American Journal of Botany
Celebrating 100 years 1914–2014

American Journal of Botany 101(12): 2022–2035, 2014.

AJB CENTENNIAL REVIEW

NEUROSPORA CRASSA: LOOKING BACK AND LOOKING FORWARD AT A MODEL MICROBE[1]

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Investigation of the red bread mold that contaminated French bakeries nearly two centuries ago has led to a wealth of discoveries that have impacted our understanding of genetic, biochemical, and molecular mechanisms in microbes, from Mendelian genetics and the gene–enzyme relationship to circadian rhythm and plant cell wall degradation. Early *Neurospora* research focused on elucidating mechanisms of genetic recombination and gene action and later progressed to addressing complex biological questions of eukaryotic microbes. Here we review the evolution of the filamentous fungus *Neurospora* as a model microbe over the past century. We discuss the origins of *Neurospora* as a model microbe, the immediate scientific impacts from work in this filamentous fungus, and how the introduction of other model organisms (i.e., *Escherichia coli* and *Saccharomyces cerevisiae*) redirected the focus of *Neurospora* research. *Neurospora* has and continues to inform our understanding of a myriad of basic scientific concepts and now has the opportunity to forge into the applied biosciences and biotechnology.

Key words: biochemical mutants; biotechnology; circadian rhythm; Fungal Genetics Stock Center; gene silencing; genetics; heterokaryosis; *Neurospora crassa*; photobiology; population.

BRIEF HISTORY OF *NEUROSPORA* RESEARCH

Neurospora has endured as a model organism for nearly a century, albeit an evolving model. The evolution of *Neurospora* as a model system began nearly two centuries ago when it was first described by Payen in 1843 as a contaminant in French bakeries (champignons rouges) and later by Went in 1901 for its use in production of fermented soybean or peanut cake (then known as *Monilia sitophila*). Nearly one century passed from the initial description of the "red-bread mold" until its reclassification as the new genus *Neurospora* based on the observation of a complete sexual cycle. The foundational work of Shear and Dodge (1927) described three species of *Neurospora*: *N. crassa*, *N. sitophila* (both heterothallic), and *N. tetrasperma* (pseudohomothallic). Today, based on phylogenetic analyses and biological species recognition concepts, at least 43 species are recognized, of which 13 are heterothallic, 2 are pseudohomothallic, and 28 are homothallic (Davis, 2000; Dettman et al., 2003; Villalta et al., 2009; Nygren et al., 2011). *Neurospora* genetics were pioneered by Shear and Dodge (1927); key insights from their work included how to enable mating and sexual spore activation and the presence of two mating types via observation of 1:1 segregation of mating-type genes in progeny from a cross.

Attributes of *Neurospora*, such as its minimal requirements, its incredibly fast-growth (~3 mm/h), a short life cycle, and its haploid genetics, made it an attractive alternative to *Drosophila* for Mendelian genetic studies. Following advances in topics of classical genetics (Shear and Dodge, 1927; Lindegren, 1932, 1933; Lindegren and Lindegren, 1937, 1939) and elucidation of a defined culture medium (Butler et al., 1941), *Neurospora* was selected by Beadle and Tatum to develop biochemical mutants. In these landmark studies, rather than investigating gene action indirectly by determining the biochemical basis of already known hereditary traits (e.g., *Drosophila* eye color), Beadle and Tatum (1941) instead asked how genes control known biochemical reactions (e.g., vitamin or amino acid biosynthesis in *Neurospora*). Results from this work supported the emerging "one gene–one enzyme" hypothesis, and led to adoption of *Neurospora* as a model system for studying biochemical genetics. Hundreds of auxotrophic mutants of *Neurospora* were selected and used for studying genetic recombination. However, it was not long before interest was diverted to a new microbe, the gram-negative bacterium *Escherichia coli*. Much of the early work on gene action in *Neurospora* was repeated and further supported in this bacterium. After genetic recombination was characterized in *E. coli* and due to the facile and well-behaved cultures, *E. coli* was quickly adopted as the model for biochemical genetics in the 1950s.

While *E. coli* functioned as an excellent model for elucidating simple metabolic reactions and linear biochemical pathways, this microbe could not function as a model for more complex eukaryotic systems. Thus, the role of *Neurospora* as a model system evolved; investigations focused on eukaryote-specific genetics, including mitochondrial inheritance (Mitchell and Mitchell, 1952), meiotic recombination (Barratt et al., 1954;

[1]Manuscript received 27 August 2014; revision accepted 20 October 2014.

We thank the Fungal Genetics Stock Center at Kansas State University, Manhattan, Kansas for ongoing support of *Neurospora* research.

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doi:10.3732/ajb.1400377

Catcheside et al., 1964), gene conversion (Mitchell, 1955), meiotic drive (Turner and Perkins, 1979), and metabolic pathway regulation (Gross, 1965; Marzluf and Metzenberg, 1968; Carsiotis et al., 1974; Chaleff, 1974). During this phase of evolution of *N. crassa* as a model organism, significant advances in domestication of *Neurospora* were made, including development of long-term strain storage conditions (Perkins, 1962) and a convenient cultivation medium (Vogel, 1956). Standard isogenic and high-fertility strains of both mating types were prepared and designated as the Oak Ridge (OR) genetic background (Case et al., 1965; Mylyk et al., 1974). The OR background strains were widely adopted in the *Neurospora* community, and OR was the genetic background of choice for most future studies.

Competition with other model systems was not finished for *Neurospora*. In the 1960s and 1970s, the yeast *Saccharomyces cerevisiae* found favor with many research groups as a model eukaryote due to its ease of culturing, genetics, and stable haploid and diploid phases. During this period, the *S. cerevisiae* research community exploded and rapidly developed a toolbox of methods and techniques that facilitated advances in biochemistry, cell and molecular biology, and genetics. So, yet again, *Neurospora* evolved as a model system. *Neurospora* is not only more developmentally complex than *Saccharomyces*, but also undergoes several biological processes that are good models for multicellular eukaryotes. Thus, investigations in *Neurospora* refocused on complex biological processes, including cell development and differentiation (Berlin and Yanofsky, 1985a, b), gene silencing and DNA methylation (Selker et al., 1987; Cambareri et al., 1989; Romano and Macino, 1992; Shiu et al., 2001; Tamaru and Selker, 2001), and circadian rhythm and photobiology (Pittendrigh et al., 1959; Sargent and Briggs, 1967; Feldman and Hoyle, 1973).

