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Circadian rhythms in *Neurospora crassa*: Downstream effectors

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ABSTRACT

The circadian rhythm in *Neurospora crassa* is exhibited as alternating areas of conidiating and non-conidiating mycelia growth. A significant role in this circadian rhythm is played by the *frq* (frequency) and *wc* (white-collar) genes, comprising the "FWC" oscillator. Strains lacking the FWC can be restored to rhythmicity, which has been attributed to a second oscillator, called the FLO (*frq*-less oscillator). This study reports additional conditions that allow this rhythmicity to occur. Rhythmicity was restored to mutants lacking either the *frq*, or *wc-1*, or *wc-2* genes in D/D (constant darkness) or L/L (constant light) by the addition of low levels of menadione, a known stimulator of ROS (reactive oxygen species). Additional studies are reported on the rhythm effects from caffeine, a known cAMP phospho-diesterase inhibitor as well as the effects from mutations in the *csp-1* gene, the *rco-1* gene, and other genes. A theme ties all of these "downstream effects" together, i.e., they affect either components thought to be part of the conidiation process itself, or the RAS–cAMP–protein kinase pathway. Since mutations in these components unexpectedly had rhythm effects, this suggests that these components may be good candidates for some part of the *frq*-less oscillator.

1. Introduction

Circadian rhythms are widely found in nature. Although often referred to as the biological clock, that term can be applied to other types of rhythms or phenomena. Circadian rhythms have several features; (1) they have approximately a 1 day period, as the term circadian states; (2) they are free-running, self-sustaining oscillations under constant conditions, such as continuous darkness (D/D); (3) they can be phase-shifted by pulses of light, or temperature, or chemicals; and (4) they have roughly the same period at different incubation temperatures, a phenomena named temperature-compensation.

The existence of a circadian rhythm in the fungus *Neurospora crassa* was originally documented in 1959 (Pittendrigh et al., 1959) and then fully characterized by a series of papers a few years later (Sargent et al., 1966; Sargent and Briggs, 1967; Sargent and Woodward, 1969). The *Neurospora* rhythm possesses all of the four qualities listed above, but in addition it is relatively easy to assay, and it is relatively easy to get mutants in this organism. The *Neurospora* rhythm is expressed in terms of a conidiation (asexual spore forming) rhythm that can be seen as a series of bands of conidiating regions when the organism is grown on the surface of an agar medium. The *Neurospora* "system" has been well studied and has been reviewed extensively (Dunlap, 2006; Dunlap and Loros, 2004; Lakin-Thomas and Brody, 2004) as well. A general picture has emerged of the *Neurospora* "clock-works" of an oscillator composed of several components such as the *frq* (frequency) gene, the two *wc* (white-collar) genes, and others. This oscillator is described in shorthand as the FWC oscillator, after its component parts. Null mutations in any of these three genes lead to an arrhythmic phenotype (Dunlap and Loros, 2006). Numerous studies (reviewed in Lakin-Thomas and Brody, 2004; Lombardi et al., 2007; Loros and Feldman, 1986) have indicated that the idea that this FWC oscillator is the only oscillator may have been a bit too reductionist in nature. These other studies have indicated that these circadian rhythm null mutants can, under certain conditions, express a conidiation rhythm, albeit not one with all of the circadian properties listed above. These findings have lead to the postulate of another oscillator named the FLO (*frq*-less oscillator). This FLO, along with the FWC, may be a more complete description of the "clock-works" of this organism, pending further experimental evidence. The complexities of the system and the possibility of even other oscillators have been discussed previously (Lakin-Thomas and Brody, 2004).

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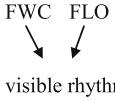
A list of the conditions that restore a conidiation rhythm to a null mutant are given below:

(1) addition of farnesol to the media (Granshaw et al., 2003);
(2) addition of menadione to the media (this report);
(3) double mutants between nulls and *vvd*, grown in L/L (Schneider et al., 2009);
(4) double mutants between nulls and the *cel* mutation, grown in the presence of the fatty acid linoleic acid (18:2). (Lakin-Thomas and Brody, 2000);
(5) double mutants between nulls and the choline mutant, grown under conditions of low choline (Lakin-Thomas and Brody, 2000);
(6) growth of frq^9 strains in extra-long growth tubes (Loros and Feldman, 1986);
(7) double mutants between nulls and the *ult* mutation (Lombardi et al., 2007);
(8) double mutants between frq^{10} and *sod-1* (Yoshida et al., 2008).

Currently, there remain several open questions about this "FLO". (1) What are the molecular components of the FLO? (2) What is the relationship to the FWC oscillator, i.e., what is the molecular architecture of the system?

