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COMMENTARY

Linking Approaches in the Study of Fungal Pathogenesis: A Commentary

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The study of fungal pathogenicity has occupied pathologists and mycologists for more than a century. It is widely recognized that fungi are the most damaging of plant pathogenic microbes and that fungal infections in immunocompromised individuals are on the rise (Sternberg, 1994). A central question in fungal pathogen research is: What distinguishes pathogenic fungi from their saprophytic relatives? How we attempt to answer this question is important for pursuing various strategies for controlling fungal growth. Although there are differences between animal and plant mycoses, particularly in the host response, mycologists are aware that there are some intriguing similarities in the pathogenic mechanisms employed by fungal pathogens of both kingdoms (Cole and Hoch, 1991). In this article we discuss fungal pathogenesis of both plants and animals and specifically evaluate two approaches that have been used to investigate fungal pathogenic mechanisms.

Degrading the Host

There is a rich history of biochemistry and cytology implicating degradative extracellular enzymes in fungal pathogenicity toward animals (e.g., see Ray and Payne, 1988) and plants (e.g., see Xu and Mendgen, 1997). The hypothesis is that pathogenic fungi may contain specific forms of extracellular enzymes which breakdown host polymers and advance the establishment of disease. The appeal of this hypothesis is understandable because filamentous fungi are naturally invasive and have an absorptive metabolism requiring the secretion of polymer degrading enzymes. Despite apparently convincing biochemical evidence, the demonstration that degradative enzymes are important factors in fungal pathogenesis has not been accomplished.

For phytopathogens, the plant cell wall is the major barrier to infection and is composed of an array of polysaccharides and proteins (Walton, 1994). Fungal mutants defective in cellulases, pectinases, polygalacturonases, xylanases, cutinases, and proteinases have been evaluated for their effects on pathogenesis. Early studies employing genetically uncharacterized variants are difficult to evaluate (for review see Cooper, 1987). However, recent approaches, employing targeted gene-disruption mutants, can be interpreted more easily. Very surprisingly, disruptions of genes encoding cutinases (Stahl and Schafer, 1992; Sweigard *et al.,* 1992, van Kan *et al.,* 1997), xylanases (Apel *et al.,* 1993; Abel-Birkhold and Walton, 1996), and other degradative enzymes (Scott-Craig *et al.,* 1990; Sposato *et al.,* 1995; Murphy and Walton, 1996) have not had dramatic effects on pathogenicity. In one case, combining two or even three mutations resulting in extracellular enzyme deficiencies has also failed to produce a nonpathogenic phenotype (Abel-Birkhold and Walton, 1996). Clearly, the results have surprised and in some cases divided plant pathologists (see for example, Rogers *et al.,* 1994; Stahl *et al.,* 1994).

In the study of human and animal mycoses, the litera-

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ture contains numerous reports of secreted fungal proteases that have been implicated in colonization and invasion. For example *Candida albicans* contains a family of at least seven aspartyl proteases (Monod *et al.*, 1994; Gow *et al.*, 1995; White *et al.*, 1995). Although molecular genetic systems have developed more slowly in some of these fungi, genetically engineered disruptions of genes encoding proteolytic enzymes have so far failed to provide unambiguous evidence for their roles in virulence. Disruption of an alkaline protease (ALP) gene of the human pathogen, *Aspergillus fumigatus*, produced a strain whose virulence was indistinguishable from the wild-type strain in a mouse model of pulmonary aspergillosis (Tang *et al.*, 1993; Jaton-Ogay *et al.*, 1994). Furthermore, double mutants lacking the ALP and either a secreted metalloprotease or a cytotoxin, restrictocin, were not attenuated in virulence (Jaton-Ogay *et al.*, 1994; Smith *et al.*, 1994). *C. albicans*, *C. tropicalis*, and *C. parasilosis* also produce a secreted acid protease (ACP) (Viragh *et al.*, 1993; Togni *et al.*, 1994). Disruption of the ACP gene in *C. parasilosis* did not have an appreciable affect on virulence in a mouse disease model (Sanglard *et al.*, 1992; Togni *et al.*, 1994).

