Review

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Fungal physiology and the origins of molecular biology

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Molecular biology has several distinct origins, but especially important are those contributed by fungal and yeast physiology, biochemistry and genetics. From the first gene action studies that became the basis of our understanding of the relationship between genes and proteins, through chromosome structure, mitochondrial genetics and membrane biogenesis, gene silencing and circadian clocks, studies with these organisms have yielded basic insight into these processes applicable to all eukaryotes. Examples are cited of pioneering studies with fungi that have stimulated new research in clinical medicine and agriculture; these studies include sexual interactions, cell stress responses, the cytoskeleton and pathogenesis. Studies with the yeasts and fungi have been effective in applying the techniques and insights gained from other types of experimental systems to research in fungal cell signalling, cell development and hyphal morphogenesis.

The early years of biochemical genetics

In the late 1940s Jackson W. Foster published a treatise on fungal physiology in which he marvelled at the progress that had been made in the most recent years, in the decade following his graduate study in S. A. Waksman's laboratory. This text (Foster, 1949) summarized the field, reviewing organic acid metabolism, the chemical nature of the mould mycelium, nutrition, nitrogen metabolism, advances in penicillin production, and new methodologies in mould metabolism. He was determined not merely to catalogue composition and observations, but to provide a comparison of active biochemical systems, or 'transformations effected by fungi'. His approach was modern in spirit, focusing on experimentation, but even more so when he excitedly described the recent accomplishments in biochemical genetics and the Beadle and Tatum work (Beadle & Tatum, 1941). His interests in the text were concentrated on biochemical activities unique to fungi, but he also recognized the potential usefulness and importance of fungi as model systems for larger questions in biology. In this text, Foster presented a pattern that has been repeated to the present: a dynamic and an occasional conflict between the study of fungi for their inherent, unique activities or for their usefulness and accessibility to study questions that apply to all of biology. Do we study fungi to solve problems in technology, agriculture and human health or to ask and answer larger questions about cell biology, genetics, development and biochemistry? Do we study the physiology of applied science or the molecular biology of model systems?

Despite Foster's enthusiasm and the acclaim eventually awarded to the Beadle and Tatum gene action studies with

Neurospora crassa, as Norman Horowitz reminded us, this experimental approach and its reductionist interpretation were not widely accepted at the time (Horowitz, 1991). Geneticists and biologists in general were uncomfortable with simple interpretations of complex phenomena; the idea that a mutant phenotype was anything more than a terminal disturbance of a complex metabolic process encountered severe resistance. Further, an important criticism of the general applicability of the one-gene, one-enzyme hypothesis was the question of whether auxotrophic mutants, reparable by supply of a single growth factor, were representative of mutations in general. Rowland Davis points out that given the absence of knowledge about the structure of proteins, the identity of the genetic material, the organization of chromosomes, and most metabolic pathways, it was not clear how to trace causal relations between mutation and phenotype of complex organisms (Davis, 2000). Nevertheless, it was this determined attempt to trace these causal relations by Beadle and Tatum and their colleagues and students, through genetic, physiological and biochemical studies, that led to the generalized understanding of gene action and interaction as well as to methodological techniques for mutant generation and analysis that were formative and essential to the new field of biochemical genetics. Tatum and C. H. Gray showed that biochemical mutations also could be induced by irradiation in bacteria, thereby providing suitable (and essential) genetic markers that led directly to the work of Tatum's student, Joshua Lederberg, who demonstrated sexuality and recombination in *Escherichia coli*.

The subsequent studies depended upon isolation and characterization of *N. crassa* mutant strains by new recruits

to the Beadle laboratory, Norman Horowitz, Herschel Mitchell, Mary Houlahan (Mitchell), David Bonner and Francis Ryan, among others. These workers generated a body of evidence supporting the one-gene, one-enzyme hypothesis, and their effort was essential for Beadle's generalization that '... a given enzyme will usually have its final specificity set by one and only one gene' (Horowitz, 1991). Nevertheless, it was not until 1948 that H. Mitchell and J. Lein showed directly that a mutation in the *N. crassa* tryptophan synthetase locus could lead to an enzyme deficiency. The original mutant strain of *N. crassa* upon which the first Beadle and Tatum paper was based turned out not to be due to a null mutation as reported; instead, scientists at the Merck Research Laboratories discovered that the expression of the gene depended upon the pH of the growth medium. This unexpected result led to modifications of the continuous mutant screen in the Beadle laboratory to also include simultaneous high-temperature incubation. This led to another very important discovery and the use by Mitchell and Houlahan in 1946 of temperature-sensitive conditional mutant strains, which proved to be even more useful in genetics than the original nonconditional auxotrophic strains.

Other workers in the 1950s asked, if an auxotrophic nutritional requirement could be attributed to loss of activity in a particular enzyme, was the expression of the mutant gene blocked, or was the gene producing an inactive form of the enzyme? Bonner and Charles Yanofsky showed that the mutant form of *N. crassa* tryptophan synthetase could be immunoprecipitated with antibodies to the wild-type protein and in comparable quantity, clearly supporting the latter alternative. Norman Giles and his colleagues in the 1950s began to study the basis of reversion in biochemical mutants of *N. crassa*, interallelic complementation, and gene structure and organization. The Giles laboratory mounted a decades-long study of gene organization and regulation in the quinic acid gene cluster of *N. crassa* (and other fungi), much later showing that the quinic acid cluster represents a group of adjacent coding sequences whose expression is regulated at the level of transcription and is under the combined control of quinic acid and the qa-1 protein. This group found that a single enzyme, a product of one gene, catalysed two distinct reactions in the same pathway (Giles *et al.*, 1991).