As to this second question, several possibilities exist: (a) the two oscillators could be a series configuration, i.e., FWC → FLO visible rhythm. The opposite configuration, i.e., FLO → FWC → visible rhythm does not fit the data; or (b) the oscillators could be in parallel configuration, i.e., 

this latter idea would require an additional postulate, i.e., some type of "integrator" to put the two rhythmic signals together. Therefore, for heuristic purposes, we shall just use the first possibility, and consider those conditions which restore a rhythm to a null mutant to be downstream from the FWC and called "downstream effectors". For each of the categories (a) and (b) above, there exists the possibilities of "cross-talk" between the two oscillators.

This report presents some new ways to restore the rhythm to null mutants, as well as additional data on some new mutants. Taken together, these new data suggest that some of the components implicated in the conidiation process itself may be good candidates for the components of the FLO.

2. Materials and methods

2.1. Strains

The *bd* strain or the *bd csp-1* strains were used as controls. The *bd* mutation has been characterized as a mutation that permits conidiation even in the presence of high CO_2 concentrations in closed cultures (Perkins et al., 2001) and has recently been found to be a mutation in the *ras-1* gene (Belden et al., 2008). The *csp-1* mutation shows improper separation of conidia from hyphae (Perkins et al., 2001) preventing self-contamination of cultures during handling. The *csp-1* gene has been found to code for a light-inducible transcription factor (Lambreghts et al., 2009).

Two alleles at the *vvd* locus were used: P4246 (abbreviated as vvd^P) and SS-692 (abbreviated as vvd^{SS}). The details of these strains are in a previous publication (Schneider et al., 2009).

The *frq* and *wc* knockout strains were detailed previously (Lombardi et al., 2007), and the *ult* mutation described previously (Lombardi et al., 2007). The *rco-1* strain was obtained from Dan Ebbole (Texas A&M) and from the FGSC. The *csp-1* KO strain (FGSC #11348) was obtained from Jay Dunlap (Dartmouth Univ.) The *bd csp-2* strain was constructed in this lab in 1988 from a cross of *bd* to *csp-2* (FGSC #591) and then was re-isolated as indicated in Section 3. The *ghh* (grainy-head homolog) mutants were obtained from the FGSC as #(s) NCU 06095.2 a (FGSC #13563) and FGSC #13564 A.

Crosses were carried out using standard genetics procedures for *Neurospora*. Details of methods can be found in 'Supplementary Methods' of a previous publication (Lombardi et al., 2007). Strains not described above were obtained from the FGSC or have been described previously.

2.2. Growth conditions and media

The general growth conditions were as previously described (Granshaw et al., 2003). Cultures were grown either in 150 mm disposable Petri dishes (plates) or in 27 cm long (1.1 cm internal diameter) glass growth tubes (race tubes). All growth media contained 2% agar, 2% Vogel's 50× minimal medium (Vogel, 1956), a carbon source, and a trace of food coloring. The carbon source was maltose (0.5%), unless otherwise specified. Auxotrophic strains were supplemented with 0.01 mg/ml pantothenate. After sterilization for 20 min, the media were dispensed into plates (50 ml/plate) or race tubes (9 ml/tube) and allowed to cool for 1 day before inoculation. Cultures were inoculated with conidia taken from slant cultures. After inoculation, cultures were either incubated immediately in various constant light (L/L) conditions or were incubated overnight in 5.4 μmol/m^2/s light before transfer to constant darkness (D/D). Illumination was provided by cool-white fluorescent bulbs (Phillips Alto 17 w, F17T8/TL735). Light intensity for most experiments was measured with a Weston light meter or Fisher Scientific light meter in lux and converted to μmol/m^2/s using the conversion factors found in (Thimijan and Heins, 1983).

Ascorbate, and caffeine were added to the media in powder form before autoclaving, while menadione was added as a solution (1.0 g/60 ml ethanol) kept in a brown bottle, covered with aluminum foil.

Period and growth rate calculations: the growth fronts of cultures were marked once a day under red safelight (for cultures in D/D) or under lighting conditions similar to growth conditions (for cultures in L/L). After growth had finished, the centers or peaks of bands were marked on the cultures and the growth marks and band marks were transferred to paper. The paper record was scanned and the growth rates and periods were calculated using software developed by Fred Hajjar and Mike Ferry that calculates every period and growth rate between adjacent band marks and growth marks. Values from at least five replicate cultures were averaged for each condition.

2.3. Light/dark entrainment

Cultures were grown under cool-white fluorescent lights for artificial light cycles of 12:12 L/D (12 h light and 12 h dark).

2.4. Imaging and densitometry

For the images in the figures, plates were illuminated from above and pictures were taken with a digital camera from directly above. The density profiles in all figures were obtained by using the Plot Profile function of ImageJ software (NIH) to obtain pixel densities, which were then analyzed with either the MatLab moving average smoothing function or R-loess. The dimensions of the profiles were about 1400 pixels and were smoothed using a smoothing window of 41, or about 3% of the profile.