Genetic Engineering of Filamentous Fungi

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Filamentous fungi are important in medicine, industry, agriculture, and basic biological research. For example, some fungal species are pathogenic to humans, whereas others produce β-lactam antibiotics (penicillin and cephalosporin). Industrial strains produce large amounts of enzymes, such as glucosamylose and proteases, and low molecular weight compounds, such as citric acid. The largest and most economically important group of plant pathogens are fungi. Several fungal species have biological properties and genetic systems that make them ideally suited for basic biological research. Recently developed techniques for genetic engineering of filamentous fungi make it possible to alter their detrimental and beneficial activities in novel ways.

THE FUNGI ARE A DIVERSE GROUP OF ORGANISMS WHOSE beneficial and detrimental biological activities have a major impact on human affairs (*1*). For example, fungi are used to produce the important antibiotics penicillin and cephalosporin. By contrast, some fungal species synthesize and excrete highly toxic metabolites, such as aflatoxins. Fungi are used as food (for example, mushrooms) and in food preparation (for example, bread), production of food additives (for example, citric acid) and fermentation of beverages (for example, beer and wine). However, billions of dollars of crop losses are annually attributed to diseases and post-harvest food spoilage caused by fungi. Crop devastation by fungi has led to starvation of large populations and to major social displacements, as during the potato famine in Ireland. Fungal pathogens have gained in medical importance as the number of immunologically compromised patients has climbed as a result of AIDS and owing to increased use of chemical and radiation therapies in the treatment of cancer and in association with organ transplants. Because of their relative simplicity, several fungal species have been used as "model" biological systems. The importance of fungi in medicine, industry, agriculture, and science has led to their intensive investigation for many years with the aim of understanding and controlling their desirable and undesirable activities.

Fungi grow as either unicellular yeasts or multicellular filaments. Yeasts multiply vegetatively by budding, as in *Saccharomyces cerevisiae*, or by fission, as in *Schizosaccharomyces pombe*. By contrast, filamentous fungi form multinucleated, tubular filaments called hyphae that are functionally coencytial and grow by apical extension (Fig. 1). Solutes and macromolecules are transported more or less freely between cells through perforate septa, but organellar movement may be restricted. Multicellular structures are formed during the asexual and sexual reproductive phases of filamentous fungi.

The filamentous growth habit of fungi underlies their ability to compete in nature with other microorganisms. Fungi can quickly colonize large areas and secrete chemicals to favor fungal growth. As nutrients are depleted near the center of a fungal colony, hyphal tips extend to exploit new resources. Hyphae can extend considerable distances over non-nutritive substrates by using endogenous stores. The extension of hyphal tips generates a mechanical force capable of disrupting even complex polymeric substrates such as wood. Extracellular hydrolytic enzymes secreted by fungi release low molecular weight, readily metabolized compounds from polymers. The metabolic diversity of fungi is extraordinary, making it possible for them to degrade even many refractory man-made compounds.

The most intensively studied fungus is the unicellular yeast *S. cerevisiae*. However, filamentous fungi, most notably *Neurospora crassa* and *Asperigillus nidulans*, have been used for fundamental physiological, biochemical, and genetic studies that have contributed to our understanding of the mechanisms controlling eukaryotic growth, differentiation, and development. The molecular genetic systems of these organisms have served as the basis for development of similar systems in less tractable but economically important species. In this article, we review the development of DNA-mediated transformation procedures for filamentous fungi, focusing on *N. crassa* and *A. nidulans*. We describe the ways in which these procedures have been used to modify the genetic compositions of target organisms and discuss how they may be used to modify the biological activities of economically important fungi.

