A Nitrate-Induced *frq*-Less Oscillator in *Neurospora crassa*

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Abstract When nitrate is the only nitrogen source, *Neurospora crassa*'s nitrate reductase (NR) shows endogenous oscillations in its nitrate reductase activity (NRA) on a circadian time scale. These NRA oscillations can be observed in darkness or continuous light conditions and also in a *frq*^{9} mutant in which no functional FRQ protein is formed. Even in a *white-collar-1* knockout mutant, NRA oscillations have been observed, although with a highly reduced amplitude. This indicates that the NRA oscillations are not a simple output rhythm of the white-collar–driven *frq* oscillator but may be generated by another oscillator that contains the *nit-3* autoregulatory negative feedback loop as a part. In this negative feedback loop, a product in the reaction chain catalyzed by nitrate reductase, probably glutamine, induces repression of the nitrate reductase gene and thus downregulates its own production. This is the first example of an endogenous, nutritionally induced daily rhythm with known molecular components that is observed in the absence of an intact FRQ protein.

Key words *Neurospora crassa*, circadian rhythm, nitrate reductase, oscillatory feedback loops, multiple oscillators, *frq*-less oscillator (FLO)

Circadian rhythms play central roles in the daily and seasonal adaptation of organisms to their environment (Bünning, 1963; Dunlap et al., 2003; Edmunds, 1988). Considerable progress has been made by using model organisms such as *Drosophila* (Hall, 1998; Young, 1998) and *Neurospora* (Davis, 2000; Lakin-Thomas et al., 1990; Loros and Dunlap, 2001; Nakashima and Onai, 1996) to identify and understand the molecular processes responsible for these rhythms. For the organisms investigated so far, all circadian "pacemakers" have been found to be composed of transcriptional-translational negative feedback loops in which clock proteins inhibit their own expression (Dunlap, 1999). In *Neurospora crassa*, the negative feedback loop of the *frequency* (*frq*) gene (Aronson et al., 1994b) has been found to be a central component in the organism's circadian clock (Loros and Dunlap, 2001), influencing properties such as temperature and pH compensation (Ruoff et al., 2000). In *frq* null strains (e.g., *frq*^{9}), no functional FRQ protein is made, and unlike in wild-type strains, no immediate sporulation rhythm is observed (Loros and Feldman, 1986). The fact, however, that rhythmic conidiation in *frq*^{9} (or other *frq*-less strains) may appear after several days indicates that in the absence of the *frq* gene, other oscillatory states, termed *frq*-less oscillators (FLOs; Granshaw et al., 2003; Lakin-Thomas and Brody, 2000, in press; McWatters et al., 1999; Merrow et al., 1999), may be formed after the system has passed an "induction period," similar to

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what is observed in chemical oscillators (Field and Burger, 1985).

In the model plant *Arabidopsis thaliana*, several genes are known to regulate circadian rhythms, but their functions within a central oscillator have not been established (McClung, 2001). It has been argued (Lillo et al., 2001) that circadian rhythms and their functionality can be perceived without postulating a central molecular chronometer. For example, the light-induced circadian nitrate reductase (NR) rhythm observed in several higher plants (Deng et al., 1989; Lillo, 1984; Lillo and Ruoff, 1989; McClung and Kay, 1994) can be understood as the result of an autonomous negative feedback loop in which probably glutamine, a product in the reaction chain initialized by NR, inhibits transcription of the NR gene (Lillo et al., 2001). Because higher plant and *Neurospora* NRs show similar features, including negative feedback inhibition by glutamine (Fu and Marzluf, 1988; Marzluf, 1997), we wondered whether *N. crassa* nitrate reductase activity (NRA) might also show circadian oscillations and, if so, to what extent an NRA rhythm might be under the control of the *frq* oscillator. In this report, we show that NRA oscillations in *Neurospora* occur on a 24-h time scale both under light and dark conditions when nitrate is the only nitrogen source. The NRA rhythm is also found in a frq^9 mutant and is thus independent of a functional FRQ protein. Even in a *wc-1* knockout mutant ($wc\text{-}1^{ko}$), we have observed NRA oscillations, although with a highly reduced amplitude.