Nitrogen metabolism

These gene action studies and their methodologies set the stage for subsequent investigations in the 1960s and 1970s into nitrogen metabolism (D. J. Cove, George Marzluf, Claudio Scazzocchio, among others), pyrimidine metabolism (Alan Radford) and polyamine synthesis (Rowland Davis). Studies of nitrogen metabolism were prominent in the 1950s and 1960s, when it was assumed that reduction of nitrite to ammonia occurred through discrete two-electron steps through enzymes and intermediates that were proving difficult to isolate. It was subsequently

learned that the hypothetical intermediate steps did not exist and the entire pathway was catalysed by only two multidomain redox enzymes, nitrate and nitrite reductases, whose regulation of expression and activity was to absorb the Cove, Marzluf and Scazzocchio groups for years. Nitrite reductase proved to be unusual in its ability to perform a six-electron transfer in a single enzymic step. The use of secondary nitrogen sources, such as nitrate, purines and amino acids, requires *de novo* synthesis of permeases and catabolic enzymes, and the genes for these enzymes are highly regulated in both induction and repression. Global regulatory genes, such as *nit-2* and *areA*, respond to nitrogen limitation through expression of *trans*-acting proteins that mediate this response to nitrogen limitation. Pathway-specific regulatory genes promote the expression only of genes whose enzymes are appropriate to the substrate to be catabolized (Marzluf, 2004).

Antibiotics and toxins

The discovery of penicillin, its chemical characterization, and its large-scale production (by H. W. Florey and E. Chain, and many others) in the 1940s led to an energetic new effort to discover and characterize additional antibacterial antibiotics, an effort which preoccupied microbiologists, mycologists and chemists and which led to rapid discoveries and the development of streptomycin and chloramphenicol, among many other drugs. The first antifungal antibiotic, griseofulvin, was isolated in 1939. Cycloheximide, also known as actidione, was discovered in 1946. The first member of the polyene macrolide antifungal antibiotics, nystatin, was discovered by Elizabeth Hazen and Rachel Brown in 1950. (Hazen and Brown were co-founders of a grants programme of the Research Corporation that contributed many millions of dollars of nystatin royalty income to mycological research and training.) Amphotericin B, a polyene macrolide still in use for treatment of the most severe fungal infections, was discovered and marketed in the mid-1950s.

The isolation and chemical characterization of mycotoxins was one of the major achievements of mycologists and chemists in the 1960s. Mycotoxins have caused problems as long as grain crops have been grown and stored, but their chemical nature was not determined until the 1950s and 1960s. In the previous decades in the USSR, a lethal disease in humans and livestock, alimentary toxic aleukia, was shown to be caused by consumption of mould-contaminated grain, and it was subsequently determined that the toxic agent was a member of the trichothecene group, potent inhibitors of eukaryotic protein synthesis. In the early 1960s the loss of a large number of animals in England was associated with consumption of peanut meal incorporated into the animal diets, a dietary ingredient that was shown to be contaminated with *Aspergillus flavus* or *A. parasiticus*. The toxic agent was characterized and described as aflatoxin B₁, itself a relatively harmless substance until metabolized by the liver cytochrome P450 system, thus creating one of the most potent carcinogens known. The aflatoxins have remained an

important public health problem, especially as contaminants of maize and tree nuts, along with other, more recently discovered classes of mycotoxins. G. A. Payne, Nancy P. Keller, D. Bhatnagar and colleagues have studied the biosynthetic pathways and molecular genetics of the polyketide aflatoxins produced by *A. flavus* and *A. parasiticus*, and they used a variety of strategies to identify and clone genes for these pathways and to examine their genetic regulation (Brown *et al.*, 1999). (This general area of research experienced an ignominious misapplication of science to politics in the early 1980s when the US government, citing a university-supplied analysis of suspect US military samples, accused the USSR of employing three toxins of the trichothecene family as biological warfare agents in Southeast Asia and Afghanistan. The toxic 'Yellow Rain' which supposedly carried the aerial toxin proved to be only beehive waste and pollen.)

Senescence

David Gottlieb and James Van Etten in the 1960s became interested in using fungi as models for ageing, and they examined changes in a number of physiological properties, biosynthetic and respiratory activities, and composition of *Penicillium*, *Rhizoctonia* and *Sclerotium* during culture growth. A striking finding was that of self-limited growth of mycelial (surface culture) mats, a phenomenon eventually explained by a factor produced in older, internal cells that inhibited growth of younger, peripheral cells. In 1953 G. Rizet described the basic phenomenon of senescence in *Podospora anserina*. Later, L. Belcour, Karl Esser and their colleagues became interested in the genetic and physiological (and eventually, molecular) basis of senescence in *P. anserina*, an organism that exhibited a genetically determined timing of onset of senescence and cell death (Kück *et al.*, 1981). The genetic factor responsible for the strain-specific time-of-onset phenotype was inherited through the protoperithelial or maternal parent. These workers and others, such as Donald Cummings, determined in the 1980s that *P. anserina* senescence is caused by circular, multimeric plasmid-like DNA elements that are amplifications of excised, non-overlapping regions of the mitochondrial genome (Cummings *et al.*, 1979). The stopper mutant strains of *N. crassa*, which show a stop-start, cell death phenotype, also have deficiencies in mitochondrial cytochromes and develop abnormal mitochondrial DNAs with large deletions or rearrangements. Ragged mutants of *Aspergillus amstelodami* show an erratic growth resembling the stopper mutants, and rearrangements of mitochondrial DNA are correlated with this phenotype.

