Letters in
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PERIOD-1 is a Component of the Neurospora Metabolic Oscillator

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Abstract

Neurospora crassa has been a successful model organism in characterizing eukaryotic circadian rhythms. *N. crassa* has at least three known oscillators that affect circadian clock. The FRQ/WC dependent oscillator (FWO), the WC-dependent oscillator (WC-FLO), and a minimum of one FRQ/WC-independent oscillators (FLO). FWO has been extensively characterized, however, little is known about the molecular mechanisms of the FLO. Previous studies by other groups have shown that the *prd-1* gene might be a key component of the FLO. To test this idea, we investigated the roles of *prd-1* in nutrient compensation; the ability to maintain the rhythm in diverse range of available nutrients. First, we will create isogenic strains that contain *prd-1* mutants by crossing the *prd-1* mutant N272_012 (25 hour period) with the wild type (WT) N4720 (22 hour period). Second, we will characterize the clock phenotypes of *prd-1* bearing strains. Third, we will test the effects of different carbon source in the media by growing the *prd-1* mutants on a high glucose media and a low glucose media. Our results show that the developmental rhythms of wild type strains are compensated in different nutrient conditions as predicted, whereas, those of *prd-1* mutants are not compensated. Our data support that *prd-1* is a component of FLO.

Introduction

Circadian rhythms are biological processes that govern an organism's cycle. Circadian Rhythms are present in every domain. In order to be considered as an authentic circadian rhythm, a biological rhythm must meet three conditions. First, the period of the rhythm must be anout 24 hours in constant conditions. Second, the rhythm must be locked in to the local time. Third, the period of the rhythm maintained in a wide range of physiological temperatures. Circadian Rhythm has been shown to be a fitness trait.

Neurospora crassa belongs to the phylum Ascomycota, the main characteristic of all Ascomycota is the ascus that develops ascospores. It produces ascospores by sexual reproduction, but upon environmental signals it is able to reproduce asexually producing conidia. *N. crassa* has been a successful model organism to study the circadian clock. Circadian rhythms were first reported in *N. crassa* in 1959 (Pittendrigh 1957). Under constant conditions it has a period of approximately 22.5h. It was entrainable by a 24h light cycle and was capable of temperature compensation. The first known molecular component of the circadian clock of *N. crassa* was *frequency (frq),* a single locus that showed characteristics of long, short, and arrhythmic periods that altered temperature compensation (Gardner 1980; Loros 1986). The mechanism involved in *frq* is a central time-delayed negative feedback loop. Positive functions activate negative responses, which loop back to inhibit the positive function (Aronson 1994; Garceau 1997). Expression of *frq* is regulated by *white collar-1 and white collar-2* (Crosthwaite 1997) proteins that are photoreceptors. The WCC (white collar complex) binds to *frq* to allow expression in the morning. The binding of *frq* and the WCC induces phosphorylation, which removes the WCC from *frq* (Schafmeier et al., 2005, 2008; He et al., 2006; Hong et al., 2008). The negative responses are successful when *frq* expression declines. In addition to *frq* and the WCC, other components making up the circadian clock of *N. crassa* include FRH (*frq*-interacting RNA helicase) that upon translation binds to stoichiometrically to *frq.* Another molecular feedback loops besides the transcription translation based oscillators is a metabolic oscillator, in which an energy source drives the positive and negative loops. Although metabolic oscillators are known to exist in *N. crassa*, little is known about these feedback mechanisms. A gene thought to be a component of the metabolic oscillator of *N. crassa* is the *prd-1* gene

that will be studied and tested during this experiment revealing if it is a component of the metabolic oscillator.

Materials & Methods

Fungal Strains

The wild type and mutant strains of *N. crassa,* N4720 and N272_012 respectively, were obtained from Dr. Lee's laboratory at Rutgers University – Camden. We chose these two strains because the N4720 strain was wildtype and had a period of about 22h, and the N272_012 strain was mutant and had a period of about 25h.