MATERIALS AND METHODS

The *N. crassa* strains *bd* frq^+ and *bd* frq^9 (both mating type A) were obtained from the Fungal Genetic Stock Center (http://www.fgsc.net). In all experiments, ammonium-free Vogel's medium (Vogel, 1956) was used. A 10× medium was prepared by replacing the normally used NH_4NO_3 by $NaNO_3$, such that the nitrate concentration in the 1× solution was 25 mM. In the trace element solution, the normally used $(NH_4)_2Fe(SO_4)_2$ was replaced by an equimolar amount of $FeSO_4 \bullet 7H_2O$. Inoculation of approximately 1×10^8 conidia/L was performed in Petri dishes (90-mm diameter) containing 20 mL of 1× Vogel's medium with 2% sucrose, and the culture was exposed to white fluorescent light (25 μmol $s^{-1}m^{-2}$) at 30 °C. After 36 h of continuous light exposure, mycelial discs were cut out with a cork borer (1-cm diameter), and Petri dishes containing 20 mL 1× Vogel's medium and low sucrose (0.005%) were loaded with 3 mycelial discs each. The Petri dishes with the discs were then placed in darkness at 25 °C. Two time series with a 12-h time difference were prepared by inoculating series 1 and 2 at 0800 h and 2000 h, respectively, on day 1. Harvesting of the mycelial discs started on day 3 and continued over the following days from 0800 h until 2000 h with 4-h intervals. Harvesting in dark or light conditions was done by gently placing the discs on filter paper to remove excess liquid. Each set of discs was wrapped in aluminum foil, rapidly frozen in liquid nitrogen, and stored at –70 °C.

The slightly modified nitrate reductase activity assay (Lillo, 1983) was performed as follows: 1 mL ice-cold extraction buffer (0.1 M HEPES [pH 7.5], 1 mM EDTA, 3% (w/v) polyvinylpolypyrrolidone, 7 mM cysteine) was added to a 3-mL precooled glass homogenizer along with the mycelial discs, which then were rapidly homogenized on ice. In a 2-mL reaction tube, 650 μL of assay buffer (50 mM HEPES-KOH [pH 7.5], 2 mM KNO_3, 200 μM NADPH, 2 mM EDTA) was kept at 30 °C, and 100 μL of the extract was added and vortexed. After 10 min at 30 °C, 700 μL of the color-developing reagent (1% (w/v) sulfanilamide and 0.02% (w/v) N-(1-naphtyl)-ethylenediamine dihydrochloride in 1/10 diluted HCl) was added and vortexed again. Tubes were centrifuged for 30 min at 12,000 rpm, and the amount of nitrite formed was measured as the absorbance at 540 nm. For each extract, the NRA assay was performed in triplicate.

Amounts of total protein in extracts were measured in duplicate using the Bio-Rad Protein Assay with IgG as a standard. NRA levels are given in μmol-formed NO_2^- per mg protein per hour. The NRA rhythm was analyzed by fitting the average NRA levels from repeated time courses to a variable-amplitude square sinus function $g(t)$,

$$g(t) = \lambda_1 + \lambda_2 e^{-\lambda_3 \cdot t} \sin^2((\pi / P) + \phi), \quad (1)$$

using the program KaleidaGraph (Synergy Software, Reading, MA). The λ_is, as well as P (period) and ϕ, are adjustable parameters, and t is time.

RESULTS AND DISCUSSION

Figure 1A,B shows the observed NRA rhythm of *bd* and frq^9 mutants in continuous darkness (DD). Because *N. crassa* NR is photoactivated like plant NR