Neurospora is now considered the model filamentous fungus. Specifically, *N. crassa* has been the subject of extensive investigation and further development since the advent of molecular genetics. The rise of modern molecular biology techniques and transformation of genetic material in *Neurospora* (Mishra and Tatum, 1973; Case et al., 1979) led to the discovery of gene silencing (Selker et al., 1987) and launched a revolution of epigenetic studies. As DNA manipulation and sequencing technology improved, the *Neurospora* community kept pace with identifying key insights from the genome, including identification of the 5S RNA genes (Free et al., 1979), characterization of telomeric sequences (Schechtman, 1987), and identification of coding sequences of many genes via expressed sequence tag (EST) libraries (Nelson et al., 1997), and followed by whole genome sequencing of *N. crassa* (Galagan et al., 2003), a first among filamentous fungal species. In recent years, molecular tools have been developed for targeted gene disruption and integration of recombinant DNA (Ninomiya et al., 2004), inducible and constitutive promoters (McNally and Free, 1988; Kupper et al., 1990; Campbell et al., 1994; Colot et al., 2006; Hurley et al., 2012; Lamb et al., 2013), and selectable markers (Orbach et al., 1986; Avalos et al., 1989; Staben et al., 1989; Austin et al., 1990; Yamashiro et al., 1992; Colot et al., 2006). These tools enabled production of the whole genome deletion library, which is an invaluable genetic resource to the *Neurospora* community (Colot et al., 2006; Dunlap et al., 2007), and has facilitated protein expression and thus protein biochemical studies (Honda and Selker, 2009).

Despite being challenged by the development of other model organisms more amenable for answering some early biochemical and genetic questions, *Neurospora* research has evolved and found a niche investigating more complex biological questions of higher eukaryotes. Current research continues to provide valuable insights into several areas of biology that is applicable for fungi, as well as across all kingdoms of life. In particular, research efforts are focused on circadian rhythm, epigenetics, gene expression and regulation, genomics, environmental sensing, morphogenesis, and as a model for cell and developmental processes. Numerous comprehensive reviews on *Neurospora* biology have been published: heterokaryosis, cell and developmental biology, circadian rhythm and photobiology, mitochondria, epigenetics, natural populations, mating types, and classical genetics (Perkins and Barry, 1977; Glass and Nelson, 1994; Glass et al., 2000; Turner, 2001; Kennell et al., 2004; Jinhu and Yi, 2010; Crosthwaite, 2011; Riquelme et al., 2011; Baker et al., 2012; Aramayo and Selker, 2013).

CLASSICAL GENETICS

At the start of the 20th century, the groundwork for classical genetic studies was made by Thomas Hunt Morgan in the model system *Drosophila melanogaster* using random strand analysis. Since only one quarter of the meiotic data are captured in this organism, an alternate model system was pursued. All meiotic data can be captured by tetrad analysis of *Neurospora*, and the products of meiosis (ascospores) are contained within a sac (ascus) in the order of formation. This meiotic property of the haploid fungus lent itself as a superior model for classical genetic studies and enabled key discoveries in genetic recombination, including 1st and 2nd meiotic division segregation (Lindegren, 1932) and that crossovers occur at the 4-strand stage of meiosis (Lindegren, 1933). The characteristic ordered ascus was used to investigate the frequency of 2nd division segregation, which informed chromosome centromere mapping. Additionally, the linear ascus was essential in determining that crossovers may involve two, three, or all four chromatids (Lindegren and Lindegren, 1937). Most classical genetic studies do not require an ordered ascus; however, analysis of all meiotic products is essential for elucidating complete genetic recombination events that occurred during meiosis. Full tetrad analysis by random spore analysis enabled classification of linkage groups and genetic mapping within all seven linkage groups of *N. crassa* (Lindegren, 1936; Lindegren and Lindegren, 1939; Houlahan et al., 1949; Barratt et al., 1954), as well as the discovery of gene conversion (Mitchell, 1955).

Around the same time that the seven linkage groups were identified, the chromosomes of *Neurospora* were visualized via cytogenetic light microscopy; meiotic chromosome behavior was deemed similar to that of higher eukaryotes (McClintock, 1945). *Neurospora* once again proved to be a valuable resource by facilitating cytogenetic studies as a haploid microbe with low chromosome number, and most importantly, a life cycle that permits survival of all meiotic products (Perkins and Barry, 1977). Of particular interest, viable vs. inviable meiotic products can be evaluated by visual inspection (i.e., viable ascospores exhibited black pigmentation, while inviable spores were white), which enabled simple detection of chromosomal aberrations. The ratio of black to white ascospores is characteristic of and can be interpreted as different chromosomal aberrations (e.g., reciprocal translocation, insertional translocation, quasiterminal translocation, or pericentric translocations) detected via ascospore abortion and cytogenics (McClintock, 1945; Singleton, 1948). Since then, this technique has been used to

detect hundreds of chromosomal rearrangements. Strains carrying these rearrangements have been used for many genetic studies, for example, for determining dominance and dosage effects in gene regulation (Metzenberg et al., 1974) and for identifying genes involved in heterokaryon incompatibility (Newmeyer and Taylor, 1967; Mylyk, 1975; Perkins, 1975).

BIOCHEMICAL MUTANTS

Arguably the most important contribution to the field of genetics by *Neurospora* was the revolutionary biochemical mutant screen of Beadle and Tatum (1941), which enabled investigation of "lethal" mutations. Biochemical mutants were identified by screening for variants of *N. crassa* that were able to grow on complete medium and unable to grow on minimal medium (Fig. 1). Selected mutants were then subjected to a panel of nutritional supplements (i.e., amino acids, vitamins, and carbon sources) and evaluated for restored growth. In the initial experiment, three mutants were identified: vitamin B_1, vitamin B_6, and para-aminobenzoic acid. The vitamin B_6 deficient strain was further characterized as a single-gene mutation through segregation analysis (Beadle and Tatum, 1941). This elegant mutant screen to identify gene reactions that influence known biochemical reactions gave rise to the one gene–one enzyme hypothesis, which was further investigated by examining full biosynthetic pathways (Srb and Horowitz, 1944; Tatum and Bonner, 1944).