The birth of mitochondrial genetics

In 1949 Boris Ephrussi observed some spontaneously occurring mutant strains of *Saccharomyces cerevisiae* that exhibited slow colony growth, and later these *p petite* strains were shown to be defective in normal respiratory activity. This *p petite* phenotype was not transmitted through sexual

crosses with any known marker gene; the trait was lost upon mating but could be recovered after several generations of mitotic growth. At almost the same time, Mitchell and Mitchell in 1952 isolated a mutant strain of *N. crassa* that grew slowly, initially called *poky*, and they determined that this slow-growth trait was transmitted only through the protoperithelial parent in a sexual cross, the maternal parent that contributes both nucleus and cytoplasm. The trait did not show segregation among ascospores in tetrads. The maternally inherited pattern of slow growth in this *mi-1* strain was later associated with defective respiratory activities and loss of two of the three major cytochrome bands (Lambowitz *et al.*, 1972). These findings presented the first evidence of non-Mendelian inheritance or of cytoplasmic inheritance of a genetic trait. Subsequent work by David Luck and others in the 1960s using other slow-growing strains of *N. crassa* showed that the trait could be transferred from one strain to another in microinjection experiments, and the cytoplasmic element responsible for the trait was the mitochondrial fraction. Luck also performed labelling experiments to test possible explanations for the origin of mitochondria, and he determined that mitochondria arose from pre-existing mitochondria, not from accretion of other cellular components. Anaerobic yeast cells appeared to have no mitochondria, leading to the view that mitochondria assembled *de novo* upon aerobic growth. However, Gottfried Schatz and colleagues showed that anaerobic yeast cells do possess rudimentary mitochondria, and these organelles become visible cytologically if certain lipids are provided to the cultures. A number of groups working with *S. cerevisiae* and *N. crassa* determined that mitochondria possess quasi-autonomous genetic systems, with circular DNAs, ribosomes and tRNAs that are distinct from the cytoplasmic protein synthesis system, and sensitivity to antibiotics such as chloramphenicol that have no effect upon cytoplasmic protein synthesis. The slow-growth phenotypes of *N. crassa* and *S. cerevisiae* mutant strains and the loss of mitochondrial respiratory system components like cytochromes proved to be caused by loss or rearrangement of mitochondrial DNA or defective gene expression and protein synthesis in mitochondria. In *N. crassa*, for example, R. Brambl found that several mitochondrial tRNAs were not synthesized in *mi-1*, apparently a consequence of a mutant transcriptional promoter for a region of the mitochondrial genome that also encoded the small rRNA, as shown by Alan Lambowitz, explaining also the low concentration of mitochondrial ribosomes in this mutant strain.

Alexander Tzagoloff, L. A. Grivell, Nancy Martin and G. Bernardi, and many others, studied the organization and the expression of mitochondrial genes in *S. cerevisiae* and the processing and splicing of transcripts (Tzagoloff & Myers, 1986; Grivell, 1995). A. Lambowitz, E. Agsteribbe and U. L. RajBhandary performed early studies of the *N. crassa* mitochondrial genome, and C. Scazzocchio, G. Turner and H. Küntzel studied that of *Aspergillus nidulans*.

Several laboratories in the 1970s working with *S. cerevisiae*, such as those of Schatz and Tzagoloff, or with *N. crassa*,

such as those of Walter Neupert, Sigurd Werner and Walter Sebald, showed that the proteins encoded by mitochondrial genes provided one to several subunit peptides to each of the respiratory membrane complexes and to the ATP synthase, whereas the remainder of the subunit peptides were encoded by nuclear genes, synthesized on cytosolic ribosomes and imported into mitochondria for assembly with their mitochondrially synthesized counterparts. In the next two decades the Schatz and Neupert laboratories in particular were preoccupied with the process of protein import into the organelles and with determining the structure and function of the surprisingly complicated translocase complexes of the outer and inner mitochondrial membranes (Hoppins *et al.*, 2004; Neupert, 1997). These comprehensive studies with fungal systems provided the foundation for studies of mammalian mitochondria and of the role of mitochondria in human disease.

An important level of regulation of gene expression in mitochondria appears to be post-transcriptional, a gene expression entirely dependent upon nuclear-encoded enzymes and regulatory factors (Brambl, 2004). Thomas Fox and colleagues have found that in *S. cerevisiae*, translation of at least five of the eight major mitochondrial mRNAs for subunits of cytochrome *b* and cytochrome *c* oxidase requires the action of one or more nuclear-encoded, gene-specific translational activator proteins (Costanzo & Fox, 1990). The proteins seem to function by interacting with the mitochondrial ribosome, with specific regions of the 5'-leaders, and with the inner membrane. The Brambl laboratory found evidence of translational control of mitochondrial gene expression in *N. crassa*. The three genes for the mitochondrial cytochrome *c* oxidase subunits are transcribed at sharply divergent times during conidial germination in *N. crassa*. Nevertheless, all three subunit peptides are first synthesized at the same point late in germination, at the time when processed transcripts for the three genes are simultaneously recruited into the mitochondrial polyribosomes (Bittner-Eddy *et al.*, 1994). The 5'-leaders of these mitochondrial mRNAs have conserved stem-loop structures that bind proteins that may regulate their recruitment into the translational apparatus (Kleidon *et al.*, 2003). The nuclear gene *cya-5* of *N. crassa* encodes such a candidate mRNA-binding protein that is required for the mitochondrial translation of stable, mature transcripts for subunit 1 of cytochrome *c* oxidase, a finding that is parallel to the finding in yeast.

Mating systems

In the 1940s and 1950s John Raper discovered that sexual reproduction in *Achlya* species is initiated and controlled by diffusible substances – hormones – that are secreted reciprocally by the interacting mycelia. The isolation and structural determination of the steroidal hormone A, antheridiol, was accomplished by Alma Barksdale. In the Mucorales (during the 1970s), H. van den Ende, J. D.