Several reasons can be offered for why the majority of mutants containing gene-disruptions in degradative enzymes remain pathogenic. First, genetic redundancy appears to be common for degradative enzymes and multiple xylanases (Abel-Birkhold and Walton, 1996), cutinases (Yao and Koller, 1995), and proteases (Murphy and Walton, 1996) are common in plant pathogens. Recently, it was proposed that fungal phytopathogens release "a cocktail" of degradative enzymes to attack their hosts (Knogge, 1996). This cocktail hypothesis will be difficult to prove, as the task of assembling combinatorial knockout mutants for different enzyme activities is daunting given the limited tools for genetic analysis in many of these fungi. In addition some pathogenicity assays or infection models may not be sophisticated enough to detect subtle differences in pathogenesis.

Degradative enzyme activities are usually discovered during fungal growth in axenic culture. While this approach facilitates the identification of secreted enzymes, there is a distinct possibility that some forms of enzymatic activity are expressed only during specific stages of pathogenesis. Although some reports suggest this might occur in fungi (e.g., Yao and Koller, 1995), the idea comes from the discovery of two collections of pectinases in the bacterial plant pathogen *Erwinia chrysanthemi*. One group of pectinase genes is expressed during culturing of the bacteria in axenic media and are dispensable for pathogenicity. Only when all of these axenically expressed genes are disrupted were a second group of bacterial pectinases discovered that are only expressed in plants (Kelemu and Collmer, 1993).

Last, the possibility exists that some processes, once ascribed to the activity of fungal degradative enzymes, may be accomplished by other means. For example, although plant tissue maceration must surely require some combination of degradative enzymes, the penetration of the plant cell wall may not. The rice blast fungus *Magnaporthe grisea* is able to directly penetrate plant cell walls using a specialized cell called an appressorium (Bourett and Howard, 1990; Heath *et al.*, 1990). The appressorium is able to generate enormous turgor pressure and can penetrate inert polymer films (Howard *et al.*, 1991). Reductions in cell turgor prevent penetration. These studies in the rice blast fungus demonstrate that the mechanical forces exerted by fungal cell turgor can be substantial. Detailed investigations in other pathogens may reveal a wider role for fungal cell turgor in penetration and invasive growth.

Although many fungal species are adept at degrading complex polymers and exploiting the nutrient-rich environment of wounded and/or debilitated hosts, only a minority (less than 10%) are considered pathogens. Pathogens are generally distinguished by their ability to circumvent host resistance and exploit a unique niche for growth and reproduction. Thus research in animal and plant mycoses has also focused on fungal cell differentiation events that accompany pathogenic growth. There is considerable cytological and biochemical evidence to suggest that pathogenic fungi may utilize specific regulatory pathways. In this scenario, the expression of toxic molecules, proteins, and enzymes are a consequence of infection-related morphogenesis.

Pathogenesis and Fungal Cell Differentiation

The most elaborate forms of infection-related morphogenesis are found in obligate phytopathogens (reviewed by Mendgen and Deising, 1993). Obligate and nonobligate phytopathogens from diverse fungal taxa (Ascomycetes, Basidiomycetes, and Oomycetes) have evolved the ability to differentiate an infectious cell called an appressorium in response to varied signals provided by the plant surface. Biochemical studies have shown that appressorium formation involves the synthesis of new cell wall layers, specialized cell divisions, changes in the cytoskeleton, and changes in the pattern of gene expression. Both calcium and cAMP

have been implicated as potential second messengers in appressorium differentiation (for review see Staples and Hoch, 1987).