This initial biochemical mutant screen led to the elucidation of genes involved in biosynthetic pathways under the theory that a mutation in a gene resulting in loss of enzyme activity of an intermediate biosynthetic reaction would result in (1) auxotrophy for the whole biosynthetic pathway that can be recovered by the final product or any intermediate downstream in the biochemical pathway and (2) accumulation of the metabolic intermediate acting as substrate for the reaction. The tryptophan biosynthetic pathway was interrogated with two genetically distinct tryptophan auxotrophs (tryptophanless). One strain accumulated the metabolic intermediate, anthranilic acid, which restored growth of the second strain on minimal medium. This result indicated that the gene in the latter strain acts upstream in the tryptophan biosynthetic pathway (Tatum and Bonner, 1944). Similarly, the arginine biosynthetic pathway was characterized. Arginine auxotrophs (designated argininеless) were selected using this method with arginine as the nutrient supplement and characterized by complementation studies and outcrossing (Srb and Horowitz, 1944). Seven genetically distinct, single-gene loci were identified. A representative mutant strain for each of the seven genes was further interrogated with different minimal medium supplements of metabolic intermediates known for the arginine biosynthesis pathway: ornithine and citrulline. Results from this experiment indicated at least seven reactions were involved in arginine biosynthesis and gene order

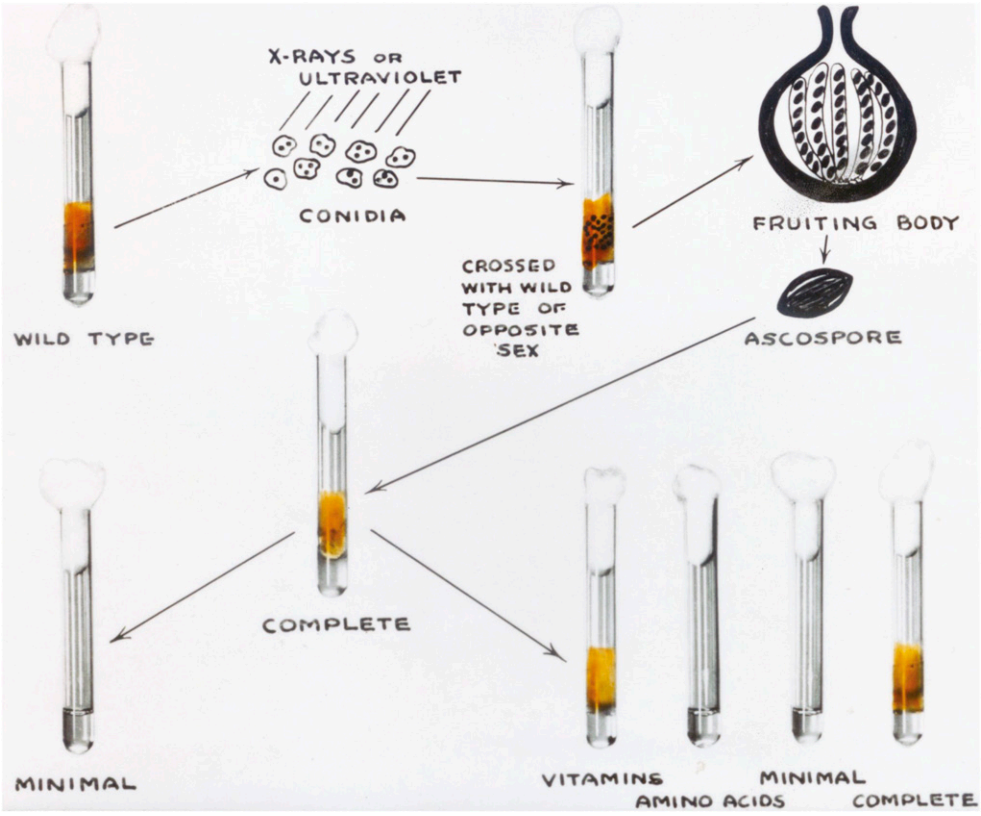

Fig. 1. Beadle and Tatum's experimental design for producing and isolating biochemical mutants. Wild-type *Neurospora* conidia were mutagenized with x-rays or ultraviolet light and subsequently mated with nonmutagenized *Neurospora*. The resulting ascospore progeny were transferred individually into complete medium. Mutant strains were tested for their ability to grow on minimal medium. Mutants that were unable to grow on minimal medium were tested for restoration of growth on minimal medium supplemented with a vitamin or an amino acid. Minimal medium and complete medium were used as experimental controls. Courtesy of the Archives, California Institute of Technology.

in relation to ornithine and citrulline synthesis was determined (Srb and Horowitz, 1944).

Some early criticism regarding use of nutritional supplements to recover auxotrophic mutants instead of restoring enzyme function to support the one gene–one enzyme hypothesis led to the identification of temperature-sensitive mutants. These conditional mutants exhibited a wild-type phenotype at a lower (permissive) temperature, but had an auxotrophic phenotype at a higher (restrictive) temperature: riboflavin-requiring (Beadle and Tatum, 1945; Mitchell and Houlahan, 1946; Miller and McElroy, 1948) and tyrosine-requiring (Horowitz and Shen, 1952; Horowitz and Fling, 1953). In addition to providing further evidence supporting the one gene–one enzyme hypothesis, these studies laid the groundwork for identifying and studying essential genes.

As a brief aside, additional support for the one gene–one enzyme hypothesis came from characterization of missing enzymatic activities in mutant strains that could be purified from wild-type strains of *Neurospora*. The cell-free extracts from two tryptophan auxotrophs were deficient in the enzymatic activity for tryptophan desmolase (Yanofsky, 1952). Similarly, strains auxotrophic for α-amino nitrogen due to a single gene mutation (*am*) were deficient in NADP-glutamate dehydrogenase activity (Fincham, 1954). Over time, the original one gene–one enzyme hypothesis evolved to the one gene–one polypeptide hypothesis, which accounts for findings that some proteins are composed of several polypeptide chains encoded by separate genes (Yanofsky, 1952; Fincham, 1954).

Most biochemical genetic studies transitioned to investigation in *E. coli*, in which genes for biochemical pathways are often coordinately regulated in operons. Unlike prokaryotes, eukaryotes do not typically use this type of gene regulation; hence, *Neurospora* and other fungi were used to investigate alternate forms of gene regulation used by eukaryotes. Specifically, *Neurospora* biochemical mutant selection enabled key findings in gene regulation and compartmentalization. Coordinated control of unlinked genes was identified in a leucine auxotroph (*leu-3*); the gene product of *leu-3* is a positive regulator of leucine biosynthetic enzymes (Gross, 1965). Additionally, positive regulation of sulfur metabolism was identified in a similar experiment using the sulfur auxotroph *cys-3*. The gene product of *cys-3* acts as a direct, positive regulator for three sulfur acquisition enzymes: aryl sulfatase, choline sulfatase, and sulfate permease (Marzluf and Metzenberg, 1968). Two of the best-studied metabolic pathways in *Neurospora* are aromatic amino acid biosynthesis and quinic acid catabolism. These pathways use a common metabolic reaction (dehydroquinase), but do not cross react (Giles et al., 1967). Pathway separation is accomplished via (1) tight substrate inducible regulation of quinic acid catabolism, which is organized as a coordinately controlled and linked quinic acid pathway gene cluster (Chaleff, 1974), and (2) aggregated aromatic amino acid biosynthetic enzymes (i.e., one polypeptide as part of a multidomain enzyme) that allows channeling of metabolic intermediates directly into subsequent biosynthetic reactions (Catcheside et al., 1985).