Bu'Lock, G. W. Gooday and colleagues found that trisporic acid in mated cultures was a substance required for the sexual cycle and that it is cooperatively synthesized from sex-specific precursors by the interacting (+) and (–) strains. In the 1930s, Ö. Winge may have been the first to observe mating-type switching in *S. cerevisiae*, and later he showed that homothallism versus heterothallism is caused by a single pair of Mendelian alleles, now called *HO* and *ho*, respectively. In the mid-1950s, L. J. Wickerham and T. E. Brock found that mating in another yeast, *Hansenula wingei*, depends upon chemically distinct agglutinins on each of the haploid mating cells. Brock, C. E. Ballou and other workers characterized the constitutive sexual mating system in *H. wingei*, which is mediated by two cell surface macromolecules, a glycoprotein and a protein, that provide a specific recognition and agglutination between cells of opposite mating type. Other yeasts, species of *Pichia* and *Saccharomyces*, also were found to have strong constitutive agglutination. J. D. Levi and Brock in the 1950s and J. Kurjan and others in the 1970s found that fusion of haploid cells of opposite mating type in *S. cerevisiae* requires reciprocal secretion of diffusible mating-type-specific peptides, leading to cell cycle arrest in G₁, change in cell shape, and development of agglutinins on the cell surface. In the 1970s and 1980s, Ira Herskowitz, Y. Oshima, Jasper Rine, Kim Nasmyth and others found that mating type interconversion in yeast occurs by transposition of copies of the *a* or α mating-type genes from inactive, storage loci to an active locus, *MAT* (Rine & Herskowitz, 1987). The lack of expression in these storage loci is caused by *trans*-acting regulators encoded by *SIR* genes. The gene products that are encoded by both *MATa* and *MAT α* are proteins that regulate the switch to the alternate mating-type. The replacement of genetic material being expressed at the *MAT* locus by sequences in a distant storage locus represents one of the first molecular explanations of gene conversion, a process first described in *N. crassa* in 1955 by H. Mitchell.

The *A* and *a* mating-type loci in *N. crassa* also regulate heterokaryon formation and heterogenic incompatibility, a process that causes death of the *A* and *a* hyphae at the point of fusion. The *A* and *a* mating-type genes of *N. crassa* were the first to be isolated from a filamentous ascomycete in the late 1980s, by several cooperating groups, by S. J. Volmer, Chuck Staben and C. Yanofsky and by Louise Glass and Robert Metzenberg. These *A* and *a* mating-type genes consist of dissimilar DNA sequences at the same chromosomal locus, and haploid strains contain a single copy of either *A* or *a* sequence, unlike *S. cerevisiae*. These genes encode transcriptional activators that regulate genes in a cell-specific signalling pathway and that regulate expression of pheromones and pheromone receptors (Glass & Nelson, 1994).

Several groups in the 1980s and 1990s, including Robert Ullrich and Charles Novotny (using *Schizophyllum commune*), Lorna Casselton (*Coprinus cinereus*), and Regine Kahmann and Flora Banuett (*Ustilago maydis*), studied the

complicated, multiallelic mating systems of the basidiomycetes (Casselton & Kües, 1994; Kämper *et al.*, 1994). They found that mating interaction in this group of fungi requires coordinated activities of two gene complexes, one of which encodes homeodomain transcription factors that heterodimerize upon mating to generate an active transcriptional regulator that drives other mating responses, and the other of which encodes peptide pheromones and pheromone receptors. The homeodomain proteins 1 and 2 of the basidiomycete fungi resemble the $\alpha 1$ and $\alpha 2$ homeodomain mating-type proteins of *S. cerevisiae*, reflecting a conservation of the mechanisms that regulate mating and sexual development in basidiomycetes and unicellular ascomycete yeasts. The ascomycete proteins act as transcriptional repressors, but they also require additional proteins to complete this function; it is not known how the counterparts in the basidiomycetes regulate transcription in the mated cells.

Studies of mating-type switching in *S. cerevisiae* and of mycelial heterothallic mating-type loci of the ascomycetes *N. crassa* and *P. anserina*, along with those of the basidiomycetes, gave rise to an amazement that complex morphology and complex behaviour could be the result of such simple, single genetic specifications. The mating processes of these fungi have been especially useful for understanding cell signalling pathways in higher organisms, including mammals, and for understanding the roles of protein–protein interactions in regulation of gene expression.

Chitin synthesis: the chitosome

In 1974 José Ruiz-Herrera, working with *Mucor rouxii*, began a series of studies (that subsequently included Salomón Bartnicki-García and still later Charles Bracker) that led to the discovery, isolation and characterization of chitosomes, the intracellular microvesicles that deliver chitin synthetase to the cell surface of a wide range of fungi (Bracker *et al.*, 1976). They are the major reservoir of zymogenic chitin synthetase in fungi, and they have a unique size, a low buoyant density and a shell that is shed upon chitosome activation by substrate. The initial discovery that visible chitin microfibrils could be made from UDP-GlcNAc validated the then-uncertain sugar nucleotide pathway hypothesized for chitin synthesis, and it showed that the crystalline fibrous component of the fungal cell wall could be assembled *in vitro* by these microvesicles. The smallest component of the chitosome capable of synthesizing chitin *in vitro* is the reversibly dissociated 16S subunit (~500 kDa), but the catalytic subunit of chitin polymerization has not yet been identified. This discovery of exocytotic chitosomes and a particulate chitin synthetase activity contrasted with Enrico Cabib's alternative description of a plasma-membrane-bound chitin synthetase in yeast (Cabib *et al.*, 1996). Eventually, it was shown that yeast cells do contain chitin synthetase in conventional chitosomes with a low buoyant

density, and also in another population of large vesicles, derived from the plasma membrane. The rapid growth of the fungal hyphal tip is dependent upon a polarized secretory apparatus, with an accumulation of at least two populations of vesicles at the tip: the microvesicles containing chitin synthesis zymogen, and the macrovesicles containing enzymes and polymers that contribute the amorphous phase of the fungal cell wall.