Getting DNA into Cells

Genetic engineering of filamentous fungi depends on genomic incorporation of exogenously added DNA. DNA-mediated transformation procedures have been modeled after those developed for *S. cerevisiae* (*2*). Cell walls are usually removed by treatment of germinated spores or hyphae with cell wall–degrading enzymes to produce osmotically sensitive cells (OSCs) (*3*). Digestion of fungal cell walls has sometimes been difficult, but the introduction of Novozym 234, a commercially available hydrolytic enzyme mixture secreted by the filamentous fungus *Trichoderma harzianum*, has largely eliminated this problem. The hyphae of most fungi are highly susceptible to this enzyme preparation, which has been widely used to produce OSCs. Once cell walls have been perforated or removed, transforming DNA is added in the presence of Ca^{2+}, and OSCs are induced to fuse by addition of polyethylene glycol (PEG). DNA molecules are apparently internalized during fusion, as no transformation occurs when PEG is omitted (*4*). Fused OSCs are plated on an osmotically balanced regeneration medium that selects for cells expressing the functions supplied specifically by the added DNA

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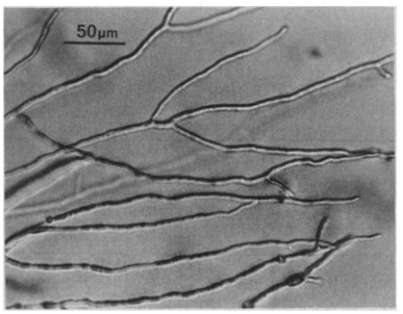

Fig. 1. Growth habit of filamentous fungi. *A. nidulans* hyphae were grown in agarose-solidified medium and photographed at ×100 by using differential interference contrast optics.

molecules. OSC viability ranges from <0.1% to >30%. Even though very low viabilities may be encountered, large numbers of OSCs can usually be obtained without difficulty. DNA can also be introduced into OSCs by electroporation (*5*). In addition, intact cells can be treated with Li^+ or Cs^+ before addition of DNA and treatment with PEG (*6*). Transformation procedures involving particle bombardment (*7*) or partial cell breakage by blending with glass beads (*8*) could also be useful with filamentous fungi.

Selectable Transformation Markers

Selection of transformants from the background of nontransformed cells depends on expression of genes conferring readily selectable dominant phenotypes. Transformation of a filamentous fungus was originally reported by Mishra and Tatum in 1973 (*9*). They treated an inositol-requiring *N. crassa* mutant strain with DNA from the wild type and obtained inositol-independent colonies. However, molecular techniques were not available for characterization of the putative transformants. Case *et al.* (*10*) first confirmed DNA-mediated transformation of *N. crassa* by using the *qa-2* gene, which had been cloned by virtue of its expression in *Escherichia coli* (*11*). Transformants were shown to contain chromosomally integrated plasmid molecules. Subsequently, many *Neurospora* genes have been recruited as selectable markers for transformation (*12*), including *pyr-4* (*13*), *trp-1* (*14*), *am* (*15*), and *Bml* (benomyl resistance) (*16*).

Transformation of *A. nidulans* was first reported by Ballance *et al.* (*13*), who used the *N. crassa pyr-4* gene to complement an *A. nidulans pyrG* mutation. Shortly thereafter, Tilburn *et al.* (*17*) used the *A. nidulans amdS* gene, encoding acetamidase (*18*), and Yelton *et al.* (*19*) used the *A. nidulans trpC* gene for transformation. In each case, transforming DNA became integrated into the genome, often at homologous sites. Subsequently, other *A. nidulans* genes were used as transformation markers, including *argB* (*20*), *prn* genes (*21*), *pyrF* (*22*), *pabaA* (*4*), *niaD* and *niiA* (*23*), and $BenA^R$ (benomyl resistance) (*24*). Transformation frequencies and the types of integration events obtained vary with the selective marker used.

