient amounts of the *alcR*$^+$ gene product derived from the multiple copies of the integrated *alcR*$^+$ were presumably able to relieve the titration effect caused by the multiple *cis*-acting regulatory sites of *alcA*$^+$ in the expression cassette.

CHAPTER II

CLONING AND CHARACTERIZATION OF THE *TRPC*$^+$ GENE FROM AN AFLATOXIGENIC STRAIN OF *ASPERGILLUS PARASITICUS*

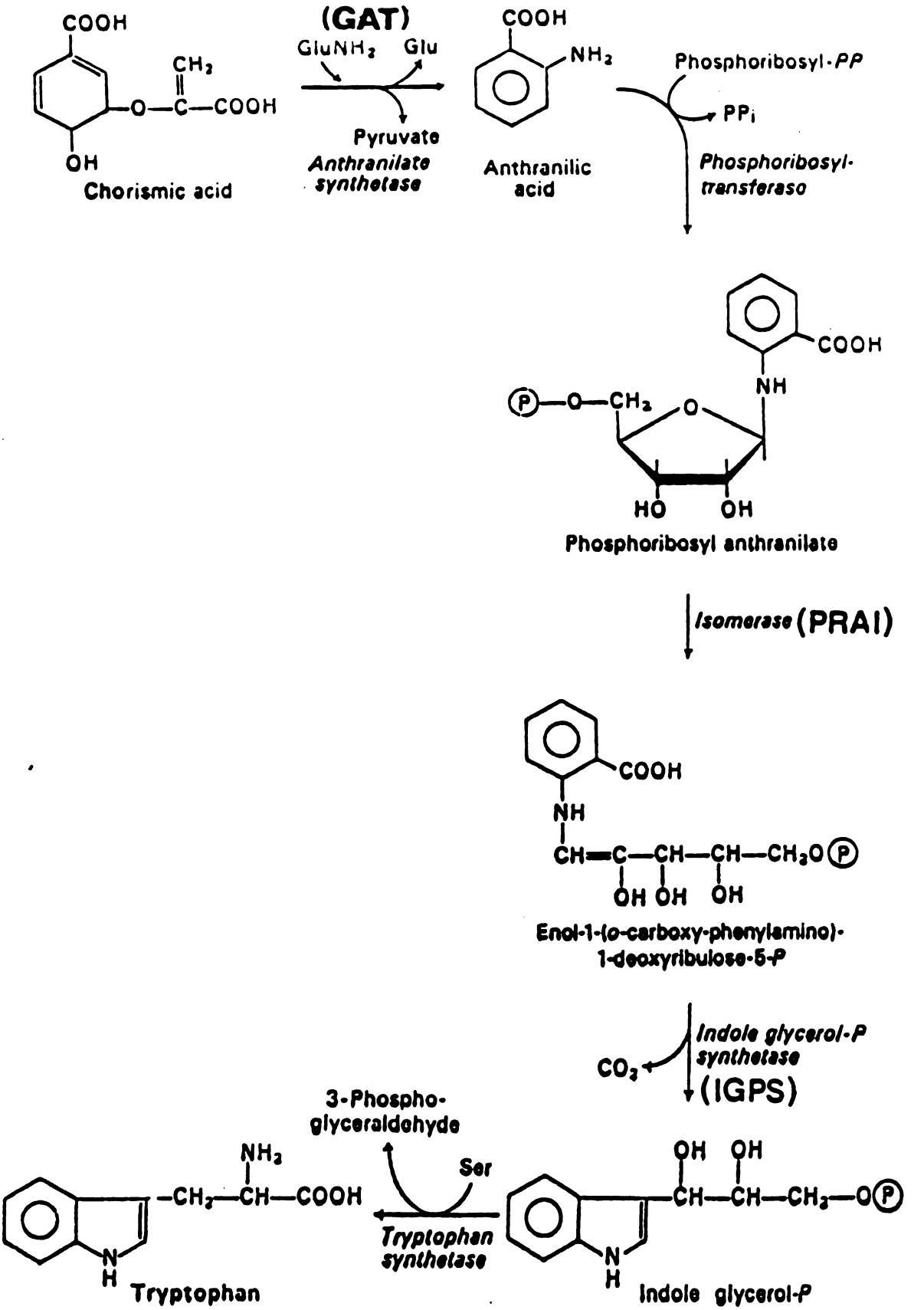
I. INTRODUCTION

The five biosynthetic steps from chorismate to tryptophan, catalyzed by seven enzymatic activities (Figure 9), appear to be the same in bacteria, yeasts, and filamentous fungi (Hütter et al., 1986). The seven enzymatic functions of *Escherichia coli* are encoded by 5 genes (*trpA*$^+$ through *trpE*$^+$) in a single operon and are designated domain A through G (Hütter et al., 1986). In the filamentous fungi *Neurospora crassa* and *Aspergillus nidulans*, four unlinked genes encode four polypeptides, of which two are monofunctional, one is bifunctional, and one is trifunctional. The activities of the fungal trifunctional gene correspond to the domains G, C, and F of *E. coli*, which encode glutamine amidotransferase (GAT), indoleglycerolphosphate synthase (IGPS), and phosphoribosylanthranilate isomerase (PRAI), respectively. In *E. coli*, the *trpC*$^+$ gene product is a bifunctional polypeptide with IGPS and PRAI activities.

The *trpC*$^+$ gene has been used previously as a selectable marker for several molecular studies in filamentous fungi. There is considerable conservation of domain structure of this gene throughout the prokaryotic (Crawford, 1975, 1989) and eukaryotic worlds (Hütter et al., 1986). The genes that encode PRAI activity of different filamentous fungi including *N. crassa* (Schechtman and Yanofsky, 1983), *A. nidulans* (Yelton et al., 1983), *Aspergillus niger* (Kos et al., 1985), *Cochliobolus heterostrophus* (Turgeon et al., 1986), *Schizophyllum commune* (Muñoz-Rivas, et al., 1986a), *Penicillium chrysogenum* (Sánchez et al., 1986), and *Phycomyces blakesleeanus* (Revuelta and Jayaram, 1987) have been cloned by complementation of the

PRAI deficiency in *E. coli trpC* mutants. Therefore, the *A. parasiticus trpC*$^{+}$ gene could be selected in suitable *E. coli* mutant strains based on the same strategy.

Figure 9. The biosynthetic pathway of tryptophan from chorismic acid.


COOH
CH2
O—C—COOH
OH
Chorismic acid
(GAT)
GluNH2
Glu
Pyruvate
Anthranilate synthetase
COOH
NH2
Anthranilic acid
Phosphoribosyl-PP
PPi
Phosphoribosyl-transferase
COOH
NH
P—O—CH2
O
HO
OH
Phosphoribosyl anthranilate
Isomerase (PRAI)
COOH
NH
CH=C—CH—CH—CH2O P
OH OH OH
Enol-1-(o-carboxy-phenylamino)-1-deoxyribulose-5-P
CO2
Indole glycerol-P synthetase
(IGPS)
3-Phospho-glyceraldehyde
NH2
-CH2—CH—COOH
N
H
Tryptophan
Ser
Tryptophan synthetase
OH OH
CH—CH—CH2—O P
N
H
Indole glycerol-P

II. MATERIALS AND METHODS

A. Bacterial and fungal strains

The *E. coli* K-12 strain DH5 (F⁻ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* $r_k^- m_k^+$) (Hanahan, 1983) was used for plasmid library construction. DH5 and *E. coli* HB101 [*hsdS20* ($r_B^- m_B^-$) *recA13 ara14 proA2 lacY1 galK2 rpsL20 Sm^r xyl5 mtl1 supE44*] were used to propagate plasmids. *E. coli* JA209 (*trpA36 argH metE xyl recA56 str^r glyH*) (Clarke and Carbon, 1978), JA300 (*trpC1117 thr leuB6 thyA thi hsdR hsdM str*) (Tschumper and Carbon, 1980), MC1066 [*trpC9830 Δlac(IPOZYA)X74 galU galK strA hsdR leuB6 pyrF74::Tn5 (km^r)*] (Casadaban et al., 1983) as well as mutants of strain W3110 (Schechtman and Yanofsky, 1983), each carrying a different mutation in the *trp* operon (Table 7), were used for complementation assays. *E. coli* NM539 [*supF hsdR* ($r_k^- m_k^+$) (P_2)] (Frischauf et al., 1983) and strain LE392 (F⁻ *hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55* λ^-) (Murray et al., 1977) were the host for amplification and propagation of the lambda phage library, respectively.

The wild-type aflatoxigenic strain *A. parasiticus* NRRL 5862 (SU-1) (Bennett and Papa, 1988) was the source of DNA for genomic library construction. *A. nidulans* mutant strain FGSC 237 (*pabaA1 yA2 trpC 801*, deficient in all trifunctional *trpC*⁺ activities) (Käfer, 1977) was the host for studies of heterologous gene expression.

Table 7. Complementation of *E. coli* tryptophan auxotrophs by plasmid pLH23.

Recipient strain[a]	Deficient domain(s)[b]	Growth on minimal medium[c]	
		with TRP	without TRP
MC1066 *trpC9830*	F	+	+
JA300 *trpC1117*	F	+	+
W3110 *trpC9830*	F	+	+
W3110 *trpC9941*	C	+	+
W3110 Δ*trpC10-16*	C, F	+	+
W3110 Δ*trpE5*	E	+	-
W3110 Δ*trpLD102*	E, D	+	-
W3110 *trpB9578*	B	+	-
JA209 *trpA36*	A	+	-

[a]: Strain JA300 *trpC1117* and JA209 *trpA36* were purchased from American Type Culture Collection (ATCC); strain MC1066 *trpC9830* was obtained from W. E. Timberlake (University of Georgia, Athens, GA); all the W3110 strains were obtained from C. Yanofsky (Stanford University, Stanford, CA).

[b]: Enzymatic activity encoded by each domain: A, subunit of tryptophan synthase, which catalyzes the conversion of indole-3-glycerophosphate to indole; B, subunit B of tryptophan synthase, which catalyzes the conversion of indole to tryptophan; C, IGPS; D, phosphoribosyl transferase; E, anthranilate synthase (with ammonia as the amino group donor); F, PRAI; G, GAT, interacts with the E domain in the glutamine-dependent anthranilate synthase.

[c]: Cells of each strain were transformed with pLH23, plated on M9 minimal agar, and incubated overnight at 37°C. Colonies which appeared were transferred to M9 minimal medium with or without tryptophan (TRP).

B. Growth media

For growing *A. parasiticus*, Czapek Dox broth (agar) and a synthetic low salt medium (Reddy et al., 1971, 1979) were used as minimal media; potato dextrose broth (agar) supplemented with 0.5% yeast extract (PDY) (Bennett and Papa, 1988) was used as a complete medium. Standard complete and minimal media for *E. coli* (Maniatis et al., 1982; Miller, 1972) and for *A. nidulans* (Pontecorvo, 1953; Barratt et al., 1965) were used where appropriate.

C. Preservation of cultures

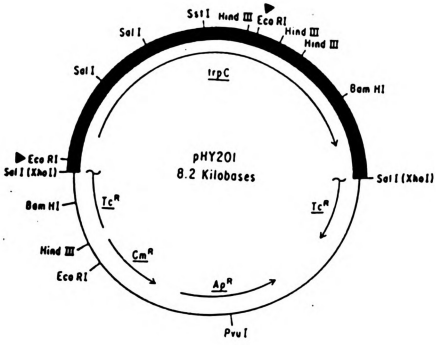
E. coli strains were routinely stored as frozen (-70°C) stock with 50% glycerol. For storage of fungal strains, young cultures germinated from single spores (conidia) that were isolated by a serial dilution and plating technique (Raper and Fennell, 1965) were transferred onto the center of Petri plates with appropriate media. They were grown for 7 to 10 days at 28-30°C, until the surface of the media was covered with conidia. Suspensions of conidia were prepared by adding sterile solution of 0.01% (v/v) Triton X-100 and lightly scraping plates with an inoculating loop. Suspensions were filtered through a sterile cheese cloth, glass wool or Miracloth (Calbiochem, San Diego, CA) and washed 2 to 4 times with distilled water by centrifugation at about 800 xg. For long-term preservation, stock cultures were generally maintained as frozen (-70°C) spore suspensions in 15-20% glycerol or on silica gel granules as described for *Neurospora* (Perkins, 1962; Davis and de Serres, 1970). In brief, conidia were suspended in sterile, reconstituted dry

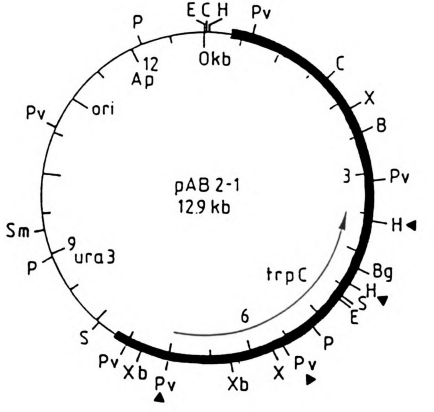
skim milk (Difco Laboratories, Detroit, MI). Small, screw-cap vials were half filled with silica gel granules (6-12 mesh, grade 40, desiccant, activated, without indicator, Davison Chemical Corp., Baltimore, MA) and sterilized in a 180-200°C oven for 2 hr. Samples of the spore suspension were transferred to the vials, cooled with ice, and the vials were sealed. Silica gel stocks were stored at room temp. They were reactivated by sprinkling granules onto the surface of agar-solidified growth medium. For shorter term storage, cultures grown on Petri dishes or slants for one week were sealed with tape or Parafilm and stored at 4°C. Alternatively, short slants were covered with sterile mineral oil (heavy white oil, Sigma Chemical Co., St. Louis, MO) and stored at 4°C.

D. Vectors

Plasmids pHY201 (Figure 10) and pHY101 (Yelton et al., 1984), both containing a complete trifunctional *A. nidulans trpC*$^+$ gene, as well as plasmid pAB2-1 (Figure 10; Kos et al., 1985), containing an intact *A. niger trpC*$^+$ gene, were sources of probes in heterologous hybridization study. Plasmid pHY101 was constructed by ligating a 4.3-kb *Xho*I fragment, which contains the entire *A. nidulans trpC*$^+$ gene, with the *Xho*I-digested pACYC177 (Chang and Cohen, 1978).

Figure 10. Restriction maps of *trpC*$^+$-containing plasmids pHY201 and pAB2-1. Plasmid pHY201 (Yelton et al., 1984) was constructed by ligating a 4.3-kb *Xho*I fragment containing a complete wild-type copy of the *A. nidulans trpC*$^+$ gene to *Sal*I-digested pBR329 DNA, thereby interrupting the tetracycline-resistance (Tc^R) gene. The plasmid confers resistance to ampicillin (Ap^R) and chloramphenicol (Cm^R) to *E. coli*. Plasmid pAB2-1 (Kos et al., 1985) was isolated from an *A. niger* gene bank by complementation of an *E. coli trpC* mutant. It consists of the yeast vector YIp5 with a 7.2-kb *A. niger* DNA inserted at the *Bam*HI site. The 3.3-kb *Pvu*II-*Hin*dIII fragment contains the complete *A. niger trpC*$^+$ gene. Small solid triangles indicate restriction sites used to generate probes for heterologous hybridization analyses. Thin lines, vector DNA sequences; heavy lines, fungal DNA sequences. Abbreviations are: E, *Eco*RI; H, *Hin*dIII; C, *Cla*I; Pv, *Pvu*II; X, *Xho*I; B, *Bam*HI; Bg, *Bgl*II; S, *Sal*I; P, *Pst*I; Xb, *Xba*I; Sm, *Sma*I.


Sst I
Hind III
Eco RI
Hind III
Hind III
Sal I
Sal I
trpC
Bam HI
Eco RI
Sal I (Xho I)
pHY201
8.2 Kilobases
Sal I (Xho I)
Bam HI
Tc^R
Tc^R
Hind III
Eco RI
Cm^R
Ap^R
Pvu I


E C H
Pv
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12
Ap
0kb
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Pv
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9
ura3
Sm
Pv
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trpC
6

Plasmid pRK9 (Schechtman and Yanofsky, 1983) was used for the construction of the *A. parasiticus* genomic DNA library. pRK9 is a pBR322 (Balbás et al., 1986) derivative with a unique *Bam*HI site created by replacing the 380-base-pair (bp) *Eco*RI-*Bam*HI fragment of pBR322 with a 96-bp *Serratia marcescens trp* operon promoter. The bacterial plasmid pUC19 (Yanisch-Perron et al., 1985) and the yeast *Saccharomyces cerevisiae* vector YIp5 (Botstein et al., 1979) were used for sub-cloning.

E. General Procedures

Preparation of bacteria and plasmid DNA, restriction enzyme digestions, agarose gel electrophoresis, and hybridization analyses were performed by standard procedures (Maniatis et al., 1982; Ausubel et al., 1987). Phage DNA was purified according to the procedure of Carlock (1986). Using a kit purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), DNA probes were radio-labeled with ^{32}-P (New England Nuclear Corp., Wilmington, DE; or Amersham Corp., Arlington Heights, IL) by the random primer technique (Feinberg and Vogelstein, 1983, 1984) to a specific activity of greater than 10^8 cpm/μg of DNA. Unincorporated radioactive precursors were separated from the probes by Sephadex G 50-80 (Sigma) exclusion column chromatography as described by Maniatis et al. (1982). Filters with DNAs or RNAs were hybridized in 6x SSC (1x SSC is 0.15 M sodium chloride and 0.015 M sodium citrate), 5x Denhart solution (1x Denhart solution contains 1% Ficoll, 1% bovine serum albumin and 1% polyvinylpyrro-lidone), 40% formamide, 0.1% sodium dodecyl sulfate (SDS), 5 mM

ethylenediamine tetraacetic acid (EDTA) and 100 μg/ml denatured salmon sperm DNA at 65°C for higher stringency, and at 37 or 42°C for lower stringency. Filters were washed twice in 0.1% SDS and 2x SSC at room temperature for 40 min followed by a final wash in 0.1% SDS and 0.1% SSC at 65°C for one hour. Autoradiograms were obtained by exposing filters to Kodak X-OMAT XAR5 diagnostic X-ray film (Eastman Kodak Company, Rochester, NY) with or without Cronex Lightning plus intensifying screens (E. I. Du Pont Nemours & Co., Wilmington, DE) at -70°C for variable time.

Restriction enzymes and other enzymes for manipulation of recombinant DNA were purchased from Bethesda Research Laboratories Inc. (Gaithersburg, MD), Boehringer Mannheim Biochemicals, New England Biolabs (Bevery, MA), New England Nuclear Corp. or Promega Biotec, and were used according to the instructions of the suppliers. Novozym 234 used for production of fungal protoplasts was purchased from Novo Laboratories, Inc. (Wilton, CT). Nitrocellulose and Nytran nylon membranes were purchased from Schleicher & Schuell Inc., (Keene, NH); Gene Screen Plus membrane was supplied by New England Nuclear Corp. All chemicals were of analytical grade and were purchased from Sigma Chemical Co., Aldrich Chemical Co. (Milwaukee, WI), or Fluka Chemical Corp. (Ronkokoma, NY).

F. Isolation and manipulation of genomic DNA of *A. parasiticus*

High-molecular-weight chromosomal DNA of *A. parasiticus* was prepared by a procedure modified from that of Cihlar and Sypherd (1980). Mycelia of *A.*

parasiticus NRRL 5862 were harvested from YES medium (2% yeast extract and 6% sucrose), lyophilized and blended into a powder with a Waring blender and then ground in a mortar and pestle with glass powder. The resulting mycelial powder was suspended in TSE buffer (100 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, 100 mM EDTA) and incubated with 1% SDS at 37°C for 1 hr with occasional gentle shaking. The suspension was centrifuged (5,000 x g, 10 min) to remove cell debris. The supernatant was extracted with equal volume of TSE-saturated phenol for 1 hr at room temp with gentle shaking. After centrifugation (7,500 x g, 10 min), the aqueous layer was removed and extracted with equal volume of TSE-saturated phenol-chloroform-isoamylalcohol (25:24:1). The nucleic acids were then precipitated from the aqueous phase with ethanol, dried under vacuum, and resuspended in TE (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA). Contaminating RNA and proteins were removed by treatment with RNase A and proteinase K (37°C, 1 h each). The enzymes were removed by phenol extraction and the genomic DNA was recovered by ethanol precipitation. With this protocol yields were normally greater than 1 mg of *A. parasiticus* DNA from 10 g wet mycelia. The majority of the genomic DNA migrated more slowly than phage lambda DNA in agarose gel electrophoresis (larger than 50 kilobase [kb]) and was readily digested with restriction enzymes.

G. Preparation of RNA from *A. parasiticus*

Total RNA was isolated by the hot phenol method of Maramatsu (1973). Frozen mycelia of *A. parasiticus* NRRL 5862 were ground into powder with a

mortar and pestle under liquid nitrogen. The mycelial powder was suspended in RNA extraction buffer (50 mM NaOAc, 1.0 mM EDTA, 1% SDS, pH 5.0) pre-warmed to 65°C. An equal volume of phenol saturated with extraction buffer and prewarmed to 65°C was added immediately and the mixture was incubated at 65°C for 30 min with gentle shaking. The aqueous phase was recovered by centrifugation (5,000 rpm, 10 min), extracted with an equal volume of 65°C, buffer-saturated phenol, followed by an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) mixture and finally with water-saturated ether. Total RNA was then precipitated from the aqueous phase by adding 1/6 volume of 3 M NaOAc (pH 5.2) and 2.5 volume of absolute ethanol at -20°C. The RNA was collected by centrifugation (20,000 rpm, 20 min), dried under vacuum and resuspended in RNase-free water.

Poly(A)$^+$ messenger RNAs were separated from total RNA by oligo(dT) cellulose (Boehringer Mannheim) affinity column chromatography, done by using standard procedures (Maniatis et al., 1982).

H. Construction of genomic DNA libraries

For construction of the phage lambda library, high-molecular-weight genomic DNA from *A. parasiticus* was partially digested with restriction endonuclease *Sau*3A1. DNA restriction fragments (15 to 20 kb) were isolated by fractionation twice on 5-25% sodium chloride gradients (Kaiser and Murray, 1985) and ligated to phage λEMBL3 *Bam*H1 arms (Promega Biotec). Recombinant phage DNAs were packaged according to the protocol of the supplier (Promega Biotec). Libraries

were amplified by the procedure of Maniatis et al. (1982), distributed into 1.5-ml tubes with screw cap, and stored at 4°C with a drop of chloroform.

For construction of the plasmid library, genomic DNA fragments with an average size of 5 to 10 kb were generated by partial digestion with *Sau*3A1 followed by fractionation twice on 5-24% sodium chloride gradients, and then ligated with *Bam*H1-digested, phosphatase-treated plasmid pRK9 (Schechtman and Yanofsky, 1983). *A. parasiticus* DNA fragments were inserted into the unique *Bam*HI site in the *S. marcescens trp* leader region of pRK9 in order to maximize expression of the gene cloned downstream from the promoter. The recombinant plasmids were used to transform competent cells of *E. coli* DH5 (Bethesda Research Laboratories) to ampicillin resistance. The primary plasmid library was amplified as described by Vogeli et al. (1985) and was stored as 1-ml aliquots at -70°C with 15% glycerol.

I. Lytic complementation of *E. coli trpC* mutants

Lytic complementation was performed by the procedure of Davis et al. (1980). *E. coli trpC* mutants MC1066 and W3110 *trpC*9830, defective in PRAI activity, were infected with 10^7 recombinant phages from the *A. parasiticus* genomic DNA library (multiplicity of infection, < 0.001) and plated on M9 medium lacking tryptophan. Plaques appeared after overnight incubation at 37°C. Phage clones were further purified by reinfecting the susceptible host under selective conditions to isolate single plaques.

J. Transformation

E. coli strains were transformed by the hexamine cobalt chloride method (Hanahan, 1983). For transformation of *A. nidulans*, the procedure of Oakley et al. (1987) was used with the following slight modifications: young germlings for production of protoplasts were prepared from frozen conidia stocks. Protoplasting solution was filtered-sterilized through a 0.45 μm Millex-HA Millipore filter (Millipore Products Division, Bedford, MA) and the final protoplast preparation was resuspended in 0.6 M KCl, 0.05 M $CaCl_2$ and 10 mM Tris hydrochloride, pH 7.5.

K. Laboratory safety

Guidelines for the recombinant DNA researches as established by the National Institutes of Health (1986) were observed throughout the experiments. Glasswares, equipments, waste media and cultures that were suspect of contamination with aflatoxins or their toxic precursors were routinely soaked in commercially available 10% NaOCl solution for at least 30 min followed by treatment with acetone and autoclaving to destroy these toxins (Castegnaro et al., 1980; Yang, 1972; Fischbach and Campbell, 1965; Stoloff and Trager, 1965).

III. RESULTS

A. Isolation of *A. parasiticus trpC*$^+$ gene from recombinant phage libraries

Two primary phage libraries were constructed (Table 8). Library I contained 1.2 x 10^5 recombinant phage clones. It was generated during the preliminary test process to determine the optimal packaging condition for the phage particles. Library II contained 2.1 x 10^5 recombinant phage clones, and was generated using the optimal packaging condition. Restriction digestion analysis of DNA from 20 randomly selected clones from both libraries indicated that 99% of them contained *A. parasiticus* DNA inserts averaging 15 kb. Assuming that the genomic size of *A. parasiticus* is comparable to that of the *A. nidulans* (Timberlake, 1978) and *N. crassa* (Krumlauf and Marzluf, 1979) genomes, the probability of finding any given DNA sequence in the library was greater than 99.9% (Clarke and Carbon, 1976). The primary library was amplified in *E. coli* NM539, which supports only the growth of recombinant phage particles (Frischauf et al., 1983). The amplified libraries consisted of 5.1 x 10^9 (library I) and 6.7 x 10^9 (library II) plaque-forming units (PFU) in total. Their titers decreased only 2 to 3-fold after storage at 4°C for approximately three years.

E. coli MC1066 and W3110 carrying the *trpC*9830 missense mutation were used as recipients for lytic complementation. Plaques appeared after infection followed by overnight incubation (Table 9). Twenty phage isolates were purified and replated on both host strains under selective conditions, i.e., without

Table 8. Characteristics of the *A. parasiticus* genomic DNA libraries.

	λEMBL3 library[1] (PFU)		Plasmid pRK9 library (CFU)
	I	II	
Before amplification	1.2×10^5	2.1×10^5	3.7×10^4
After amplification	5.1×10^9	6.7×10^9	3.2×10^{10}
Average DNA insert (kb)	15	15	6.0
N[2]	7979	7979	2.4×10^4

[1]: Library I was created during the preliminary packaging test; library II was generated using the optimal packaging condition as determined empirically.

[2]: The number of recombinant phage plaques or bacterial colonies harboring recombinant plasmids that are required to represent 99% of the *A. parasiticus* genome (approximately 2.6×10^7 base pairs) as calculated by the procedure of Clark and Carbon (1976).

Table 9. Lytic complementation of *E. coli trpC* mutants with recombinant lambda EMBL3 phages from the *A. parasiticus* genomic DNA libraries*.

	E. coli trpC9830 mutants	
Phage library	MC 1066	W3110
I	16	215
II	70	320

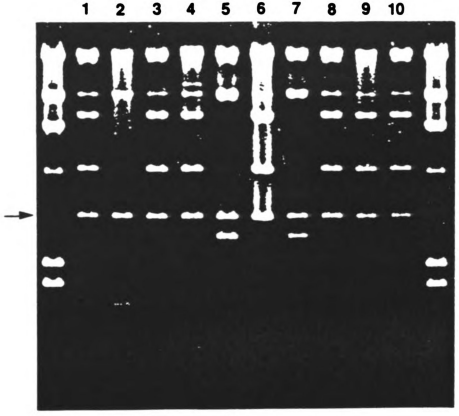
* *E. coli trpC* mutants were infected with phage particles from the amplified genomic libraries at a multiplicity of infection < 0.001, and plated onto M9 minimal medium. Plaques appeared after overnight incubation at 37°C were scored. Each number represents an average of results from two independent experiments.

tryptophan supplement in the media. All 20 phage isolates were able to complement the *E. coli* mutants at a high frequency. Restriction analysis of DNA purified from 10 phage clones revealed 3 different types of restriction patterns (Figure 11). One phage representing each restriction pattern, designated λAptrpM1, λAptrpM5 and λAptrpW9 was selected for further analysis. Restriction endonuclease analysis (Figure 12) of the *A. parasiticus* DNA inserts from these 3 clones showed that the inserts were approximately 15 kb in size and shared a common 10.5-kb region which presumably contained the complementing activity for *trpC*9830 mutation. The DNA restriction maps were contiguous, indicating that no identifiable DNA rearrangements occurred during lytic complementation.

B. Localization and organization of the *A. parasiticus trpC*⁺ gene

To identify which portion of the DNA insert contained the *A. parasiticus trpC*⁺ gene, DNAs from the 3 phage clones were digested with restriction enzymes, fractionated by electrophoresis on 0.9% agarose gels, blotted onto Nytran nylon membranes, and hybridized under low-stringency conditions with DNA probes (Figure 10) prepared from the *A. nidulans trpC*⁺ gene. The hybridization patterns (Figure 13A, with λAptrpW9 as an example) were consistent with the restriction maps of the three phage clones shown in Figure 12. Both amino- and carboxyl-terminus probes hybridized strongly, suggesting that extensive sequence similarities existed between *trpC*⁺ genes of *A. nidulans* and *A. parasiticus*. A similar experiment (data not shown) using DNA probes (Figure 10)

Figure 11. Restriction endonuclease digestion of DNAs from 10 randomly selected recombinant lambda EMBL3 clones with DNA inserts containing the *A. parasiticus trpC*$^{+}$ gene. Purified DNA (5 μg) from each phage clones was digested with *SalI* restriction endonuclease. The digestion mixtures were separated by electrophoresis in a 0.9% agarose gel. Three restriction patterns were identified: pattern I consists of phage clones No. 1, 3, 4, 6, 8, 9, and 10; pattern II consists of clones No. 5 and 7; pattern III consists of only clone No. 2. A 3.3-kb DNA insert fragment common to all phage clones is indicated by an arrow. Molecular size markers (*Hind*III digest of lambda DNA) are indicated on the leftmost and rightmost lanes.


1
2
3
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6
7
8
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10

Figure 12. Restriction endonuclease maps of three *A. parasiticus* DNA inserts (open bars) in λEMBL3 which complement the *E. coli trpC*9830 mutation. Restriction sites are: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; X, *Xba*I.