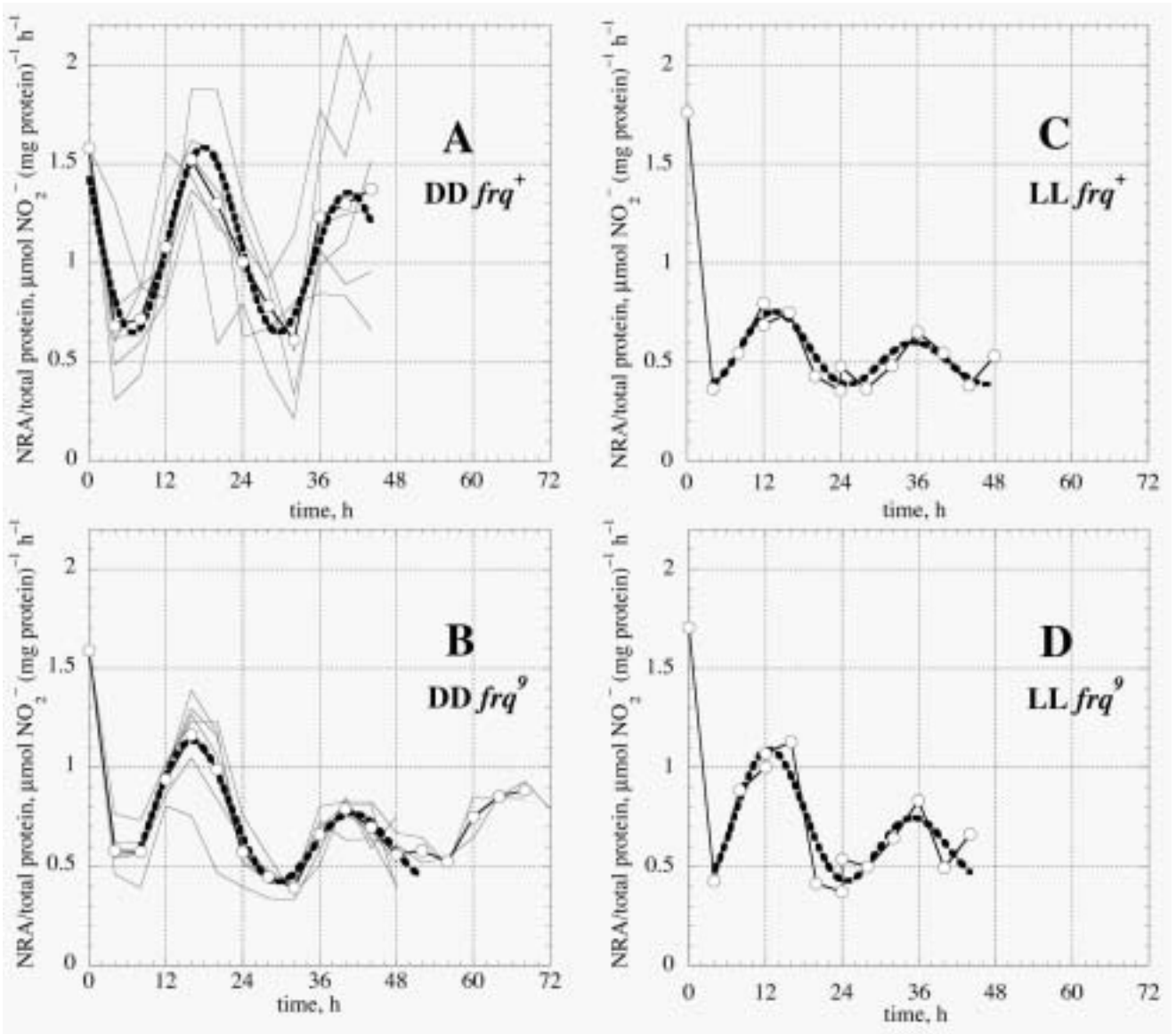

Figure 1. Nitrate reductase activity (NRA) shows endogenous oscillations with a period length of approximately 24 h in DD, in LL, and in the absence of a functional FRQ protein. (A, B) NRA rhythm in DD of (A) frq^+ ($n = 6$) and (B) frq^9 ($n = 6$ for 0-48 h; $n = 2$ for 0-72 h) at 25 °C. Time $t = 0$ represents the transition from LL with high sucrose to DD with low sucrose. (C, D) Examples of NRA rhythm in LL of (C) frq^+ ($n = 1$) and (D) frq^9 ($n = 1$) at 25 °C. Time $t = 0$ represents the transition in which discs were transferred from high sucrose to low sucrose. Open circles in (A, B) represent average NRA/total protein values. The average values at $t = 0$ for (A) and (B) were 1.58 ± 0.41 and 1.59 ± 0.22 µmol NO_2^- (mg protein)$^{-1}$h^{-1}, respectively. Thick dashed lines are curve fits of equation (1) to the averaged experimental data; *R*-values of the curve fits: (A) 0.9328, (B) 0.9708, (C) 0.8880, and (D) 0.8966.