Transformation of many other fungal species, including medically, industrially, and agriculturally significant species, rapidly followed the successes with *N. crassa* and *A. nidulans* (*25*). However, appropriate auxotrophic mutant strains do not exist for most fungal species. Thus, transformation with plasmids containing metabolic genes is usually not feasible and dominant selectable markers that do not require corresponding mutant strains are therefore of particular value. The *Bml* ($BenA^R$) and *amdS* genes have been useful in this context. Similarly, plasmids containing hygromycin or bleomycin resistance genes fused to fungal promoters have been incorporated into a number of transformation plasmids (*26*). The existence of these plasmids means that most fungal species, even those that have not previously been subjected to laboratory investigation, can now be genetically modified by transformation.

Fates of Selective Markers

Transforming DNAs could in theory be maintained extrachromosomally, and autonomously replicating fungal transformation vectors have been reported (*27*). They have not found wide use as molecular cloning vectors, however, perhaps because it is difficult to maintain the vectors in the extrachromosomal state. Genetic selections cannot be applied to individual nuclei, because the fungal thallus is coencytial. Nuclei lacking the plasmid carrying the selective marker continue to divide by cross-feeding with nuclei that contain the plasmid. As metabolically incompetent nuclei accumulate in the common cytoplasm they are expected to become a drain on the organism, ultimately leading to cessation of growth (*28*). The appearance of a high proportion of "abortive" colonies in primary transformation plates (*13, 17, 19*) could result from unstable plasmid replication. No methods are currently available to prevent plasmid integration and thereby to maintain strains carrying autonomous plasmids. Plasmids readily integrate at either homologous or heterologous sites, so construction of molecules lacking genomic homology is of no use. Addition of a centromere to plasmids could increase mitotic stability and prevent chromosomal integration (*29*). However, centromeres have not yet been isolated from filamentous fungi and *S. cerevisiae* centromeres do not display detectable activity (*30*).

Genomic integration of circular plasmids occurs in several ways. Some transformants appear to arise from simple gene conversion or double crossover events, in that no integrated plasmid sequences are detected (Fig. 2A) (*19, 31*). In other transformants, however, plasmid molecules are incorporated into the genome. Integration sometimes occurs at homologous sites, leading to formation of tandem reiterations of the target region separated by plasmid sequences (Fig. 2B). Integration also occurs at heterologous sites, with insertion of single or multiple plasmid copies (Fig. 2C). It is not known whether nominally heterologous integration events

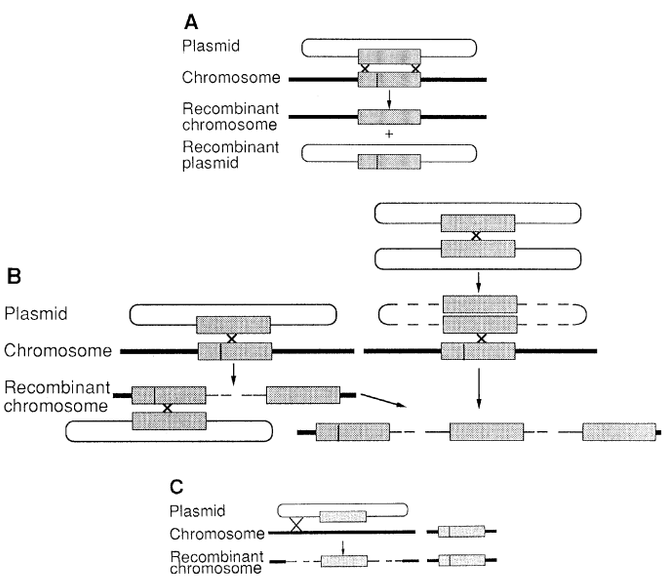

Fig. 2. Transformation with circular plasmid DNA. Thin lines represent plasmid DNA and thick lines represent chromosomal DNA. Shaded rectangles represent genes. Vertical lines within genes represent mutations. (**A**) Double crossover or gene conversion event at the homologous chromosomal site. (**B**) Single crossover events at the homologous chromosomal site. Integration of multiple plasmid copies could result from recombination events occurring before (upper right) or after (lower left) plasmid integration. (**C**) Heterologous integration event. Recombination within the gene sequence is expected to lead to its inactivation.

occur at limited regions of homology. Heterologous integration can be used to mutagenize and tag genes physically, as with transposons, thereby facilitating their cloning. Rearrangements in integrated plasmids may occur at readily detectable frequencies. The frequency with which the various types of integration events occur varies according to the plasmids and recipient strains or species used.