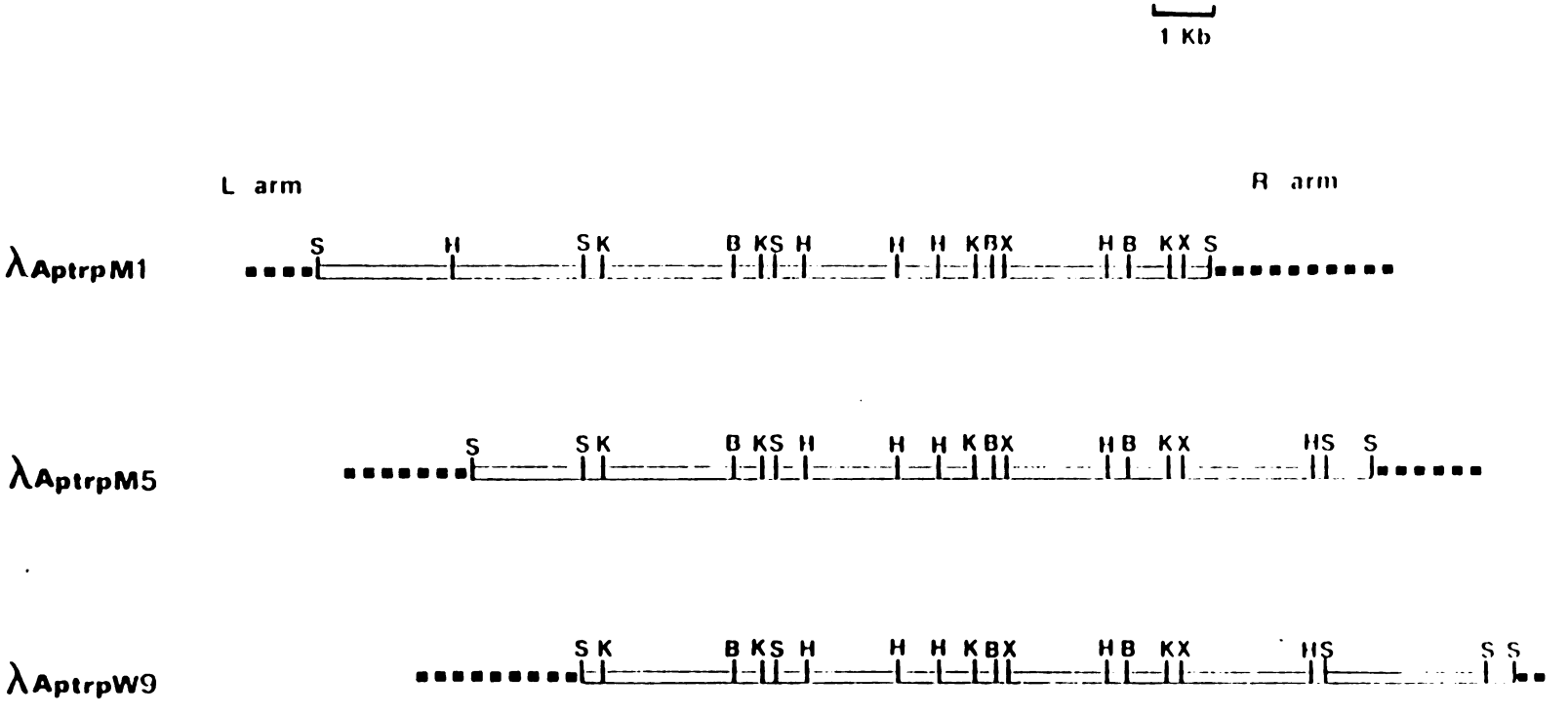
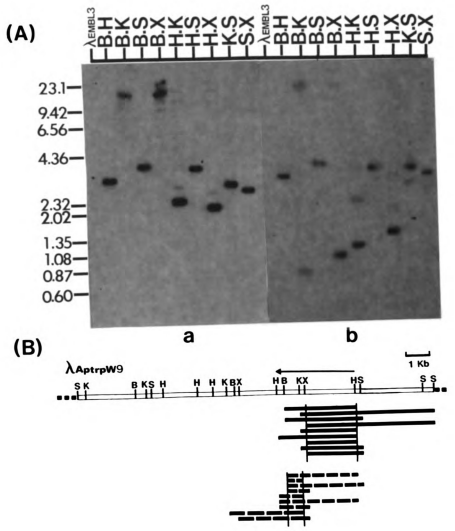

1 Kb
L arm
R arm
λAptrpM1
λAptrpM5
λAptrpW9

Figure 13. Southern hybridization of lambda clone λAptrpW9 with *A. nidulans trpC*$^+$ probes. (A) DNA was digested with pairs of restriction enzymes as indicated, blotted to a nylon membrane, and hybridized under conditions of low stringency with the radiolabeled amino-terminal portion (gel a) or carboxyl-terminal portion (gel b) of the *A. nidulans trpC*$^+$ gene. A 2.5-kb *Eco*RI fragment purified from plasmid pHY201 was used as an N-terminus probe; a 1.8-kb *Eco*RI-*Xho*I fragment purified from plasmid pHY101 was used as a C-terminus probe. Both pHY201 and pHY101 (obtained from Dr. W. E. Timberlake, University of Georgia) contain complete trifunctional *A. nidulans trpC*$^+$ gene. Molecular sizes in kb are indicated. (B) Diagram of the location and orientation of the *A. parasiticus trpC*$^+$ gene as determined from hybridization data. Solid bars indicate regions which hybridize with the N-terminus probe; dashed bars indicate regions which hybridize with the C-terminus probe. The minimal overlapping regions are delimited with vertical lines. The arrow shows the predicted direction of transcription of the *A. parasiticus trpC*$^+$ gene. Restriction sites are as follows: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; X, *Xba*I.

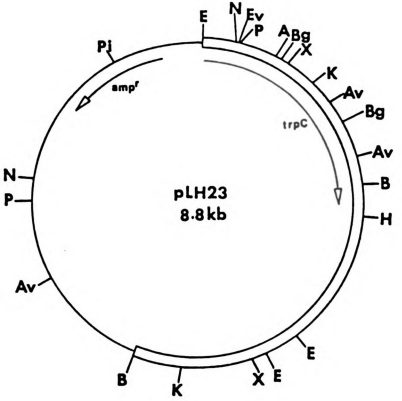

(A)
λEMBL3
B.H
B.K
B.S
B.X
H.K
H.S
H.X
K.S
S.X
λEMBL3
B.H
B.K
B.S
B.X
H.K
H.S
H.X
K.S
S.X
23.1
9.42
6.56
4.36
2.32
2.02
1.35
1.08
0.87
0.60
a
b
(B)
λAptrpW9
1 Kb
S K
B K S H
H H K B X
H B K X
H S
S S

prepared from the cloned *trpC*$^+$ gene of *A. niger* (Kos et al., 1985) was consistent with these findings. Southern hybridization with N- and C-terminus probes also suggested that the *trpC*$^+$ gene was transcribed in the direction indicated by the arrow in Figure 13B. The *A. parasiticus trpC*$^+$ gene, therefore, has the same structural organization as those of *A. nidulans*, *A. niger* and other filamentous fungi. This result is consistent with previous evidence that enzyme activities encoded by the *trpC*$^+$ gene are highly conserved in filamentous fungi (Hütter et al., 1986).

C. Complementation of an *A. nidulans trpC* mutant by the cloned *A. parasiticus trpC*$^+$ gene

A 3.4-kb *Hin*dIII fragment thought to contain the complete *A. parasiticus trpC*$^+$ gene was subcloned in opposite orientations into the unique *Hin*dIII site of the *E. coli* plasmid pUC19 and the vector YIp5. The resulting recombinant plasmids designated pJH34 and pAP34 (derivatives of pUC19) and YAP43H and YAPH34 (derivatives of YIp5) were used to transform *A. nidulans* FGSC 237, a mutant deficient in all three enzyme activities encoded by the *trpC*$^+$ gene (a detailed restriction map of plasmid pJH34 is shown in Figure 14). Each of the plasmids was able to transform this *trpC* mutant to tryptophan prototrophy (Table 10). Transformation frequencies were approximately 2-fold less than that for plasmid pHY201 which contains an intact *A. nidulans trpC*$^+$ gene, suggesting that the *A. parasiticus trpC*$^+$ gene was expressed at a lower efficiency in the heterologous host. The successful transformation with both types of plasmids containing

Figure 14. Restriction endonuclease maps of plasmids containing the *A. parasiticus trpC*$^{+}$ gene. The single lines represent sequences derived from vector pRK9 (plasmid pLH23) or pUC19 (plasmid pJH34). The double lines indicate *A. parasiticus* DNA inserts. The longer arrows show the coding regions and directions of transcription. The striped region in pJH34 was deleted to create plasmid pIV238. Only selected restriction sites are shown for pLH23 outside of the *trpC*$^{+}$ coding region. Plasmids are not drawn to scale. Abbreviations: A, *Acc*I; Av, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; N, *Nde*I; Pi, *Pvu*I; P, *Pvu*II; S, *Sac*I; X, *Xba*I; Xh, *Xho*I. The following restriction enzymes have no cutting sites on the 3.4-kb *Hin*dIII insert in plasmid pJH34: *Bgl*I, *Cla*I, *Eco*RI, *Nco*I, *Nru*I, *Pst*I, *Pvu*I, *Sal*I, *Sma*I, *Sph*I, and *Sst*I.


E
N
Ev
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H
Pi
ampr
trpC
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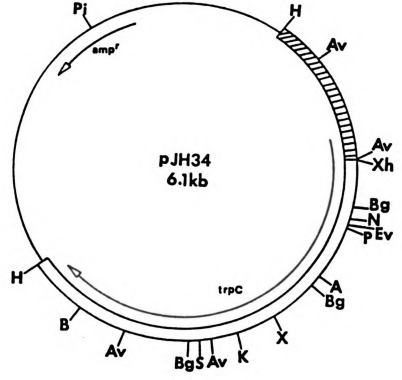
pLH23
8.8kb
Av
B
K
X
E
E


Pi
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Xh
pJH34
6.1kb
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trpC
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Av
K

Table 10. Transformation of an *A. nidulans trpC* mutant with the *A. parasiticus* *trpC*$^+$ gene.

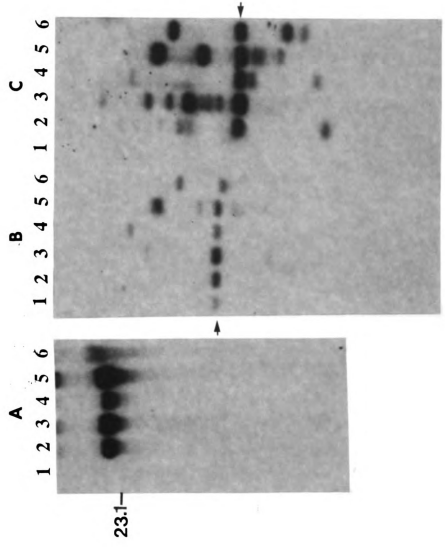
Plasmid	Transformation frequency[a] (Transformants/μg of DNA)
No DNA	0
pUC19	0
pRK9	0
YIp5	0
pLH23	0
pIV238	0
pJH34	15
pAP34	12
YAPH34	10
YAP43H	13
pHY201	26

[a]Protoplasts of *A. nidulans* FGSC 237 were transformed with 10 μg of each of the plasmid DNAs and plated onto minimal medium. Stable Trp$^+$ transformants were scored after incubation at 37°C for 4 days.

identical *A. parasiticus* DNA inserts in different orientations suggested that the complementing activity was encoded by the DNA insert, not by the vector sequences.

DNA was purified from five *A. nidulans* FGSC 237 Trp$^+$ isolates which had been transformed with pJH34 and subjected to Southern hybridization analyses using ^{32}P-labeled pUC19, pJH34, or a 3.4-kb *Hin*dIII restriction fragment excised from pJH34 (Figure 14) as probes. In all cases, the pUC19 probe hybridized to undigested *A. nidulans* DNA at the position of high-molecular-weight chromosomal DNA (Figure 15A), indicating that the vector sequences had integrated into the host genome. Hybridization to lower molecular weight fragments did not occur, suggesting that unintegrated copies of pJH34 were not detectable in the transformants. However, the data did not rule out the possibility of hybridization of the probe to concatemers of unintegrated transforming plasmids. There was no homology between pUC19 and *A. nidulans* DNA that had not been transformed with pJH34 (Figure 15A, lane 1). The 3.4-kb *Hin*dIII probe thought to carry the complete *A. parasiticus trpC*$^+$ gene hybridized with several (including a 3.4-kb) chromosomal DNA *Hin*dIII fragments from the *A. nidulans* transformants (Figure 15B), indicating the presence of the intact 3.4-kb *Hin*dIII fragment of pJH34, i.e, *A. parasiticus trpC*$^+$ sequences. The *A. parasiticus trpC*$^+$ gene also hybridized with a 3.4-kb *Hin*dIII fragment of *A. nidulans* FGSC 237. The identity of this 3.4-kb *Hin*dIII fragment in untransformed cells is unclear. Presumably it could be generated from *Hin*dIII sites within the *A. nidulans trpC*$^+$ gene and its flanking region. When the pUC19 probe was hybridized to the *A. nidulans* DNA cut with *Hin*dIII, one or more chromosomal DNA

Figure 15. Southern hybridization analysis of five *A. nidulans* Trp$^+$ transformants. (A) Undigested genomic DNAs were electrophoresed in a 0.7% agarose gel, blotted onto a nitrocellulose membrane and hybridized with radiolabeled pUC19. The band at the top of lane 5 was caused by DNA molecules that did not migrate into the gel. (B and C) DNAs were digested with *Hin*dIII, blotted onto a nitrocellulose membrane, and hybridized with a radiolabeled 3.4-kb *Hin*dIII fragment excised from pJH34 (B) or pUC19 (C). Blots were hybridized and washed under high-stringency conditions. Fragments indicated by arrows are 3.4 kb (B) and 2.7 kb (C), respectively. Lanes 1, *A. nidulans* FGSC 237; lanes 2 through 6, *A. nidulans* FGSC 237 transformed with pJH34.

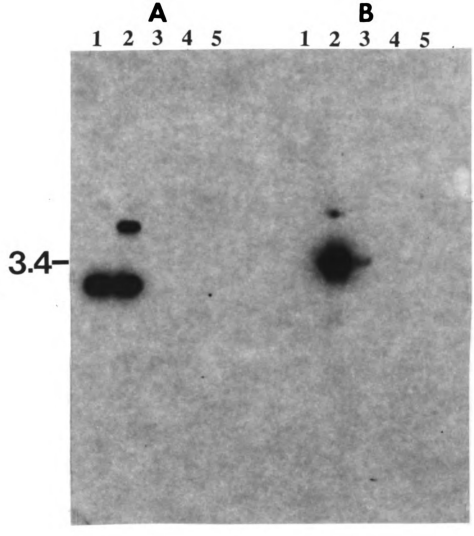

A
1 2 3 4 5 6
B
1 2 3 4 5 6
C
1 2 3 4 5 6
23.1

fragments in the transformants were detected (Figure 15C), demonstrating again the presence of vector sequences of pJH34 in the transformants. No hybridization was seen with genomic DNA of the untransformed host. The presence of the *A. parasiticus* 3.4-kb *Hin*dIII fragment concurrent with the Trp$^+$ phenotype in the transformants suggested that the *A. parasiticus trpC*$^+$ gene was contained on the 3.4-kb *Hin*dIII fragment and was functionally expressed in *A. nidulans*.

To prove that the cloned DNA insert in pJH34 was indeed derived from the *A. parasiticus* genome, *A. parasiticus* DNA was digested with restriction enzyme *Hin*dIII, and the gel-fractionated digests were probed with ^{32}P-labeled pUC19 or the 3.4-kb *Hin*dIII insert that was excised from pJH34 (Figure 16). The 3.4-kb *Hin*dIII DNA probe hybridized to a 3.4-kb fragment from *A. parasiticus* genomic DNA (panel B, lane 3). No hybridization to genomic DNA of either *E. coli trpC*9830 or phage λEMBL3 was observed, indicating the absence of a DNA sequence from *E. coli* or phage lambda that was able to complement the *E. coli trpC*9830 mutation. The genome of *A. parasiticus* showed no detectable homology to pUC19 under the wash stringency used in this experiment (panel A, lane 3).

To define more precisely the coding region in the cloned 3.4-kb *Hin*dIII insert that was essential for the trifunctional activities, a 930-bp *Hin*dIII-*Xho*I fragment at the beginning of the insert in pJH34 (Figure 14) was deleted. The remaining *trpC*$^+$ DNA fragment was purified and subcloned into *Hin*dIII-*Sal*I-digested pUC19. The resulting plasmid (pIV238) was used to transform an *A. nidulans trpC* mutant (FGSC 237). This plasmid failed to complement the *trpC* mutation (Table 10),

Figure 16. Hybridization of plasmid pJH34 to *A. parasiticus* chromosomal DNA fragments. Each DNA sample was digested with *Hin*dIII, blotted and hybridized under high stringency conditions and probed with ^{32}P-labeled pUC19 (panel A) or a 3.4 kb *Hin*dIII fragment excised from pJH34 containing the complete *trpC*$^{+}$ gene of *A. parasiticus* (panel B). Lane 1, pUC19; lane 2, pJH34; lane 3, *A. parasiticus* NRRL 5862 genomic DNA; lane 4, *E. coli* W3110 *trp*C9830 genomic DNA; lane 5, phage λEMBL3 DNA. A larger fragment in lane A2 is the result of an incomplete digestion. The position of the 3.4-kb fragment is indicated on the left.


A
1 2 3 4 5
B
1 2 3 4 5
3.4–

indicating that the deleted 930-bp *Hin*dIII-*Xho*I fragment was necessary for complete *trpC*$^+$ function.

The five Trp$^+$ transformants were tested for mitotic stability by growing colonies from single conidia on minimal medium for two rounds and then replica-plating more than 50 isolates onto selective and nonselective media. No Trp$^-$ colonies were found with any of the transformants, indicating that the transforming plasmid was stably inherited through integration into the host chromosome.

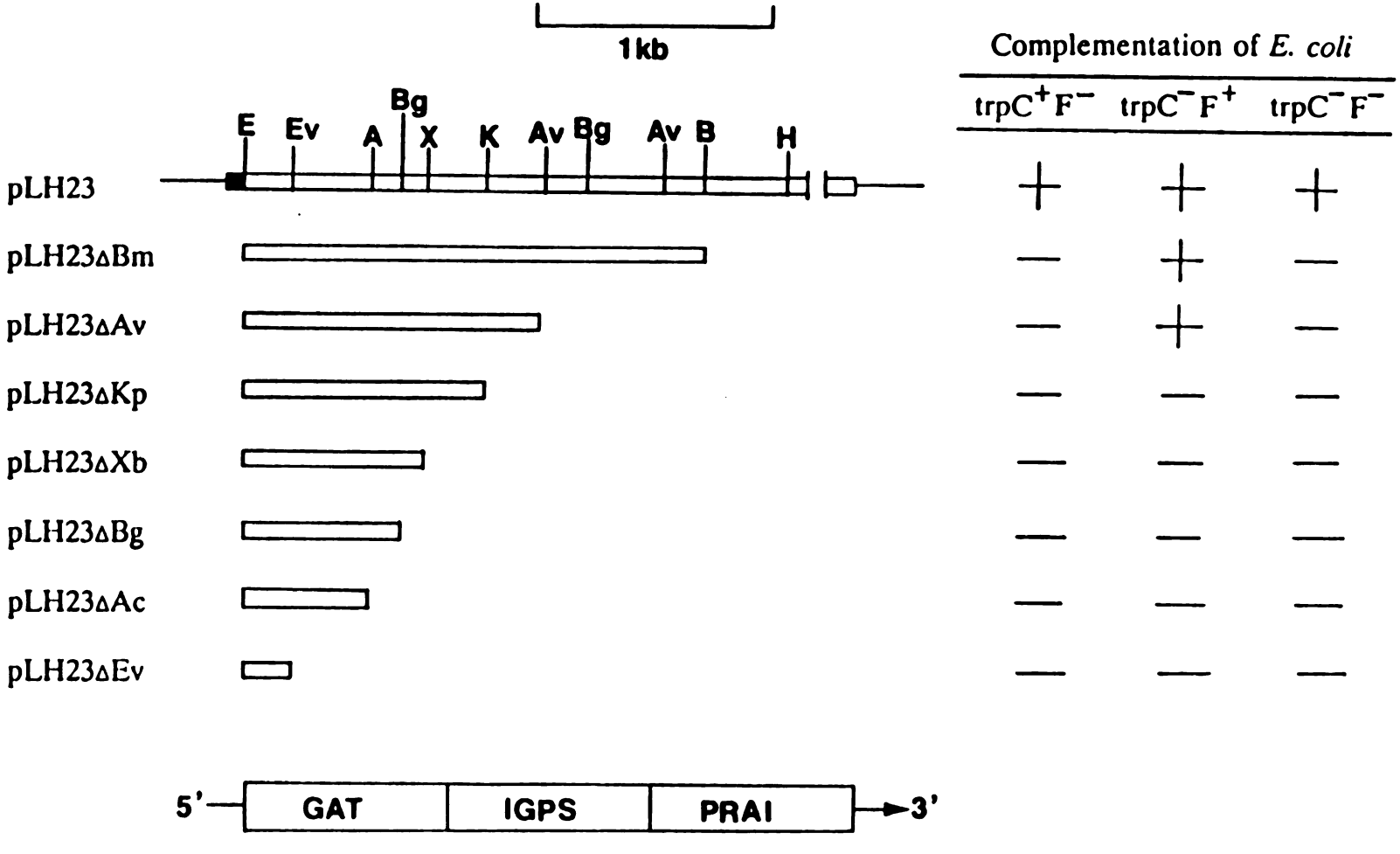
D. Isolation, complementation analysis, and deletion mapping of plasmid pLH23

E. coli trpC mutants were transformed with DNAs pooled from the *A. parasiticus* plasmid library. Trp$^+$ isolates were obtained and screened by using radiolabeled pJH34 as a probe. A plasmid designated pLH23 (Figure 14), which contained a 4.9-kb *A. parasiticus* DNA insert, was recovered. On the basis of a comparison by restriction enzyme analysis of this insert and the *trpC*$^+$ gene isolated from the lambda library, the insert was thought to encode an incomplete *A. parasiticus trpC*$^+$ gene with a short deletion in the 5' end. Consistent with this, plasmid pLH23 was unable to transform *A. nidulans trpC* mutants to Trp$^+$ phenotype. However, pLH23 complemented all *E. coli* strains with a deficiency in PRAI, IGPS or both activities (Table 7), whereas pJH34 and pAP34 complemented only strains with PRAI deficiency, but not strains with IGPS activity (data not shown). These results indicated that the regions within the *A. parasiticus trpC*$^+$ gene allowed for expression of the PRAI gene sequences, but not of the IGPS gene sequences in *E. coli*

and that *S. marcescens trp* promoter from pRK9 allowed for transcription of the *A. parasiticus trpC*$^+$ (IGPS) sequence in *E. coli*. Plasmid pLH23 did not complement *E. coli* strains with deficiencies in domain A, B, D, or E (Table 7).

To confirm the functional organization of the *A. parasiticus trpC*$^+$ gene as revealed by heterologous hybridization, I determined the relative locations of the IGPS and PRAI domains on pLH23 by deletion mapping. Plasmids were constructed with deletions in the *trpC*$^+$ gene and analyzed by restriction endonuclease digestion to confirm the desired deletions. The resulting deletion plasmids were then tested for Trp function in various Trp$^-$ mutants of *E. coli* (Figure 17). The deduced domain organization of the *A. parasiticus trpC*$^+$ polypeptide (Figure 17, bottom) was fully consistent with that obtained from heterologous hybridizations and was similar to that described for other filamentous fungi. These data also showed that a 280-bp *Hin*dIII-*Bam*HI fragment located at the 3' end of the insert was necessary for PRAI activity. This result, together with the inferences that both a 930-bp *Hin*dIII-*Xho*I fragment on pJH34 (see previous section) and a minimum of 2.3 kb are necessary for the complete *trpC*$^+$ function in filamentous fungi (Choi et al., 1988; Kos et al., 1988; Mullaney et al., 1985; Revuelta and Jayaram, 1987; Sánchez et al., 1986; Schechtman and Yanofsky, 1983), suggested that the *A. parasiticus trpC*$^+$ gene was initiated near the 3' end of the 930-bp *Hin*dIII-*Xho*I fragment and terminated within the 280-bp *Hin*dIII-*Bam*HI fragment as indicated by the longer arrow on plasmid pJH34 in Figure 14.

Figure 17. Localization of the Trp functions in pLH23 by deletion mapping. Deletions were constructed by digesting pLH23 with suitable restriction enzymes, recovering the remaining fragment containing the *Serratia trp* promoter, and recircularizing by ligation. Open blocks represent the *A. parasiticus trpC*$^+$ DNA sequences which remained in the final plasmid constructs; single lines are vector pRK9 sequences. The solid block indicates the *Serratia trp* promoter region. The ability of each of these plasmids to complement the three *E. coli* tryptophan auxotrophs is given on the right-hand side. The deduced domain organization of the trifunctional *A. parasiticus trpC*$^+$ gene product is depicted at the bottom. Restriction sites are shown only for regions containing the Trp functions. For abbreviations, see legend to Figure 14. IGPS is encoded by *trpC*$^+$, and PRAI is encoded by *trpF*$^+$.


1kb
E
Ev
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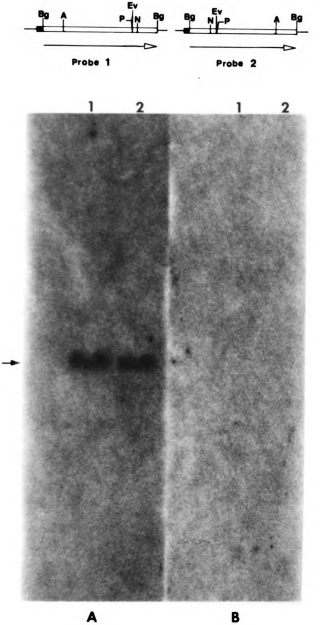
Av
B
H
pLH23
pLH23ΔBm
pLH23ΔAv
pLH23ΔKp
pLH23ΔXb
pLH23ΔBg
pLH23ΔAc
pLH23ΔEv
Complementation of E. coli
trpC+F−
trpC−F+
trpC−F−
5'
GAT
IGPS
PRAI
3'

E. Analysis of *A. parasiticus trpC*$^+$ transcript

To determine the transcriptional pattern and direction of the cloned *trpC*$^+$ gene, filters with poly(A)$^+$ RNAs purified from *A. parasiticus* mycelia grown in minimal and rich medium were hybridized to ^{32}P-labeled RNA probes generated with an SP6 transcription system (Riboprobe System II, Promega Biotec). Probe 1 but not probe 2 hybridized to a single species of poly(A)$^+$ RNA, 2.7 kb in length, which was approximately the same size as *trpC*$^+$ transcripts from other filamentous fungi (Figure 18) (Choi et al., 1988; Kos et al., 1988; Mullaney et al., 1985; Revuelta and Jayaram, 1987; Sánchez et al., 1986; Schechtman and Yanofsky, 1983). The direction of transcription of the *A. parasiticus trpC*$^+$ gene (Figure 14, longer arrow) must therefore have been the same as that of the template which gave rise to probe 2. These data were fully consistent with those obtained by heterologous hybridization analysis of phage clones and by deletion mapping of pLH23. Transcriptional analysis did not show significant differences in the level of the *trpC*$^+$ mRNA in cell grown in minimal medium or in rich medium, indicating a substantial constitutive expression of the *A. parasiticus trpC*$^+$ gene.

Figure 18. Northern analysis of the *A. parasiticus trpC*$^+$ transcript. Poly (A)$^+$ RNA isolated from *A. parasiticus* NRRL 5862 grown in minimal medium (Czapek Dox broth; lane 1, 10 μg) or rich medium (Czapek Dox broth supplemented with 100 μg of L-tryptophan per ml; lane 2, 10 μg) was fractionated on 1.2 % agarose gels containing 2.2 M formaldehyde (Fourney et al., 1988), blotted onto a Gene Screen Plus membrane and hybridized to ^{32}P-labeled probe 1 (A) or probe 2 (B) generated with an SP6 transcription system (Riboprobe System II; Promega Biotec). The templates for SP6 RNA polymerase were prepared by inserting a 0.5-kb *Bgl*II fragment purified from the *trpC*$^+$-coding region in plasmid pJH34 in the two possible orientations into *Bam*HI-digested Riboprobe plasmid vector pSP64. The resulting recombinant plasmids were linearized with *Ava*I and subjected to in vitro transcription with SP6 RNA polymerase by the procedure of Melton et al. (1984). Specific activities were >10^8 cpm/μg of RNA. A solid bar in the diagram indicates the SP6 promoter region. The size of the RNA transcript was estimated with a 0.24- to 9.5-kb RNA ladder from Bethesda Research Laboratories. The 2.7-kb *A. parasiticus trpC*$^+$ transcript is indicated by an arrow. For abbreviations of restriction enzymes, see legend to Figure 14.


Bg
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B

IV. DISCUSSION

Several lines of evidence demonstrate that the complete *A. parasiticus trpC*$^+$ gene is present on the plasmid pJH34. First, pJH34 was capable of complementing different strains of *E. coli trpC* mutant lacking PRAI activity. Hybridization experiments revealed that *A. parasiticus* DNA was inserted in pJH34 (Figure 16). A truncated version of this plasmid (pLH23) obtained independently not only complemented the PRAI mutation but also an IGPS mutation (Table 7). Southern blot analysis showed that the 3.4-kb insert of pJH34 was homologous with the *trpC*$^+$ gene of *A. nidulans* (Figure 13) and *A. niger* (data not shown). Finally, transformation of an *A. nidulans trpC* mutant deficient in GAT, IGPS, and PRAI activities with pJH34 resulted in tryptophan prototrophy (Table 10). Plasmid pJH34 was present in DNA prepared from these Trp$^+$ transformants. The ability of pJH34 to restore prototrophic growth of an *A. nidulans trpC* mutant lacking the GAT, IGPS and PRAI enzymatic activities further indicated that the *A. parasiticus trpC*$^+$ gene codes for a polypeptide with the same enzymatic activities as the *A. nidulans trpC*$^+$ gene product. On the basis of these observations, it is concluded that pJH34 carries a functional *A. parasiticus trpC*$^+$ gene which can be expressed in both *E. coli* as well as homologous and heterologous fungal cells.

Deletion mapping and Southern hybridization analyses using cloned *trpC*$^+$ genes from other filamentous fungi as probes allow prediction of the direction of transcription of the *A. parasiticus trpC*$^+$ gene and its functional organization. The data suggested that organization of the *A. parasiticus trpC*$^+$ gene was identical to that of the *trpC*$^+$ (*trp-1*$^+$) genes of other filamentous fungi in which the gene product

is a trifunctional polypeptide harboring *trpG*$^+$ (GAT), *trpC*$^+$ (IGPS), and *trpF*$^+$ (PRAI) activities arranged in the order of NH_2-GAT. IGPS. PRAI-COOH.

Plasmids carrying the 3.4-kb *Hin*dIII insert of *A. parasiticus* were also capable of complementing different *E. coli* mutants lacking PRAI activity. The observation that complementation of a *trpC* mutation in *E. coli* was not dependent on the cloning vector or the orientation of the insert in the vector (Table 10) suggested that expression of the gene in *E. coli* was independent of vector DNA sequences. This implies that the sequences required for transcription and translation initiation in *E. coli* occur fortuitously within the coding sequence of the *A. parasiticus trpC*$^+$ gene. The PRAI activity encoded by the *A. parasiticus trpC*$^+$ was apparently initiated in *E. coli* from within the coding region and not from the *A. parasiticus* promoter, since the 5' end of the gene could be eliminated (plasmid pIV238) and the PRAI activity was still retained. In support of this inference clone λAptrpM1 (Figure 12), which contained only the C-terminal portion of the *A. parasiticus trpC*$^+$ gene, was able to complement the PRAI deficiency in *E. coli*. The fact that pJH34 encoded a complete *A. parasiticus trpC*$^+$ gene but that only PRAI activity could be expressed in *E. coli* also supported this inference. This same pattern was observed for expression of the *A. nidulans trpC*$^+$ gene (Yelton et al., 1984) and the *N. crassa trp-1*$^+$ gene (Schechtman and Yanofsky, 1983). Expression of both PRAI and IGPS activities encoded by pLH23 in *E. coli* suggested that transcription from this construct was initiated at the *Serratia trp* promoter. However, the *A. parasiticus* DNA insert must have provided a Shine-Dalgarno sequence (ribosome binding site) for translation because both pRK9 and the *Serratia trp* promoter lack this sequence (Schechtman and Yanofsky, 1983).