Compartmentalization is an alternate approach to prevent cross talk between metabolic pathways that use common metabolic intermediates. Compartmentalization of enzyme pools was first investigated in *Neurospora* using two arginine auxotrophs (*arg-2* and *arg-3*) that encode distinct carbamyl phosphate synthases. Channeling of carbamyl phosphate into separate biosynthetic pathways (arginine and pyrmidine) (Williams et al., 1971) was evaluated by determining intracellular localization of ARG-2 and ARG-3, which indicated spatial separation of these two enzymes by the mitochondrial membrane (Bernhardt and Davis, 1972). Later studies demonstrated spatial separation or compartmentalization of metabolic intermediates of arginine anabolism and catabolism (Weiss, 1973). In conjunction with work on compartmentalization of metabolic processes were elegant investigations on mitochondrial protein import, where comparative analyses of *Neurospora* and *S. cerevisiae* played important roles in dissecting this process (Herrmann and Neupert, 2000).

The carefully constructed, novel approach of Beadle and Tatum for identifying single gene mutants deficient for a specific nutrient has been an invaluable tool for *Neurospora* biochemical genetic studies and for evaluating the one gene–one enzyme hypothesis. This methodology has been applied to countless organisms and has led to elucidation of complete biosynthetic pathways.

HETEROKARYOSIS

Fungi are capable of fusing with each other to form a heterokaryon, in which two or more genetically distinct nuclei share a common cytoplasm. Fungal heterokaryon formation was discovered early in the 20th century (Hansen, 1938, 1942; Beadle and Coonradt, 1944). Heterokaryosis is believed to be beneficial for haploid fungi to form functional diploids, exchange genetic material via the parasexual cycle, or synergize individual traits to cooperatively exploit available resources (Glass et al., 2000). Improved growth fitness (heterokaryotic vigor) was observed for the heterokaryon of two *N. tetrasperma* dwarf mutant strains (Dodge, 1942). In addition to being beneficial for fungal fitness, heterokaryosis has enabled many genetic studies in *Neurospora*, including allelism, nuclear selection, and heterokaryon incompatibility.

Early studies in *Neurospora* took advantage of heterokaryon formation to test allelism of mutant strains. Forced heterokaryons of mutant and wild-type strains informed whether mutant alleles were recessive or dominant to the wild-type alleles. Additionally, heterokaryons were used to determine functional complementation of mutant strains (i.e., allelic and nonallelic genes) (Beadle and Coonradt, 1944). For many heterokaryons, the nuclear proportions of the two complementing nuclei remained constant over an extended period of growth, which was a useful property for investigating gene dosage (Pittenger et al., 1955; Davis, 1959; Pittenger and Brawner, 1961). In other cases, nuclear optimization resulted in nonadaptive increases in one nuclear type in a heterokaryon. These heterokaryons exhibited cyclic, labile, and/or submaximal growth rates, and in the extreme case growth stopped altogether when the strain reached homozygosity (Ryan and Lederberg, 1946; Holloway, 1955). Although heterokaryosis proved to be a useful genetic tool, the mechanism of heterokaryon formation and maintenance was not well understood. Heterokaryon incompatibility (HI) complicated genetic analysis of mutants prepared from many different wild-type strains. Consequently, a standard, isogenic wild-type strain set of both mating types (i.e., OR74A and 74 ORs6a) was constructed and adopted early in the development of *Neurospora* as a model system for future strain manipulation (Case et al., 1965; Newmeyer et al., 1987). In addition, intensive research efforts ensued to better characterize and understand heterokaryosis.

HI was first identified between fusions of strains of opposite mating type (Beadle and Coonradt, 1944). Not long after, the same phenomenon was described between strains of the same

mating type, but under different genetic control (Garnjobst, 1953). The general understanding of HI is that individuals that are genetically different at any heterokaryon incompatibility (*het*) locus may undergo hyphal fusion; however, the fusion cell is rapidly compartmentalized by septal plugging and undergoes cell death (Garnjobst and Wilson, 1956). It is hypothesized that HI serves as a self/nonself recognition system, which acts as fungal immune system to prevent transfer of deleterious genetic elements (Paoletti and Saupe, 2009). Initial genetic studies used forced heterokaryons to identify five *het* loci that controlled heterokaryon stability, including the mating type locus, *het-c*, *-d*, *-e*, and *-i* (Garnjobst, 1953; Pittenger and Brawner, 1961). Since then, 11 *het* loci have been identified and mapped in *N. crassa* (Mylyk, 1975; Perkins, 1975). In *N. crassa*, HI is mediated both by allelic (same locus) and nonallelic interactions (alleles at one locus interacting with alleles at another locus) (Glass and Kaneko, 2003). Current research is aimed at determining all *het* loci and alleles in *N. crassa* using population genomics approaches, as well as physiology associated with allelic and nonallelic incompatibility (Glass et al., 1988; Jacobson, 1992; Saupe et al., 1996; Saupe and Glass, 1997; Shiu and Glass, 1999; Mir-Rashed et al., 2000; Smith et al., 2000; Sarkar et al., 2002; Kaneko et al., 2006; Micali and Smith, 2006; Hall et al., 2010).

CIRCADIAN RHYTHM AND PHOTOBIOLOGY

An organism's ability to perceive and respond to environmental light is tightly coupled to its internal ability to measure time of day, information supplied by an intracellular-based circadian clock that allows an organism to anticipate daily changes in the environment due to Earth's rotation. All clocks, including that of *Neurospora*, have formal properties. These include free running rhythms of some observable phenotypic output in the absence of external cues, with a period length close to a day, and the ability to be entrained by environmental stimuli including light and temperature changes. Even so, the clock has the ability to compensate the period of oscillation against these changes such that the clock can reset its phase via entrainment by a stimulus, but maintain its approximately 24-h periodicity in the face of a changing environment.

Although biological clocks have been studied for well over a century, modern understanding that clocks had a cellular basis began with a description of formal properties in unicellular microbes such as *Gonyaulax* (Hastings and Sweeney, 1958). This work was rapidly followed by a description of a circadian clock controlling daily asexual developmental in *Neurospora* (Pittendrigh et al., 1959), followed again some years later by a report of a strain (*timex*) that showed a clear and easily monitored daily banding of conidiation that met the formal criteria for circadian rhythmicity (Sargent et al., 1966). The development of "race tubes" (Fig. 2) allowed visualization of rhythmicity of asexual development in real time. The *timex* phenotype was later shown to be due to a single gene that became known in the literature as *band* (*bd*), which was eventually identified as a mutation in *ras-1* that amplified clock-regulated development (Belden et al., 2007). The *bd* mutation was critical for *Neurospora* becoming a pioneer as a genetic model system for clocks that allowed eventual dissection of the genetic, molecular, and biochemical underpinnings of rhythmicity in eukaryotes (Feldman and Waser, 1971; Konopka and Benzer, 1971).