Genomic integration of linear molecules also occurs, often by a process formally equivalent to a double crossover event (Fig. 3A) (*31, 32*). However, circularization of linear molecules prior to integration can produce tandem duplications (Fig. 3B) (*31*).

Integrated plasmids are mitotically stable. For example, a tandem duplication of the *A. nidulans argB* locus separated by plasmid sequences, containing or lacking a yeast centromere, was not detectably lost during growth for ≥50 generations (*30*). This high degree of mitotic stability is desirable for genetic engineering of industrial strains, because introduced gene copies will be retained during large-scale fermentations. By contrast, introduced DNA sequences are often meiotically unstable. For example, in *A. nidulans*, tandemly repeated sequences are lost at variable, but readily detectable, frequencies after selfing or out-crossing (*17, 33*). During selfing, loss of sequences appears to result at least partially from unequal crossing over, because progeny with additional copies of the reiterated sequences are also obtained. In *N. crassa*, introduced sequences are often hypermethylated. Duplicated sequences are eliminated at high frequency during the sexual phase by a process called repeat-induced point mutation (RIP) (*34*). This process has been used to inactivate functional genes in *N. crassa* where more traditional methods for gene disruption are inefficient (*35*). Most economically important filamentous fungi lack a sexual phase. Thus, with many species the behavior of inserted DNA sequences in meiosis is irrelevant.

Current Applications of Transformation Technology

Transformation technology has led to new ways of altering the biological characteristics of fungi and has been used extensively in basic studies of the mechanisms controlling growth, metabolism, and development (*36*) and for isolation and manipulation of genes of potential importance in medicine, industry, or agriculture.

Gene cloning. Efficient techniques for cloning genes by genetic complementation of mutations have been developed for *A. nidulans* and *N. crassa*. *A. nidulans* plasmid (*37*) and cosmid (*33*) vectors have been constructed that permit (i) cloning of random chromosomal DNA fragments, (ii) transformation of mutant strains by primary selection for prototrophy or drug resistance, and (iii) testing for complementation of mutations identifying genes to be cloned. Complementing DNA molecules can be recovered in two ways. First, with cosmid clones, genomic DNA is treated with a bacteriophage lambda in vitro packaging extract, and cosmids are recovered by transduction of *E. coli* (*33*). Second, with either cosmid or plasmid clones, genomic DNA is partially digested with a restriction enzyme, ligated at low concentration, and used to transform *Escherichia coli* cells. A rapid procedure for mapping mutation-complementing DNA fragments in cloned inserts has been developed (*38*). These approaches have been used to clone and map numerous *A. nidulans* genes that were previously identified only by their mutant phenotype.

Cosmid vectors have also been used to clone many genes from *N. crassa*. Problems with DNA rearrangements have impeded the direct complementation-recovery approach used with *A. nidulans*, but may be overcome by use of *E. coli* strains that are tolerant of methylated DNA (*39*). Alternatively, ordered collections of genomic clones

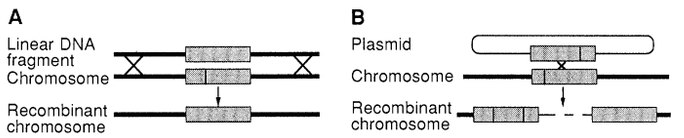

Fig. 3. Transformation with linear DNA fragments. Representation of DNA sequences is as in Fig. 2. (**A**) Double crossover or gene conversion event at the homologous chromosomal site. (**B**) Single crossover event of circularized DNA at the homologous chromosomal site.

constructed with drug resistance cosmids (*40*) may be used. In this case, DNA from dichotomously divided pools of clones is tested for its ability to complement a mutation until a single complementing clone is identified (sib selection). The method is quite rapid and efficient once DNA has been isolated from the many subcollections needed.