Spores and spore germination

Research into the physiology of fungal spores began with Alfred Sussman, Paul Allen and D. Gottlieb, building upon cytological studies of Lillian Hawker, P. H. Gregory, C. T. Ingold and others (Weber & Hess, 1976). A seemingly inordinate amount of effort was devoted, especially by the physiologists and mycologists, to defining dormancy, dormancy subtypes and ontogenetic diversity of spores, and even to descriptions of spore germination itself and its ultrastructural correlates (Turian & Hohl, 1981). Since the early discovery of the requirement for heat activation of ascospores of *N. crassa* and *Ascobolus* by B. O. Dodge, several workers, such as Sussman and David Cotter, have attempted to understand the physical basis of this activation, a process that remains unexplained.

In the 1960s several groups began investigating the process of fungal spore germination, in the context of developmental biology. These fungal workers, like those using echinoderm and amphibian eggs, plant seeds or bacterial spores, searched for controls of the resumption of transcriptional and translational activities characteristic of the activation of dormant cells. These studies asked if the regulation of dormancy and activation was controlled at the level of transcription or translation. J. Van Etten and his colleagues – and others, such as Richard Staples and T. Yanagita – compared components of protein synthesis systems extracted from dormant and germinated spores, since it was known that protein synthesis is essential for spore germination, finding that all components were present in the dormant spores, but with generally lower levels of enzyme activities. One of the more attractive questions remained, however: do the dormant spores contain mRNA? Is new transcription required for germination, or if present, does the dormant cell's mRNA encode sufficient information for germination? James Lovett, P. Allen, Larry Dunkle and Richard Staples, along with Van Etten and Brambl, used diverse approaches to answer this question (Brambl *et al.*, 1978). (The highly influential generalization in developmental biology, that cell differentiation is associated with differential gene expression, was expected to apply to spore germination, but it was not clear where this gene expression occurred, during either spore formation or spore germination.) The question seemed to be clearly answered by these workers when mRNA was detected in polyribosomes in dormant spores. The Brambl and Van Etten laboratories separately showed that this mRNA encoded proteins qualitatively

different from the same mRNA fraction of germinated spores. However, it was only after this finding that the Brambl group along with Peter Russell showed that an additional RNA was synthesized early in *N. crassa* conidial germination that was essential for germination (Brambl *et al.*, 1987).

Gottlieb, Sussman and their colleagues measured respiration during germination, particularly in terms of carbohydrates used as substrates for germination, self-inhibitors of germination, and enzymic components of pathways such as glycolysis and the TCA cycle. Using well-characterized inhibitors and techniques to measure oxygen consumption and respiratory enzyme activity, it was easy to establish that aerobic respiration was essential to spore germination. Many years later, Brambl examined the mitochondrial cytochrome electron-transport chain in the dormant pycnidiospores of *Botryodiplidia theobromae* and found that, based upon enzyme activity and upon low-temperature spectroscopy, cytochromes *bc1* and *a* were absent but were assembled upon germination, concomitantly with the development of aerobic respiration. Subsequently, it was established that the cytochrome *c* oxidase was assembled early in germination from several enzyme subunit precursors synthesized and stored in the cytoplasm during dormancy and imported into mitochondria upon initiation of germination for assembly with mitochondrially synthesized subunits (Brambl, 1981, 1985). In the dormant ascospores of *N. crassa*, evidence exists for a similar pattern of assembly of the ATP synthase from precursors of subunits accumulated in cytoplasm. The mitochondrial ATP synthase was also absent in the dormant spores of *B. theobromae*, but its assembly appears to depend upon *de novo* translation of mRNA for the nucleus-encoded subunits of this enzyme. The dormant, nonhydrated conidia of *N. crassa* contain a complete mitochondrial respiratory system and electron-transport system that is active immediately upon spore activation, illustrating the diversity of mechanisms among spore types.

Respiration

Studies of the physiology and biochemistry of respiration began with manometric studies and O. Warburg's influential but erroneous idea of oxygen activation by iron, which he considered to be the chief catalyst in cellular oxidation–reduction reactions. In 1925 D. Keilin used a hand spectroscope to describe changes in absorption properties of the cytochromes *a*, *b* and *c* under different metabolic conditions. Warburg then showed that 'Atmungsferment', the yeast enzyme that reacts with oxygen, was identical to cytochrome *a₃*, the catalytic component of cytochrome *c* oxidase. Subsequently, most progress on understanding of the respiratory system, the relationship between the processes of oxidation and phosphorylation, depended upon studies of mitochondria prepared from animal tissues, the organelles which had just been shown by A. L. Lehninger to be the subcellular

structures in which oxidative phosphorylation occurred. However, the realization that large blocks of commercially prepared, inexpensive Red Star brand baker's yeast would provide readily accessible cell extracts brought biochemists to study yeast mitochondria. In the early 1950s, B. Ephrussi's discovery of the *petite* mutation in yeast, with a deficiency of respiratory functions, and the discovery of DNA in yeast mitochondria further helped bring yeast and other fungi back to the centre of respiration research.

Transport systems

Electrophysiological studies were begun in the early 1960s by Carolyn and Clifford Slayman, who showed that glucose import, as well as other transport systems, is driven by a transmembrane proton gradient (Slayman & Slayman, 1962). Thus, the fungal plasma membrane potential is maintained by a proton flux, generated by the plasma-membrane ATPase, rather than sodium and potassium fluxes as in animal cells. Barry and Emma Jean Bowman, initially working with Carolyn Slayman, began a long-term study of the vacuolar ATPase of *N. crassa*. The Slayman laboratories, with A. Lambowitz, also characterized the differences in concentrations of respiratory cytochrome components of wild-type and *poky* strains of *N. crassa*, and described the cyanide-resistant respiration of this organism, a study that led eventually to the identification of the SHAM-sensitive, CN-insensitive alternate oxidase.