Complementation of *E. coli* mutations is a common practice to isolate eukaryotic genes. However, since *E. coli* cells can not process eukaryotic intervening sequences (introns), in principle, only eukaryotic genes lacking introns can be cloned in this way. Consistent with this inference, available DNA sequences of the filamentous fungal *trpC*$^+$ genes isolated by complementation of *E. coli* mutations do not contain introns (Choi et al., 1988; Kos et al., 1988; Mullaney et al., 1985; Revuelta and Jayaram, 1987; Schechtman and Yanofsky, 1983). Moreover, attempts to clone the *A. parasiticus pyrG*$^+$ gene by complementation of *E. coli pyrF* mutants (ATCC 35673, DB6656 [Bach et al., 1979] and MC1066) were not successful, presumably due to the existence of intron(s). In support of this view, genes in the pyrimidine biosynthetic pathway of *N. crassa* (Newbury et al., 1986) and *P. anserina* (Béguert et al., 1984) isolated by complementation of *E. coli* mutations had no introns, whereas *A. nidulans pyrG*$^+$ gene which had one intron could not complement the *E. coli pyrF* mutation (Oakley et al., 1987).

Expression of the trifunctional *trpC*$^+$ genes is regulated differently among fungi. For instance, in *A. nidulans*, the level of the *trpC*$^+$ transcript from cultures grown in a minimal medium is considerably higher than those grown in tryptophan-rich medium (Yelton et al., 1983). In contrast, cultures of *C. heterostrophus* (Turgeon et al., 1986) and *P. blakesleeanus* (Revuelta and Jayaram, 1987) grown in minimal or rich media show no significant differences in the amount of *TRP1*$^+$ mRNA. In *A. parasiticus*, the amount of the *trpC*$^+$ mRNA did not appear to be affected by the presence or absence of tryptophan in the culture medium.

There is increasing interest in studies of heterologous gene expression. Apart from the practical applications of developing new gene transfer systems,

heterologous gene expression studies are of interest from the evolutionary standpoint in order to see how conserved transcription and regulatory signals are between the major taxonomic groups of fungi. Studies with ascomycete fungi (see Table 5 for summary) have shown that it is not only possible to transfer genes between different fungal species but that these genes can still be metabolically regulated, thus there is conservation of both transcriptional and regulatory signals. The demonstration in this study that the *A. parasiticus trpC*$^{+}$ gene was able to complement the *A. nidulans trpC* mutant in transformation supported this generalization. It will be interesting to see if *trpC*$^{+}$ genes from different fungi can be expressed in other heterologous fungal hosts. Casselton and de la Fuente Herce (1989) had shown that a *trp-2* mutant of the basidiomycete *C. cinereus* (corresponding to the *trpC* mutant in *Aspergillus*) could be complemented by *trpC*$^{+}$ genes from two other basidiomycete species, *S. commune* and *P. chrysosporium*, but not by the *trpC*$^{+}$ gene from the ascomycete *A. nidulans*. Cotransformation experiments indicated that the *A. nidulans trpC*$^{+}$ gene had integrated into the *Coprinus* genome but was not expressed. Too little sequence data are available at present to understand the restraints which may limit gene expression across different taxonomic groups of fungi. It should be noted that the ascomycete gene lacks the CAAT and TATAAA motifs characteristic of the promoters of higher eukaryotic genes (Hamer and Timberlake, 1987), whereas these motifs are found in the promoter of the phycomycete gene (Revuelta and Jayaram, 1987).

To my knowledge, this report represents the first successful cloning of a gene from an aflatoxin-producing strain of *A. parasiticus*. This is particularly significant in the task of analyzing the aflatoxin biosynthetic pathway of this fungus

at the molecular level. For example, there exist several *A. parasiticus* mutants blocked at various stages of aflatoxin biosynthesis (Bennett and Papa, 1988). The *trpC*$^+$ gene vector could be used in complementation studies to isolate aflatoxin biosynthetic genes. This cloned *trpC*$^+$ gene will offer a useful selectable marker in parasexual analysis and for development of a DNA-mediated transformation system for *A. parasiticus*.

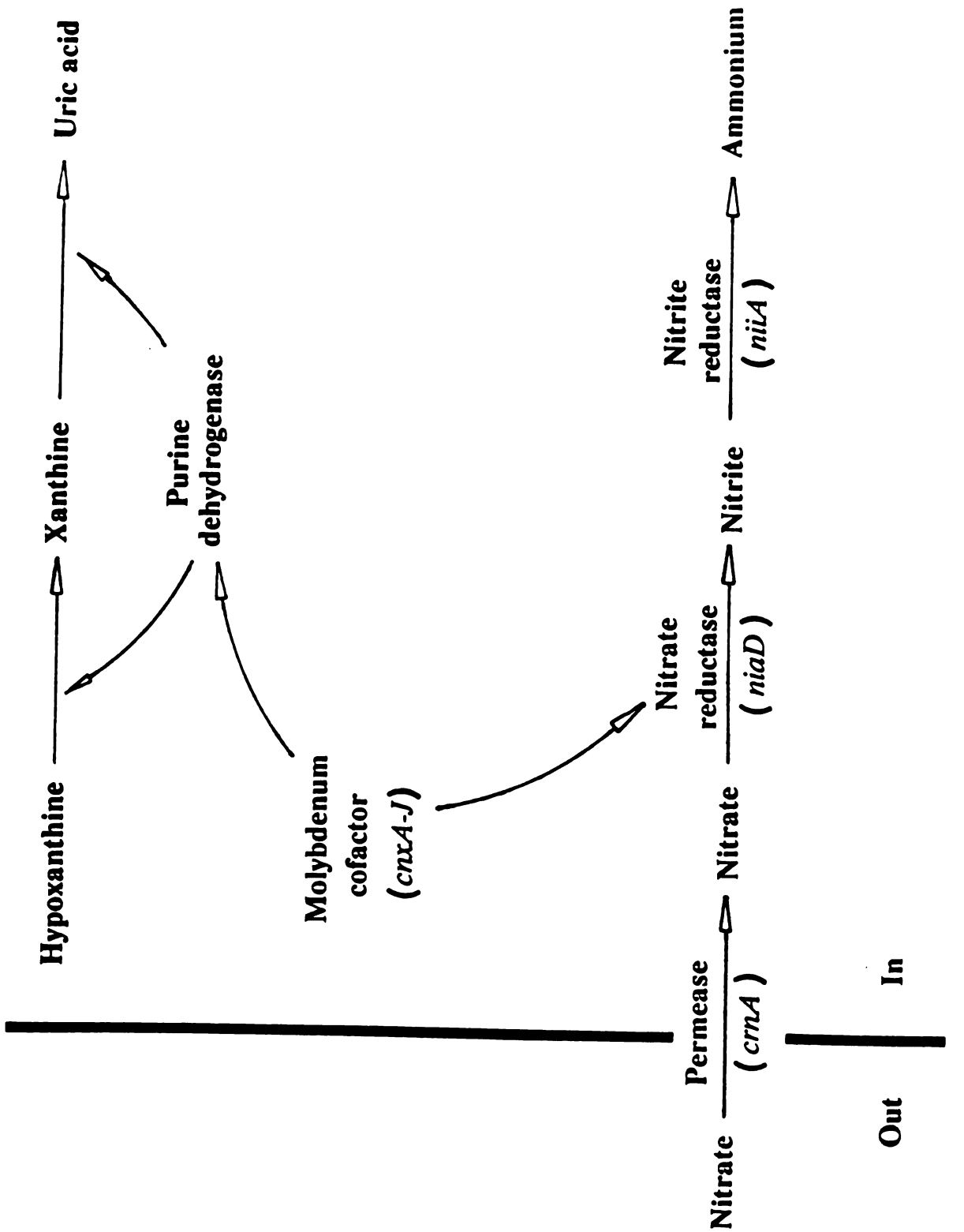
CHAPTER III

ISOLATION OF NITRATE-NONUTILIZING MUTANTS AND CLONING OF THE NITRATE REDUCTASE STRUCTURAL GENE (*NIAD*$^+$) FROM *ASPERGILLUS PARASITICUS*

I. INTRODUCTION

The assimilation of nitrate has been extensively studied at the biochemical and genetic levels in a variety of eukaryotic and prokaryotic organisms (Wray and Kinghorn, 1989). In filamentous fungi, the nitrate reduction pathway has been well-characterized in *N. crassa* (Marzluf, 1981; Marzluf et al., 1985) and *A. nidulans* (Cove, 1979). Extracellular nitrate is taken up by a permease (the product of *crnA*$^+$ in *A. nidulans*). Two enzymes are then induced to reduce nitrate to ammonium: nitrate reductase (*A. nidulans niaD*$^+$ and *N. crassa nit-3*$^+$ gene products), which converts nitrate to nitrite, and nitrite reductase (*A. nidulans niiA*$^+$ and *N. crassa nit-6*$^+$ gene products), which reduces nitrite to ammonium (Figure 19). Numerous genes, however, control the nitrate assimilation process in these fungi (at least 11 genes in *A. nidulans* and eight in *N. crassa*), and their regulation is complex (Cove, 1979; Marzluf et al., 1985). In general, the genes necessary for nitrate assimilation in *Aspergillus* and *Neurospora* appear to be similar in function. For example, both the nitrate and nitrite reductase structural polypeptides are encoded by single genes in these fungi. Both fungi also require several genes (at least five in *A. nidulans* and four in *N. crassa*) for the synthesis of a molybdenum-containing cofactor that is part of the nitrate reductase complex. This cofactor is also essential for purine dehydrogenase (Pateman et al., 1964) activity in the purine catabolism pathway. In addition to these structural genes, two regulatory genes are known in *A. nidulans* and *N. crassa*. The product of the nitrate-assimilation pathway-specific regulatory gene (the *A. nidulans nirA*$^+$ and the *N. crassa nit-4*$^+$ gene) controls the induction

Figure 19. The nitrate utilization pathway in *Aspergillus* spp. (modified from Correll et al., 1987). Extracellular nitrate is transported into the cell by a permease encoded by the *crnA*$^+$ gene, converted into nitrite by the action of nitrate reductase encoded by *niaD*$^+$ gene, and finally converted into ammonium ion by nitrite reductase, the *niiA*$^+$ gene product. Nitrate reductase shares a common molybdenum cofactor (encoded by a number of *cnx*$^+$ genes) with the hypoxanthine and xanthine (purine) dehydrogenase.


Uric acid
Xanthine
Hypoxanthine
Purine dehydrogenase
Molybdenum cofactor (cnxA-J)
Nitrate reductase (niaD)
Nitrite
Nitrite reductase (niiA)
Ammonium
Nitrate
Permease (crnA)
Nitrate
Out
In

of nitrate reductase and nitrite reductase. The gene product of a second locus, the major nitrogen regulatory gene (the *A. nidulans areA*$^+$ and the *N. crassa nit-2*$^+$ gene), represses the synthesis of both nitrate reductase and nitrite reductase whenever a preferred nitrogen source such as ammonium or glutamine is present (Cove, 1979; Marzluf et al., 1985).

An advantage of the nitrate system for genetic transformation is the ease with which recipient mutants can be generated on the basis of resistance to chlorate (Cove, 1976a; Tomsett and Garrett, 1980; Birkett and Rowlands, 1981; Correll et al., 1987; Newton and Caten, 1988). The positive screening of mutants deficient in nitrate reductase activity through the resistance to chlorate has been successfully used to develop a transformation system in a number of filamentous fungi (summarized in Table 5). This procedure is of general applicability and is particularly suitable for fungi without uninucleate spores such as *A. parasiticus*. In addition, the nitrate-nonutilizing, chlorate-resistant mutants can be easily characterized by their ability to grow on various nitrogen sources (Table 12). In filamentous fungi that have been investigated (Cove, 1979; Tomsett and Garrett, 1980; Birkett and Rowlands, 1981; Newton and Caten, 1988; Klittich and Lesile, 1988; Unkles et al., 1989a, 1989b), the nitrate reductase is encoded by a single gene, and mutants having a lesion in this gene can be recovered at a high frequency. Furthermore, the conserved homology among the nitrate reductase genes (Kinghorn and Campbell, 1989) has allowed identification of this gene from filamentous fungi using cloned heterologous genes as probes (Unkles et al., 1989a,b). Thus, it seems feasible that the nitrate reductase gene and appropriate mutants can be obtained and used to develop a transformation system for *A. parasiticus*.

II. MATERIALS AND METHODS

A. Fungal and bacterial strains

Escherichia coli strains HB101 or DH5α (Bethesda Research laboratories, Gaithersburg, MD) were used to propagate plasmids; strain LE392 was used for propagation of phage. The following parental strains of *A. parasiticus* were used for selection of nitrate-nonutilizing mutants: *A. parasiticus* NRRL 5862, a wild-type aflatoxigenic strain (Bennett and Papa, 1988); *A. parasiticus* ATCC 24690, a brown-spored mutant which accumulates a brick-red pigment, norsolorinic acid (Lee et al., 1970); *A. parasiticus* ATCC 36537, a white-spored mutant which accumulates a yellow pigment, versicolorin A (Lee et al., 1975). Both *A. parasiticus* ATCC 24690 and 36537 were derived from *A. parasiticus* NRRL 5862 and produced no detectable aflatoxins. *A. nidulans* FGSC A691 (*biA1; niaD15*), a nitrate reductase mutant obtained from the Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas), was used in experiments for heterologous gene complementation.

B. Media

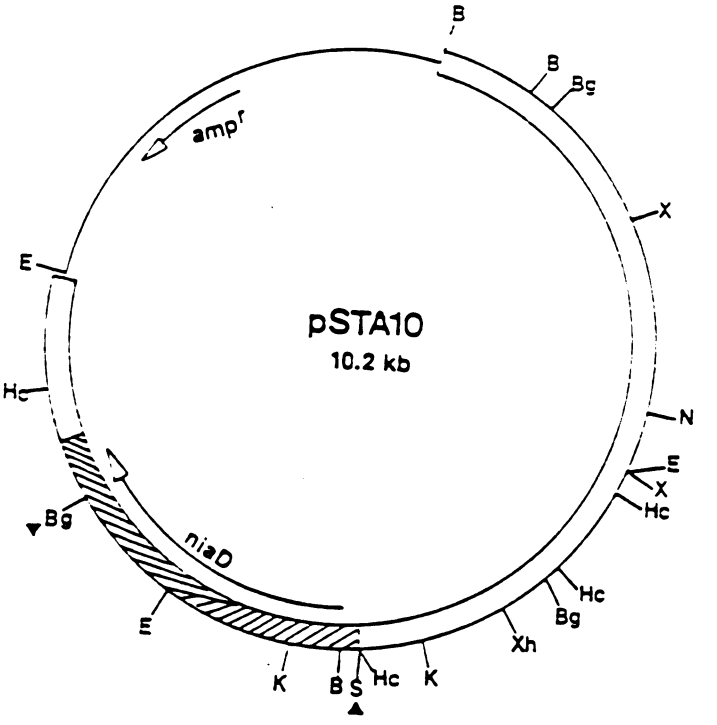
Media for growth of *E. coli*, *A. nidulans* and *A. parasiticus* were previously described (Chapter II, Horng et al., 1989). Biotin (10 μg/ml) was added to media for growth of *A. nidulans* FGSC A691. The synthetic low salt medium (SLS) (Reddy et al., 1971, 1979) and potato dextrose broth supplemented with 0.5% yeast extract (PDY) were used to grow *A. parasiticus* for protoplast preparation. A

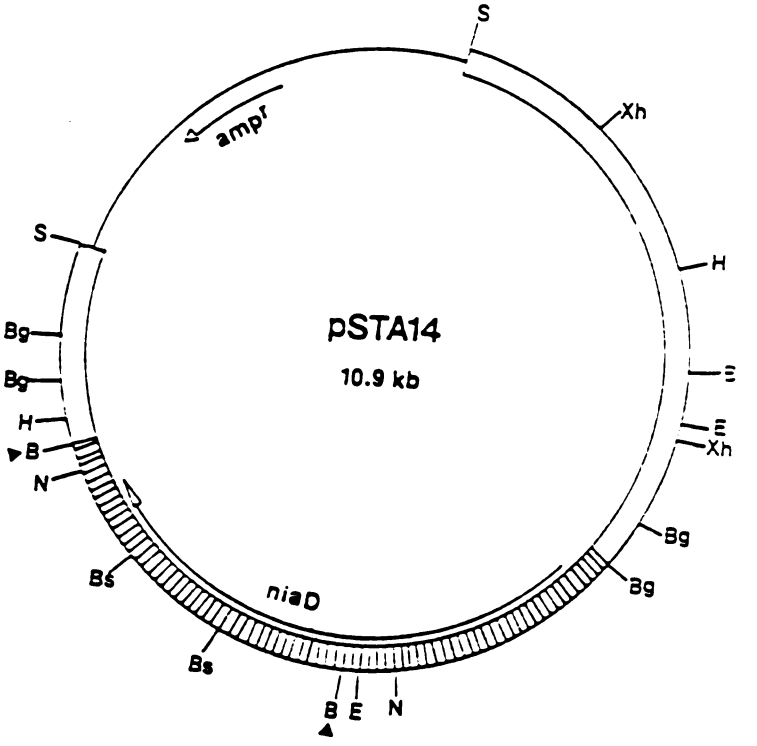
modified minimal medium for *A. nidulans* (Pontecorvo, 1953; Barratt et al, 1965) was used to select and propagate *A. parasiticus* mutants impaired in nitrate assimilation. This medium (MMG) consists of sodium L-glutamate (1.69 g), potassium chloride (0.52 g), magnesium sulfate.7 H_2O (0.52 g), potassium dihydrogen phosphate (1.52 g), glucose (10 g), zinc sulfate (250 mg), ferric sulfate (50 mg), agar (1.5%) and distilled water (1000 ml). The pH was adjusted to 6.5 with sodium hydroxide before sterilization. For growth tests of the chlorate-resistant mutants, the sodium L-glutamate was replaced with one of the following nitrogen sources: sodium nitrate (0.85 g/l); sodium nitrite (0.69 g/l); ammonium tartrate (1.84 g/l); hypoxanthine (0.1 g/l); uric acid (0.1 g/l). When specified, potassium chlorate was added to selective media at a concentration of 470 mM before autoclaving.

C. Vectors

Plasmids pSTA10 and pSTA14 (Figure 20; kindly supplied by J. R. Kinghorn, Plant Molecular Genetics Unit, U.K.) containing the *niaD*$^+$ gene of *A. niger* (Unkles et al., 1989b) and *A. oryzae* (Unkles et al., 1989a), respectively, were sources of probes used in heterologous hybridization experiments to isolate the analogous gene from *A. parasiticus*.

Figure 20. Restriction maps of *niaD*$^+$-containing plasmids pSTA10 and pSTA14. Plasmid pSTA10 (Unkles et al., 1989b) consists of a 7.5-kb *A. niger* genomic DNA fragment, which harbors the entire *niaD*$^+$ gene, inserted at the *Bam*HI site of plasmid pUC8. Plasmid pSTA14 (Unkles et al., 1989a) was constructed by ligating an 8.2-kb *Sal*I fragment, which contained the complete *A. oryzae niaD*$^+$ gene, with the *Sal*I-digested pUC18. One of the *Bam*HI sites on the polylinker in pSTA10 was disrupted during the cloning process. No cutting sites were found for enzymes *Acc*I, *Hin*dIII, *Pst*I, *Sma*I or *Xma*I on plasmid pSTA10, and for enzymes *Bsc*I, *Pst*I, *Sma*I, *Xba*I, or *Xma*I on plasmid pSTA14. The approximate start position for *niaD*$^+$ of *A. niger* and *A. oryzae* are determined by hybridization of restriction endonuclease-digested, Southern-blotted plasmids to an extreme 5' end of the *A. nidulans niaD*$^+$ fragment. The arrows indicate the direction of transcription as judged by hybridization to a 3' end of the *A. nidulans niaD*$^+$ probe. The approximate positions of the *niaD*$^+$ genes within both plasmids are indicated by solid bars; vector DNA sequences are represented by single lines. Small solid triangles indicate restriction sites used to generate DNA probes in heterologous hybridization analyses. Abbreviations are: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bss*HII; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; K, *Kpn*I; N, *Nru*I; S, *Sal*I; X, *Xba*I; Xh, *Xho*I.


pSTA10
10.2 kb
ampr
niaD
B
Bg
X
N
E
Hc
K
S


pSTA14
10.9 kb
ampr
niaD
S
Xh
H
Bg
N
Bs
E

D. General procedures

Standard recombinant DNA techniques were performed as previously described (Chapter II; Horng et al., 1989; Maniatis et al., 1982). Where specified, nonradioactive DNA probes generated with the ECL gene detection system (Amersham Corp.) were also used in Southern hybridization analyses according to the procedures of the supplier.

E. Isolation and analysis of nitrate-nonutilizing mutants

A. parasiticus spontaneous mutants defective in nitrogen assimilation were isolated by positive selection for resistance to chlorate on a minimal medium with sodium L-glutamate as the sole nitrogen source (Cove, 1979). Approximately 10^7 spores from frozen stocks were spread with a glass rod onto a single 85-mm Petri dish containing MMG supplemented with 470 mM potassium chlorate. Plates were incubated at 30°C. Chlorate-resistant colonies appeared were transferred with toothpicks onto fresh MMG plates supplemented with chlorate to confirm the chlorate resistance. Stable resistant mutants were subject to single spore purification (Roper and Fennel, 1965) on selective medium for three rounds before preservation as frozen spore stocks. For classification of mutants, chlorate-resistant colonies were tested for growth on media with chlorate and various nitrogen sources.

F. Assay of nitrate reductase

Mycelium for enzyme extraction was obtained from cultures inoculated with approximately 10^6 spores and grown overnight at 30°C in PDY. The mycelium was washed and then transferred to fresh minimal medium containing 10 mM nitrate as the sole nitrogen source and shaken for 6 more hours to induce the nitrate reductase activity.

Cell-free extracts were prepared by grinding frozen mycelium in liquid nitrogen and resuspending the powder (1 g) in cold 0.1 M phosphate buffer (pH 7.0). The resulting slurry was centrifuged at 4°C for 30 min (15,000 x g). The supernatant was used as a crude enzyme extract. Nitrate reductase activity was assayed with the colorimetric procedure described by Cove (1966). An assay mixture was made as follows: 200 µl sodium nitrate (337.5 mg/ml); 100 µl NADPH (tetrasodium salt) (16 mg/ml); 10 µl FAD (disodium salt) (1.05 mg/ml); 100 µl phosphate buffer (pH 7.75) (0.3 M); 100 µl cell extract and distilled water to give a final volume of 3 ml. After 20 min at room temperature, 0.5 ml of a 1% solution of sulfanilamide (dissolved in 25% HCl) was added followed by 0.5 ml of a 0.02% aqueous solution of N-(1-naphthyl)ethylenediamine hydrochloride. The resultant pink color was proportional to the amount of nitrite present, and was estimated by determining the absorbance of the assay mixture spectrophotometrically (540 nm). Control tubes lacking NADPH were used to correct for nitrite present in the extracts, and any turbidity caused by the samples.

G. Preparation of protoplasts from *A. parasiticus*

Protoplasts of *A. parasiticus* were prepared by a modification of the procedure of Yelton et al. (1984). Approximately 10^8 spores from frozen stocks were inoculated into a one-liter Erlenmeyer flask with 400 ml of SLS or PDY broth. Mycelia were harvested onto a disc of Miracloth (Calbiochem, San Diego) after overnight growth (16-20 h) at 37°C with shaking. The mycelia were washed with sterile distilled water followed by 0.6 M $MgSO_4$, and resuspended in an osmotic buffer (0.8-1.2 M $MgSO_4$ in 0.1 M sodium phosphate buffer, pH 5.8; 5 ml/g wet mycelium). A filter-sterilized hydrolytic enzyme solution consisting of Novozym 234 (5 mg/ml), β-glucuronidase (Sigma, 0.2 ml/g wet mycelium) and bovine serum album (12 mg/g wet mycelium) was added. The mixture was incubated at 30°C with gentle shaking for 2-3 h. Samples were withdrawn at intervals to check the progress of digestion and possible contamination. The digestion mixture was transferred into sterile centrifuge tubes and carefully overlaid with equal volume of ST buffer (1.2 M sorbitol in 10 mM Tris hydrochloride, pH 7.5). After centrifugation (4,000 x g for 15 min at 4°C using Sorval SS-34 fixed-angle rotor), the protoplasts which banded at the buffer interface were withdrawn with a sterile Pasteur pipette, washed three times by suspending in STC buffer (ST plus 10 mM $CaCl_2$) and centrifugation (2000-3000 rpm at room temperature for 5 min each) in a table-top centrifuge. Protoplasts were resuspended in a suitable volume of STC buffer to give a final concentration of approximately 10^8/ml as determined with a hemacytometer. The regeneration frequency of the protoplasts was determined by calculating the portion of the protoplasts that were capable of forming colonies in

MMG with 1.2 M sorbitol or 0.6 M KCl as osmotic stabilizers. This was generally found to be in the range of 5-15%, depending on the fungal strains used.

H. Transformation of fungi

For transformation of *A. parasiticus* a suitable amount (typically 5-10 μg in TE buffer) of DNA was mixed with approximately 10^7 protoplasts in a volume of 100-200 μl of STC buffer, followed by 50 μl of PEG solution [50% PEG 4000 (Fluka Chemical Corp. or Sigma Chemical Co.), 10 mM $CaCl_2$, 10 mM Tris hydrochloride, pH 7.5]. The mixture was allowed to stand on ice for 20 min. One ml of PEG solution was added, mixed thoroughly and incubated at room temperature for 30 min. The transformation mixture was then diluted with ten volumes of STC buffer, added to 400 ml of melted (precooled to 45 to 50°C) selection medium (MMG supplemented with 1.2 M sorbitol and 10 mM nitrate instead of glutamate as the sole nitrogen source). The mixture was poured into 15 to 20 Petri plates, and incubated at 30°C. Control protoplasts were treated as above but with vector DNA only (no insert) or without addition of DNA.

A. nidulans was transformed by a modified procedure of Oakley et al. (1987) as described previously (Chapter II).

III. RESULTS

A. Chlorate-resistant mutants of *A. parasiticus*

Spontaneous chlorate-resistant mutants of various colony sizes were readily recovered at a frequency of approximately one in 10^6 viable spores from all three strains of *A. parasiticus* tested after incubation for 7 to 10 days at 30°C (Table 11). Growth of wild-type strains was restricted on 470 mM chlorate. However, all nitrate-nonutilizing mutants were resistant to chlorate and showed wild-type growth on MMG. After transfer onto a fresh chlorate medium containing nitrate as the sole nitrogen source, those that grew as thin, expansive, nitrogen-starved colonies with little or no aerial mycelium were considered nitrate-nonutilizing mutants.

Selected mutants derived from all three *A. parasiticus* strains were isolated and purified on chlorate-containing minimal medium with glutamate as the sole source of nitrogen. The ability of these mutants to grow on nitrate, nitrite, ammonium, hypoxanthine, uric acid or glutamate as the sole nitrogen source was assessed. Chlorate resistant mutants could be divided into three phenotypic classes (Figure 21 and Table 12). These classes presumably represent a mutation at a nitrate reductase structural gene (*niaD*$^+$), a nitrate-assimilation pathway-specific regulatory gene (*nirA*$^+$), or genes (*cnx*$^+$) that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity. The phenotype designations used for *A. nidulans* have been adopted for comparable phenotypes in *A. parasiticus*. On MMG medium a high frequency of the *niaD*$^+$

Table 11. Identification of nitrate non-utilizing mutants from *A. parasiticus* on the basis of growth on different nitrogen sources.

Genotype	Putative mutation	Growth on nitrogen sources[b]					
		NO_3	NO_2	NH_4	HX	UA	MSG
niaD[a]	Nitrate reductase structural gene	-	+	+	+	+	+
niiA[a]	Nitrite reductase structural gene	-	-	+	+	+	+
cnx[a]	Molybdenum cofactor genes	-	+	+	-	+	+
crnA[a]	Permease gene for nitrate uptake	+	+	+	+	+	+
areA[a]	Major nitrogen regulatory gene	-	-	+	-	-	-
nirA	Pathway-specific regulatory gene	-	-	+	+	+	+
WT[a,d]	None	+	+	+	+	+	+

[a]: Mutants of this type were not isolated in this experiment but were included for completeness.

[b]: Growth on basal medium with various nitrogen sources at a concentration of 10 mM except for hypoxanthine (HX) and uric acid (UA) which were added at a concentration of 100 mg/l; +: typical wild-type growth, -: thin, expansive growth with no aerial mycelium; MSG= monosodium L-glutamate.

[c]: Several mutants are possible which are defective in the synthesis of the molybdenum cofactor required for nitrate reductase and purine dehydrogenase activities.

[d]: Wild-type strain.

phenotype was found among the chlorate-resistant mutants (Table 12). All three parental strains were able to overcome the inhibitory effect of chlorate when grown on media with uric acid or ammonium as a sole source of nitrogen, but not with the other nitrogen sources tested (Figure 21).

To confirm that the *niaD* mutants were deficient in nitrate reductase, enzyme activities were measured in crude extracts of selected mutants and wild-type strains. Only the wild-type strains, when induced, displayed nitrate reductase activity. No attempt was made to further characterize the *nirA* or the *cnx* mutants.

On testing selected *niaD* mutant strains for reversion to nitrate utilization, it was found that the frequency was generally less than one in 10^7 viable spores. Thus, the *niaD* mutants were suitable recipient strains for transformation, because transformation frequencies could be expected to exceed the spontaneous reversion frequency.

B. Cloning the *A. parasiticus niaD*$^+$ gene

To test if heterologous *niaD*$^+$ genes could be used as probes to identify the corresponding gene from *A. parasiticus*, internal restriction fragments from the *niaD*$^+$ genes of *A. niger* and *A. oryzae* (Figure 20) were purified, radiolabeled, and used to probe Southern blots of genomic DNA digests from two strains of *A. parasiticus*. Homology among *niaD*$^+$ genes of these three fungi were detected when low stringency hybridization and washing conditions were used (Figure 22),

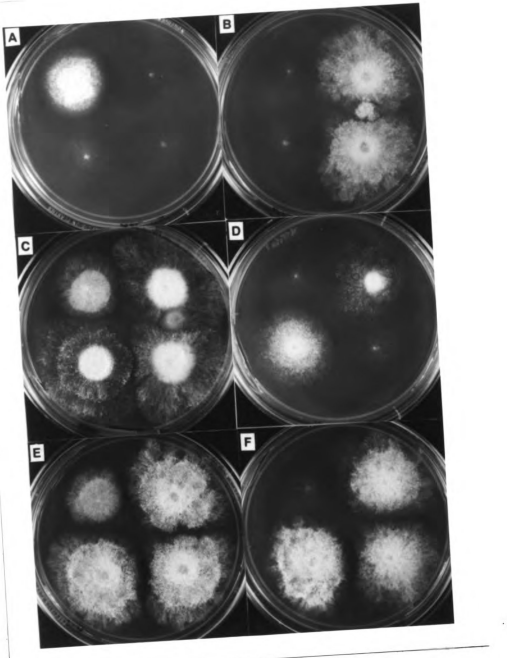
Table 12. Comparison of chlorate-resistant mutant types recovered from three strains of *A. parasiticus*[a]

Strain	Number[b]	Types of mutant		
		niaD	*nirA*	*cnx*
NRRL 5862	50	25	15	10
ATCC 24690	62	34	25	3
ATCC 36537	154	70	75	9

[a]: Chlorate-resistant mutants were recovered from basal media with 10 mM L-glutamate as the sole nitrogen source and supplemented with 470 mM potassium chlorate.

[b]: Numbers of chlorate-resistant mutants analyzed. Each number represents an average of results from two independent isolations.