Genetic analysis of the *Neurospora* circadian clock identified several mutant alleles that clustered to a single locus, *frequency* (*frq*), that resulted in period shortening, lengthening, or loss of rhythmicity of asexual development, as well as altered temperature compensation (Feldman and Hoyle, 1973; Loros et al., 1986). These *frq* mutants facilitated the cloning of the underlying gene that encoded a large and novel protein of then unknown function (McClung et al., 1989). Revealingly, both the *frq* transcript and FRQ protein were found to be rhythmically expressed with a periodicity matching the appropriate wild-type or mutant strain. Inhibition or alteration of *frq* expression resulted in loss of rhythmicity or alteration of the periodicity of the clock, solidifying *frq*'s role as a primary driver of the clock in *Neurospora* (Aronson et al., 1994; Garceau et al., 1997; Liu et al., 1997). Recent work has shown that noncoding antisense RNA at the *frq* locus is required for maintenance of the circadian cycle (Xue et al., 2014), a regulatory mechanism that may be conserved in animal circadian systems.

Another avenue aimed at understanding rhythmicity in *Neurospora* was the isolation of messenger RNAs that were rhythmically regulated by the circadian oscillator, yet not directly involved in functioning of the clock, so-called clock-controlled genes (ccg's) (Loros et al., 1989). Studying genes with daily cyclic expression revealed the major means that clocks use to transfer information about time-of-day to coordinate cellular and metabolic activities (Bell-Pedersen et al., 1996).

The circadian clock can be entrained by exposure to light. The isolation of mutant strains in *Neurospora* defective in light-regulated carotenogenesis identified two loci called *white collar* (*wc*)-1 and *wc*-2, which began research on photobiology in fungi (Perkins et al., 1962; Harding and Turner, 1981; Degli-Innocenti and Russo, 1984). From the late 1980s, several laboratories found light-induced transcription of specific mRNAs (Nelson et al., 1989; Sommer et al., 1989). In *Neurospora*, expression of ~6% of its genes are rapidly induced in response to light exposure, which virtually all require *wc*-1 and *wc*-2 (Chen et al., 2009). WC-1 and WC-2 were shown to bind to the promoter region of a light-induced gene via a zinc-finger domain (Ballario et al., 1996) and possibly to act as a heterodimeric complex (White Collar Complex; WCC) through PAS domain interaction (Linden and Macino, 1997), leading to the conclusion that they act as transcriptional regulators.

An attempt to discover whether these genes participated in photo-entrainment of the circadian clock via induction of *frq* yielded the surprising result that *wc-1* and *wc-2* were required for expression of *frq* in the dark (Crosthwaite et al., 1997), establishing that the WCC had both light and dark functions in the cell. The WCC was then shown to bind flavin adenine dinucleotide as a cofactor and to act as the blue-light photoreceptor in *Neurospora*, mediating general light responses including entrainment of the circadian clock (Froehlich et al., 2002; He et al., 2002). Current thought tells us that most fungi have the ability to sense light using a broad array of photoreceptors, the most widely conserved being orthologs of *wc-1* and *wc-2* (Idnurm et al., 2010).

GENE SILENCING

Gene silencing mechanisms are hypothesized to be genome defense systems that monitor the content and arrangement of the genome. *Neurospora* has provided a wealth of information and discoveries on gene silencing systems. Four silencing mechanisms have been identified in *Neurospora* that all rely on

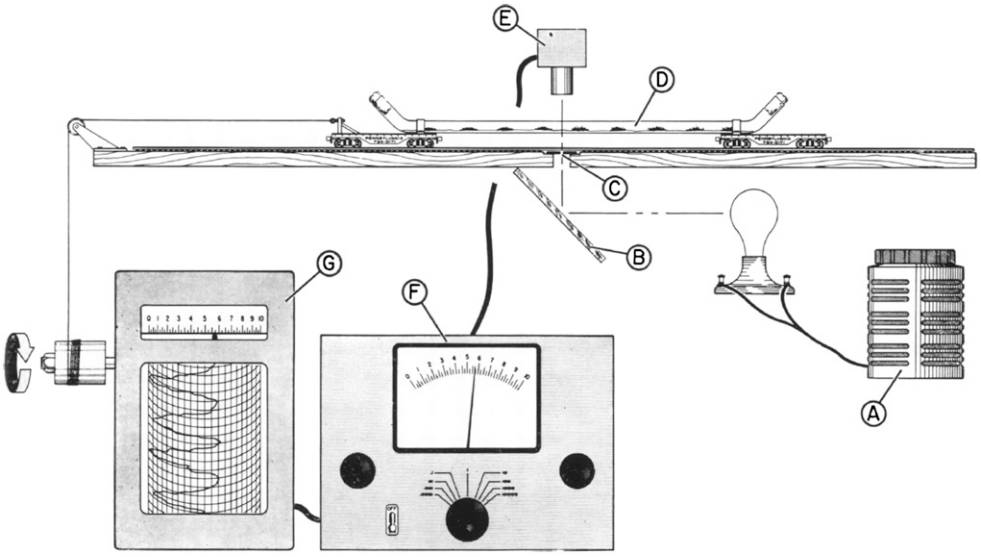

Fig. 2. Device for recording growth and conidial banding patterns, which includes a (A) powerstat, (B) mirror, (C) light slit, (D) growth tube, (E) multiplier photometer sensing element, (F) multiplier photometer, and (G) graphic ammeter. Reprinted with permission from Sargent et al. (1966; *Plant Physiology*).

recognition of homologous and repetitive DNA: repeat-induced point mutation (RIP), DNA methylation, quelling, and meiotic silencing by unpaired DNA (MSUD) (Aramayo and Selker, 2013). Early evidence for gene silencing included instability of duplicated chromosome segments and integrated genomic or cloned DNA during the sexual phase of the life cycle (Mishra and Tatum, 1973; Raju and Perkins, 1978; Grant et al., 1984; Case, 1986; Selker, 1990). This phenomenon was molecularly characterized by Selker and colleagues and named rearrangement induced premeiotically (RIP, later renamed repeat-induced point mutations) (Selker et al., 1987; Cambareri et al., 1989). Specifically, duplicated DNA sequences, regardless of origin, are detected and mutated in the sexual phase of the *Neurospora* life cycle. This foundational study identified de novo methylation and sequence alteration of the duplicated sequences. RIP permanently inactivates both copies of duplicated sequences at the stage of dikaryotic cell proliferation prior to karyogamy and meiosis, regardless of the location of the duplicated sequences within the genome (i.e., duplications at linked or unlinked loci) (Selker et al., 1987; Cambareri et al., 1989; Cambareri et al., 1991). RIP occurs at a higher rate when DNA is linked, especially for tandem and/or longer duplications (i.e., 400 bp for linked, 1 kbp for unlinked) (Cambareri et al., 1989; Stadler et al., 1991). Further molecular studies showed that RIP specifically induces G-C to A-T transition mutations (Singer et al., 1995). It was hypothesized that RIP was a mechanism to prevent transposons from accumulating in the genome. On the other hand, RIP was suggested to limit divergent evolution of duplicated genes (Selker, 2002). Since its discovery, RIP has been employed as a genetic tool to make gene disruptions and for generating partial-function mutants (Dunlap et al., 2007).