Four amino acid transport systems were identified (by Gabriel Lester, Martin Pall, Gib DeBusk and others) and shown to have broad substrate specificities, like those of animal systems, rather than the highly specific permeases of bacteria. Amino acid biosynthesis is controlled by feedback inhibition of biosynthetic enzymes and by compartmentalization in organelles. Matthew Sachs and others have explored cross-pathway controls of amino acid biosynthesis, in which starvation for a single amino acid causes expression of genes for biosynthetic enzymes of other amino acids in addition to those of the missing amino acid. Absolute levels of free amino acids do not cause this control, which instead is the result of lowering the ratio of charged tRNA to uncharged tRNA, the latter of which limits polypeptide synthesis.

Stress responses and heat-shock proteins

The heat-shock response is a cellular reaction to stressful, but sublethal high temperature, and it features elevated expression of genes whose products, the heat-shock proteins, help cells survive this stress. This response is universal, occurring in all organisms, but it was best characterized in studies in the 1980s in *S. cerevisiae* by Susan Lindquist, Elizabeth Craig and their colleagues (Plesofsky, 2004). The synthesis of these proteins is not confined to the stress response, since they or relatives are essential proteins that are required for metabolism during normal cell growth and development. They have come to

be seen as molecular chaperones, required for folding and assembly of proteins and protein complexes, with certain chaperones, such as Hsp70 and Hsp60, having specific functions that depend upon cellular localization. Distinct forms of Hsp70 are present in the cytoplasm, the endoplasmic reticulum and mitochondria, where they participate in synthesis and trans-membrane movement and folding of substrate proteins, whereas the Hsp60 is confined to the mitochondrion. Nora Plesofsky and her colleagues have studied the expression of the *N. crassa* gene for Hsp30, a protein related to the vertebrate lens α -crystallin, and its function in apparently stabilizing activities of hexokinase and of protein complexes of the outer mitochondrial membrane, such as the MOM complex for import of proteins into mitochondria.

Lignin degradation

Several laboratories led by T. K. Kirk, Dan Cullen and Michael Gold beginning in the 1970s have provided a detailed biochemical understanding of lignin degradation, presenting an example of fungal physiology becoming molecular biology (Cullen & Kersten, 2004). The white-rot basidiomycetes, such as *Phanerochaete chrysosporium*, are unique in their ability to efficiently mineralize lignin, which itself is unsuitable as a sole carbon source, while simultaneously degrading cellulose and hemicellulose. The enzymes that accomplish lignin degradation have an extremely high oxidation potential, and they are extracellular and non-specific. The lignin peroxidase, manganese peroxidase and hydrogen peroxide (a product of glyoxal oxidase) generate highly reactive free radicals that cause a series of spontaneous cleavage reactions. (There is no role for laccases in lignin degradation in *P. chrysosporium*, but they evidently function in related organisms.) In *P. chrysosporium*, previously not known to be accessible experimentally, the recent development of transformation systems, site-specific mutagenesis and heterologous expression systems have led to enormous progress in understanding of lignin degradation. The multiple gene sequences encoding these individual enzymes have been analysed with respect to their structure, evolution and regulation of expression.

Polarized growth and the role of microtubules

Studies of the cytoskeleton in *A. nidulans* and its components begun by N. Ronald Morris and his colleagues have had a large influence throughout eukaryotic biology (May *et al.*, 1985). They found that the cytoskeletal systems that orient and move nuclei in germinating conidia are highly conserved from fungi to animal cells. In early experiments they found that nuclear migration in these conidia was blocked by the microtubule inhibitor benomyl. That the inhibition was due to its effect on microtubules was shown with mutants having genetic lesions in β -tubulin which produce either resistance to benomyl or high sensitivity. Subsequently, dynactin, dynein (a minus-end

microtubule-stimulated ATPase-motor), and other components on the plus ends of the astral microtubules interact with the cell cortex to generate forces that act on the minus ends of microtubules embedded in a γ -tubulin (discovered in *A. nidulans*)-containing structure in the nucleus, the nuclear microtubule organizing centre or the spindle pole body (Oakley, 1985).

Hyphal morphogenesis and the mechanism of polarized growth of fungi at the hyphal tip has deeply interested mycologists and cytologists for decades, and until recently the components of this system were unknown. In the 1960s Tatum and his colleagues generated and characterized a number of morphological mutants which, decades later, with techniques of molecular genetics, were shown to be mutant in signalling pathways, such as protein kinases, or components of the cytoskeleton or dynein motor complexes. With abundant vesicles contributing to a highly structured tip growth apparatus, the Spitzenkörper, it has been assumed that this apparatus functioned to incorporate cell wall components into vesicles and to deliver these vesicles to the plasma membrane at the hyphal tip for exocytosis and assembly of the wall components. Salomón Bartnicki-García, Berl Oakley, Steven Harris, Michelle Momany and others have contributed both mathematical models and experimental evidence showing that microtubules and actin microfilaments have important roles in this vesicle traffic (Harris *et al.*, 1999). Recently, generation of fluorescent fusions of proteins involved in tip growth has made it possible to study their distribution by time-lapse microscopy. The components of a stable, highly structured but dynamic tip growth apparatus are held together by actin cables. The microtubules are important for long-distance delivery of vesicles to the Spitzenkörper; actin microfilaments organize vesicles within the Spitzenkörper (which may be a vesicle supply centre), control their delivery to the apical plasma membrane of the hypha, and position the tip growth apparatus. Endocytosis of secretory components occurs at a ring of the plasma membrane just below the apex.