Figure 21. Growth of wild-type and nitrate-nonutilizing mutant strains of *A. parasiticus* ATCC 36537 on media with various nitrogen sources. The arrangement of colonies on each plate is: upper left, wild-type strain ATCC 36537; upper right, *niaD* mutant; lower left, *nirA* mutant; lower right, *cnx* mutant. **A,** Nitrate medium (although difficult to see, all mutant strains on this plate showed thin, spidery growth with colonial diameter similar to the wild-type strain). **B,** Nitrite medium; note thin growth of the *nirA* mutant. **C,** Ammonium medium; note less growth of the wild-type strain. **D,** Hypoxanthine medium; note thin growth of the *cnx* mutant. **E,** Uric acid medium; note less growth of the wild-type strain. **F,** Glutamate medium; note thin growth of the wild-type strain. All but plate A contain 470 mM potassium chlorate. Pictures were taken after incubation for 7 days at 30°C.


A
B
C
D
E
F

indicating that it was feasible to clone the *A. parasiticus niaD*$^+$ gene by heterologous hybridization.

An amplified *A. parasiticus* genomic DNA library in phage lambda (Chapter II; Horng et al., 1989) was screened by *in situ* plaque hybridization for the nitrate reductase structural gene using probes prepared from *niaD*$^+$ genes of *A. niger* and *A. oryzae*. Three phage clones which hybridized strongly to these probes were identified and plaque-purified. Restriction digestion of DNAs purified from these clones with *Sal*I (which releases the DNA insert from the recombinant phage genome) indicated that they contained unique DNA inserts, approximately 17 kb in length, with overlapping regions. These clones were further analyzed by restriction digestion and Southern hybridization using internal DNA fragments from the *niaD*$^+$ gene of *A. oryzae* or *A. niger* as probes. The following unique fragments were identified which hybridized strongly to the *A. niger niaD*$^+$ probe (Figure 23): a 5.2-kb *Eco*RI (from clone #1), a 6.2-kb *Hin*dIII (from clones #2 and #3), a 5.4-kb *Sal*I (from clone #1), and an 8.2-kb *Sal*I (from clone #2 and #3) fragments. The same fragments also hybridized strongly to the *A. oryzae niaD*$^+$ probe (data not shown) and were detected in the genomic DNA blots (Figure 22, data for *Sal*I digests were not shown), suggesting that DNA inserts in these three phage clones were not rearranged during the cloning process. The *A. niger niaD*$^+$ gene probe hybridized strongly to the middle region of the 8.2-kb *Sal*I fragment (Figure 24), suggesting that this fragment is large enough to encode a complete copy of the *A. parasiticus niaD*$^+$ gene. This fragment was subcloned into vector pUC19 to yield plasmid pSL82 (Figure 24).

Figure 22. Identification of the *A. parasiticus* genomic DNA fragments containing the *niaD*$^+$ gene by Southern hybridization. Genomic DNAs from (1) *A. parasiticus* ATCC 36537 and (2) *A. parasiticus* NRRL 5862 were digested with restriction enzymes *Bam*HI (B), *Eco*RI (E) and *Hin*dIII (H). Restriction fragments were separated by electrophoresis through a 0.7% agarose gel, transferred onto a nitrocellulose membrane, and probed with a 1.7-kb ^{32}P-labeled *SalI-BglII* fragment purified from plasmid pSTA10 which contains the *A. niger niaD*$^+$ gene (**panel a**), or with a 2.0-kb ^{32}P-labelled *Bam*HI fragment purified from plasmid pSTA14 containing the *A. oryzae niaD*$^+$ gene (**panel b**). Hybridization (in 6x SSC, 5x Denhardt solution, 40% formamide, 0.1% SDS, 5 mM EDTA, 100 μg salmon sperm DNA per ml, 37°C) and washings (twice in 2X SSC-o.1% SDS at room temp for 40 min, followed by a final wash in 2x SSC-0.1% SDS at 42°C for 1 h) of blots were done under less stringent conditions. Molecular size markers in kilobases are indicated.

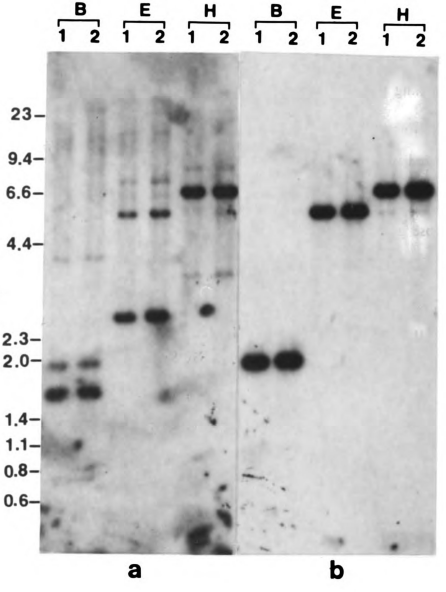

B
E
H
B
E
H
1 2 1 2 1 2 1 2 1 2 1 2
23
9.4
6.6
4.4
2.3
2.0
1.4
1.1
0.8
0.6
a
b

Figure 23. Southern analysis of lambda EMBL3 clones containing the *A. parasiticus niaD*$^+$ gene. Purified DNAs from three phage clones (1, 2 and 3) were digested with restriction enzymes *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), and *Sal*I (S), fractionated by electrophoresis in a 0.9% agarose gel, and transferred onto a nitrocellulose membrane. The blot was probed with a 1.7-kb *Sal*I-*Bgl*II fragment excised from plasmid pSTA10 which contained majority of the coding sequence for the *A. niger niaD*$^+$ gene. The probe was non-radioactively labeled by the ECL gene detection system from Amersham Corp. Due to a nonspecific binding region between top portions of lanes E2 and H2, an approximately 20-kb fragment on lane H1 which hybridized strongly to the probe was invisible. Light bands on lanes S1, S2 and S3 were caused by incomplete digestions. Molecular size markers in kb are indicated.

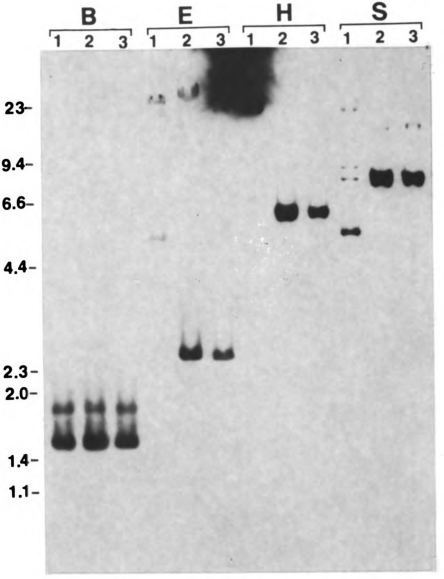
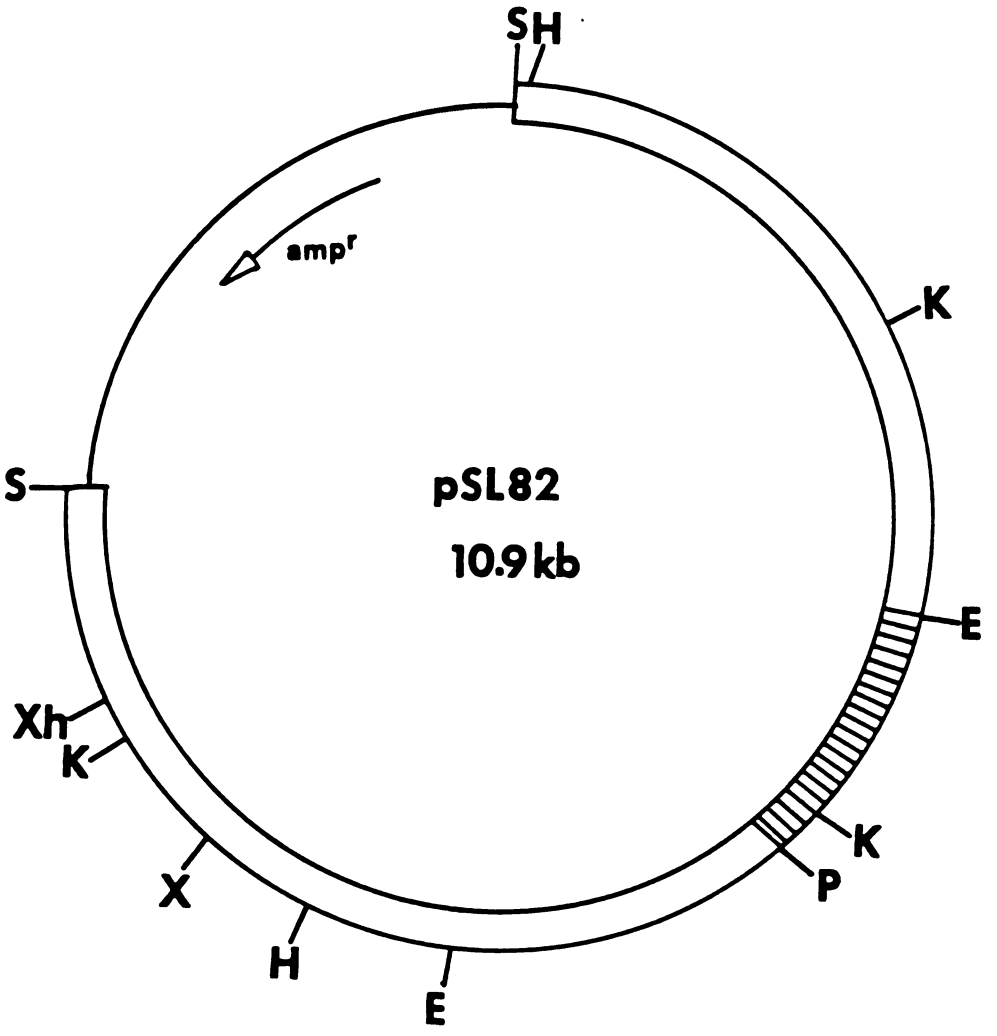

B
E
H
S
1 2 3 1 2 3 1 2 3 1 2 3
23-
9.4-
6.6-
4.4-
2.3-
2.0-
1.4-
1.1-

Figure 24. Restriction endonuclease map of plasmid pSL82. The double lines represent the 8.2-kb *SalI* DNA insert from *A. parasiticus*. Plasmid pUC19 is represented by a single line. A striped block represents the minimal coding region of the *A. parasiticus niaD*$^+$ gene which hybridizes strongly to the *A. niger niaD*$^+$ gene. Abbreviations for restriction enzymes are: E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; X, *Xba*I; Xh, *Xho*I.


SH
ampr
K
S
pSL82
10.9kb
E
Xh
K
K
P
X
H
E

IV. DISCUSSION

The wild-type strain of *A. parasiticus* is capable of utilizing nitrate as the sole nitrogen source. Nitrate-nonutilizing mutants of this fungus were readily obtainable by selecting spontaneous mutants that were resistant to chlorate. The genotype of these mutants could be easily characterized by growth tests on various nitrogen sources. Only three types of mutants (*niaD*, *nirA* and *cnx*) were isolated when glutamate was used as the sole nitrogen source in the chlorate selection medium. A significant portion (approximately 50%) of these chlorate-resistant mutants were *niaD* mutants. This high proportion of *niaD* mutants was consistent with results from Cove (1976b) who found that nitrogen sources had significant effects on relative frequencies and types of chlorate-resistant mutants recovered. Glutamate favored the selection of *niaD* mutants. Using L-arginine as the sole nitrogen source for selection, Papa (1986) also recovered three classes of chlorate-resistant mutants with similar phenotypes from *A. flavus*, an aflatoxigenic fungus closely-related to *A. parasiticus*. Therefore, nitrate assimilation in *A. parasiticus* appears to be very similar to that in *A. nidulans* in that both species are sensitive to chlorate and produce spontaneous mutants with similar physiological characteristics.

The *cnx*$^+$ loci encoding molybdenum cofactor in *A. parasiticus* were not further analyzed in this study, nor was the *nirA*$^+$ locus. Depending on species, there are 4 to 7 distinct *cnx*$^+$ loci in filamentous fungi (Unkles, 1989). These are generally distinguished from each other by complementation tests.

The structural gene for the nitrate reductase of *A. parasiticus* was isolated on the basis of its homology with analogous genes from *A. niger* and *A. oryzae*. The

identity of this gene is being characterized by subcloning and transformation of heterologous and homologous *niaD* mutants. Preliminary experiments indicated that plasmid pSL82 or DNA fragments carrying the cloned *A. parasiticus niaD*$^+$ gene were able to transform a putative *niaD* mutant derived from *A. parasiticus* ATCC 36537 at a frequency of approximately 100 transformants/μg DNA. However, attempted transformations of *A. nidulans* FGSC A691 with pSL82 were not successful. The reason for these failures remains unclear.

Chlorate resistance provides a system for the positive selection of mutations with an auxotrophic phenotype in several different genes and has wide potential applications. The procedure is economical in terms of labor and materials and, furthermore, does not require the use of mutagens, thereby avoiding problems resulting from the mutagenic induction of chromosomal aberrations or multiple mutations. The procedure is particularly suitable for isolation of mutants from genetically-undeveloped fungal strains such as *A. parasiticus*. This study has demonstrated that chlorate resistant mutants can be easily isolated from three strains of *A. parasiticus*, including two blocked mutants that are impaired in aflatoxin biosynthesis. These mutants are being used as recipient strains to develop a genetic transformation system for cloning genes associated with aflatoxin biosynthesis. In addition, chlorate resistance should be useful as a general marker in genetic mapping analyses to construct linkage maps. Furthermore, the *niaD*$^+$ gene represents a selectable marker which can be selected and counterselected. This feature will be extremely useful in designing gene replacement and disruption experiments (see Chapter I for discussion) for *A. parasiticus*. Moreover, chlorate resistant mutants of

A. flavus have been successfully used in studies of vegetative (heterokaryon) compatibility (Papa, 1986; Bayman and Cotty, 1989).

CHAPTER IV

ISOLATION OF A DNA FRAGMENT FROM A BENOMYL-RESISTANT MUTANT OF *ASPERGILLUS PARASITICUS* WITH HOMOLOGY TO THE *NEUROSPORA CRASSA TUB-2*$^{+}$ GENE

I. INTRODUCTION

The tubulins are a family of the principal protein subunits of microtubules in the eukaryotic cell cytoplasm. Tubulins polymerize into a diverse number of microtubule arrays whose assembly and functional properties are defined both by specific programs of cellular differentiation and by cell cycle determinants. Microtubules represent the principal structural components of mitotic and meiotic spindles. They participate in several aspects of intracellular transport, in maintenance of various cell surface properties, and establish overall cell shape and internal cytoplasmic architecture (Roberts and Hyams, 1979; Borisy et al., 1984).

Tubulin amino acid sequences are highly conserved among evolutionarily diverse organisms (Cleveland and Sullivan, 1985). This has allowed tubulin genes to be cloned and characterized from many different organisms. Multiple genes encoding different tubulin proteins are common in eukaryotes. For example, in the filamentous fungus *A. nidulans*, *tubA*$^+$ encodes two α-tubulin polypeptides and *tubB*$^+$ encodes the third α-tubulin. The β-tubulins parallel the α-tubulins in that the *benA*$^+$ locus codes for two β-tubulin polypeptides, and the *tubC*$^+$ locus codes for another (Morris, 1986). Recently, the γ-tubulin polypeptide, a new member of the tubulin superfamily encoded by the *mipA*$^+$ locus, was discovered in *A. nidulans* by Oakley and Oakley (1989). Fewer tubulin genes have been found in yeasts and other filamentous fungi. For example, two α-tubulin genes and one β-tubulin gene have been identified in the fission yeast *Schizosaccharomyces pombe* (Hiraoka et al., 1984; Toda et al., 1984), and in the budding yeast *Saccharomyces cerevisiae* (Neff et al., 1983; Schatz et al., 1986). *Neurospora crassa* contains only one β-tubulin

gene (Orbach et al., 1986). The dimorphic, pathogenic fungus *Histoplasma capsulatum* contains a single α- and a single β-tubulin gene (Harris et al., 1989).

Polymerization of tubulin monomers into microtubules is inhibited by the antimitotic fungicide benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate]. Benomyl resistance is caused by alternations in tubulins which apparently inhibit or reduce binding of benomyl to the tubulin protein (Davidse, 1986). In fungi, most mutations conferring resistance to benomyl map in the β-tubulin structural genes (Hiraoka et al., 1984; Neff et al., 1983; Sheir-Neiss et al., 1978; Thomas et al., 1985; Orbach et al., 1986). A mutant allele of the β-tubulin gene has been cloned from a benomyl-resistant strain of *N. crassa*, and was successfully used as a dominant selectable marker to transform this fungus (Orbach et al., 1986) and several others (summarized in Table 5).

Preliminary investigations have demonstrated that growth of *A. parasiticus* is completely inhibited by benomyl at a concentration of 5 μg/ml. Thus, this fungus potentially can be transformed to benomyl resistance. However, repeated transformations of *A. parasiticus* using the *N. crassa* benomyl resistance gene as a selectable marker were not successful, indicating that this marker either was not expressed or was insufficiently expressed in *A. parasiticus*. An attempt was thus initiated to clone the native β-tubulin gene from a benomyl-resistant strain of *A. parasiticus*, and to use it as a dominant selectable marker for transformation of this fungus. A similar strategy was used to develop a transformation system for *A. flavus* (Seip et al., 1989).

II. MATERIALS AND METHODS

A. General procedures

Purification of DNAs from fungi, phage particles and plasmids were previously described (Horng et al., 1989; Chapter II). Standard recombinant DNA procedures were performed as described by Maniatis et al. (1982).

B. Isolation of benomyl-resistant mutants

Approximately 10^8 spores of *A. parasiticus* ATCC 36537 were irradiated with ultraviolet (uv) light (254 nm) resulting in a 10% survival level. The treated spore suspension was spread onto a single Petri dish containing Czapek-Dox agar medium supplemented with benomyl (5 μg/ml) (DuPont Co, technical grade, 98% pure). Resistant colonies appeared after incubation at 30°C and were purified through single spore isolation. The level of resistance of these isolates to benomyl was determined on Czapek-Dox agar plates supplemented with various concentrations of benomyl.

C. Construction of a genomic DNA library

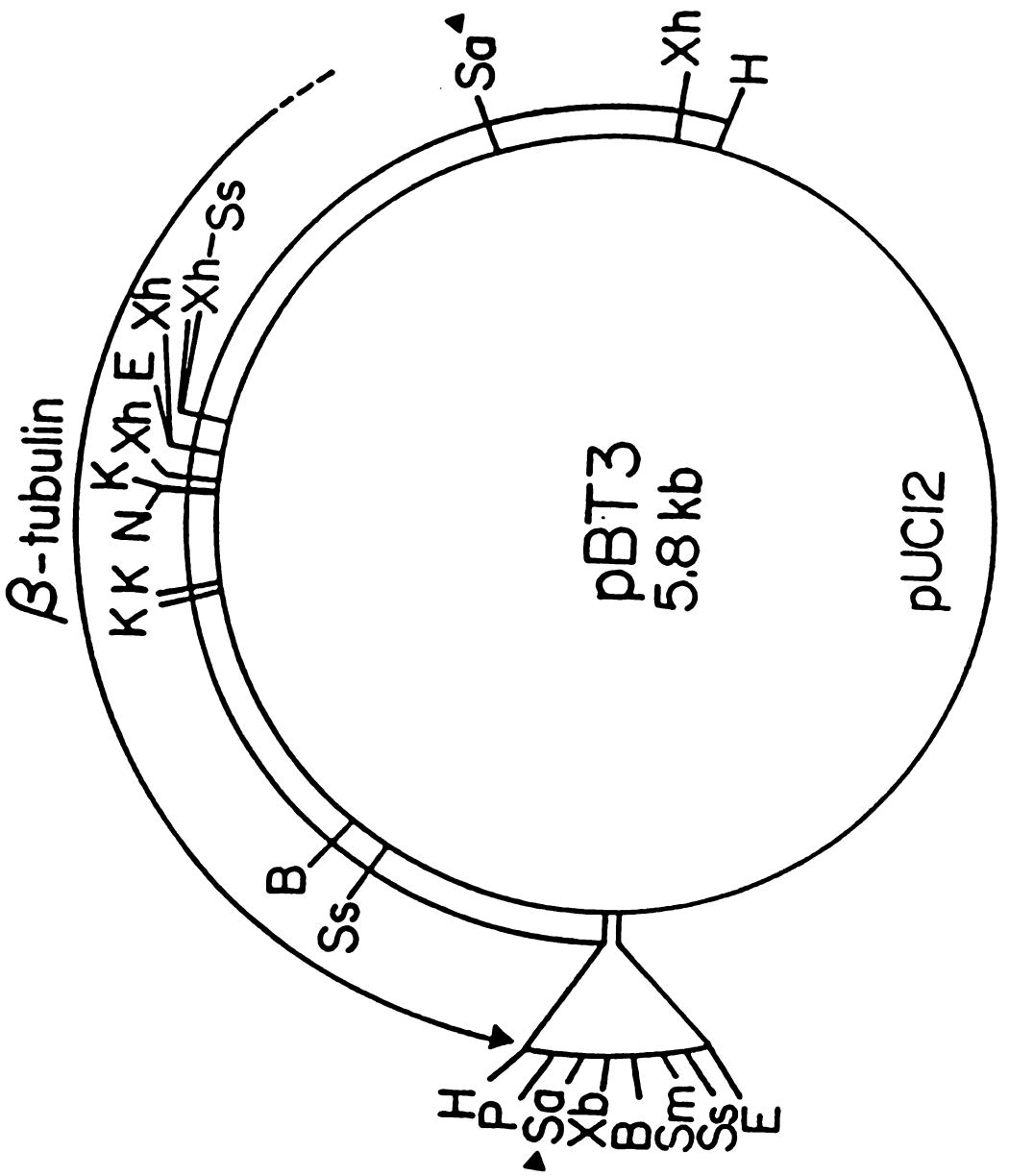
Chromosomal DNA was purified from a benomyl resistant isolate (20 μg/ml) of *A. parasiticus* ATCC 36537, and used to construct a genomic DNA library in the lambda vector EMBL3 following the procedures as described previously (Horng et al., 1989; Chapter II).

D. Conditions for Southern analysis and plaque hybridization

A 2.6-kb *Sal*I fragment excised from plasmid pBT3 (Figure 25) containing the cloned mutant allele of the *N. crassa* β-tubulin gene (Orbach et al., 1986) was used as a heterologous probe to identify the analogous β-tubulin gene of *A. parasiticus*. Southern hybridization analysis was conducted with the radiolabeled *N. crassa* β-tubulin gene probe and *A. parasiticus* genomic DNA on a nitrocellulose filter (BA 85, Schleicher & Schuell). The hybridization reaction was performed at 42°C in 6x SSC-5x Denhardt solution-40% formamide-0.1% SDS-100 μg of denatured salmon sperm DNA per ml. Filters were washed twice for 20 min at room temp in 2x SSC-0.1% SDS and then twice for 30 min at 63°C in 0.1x SSC-0.1% SDS.

The *A. parasiticus* lambda DNA library was screened by *in situ* plaque hybridization with the *N. crassa* β-tubulin probe as described previously (Chapter III). Filters with phage DNAs were hybridized at 45°C and washed at 52°C in the same solutions as described for genomic DNA blots.

Figure 25. Restriction endonuclease map of plasmid pBT3. The double lines represent the 3.1-kb *N. crassa* DNA insert. Plasmid pUC12 is represented by a single line. The pUC12 polylinker region is expanded on the left side of the plasmid. The line with an arrow, above the *N. crassa* DNA, represents the β-tubulin transcript. The dashes at the 5' end indicate that this end has not been determined precisely. Restriction sites indicated by solid triangles are used to prepare the DNA probe for use in heterologous hybridization. Restriction sites are abbreviated as follows: E, *Eco*RI; Ss, *Sst*I; Sm, *Sma*I; B, *Bam*HI; Xb, *Xba*I; Sa, *Sal*I; P, *Pst*I; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; Xh, *Xho*I. The map is complete for these enzymes. 1° represents 16 bp (Orbach et al., 1986).


β-tubulin
K K N K Xh E Xh
Xh-Ss
Sa
Xh
H
pBT3
5.8 kb
pUC12
B
Ss
H P Sa Xb B Sm Ss E

E. Transformation of *A. parasiticus*

Protoplasting and transformation of *A. parasiticus* were performed essentially as described previously (Chapter III). Benomyl-resistant transformants were selected by two methods. First, portions of the transformation mix were added to Czapek-Dox top agar (1%) containing 1.2 M sorbitol, and plated onto agar (1.5%) plates of similar composition, excluding the sorbitol and including benomyl at 5 μg/ml. Alternatively, portions of the transformation mixture were spread onto Czapek-Dox agar plates (1.5% agar) containing 1.2 M sorbitol. After incubation at 30°C overnight, top agar (1%) containing 5 μg/ml benomyl was gently overlaid on the surface of the medium. Benomyl was made fresh as a 0.5 mg/ml stock solution in 100% ethanol, and was added without further sterilization after the medium had been autoclaved.

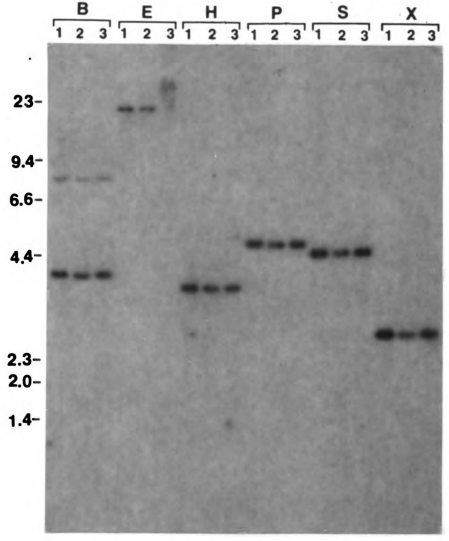
III. RESULTS AND DISCUSSION

Although the *N. crassa* benomyl resistance gene has been successfully used as a dominant selectable marker in transformation of several filamentous fungi (see Table 5), preliminary transformation studies on *A. parasiticus* with plasmid pBT3 and cosmid pSV50 (Vollmer and Yanofsky, 1986), both containing the mutant allele of the *N. crassa tub-2*$^{+}$ gene conferring resistance to benomyl (Orbach et al., 1986), suggested that this marker was not sufficiently expressed in *A. parasiticus* for selection of transformants. Similar results were also found (Woloshuk et al., 1989) in attempts at transformation of *A. flavus* with the benomyl resistance genes from *N. crassa* and *A. nidulans*.

DNA restriction fragments with strong homology to the cloned *N. crassa tub-2*$^{+}$ gene were identified in the genome of *A. parasiticus* using Southern analysis under high-stringency hybridization and washing conditions (Figure 26). These data indicated that it was feasible to identify the analogous β-tubulin gene in an *A. parasiticus* genomic DNA library using this heterologous probe.

A strain of *A. parasiticus* resistant to 20 μg/ml of benomyl was isolated by repeated mutagenesis with uv irradiation. Hybridization analysis failed to detect any difference in distribution of restriction fragments between the benomyl-resistant mutants and the wild-type strain of *A. parasiticus*. A genomic DNA library (1.4 x 10^5 PFU) was constructed in the vector lambda EMBL3 using genomic DNA purified from this strain. A total of 12,000 plaques from the library were screened at a density of 2,400 plaques per 82-mm Petri plate using a 2.6-kb *Sal*I restriction fragment excised from plasmid pBT3 as probe.

Figure 26. Identification of restriction DNA fragments from the *A. parasiticus* chromosome with homologies to the *N. crassa* β-tubulin gene. Purified genomic DNAs from *A. parasiticus* ATCC 36537 (lane 1), and its benomyl-resistant isolates (lane 2, resistant to 10 μg/ml benomyl; lane 3, resistant to 20 μg/ml benomyl) were digested with various restriction enzymes, fractionated by electrophoresis through a 0.8% agarose gel, and transferred onto a nitrocellulose membrane. The blot was hybridized with a radiolabeled 2.6-kb *Sal*I fragment excised from plasmid pBT3. The aberration in lane E3 was caused by an incomplete digestion of the genomic DNA by *Eco*RI. Abbreviations are: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xba*I. Molecular size markers in kb are indicated.


B
E
H
P
S
X
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
23-
9.4-
6.6-
4.4-
2.3-
2.0-
1.4-

Three plaques that hybridized strongly were recovered and purified. Restriction enzyme analyses of DNAs purified from these clones indicated that two of them were identical and shared few common restriction fragments with the third clone for most of the restriction enzymes tested (data not shown). The same restriction fragments present in the two identical phage clones, but not in the third clone, were also detected in the *A. parasiticus* chromosome, suggesting that the two identical phage clones probably contain a complete copy of the putative mutant allele of the *A. parasiticus* β-tubulin gene (designated *benr*), with the copy in the third clone being incomplete or rearranged. However, a 7.4-kb *Nsi*I fragment and a 12-kb *Sma*I fragment that hybridized strongly to the *N. crassa* β-tubulin probe were present in all three phage clones (data not shown). These fragments are being subcloned into plasmid pUC19. Further analyses of these plasmids by transformation of *A. parasiticus* and by DNA sequencing will provide insight into the identity and structure of the cloned β-tubulin gene.

Attempted transformations of *A. parasiticus* with DNAs purified from the three phage clones were not successful. The reasons for this failure remain unclear. However, there are several possible explanations. First, with the high-molecular-weight phage DNA, the transformation frequency may be too low to detect. Second, it is possible that the cloned gene did not confer resistance to benomyl, or conferred insufficient resistance for selection of transformants. Mutated β-tubulin genes are not responsible for all cases of benomyl resistance. For example, in the fungus *Sporobolomyces roseus* resistance to benomyl can be caused by the differential uptake of this fungicide by the sensitive and resistant strains (Nachmias and Barash, 1976). Alternatively, the cloned gene may not represent a mutated β-

tubulin gene, but an α- or a γ-tubulin gene which may not confer resistance to benomyl. This is reasonable since all the α- and β-tubulins reported to date share 36-42% identity with each other, and the γ-tubulin from *A. nidulans* is 33.3% identical to the corresponding β-tubulins (Oakley and Oakley, 1989). However, the hybridization reaction used to identify this gene was conducted under high-stringency conditions which tended to rule out this possibility. The screening procedure, in theory, should allow only β-tubulin gene to be isolated, unless the homology between the *N. crassa* β-tubulin gene and the *A. parasiticus* α- or γ-tubulin gene is stronger than that between the *N. crassa* β-tubulin gene and the *A. parasiticus* β-tubulin gene. Finally, the method and time of application of selective pressure in the current transformation protocol may not be appropriate for selection of benomyl-resistant transformants in *A. parasiticus*. Orbach et al. (1986) noted that if benomyl was included in the overlaying top agar, the transformation frequency for benomyl resistance of *N. crassa* was reduced 4- to 10-fold, suggesting that transformed cells require a growth period to allow expression of the benomyl resistance gene before selection is applied.

In conclusion, a systematic analysis is required to characterize the nature of the mutation in the benomyl-resistant mutants and the putatively cloned β-tubulin gene before they can be used in developing a transformation system for *A. parasiticus*.

CONCLUSIONS

Selectable markers are necessary for development of genetic transformation systems for cloning genes involved in the aflatoxin biosynthesis from *A. parasiticus*. Three such genetic markers, *trpC*$^+$, *niaD*$^+$, and *ben*^{r}, have been isolated and characterized from this fungus in this study, while a fourth marker, *pyrG*$^+$ was also isolated in the lab.