In conjunction with RIP, *Neurospora* has contributed significantly to the understanding of DNA methylation. Unlike plants and mammals, DNA methylation is not essential for most fungi, including *Neurospora* (Antequera et al., 1984; Tamaru and Selker, 2001). Recent studies in *N. crassa* have demonstrated that DNA methylation relies on histone methylation, which suggests that histones may be used as cofactors by proteins that require access to DNA (Tamaru and Selker, 2001; Honda and Selker, 2008; Honda et al., 2010; Lewis et al., 2010), a conserved feature in eukaryotic regulation of DNA methylation. The important finding that epigenetic inheritance is dependent on histone methylation has opened new areas of epigenetic research and has provided valuable insight into other areas of research, including cancer genetics (Sharma et al., 2010).

Another similar, but distinct mechanism for gene silencing, dubbed quelling, was identified shortly after RIP. Unlike RIP, which occurs during the sexual cycle, quelling silences duplicated DNA during the vegetative cycle (Romano and Macino, 1992). This form of gene silencing is significantly less potent; many gene duplications are stable during vegetative growth but are silenced during the sexual cycle (Selker, 1990). Unlike RIP, quelling is reversible. Gene silencing via quelling occurs post-transcriptionally and is due to reduction in messenger RNA levels (Romano and Macino, 1992; Cogoni et al., 1996; Cogoni and Macino, 1997). At the time, the mechanistic relation of quelling to cosuppression in plants and animals was not obvious (Napoli et al., 1990). However, a landmark study in *Caenorhabditis elegans*, which identified double-stranded RNA (dsRNA) as a potent trigger for gene silencing (dubbed RNA interference [RNAi]), preceded the implication of dsRNA in quelling. The involvement during quelling of RNA-dependent RNA polymerase, which synthesizes dsRNA from single-stranded RNA, was revealed via a classical forward genetic approach (Cogoni and Macino, 1997; Lee et al., 2010b). This discovery was foundational in relating the conserved mechanism of RNAi across all eukaryotic kingdoms. In *Neurospora*, the DNA-dependent RNA polymerase (QDE-1) produces aberrant RNA (aRNA) from repetitive DNA loci (Lee et al., 2010b), which is converted to dsRNA by the RNA-dependent RNA polymerase (Cogoni and Macino, 1997) and then to siRNA by Dicer proteins (Catalanotto et al., 2004). A resulting siRNA–Argonaute protein QDE-2 complex mediates posttranscriptional gene silencing (Maiti et al., 2007). Mechanistic studies of quelling in *Neurospora* is an active area of investigation (Chang et al., 2012) and

includes investigations into the involvement of homologous recombination in quelling (Zhang et al., 2013) and the relationship between histone modifications and RNAi (Zhang et al., 2014). Like RIP, RNAi has been used as a tool for functional genomic studies and has made a significant impact in the biotechnological and medical fields for use as gene-specific therapeutics and technologies (Dunlap et al., 2007; Giering et al., 2008).

The final known gene-silencing mechanism in *Neurospora* (meiotic silencing by unpaired DNA [MSUD]) silences gene expression of unpaired homologous chromosomes during the sexual cycle postnuclear fusion (Aramayo and Metzenberg, 1996; Shiu et al., 2001), which was earlier described as a meiotic transvection phenomenon (Aramayo and Metzenberg, 1996). The effects of MSUD were beautifully shown by using strains carrying a duplication of a gene encoding a component of the microtubule cytoskeleton (β-tubulin). This strain was normal during vegetative growth, but during the sexual cycle, silencing of both copies of the β-tubulin gene resulted in disruption of the spindle, thereby disrupting meiosis and ascospore formation (Shiu et al., 2001). Mutational analyses showed that MSUD is mechanistically related to RNAi (Shiu et al., 2001, 2006; Lee et al., 2010a; Xiao et al., 2010; Hammond et al., 2013). Studies suggest that MSUD occurs via a two-step mechanism by which unpaired segments of chromosomes are detected and then transcripts resulting from those unpaired genes are processed by mechanisms related to RNAi (Hammond et al., 2013). The full mechanism of MSUD has yet to be elucidated and is an area of continuing research.

NEUROSPORA POPULATIONS AND THE FUNGAL GENETICS STOCK CENTER

The systematic global collection of wild isolates of *Neurospora* initiated by Perkins in 1968 has significantly contributed to the understanding of genus distribution, population structure, meiotic drive elements, and heterokaryon incompatibility, as well as provided variants for laboratory investigation (Perkins et al., 1976; Perkins and Turner, 1988; Turner, 2001). Over 4600 *Neurospora* isolates from 735 sites were collected by Perkins worldwide, mostly from newly burnt vegetation, but also from unburned substrate and soil, and from tropical climates in particular. It is thought that fire produces ideal substrates for *Neurospora* and enables activation of sexual spores (ascospores). Wild isolates were subjected to fertility tests for species classification. The *Neurospora* genus is diverse in breeding, containing both homothallic inbreeders and heterothallic outbreeders (Perkins and Turner, 1988). Populations of *N. crassa* possess high genetic variability, which has been used extensively for research investigations of protein polymorphisms, recessive genes expressed in the sexual diplophase (i.e., recessive lethal genes), heterokaryon incompatibility and more recently, population genomics studies (Perkins and Turner, 1988; Ellison et al., 2011, 2014; Palma-Guerrero et al., 2013). Both meiotic drive elements (spore killer) and resistance factors have been identified in *Neurospora* populations (Turner and Perkins, 1979; Campbell and Turner, 1987; Turner, 2001). A variety of morphologically distinct isolates were also collected (Perkins et al., 1976). The Fungal Genetics Stock Center (FGSC), discussed next, has been critical to maintaining the collection of wild and laboratory isolates, a selection of which are depicted in Fig. 3.

As the *Neurospora* community grew over the last century, the collaborative nature of the community was evident. As the first generation of *Neurospora* researchers reached the ends of their careers, there was a general sentiment that the materials generated during this era should be maintained through a formal and open collection of strains. Funding from the U.S. National Science Foundation and coordination by the Genetics Society of America (GSA) gave rise to the Fungal Genetics Stock Center in 1960. At the time of inception, a survey made by the GSA Committee on the Maintenance of Genetic Stocks identified 21 laboratories using *Neurospora*. Among these laboratories, as many as 9000 different stocks were being used. These strains included mutants at approximately 120 different genetic loci, as well as numerous unmapped mutants and smaller numbers of wild-type strains.