Dimorphism: yeast versus hyphal growth

The phenomenon of fungal dimorphism was described first in early studies in *Mucor rouxii* by workers such as S. Bartnicki-García, G. W. Gooday and Roger Storck (Stewart & Rogers, 1978). Originally, dimorphism seemed to be a useful model for morphogenesis and regulation of apical growth, but more recently it has also become important in terms of growth of clinically and agriculturally important fungi. The conversion of cells from a yeast-like to a mycelial habit of growth was studied initially in terms of the inducing cultural or environmental conditions. The yeast form of *M. rouxii* results from growth under anaerobiosis in an atmosphere containing at least 30 % CO₂; in the absence of CO₂ and regardless of O₂, growth is mycelial. A causal relationship seemed to exist between respiratory or oxidative metabolism and mycelial growth,

but only later did it become evident that the concentration and nature of the carbon source is the primary determinant of yeast-like growth in *M. rouxii*. Disruption of mitochondrial functions also stimulates yeast-like growth. In *M. rouxii*, cAMP levels decrease during the yeast-to-hyphal transition induced by exposure to O₂ and this transition was blocked by addition of dibutyryl-cAMP. Certain amino acids, such as cysteine or proline, induce yeast-like growth in fungi such as *Candida albicans* and *Histoplasma capsulatum*, fungal pathogens in which dimorphism is important as a clinical phenomenon.

In 1992 G. R. Fink's group reported that in an atypical, non-laboratory strain of *S. cerevisiae*, nitrogen starvation (or poor carbon sources and some physical stresses) caused formation of filamentous chains of cells called pseudohyphae, with a resulting filamentous growth that differs morphologically and behaviourally according to diploidy or haploidy (Roberts & Fink, 1994). This pseudohyphal or invasive growth requires two signalling pathways, a MAP kinase cascade and a cAMP-dependent pathway, involving as a central element the GTP-binding protein Ras2. Pathogenic *C. albicans* may grow reversibly as a budding yeast or as pseudohyphae, and it also develops true hyphae (without constrictions that mark septa) and germ tubes, in which growth depends upon apical extension. In *C. albicans*, hyphal development is induced by stresses such as nitrogen starvation, high temperature and high pH.

In the basidiomycete *Ustilago maydis*, the cause of maize smut, the haploid, yeast-like cells undergo mating and develop a dikaryotic filamentous cell; a haploid yeast phase can undergo direct conversion to a haploid hyphal phase. Flora Banuett and her colleagues have studied the *U. maydis* yeast-like non-pathogenic form and filamentous pathogenic form, finding that the *fuz1* gene encodes a Zn-finger domain protein that through interactions with other proteins regulates specific transcriptional activity and morphogenesis (Banuett & Herskowitz, 1994). Disruption of genes encoding adenylate cyclase or the catalytic subunit of cAMP-dependent protein kinase led to constitutive filamentous growth, which could be suppressed by the lack of MAPKK (Fuz7), leading to the suggestion that morphogenesis in this organism is regulated by both cAMP and MAP kinase signal transduction pathways, reminiscent of the much earlier observations with the phycomycete *M. rouxii*.

Signalling

Our understanding of pathways of cell signalling depends largely upon studies with *S. cerevisiae*, and these studies have guided research in the filamentous fungi. The trimeric G-protein-linked pathways permit responses to extracellular signals, such as light, chemoattractants and hormones. The G protein $\beta\gamma$ dimer functions in many roles in the signalling process, including membrane localization of the G protein α subunit. The G β subunit in yeast functions as a regulator of the pheromone response in haploid cells by

activation of the MAP kinase cascade. Deletion of this subunit gene in *Cryptococcus neoformans* causes sterility, and deletion in other species leads to disruption of conidiation, hyphal growth, pigmentation, defects in the cAMP glucose-sensing pathway and aberrant reproductive structures. Katherine Borkovich and colleagues have shown that the G protein $\beta\gamma$ dimer is essential for normal asexual sporulation and female fertility in *N. crassa* (Kays & Borkovich, 2004). An understanding of regulation of non-senescent, necrotic and apoptotic death in mycelial fungi has been slow to develop, although recently, Nora Plesofsky and her colleagues have shown that regulated cell death resulting from physical and nutritional stress in *N. crassa* may be signalled by stress-specific sphingolipids through the OS-2 MAP kinase pathway (Plesofsky *et al.*, 2008).

Circadian rhythms

Fungi, especially *N. crassa*, have been useful in the study of circadian rhythms, in part because the conidiation banding patterns could be readily seen in growth tubes and measured. The characteristics of the circadian rhythm include its entrainment by light, its free-running or persistent near-24 h expression in darkness, and a period that is not influenced by temperature. Colin Pittendrigh and Malcolm Sargent and their colleagues in the 1960s were among the first to study the *N. crassa* circadian rhythm of conidiation, to demonstrate phase-shifting behaviour by blue light, and to isolate mutants in the periodicity of conidiation. The metabolic source of the rhythms, the oscillator, remains undetermined, as does the mechanism of its coupling to its developmental and metabolic expression. A long-term study initiated in the mid 1970s by Jay Dunlap and Jennifer Loros and their colleagues has yielded a number of mutants at the *frq* (frequency) locus, as well as several other loci that affect expression of the clock, in which period lengths were up to 5 to 7 h shorter or longer than wild-type (22 h) or in which no rhythm was expressed. Transcripts of the wild-type *frq* gene exhibit a 22 h rhythm in abundance, and cellular rhythm is lost if *frq* is expressed constantly. The protein translated from the *frq* mRNA localizes to the nucleus, where it negatively regulates *frq* transcription. The products of the *white collar* genes participate as transcription activators in light-sensing mechanism of the clock, including induction of the *frq* gene, which mediates light entrainment of the clock. The *white collar-1* protein, with a chromophore flavin adenine dinucleotide, is probably the blue-light photoreceptor for light responses in *N. crassa* (Dunlap *et al.*, 2004).