A transformation system based on the cloned *trpC*$^+$ gene as a selectable marker can be developed once suitable *trpC* mutants of *A. parasiticus* are available to serve as recipient strains. Attempts to isolate a *trpC* auxotroph from *A. parasiticus* by traditional mutagenesis and screening techniques were not successful, presumably due to the existence of multiple nuclei in its conidia and the coenocytic nature of the fungal cytoplasm. Gene disruption and replacement techniques as previously described offer an alternative strategy to obtain such a mutant. Plasmid pLH23 (Figure 14) and its deletion derivatives (Figure 17) that carry different truncated versions of the *A. parasiticus trpC*$^+$ gene are well suited for this purpose. This approach requires an established transformation system with an appropriate selectable marker for the primary selection. Successful development of transformation systems based on *niaD*$^+$ or the benomyl resistance marker should make this approach feasible.

The gene coding for nitrate reductase (*niaD*$^+$) provides another potential selectable marker to develop a transformation system for *A. parasiticus*. The *niaD*$^+$ gene has been cloned and mutants deficient in nitrate reductase activity have been isolated from *A. parasiticus*. A homologous transformation system has been developed based on the *niaD*$^+$ as the selectable marker. This is a transformation system of general applicability since this investigation has demonstrated that the

recipient mutants can be easily selected from wild-type and blocked mutant strains of *A. parasiticus* by virtue of their resistance to chlorate.

A gene (*ben*^{r}) has been cloned from a benomyl-resistant strain of *A. parasiticus* by virtue of its homology to the *N. crassa* β-tubulin gene. The *ben*^{r} gene potentially encodes a mutant allele of the β-tubulin gene which confers resistance to the fungicide benomyl, and can be used as a dominant selectable marker in transformation of *A. parasiticus* since this fungus is reasonably sensitive to benomyl. Comparison of DNA sequences between β-tubulin genes isolated from wild-type and the benomyl-resistant strains of *A. parasiticus* will shed light on the nature of the cloned benomyl resistance marker.

This work represents the first systematic effort to explore the aflatoxigenic *A. parasiticus* by the modern molecular genetic techniques. The wild-type *A. parasiticus* genomic DNA libraries constructed and preserved in this study are of sufficient quality to allow successful isolation of three genes (*trpC*$^+$, *niaD*$^+$, and *pyrG*$^+$), and should provide stable sources for obtaining more genetic markers from this fungus. Genes isolated in this study can be used as general markers for routine genetic analyses. Further characterization of these cloned genes by DNA sequence analysis will provide insight into the structure, organization, and nature of the regulatory elements for genes of this fungus, which should help in designing cloning and expression vectors to analyze the aflatoxin biosynthetic pathway.

LIST OF REFERENCES

REFERENCES

1. Adams, T. H., M. T. Boylan, and W. E. Timberlake. 1988. *brlA* is necessary and sufficient to direct conidiophore development in *Aspergillus nidulans*. Cell 54:353-362.

2. Adye, J., and R. I. Mateles. 1964. Incorporation of labelled compounds into aflatoxins. Biochim. Biophys. Acta 86:418-420.

3. Akins, R. A., and A. M. Lambowitz. 1985. General method for cloning *Neurospora crassa* nuclear genes by complementation of mutants. Mol. Cell. Biol. 5:2272-2278.

4. Alderson, T., and C. Scazzocchio. 1967. A system for the study of interlocus specificity for both forward and reverse mutations in at least eight gene loci in *Aspergillus nidulans*. Mutat. Res. 4:567-577.

5. Alic, M., J. R. Kornegay, D. Pribnow, and M. H. Gold. 1989. Transformation by complementation of an adenine auxotroph of the lignin-degrading basidiomycete *Phaerochaete chrysosporium*. Appl. Environ. Microbiol. 55:406-411.

6. Anderson, M. S., and M. F. Dutton. 1979. The use of cell free extracts derived from fungal protoplasts in the study of aflatoxin biosynthesis. Experientia 35:21-22.

7. Anderson, M. S., and M. F. Dutton. 1980. Biosynthesis of versicolorin A. Appl. Environ. Microbiol. 40:706-709.

8. Andrianopoulos, A., and M. J. Hynes. 1988. Cloning and analysis of the positively acting regulatory gene *amdR* from *Aspergillus nidulans*. Mol. Cell. Biol. 8:3532-3541.

9. Anné, J., H. Eyssen, and P. de Somer. 1974. Formation and regeneration of *Penicillium chrysogenum* protoplasts. Arch. Microbiol. 98:159-166.

10. Applebaum, R. S., and E. H. Marth. 1981. Biogenesis of the C_{20} polyketide, aflatoxin. Mycopathologia 76:103-114.

11. Aramayo, R., T. H. Adams, and W. E. Timberlake. 1989. A large cluster of highly expressed genes is dispensable for growth and development in *Aspergillus nidulans*. Genetics 122:65-71.

12. Arnau, J., F. J. Murillo, and S. Torres-Martínez. 1988. Expression of Tn5-derived kanamycin resistance in the fungus *Phycomyces blakesleeanus*. Mol. Gen. Genet. 212:375-377.

13. Arst, H. N., Jr., and C. Scazzocchio. 1975. Initiator constitutive mutation with an "up-promoter" effect in *Aspergillus nidulans*. Nature 254:31-34.

14. Arst, H. N., Jr., and C. Scazzocchio. 1985. Formal genetics and molecular biology of the control of gene expression in *Aspergillus nidulans*, pp. 309-343. *In* Bennett, J. W., and L. L. Lasure (eds.), Gene manipulations in fungi. Academic Press, Inc., New York, NY.

15. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K, Struhl. 1987. Current Protocols in molecular biology. John Wiley & Sons, New York.

16. Ayer, W. A., L. Pena-Rodriguez, and J. C. Vederas. 1981. Identification of sterigmatocystin as a metabolite of *Monocillium nordinii*. Can. J. Microbiol. 27:846-847.

17. Bach, M. L., F. Lacroute, and D. Botstein. 1979. Evidence for transcriptional regulation of orotidine-5'-phosphate decarboxylase in yeast by hybridization of mRNA to the yeast structural gene cloned in *E. coli*. Proc. Natl. Acad. Sci. USA 76:386-390.

18. Bachmann, B. J., and D. M. Bonner. 1959. Protoplasts from *Neurospora crassa*. J. Bacteriol. 78:550-556.

19. Balbás, P., X. Soberón, E. Merino, M. Zurita, H. Lomeli, F. Valle, Flores, N, and F. Bolivar. 1986. Plasmid vector pBR322 and its special purpose derivatives- a review. Gene 50:3-40.

20. Ballance, D. J., and G. Turner. 1985. Development of a high-frequency transforming vector for *Aspergillus nidulans*. Gene 36:321-331.

21. Ballance, D. J., and G. Turner. 1986. Gene cloning in *Aspergillus nidulans*: isolation of the isocitrate lyase gene (*acuD*). Mol. Gen. Genet. 202:271-275.

22. Ballance, D. J., F. P. Buxton, and G. Turner. 1983. Transformation of *Aspergillus nidulans* by the orotidine-5'-phosphate decarboxylase gene of *Neurospora crassa*. Biochem. Biophys. Res. Commun. 112:284-289.

23. Banks, G. R. 1983a. Transformation of *Ustilago maydis* by a plasmid containing yeast 2-micron DNA. Curr. Genet. 7:73-77.

24. Banks, G. R. 1983b. Chromosomal DNA sequences from *Ustilago maydis* promote autonomous replication of plasmids in *Saccharomyces cerevisiae.* Curr. Genet. 7:79-84.

25. Banks, G. R., and S. Y. Taylor. 1988. Cloning of the *PYR3* gene of *Ustilago maydis* and its use in DNA transformation. Mol. Cell. Biol. 8:5417-5424.

26. Barnes, D. E., and D. W. MacDonald. 1986. Behavior of recombinant plasmids in *Aspergillus nidulans*: structure and stability. Curr. Genet. 10:767-775.

27. Barratt, B. W., G. B. Johnson, and W. N. Ogata. 1965. Wild-type and mutant stocks of *Aspergillus nidulans*. Genetics 52:233-246.

28. Bartnicki-Garcia, S. 1968. Cell wall chemistry and taxonomy of fungi. Annu. Rev. Microbiol. 22:87-108.

29. Bayman, P., and P. J. Cotty. 1989. Vegetative compatibility groups in *Aspergillus flavus*. APS Annu. Meet. Abst. No. 406.

30. Bégueret, J., V. Razanamparany, M. Perrot, and C. Barreau. 1984. Cloning gene *ura5* for the orotidylic acid pyrophosphorylase of the filamentous fungus *Podospora anserina*: transformation of protoplasts. Gene 32:487-492.

31. Bej, A. K., and M. H. Perlin. 1989. A high efficiency transformation system for the basidiomycete *Ustilago violacea* employing hygromycin resistance and lithium-acetate treatment. Gene 80:171-176.

32. Bennett, J. W. 1979. Aflatoxins and anthraquinones from diploids of *Aspergillus parasiticus*. J. Gen. Microbiol. 113:127-136.

33. Bennett, J. W. 1981. Loss of norsolorinic acid and aflatoxin production by a mutant of *Aspergillus parasiticus*. J. Gen. Microbiol. 124:429-432.

34. Bennett, J. W. 1982. Genetics of mycotoxin production with emphasis on aflatoxins, pp. 549-561. *In* Krumphanzl, V., B. Sikyta, and Z. Vanek (eds.), Overproduction of microbial products. Academic Press, Inc., London.

35. Bennett, J. W., and S. B. Christensen. 1983. New perspectives on aflatoxin biosynthesis. Adv. Appl. Microbiol. 29:53-92.

36. Bennett, J. W., and E. Deutsch. 1986. Genetics of mycotoxin biosynthesis, pp. 51-64. *In* Steyn, P. S., and R. Vleggaar (eds.), Mycotoxins and phycotoxins. Elsevier Science Publishers, Amsterdam.

37. Bennett, J. W., and L. A. Goldblatt. 1973. The isolation of mutants of *Aspergillus flavus* and *A. parasiticus* with altered aflatoxin producing ability. Sabouraudia 11:235-241.

38. Bennett, J. W., and L. L. Lasure. 1985a. Gene manipulations in fungi. Academic Press, Inc., New York, NY.

39. Bennett, J. W., and L. L. Lasure. 1985b. Conventions for gene symbols, pp. 537-544. *In* Bennett, J. W., and L. L. Lasure (eds.), Gene manipulations in fungi. Academic Press, Inc., New York.

40. Bennett, J. W., and L. S. Lee. 1979. Mycotoxins- their biosynthesis in fungi: aflatoxins and other bisfuranoids. J. Food Prot. 42:805-809.

41. Bennett, J. W., and K. E. Papa. 1988. The aflatoxigenic *Aspergillus* spp. Adv. Plant Pathol. 6:263-280.

42. Bennett, J. W., L. S. Lee, and A. F. Cucullu. 1976. Effect of dichlorvos on aflatoxin and versicolorin A production in *Aspergillus parasiticus*. Bot. Gaz. 137:318-324.

43. Bennett, J. W., L. S. Lee, S. M. Shoss, and G. H. Boudreaux. 1980a. Identification of averantin as an aflatoxin B_1 precursor: placement in the biosynthetic pathway. Appl. Environ. Microbiol. 39:835-839.

44. Bennett, J. W., P. -M. Leong, S. Kruger, and D. Keyes. 1986. Sclerotial and low aflatoxigenic morphological variants from haploid and diploid *Aspergillus parasiticus*. Experientia 42:848-851.

45. Bennett, J. W., R. B. Silverstein, and S. J. Kruger. 1981a. Isolation and characterization of two nonaflatoxigenic classes of morphological variants of *Aspergillus parasiticus*. J. Amer. Oil Chem. Soc. 58:952A-955A.

46. Bennett, J. W., C. H. Vinnett, and W. R., Jr., Goynes. 1980b. Aspects of parasexual analysis in *Aspergillus parasiticus*. Can. J. Microbiol. 26:706-713.

47. Bennett, J. W., D. G. Wheeler, and J. J. Dunn. 1981b. Genetic analysis of aflatoxin production by *Aspergillus parasiticus*, pp. 417-422. *In* Moo-Young, M., C. Vezina, and K. Singh (eds.), Advances in biotechnology. Vol. III. Fermentation products. Pergamon Press, Toronto.

48. Berdy, J. 1980. Bleomycin type antibiotics, pp. 459-491. Handbook of antibiotic compounds, Vol. IV. CRC Press, Boca Raton, Florida.

49. Bergès, T., and C. Barreau. 1989. Heat shock at an elevated temperature improves transformation efficiency of protoplasts from *Podospora anserina*. J. Gen. Microbiol. 135:601-604.

50. Beri, R. K., and G. Turner. 1987. Transformation of *Penicillium chrysogenum* using the *Aspergillus nidulans amdS* gene as a dominant selective marker. Curr. Genet. 11:639-641.

51. Bernier, L., R. M. Cooper, A. K. Charnley, and J. M. Clarkson. 1989. Transformation of the entomopathogenic fungus *Metarhizium anisopliae* to benomyl resistance. FEMS Microbiol. Lett. 60:261-266.

52. Berse, B., A. Dmochowska, M. Skyrzypek, P. Weglenski, M. A. Bates, and R. L. Weiss. 1983. Cloning and characterization of the ornithine carbamoyltransferase gene from *Aspergillus nidulans*. Gene 25:109-117.

53. Betina, V. 1984. Mycotoxins- production, isolation, separation and purification. Elsevier Science Publishers, Amsterdam.

54. Bhatnagar, D., and T. E. Cleveland. 1988. Fate of the methyl group during the conversion of sterigmatocystin into O-methylsterigmatocystin and aflatoxin B_1 by cell-free preparations of *Aspergillus parasiticus*. Biochimie 70:743-747.

55. Bhatnagar, D., T. E. Cleveland, and A. R. Lax. 1989. Comparison of the enzymatic composition of cell-free extracts of non-aflatoxigenic *Aspergillus parasiticus* with respect to late stages of aflatoxin biosynthesis. Arch. Environ. Contam. Toxicol. 18:434-438.

56. Bhatnagar, D., S. P. McCormick, L. S. Lee, and R. A. Hill. 1987. Identification of O-methylsterigmatocystin as an aflatoxin B_1 and G_1 precursor in *Aspergillus parasiticus*. Appl. Environ. Microbiol. 53:1028-1033.

57. Bhatnagar, D., A. H. J. Ullah, and T. E. Cleveland. 1988. Purification and characterization of a methyltransferase from *Aspergillus parasiticus* SRRC 163 involved in aflatoxin biosynthetic pathway. Preparative Biochem. 18:321-349.

58. Binninger, D. M., C. Skrzynia, P. J. Pukkila, and L. A. Casselton. 1987. DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. EMBO J. 6:835-840.

59. Biollaz, M., G. Büchi, and G. Milne. 1968. Biosynthesis of aflatoxins: the biogenesis of bisfuranoids in the genus *Aspergillus*. J. Amer. Chem. Soc. 90:5017-5020.

60. Biollaz, M., G. Büchi, and G. Milne. 1970. The biosynthesis of aflatoxins. J. Amer. Chem. Soc. 92:1035-1043.

61. Birkett, J. A., and J. A. Roper. 1977. Chromosome aberrations in *Aspergillus nidulans*, pp 293-303. *In* Smith J. E., and J. A. pateman (eds.), Genetics and physiology of filamentous fungi. Academic Press, Inc., London.

62. Birkett, J. A., and R. T. Rowlands. 1981. Chlorate resistance and nitrate assimilation in industrial strains of *Penicillium chrysogenum*. J. Gen. Microbiol. 123:281-285.

63. Blakemore, E. J. A., M. J. Dobson, M. J. Hocart, J. A. Lucas, and J. F. Peberdy. 1989. Transformation of *Pseudocercosporella herpotrichoides* using two heterologous genes. Curr. Genet. 16:177-180.

64. Blount, W. P. 1961. Turkey "X" disease. J. Brit. Turkey Federation 9:55-58.

65. Boeke, J. D., F. LaCroute, and G. R. Fink. 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. 197:345-346.

66. Borisy, G. G., D. W. Cleveland, and D. G. Murphy. 1984. Molecular biology of the cytoskeleton. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

67. Bos, C. J. 1985. Protoplasts from fungal spores, pp. 73-85. *In* Peberdy, J. F., and L. Ferenczy (eds.), Fungal protoplasts: applications in biochemistry and genetics. Marcel Dekker, Inc., New York, NY.

68. Bos, C. J., and S. M. Slakhorst. 1981. Isolation of protoplasts from *Aspergillus nidulans* conidiospores. Can. J. Microbiol. 27:400-407.

69. Bos, C. J., A. J. M. Debets, A. W. van Heusden, and H. T. A. M. Schepers. 1983. Fusion of protoplasts from conidiospores of *Aspergillus nidulans*. Experientia (Suppl.) 45:298-299.

70. Bothast, R. J., and D. I. Fennell. 1974. A medium for rapid identification and enumeration of *Aspergillus flavus* and related organisms. Mycologia 66:365-369.

71. Botstein, D., S. C. Falco, S. E. Stewart, M. Brennan, S. Scherer, D. T. Stinchcomb, K. Struhl, and R. W. Davies. 1979. Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene 8:17-24.

72. Boylan, M. T., M. J. Holland, and W. E. Timberlake. 1986. *Saccharomyces cerevisiae* centromere *CEN11* does not induce chromosome instability when integrated into the *Aspergillus nidulans* genome. Mol. Cell. Biol. 6:3621-3625.

73. Boylan, M. T., P. M. Mirabito, C. E. Willett, C. R. Zimmerman, and W. E. Timberlake. 1987. Isolation and physical characterization of three essential conidiation genes from *Aspergillus nidulans*. Mol. Cell. Biol. 7:3113-3118.

74. Bradshaw, R. E., J. W. Bennett, and J. F. Peberdy. 1983. Parasexual analysis of *Aspergillus parasiticus*. J. Gen. Microbiol. 129:2117-2123.

75. Brygoo, Y., and R. Debuchy. 1985. Transformation by integration in *Podospora anserina*. I. Methodology and phenomenology. Mol. Gen. Genet. 200:128-131.

76. Bull, J. H., and J. C. Wootton. 1984. Heavily methylated amplified DNA in transformants of *Neurospora crassa*. Nature 310:701-704.

77. Bull, J. H., D. J. Smith, and G. Turner. 1988. Transformation of *Penicillium chrysogenum* with a dominant selectable marker. Curr. Genet. 13:377-382.

78. Bu'Lock, J. D. 1961. Intermediary metabolism and antibiotic synthesis. Adv. Appl. Microbiol. 3:293-342.

79. Bu'Lock, J. D. 1986. Genetic aspects of mycotoxin formation, pp. 1-12. *In* Kleinkauf, H., H. V. Dohren, H. Dornauer, and G. Nesemann (eds.), Regulation of secondary metabolite formation. VCH Verlagsgesellschaft mbH, Weinheim.

80. Bu'Lock, J. D., J. P. Mooney, and C. E. Wright. 1986a. Regulation of mycotoxin production by *Fusarium graminearum*: complementation effects between two mutant types. Biotechnol. Lett. 8:323-326.

81. Bu'Lock, J. D., C. E. Wright, and J. E. Mooney. 1986b. Use of a protoplast fusion test to establish the status of mycotoxin genes in an edible *Fusarium*. Biotechnol. Lett. 8:621-624.

82. Busby, W. F., and G. N. Wogan. 1981. Aflatoxins, pp. 3-27. *In* Shank, R. C. (ed.), Mycotoxins and N-nitroso compounds: environmental risks. Vol. II. CRC Press, Boca Raton, Florida.

83. Buxton, F. P., and A. Radford. 1983. Cloning of the structural gene for orotidine 5'-phosphate carboxylase of *Neurospora crassa* by expression in *Escherichia coli*. Mol. Gen. Genet. 190:403-405.

84. Buxton, F. P., and A. Radford. 1984. The transformation of mycelial spheroplasts of *Neurospora crassa* and the attempted isolation of an autonomous replicator. Mol. Gen. Genet. 196:339-344.

85. Buxton, F. P., D. I. Gwynne, and R. W. Davis. 1985. Transformation of *Aspergillus niger* using the *argB* gene of *Aspergillus nidulans*. Gene 37:207-214.

86. Buxton, F. P., D. I. Gwynne, S. Garven, S. Sibley, and R. W. Davis. 1987. Cloning and molecular analysis of the ornithine carbamoyl transferase gene of *Aspergillus niger*. Gene 60:255-266.

87. Cabib, E., B. Bowers, A. Sburlati, and S. J. Silverman. 1988. Fungal cell wall synthesis: the construction of a biological structure. Microbiol. Sci. 5:370-375.

88. Cambareri, E. B., B. C. Jensen, E. Schabtach, and E. C. Selker. 1989. Repeat-induced G-C to A-T mutations in *Neurospora*. Science 244:1571-1575.

89. Campbell, E. I., S. E. Unkles, J. A. Macro, C. van den Hondel, R. Contreras, and J. R. Kinghorn. 1989. Improved transformation efficiency of *Aspergillus niger* using the homologous *niaD* gen for nitrate reductase. Curr. Genet. 16:53-56.

90. Cantoral, J. M., B. Díez, J. L. Barredo, E. Alvarez, and J. F. Martín. 1987. High-frequency transformation of *Penicillium chrysogenum*. Bio/Technol. 5:494-497.

91. Caprioglio, D. R., and L. W. Parks. 1988. Purification and characterization of plasmid-like DNA from the antimycotic producing fungus, *Scytalidium flavo-brunneum*. Plasmid 20:175-181.

92. Caprioglio, D. R., and L. W. Parks. 1989. Temporal expression of transcription and relative copy number of plasmid pSFB-1 in *Scytalidium flavo-brunneum*. J. Bacteriol. 171:4876-4880.

93. Carlock, L. R. 1986. Analyzing lambda libraries. Focus 8(2):6-8.

94. Caroline, D. F. 1969. Pyrimidine synthesis in *Neurospora crassa*: gene-enzyme relationships. J. Bacteriol. 100:1371-1377.

95. Carramolino, L., M. Lozano, A. Pérez-Aranda, V. Rubio, and F. Sánchez. 1989. Transformation of *Penicillium chrysogenum* to sulfonamide resistance. Gene 77:31-38.

96. Casadaban, M., A. Martinez-Arias, S. Shapina, and J. Chow. 1983. Beta-galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. Methods Enzymol. 100B:293-308.

97. Case, M. E. 1986. Genetical and molecular analyses of *QA-2* transformants in *Neurospora crassa*. Genetics 113:569-587.

98. Case, M. E., M. Schweizer, S. R. Kushner, and N. H. Giles. 1979. Efficient transformation of *Neurospora crassa* by utilizing hybrid plasmid DNA. Proc. Natl. Acad. Sci. USA 76:5259-5263.

99. Casselton, L. A., and A. de la Fuente Herce. 1989. Heterologous gene expression in the basidiomycete fungus *Coprinus cinereus*. Curr. Genet. 16:35-40.

100. Castegnaro, M., M. Friesen, J. Michelon, and E. A. Walker. 1981. Problems related to the use of sodium hypochlorite in the detoxification of aflatoxin B_1. Amer. Ind. Hyg. Assoc. J. 42:398-401.

101. Castegnaro, M., D. C. Hunt, E. B. Sansone, P. L. Schuller, M. G. Siriwardana, G. M. Telling, H. P. van Egmond, and E. A. Walker. 1980. Laboratory decontamination and destruction of aflatoxins B_1, B_2, G_1, G_2 in laboratory wastes. International Agency for Research on Cancer, Lyon.

102. Chang, A. C. Y., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134:1141-1156.

103. Chattoo, B. B., F. Sherman, D. A. Azubalis, T. A. Fjellstedt, D. Mehvert, and M. Ogur. 1979. Selection of *lys2* mutants of the yeast *Saccharomyces cerevisiae* by the utilization of α-amino-adipate. Genetics 93:51-65.

104. Choi, H. T., J. L. Revuelta, C. Sadhu, and M. Jayaram. 1988. Structural organization of the *TRP1* gene of *Phycomyces blakesleeanus*: implications for evolutionary gene fusion in fungi. Gene 71:85-95.

105. Christensen, T., H. Woeldike, E. Boel, S. B. Mortensen, K. Hjortshoej, L. Thim, and M. T. Hansen. 1988. High level expression of recombinant genes in *Aspergillus nidulans*. Bio/Technol. 6:1419-1422.

106. Chu, S. B., and M. Alexander. 1972. Resistance and susceptibility of fungal spores to lysis. Trans. Br. Mycol. Soc. 58:489-497.

107. Cihlar, R., and P. S. Sypherd. 1980. The organization of the ribosomal RNA genes in the fungus *Mucor*. Nucleic Acids Res. 8:793-804.

108. Clark, L., and J. Carbon. 1976. A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire *E. coli* genome. Cell 9:91-99.

109. Clarke, L., and J. Carbon. 1978. Functional expression of cloned yeast DNA in *Escherichia coli*: specific complementation of argininosuccinate lyase (*argH*) mutations. J. Mol. Biol. 120:517-532.

110. Cleveland, D. W., and K. F. Sullivan. 1985. Molecular biology and genetics of tubulin. Annu. Rev. Biochem. 54:331-365.

111. Cleveland, T. E. 1989. Conversion of dihydro-O-methylsterigmatocystin to aflatoxin B_2 by *Aspergillus parasiticus*. Arch. Environ. Contam. Toxicol. 18:429-433.

112. Cleveland, T. E., and D. Bhatnagar. 1987. Individual reaction requirements of two enzyme activities, isolated from *Aspergillus parasiticus*, which together catalyze conversion of sterigmatocystin to aflatoxin B_1. Can. J. Microbiol. 33:1108-1112.

113. Cleveland, T. E., and D. Bhatnagar. 1988. Construction of a cDNA library from *Aspergillus parasiticus* mRNA isolated at the time of first expression of enzymes catalyzing aflatoxin biosynthesis. APS Annu. Meet. Abst. No. 608.

114. Cleveland, T. E., D. Bhatnagar, C. J. Foell, and S. P. McCormick. 1987. Conversion of a new metabolite to aflatoxin B_2 by *Aspergillus parasiticus*. Appl. Environ. Microbiol. 53:2804-2807.

115. Cleveland, T. E., A. R. Lax, L. S. Lee, and D. Bhatnagar. 1987. Appearance of enzyme activities catalyzing conversion of sterigmatocystin to aflatoxin B_1 in late-growth-phase *Aspergillus parasiticus* cultures. Appl. Environ. Microbiol. 53:1711-1713.

116. Clutterbuck, A. J. 1972. Absence of laccase from yellow-spored mutants of *Aspergillus nidulans*. J. Gen. Microbiol. 70:423-435.

117. Clutterbuck, A. J. 1977. The genetics of conidiation in *Aspergillus nidulans*, pp. 305-317. *In* Smith, J. E., and J. A. Pateman (eds.), Genetics and physiology of *Aspergillus*. Academic Press, Inc., London.

118. Colbére-Garapin, F., F. Horodniceanu, P. Kourilsky, and A. -C. Garapin. 1981. A new dominant hybrid selective marker for higher eukaryotic cells. J. Mol. Biol. 150:1-14.

119. Collis, C. M., and R. M. Hall. 1985. Identification of a Tn5 determinant conferring resistance to phleomycins, bleomycins and tallysomycins. Plasmid 14:143-151.

120. Cooley, R. N., R. K. Shaw, F. C. H. Franklin, and C. E. Caten. 1988. Transformation of the phytopathogenic fungus *Septoria nodorum* to hygromycin B resistance. Curr. Genet. 13:383-389.

121. Correll, J. C., C. J. R. Klittich, and J. F. Leslie. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. Phytopathol. 77:1640-1646.

122. Costanzo, M. C., and T. D. Fox. 1988a. Transformation of yeast by agitation with glass beads. Genetics 120:667-670.

123. Costanzo, M. C., and T. D. Fox. 1988b. Specific translational activation by nuclear gene products occurs in the 5' untranslated leader of a yeast mitochondrial mRNA. Proc. Natl. Acad. Sci. USA 85:2677-2681.

124. Cove, D. J. 1976a. Chlorate toxicity in *Aspergillus nidulans*: studies of mutants altered in nitrate assimilation. Mol. Gen. Genet. 146:147-159.

125. Cove, D. J. 1976b. Chlorate toxicity in *Aspergillus nidulans*: the selection and characterization of chlorate resistant mutants. Heredity 36:191-203.

126. Cove, D. J. 1979. Genetic studies of nitrate assimilation in *Aspergillus nidulans*. Biol. Rev. 54:291-327.

127. Crawford, I. P. 1975. Gene rearrangements in the evolution of the tryptophan synthetic pathway. Bacteriol. Rev. 39:87-120.

128. Crawford, I. P. 1989. Evolution of a biosynthetic pathway: the tryptophan paradigm. Annu. Rev. Microbiol. 43: 567-600.

129. Cullen, D., and S. A. Leong. 1986. Recent advances in the molecular genetics of industrial filamentous fungi. Trends Biotechnol. 4:285-288.

130. Cullen, D., G. L. Gray, L. J. Wilson, K. J. Hayenga, M. H. Lamsa, M. W. Rey, S. Norton, and R. M. Berka. 1987. Controlled expression and secretion of bovine chymosin in *Aspergillus nidulans*. Bio/Technol. 5:369-376.

131. Cullen D, S. A. Leong, L. J. Wilson, and D. J. Henner. 1987. Transformation of *Aspergillus nidulans* with the hygromycin-resistance gene, *hph*. Gene 57:21-26.

132. Daboussi, M. J., A. Djeballi, C. Gerlinger, P. L. Blaiseau, Bouvier, I, M. Cassan, M. H. Lebrun, D. Parisot, and Y. Brygoo. 1989. Transformation of seven species of filamentous fungi using the nitrate reductase gene of *Aspergillus nidulans*. Curr. Genet. 15:453-456.

133. Darlington, A. J., and C. Scazzocchio. 1967. Use of analogues and the substrate-sensitivity of mutants in analysis of purine uptake and breakdown in *Aspergillus nidulans*. J. Bacteriol. 93:937-940.

134. Das, S., E. Kellermann, and C. P. Hollenberg. 1984. Transformation of *Kluyveromyces fragilis*. J. Bacteriol. 158:1165-1167.

135. Davidse, L. C. 1986. Benzimidazole fungicides: mechanism of action and biological impact. Annu. Rev. Phytopathol. 24:43-65.

136. Davies, J. E., D. Kirkaldy, and J. C. Robert. 1960. Studies in mycological chemistry. Part VII. Sterigmatocystin, a metabolite of *Aspergillus versicolor* (Vuillemin) Tiraboschi. J. Chem. Soc. 1960:2169-2178.

137. Davis, B. 1985. Factors influencing protoplast isolation, pp. 45-71. *In* Peberdy, J. F., and L. Ferenczy (eds.), Fungal protoplasts: applications in biochemistry and genetics. Marcel Dekker, Inc., New York, NY.

138. Davis, M. A., C. S. Cobbett, and M. J. Hynes. 1988. An *amdS-lacZ* fusion for studying gene regulation in *Aspergillus*. Gene 63:199-212.

139. Davis, M. A., and M. J. Hynes. 1987. Complementation of *areA*⁻-regulatory gene mutations of *Aspergillus nidulans* by the heterologous regulatory gene *nit-2* of *Neurospora crassa*. Proc. Natl. Acad. Sci. USA. 84:3753-3757.

140. Davis, N. D. 1981. Sterigmatocystin and other mycotoxins produced by *Aspergillus* species. J. Food Prot. 44:711-714.

141. Davis, N. D., S. K. Iyer, and U. L. Diener. 1987. Improved method of screening for aflatoxin with a coconut agar medium. Appl. Environ. Microbiol. 53:1593-1595.