Cloned DNA fragments may complement a mutation by expression of the wild-type function or by extragenic suppression. With *A. nidulans*, proof of cloned sequence identity can be made by genetic mapping or complementation analysis in diploids or merodiploids (*33, 41*). In *N. crassa*, which does not form stable diploids, gene identity can be inferred from tight linkage of the complementing DNA fragment and the target gene. Such mapping is simplified by a set of polymorphic strains constructed by Metzenberg (*42*). RIP-mediated gene inactivation can also be used to establish gene identity. Analogous techniques should be possible with less well characterized species.

Cosmid rescue has also been used to clone genes from one species by detecting their expression in a different species. For example, a disease-determinant gene, pisatin demethylating ability (PDA), from the phytopathogenic fungus *Nectria haematacocca* was cloned by detecting its expression in *A. nidulans* (*43*). It should be possible to use identical or related strategies to isolate genes of interest from many other fungal species.

Targeted insertions and directed mutations. Insertion of plasmids at specific chromosomal sites is important for analysis of mutations generated in vitro (to avoid or test for chromosome position effects) and for making targeted in vivo mutations. Convenient techniques exist for selection of plasmid integration events at predetermined sites. For example, in *A. nidulans, yA* is required for production of spore pigment, and mutants produce yellow instead of green spores (*44*). An internal *yA* restriction fragment can be incorporated into transformation plasmids so that homologous integration results in formation of readily selectable yellow-spored mutants (Fig. 4A) (*45*). Alternatively, a strain containing one mutant allele can be transformed with a plasmid containing a different mutant allele (Fig. 4B) (*46*). In this case, only homologous recombination events regenerate a selectable wild-type allele. In a related approach, a linear DNA fragment containing one mutant allele linked to a sequence to be inserted can be used to transform a strain containing a different mutant allele (Fig. 4C) (*47*). Transformants containing a regenerated wild-type allele frequently contain the linked sequence.

Directed inactivation of preselected genes can be accomplished with many filamentous fungi. For example, Miller *et al.* (*31*) showed that direct and indirect gene replacement techniques modeled after the yeast procedures of Scherer and Davis (*48*) and Rothstein (*49*) are effective with *A. nidulans*. As in yeast, linearization of circular plasmids by cutting within the target sequence increases the frequency of recombination at the desired site. These techniques have been used to prove the identity of newly cloned genes (*41*) and in attempts to determine the physiological functions of cloned genes with unknown functions (*50*).

With yeast it is possible to make a directed mutation of an essential gene in a diploid and then to prove it is essential by tetrad

analysis (*51, 52*). An analogous approach has not been used with filamentous fungi because premeiotic diploid nuclei exist only transiently in specialized reproductive cells. In some species, such as *A. nidulans*, stable vegetative diploids can be formed, but these cannot be induced to produce meiotic spores. Two alternative approaches have thus been adopted. In the first, one putatively essential gene in a vegetative diploid is inactivated by site-directed mutagenesis. The resultant strain is then "haploidized" (*53*), and haploids are tested for the presence of both the mutated gene and the marker gene used to make the mutation. Absence of the mutated and marker genes in all haploids, accompanied by normal segregation of other markers, shows that the gene is essential. In the second approach (*54*), a haploid strain is transformed with a plasmid that disrupts the target gene. Primary transformants are most often heterokaryotic, and nuclei containing potentially lethal mutations can thus be maintained. However, in most fungi the asexual spores are uninucleate or homokaryotic so that masked genotypes are exposed. If the mutation leads to cell death any time during spore germination, it can be detected by observing abortive growth of germinating spores from the heterokaryon. In *N. crassa*, pre-meiotic mutation of essential genes by RIP (*34*) leads to 2:2 segregation of viable versus inviable ascospores in many asci.