Infection-related morphogenesis is also a feature of many human fungal pathogens. Many of these fungi are capable of dimorphic growth and alternate their growth morphology between a yeast-like budding state and a filamentous hyphal-form. Some of these fungi produce a range of asexual propagules. For example, *Coccidiodes immitis* undergoes specialized forms of spore production (spherule formation) during pathogenesis (Sun and Huppert, 1976). Host signals that trigger these morphogenetic changes are unclear, but in culture these morphological transitions can be triggered in response to environmental signals such as nutrients, temperature, serum factors, pH, or oxygen tension. Studies of dimorphism have also documented changes in cell physiology, wall composition, cell cycle progression, and growth polarity (for reviews see San-Blas and San-Blas, 1984; Odds, 1985; Maresca and Kobayashi, 1989). Recently, evidence suggests that dimorphism is also accompanied by changes in pathogen gene expression (Keath and Abidi, 1994; Maresca *et al.*, 1994; Gow *et al.*, 1995; White *et al.*, 1995).

Regulatory Genes and Signal Transduction in Fungal Pathogenesis

Like the studies of fungal extracellular enzymes, gene knockouts have been used to directly test the hypothesis that specific regulatory networks are required for fungal pathogenesis. In these studies "candidate" genes are chosen for cloning and mutagenesis based on physiological studies of the pathogen and from inferences gained from studies in nonpathogens. For example, the presence of rich nutrient sources can repress the expression of numerous putative pathogenicity genes, such as proteases, cutinases, xylanases, etc. In particular, nitrogen starvation is known to regulate morphogenesis in several species of fungi and the expression of some genes during pathogenic growth (VandenAckerveken *et al.*, 1994; Lau and Hamer, 1996). However, the cloning, functional analysis, and disruption of the major transcriptional activator for nitrogen regulation (*NUT1*) in the fungal pathogen *Magnaporthe grisea* showed that it was dispensable for pathogenicity (Froeliger and Carpenter, 1996). This result rules out nitrogen metabolite derepression as an explanation for the regulation of pathogenicity genes in *M. grisea* and provided the impetus for a genetic screen which identified two potentially novel regulatory genes (Lau and Hamer, 1996).

Knock-outs of candidate genes have provided evidence for the involvement of specific signal transduction pathways in fungal pathogenesis. The mitogen-activated protein (MAP) kinase cascades in the budding and fission yeast phermone signal transduction pathways are functionally conserved and are found in multicellular eukaryotes (Sugimoto *et al.*, 1991; Neiman *et al.*, 1993; Herskowitz, 1995). The first evidence that this signaling pathway may have an additional function in fungi came with the discovery that components of this signaling pathway are also required for invasive growth and pseudohyphal formation in *S. cerevisiae* (Liu *et al.*, 1993).

In the corn smut pathogen *Ustilago maydis*, a pathogenic, filamentous, dikaryotic stage is formed by the fusion of nonpathogenic haploid yeast cells of opposite mating type. One mating-type locus encodes peptide pheromones and receptors needed for cell fusion while a second locus encodes transcriptional regulators needed for pathogenicity as well as the mating response (for review see Kahmann *et al.*, 1995). Insertional mutagenesis of Fus7, a candidate protein kinase related to the budding yeast MAP-kinase kinase Ste7, showed that Fus7 has a distinct role in pathogenesis in addition to its role in the mating response (Banuett and Herskowitz, 1994). Similarly a candidate transcription factor Prf1 related to the fission yeast transcription factor Ste11, also has a specific role in pathogenesis, most likely as an activator of gene expression (Hartmann *et al.*, 1996).