142. Davis, R. H., and F. J. de Serres. 1970. Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. 17:79-143.

143. Davis, R. W., D. Botstein, and J. R. Roth. 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

144. de Graaff, L., H. van den Broeck, and J. Visser. 1988. Isolation and transformation of the pyruvate kinase gene of *Aspergillus nidulans*. Curr. Genet. 13:315-321.

145. de Ruiter-Jacobs, Y. M. J. T., M. Broekhuijsen, S. E. Unkles, E. I. Campbell, J. R. Kinghorn, R. Contreras, P. H. Pouwels, and C. A. M. J. J. van den Hondel. 1989. A gene transfer system based on the homologous *pyrG* gene and efficient expression of bacterial genes in *Aspergillus oryzae*. Curr. Genet. 16:159-163.

146. Dean, F. M. 1963. Naturally occurring oxygen ring compounds. Butterworths, London.

147. Debuchy, R., E. Coppin-Raynel, D. Le Coze, and Y. Brygoo. 1988. Chromosome walking towards a centromere in the filamentous fungus *Podospora anserina*: cloning of a sequence lethal at a low-copy state. Curr. Genet. 13:105-111.

148. Delorme, E. 1989. Transformation of *Saccharomyces cerevisiae* by electroporation. Appl. Environ. Microbiol. 55:2242-2246.

149. Detroy, R. W., E. B. Lillehoj, and A. Ciegler. 1971. Aflatoxins and related compounds, pp 4-178. *In* Ciegler, A., S. Kadis, and S. J. Ajl (eds), Microbial toxins, Vol. VI. Fungal toxins. Academic Press, Inc., New York, NY.

150. De Vogel, P., R. Van Rhee, and W. A. A. Blanche-Koelensmid. 1965. A rapid screening test for aflatoxin-synthesizing aspergilli of the *flavus-oryzae* group. J. Appl. Bacteriol. 28:213-220.

151. de Vries, O. M. H., and J. G. H. Wessels. 1972. Release of protoplasts from *Schizophyllum commune* by a lytic preparation from *Trichoderma viride*. J. Gen. Microbiol. 73:13-22.

152. Dhawale, S. S., and G. A. Marzluf. 1985. Transformation of *Neurospora crassa* with circular and linear DNA and analysis of the fate of the transforming DNA. Curr. Genet. 10:205-212.

153. Dhawale, S. S., J. V. Paietta, and G. A. Marzluf. 1984. A new, rapid and efficient transformation procedure for *Neurospora*. Curr. Genet. 8:77-79.

154. Diallinas, G., and C. Scazzocchio. 1989. A gene coding for the uric acid-xanthine permease of *Aspergillus nidulans*: inactivational cloning, characterization, and sequence of a cis-acting mutation. Genetics 122:341-350.

155. Dickinson, L., M. Harboe, R. van Heeswijck, P. Stroman, and L. P. Jepsen. 1987. Expression of active Mucor miehei aspartic protease in *Mucor circinelloides*. Carlsberg Res. Commun. 52:243-252.

156. Dickman, M. B. 1988. Whole cell transformation of the alfalfa fungal pathogen *Colletotrichum trifolii*. Curr. Genet. 14:241-246.

157. Dickman, M. B., and J. F. Leslile. 1989. Regulation of nitrogen metabolism in *Fusarium* by a homologous *Neurospora* gene. APS Annu. Meet. Abst. No. 296.

158. Diener, J. S., and N. D. Davis. 1969. Aflatoxin formation by *Aspergillus flavus*, pp. 13-54. *In* Goldblatt, L. A. (ed.), Aflatoxin: scientific background, control and implications. Academic Press, Inc., New York, NY.

159. Díez, B., E. Alvarez, J. M. Cantoral, J. L. Barredo, and J. F. Martín. 1987. Selection and characterization of *pyrG* mutants of *Penicillium chrysogenum* lacking orotidine-5'-phosphate decarboxylase and complementation by the *pyr4* gene of *Neurospora crassa*. Curr. Genet. 12:277-282.

160. Donkersloot, J. A., D. P. H. Hsieh, and R. I. Mateles. 1968. Incorporation of precursors into aflatoxin B_1. J. Amer. Chem. Soc. 90:5020-5021.

161. Donkersloot, J. A., R. I. Mateles, and S. S. Yang. 1972. Isolation of averufin from a mutant of *Aspergillus parasiticus* impaired in aflatoxin biosynthesis. Biochem. Biophys. Res. Commun. 47:1051-1055.

162. Dorner, J.W., R. J. Cole, and U. L. Diener. 1984. The relationship of *Aspergillus flavus* and *Aspergillus parasiticus* with reference to production of aflatoxins and cyclopiazonic acid. Mycopathologia 87:13-15.

163. Doy, C. H., J. A. Pateman, J. E. Olsen, H. J. Kane, and E. H. Creaser. 1985. Genomic clones of *Aspergillus nidulans* containing *alcA*, the structural gene for alcohol dehydrogenase and *alcR*, a regulatory gene for ethanol metabolism. DNA 4:105-114.

164. Duncan, J. S., and J. D. Bu'Lock. 1985. Degeneration of zearalenone production in *Fusarium graminearum*. Exp. Mycol. 9:133-140.

165. Duncan, K., and J. R. Coggins. 1984. Subcloning of the *Escherichia coli* genes *aro5* (5-enolpyruvyl shikimate 3-phosphate synthase) and *aroB* (3-dehydroquinate synthase). Biochem. Soc. Trans. 12:274-275.

166. Duncan, K., and J. R. Coggins. 1986. The *serC-aroA* operon of *Escherichia coli*: a mixed function operon encoding enzymes from two different amino acid biosynthetic pathways. Biochem. J. 234:49-57.

167. Dunne, P. W., and B. R. Oakley. 1988. Mitotic gene conversion, reciprocal recombination and gene replacement at the *benA*, beta-tubulin, locus of *Aspergillus nidulans*. Mol. Gen. Genet. 213:339-345.

168. Dutton, M. F. 1988. Enzymes and aflatoxin biosynthesis. Microbiol. Rev. 52:274-295.

169. Dutton, M. F., and M. S. Anderson. 1978. The use of fungal protoplasts in the study of aflatoxin biosynthesis. Experientia 34:22-24.

170. Dutton, M. F., and M. S. Anderson. 1982. Role of versicolorin A and its derivatives in aflatoxin biosynthesis. Appl. Environ. Microbiol. 43:548-551.

171. Dutton, M. F., K. Ehrlich, and J. W. Bennett. 1985. Biosynthetic relationship among aflatoxins B_1, B_2, M_1, and M_2. Appl. Environ. Microbiol. 49:1392-1395.

172. Emerson, S., and M. R. Emerson. 1958. Production, reproduction and reversion of protoplastlike structures in the osmotic strain of *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 44:668-671.

173. Enns, R. K., and R. S. Criddle. 1977. Affinity labelling of yeast mitochondrial adenosine triphosphatase by reduction with [^{3}H]borohybride. Arch. Biochem. Biophys. 182:587-600.

174. Esser, K., and G. Mohr. 1986. Integrative transformation of filamentous fungi with respect to biotechnological application. Process Biochem. 21:153-159.

175. Farkaš, V. 1985. The fungal cell wall, pp. 3-29. *In* Peberdy, J. F., and L. Ferenczy (eds.), Fungal protoplasts: application in biochemistry and genetics. Marcel Dekker, Inc., New York, NY.

176. Farman, M. L., and R. P. Oliver. 1988. The transformation of protoplasts of *Leptosphaeria maculans* to hygromycin B resistance. Curr. Genet. 13:327-330.

177. Faugeron, G., C. Goyon, and A. Gregoire. 1989. Stable allele replacement and unstable non-homologous integration events during transformation of *Ascobolus immersus*. Gene 76:109-119.

178. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.

179. Feinberg, A. P., and B. Vogelstein. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Addendum. Anal. Biochem. 137:266-267.

180. Felenbok, B., D. Sequeval, M. Mathieu, S. Sibley, D. I. Gwynne, and R. W. Davies. 1988. The ethanol regulon in *Aspergillus nidulans*: characterization and sequence of the positive regulatory gene *alcR*. Gene 73:385-396.

181. Fernández-Larrea, J., and U. Stahl. 1989. Transformation of *Podospora anserina* with a dominant resistance gene. Curr. Genet. 16:57-60.

182. Fincham, J. 1988. Hazards in sexual transformation. Nature 331:207-208.

183. Fincham, J. R. S. 1989. Transformation in fungi. Microbiol. Rev. 53:148-170.

184. Fincham, J. R. S., I. F. Connerton, E. Notarianni, and K. Harrington. 1989. Premeiotic disruption of duplicated and triplicated copies of the *Neurospora crassa am* (glutamate dehydrogenase) gene. Curr. Genet. 15:327-334.

185. Fischbach, H., and A. D. Campbell. 1965. Note on detoxification of the aflatoxins. J. Assoc. Off. Anal. Chem. 48:28.

186. Floyd, J. C., and J. W. Bennett. 1981. Preparation of ^{14}C-labeled aflatoxins and incorporation of unlabeled aflatoxins in a blocked versicolorin A-accumulating mutant of *Aspergillus parasiticus*. J. Amer. Oil Chem. Soc. 58:956A-959A.

187. Floyd, J. C., J. C. III., Mills, and J. W. Bennett. 1987. Biotransformation of sterigmatocystin and absence of aflatoxin biotransformation by blocked mutants of *Aspergillus parasiticus*. Exp. Mycol. 11:109-114.

188. Fotheringham, S., and W. K. Holloman. 1989. Cloning and disruption of *Ustilago maydis* genes. Mol. Cell. Biol. 9:4052-4055.

189. Fourney, R. M., J. Miyakoshi, R. S. III., Day, and M. C. Paterson. 1988. Northern blotting: efficient RNA staining and transfer. Focus 10:5-7.

190. Fox, T. D., J. C. Sanford, and T. W. McMullin. 1988. Plasmids can stably transform yeast mitochondria lacking endogenous mtDNA. Proc. Natl. Acad. Sci. USA 85:7288-7292.

191. Fraley, R., and D. Papahadjopoulos. 1981. New generation liposomes: the engineering of an efficient vehicle for intracellular delivery of nucleic acids. Trends Biochem. Sci. 6:77-80.

192. Frederick, G. D., D. K. Asch, and J. A. Kinsey. 1989. Use of transformation to make targeted sequence alterations at the *am* (GDH) locus of *Neurospora*. Mol. Gen. Genet. 217:294-300.

193. Frischauf, A. -M., H. Lehrach, A. Poustka, and N. Murray. 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. 170:827-842.

194. Fromm, M., J. Gallis, L. P. Taylor, and V. Walbot. 1987. Electroporation of DNA and RNA into plant protoplasts. Methods Enzymol. 153:351-366.

195. Fu, Y. -H., and G. A. Marzluf. 1987. Molecular cloning and analysis of the regulation of *nit-3*, the structural gene for nitrate reductase in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 84:8243-8247.

196. Garber, R. C., J. J. Lin, and O. C. Yoder. 1986. Mitochondrial plasmids in *Cochliobolus heterostrophus*, pp 105-118. *In* Wickner, R. B., A. Hinnebusch, A. M. Lambowitz, I. C. Gunsalus, and A. Hollander (eds.), Extrachromosomal elements in lower eukaryotes. Plenum Press, New York.

197. Garcia Acha, I., and J. R. Villanueva. 1963. Differences in the mode of action of strepzyme and *Helix pomatia* enzyme preparations on *Trichotecium roseum* spores. Nature 200:1231.

198. Garcia Acha, I., and J. R. Villanueva. 1964. Protoplasts from conidia of *Fusarium culmorum*. Can. J. Microbiol. 10:99-101.

199. Garcia Acha, I., F. Lopez-Belmonte, and J. R. Villanueva. 1966. Preparation of protoplast-like structures from conidia of *Fusarium culmorum*. Antonie van Leeuwenhoek J. Microbiol. Serol. 32:299-311.

200. Gatignol, A., M. Baron, and G. Tiraby. 1987. Phleomycin resistance encoded by the *ble* gene from transposon Tn5 as a dominant selectable marker in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 207:342-348.

201. Geisen, R., and L. Leistner. 1989. Transformation of *Penicillium nalgiovense* with the *amdS* gene of *Aspergillus nidulans*. Curr. Genet. 15:307-309.

202. Glazebrook, J. A., K. Mitchell, and A. Radford. 1987. Molecular genetic analysis of the *pyr-4* gene of *Neurospora crassa*. Mol. Gen. Genet. 209:399-402.

203. Gold, S. E., and N. T. Keen. 1989. Cloning and sequence analysis of gene(s) encoding β-tubulin in the *Hemiascomycete* plant pathogen *Geotrichum candidum*. APS Annu. Meet. Abst. No. 548.

204. Goldblatt, L. A. 1969. Aflatoxin: scientific background, control and implications. Academic Press, Inc., New York, NY.

205. Gomi, K., Y. Iimura, and S. Hara. 1987. Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans argB* gene. Agr. Biol. Chem. 51:2549-2555.

206. Goosen, T., G. Bloemheuvel, C. Gysler, D. A. de Bie, H. W. J. van den Broek, and K. Swart. 1987. Transformation of *Aspergillus niger* using the homologous orotidine-5'-phosphate decarboxylase gene. Curr. Genet. 11:499-503.

207. Goyon, C., and G. Faugeron. 1989. Targeted transformation of *Ascobolus immersus* and de novo methylation of the resulting duplicated DNA sequences. Mol. Cell. Biol. 9:2818-2827.

208. Grant, D. M., A. M. Lambowitz, J. A. Rambosek, and J. A. Kinsey. 1984. Transformation of *Neurospora crassa* with recombinant plasmids containing the cloned glutamate dehydrogenase (*am*) gene: evidence for autonomous replication of the transforming plasmid. Mol. Cell. Biol. 4:2041-2051.

209. Gray, G. L., K. Hayenga, D. Cullen, L. J. Wilson, and S. Norton. 1986. Primary structure of *Mucor miehei* aspartyl protease: evidence for a zymogen intermediate. Gene 48:41-53.

210. Griffin, D. H., M. DeVit, and R. Tuori. 1989. Protoplast formation and transformation of *Hypoxylon mammatum*. APS Annu. Meet. Abst. No. 549.

211. Grigg, G. W., R. M. Hall, N. K. Hart, D. R. Havulak, J. A. Lamberton, and A. Lane. 1985. Amplification of the antibiotic. Effects of the bleomycins, phleomycins and tallysomycins: its dependence on the nature of the variable basic groups. J. Antibiot. 38:99-110.

212. Groopman, J. D., L. G. Cain, and T. W. Kensler. 1988. Aflatoxin exposure in human populations: measurements and relationship to cancer. CRC Crit. Rev. Toxicol. 19:113-145.

213. Gunasekaran, M. 1981. Optimum culture conditions for aflatoxin B_2 production by a human pathogenic strain of *Aspergillus flavus*. Mycologia 73:697-704.

214. Gussack, G., J. W. Bennett, S. Cavalier, and L. Yatsu. 1977. Evidence for the parasexual cycle in a strain of *Aspergillus flavus* containing virus-like particles. Mycopathologia 61:159-165.

215. Gwynne, D. I., F. P. Buxton, S. Sibley, R. W. Davies, R. A. Lockington, C. Scazzocchio, and H. M. Sealy-Lewis. 1987a. Comparison of the cis-acting control regions of two coordinately controlled genes involved in ethanol utilization in *Aspergillus nidulans*. Gene 51:205-216.

216. Gwynne, D. I., F. P. Buxton, S. A. Williams, S. Garven, and R. W. Davies. 1987b. Genetically engineered secretion of active human interferon and a bacterial endoglucanase from *Aspergillus nidulans*. Bio/Technol. 5:713-719.

217. Gwynne, D. I., F. P. Buxton, S. Williams, A. M. Sills, J. A. Johnstone, J. K. Buch, Z. -M. Guo, D. Drake, M. Westphal, and R. W. Davies. 1989. Development of an expression system in *Aspergillus nidulans*. Biochem. Soc. Trans. 17:338-340.

218. Hahm, Y. T., and C. A. Batt. 1988. Genetic transformation of an *argB* mutant of *Aspergillus oryzae*. Appl. Environ. Microbiol. 54:1610-1611.

219. Hamasaki, T., T. Nakagomi, Y. Hatsuda, K. Fukuykama, and Y. Katsube. 1977. 5,6-Dimethylsterigmatocystin, a new metabolite from *Aspergillus multicolor*. Tetrahedron Lett. 32:2765-2766.

220. Hamer, J. E., and W. E. Timberlake. 1987. Functional organization of the *Aspergillus nidulans trpC* promoter. Mol. Cell. Biol. 7:2352-2359.

221. Hamlyn, P. F., R. E. Bradshaw, F. M. Mellon, C. M. Santiago, J. M. Wilson, and J. F. Peberdy. 1981. Efficient protoplast isolation from fungi using commercial enzymes. Enzyme Microb. Technol. 3:321-325.

222. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166:557-580.

223. Hara, S., D. I. Fennell, and C. W. Hesseltine. 1974. Aflatoxin-producing strains of *Aspergillus flavus* detected by fluorescence of agar medium under ultraviolet light. Appl. Microbiol. 27:1118-1123.

224. Harkki, A., J. Uusitalo, M. Bailey, M. Penttilä, and J. K. C. Knowles. 1989. A novel fungal expression system: secretion of active calf chymosin from the filamentous fungus *Trichoderma reesei*. Bio/Technol. 7:596-603.

225. Harris, G. S., E. J. Keath, and J. Medoff. 1989. Characterization of alpha and beta tubulin genes in the dimorphic fungus *Histoplasma capsulatum*. J. Gen. Microbiol. 135:1817-1832.

226. Hearn, V. M., E. V. Wilson, and D. W. R. Mackenzie. 1980. The preparation of protoplasts from *Aspergillus fumigatus* mycelium. Sabouraudia 18:75-77.

227. Heathcote, J. G., and J. R. Hibbert. 1978. Aflatoxins: chemical and biological aspects. Elsevier Science Publishers, Amsterdam.

228. Henderberg, A., J. W. Bennett, and L. S. Lee. 1988. Biosynthetic origin of aflatoxin G_1: confirmation of sterigmatocystin and lack of confirmation of aflatoxin B_1 as precursors. J. Gen. Microbiol. 134:661-667.

229. Henson, J. M., N. K. Blake, and A. L. Pilgeram. 1988. Transformation of *Gaeumannomyces graminis* to benomyl resistance. Curr. Genet. 14:113-117.

230. Hertzberg, R. P., M. J. Caranfa, and S. M. Hecht. 1985. DNA methylation diminishes bleomycins-mediated strand scission. Biochemistry 24:5285-5289.

231. Hesseltine, C. W., W. G. Sorenson, and M. Smith. 1970. Taxonomic studies of the aflatoxin-producing strains in the *Aspergillus flavus* group. Mycologia 62:123-132.

232. Hinnen, A., J. B. Hicks, and G. R. Fink. 1978. Transformation of yeast chimeric ColE1 plasmid carrying *LEU2*. Proc. Natl. Acad. Sci. USA 75:1929-1933.

233. Hiraoka, Y., T. Toda, and M. Yanagida. 1984. The *NDA3* gene of fission yeast encodes β-tubulin: a cold sensitive *nda3* mutation reversibly blocks spindle formation and chromosome movement in mitosis. Cell 39:349-358.

234. Hirth, K. -P., C. A. Edwards, and R. A. Firtel. 1982. A DNA-mediated transformation system for *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. USA 79:7356-7360.

235. Holden, D. W., J. Wang, and S. A. Leong. 1988. DNA-mediated transformation of *Ustilago hordei* and *Ustilago nigra*. Physiol. Mol. Plant Pathol. 33:235-239.

236. Holzapfel, C. W., I. F. H. Purchase, P. S. Steyn, and L. Gouws. 1966. The toxicity and chemical assay of sterigmatocystin, a carcinogenic mycotoxin, and its isolation from two new fungal sources. S. A. Medical J. 40:1100-1101.

237. Horng, J. S., J. E. Linz, and J. J. Pestka. 1989. Cloning and characterization of the *trpC* gene from an aflatoxigenic strain of *Aspergillus parasiticus*. Appl. Environ. Microbiol. 55:2561-2568.

238. Hsiao, C. -L., and J. Carbon. 1979. High-frequency transformation of yeast by plasmids containing the cloned yeast *ARG4* gene. Proc. Natl. Acad. Sci. USA 76:3829-3833.

239. Hsieh, D. P. H. 1973. Inhibition of aflatoxin biosynthesis of dichlorvos. J. Agric. Food Chem. 21:468-470.

240. Hsieh, D. P. H. 1986. The role of aflatoxin in human cancer, pp. 447-456. *In* Steyn, P. S., and R. Uleggar (eds.), Mycotoxins and phycotoxins. Elsevier Science Publishers, Amsterdam.

241. Hsieh, D. P. H. 1989. Carcinogenic potential of mycotoxins in foods, pp. 11-30. *In* Taylor, S. L., and Scanlan, R. A. (eds.), Food toxicology: a perspective on the relative risks. Marcel Dekker, Inc., New York.

242. Hsieh, D. P. H., and R. I. Mateles. 1970. The relative contribution of acetate and glucose to aflatoxin biosynthesis. Biochim. Biophys. Acta 208:482-486.

243. Hsieh, D. P. H., and R. I. Mateles. 1971. Preparation of labeled aflatoxins with high specific activities. Appl. Microbiol. 22:79-83.

244. Hsieh, D. P. H., M. T. Lin, and R. C. Yao. 1973. Conversion of sterigmatocystin to aflatoxin B_1 by *Aspergillus parasiticus*. Biochem. Biophys. Res. Commun. 52:992-997.

245. Hsieh, D. P. H., M. T. Lin, R. C. Yao, and R. Singh. 1976. Biosynthesis of aflatoxin: conversion of norsolorinic acid and other hypothetical intermediates into aflatoxin B_1. J. Agr. Food Chem. 24:1170-1174.

246. Hsieh, D. P. H., R. Singh, R. C. Yao, and J. W. Bennett. 1978. Anthraquinones in the biosynthesis of sterigmatocystin by *Aspergillus versicolor*. Appl. Environ. Microbiol. 35:980-982.

247. Huang, D., S. Bhairi, and R. C. Staples. 1989. A transformation procedure for *Botryotinia squamosa*. Curr. Genet. 15:411-414.

248. Huge-Jensen, B., F. Andreasen, T. Christensen, M. Christensen, L. Thim, and E. Boel. 1989. *Rhizomucor miehei* triglyceride lipase is processed and secreted from transformed *Aspergillus oryzae*. Lipids 24:781-785.

249. Hütter, R., P. Niederberger, and J. A. DeMoss. 1986. Tryptophan biosynthetic genes in eukaryotic microorganisms. Annu. Rev. Microbiol. 40:55-77.

250. Hynes, M. J. 1979. Fine structure mapping of the acetamidase structural gene and its controlling region in *Aspergillus nidulans*. Genetics 91:381-392.

251. Hynes, M. J. 1986. Transformation of filamentous fungi. Exp. Mycol. 10:1-8.

252. Hynes, M. J. 1989. Complementation of an *Aspergillus nidulans* mutation by a gene from the basidiomycete *Coprinus cinereus*. Exp. Mycol. 13:196-198.

253. Hynes, M. J., and M. A. Davis. 1986. The *amdS* gene of *Aspergillus nidulans*: control by multiple regulatory signals. BioAssays 5:123-128.

254. Hynes, M. J., C. M. Corrick, J. M. Kelly, and T. G. Littlejohn. 1988. Identification of the sites of action for regulatory genes controlling the *amdS* gene of *Aspergillus nidulans*. Mol. Cell. Biol. 8:2589-2596.

255. Hynes, M. J., C. M. Corrick, and J. A. King. 1983. Isolation of genomic clones containing the *amdS* gene of *Aspergillus nidulans* and their use in the analysis of structural and regulatory mutations. Mol. Cell. Biol. 3:1430-1439.

256. Iimura, K., K. Gomi, and H. Uzu. 1987. Transformation of *Aspergillus oryzae* through plasmid-mediated complementation of the methionine-auxotrophic mutation. Agr. Biol. Chem. 51:323-328.

257. Iimura, Y., K. Gotoh, K. Ouchi, and T. Nishima. 1983. Transformation of yeast without the spheroplasting process. Agr. Biol. Chem. 47:897-901.

258. Innis, M. A., M. J. Holland, P. C. McCabe, G. E. Cole, V. P. Wittman, R. Tal, W. K. Watt, D. H. Gelfand, J. P. Holland, and J. H. Meade. 1985. Expression, glycosylation, and secretion of an *Aspergillus* glucoamylase by *Saccharomyces cerevisiae*. Science 228:21-26.

259. Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153:163-168.

260. Jeenah, M. S., and M. F. Dutton. 1983. The conversion of sterigmatocystin to O-methylsterigmatocystin and aflatoxin B_1 by a cell-free preparation. Biochem. Biophys. Res. Commun. 116:1114-1118.

261. Jelinek, C. F., A. E. Pohland, and G. E. Wood. 1989. Worldwide occurrence of mycotoxins in foods and feeds- an update. J. Assoc. Off. Anal. Chem. 72:223-230.

262. Jiménez, A., and J. Davies. 1980. Expression of a transposable antibiotic resistance element in *Saccharomyces*. Nature 287:869-871.

263. John, M. A., and J. F. Peberdy. 1984. Transformation of *Aspergillus nidulans* using the *argB* gene. Enzyme Microb. Technol. 6:386-389.

264. Johnston, S. A., P. Q. Anziano, K. Shark, J. C. Sanford, and R. A. Butow. 1988. Mitochondrial transformation in yeast by bombardment with microprojectiles. Science 240:1538-1541.

265. Johnstone, I. L. 1985. Transformation of *Aspergillus nidulans*. Microbiol. Sci. 2:307-311.

266. Johnstone, I. L., and A. J. Clutterbuck. 1986. An *Aspergillus* DNA sequence giving a high frequency of unstable transformants. Heredity 57:131.

267. Johnstone, I. L., S. G. Hughes, and A. J. Clutterbuck. 1985. Cloning an *Aspergillus nidulans* developmental gene by transformation. EMBO J. 4:1307-1311.

268. Jones, I. G., and H. M. Sealy-Lewis. 1989. Chromosomal mapping and gene disruption of the *ADHIII* gene in *Aspergillus nidulans*. Curr. Genet. 15:135-142.

269. Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff. 1979. A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. Mol. Gen. Genet. 177:65-72.

270. Käfer, E. 1977. The anthranilate synthetase enzyme complex and the trifunctional *trpC* gene of *Aspergillus*. Can. J. Genet. Cytol. 19:723-738.

271. Kaiser, K., and N. E. Murray. 1985. The use of phage lambda replacement vectors in the construction of representative genomic DNA libraries, pp. 1-47. *In* Glover, D. M. (ed.), DNA cloning, a practical approach, Vol. I. IRL Press, Ltd., Oxford.

272. Kaster, K. R., S. G. Burgett, and T. D. Ingolia. 1984. Hygromycin B resistance as dominant selectable marker in yeast. Curr. Genet. 8:353-358.

273. Kaster, K. R., S. G. Burgett, R. N. Rao, and T. D. Ingolia. 1983. Analysis of a bacterial hygromycin B resistance gene by transcriptional and translational fusions and by DNA sequencing. Nucleic Acids Res. 11:6895-6911.

274. Katz, M. E., and M. J. Hynes. 1989a. Characterization of the *amdR*-controlled *lamA* and *lamB* genes of *Aspergillus nidulans*. Genetics 122:331-339.

275. Katz, M. E., and M. J. Hynes. 1989b. Gene function identified by interspecific transformation. Gene 78:167-171.

276. Katz, M. E., and M. J. Hynes. 1989c. Isolation and analysis of the acetate regulatory gene, *facB*, from *Aspergillus nidulans*. Mol. Cell. Biol. 9:5696-5701.

277. Keesey, J. K., Jr., and J. A. DeMoss. 1982. Cloning of the *trp-1* gene from *Neurospora crassa* by complementation of a *trpC* mutation in *Escherichia coli*. J. Bacteriol. 152:954-958.

278. Kelly, J. M., and M. J. Hynes. 1985. Transformation of *Aspergillus niger* by the *amdS* gene of *Aspergillus nidulans*. EMBO J. 4:475-479.

279. Kelly, J. M., and M. J. Hynes. 1987. Multiple copies of the *amdS* gene of *Aspergillus nidulans* cause titration of *trans*-acting regulatory proteins. Curr. Genet. 12:21-31.

280. Kiessling, K. -H. 1986. Biochemical mechanism of action of mycotoxins. Pure Appl. Chem. 58:327-338.

281. Kim, S. Y., and G. A. Marzluf. 1988. Transformation of *Neurospora crassa* with the *trp-1* gene and the effect of host strain upon the fate of the transforming DNA. Curr. Genet. 13:65-70.

282. Kinghorn, J. R., and E. I. Campbell. 1989. Amino acid sequence relationships between bacterial, fungal, and plant nitrate reductase and nitrite reductase proteins, pp. 385-404. *In* Wray, J. L., and J. R. Kinghorn (eds.), Molecular and genetic aspects of nitrate assimilation. Oxford Science Publications, Oxford.

283. Kinnaird, J. H., M. A. Keighren, J. A. Kinsey, M. Eaton, and J. R. S. Fincham. 1982. Cloning of the *am* (glutamate dehydrogenase) gene of *Neurospora crassa* through the use of a synthetic DNA probe. Gene 20:387-396.

284. Kinsey, J. A. 1989. Restricted distribution of the Tad transponson in strains of *Neurospora*. Curr. Genet. 15:271-275.

285. Kinsey, J. A., and J. Helber. 1989. Isolation of a transposable element from *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 86:1929-1933.

286. Kinsey, J. A., and J. A. Rambosek. 1984. Transformation of *Neurospora crassa* with the cloned *am* (glutamate dehydrogenase) gene. Mol. Cell. Biol. 4:117-122.

287. Kistler, H. C., and U. K. Benny. 1988. Genetic transformation of the fungal plant wilt pathogen, *Fusarium oxysporum*. Curr. Genet. 13:145-149.

288. Klein, T. M., E. D. Wolf, R. Wu, and J. C. Sanford. 1987. High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327:70-73.

289. Klinch, M. A., and Pitt, J. I. 1985. The theory and practice of distinguishing species of the *Aspergillus flavus* group, pp. 211-220. *In* Samson, R. A., and J. I. Pitt (eds.), Advances in *Penicillium* and *Aspergillus* systematics. Plenum Press, New York.

290. Klittich, C. J. R., and J. F. Leslie. 1988. Nitrate reduction mutants of *Fusarium moniliforme (Gibberella fujikuroi)*. Genetics 118:417-423.

291. Knight, D. E., and M. C. Scrutton. 1986. Gaining access to the cytosol: the technique and some applications of electropermeabilization. Biochem. J. 234:496-507.

292. Kolar, M., P. J. Punt, C. A. M. J. J. van den Hondel, and H. Schwab. 1988. Transformation of *Penicillium chrysogenum* using dominant selection markers and expression of an *Escherichia coli lacZ* fusion gene. Gene 62:127-134.

293. Kos, A., J. Kuijvenhoven, K. Wernars, C. J. Bos, H. W. J. van den Broek, P. H. Pouwels, and C. A. M. J. J. van den Hondel. 1985. Isolation and characterization of the *Aspergillus niger trpC* gene. Gene 39:231-238.

294. Kos, T., A. Kuijvenhoven, H. G. M. Hessing, P. H. Pouwels, and C. A. M. J. J. van den Hondel. 1988. Nucleotide sequence of the *Aspergillus niger trpC* gene: structural relationship with analogous genes of other organisms. Curr. Genet. 13:137-144.

295. Kronstad, J. W., and S. A. Leong. 1989. Isolation of two alleles of the *b* locus of *Ustilago maydis*. Proc. Natl. Acad. Sci. USA 86:978-982.