(Ninnemann, 1997; Roldán and Butler, 1980) and because plant NRA rhythms are generally light induced (Deng et al., 1989; Lillo, 1984, 1993; Lillo and Ruoff, 1989; McClung and Kay, 1994), we tested whether the *Neurospora* NRA rhythm may also occur under continuous light (LL) conditions. LL does not abolish the NRA rhythm, as the results in Figure 1C,D show, in contrast to *Neurospora*'s sporulation rhythm and FRQ oscillations (Collett et al., 2002). In both DD and LL, the NRA period length is about 24 h, but in LL (+ low glucose), the rhythm is phase advanced compared to DD. Because the NRA oscillations are also found in frq^9, which is a *frq* null allele (Aronson et al., 1994a), the NRA rhythm is not an output rhythm of the *frq* oscillator but is self-sustained. That NR oscillations can persist even in the absence of a functional white-

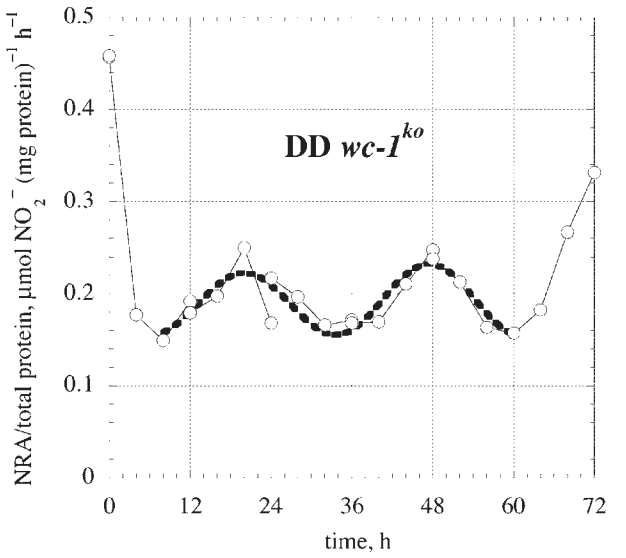

Figure 2. Observed nitrate reductase activity (NRA) oscillations in the absence of a functional white-collar complex (n = 1). Note the strongly reduced amplitude and the slight phase delay in the NRA rhythm onset in *wc-1*^{ko} compared with the NRA oscillations in *frq*$^{+}$ and *frq*^{9} (Fig. 1). Dashed line is the curve fit of equation (1) to the experimental data; R-value: 0.8612. T = 25 °C. Time t = 0 represents the transition from LL with high sucrose to DD with low sucrose.

collar complex (WCC) is indicated by results using a *wc-1*^{ko} mutant. In this mutant, light sensing and *frq* and other gene transcriptions through the WCC are abolished (Lee et al., 2003). NRA oscillations were observed in this mutant, although with a strongly reduced amplitude (Fig. 2). However, this result is somewhat preliminary because from four independent experiments, oscillations were seen in only two of the cases (one shown in Fig. 2). In the other two cases, it was difficult to judge whether there was a rhythm. We do not know the reason for the large reduction in NRA amplitude and levels in *wc-1*^{ko}. It may be related to the loss of the light sensor WC-1 (Cheng et al., 2003; Froehlich et al., 2002) or may indicate that WCC-dependent processes have an influence on *nit-3* transcription and/or translation. Although NR contains a blue light absorbing flavin, which may affect the influence of light on the NR oscillator, the suggested role that NR is a blue light receptor (Ninnemann, 2001) is still not clearly established (Belozerskaya et al., 1982).

Compared with DD, the LL NRA rhythm is phase advanced by 4 to 6 h (Figs. 1, 3A). The cause for the phase advance in LL lies in an approximately twice as rapid decrease of NRA after the transfer to low-sucrose light conditions (Fig. 3B), while the time interval between the first NRA minimum and the following maximum appears unaltered in DD and LL (≈ 10 h).

In the absence of favored nitrogen sources such as ammonium or glutamine, *Neurospora* readily takes up nitrate. Utilization of nitrate requires de novo synthesis of nitrate reductase and nitrite reductase, as well as nitrogen derepression and specific induction by nitrate ion (Marzluf, 1997). Glutamine, a downstream reaction product of nitrate reductase, appears to be the critical metabolite, which represses transcription of nitrate reductase (Premakumar et al., 1979). The actual repression mechanism is not completely understood. Glutamine somehow activates the key regulator NMR (nitrogen metabolite regulation), which binds directly to NIT2, which is one of the two recognized positive regulators of nitrate reductase transcription (Fu and Marzluf, 1988; Marzluf, 1997; Pan et al., 1997). Thus, in the presence of only ammonium (or glutamine), the NR oscillator is not expressed, but (in *frq*$^{+}$) the FRQ/WCC-driven circadian conidiation rhythm should still be operative, probably even in a *nit-3* knockout mutant.