We picked 195 spores, and of those picked, 73 successfully germinated. Of the successful germinated spores there was a clear phenotypic 1:1 ratio of normal growth and slow growth. We separated the media slants into fast growth (N308_01 – N308_37) and slow growth (N308_38 – N308_73). From there we believed the fast growth strains were the WT and the slow growth strains were mutant. We then inoculated an individual race tube with each strain. We focused on band minus strains, and were able to analyze 16 strains. When we determined if the strain was WT or mutant we used the application ChronOSX to evaluate the approximate period of the individual strains.

Race Tube Media

Low glucose liquid (LG) media was used in the first and second race tube experiment, and high glucose liquid media (HG) was used in the second experiment. In order to determine which strains were wild type and mutant we used LGLM by adding 40ml of Vogel's salts to a beaker then adding distilled H2O to 2L. 3.4g L-Arginine, 2g D-Glucose, and 1000µL Biotin were then added to the beaker and medium heat was applied while spinning. The pH was then adjusted 5.8 by adding HCl. After the pH was adjusted, 30g of Agar were then added and the solution continued to be spun on medium heat until the solution was dissolved. Both high and low glucose media was used in the second racetube experiment with the only difference being 40g of D-Glucose was used in HGLM. The solution was then pumped into the racetubes. The ends of the racetubes were covered with tinfoil, allowed to dry, and the media was flipped before inoculation.

Race Tube Inoculation and Growth Rate Measurement

To inoculate the race tubes we used a 10% milk formula by mixing 100ml of distilled H2O, 10g milk powder (fat free), stirred, and separated into 125ml flasks and autoclaved for 10min. We then added milk solution to the slants and pipetted the milk/conidia mixture into mycelia plates. The plates were allowed to sit in order to let allow the conidia to form. We then punched out holes into each mycelia plate and transferred those conidia cutouts to race tubes. The race tubes used were placed in DD conditions and checked each day at the same time.

Results

Of the 73 race tubes we inoculated in LG we found a clear 1:1 ratio of wildtype (N308_01-N308_37) and mutant (N308_38-N308_73) in their growth rates. Wild type strains grew normal in minimal media slants. Mutant strains grew slower than wild type. This growth phenotype was apparent at the third day of culture. One week later, there was no difference between two groups in asexual development. We did not measure the rate of cell mass. However, we measure the growth rate on race tubes. Wild type strains grew faster than prd-1 mutants (wild type 41.9±1.12 mm, *prd-1* mutant 32.2±0.96 mm, $p<0.001$) (Figure 1).

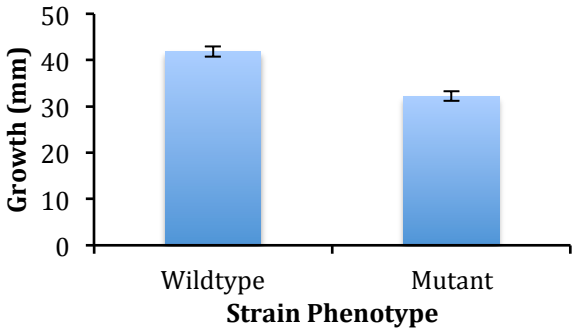

Figure 1. Growth rate of the strains on race tubes. The error bars represent one standard error of the mean.

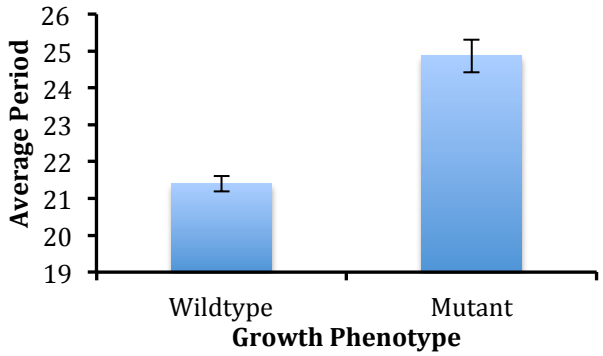

Figure 2. Circadian period. The error bars represent one standard error of the mean.