Nondirected mutations. Plasmids lacking homology with the host genome apparently integrate at random sites and thus provide a convenient method for physically tagging chromosomal DNA sequences. Insertional inactivation of genes by these plasmids can be used to clone the genes, just as with transposon tagging. A strain is transformed with an appropriate plasmid, and transformants are plated under conditions that will not select against desired mutations. The collection is screened for mutants. DNA hybridization analysis is then used to select those mutants containing one or a few plasmid copies. A genomic library is finally constructed and screened by hybridization with a DNA fragment that is unique to the plasmid used to make the disruptions. Corresponding wild-type clones can then be isolated from appropriate libraries. This approach to gene cloning does not depend on previous isolation of mutants as does the genetic complementation technique.

Promoter fusions. The *E. coli* β-galactosidase structural gene (*lacZ*) serves as a useful reporter in filamentous fungi whose endogenous β-galactosidases are repressed by glucose (*46, 55*). Transcriptional or translational fusions of promoters and associated regulatory sequences can be made with *lacZ*, and the activities of the resultant constructs can be measured by assaying β-galactosidase on plates or in protein extracts. For systematic mutational studies of regulatory sequences, plasmids containing fusions can be integrated at a preselected chromosomal site (see above) to avoid position effects, which can be pronounced in filamentous fungi (*31*).

Similarly, patterns of gene expression can be altered by fusion of structural genes to regulable promoters. For example, *A. nidulans* regulatory genes have been fused to the *A. nidulans alcA* promoter to investigate the functions of their products (*54, 56*). The *alcA* gene encodes catabolic alcohol dehydrogenase and the promoter is subject to substrate induction and carbon catabolite repression (*57*). Genes fused to this promoter can be caused to be overexpressed or expressed at the wrong time during the life cycle or in the incorrect cell type. The results of such misexpression have been useful in making inferences about gene function. In a converse approach, *cis*-acting regulatory sequences from genes of interest that do not lend themselves to genetic selection schemes (for example, developmental regulatory genes) are fused to structural genes whose activities can be readily detected (for example, *lacZ* or *alcA*). Mutations that increase or decrease the levels of expression of the reporter gene can be identified following introduction of such constructs into the genome in one or more copies.

Titrations of regulatory gene products. Numerous copies of selected DNA sequences can be introduced into fungal genomes by transformation (*58*). Hynes *et al.* (*59*) showed that multiple copies of some cis-acting regulatory sequences titrate away their corresponding trans-acting transcriptional regulators, leading to an inability to induce genes in the regulon. This approach permits identification and mapping of cis-acting regulatory sequences and identification of genes that are subject to control by common trans-acting factors. Titrations are not expected to be useful in those instances where there is a large excess of trans-acting factor or where the trans-acting regulatory gene is autogenously controlled.

Genetic rearrangements. Genetic rearrangements can be encouraged by directed genome manipulations. For example, an exogenous copy of a gene may be introduced on the same chromosome arm and in the same orientation as the endogenous copy, but at some distance away from it. During meiosis, this can lead to pairing and unequal crossing over, which results in loss of the DNA between the duplicated sites (*31, 33, 60*). Similarly, duplications of sequences on different chromosomes may lead to an increased translocation frequency as a result of recombination between the repeated sequences (*61*). These approaches have not yet been extensively explored with filamentous fungi, but could be of considerable value in genetic engineering projects where major chromosomal alterations may be desirable.

Future Prospects

Basic science. Filamentous fungi can be manipulated by sophisticated modern genetics to study a wide variety of eukaryotic regulatory mechanisms. Gene systems encoding enzymes for carbon and nitrogen utilization are subject to general (for example, ammonium or catabolite repression) and pathway-specific controls whose interactions are of substantial interest (*21*). There are many examples of gene clustering in filamentous fungi, and this pattern of organization may be related to mechanisms regulating gene expression (*21, 62*). These organisms are exceedingly well suited for studies of multicellular development (*63*), cell-cell communication and cell interactions (*64*), organelle and chromosome movement (*65*) and thigmo-, gravi-, chemo-, and phototropic responses (*66*). They are also excellent subjects for investigations of recombination, mutagenesis, DNA repair, and population dynamics. They will continue to serve as convenient models for larger eukaryotes.