One benefit of this early coordination has been that most researchers adopted a common genetic background. A simple "key sort punchcard" by the McBee Corporation was employed to allow rudimentary sorting based on genetic characteristics (i.e., amino acid auxotrophs, nucleic acid auxotrophs, vitamin auxotrophs, other metabolic variants, antimetabolite resistance, and morphological and visible variants) of the growing number of strains deposited in the collection (Davis and de Serres, 1970); approximately 400 strains populated the collection in 1960. Now nearly 21 000 *Neurospora* variants, mutants, and gene deletion strains are maintained and distributed by the FGSC, as well as vectors for cloning, expression, epitope-tagging, and fluorescent-protein labeling (McCluskey, 2011). The impact of this collection is undeniable. Despite holdings of only 1300 strains, the FGSC distributed over 6300 individual cultures between October 1960 and November 1966. Today *Neurospora* strains have been distributed worldwide to over 35 countries at a rate of more than 1500 strains annually (McCluskey, 2011).

Along with the creation of the FGSC, a biannual *Neurospora Newsletter* (later *Fungal Genetics Newsletter* then *Fungal Genetics Reports*) and a biennial *Neurospora* Information Conference (now Fungal Genetics Conference, which alternates yearly with the *Neurospora* Conference) were established, as well as an advisory committee, called the *Neurospora* Policy Committee. Over time, the FGSC expanded its repository to include several other species of fungi. The FGSC currently holds stock of 25 311 strains from 123 different fungal species.

MOLECULAR GENETICS

The era of molecular genetics, specifically DNA cloning, transformation and associated molecular biology tools, transformed genetic research. These technological advances reignited *Neurospora* research in the 1980s. Restriction-fragment length polymorphism (RFLP) methodology was developed to allow efficient mapping of cloned DNA (Metzenberg et al., 1985). Cosmid, plasmid, and lambda phage DNA libraries were constructed and employed to clone nuclear genes of *N. crassa* first via selection by complementation of auxotrophies and then by dominant selectable markers (Akins and Lambowitz, 1985; Orbach et al., 1986; Vollmer and Yanofsky, 1986). As discussed above, DNA cloning and transformation led to discovery of gene silencing, which has made a significant impact in eukaryotic epigenetics. In addition to epigenetics, cell development and differentiation and circadian rhythm and photobiology benefited from the reinvigoration of *Neurospora* research

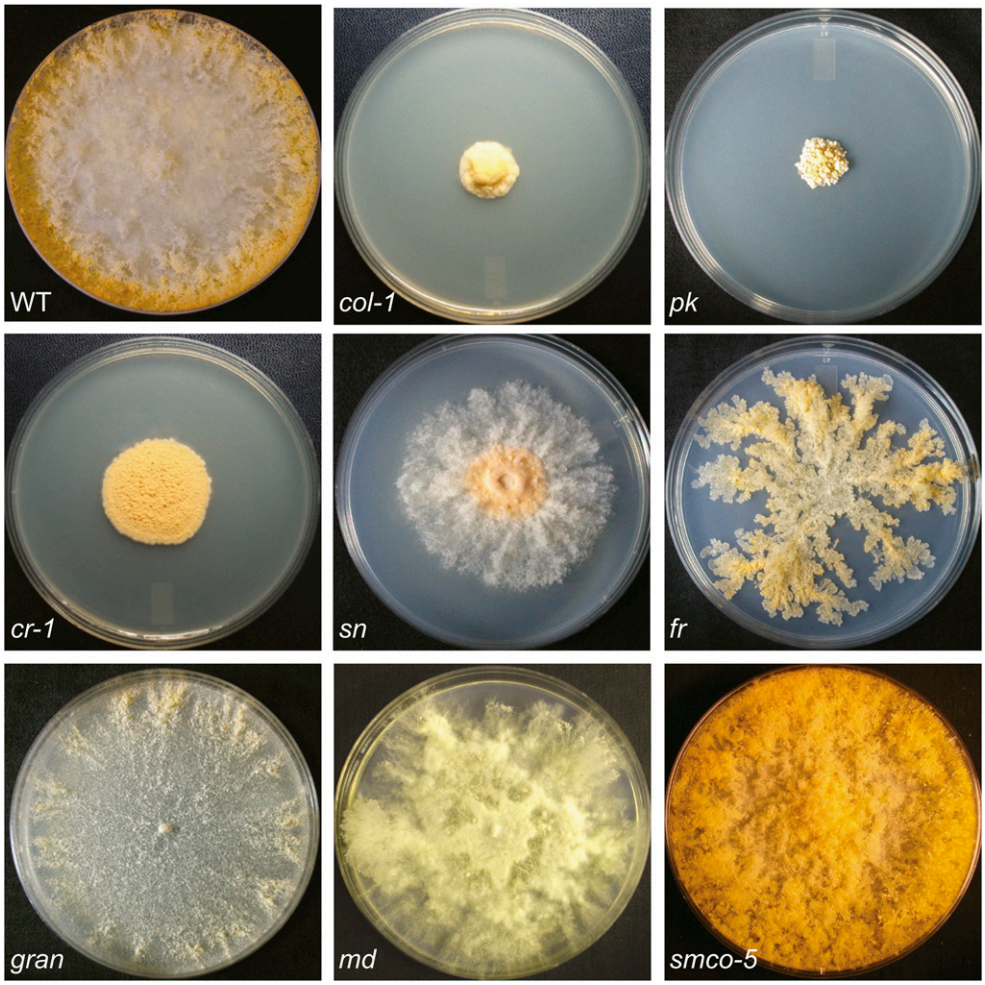

Fig. 3. Morphological mutants of *Neurospora* grown on Vogel's minimal medium for 5 d at 25°C. WT, wild-type; *col-1*, *colonial-1*; *pk*, *peak*; *cr-1*, *crisp-1*; *sn*, *snowflake*; *fr*, *frost*; *gran*, *granular*; *md*, *mad*; *smco-5*, *semicolonial-5*. Courtesy of the Fungal Genetics Stock Center.

by molecular genetics. For example, genes that exhibited increased expression during conidiation (Berlin and Yanofsky, 1985b), as well as clock-controlled genes (Loros et al., 1989) were detected by RNA hybridization and mapped via RFLP analyses.