Table1

Genotype	Media	Average Period	STER
Control (WT)	HG	19.75	0.65
	LG	21.95	0.21
prd-1 Mutant	HG	22.03	1.02
	LG	29.04	0.75

HG: High glucose, LG: Low glucose, STER: standard error

As expected the *prd-1* mutant strains showed a longer period in comparison to those of wild type strains (wild type 21.4±0.21 and the mutant period was 24.9±0.44, $p<0.001$)(Figure 2). To test if the periods of the strains

are compensated when they were grown at different media composition, we tested the periods of the strains in two different media, high glucose (HG) and low glucose (LG) (Materials and Methods). Our data shows that there is a significant change in periods when they were grown in high glucose media in both genotypes (Table 1 and Figure 3). The difference is more pronounced in the *prd-1* genotype that that of the wild type.

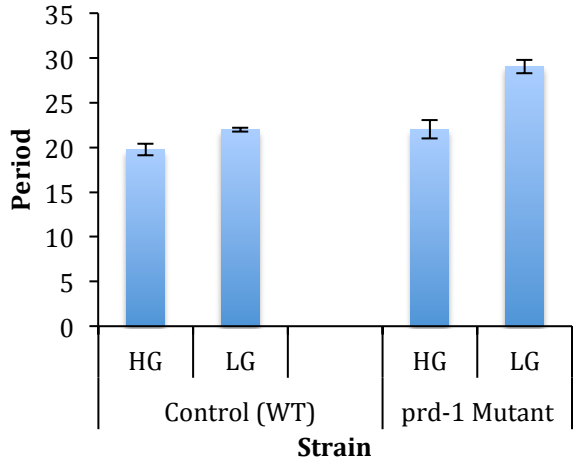

Figure 3. Circadian periods of the wild type strains and prd-1 mutants. HG: high glucose. LG: low glucose. The T-Test for WT vs prd-1 in HG was 1.01E-01 and 6.02E-07 in LG. The average T-Test for WT HG vs WT LG was 8.16E-03, and 4.40E-04 for prd-1 mutant HG vs prd-1 mutant LG. The period difference in WT HG vs WT LG was 2.20. The difference between prd-1 HG and prd-1 LG was 7.02.

Discussion

We hypothesized that if *prd-1* is involved in the metabolic oscillation, the *prd-1* mutant strains may show a significant change in its circadian period. To remove the genotype effects other than *prd-1*, we prepared genetically isogenic strains by back crossing the classical *prd-1* mutant strain to the wild type strain. We tested our hypothesis by measuring the periods of the two groups of strains, wild type and *prd-1* bearing progeny. Our data shows that the periods of both genotypes were shortened in high nutrient condition (Table 1 and Figure 3). The period change in wild type was unexpected one since it has been reported that the circadian oscillator should have a mechanism to compensate the nutrient change. However the change in period of wild type is small in comparison to that in prd-1 mutant, 2.2 hr, vs 7.02 hr. The significant media-dependent period difference supports our hypothesis that the *prd-1* is a component of FLO.

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References:

Aronson, B. D., K. A. Johnson, et al. (1994). "Negative feedback defining a circadian clock: autoregulation of the clock gene frequency." Science **263**(5153): 1578-1584.

Crosthwaite, S. K., J. C. Dunlap, et al. (1997). "Neurospora wc-1 and wc-2: transcription, photoresponses, and the origins of circadian rhythmicity." Science **276**(5313): 763-769.

Garceau, N. Y., Y. Liu, et al. (1997). "Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY." Cell **89**: 469-476.

Gardner, G. F. and J. F. Feldman (1980). "The frq locus in Neurospora crassa: a key element in circadian clock organization." Genetics **96**(4): 877-886.

Loros, J. J., A. Richman, et al. (1986). "A recessive circadian clock mutation at the frq locus of Neurospora crassa." Genetics **114**(4): 1095-1110.

Pittendrigh, C. and V. Bruce (1957). An oscillator model of biological clocks. Rhythmic and Synthetic Processes in Growth. D. Rudnick. Princeton, NJ, Princeton University Press**:** 75-109.