Medicine. Fungi are medically important as pathogens and as

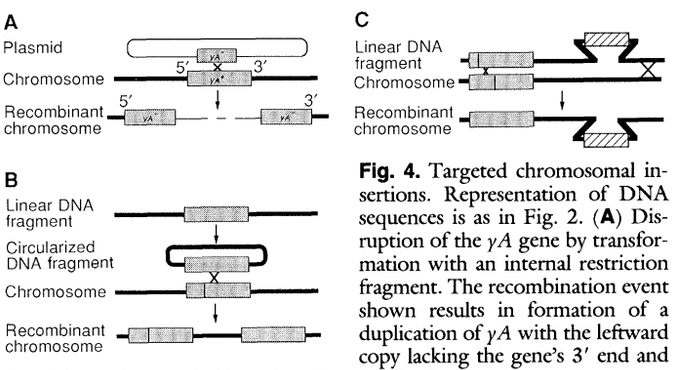

Fig. 4. Targeted chromosomal insertions. Representation of DNA sequences is as in Fig. 2. (**A**) Disruption of the *γA* gene by transformation with an internal restriction fragment. The recombination event shown results in formation of a duplication of *γA* with the leftward copy lacking the gene's 3′ end and the rightward copy lacking the 5′ end. (**B**) Recombination between a circular plasmid containing one mutant allele and the chromosome containing a different mutant allele. Only recombination in the interval between the two mutations leads to formation of a wild-type allele. (**C**) Recombination between a linear DNA fragment containing one mutant allele and the chromosome containing a different mutant allele. The rightward crossover can occur anywhere distal to the chromosomal mutation.

antibiotic producers. Two recent technological advances for some medically important species, transformation (*67*) and electrophoretic separation of chromosomes (*68*), will make it easier to manipulate their genetic systems. Advances in our understanding of the mechanisms of pathogenesis and virulence are expected. The close relationship of thoroughly investigated species, such as *A. nidulans*, and commercial antibiotic producers, such as *Penicillium chrysogenum* and *Cephalosporium acremonium*, will facilitate genetic engineering approaches for quantitative and qualitative modification of antibiotic production (*69*).

Industry. Genetic engineering of industrially important fungi may be used to enhance product production—for example, by improving secretion efficiency or by relieving specific rate-limiting steps in selected metabolic pathways. It may be possible to produce new materials by introducing genes encoding enzymes that catalyze novel biochemical reactions into existing production strains. Genetically modified strains that have enzymatic activities derived from several species might be developed to detoxify refractory compounds in industrial effluents and waste dumps. Similarly, large quantities of specific enzymes for use in processes as diverse as food preparation and paper manufacture might be synthesized by genetically engineered fungi. Regulatory concerns should be minimized because these strains are generally regarded as safe (GRAS) producers of materials consumed by humans.

Agriculture. Fungi, the most important class of plant pathogens, also affect plant productivity in positive ways. For example, symbiotic mycorrhizae increase the ability of plant roots to obtain limiting nutrients. Genetic engineering provides a new opportunity to study mechanisms regulating symbiosis and pathogenesis by allowing the isolation and controlled expression of symbiosis- and pathogenicity-related genes. It may even be possible to study the regulation and activities of agriculturally important genes in laboratory models (*70*). As the mechanisms controlling the biological activities of filamentous fungi become better understood, development of novel approaches toward the control of these activities is expected.

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71. We thank N. Giles, C. Mims, C. Glover, and our colleagues in the laboratory for critical reviews of the manuscript. Our research with *A. nidulans* has been supported by grants from the National Institutes of Health, National Science Foundation, and the U.S. Department of Agriculture (CRGO).