296. Kronstad, J. W., J. Wang, S. F. Covert, D. W. Holden, G. L. McKnight, and S. A. Leong. 1989. Isolation of metabolic genes and demonstration of gene disruption in the phytopathogenic fungus *Ustilago maydis*. Gene 79:97-106.

297. Kross, J., W. D. Henner, S. M. Hecht, and W. A. Haseltine. 1982. Specificity of deoxyribonucleic acid cleavage by bleomycin, phleomycin and tallysomycin. Biochemistry 21:4310-4318.

298. Krumlauf, R., and G. A. Marzluf. 1979. Characterization of the sequence complexity and organization of the *Neurospora crassa* genome. Biochemistry 18:3705-3713.

299. Kück, U. 1989. Mitochondrial DNA rearrangements in *Podospora anserina*. Exp. Mycol. 13:111-120.

300. Kunze, G., R. Bode, H. Rintala, and J. Hofemeister. 1989. Heterologous gene expression of the glyphosate resistance marker and its application in yeast transformation. Curr. Genet. 15:91-98.

301. Kurtzman, C. P., B. W. Horn, and C. W. Hesseltine. 1987. *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamarii*. Antonie van Leeuwenhoek 53:147-158.

302. Kurtzman, C. P., M. J. Smiley, C. J. Robnett, and D. T. Wicklow. 1986. DNA relatedness among wild and domesticated species in the *Aspergillus flavus* group. Mycologia 78:955-959.

303. Laborda, F., I. Garcia Acha, and J. R. Villanueva. 1974. Studies on a strepzyme capable of obtaining protoplasts from *Fusarium culmorum* conidia. Trans. Br. Mycol. Soc. 62:509-518.

304. Larimer, F. W., C. C. Morse, A. K. Beck, K. W. Cole, and F. H. Gaertner. 1983. Isolation of the *ARO1* cluster gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 3:1609-1614.

305. Last, R. L., and G. R. Fink. 1988. Tryptophan-requiring mutants of the plant *Arabidopsis thaliana*. Science 240:305-310.

306. Lazdins, I. B., and D. J. Cummings. 1982. Autonomously replicating sequences in young and senescent mitochondrial DNA from *Podospora anserina*. Curr. Genet. 6:173-178.

307. Leaich, L. L., and K. E. Papa. 1975. Identification of diploids of *Aspergillus flavus* by the nuclear condition of conidia. Mycologia 67:674-678.

308. Le Chevanton, L., and G. Leblon. 1989. The *ura5* gene of the ascomycete *Sordaria macrospora*: molecular cloning, characterization and expression in *Escherichia coli*. Gene 77:39-49.

309. Le Chevanton, L., G. Leblon, and S. Lebilcot. 1989. Duplications created by transformation in *Sordaria macrospora* are not inactivated during meiosis. Mol. Gen. Genet. 218:390-396.

310. Lee, L. S., J. W. Bennett, A. F. Cucullu, and R. L. Ory. 1976. Biosynthesis of aflatoxin B_1: conversion of versicolorin A to aflatoxin B_1 by *Aspergillus parasiticus*. J. Agr. Food Chem. 24:1167-1170.

311. Lee, L. S., J. W. Bennett, A. F. Cucullu, and J. B. Stanley. 1975. Synthesis of versicolorin A by a mutant strain of *Aspergillus parasiticus* deficient in aflatoxin production. J. Agr. Food Chem. 23:1132-1134.

312. Lee, L. S., J. W. Bennett, L. A. Goldblatt, and R. E. Lundin. 1970. Norsolorinic acid from a mutant strain of *Aspergillus parasiticus*. J. Amer. Oil Chem. Soc. 48:93-94.

313. Lemke, P. L., N. D. Davis, and G. W. Creech. 1989. Direct visual detection of aflatoxin synthesis by minicolonies of *Aspergillus* species. Appl. Environ. Microbiol. 55:1808-1810.

314. Lemke, P. A., N. D. Davis, S. K. Iyer, G. W. Creech, and U. L. Diener. 1988. Fluorometric analysis of iodinated aflatoxin in minicultures of *Aspergillus flavus* and *Aspergillus parasiticus*. J. Indust. Microbiol. 3:119-125.

315. Lennox, J. E., and C. K. Davis. 1983. Selection of and complementation analysis among aflatoxin-deficient mutants of *Aspergillus parasiticus*. Exp. Mycol. 7:192-195.

316. Leong, S. A. 1988. Recombinant DNA research in phytopathogenic fungi. Adv. Plant Pathol. 6:1-26.

317. Leslie, J. F., and M. B. Dickman. 1989. Heritability of hygromycin resistance in transformed strains of *Fusarium moniliforme*. APS Annu. Meet. Abst. No. 408.

318. Lin, M. T., and J. C. Dianese. 1976. A coconut-agar medium for rapid detection of aflatoxin production by *Aspergillus* spp. Phytopath. 66:1466-1469.

319. Lin, M. T., D. P. H. Hsieh, R. C. Yao, and J. A. Donkersloot. 1973. Conversion of averufin into aflatoxins by *Aspergillus parasiticus*. Biochem. 12:5167-5171.

320. Lockington, R. A., H. M. Sealy-Lewis, C. Scazzocchio, and R. W. Davies. 1985. Cloning and characterization of the ethanol utilization regulon in *Aspergillus nidulans*. Gene 33:137-149.

321. Madhosingh, C., and W. Orr. 1985. Zearalenone production in *Fusarium culmorum* after transformation with DNA of *F. graminearum*. Plant. Pathol. 34:402-407.

322. Maggon, K. K., S. K. Gupta, and T. A. Venkitasubramanian. 1977. Biosynthesis of aflatoxins. Bacteriol. Rev. 41:822-855.

323. Malardier, L., M. J. Daboussi, J. Julien, F. Roussel, C. Scazzocchio, and Y. Brygoo. 1989. Cloning of the nitrate reductase gene (*niaD*) of *Aspergillus nidulans* and its use for transformation of *Fusarium oxysporum*. Gene 78:147-156.

324. Malpartida, F., M. Zalacain, A. Jimenez, and J. Davies. 1983. Molecular cloning and expression in *Streptomyces lividans* of a hygromycin B phosphotransferase gene from *Streptomyces hygroscopicus*. Biochem. Biophys. Res. Commun. 117:6-11.

325. Manavathu, E. K., K. Suryanarayana, S. E. Hasnain, M. Leung, Y. F. Lau, and W. -C. Leung. 1987. Expression of *Herpes simplex* virus thymidine kinase gene in aquatic filamentous fungus *Achlya ambisexualis*. Gene 57:53-59.

326. Manavathu, E. K., K. Suryanarayana, S. E. Hasnain, and W. -C. Leung. 1988. DNA-mediated transformation in the aquatic filamentous fungus *Achlya ambisexualis*. J. Gen. Microbiol. 134:2019-2028.

327. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

328. Maramatsu, M. 1973. Preparation of RNA from animal cells. Methods Cell Biol. 7:23-51.

329. Marek, E. T., C. L. Schardl, and D. A. Smith. 1989. Molecular transformation of *Fusarium solani* with an antibiotic resistance marker having no fungal DNA homology. Curr. Genet. 15:421-428.

330. Marzluf, G. A. 1981. Regulation of nitrogen metabolism and gene expression in fungi. Microbiol. Rev. 45:437-461.

331. Marzluf, G. A., K. G. Perrine, and B. H. Hahm. 1985. Genetic regulation of nitrogen metabolism in *Neurospora crassa*, pp 83-94. *In* W. E. Timberlake (ed.), Molecular genetics of filamentous fungi. Alan R. Liss, Inc., New York, NY.

332. Mattern, I. E., S. Unkles, J. R. Kinghorn, P. H. Pouwels, and C. A. M. J. J. van den Hondel, 1987. Transformation of *Aspergillus oryzae* using the *A. niger pyrG* gene. Mol. Gen. Genet. 210:460-461.

333. May, G. S., J. Gambino, J. A. Weatherbee, and N. R. Morris. 1985. Identification and functional analysis of beta-tubulin genes by site specific integrative transformation in *Aspergillus nidulans*. J. Cell. Biol. 101:712-719.

334. Mayne, R. Y., J. W. Bennett, and J. Tallant. 1971. Instability of an aflatoxin-producing strain of *Aspergillus parasiticus*. Mycologia 63:644-648.

335. McCormick, S. P., D. Bhatnagar, and L. S. Lee. 1986. Averufanin: an aflatoxin B_1 precursor between averantin and averufin in the biosynthetic pathway. App. Environ. Microbiol. 53:14-16.

336. McCormick, S. P., D. Bhatnagar, and L. S. Lee. 1987. Averufanin is an aflatoxin B_1 precursor between averantin and averufin in the biosynthetic pathway. Appl. Environ. Microbiol. 53:14-16.

337. McKnight, G. L., and B. L. McConaughy. 1983. Selection of functional cDNAs by complementation in yeast. Proc. Natl. Acad. Sci. USA 80:4412-4416.

338. McKnight, G. L., H. Kato, A. Upshall, M. D. Parker, G. Saari, and P. J. O'Hara. 1985. Identification and molecular analysis of a third *Aspergillus nidulans* alcohol dehydrogenase gene. EMBO J. 4:2093-2099.

339. Mellon, F. M., P. F. R. Little, and L. A. Casselton. 1987. Gene cloning and transformation in the basidiomycete fungus *Coprinus cinereus*: isolation and expression of the isocitrate lyase gene (*acu-7*). Mol. Gen. Genet. 210:352-357.

340. Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. 12:7035-7056.

341. Miller, B. L., K. Y. Miller, K. A. Roberti, and W. E. Timberlake. 1987. Position-dependent and -independent mechanisms regulate cell-specific expression of the *SpoC1* gene cluster of *Aspergillus nidulans*. Mol. Cell. Biol. 7:427-434.

342. Miller, B. L., K. Y. Miller, and W. E. Timberlake. 1985. Direct and indirect replacements in *Aspergillus nidulans*. Mol. Cell. Biol. 5:1714-1721.

343. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

344. Mirabito, P. M., T. M. Adams, and W. E. Timberlake. 1989. Interactions of three sequentially expressed genes control temporal and spatial specificity in *Aspergillus* development. Cell 57:859-868.

345. Mishra, N. C. 1977. Characterization of the new osmotic mutants (*os*) which originated during genetic transformation in *Neurospora crassa*. Genet. Res. 29:9-19.

346. Mishra, N. C. 1979. DNA-mediated genetic changes in *Neurospora crassa*. J. Gen. Microbiol. 113:255-259.

347. Mishra, N. C. 1985. Gene transfer in fungi. Adv. Genet. 23:73-178.

348. Mishra, N. C., and E. L. Tatum. 1973. Non-mendelian inheritance of DNA-induced inositol independence in *Neurospora*. Proc. Natl. Acad. Sci. USA 70:3875-3879.

349. Mishra, N. C., G. Szabo, and E. L. Tatum. 1973. Nucleic acid induced genetic changes in *Neurospora*, pp. 259-268. *In* Niu, M. C., and S. J. Segal (eds.), The role of RNA in reproduction and development. Elsevier/North Holland Publishing Co., Amsterdam.

350. Mislivec, P. B., C. T. Dieter, and V. R. Bruce. 1975. Mycotoxin-producing potential of mold flora of dried beans. Appl. Microbiol. 29:522-526.

351. Moore, P. M., and J. F. Peberdy. 1976. Release and regeneration of protoplasts from the conidia of *Aspergillus flavus*. Trans. Br. Mycol. Soc. 66:421-425.

352. Morris, N. R. 1986. The molecular genetics of microtubule proteins in fungi. Exp. Mycol. 10:77-82.

353. Moss, M. O. 1977. *Aspergillus* mycotoxins, pp. 499-524. *In* Smith, J. E., and J. A. Pateman (eds.), Genetics and physiology of *Aspergillus*. Academic Press, Inc., New York, NY.

354. Mullaney, E. J., J. E. Hamer, K. A. Roberti, M. M. Yelton, and W. E. Timberlake. 1985. Primary structure of the *trpC* gene from *Aspergillus nidulans*. Mol. Gen. Genet. 199:37-45.

355. Mullaney, E. J., P. J. Punt, and C. A. M. J. J. van den Hondel. 1988. DNA mediated transformation of *Aspergillus ficuum*. Appl. Microbiol. Biotechnol. 28:451-454.

356. Muñoz-Rivas, A. M., C. A. Specht, R. C. Ullrich, and C. P. Novotny. 1986a. Isolation of the DNA sequence coding indole-3-glycerol phosphate synthetase and phosphoribosylanthranilate isomerase of *Schizophyllum commune*. Curr. Genet. 10:909-913.

357. Muñoz-Rivas, A., C. A. Specht, B. J. Drummond, E. Froeliger, C. P. Novotny, and R. C. Ullrich. 1986b. Transformation of the basidiomycete, *Schizophyllum commune*. Mol. Gen. Genet. 205:103-106.

358. Murakami, H. 1971. Classification of the koji mold. J. Gen. Appl. Microbiol. 17:281-309.

359. Murakami, H., H. Sagawa, and S. Takase. 1968a. Non-productivity of aflatoxin by Japanese industrial strains of the *Aspergillus*. III. Common characteristics of the aflatoxin-producing strains. J. Gen. Appl. Microbiol. 14:251-262.

360. Murakami, H., S. Takase, and T. Ishii. 1967. Non-productivity of aflatoxin by Japanese industrial strains of *Aspergillus*. I. production of fluorescent substances in agar slant and shaking culture. J. Gen. Appl. Microbiol. 13:323-334.

361. Murakami, H., S. Takase, and K. Kuwabara. 1968b. Non-productivity of aflatoxin by Japanese industrial strains of *Aspergillus*. II. production of fluorescent substances in rice koji, and their identification by absorption spectrum. J. Gen. Appl. Microbiol. 14:97-110.

362. Murray, N. E., W. J. Brammar, and K. Murray. 1977. Lambdoid phages that simplify the recovery of in vitro recombinants. Mol. Gen. Genet. 150:53-61.

363. Musílková, M., and Z. Fencl. 1968. Some factors affecting the formation of protoplasts in *Aspergillus niger*. Folia Microbiol. 13:235-239.

364. Nachmias, A., and J. Barash. 1976. Decreased permeability as a mechanism of resistance to methyl benzimidazol-2-yl-carbamate (MBC) in *Sporobolomyces roseus*. J. Gen. Microbiol. 94:167-172.

365. National Institute of Health, Department of Health and Human Services. 1986. Guidelines for Research involving recombinant DNA molecules. Federal Register 51:16957-16985.

366. Neff, N. F., J. H. Thomas, P, Grisafi, and D. Botstein. 1983. Isolation of β-tubulin gene from yeast and demonstration of its essential function in vivo. Cell 33:211-219.

367. Neumann, F., and P. Bierth. 1986. Gene transfer by electroporation. Amer. Biotechnol. Lab. 4:10-15.

368. Newbury, S. F., J. A. Glazebrook, and A. Radford. 1986. Sequence analysis of the *pyr-4* (orotidine 5'-P decarboxylase) gene of *Neurospora crassa*. Gene 43:54-58.

369. Newton, A. C., and C. E. Caten. 1988. Auxotrophic mutants of *Septoria nodorum* isolated by direct screening and by selection for resistance to chlorate. Trans. Br. Mycol. Soc. 90:199-207.

370. Ngindu, A., P. R. Kenya, D. M. Ocheng, T. N. Omondi, W. Ngare, D. Gatei, B. K. Johnson, J. A. Ngira, H. Nandwa, A. J. Jansen, J. N. Kaviti, and T. A. Siongok 1982. Outbreak of acute hepatitis caused by aflatoxin poisoning in Kenya. Lancet:1346-1348.

371. Nunberg, J. H., J. H. Meade, G. Cole, F. C. Lawyer, P. McCabe, V. Schweikart, R. Tal, V. P. Wittman, J. E. Flatgard, and M. E. Innis. 1984. Molecular cloning and characterization of the glucoamylase gene of *Aspergillus awamori*. Mol. Cell. Biol. 4:2306-2315.

372. Oakley, B. R., J. E. Rinehart, B. L. Mitchell, C. E. Oakley, C. Carmona, G. L. Gray, and G. S. May. 1987. Cloning, mapping and molecular analysis of the *pyrG* (orotidine-5'-phosphate decarboxylase) gene of *Aspergillus nidulans*. Gene 61:385-399.

373. Oakley, C. E., and B. R. Oakley. 1989. Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mipA* gene of *Aspergillus nidulans*. Nature 338:662-664.

374. O'Hara, E. B., and W. E. Timberlake. 1989. Molecular characterization of the *Aspergillus nidulans yA* locus. Genetics 121:249-254.

375. Oka, A., H. Sugisaki, and M. Takanami. 1981. Nucleotide sequence of the kanamycin resistance transposon Tn903. J. Mol. Biol. 147:217-226.

376. Oliver, R. P., I. N. Roberts, R. Harling, L. Kenyon, P. J. Punt, M. A. Dingemanse, and C. A. M. J. J. van den Hondel. 1987. Transformation of *Fulvia fulva*, a fungal pathogen of tomato, to hygromycin B resistance. Curr. Genet. 12:231-233.

377. Orbach, M. J., E. B. Porro, and C. Yanofsky. 1986. Cloning and characterization of the gene for beta-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant delectable marker. Mol. Cell. Biol. 6:2452-2461.

378. Orrell, J. C., C. J. R. Klittich, and J. F. Leslie. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. Phytopathol. 77:1640-1646.

379. Osiewacz, H. D., and A. Weber. 1989. DNA mediated transformation of the filamentous fungus *Curvularia lunata* using a dominant selectable marker. Appl. Microbiol. Biotechnol. 30:375-380.

380. Osmani, S. A., D. B. Engle, J. H. Doonan, and N. R. Morris. 1988a. Spindle formation and chromatin condensation in cells blocked at interphase by mutation of a negative cell cycle control gene. Cell 52:241-251.

381. Osmani, S. A., R. T. Pu, and N. R. Morris. 1988b. Mitotic induction and maintenance by overexpression of a G_2-specific gene that encodes a potential protein kinase. Cell 53:237-244.

382. Paietta, J. V., and G. A. Marzluf. 1985. Gene disruption by transformation in *Neurospora crassa*. Mol. Cell. Biol. 5:1554-1559.

383. Palmer, L. M., and D. J. Cove. 1975. Pyrimidine biosynthesis in *Aspergillus nidulans*: isolation and preliminary characterization of auxotrophic mutants. Mol. Gen. Genet. 138:243-255.

384. Panaccione, D. G., M. McKiernan, and R. M. Hanau. 1988. *Colletotrichum graminicola* transformed with homologous and heterologous benomyl-resistance genes retains expected pathogenicity to corn. Mol. Plant-Microbe Interact. 1:113-120.

385. Papa, K. E. 1973. The parasexual cycle in *Aspergillus flavus*. Mycologia 65:1201-1205.

386. Papa, K. E. 1976. Linkage groups in *Aspergillus flavus*. Mycologia 68:159-165.

387. Papa, K. E. 1977a. Genetics of aflatoxin production in *Aspergillus flavus*: linkage between a gene for a high B_2:B_1 ratio and the histidine locus on linkage group VIII. Mycologia 69:1185-1190.

388. Papa, K. E. 1977b. Mutants of *Aspergillus flavus* producing more aflatoxin B_2 than B_1. Appl. Environ. Microbiol. 33:206.

389. Papa, K. E. 1977c. Genetic analysis of a mutant of *Aspergillus flavus* producing more aflatoxin B_2 than B_1. Mycologia 69:556-562.

390. Papa, K. E. 1978. The parasexual cycle in *Aspergillus parasiticus*. Mycologia 70:766-773.

391. Papa, K. E. 1979. Genetics of *Aspergillus flavus*: complementation and mapping of aflatoxin mutants. Genet. Res. 34:1-9.

392. Papa, K. E. 1980. Dominant aflatoxin mutant of *Aspergillus flavus*. J. Gen. Microbiol. 118:279-282.

393. Papa, K. E. 1982. Norsolorinic acid mutant of *Aspergillus flavus*. J. Gen. Microbiol. 128:1345-1348.

394. Papa, K. E. 1984. Genetics of *Aspergillus flavus*: linkage of aflatoxin mutants. Can. J. Microbiol. 30:68-73.

395. Papa, K. E. 1986. Heterokaryon incompatibility in *Aspergillus flavus*. Mycologia 78:98-101.

396. Pardo, J. M., F. Malpartida, M. Rico, and A. Jimenez. 1985. Biochemical basis of resistance to hygromycin B in *Streptomyces hygroscopicus*- the producing organism. J. Gen. Microbiol. 131:1289-1298.

397. Parsons, K. A., F. G. Chumley, and B. Valent. 1987. Genetic transformation of the fungal pathogen responsible for rice blast disease. Proc. Natl. Acad. Sci. USA 84:4161-4165.

398. Pateman, J. A., D. J. Cove, B. M. Rever, and D. B. Roberts. 1964. A common cofactor for nitrate reductase and xanthine dehydrogenase which also regulates the synthesis of nitrate reductase. Nature 201:58-60.

399. Peberdy, J. F. 1985. Mycolytic enzymes, pp. 31-44. *In* Peberdy, J. F., and L. Ferenczy (eds.), Fungal protoplasts: applications in biochemistry and genetics. Marcel Dekker, Inc., New York, NY.

400. Peberdy, J. F. 1989. Fungi without coats- protoplasts as tools for mycological research. Mycol. Res. 93:1-20.

401. Peberdy, J. F., and L. Ferenczy. 1985. Fungal Protoplasts: applications in biochemistry and genetics. Marcel Dekker, Inc., New York, NY.

402. Peberdy, J. F., and R. K. Gibson. 1971. Regeneration of *Aspergillus nidulans* protoplasts. J. Gen. Microbiol. 69:325-330.

403. Peberdy, J. F., and S. Isaac. 1976. An improved procedure for protoplast isolation from *Aspergillus nidulans*. Microbios. Lett. 3:7-9.

404. Peberdy, J. F., C. E. Buckley, D. C. Daltry, and P. M. Moore. 1976. Factors affecting protoplast release in some filamentous fungi. Trans. Br. Mycol. Soc. 67:23-26.

405. Peñalva, M. A., A. Touriño, C. Patiño, F. Sánchez, J. M. Fernández-Sousa, and V. Rubio. 1985. Studies on transformation of *Cephalosporium acremonium*, pp. 59-68. *In* Timberlake, W. E. (ed.), Molecular genetics of filamentous fungi. Alan R. Liss, Inc., New York, NY.

406. Penttilä, M. E., K. M. H. Nevalainen, A. Raynal, and J. K. C. Knowles. 1984. Cloning of *Aspergillus niger* genes in yeast. Expression of the gene coding *Aspergillus* β-glucosidase. Mol. Gen. Genet. 194:494-499.

407. Penttilä, M., H. Nevalainen, M. Ratto, E. Salminen, and J. Knowles. 1987. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene 61:155-164.

408. Perkins, D. D. 1962. Preservation of *Neurospora* stock cultures with anhydrous silica gel. Can. J. Microbiol. 8:591-594.

409. Perkins, D. D., A. Radford, D. Newmeyer, and M. Bjorkman. 1982. Chromosomal loci of *Neurospora crassa*. Microbiol. Rev. 46:426-570.

410. Perrot, M., C. Barreau, and J. Bégueret. 1987. Nonintegrative transformation in the filamentous fungus *Podospora anserina*: stabilization of a linear vector by the chromosomal ends of *Tetrahymena thermophila*. Mol. Cell. Biol. 7:1725-1730.

411. Picard, M., R. Debuchy, J. Julien, and Y. Brygoo. 1987. Transformation by integration in *Podospora anserina*. II. targeting to the resident locus with cosmids and instability of the transformants. Mol. Gen. Genet. 210:129-134.

412. Picknett, T. M., and G. Saunders. 1989. Transformation of *Penicillium chrysogenum* with selection for increased resistance to benomyl. FEMS Microbiol. Lett. 60:165-168.

413. Picknett, T. M., G. Saunders, P. Ford, and G. Holt. 1987. Development of a gene transfer system for *Penicillium chrysogenum*. Curr. Genet. 12:449-455.

414. Pitt, J. I., A. D. Hocking, and D. R. Glenn. 1983. An improved medium for the detection of *Aspergillus flavus* and *A. parasiticus*. J. Appl. Bacteriol. 54:109-114.

415. Pontecorvo, G. 1953. The genetics of *Aspergillus nidulans*. Adv. Genet. 5:141-238.

416. Pratviel, G., J. Bernadou, and B. Meunier. 1986. DNA breaks generated by the bleomycin-iron III complex in the presence of KHSO5, a single oxygen donor. Biochem. Biophys. Res. Commun. 136:1013-1020.

417. Punt, P. J., R. P. Oliver, M. A. Dingemanse, P. H. Pouwels, and C. A. M. J. J. van den Hondel, 1987. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene 56:117-124.

418. Rabie, C. J., M. Steyn, and G. C. van Schalkwyk. 1977. New species of *Aspergillus* producing sterigmatocystin. Appl. Environ. Microbiol. 33:1023-1025.

419. Radford, A., F. P. Buxton, S. F. Newbury, and J. A. Glazebrook. 1985. Regulation of pyrimidine metabolism in *Neurospora*, pp. 127-143. *In* Timberlake, W. E. (ed.), Molecular genetics of filamentous fungi. Alan R. Liss, Inc., New York, NY.

420. Radford, A., S. Pope, A. Sazei, M. J. Fraser, and J. H. Parish. 1981. Liposome-mediated genetic transformation of *Neurospora crassa*. Mol. Gen. Genet. 184:567-569.

421. Raj, H. G., L. Viswanathan, H. S. R. Murthy, and T. A. Venkitasubramanian. 1969. Biosynthesis of aflatoxins by cell-free preparations from *Aspergillus flavus*. Experientia 25:1141-1142.

422. Rambosek, J., and J. Leach. 1987. Recombination DNA in filamentous fungi: progress and prospects. CRC Crit. Rev. Biotechnol. 6:357-393.

423. Randall, T., T. R. Rao, and C. A. Reddy. 1987. Transformation of *Phanerochaete chrysosporium*, a lignin-degrading white-rot basidiomycete, to G418 resistance. ASM Annu. Meet. Abst. p 142.

424. Rao, H. R. G., and P. K. Harien. 1972. Dichlorvos as an inhibitor of aflatoxin production on wheat, corn, rice, and peanuts. J. Econo. Entom. 65:988-989.

425. Rao, H. R. G., and P. K. Harein. 1973. Inhibition of aflatoxin and zearalenone biosynthesis with dichlorvos. Bull. Environ. Contam. Toxicol. 10:112-115.

426. Rao, R. N., N. E. Allen, J. N. Hobbs, W. E., Jr., Alborn, H. A. Kirst, and J. W. Paschal. 1983. Genetic and enzymatic basis of hygromycin B resistance in *Escherichia coli*. Antimicrob. Agents Chemother. 24:689-695.

427. Raper, K. B., and D. I. Fennell. 1965. The genus *Aspergillus* Williams and Wilkins, Baltimore.

428. Rasmussen, J. B., D. G. Panaccione, and R. M. Hanau. 1989. Improved transformation of *Colletotrichum graminicola* protoplasts and its use in cloning the *PYR1* gene. APS Annu. Meet. Abst. No. 551.

429. Razanamparany, V., and J. Bégueret. 1986. Positive screening and transformation of *ura5* mutants in the fungus *Podospora anserina*: characterization of the transformants. Curr. Genet. 10:811-817.

430. Razanamparany, V., and J. Bégueret. 1988. Non-homologous integration of transforming vectors in the fungus *Podospora anserina*: sequences of junctions at the integration sites. Gene 74:399-409.

431. Reddy, T. V., L. Viswanathan, and T. A. Venkitasubramanian. 1971. High aflatoxin production on a chemically defined medium. Appl. Microbiol. 22:393-396.

432. Reddy, T. V., L. Viswanathan, and T. A. Venkitasubramanian. 1979. Factors affecting aflatoxin production by *Aspergillus parasiticus* in a chemically defined medium. J. Gen. Microbiol. 114:409-413.

433. Revuelta, J. L., and M. Jayaram. 1986. Transformation of *Phycomyces blakesleeanus* to G-418 resistance by an autonomously replicating plasmid. Proc. Natl. Acad. Sci. USA 83:7344-7347.

434. Revuelta, J. L., and M. Jayaram. 1987. *Phycomyces blakesleeanus TRP1* gene: organization and functional complementation in *Escherichia coli* and *Saccharomyces cerevisiae*. Mol. Cell. Biol. 7:2664-2670.

435. Richardson, I. B., S. K. Hurley, and M. J. Hynes. 1989. Cloning and molecular characterization of the *amdR* controlled *gatA* gene of *Aspergillus nidulans*. Mol. Gen. Genet. 217:118-125.

436. Richey, M. G., E. T. Marek, C. L. Schardl, and D. A. Smith. 1989a. Transformation of filamentous fungi with plasmid DNA by electroporation. Phytopathol. 79:844-847.

437. Richey, M. G., C. L. Schardl, and D. A. Smith. 1989b. Isolation of the *ARGB* gene from *Fusarium solani f. sp. phaseoli*. APS Annu. Meet. Abst. No. 293.

438. Roberts, K., and J. S. Hyams. 1979. Microtubules. Academic Press, Inc., London.

439. Roberts, I. N., R. P. Oliver, P. J. Punt, and C. A. M. J. J. van den Hondel. 1989. Expression of the *Escherichia coli* β-glucuronidase gene in industrial and phytopathogenic filamentous fungi. Curr. Genet. 15:177-180.

440. Rodriguez, R. J., and L. W. Parks. 1980. Growth and antifungal homoazasterol production in *Geotrichum flavo-brunneum*. Antimicrob. Agents Chemother. 18:822-828.

441. Rodriguez, R. J., and O. C. Yoder. 1987. Selectable genes for transformation of the fungal plant pathogen *Glomerella dingulata f. sp. phaseoli (Colletotrichum lindemuthianum)*. Gene 54:73-81.

442. Roper, J. A. 1966. Mechanisms of inheritance. 3. the parasexual cycle, pp. 589-617. *In* Ainsworth, G. C., and A. S. Sussman (eds.), The fungi. Vol. II. The fungal organism. Academic Press, Inc., New York, NY.

443. Rosenberger, R. F. 1976. The cell wall, pp. 328-344. *In* Smith, J. E., and D. R. Berry (eds.), The filamentous fungi, Vol. II. Edward Arnold, London.

444. Rowlands, R. T., and G. Turner. 1973. Nuclear and extranuclear inheritance of oligomycin resistance in *Aspergillus nidulans*. Mol. Gen. Genet. 126:201-216.

445. Rowlands, R. T., and G. Turner. 1977. Nuclear-extranuclear interactions affecting oligomycin resistance in *Aspergillus nidulans*. Mol. Gen. Genet. 154:311-318.

446. Russel, P. J., J. A. Welsch, and S. Wagner. 1989. Transformation of *Neurospora crassa* by an integrative transforming plasmid is not enhanced by ribosomal DNA sequences. Biochim. Biophys. Acta 1008:243-246.

447. Sakai, K., and M. Yamamoto. 1986. Transformation of the yeast, *Saccharomyces carlsbergensis*, using an antibiotic resistance marker. Agric. Biol. Chem. 50:1177-1182.

448. Sakai, K., J. Sakaguchi, and M. Yamamoto. 1984. High-frequency cotransformation by copolymerization of plasmids in the fission yeast *Schizosaccharomyces pombe*. Mol. Cell. Biol. 4:651-656.

449. Salch, Y. P., and M. N. Beremand. 1989. Identification of sequences with promoter activity from *Gibberella pulicaris (Fusarium sambucinum)* transformants. APS Annu. Meet. Abst. No. 294.