Chromosomes and telomeres

An understanding of the biology of fungal chromosomes began with Barbara McClintock's demonstration that the chromosomes of *N. crassa* were like those of plants and animals. She was the first to visualize fungal chromosomes and she described the distinctive morphologies of the seven *N. crassa* chromosomes at pachytene and their behaviour

during meiosis. She described meiotic pairing in a translocation heterozygote and recorded the ascus phenotypes stemming from different modes of segregation when the translocation was heterozygous (Perkins, 1992). Twenty years later, analysis (by J. A. Huberman and colleagues) of replicating chromosomal DNA of *S. cerevisiae* by fibre autoradiography showed that replication initiates at multiple initiation sites, giving rise to replication forks, along each DNA molecule, and these replication forks move bidirectionally away from the initiation sites. Subsequent studies of yeast helped define the essential attributes of the eukaryotic chromosome, including the autonomously replicating sequences, centromeres and telomeres; the identification of proteins needed for chromosomal replication; the structure–functional analysis of centromeric sequences; and telomere dynamics and replication. Carol Newlon and her colleagues have contributed much to our knowledge of yeast chromosome replication, with the development of tools to study these processes and identification of proteins that function at the replication fork. McClintock anticipated the need for specialized structures at the ends of chromosomes to maintain their stable transmission and to prevent end-to-end fusions. Many decades later, telomeres were identified as the structures at the ends of chromosomes whose function is to stabilize these chromosomes and prevent stepwise loss of genetic material as a result of the incomplete replication of DNA strands with each round of replication. The discovery of the ribonucleoprotein telomerase, with its enzyme-associated RNA template, which recognizes and extends the G-rich strand of the terminal repeat by adding T-G polydeoxyribonucleotide repeats, came from studies of the ciliate *Tetrahymena*, but this insight was soon expanded by studies of telomeres and the proteins involved in telomere function in *S. cerevisiae* by laboratories of V. A. Zakian, B. Tye, Judith Berman, J. E. Haber, E. H. Blackburn, D. Shore, and many others (Enomoto *et al.*, 2002).

Gene silencing

Integration of transforming DNA in *N. crassa* usually occurs ectopically. When a transformant bearing both a resident gene and a homologous, ectopic sequence is crossed sexually, both copies of the sequence in the transformant nucleus, prior to nuclear fusion, undergo slight to extensive mutation. In the 1980s Eric Selker and colleagues discovered that introduced duplications of chromosomal segments led to inactivation of the relevant genes through cytosine methylation, followed by deamination, and subsequent GC→AT transitions (Galagan & Selker, 2004). This mutagenic process correlated positively with closeness of linkage of the duplications and is more severe and more likely with duplications of 1 kbp or more. This process is thought to be an inactivating defence against transposable elements and a mechanism to force long homologous sequences to diverge increasingly with

repeated rounds of sexual reproduction until they are no longer sufficiently homologous to attract this mutagenesis.

Quelling is a process discovered by Giuseppe Macino and colleagues (Catalanotto *et al.*, 2004), in which the expression of certain genes is reduced when multiple, complete or incomplete copies of these genes are introduced into the genome of *N. crassa*, much like transgene silencing in green plants. This reversible gene-silencing results from the RNA interference pathway that causes both transgene-induced as well as double-stranded RNA-induced gene silencing. A transgene-specific transcript is converted to double-stranded RNA, which is cleaved to 25-nucleotide inhibitory RNAs that block gene expression through an RNA-induced silencing complex. Slightly shorter inhibitory RNAs, originating from the rRNA locus, tRNA genes, intergenic regions of the genome or open reading frames, have been shown by Yi Liu and colleagues to be induced by the DNA-damage response. These inhibitory RNAs could help regulate DNA damage checkpoints by inhibition of protein synthesis until DNA repair.

In *N. crassa*, repeat induced point mutation and quelling destroy or silence non-single-copy DNA sequences in haploid mycelial cells. R. Metzenberg and colleagues identified a novel phenomenon, meiotic silencing, as a third mechanism for silencing expression of unpaired sequences, occurring during mating in the transient diploid zygote cell, following karyogamy (Shiu *et al.*, 2001). DNA that is unpaired early in meiosis, in prophase I, induces silencing of all other DNA that is homologous to it, paired or unpaired.

Concluding perspective: the emergence of fungal molecular biology

Fungal physiology became biochemistry, which became molecular biology. These shifts were accompanied by the diminishing importance of the purely observational, descriptive science as an end point and the growing importance of the hypothesis-driven experimental science. Two or three decades ago, students used certain filamentous fungi and yeasts to study problems in physiology or biochemistry, unaware of progress under way in laboratories where topics in genetics were being explored through use of these same fungi. Students in genetics mirrored the biochemists in their obliviousness. Nevertheless, these younger workers became aware of each other and began to appreciate the insight that might be achieved through combining the experimental design, intellectual approaches, and techniques of biochemistry and fungal genetics. Today this historical separation between eukaryotic biochemistry and genetics has now almost disappeared, these disciplines having merged today into fungal molecular biology. Further, as Jackson Foster anticipated, the most consistent ingredient in this evolution of fungal physiology and biochemistry is the incorporation of genetics (and the thinking of geneticists) into the science,

leading to molecular biology. Finally, the occasional conflict between research driven by economic or clinical needs and that driven by curiosity, seen by those who have worked in both worlds, actually repeats itself today, but it seems tempered by the many recent examples of progress in one area eventually making possible scientific insight and public support in the other.

Another author invited to prepare this review certainly would have included topics and names of individuals that are not mentioned here or given sufficient attention. I have discussed topics that others might have minimized and I have omitted topics that others may consider to be far more important, this being a disappointment or a relief, depending upon one's point of view. The individuals cited here, for the most part, are those who have had some direct or indirect influence on my own experiences and career, many of whom I began to meet as a student. This account is by no means a history of this era; but a history begins as stories inside the personal narratives of those who tell it. This is a recollection of topics I've enjoyed learning about and teaching and in some cases have had the remarkable good fortune to investigate with my own friends and laboratory colleagues.

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