DNA sequencing technologies developed by Sanger et al. (1977) enabled sequencing of the mitochondrial genome (Heckman et al., 1978) and the first nuclear gene (*am,* NADP-specific glutamate dehydrogenase) of *N. crassa* (Kinnaird and Fincham, 1983). DNA manipulation and sequencing technologies continued to improve and resulted in full genome sequencing efforts. Initial efforts to investigate the whole genome sequence of *Neurospora* used expressed sequence tags (ESTs) from different developmental stages (Nelson et al., 1997). Soon after, whole-genome sequencing efforts via shotgun sequencing and assembly ensued. The first draft of the *N. crassa* genome was released in 2003 (Galagan et al., 2003). The 43 Mb genome has provided insights into many cellular processes, including cell signaling, environmental sensing, genome defense, growth and development, and metabolism and transport (Galagan et al., 2003; Borkovich et al., 2004). Lessons learned from the *Neurospora* genome have since been applied to many other fungi, including insights using comparative genomics approaches.

To further unlock the information acquired from the genome sequence, elucidation of gene function by gene deletion was pursued. At the time of genome sequencing, few gene deletions had been constructed, in large part due to the low rate of homologous recombination in *Neurospora*. To facilitate construction of a whole-genome-deletion collection, the nonhomologous end-joining (NHEJ) pathway of DNA repair was blocked (Walker et al., 2001), allowing DNA repair to proceed via homologous recombination (Ninomiya et al., 2004). NHEJ-deficient strains and deletion vectors constructed via yeast recombineering enabled high-throughput production of a nearly complete whole-genome knockout collection by the *Neurospora* functional genomics program (Colot et al., 2006; Dunlap et al., 2007; Collopy et al., 2010). This tool is widely used in the *Neurospora* and filamentous fungal research community and has enabled elucidation of function of a large number of genes, including identification of transcription factors that regulate gene expression for specific biochemical pathways and cellular responses (Colot et al., 2006). However, not all knockout strains have a detectable phenotype and strains carrying deletions for essential genes are only viable as heterokaryons, which somewhat limits the utility of the whole-genome-deletion collection for functional analysis (Fu et al., 2011; Park et al., 2011;

Watters et al., 2011). Forward genetic screens have been and will continue to be a valuable tool for function genomic studies, especially in conjunction with whole-genome sequencing and classical gene mapping. For example, the genomes of 18 classical mutants of *N. crassa* were recently sequenced to identify the causative mutation(s) (McCluskey, 2011).

The advent of next-generation sequencing technologies has revolutionized genetic research and has enabled the collection of a surplus of genetic data. In addition to genome resequencing, RNA sequencing (RNA-seq) and chromatin-immunoprecipitation sequencing (ChIP-seq) have shed light on interactions of genes and gene expression and have informed the role of DNA and protein interaction in the regulation of gene expression and control of DNA repair, replication, and transcription. Specifically, these technologies have enabled in-depth investigation of *Neurospora* as a model for biomass deconstruction (Tian et al., 2009; Coradetti et al., 2012; Sun et al., 2012; Znameroski et al., 2012; Benz et al., 2014), circadian rhythm and photobiology (Smith et al., 2010; Sancar et al., 2011; Arthanari et al., 2014), epigenetics (Pomraning et al., 2009; Hammond et al., 2013; Jamieson et al., 2013), and population genetics (Hall et al., 2010; Ellison et al., 2011).

TOOL DEVELOPMENT AND THE APPLICATION OF *NEUROSPORA* TO BIOTECHNOLOGY

In the upcoming years, *Neurospora* will likely continue to contribute to our understanding of fundamental aspects of circadian rhythm, epigenetic and gene silencing processes, and population genetics; however, the era of next-generation sequencing will enable many of these studies in nonmodel, ecologically well-characterized microorganisms. Whole-genome sequencing of other fungi has enabled comparative genomic studies with *Neurospora* to elucidate specialization of fungal species and basic biological processes such as signaling cascades (Zelter et al., 2004; Rispail et al., 2009; Sharpton et al., 2009; Ma et al., 2010). Harnessing new tools to leverage the genomic, transcriptional and functional data available for *Neurospora* for computational modeling of genetic networks is an exciting new area for development for *N. crassa* as a model system.

Neurospora has recently found a niche role in understanding fungal deconstruction of the plant cell wall, in particular for enabling an economically viable second-generation biofuel (Glass et al., 2013). Specifically, plant biomass detection and signaling, regulation, expression, and secretion of the lignocellulolytic enzymes by *Neurospora* are being investigated (Tian et al., 2009; Coradetti et al., 2012; Sun et al., 2012; Znameroski et al., 2012; Benz et al., 2014). Novel proteins involved in plant cell wall deconstruction have been identified in *Neurospora* through comparative genomic, transcriptomic, and proteomic studies (Beeson et al., 2012; Phillips et al., 2011), and evidence suggests additional factors for plant biomass degradation are present (Coradetti et al., 2012; Benz et al., 2014). Recent studies have pushed *Neurospora* research beyond understanding fundamental scientific questions and have used *Neurospora* for applied research as a consolidated bioprocessing host for lignocellulosic biofuel production and a heterologous protein production host (Dogaris et al., 2013; Dana et al., 2014; Roche et al., 2014). With these studies, *Neurospora* continues to evolve in its role as a model microbe, now including applied biotechnological research. The rich genetic history and development of genetic and 'omic tools, sets *Neurospora* up for leading the effort of applying synthetic biology approaches pioneered in bacteria and yeast to filamentous fungi for production of biofuels, chemicals, hydrolytic enzymes, and organic acids.

The feasibility of developing synthetic biology and metabolic engineering technologies in *Neurospora* will largely depend on increasing the number of selectable markers and improving genetic tools for fine-tuning gene expression levels. Although the *cre–loxP* system for marker recycling has been established in *Neurospora* (Honda and Selker, 2009), additional markers are desirable. Negative selectable markers present in the *Neurospora* genome (e.g., *csr-1* for cyclosporin A selection [Bardiya and Shiu, 2007]) are especially of interest. The abundant data from recent transcriptomic studies can help inform promoter selection for inducible or constitutive, fine-tuning transcription levels. Additional promoter modifications, such as approaches taken for developing promoter libraries and chimeric promoters (Alper et al., 2005; Zhang et al., 2012), will support development of metabolic engineering technologies. Further development of RNA control devices, such as ribozymes and aptamers, would greatly advance gene-expression modulation and thus further enable synthetic biology and metabolic engineering applications in *Neurospora.*

The future of *Neurospora* as a model system is boundless. Over the years, *Neurospora* has been challenged by newcomer model microbes, yet has proved to be valuable as a classical genetic model and for investigation of fundamental biological processes. Avant-garde tools and technologies for genome, transcriptome, proteome, and metabolome analysis will enable continued advancement of the state of knowledge of modern biology, evolution, and genetics, and facilitate rapid advances in the applied biological sciences and biotechnology. No doubt, the scientific legacy of *Neurospora* will extend another century.

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