450. Samac, D. A., and S. A. Leong. 1988. Two linear plasmids in mitochondria of *Fusarium solani* f. sp. *cucurbitae*. Plasmid 19:57-67.

451. Samac, D. A., and S. A. Leong. 1989. Characterization of the termini of linear plasmids from *Nectria haematococca* and their use in construction of an autonomously replicating transformation vector. Curr. Genet. 16:187-194.

452. Sánchez, F., M. Lozano, V. Rubio, and M. A. Peñalva. 1987. Transformation in *Penicillium chrysogenum*. Gene 51:97-102.

453. Sánchez, F., A. Touriño, S. Traseira, A. Pérez-Aranda, V. Rubio, and M. A. Peñalva, 1986. Molecular cloning and characterization of the *trpC* gene from *Penicillium chrysogenum*. Mol. Gen. Genet. 205:248-252.

454. Sandeman, R. A., and M. J. Hynes. 1989. Isolation of the *facA* (acetyl-coenzyme A synthetase) and *acuE* (malate synthase) genes of *Aspergillus nidulans*. Mol. Gen. Genet. 218:87-92.

455. Santerre, R. F., N. E. Allen, J. N., Jr., Hobbs, R. N. Rao, and R. J. Schmidt. 1984. Expression of prokaryotic genes for hygromycin B and G418 resistance as dominant-selection markers in mouse L cells. Gene 30:147-156.

456. Saunders, G., M. F. Tuite, and G. Holt. 1986. Fungal cloning vectors. Trends. Biotechnol. 4:93-98.

457. Saunders, G., T. M. Picknett, M. F. Tuite, and M. Ward. 1989. Heterologous gene expression in filamentous fungi. Trends Biotechnol. 7:283-287.

458. Scazzocchio, C., and H. N., Jr., Arst, 1978. The nature of an initiator constitutive mutation in *Aspergillus nidulans*. Nature 274:177-179.

459. Schatz, P. J., L. Pillus, P. Grisafi, F. Solomon, and D. Botstein. 1986. Two functional alpha tubulin genes of the yeast *Saccharomyces cerevisiae* encode divergent proteins. Mol. Cell. Biol. 6:3711-3721.

460. Schechtman, M. G. 1987. Isolation of telomere DNA from *Neurospora crassa*. Mol. Cell. Biol. 7:3168-3177.

461. Schechtman, M. G., and C. Yanofsky. 1983. Structure of the trifunctional *trp-1* gene from *Neurospora crassa* and its aberrant expression in *Escherichia coli*. J. Mol. Appl. Genet. 2:83-99.

462. Schmidt, F. R., and K. Esser. 1985. Aflatoxins: medical, economic impact, and prospects for control. Process Biochem. 20:167-174.

463. Schmidt, F. R., N. D. Davis, U. L. Diener, and P. A. Lemke. 1983. Cycloheximide induction of aflatoxin synthesis in a nontoxigenic strain of *Aspergillus flavus*. Bio/Technol. 1:794-795.

464. Schmidt, F. R., P. A. Lemke, and K. Esser. 1986. Viral influences on aflatoxin formation by *Aspergillus flavus*. Appl. Microbiol. Biotechnol. 24:248-252.

465. Schroeder, H. W., and W. W. Carlton. 1973. Accumulation of only aflatoxin B_2 by a strain of *Aspergillus flavus*. Appl. Microbiol. 25:146-148.

466. Schroeder, H. W., and W. H. Kelton. 1975. Production of sterigmatocystin by some species of the genus *Aspergillus* and its toxicity to chicken embryos. Appl. Environ. Microbiol. 30:589-591.

467. Schroeder, H. W., R. J. Cole, R. D. Grigsby, and H., Jr., Hein. 1974. Inhibition of aflatoxin production and tentative identification of an aflatoxin intermediate "versiconal acetate" from treatment with dichlorvos. Appl. Microbiol. 27:394-399.

468. Schwab, H. 1988. Strain improvement in industrial microorganisms by recombinant DNA techniques. Adv. Biochem. Eng. Biotechnol. 37:129-168.

469. Sealy-Lewis, H. M., and R. A. Lockington. 1984. Regulation of two alcohol dehydrogenases in *Aspergillus nidulans*. Curr. Genet. 8:253-259.

470. Sebald, W., and J. Hoppe. 1981. On the structure and genetics of the proteolipid subunit of the ATP synthase complex. Curr. Top. Bioenerg. 12:1-64.

471. Seip, E. R., C. P. Woloshuk, G. A. Payne, and C. R. Adkins. 1988. Isolation of cloned DNA from a benomyl resistant mutant of *Aspergillus flavus* with homology to the *benA* gene of *A. nidulans*. APS Annu. Meet. Abst. No. 294.

472. Seip, E. R., C. P. Woloshuk, G. A. Payne, and C. R. Adkins. 1989. Expression of MBC (benomyl) resistance in *Aspergillus flavus* transformed with a homologous β-tubulin gene. APS Annu. Meet. Abst. No. 552.

473. Sekita, S., K. Yoshihira, S. Natori, S. Udagawa, T. Muroi, Sugiyama, Y, and H. Kurata. 1981. Mycotoxin production by *Chaetomium* spp. and related fungi. Can. J. Microbiol. 27:766-772.

474. Selker, E. U., E. B. Cambareri, B. C. Jensen, and K. R. Haack. 1987. Rearrangement of duplicated DNA in specialized cells of *Neurospora*. Cell 51:741-752.

475. Semon, D., N. R. Movva, T. F. Smith, M. El Alama, and J. Davies. 1987. Plasmid-determined bleomycin resistance in *Staphylococcus aureus*. Plasmid 17:46-53.

476. Sequin, U., and I. Scott. 1974. Carbon-13 as a label in biosynthetic studies. Science 186:101-107.

477. Sheir-Neiss, G., M. Lai, and N. Morris. 1978. Identification of a gene for β-tubulin in *Aspergillus nidulans*. Cell 15:639-647.

478. Sherwood, J. E., and S. C. Sommerville. 1989. Molecular characterization of a β-tubulin gene from *Erysiphe graminis*. APS Annu. Meet. Abst. No. 560.

479. Sietsma, J. H., and W. R. de Boer. 1973. Formation and regeneration of protoplasts from *Pythium* PRL 2142. J. Gen. Microbiol. 74:211-217.

480. Singh, A., and F. Sherman. 1974. Characteristics and relationships of mercury-resistant mutants and methionine auxotrophy of yeast. J. Bacteriol. 118:911-918.

481. Singh, A., and F. Sherman. 1975. Genetic and physiological characterization of *met15* mutants of *Saccharomyces cerevisiae*: a selective system for forward and reverse mutations. Genetics 81:75-97.

482. Singh, R., and D. P. H. Hsieh. 1976. Enzymatic conversion of sterigmatocystin into aflatoxin B_1 by cell-free extracts of *Aspergillus parasiticus*. Appl. Environ. Microbiol. 31:743-745.

483. Singh, R., and D. P. H. Hsieh. 1977. Aflatoxin biosynthetic pathway: elucidation by using blocked mutants of *Aspergillus parasiticus*. Arch. Biochem. Biophys. 178:285-292.

484. Siyan, A., T. E. Stasz, G. E. Harman, and M. Hemmat. 1989. Transformation of *Trichoderma* spp. to hygromycin B resistance. APS Annu. Meet. Abst. No. 298.

485. Skatrud, P. L., and S. W. Queener. 1984. Cloning of a DNA fragment from *Cephalosporium* which functions as an autonomous replication sequence in yeast. Curr. Genet. 8:155-163.

486. Skatrud, P. L., S. W. Queener, L. G. Carr, and D. L. Fisher. 1987. Efficient integrative transformation of *Cephalosporium acremonium*. Curr. Genet. 12:337-348.

487. Smith, J. E., and M. O. Moss. 1985. Mycotoxins: formation, analysis and significance. John Wiley & Sons, New York, NY.

488. Soliday, C. L., M. B. Dickman, and P. E. Kolattukudy. 1989. Structure of the cutinase gene and detection of promoter activity in the 5'-flanking region by fungal transformation. J. Bacteriol. 171:1942-1951.

489. Specht, C. A., A. Muñoz-Rivas, C. P. Novotny, and R. C. Ullrich. 1988. Transformation of *Schizophyllum commune*: an analysis of parameters for improving transformation frequencies. Exp. Mycol. 12:357-366.

490. Stahl, U., P. Tudzynski, U. Kuck, and K. Esser. 1982. Replication and expression of a bacterial-mitochondrial hybrid plasmid in the fungus *Podospora anserina*. Proc. Natl. Acad. Sci. USA 79:3641-3645.

491. Steyn, P. S. 1980. The Biosynthesis of Mycotoxins: a study in secondary metabolism. Academic Press, Inc., New York, NY.

492. Steyn, P. S., R. Vleggaar, and P. L. Wessels. 1980. The biosynthesis of aflatoxin and its congeners, pp. 105-155. *In* Steyn, P. S. (ed.), The biosynthesis of mycotoxins: a study in secondary metabolism. Academic Press, Inc., New York, NY.

493. Stinchcomb, D. T., M. Thomas, J. Kelly, E. Selker, and R. W. Davis. 1980. Eukaryotic DNA segments capable of autonomous replication in yeast. Proc. Natl. Acad. Sci. USA 77:4559-4563.

494. Stohl, L. L., and A. M. Lambowitz. 1983. Construction of a shuttle vector for the filamentous fungus *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 80:1058-1062.

495. Stohl, L. L., R. A. Atkins, and A. M. Lambowitz. 1984. Characterization of deletion derivatives of an autonomously replicating *Neurospora* plasmid. Nucleic Acids Res. 12:6169-6178.

496. Stoloff, L., and W. Trager. 1965. Recommended decontamination procedures for aflatoxin. J. Assoc. Off. Anal. Chem. 48:681-682.

497. Struhl, K., D. T. Stinchcomb, S. Scherer, and R. W. Davis. 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA 76:1035-1039.

498. Suárez, T., and A. Eslava. 1988. Transformation of *Phycomyces* with a bacterial gene for kanamycin resistance. Mol. Gen. Genet. 212:120-123.

499. Suzci, A., and A. Radford. 1983. ARS8 sequences in the *Neurospora* genome. Neurospora Newslett. 30:13.

500. Swenson, D. H. 1981. Metabolic activation and detoxication of aflatoxins. Rev. Biochem. Toxicol. 3:155-192.

501. TeBeest, D. O., and M. B. Dickmam. 1989. Transformation of *Colletotrichum gloeosporioides f. sp. aeschynomene*. APS Annu. Meet Abst. No. 301.

502. Thomas, G. H., I. F. Connerton, and J. R. S. Fincham. 1988. Molecular cloning, identification and transcriptional analysis of genes involved in acetate utilization in *Neurospora crassa*. Mol. Microbiol. 2:599-606.

503. Thomas, J. H., N. Neff, and D. Botstein. 1985. Isolation and characterization of mutations in the β-tubulin gene of *Saccharomyces cerevisiae*. Genetics 112:715-734.

504. Thomas, M. D., and C. M. Kenerley. 1989. Transformation of the mycoparasite *Gliocladium*. Curr. Genet. 15:415-420.

505. Tilburn, J., C. Scazzocchio, G. G. Taylor, J. H. Zabicky-Zissman, R. A. Lockington, and R. W. Davis. 1983. Transformation by integration in *Aspergillus nidulans*. Gene 26:205-221.

506. Timberlake, W. E. 1978. Low repetitive DNA content in *Aspergillus nidulans*. Science 202:773-775.

507. Timberlake, W. E. 1985. Molecular genetics of filamentous fungi. Alan R. Liss, New York, NY.

508. Timberlake, W. E. 1987. Molecular genetic analysis of development in *Aspergillus nidulans*, pp. 63-82. *In* Loomis, W. F. (ed.), Genetic regulation of development. Alan R. Liss, Inc., New York, NY.

509. Timberlake, W. E., and M. A. Marshall. 1988. Genetic regulation of development in *Aspergillus nidulans*. Trends Genet. 4:162-169.

510. Timberlake, W. E., and M. A. Marshall. 1989. Genetic engineering of filamentous fungi. Science 244:1313-1317.

511. Timberlake, W. E., M. T. Boylan, M. B. Cooley, P. M. Mirabito, E. B. O'Hara, and C. E. Willett. 1985. Rapid identification of mutation-complementing restriction fragments from *Aspergillus nidulans* cosmids. Exp. Mycol. 9:351-355.

512. Toda, T., Y. Adachi, Y. Hiraoka, and M. Yanagida. 1984. Identification of the pleiotropic cell division cycle gene *NDA2* as one of two different alpha tubulin genes in *Schizosaccharomyces pombe*. Cell 37:233-242.

513. Tomsett, A. B., and R. H. Garrett. 1980. The isolation and characterization of mutants defective in nitrate assimilation in *Neurospora crassa*. Genetics 95:649-660.

514. Torres, J., J. Guarro, G. Suarez, N. Sune, M. A. Calvo, and C. Ramirez. 1980. Morphological changes in strains of *Aspergillus flavus* Link ex Fries and *Aspergillus parasiticus* Speare related with aflatoxin production. Mycopathologia 72:171-174.

515. Torrey, G. S., and E. H. Marth. 1976. Silica gel medium to detect molds that produce aflatoxin. Appl. Environ. Microbiol. 32:376-380.

516. Townsend, C. A., K. A. Plavcan, K. Pal, and S. W. Brobst. 1988. Hydroxyversicolorone: isolation and characterization of a potential intermediate in aflatoxin biosynthesis. J. Amer. Chem. Soc. 53:2472-2477.

517. Townsend, C. A. 1986. Progress toward a biosynthetic rationale of the aflatoxin pathway. Pure Appl. Chem. 58:227-238.

518. Tschumper, G., and J. Carbon. 1980. Sequence of a yeast DNA fragment containing a chromosomal replicator and the *TRP1* gene. Gene 10:157-166.

519. Tsukuda, T., S. Carleton, S. Fotheringham, and W. K. Holloman. 1988. Isolation and characterization of an autonomously replicating sequence from *Ustilago maydis*. Mol. Cell. Biol. 8:3703-3709.

520. Tudzynski, P., and K. Esser. 1980. Transformation to senescence with plasmid like DNA in the ascomycete *Podospora anserina*. Curr. Genet. 2:181-184.

521. Tudzynski, P., and K. Esser. 1985. Mitochondrial DNA for gene cloning in eukaryotes, pp. 403-416. *In* Bennett, J. W., and L. L. Lasure (eds.), Gene manipulation in fungi. Academic Press, Inc., New York, NY.

522. Tudzynski, P., U. Stahl, and K. Esser. 1982. Development of a eukaryotic cloning system in *Podospora anserina*. I. Long-lived mutants as potential recipients. Curr. Genet. 6:219-222.

523. Turcq, B., and J. Bégueret. 1987. The *ura5* gene of the filamentous fungus *Podospora anserina*: nucleotide sequence and expression in transformed strains. Gene 53:201-209.

524. Turgeon, B. G., R. C. Garber, and O. C. Yoder. 1985. Transformation of the fungal maize pathogen *Cochliobolus heterostrophus* using the *Aspergillus nidulans amdS* gene. Mol. Gen. Genet. 201:450-453.

525. Turgeon, B. G., R. C. Garber, and O. C. Yoder. 1987. Development of a fungal transformation system based on selection of sequences with promoter activity. Mol. Cell. Biol. 7:3297-3305.

526. Turgeon, B. G., W. D. MacRae, R. C. Garber, G. R. Fink, and O. C. Yoder. 1986. A cloned tryptophane-synthesis gene from the ascomycete *Cochliobolus heterostrophus* functions in *Escherichia coli*, yeast and *Aspergillus nidulans*. Gene 42:79-88.

527. Turnbull, I. F., K. Rand, N. S. Willetts, and M. J. Hynes. 1989. Expression of the *Escherichia coli* enterotoxin subunit B gene in *Aspergillus nidulans* directed by the *amdS* promoter. Bio/Technol. 7:169-174.

528. Turner, G., and D. J. Ballance. 1985. Cloning and transformation in *Aspergillus*, pp. 259-278. *In* Bennett, J. W., and L. L. Lasure (eds.), Gene manipulations in fungi. Academic Press, Inc., New York, NY.

529. Turner, G., D. J. Ballance, M. Ward, and R. K. Beri. 1985. Development of cloning vectors and a marker for gene replacement techniques in *Aspergillus nidulans*, pp. 15-28. *In* Timberlake, W. E. (ed.), Molecular genetics of filamentous fungi. Alan R. Liss, Inc., New York, NY.

530. Turner, W. B. 1971. Fungal metabolites. Academic Press, Inc., New York, NY.

531. Turner, W. B., and D. C. Aldridge. 1983. Fungal metabolites II. Academic Press, Inc. New York, NY.

532. Tyagi, J. S., A. K. Tyagi, and T. A. Venitasubramanian. 1981. Preparation and properties of spheroplasts from *Aspergillus parasiticus* with special reference to the de novo synthesis of aflatoxins. J. Appl. Bacteriol. 50:481-491.

533. Udagawa, S., T. Muroi, H. Kurata, S. Sekita, K. Yoshihira, and S. Natori. 1979. The production of chaetoglobsins, sterigmatocystin, O-methylsterigmatocystin, and chaetocin by *Chaetomium* spp. and related fungi. Can. J. Microbiol. 25:170-177.

534. Ueda, K., S. Kobayashi, H. Sakai, and T. Komano. 1985. Cleavage of stem and loop structure DNA by bleomycin. J. Biol. Chem. 260:5804-5807.

535. Ullrich, R. C., C. P. Novotny, C. A. Specht, E. H. Froeliger, and A. M. Muñoz-Rivas. 1985. Transforming *Basidiomycetes*, pp. 39-57. *In* Timberlake, W. E. (ed.), Molecular genetics of filamentous fungi. Alan R. Liss, Inc., New York, NY.

536. Umezawa, H. 1974. Chemistry and mechanism of action of bleomycin. Fed. Proc. 33:2296-2302.

537. Unkles, S. E. 1989. Fungal biotechnology and the nitrate assimilation pathway, pp 341-363. *In* Wray, J. L., and J. R. Kinghorn (eds.), Molecular and genetic aspects of nitrate assimilation. Oxford Science Publications, Oxford.

538. Unkles, S. E., E. I. Campbell, Y. M. J. T. de Ruiter-Jacobs, M. Broekhuijsen, J. A. Macro, D. Carrez, R. Contreras, C. A. M. J. J. van den Hondel, and J. R. Kinghorn. 1989a. The development of a homologous transformation system for *Aspergillus oryzae* based on the nitrate assimilation pathway: a convenient and general selection system for filamentous fungal transformation. Mol. Gen. Genet. 218:99-104.

539. Unkles, S. E., E. I. Campbell, D. Carrez, C. Grieve, R. Contreras, W. Fibers, C. A. M. J. J. van den Hondel, and J. R. Kinghorn. 1989b. Transformation of *Aspergillus niger* with the homologous nitrate reductase gene. Gene 78:157-166.

540. Upshall, A. 1986. Filamentous fungi in biotechnology. BioTechniques 4:158-166.

541. Upshall, A., A. A. Kumar, M. C. Bailey, M. D. Parker, M. A. Favreau, K. P. Lewison, M. L. Joseph, J. M. Maraganore, and G. L. McKnight. 1987. Secretion of active human tissue plasminogen activator from the filamentous fungus *Aspergillus nidulans*. Bio/Technol. 5:1301-1304.

542. Van Alfen, N. K., A. C. L. Churchill, D. R. Hansen, l. M. Cuiffetti, and H. D. Van Etten. 1988. Transformation of *Cryphonectria (Endothia) parasitica* using a variety of fungal promoters. APS Annu. Meet. Abst. No. 290.

543. Van Brunt, J. 1986. Fungi: the perfect hosts? Bio/Technol. 4:1057-1062.

544. van den Hondel, C. A. M. J. J., R. F. M. van Gorcom, T. Goosen, H. W. J. van den Broek, W. E. Timberlake, and P. H. Pouwels. 1985. Development of a system for analysis of regulation signals in *Aspergillus*, pp. 29-38. *In* Timberlake, W. E. (ed.), Molecular genetics of filamentous fungi. Alan R. Liss, Inc. New York, NY.

545. van den Hondel, C. A. M. J. J., P. J. Punt, B. L. M. Jacobs-Meijsing, W. van Hartingsveldt, R. F. M. van Gorcom, and P. H. Pouwels. 1986. Analysis of transcription-control signals in *Aspergillus*, pp. 365-369. *In* Bailey, J. (ed.), Biology and molecular biology of plant-pathogen interactions. Springer-Verlag, Berlin.

546. van Engelenburg, F., R. Smit, T. Goosen, H. van den Broek, and P. Tudzynski. 1989. Transformation of *Claviceps purpurea* using a bleomycin resistance gene. Appl. Microbiol. Biotechnol. 30:364-370.

547. Van Etten, H. D. and H. C. Kistler. 1988. *Nectria haematococca*, mating populations I and VI. Adv. Plant Pathol. 6:189-206.

548. van Gorcom, R. F. M., P. H. Pouwels, T. Goosen, J. Visser, H. W. J. van den Broek, J. E. Hamer, W. E. Timberlake, and C. A. M. J. J. van den Hondel. 1985. Expression of an *Escherichia coli* β-galactosidase fusion gene in *Aspergillus nidulans*. Gene 40:99-106.

549. van Gorcom, R. F. M., P. J. Punt, P. H. Pouwels, and C. A. M. J. J. van den Hondel. 1986. A system for the analysis of expression signals in *Aspergillus*. Gene 48:211-217.

550. van Hartingsveldt, W., I. E. Mattern, C. M. J. van Zeijl, P. H. Pouwels, , and C. A. M. J. J. van den Hondel. 1987. Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. Mol. Gen. Genet. 206:71-75.

551. van Heeswijck, R. 1986. Autonomous replication of plasmids in *Mucor* transformants. Carlsberg Res. Commun. 51:433-443.

552. van Heeswijck, R. V., and M. I. G. Roncero. 1984. High frequency transformation of *Mucor* with recombinant plasmid DNA. Carlsberg Res. Commun. 49:691-702.

553. van Walbeek, W., P. M. Scott, and F. S. Thatcher. 1968. Mycotoxins from food-borne fungi. Can. J. Microbiol. 14:131-137.

554. Vapnek, D., J. A. Jautala, J. W. Jacobson, N. H. Giles, and S. R. Kushner. 1977. Expression in *Escherichia coli* K-12 of the structural gene for catabolic dehydroquinase of *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 74:3508-3512.

555. Vogeli, G., E. Horn, M. Laurent, and P. Nath. 1985. Recombinant DNA techniques: storage and screening of cDNA libraries with large numbers of individual colonies from initial transformations. Anal. Biochem. 151:442-444.

556. Vollmer, S. J., and C. Yanofsky. 1986. Efficient cloning of genes of *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 83:4869-4873.

557. Waldron, C., E. B. Murphy, J. L. Roberts, G. D. Gustafson, S. L. Armour, and S. K. Malcolm. 1985. Resistance to hygromycin B: a new marker for plant transformation studies. Plant Mol. Biol. 5:103-108.

558. Wan, N. C., and D. P. H. Hsieh. 1980. Enzymatic formation of the bisfuran structure in aflatoxin biosynthesis. Appl. Environ. Microbiol. 39:109-112.

559. Wang, J., D. W. Holden, and S. A. Leong. 1988. Gene transfer system for the phytopathogenic fungus *Ustilago maydis*. Proc. Natl. Acad. Sci. USA 85:865-869.

560. Ward, M., K. H. Kodama, and L. J. Wilson. 1989. Transformation of *Aspergillus awamori* and *A. niger* by electroporation. Exp. Mycol. 13:289-293.

561. Ward, M., B. Wilkinson, and G. Turner. 1986. Transformation of *Aspergillus nidulans* with a cloned, oligomycin-resistant ATP synthase subunit 9 gene. Mol. Gen. Genet. 202:265-270.

562. Ward, M., L. J. Wilson, C. L. Carmona, and G. Turner. 1988. The *oliC* gene of *Aspergillus niger*: isolation, sequence and use as a selectable marker for transformation. Curr. Genet. 14:37-42.

563. Waring, R. B., G. S. May, and N. R. Morris. 1989. Characterization of an inducible expression system in *Aspergillus nidulans* using *alcA* and tubulin-coding genes. Gene 79:119-130.

564. Webster, T. D., and R. C. Dickson. 1983. Direct selection of *Saccharomyces cerevisiae* resistant to the antibiotic G418 following transformation with a DNA vector carrying the kanamycin-resistance gene of Tn903. Gene 26:243-252.

565. Wei, D. -L., and S. -C. Jong. 1986. Production of aflatoxins by strains of the *Aspergillus flavus* group maintained in ATCC. Mycopathologia 93:19-24.

566. Weiss, B. 1965. An electron microscope and biochemical study of *Neurospora crassa* during development. J. Gen. Microbiol. 39:85-94.

567. Weiss, R. L. 1985. Expression of *Aspergillus* genes in *Neurospora*, pp. 279-292. *In* Bennett, J. W., and L. L. Lasure (eds.), Gene manipulations in fungi. Academic Press, Inc., New York, NY.

568. Weltring, K. -M., B. G. Turgeon, O. C. Yoder, and H. D. Van Etten. 1988. Isolation of a phytolexin-detoxification gene from the plant pathogenic fungus *Nectria haematococca* by detecting its expression in *Aspergillus nidulans*. Gene 68:335-344.

569. Wernars, K., T. Goosen, B. M. J. Wennekes, K. Swart, C. A. M. J. J. van den Hondel, and H. W. J. van den Broek. 1987. Cotransformation of *Aspergillus nidulans*: a tool for replacing fungal genes. Mol. Gen. Genet. 209:71-77.

570. Wernars, K., T. Goosen, L. M. J. Wennekes, J. Visser, C. J. Bos, H. W. J. van den Broek, R. F. M. van Gorcom, C. A. M. J. J. van den Hondel, and P. H. Pouwels. 1985. Gene amplification in *Aspergillus nidulans* by transformation with vectors containing the *amdS* gene. Curr. Genet. 9:361-368.

571. Wessels, J. G. H. 1986. Cell wall synthesis in apical hyphal growth. Int. Rev. Cytol. 104:37-79.

572. Whitehead, M. P., S. E. Unkles, M. Ramsden, E. I. Campbell, S. J. Gurr, D. Spence, C. van den Hondel, R. Contreras, and J. R. Kinghorn. 1989. Transformation of a nitrate reductase deficient mutant of *Penicillium chrysogenum* with the corresponding *Aspergillus niger* and *A. nidulans niaD* genes. Mol. Gen. Genet. 216:408-411.

573. Wicklow, D. T. 1983. Taxonomic features and ecological significance of sclerotia, pp. 6-12. *In* Diener, U. L., R. L. Asquith, and J. W. Dickens (eds.), Aflatoxin and *Aspergillus flavus* in corn. Auburn University, Craftmaster Printers, Opelika, Alabama.

574. Woloshuk, C. P., E. R. Seip, G. A. Payne, and C. R. Adkins. 1989. Genetic transformation system for the aflatoxin-producing fungus *Aspergillus flavus*. Appl. Environ. Microbiol. 55:86-90.

575. Wood, H. A., R. F. Bozarth, J. Adler, and D. W. Mackenzie. 1974. Proteinaceous virus-like particles from an isolate of *Aspergillus flavus*. J. Virol. 13:532-534.

576. Wootton, J. C., M. J. Fraser, and A. J. Baron. 1980. Efficient transformation of germinating *Neurospora* conidia using total nuclear fragments. Neurospora Newslett. 27:33.

577. Wöstemeyer, J., A. Burmester, and C. Weigel. 1987. Neomycin resistance as a dominantly selectable marker for transformation of the zygomycete *Absidia glauca*. Curr. Genet. 12:625-627.

578. Woudt, L. P., J. J. van den Heuvel, M. M. C. van Raamsdonk-Duin, W. H. Mager, and R. J. Planta. 1985. Correct removal by splicing of a *Neurospora* intron in yeast. Nucleic Acids Res. 13:7729-7739.

579. Wray, J. L., and J. R. Kinghorn. 1989. Molecular and genetic aspects of nitrate assimilation. Oxford Science Publications, Oxford.

580. Yabe, K., Y. Ando, and T. Hamasaki. 1988. Biosynthetic relationship among aflatoxins B_1, B_2, G_1, and G_2. Appl. Environ. Microbiol. 54:2101-2106.

581. Yabe, K., Y. Ando, J. Hashimoto, and T. Hamasaki. 1989. Two distinct O-methyltransferases in aflatoxin biosynthesis. Appl. Environ. Microbiol. 55:2172-2177.

582. Yang, C. Y. 1972. Comparative studies on the detoxification of aflatoxins by sodium hypochlorite and commercial bleaches. Appl. Microbiol. 24:885-890.

583. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103-119.

584. Yao, R. C., and D. P. H. Hsieh. 1974. Step of dichlorvos inhibition in the pathway of aflatoxin biosynthesis. Appl. Microbiol. 28:52-57.

585. Yeh, F. -S., M. C. Yu, C. -C. Mo, S. Luo, M. J. Tong, and B. E. Henderson. 1989. Hepatitis B virus, aflatoxins, and hepatocellular carcinoma in Southern Guangxi, China. Cancer Res. 49:2506-2509.

586. Yelton, M. M., J. E. Hamer, E. R. DeSouza, E. J. Mullaney, and W. E. Timberlake. 1983. Developmental regulation of the *Aspergillus nidulans trpC* gene. Proc. Natl. Acad. Sci. USA 80:7576-7580.

587. Yelton, M. M., J. E. Hamer, and W. E. Timberlake. 1984. Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. Proc. Natl. Acad. Sci. USA 81:1470-1474.

588. Yelton, M. M., W. E. Timberlake, and C. A. M. J. J. van den Hondel. 1985. A cosmid for selecting genes by complementation in *Aspergillus nidulans*: selection of the developmentally regulated *yA* locus. Proc. Natl. Acad. Sci. USA 82:834-838.

589. Yoder, O. C., and B. G. Turgeon. 1985. Molecular bases of fungal pathogenicity to plants, pp. 417-48. *In* Bennett, J. W., and L. L. Lasure (eds.), Gene manipulations in fungi. Academic Press, Inc., New York, NY.

590. Yoder, O. C., B. Valent, and F. Chumley. 1986. Genetic nomenclature and practice for plant pathogenic fungi. Phytopathol. 76:383-385.

591. Yuill, E. 1950. The numbers of nuclei in conidia of *Aspergilli*. Trans. Br. Mycol. Soc. 33:324-331.

592. Zaika, L. L., and R. L. Buchanan. 1987. Review of compounds affecting the biosynthesis or bioregulation of aflatoxins. J. Food Prot. 50:691-708.

593. Zamir, L. O., and R. Ginsburg. 1979. Aflatoxin biosynthesis: detection of transient, acetate-dependent intermediates in *Aspergillus* by kinetic pulse-labeling. J. Bacteriol. 138:684-690.

594. Zamir, L. O., and K. D. Hufford. 1981. Precursor recognition by kinetic pulse-labeling in a toxigenic aflatoxin B_1-producing strain of *Aspergillus*. Appl. Environ. Microbiol. 42:168-173.

595. Zimmermann, U. 1982. Electric field-mediated fusion and related electrical phenomena. Biochim. Biophys. Acta 694:227-277.

596. Zimmermann, U., and J. Vienken. 1982. Electric field-induced cell-to-cell fusion. J. Membrane Biol. 67:165-182.

597. Zurawski, G., D. Elseviers, V. Stauffer, and C. Yanofsky. 1978. Translational control of transcription termination at the attenuator of the *Escherichia coli* tryptophan operon. Proc. Natl. Acad. Sci. USA 75:5988-5992.