A "candidate gene" approach has been used to identify interacting signal transduction pathways involved in infection-related morphogenesis in the rice pathogen *M. grisea.* Exogenously added cAMP can stimulate appressorium formation in *M. grisea;* however, gene-disruption of a candidate cAMP-dependent protein kinase gene, *CPKA* (Mitchell and Dean, 1995), demonstrated an additional role for cAMP signaling in plant penetration (Xu *et al.*, 1997). Another signal transduction pathway required for appressorium formation involves a MAP-kinase called Pmk1 (Xu and Hamer, 1996). Pmk1 knock-out mutants fail to form appressoria and cannot grow invasively in plant cells. Interestingly, Pmk1 can function in place of the yeast MAP-kinases Fus3 and Kss1, involved in the pheromone signaling pathway in *S. cerevisiae.* In *M. grisea*, null mutations in either CpkA or Pmk1 have little or no effect on growth, sporulation, or mating ability in culture, suggesting these kinases participate in signal transduction pathways that are specific for pathogenesis.

Surprisingly, although the plant pathogens *M. grisea* and *U. maydis* come from diverse taxonomic groups, infect different hosts, and have different mechanisms of pathogen-

esis, these recent studies suggest they share components of a common signal transduction pathway for pathogenesis related to the pheromone-MAP kinase signaling pathways of budding and fission yeast. Hyphal growth and pathogenicity in the human pathogen, *C. albicans*, also appears to require homologs of kinases found in the pheromone signaling pathway (Liu *et al.*, 1994; Clarke *et al.*, 1995; Leberer *et al.*, 1996; Kohler and Fink, 1996). The conservation of this signaling pathway in such diverse fungi is not surprising, but its involvement in pathogenesis-related processes in such different fungal pathogens is quite remarkable and suggests that a common signal transduction pathway may have evolved to regulate pathogenic growth in a variety of fungi.

The protein kinases and transcription factors involved in these signaling pathways are not pathogen specific. Thus it will be important to learn what signals activate these pathways in pathogens, and what gene products are induced following their activation. It is tempting to speculate that these signaling pathways in addition to bringing about changes in morphogenesis may also stimulate changes in fungal metabolism that favor pathogenic growth. Such changes might include the production of important secondary metabolites (toxins) and the secretion of specific extracellular enzymes.

Expectations for Controlling Fungal Growth

One widely held expectation is that a study of fungal pathogenesis will yield a plethora of novel "pathogenicity genes" whose products may be used as targets for the development of antifungal chemicals. This expectation might be overly simplistic. Although there may be some biochemical pathways (e.g., for capsule synthesis in *Cryptococcus* or toxin production) unique to some pathogens, the protein sequence conservation observed from early genome sequencing projects makes it seem likely that the majority of genes in fungal pathogens will have homologs in yeast and almost certainly in nonpathogenic, saprophytic relatives. However, fungal associations, at least with plants, are ancient (Simon *et al.*, 1993; Gehrig *et al.*, 1996), and thus one expectation is that studies of pathogenicity may help to ascribe functions to some of the yeast and fungal genes whose sequences are currently without matches in GenBank.

The current findings hint that signal transduction pathways that govern pathogenic growth in fungi are conserved. This is a surprising finding given the diversity of fungi that have been studied. Thus there is the strong expectation that studies of fungal pathogenicity will uncover additional common regulatory pathways that have been adapted for pathogenic growth. These *pathways* (as opposed to "pathogenicity factors") will clearly offer potent and broad range targets for controlling fungal growth and pathogenesis.

Summary

Thus far, it has not been possible to demonstrate that fungal pathogens possess specific extracellular enzymes with important roles in pathogenesis. In contrast, candidate gene knock-out experiments have revealed regulatory networks that appear to be specific to pathogenesis. The tracings of these networks may have already been described in the well-known cell differentiation pathways of many nonpathogens. The challenge for fungal pathologists will be to link these signaling pathways to the host environment and to the expression of determinants that advance the disease state.

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Note added in proof. We recognize that the terms "virulence" and "pathogenicity" appear to have different meanings within the fields of animal (human) and plant pathogenesis. To avoid ambiguity we have used the term "virulence" when referring to animal (human) pathogens and the terms "pathogenicity" and "pathogenesis," when referring to work on phytopathogens.

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