Similar to what has been observed for higher plants (Lillo et al., 2001), the regulation circuit governing nitrate utilization in *Neurospora* (Fig. 4) is also a negative feedback loop, which, like in higher plants and algae, may be capable of acting as an autonomous daily rhythm generator. The inactivation and turnover of *Neurospora* NR and its mRNA have been found to be fairly rapid processes (Okamoto et al., 1991; Sorger et al., 1978; Walls et al., 1978). As for the FRQ negative feedback loop (Ruoff et al., 1999), the degradation of NR and its mRNA may play a role in defining the period length of the NR oscillator and its temperature behavior. In *Gonyaulax*, nitrate had a profound effect on the circadian rhythm, affecting amplitude, phase, and period (Roenneberg and Rehman, 1996).

It is intriguing that in *Neurospora*, an additional NR oscillator (besides the *frq* oscillator) comes into play when environmental conditions require assimilation of nitrate as the only nitrogen source. Interestingly, both FRQ (Garceau et al., 1997) and NRA (Fig. 1) peak at approximately the same phase relative to a light-to-dark transfer. In fact, microarray analyses (Correa et al., 2003; Nowrousian et al., 2003) show that the majority of rhythmically expressed *Neurospora* genes (2.7%-5.9%) peak at approximately the same phase with an increased transcriptional/metabolic activity during late night to early morning.

Because under nitrate-growing conditions, all assimilated nitrogen goes through the nitrate

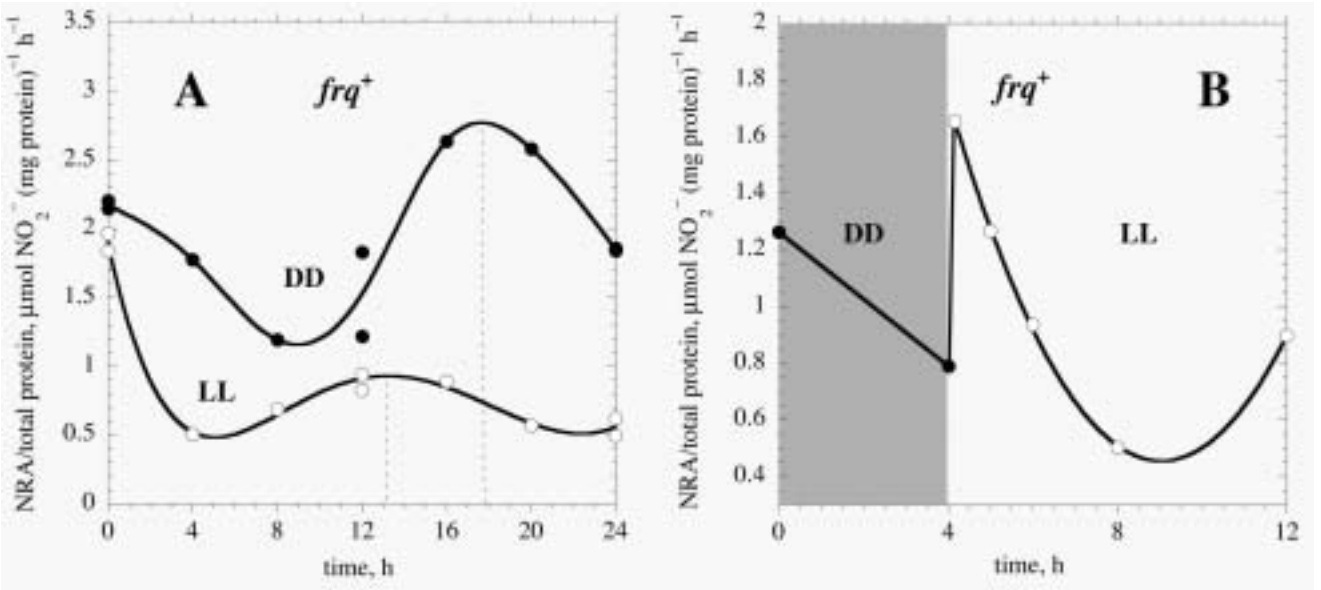

Figure 3. Kinetics of nitrate reductase activity (NRA) in DD and LL during the induction period. Time $t = 0$ represents the transition from LL with high sucrose to DD with low sucrose. (A) *frq*$^+$ mycelium discs that were cut out from the same culture (mycelium) and then tested in parallel under DD and LL conditions show a more rapid approach to the oscillatory state in LL than in DD. As a result, the LL rhythm shows a phase advance compared to the DD rhythm. (B) Effect of DD → LL transition on *frq*$^+$ NRA. After a rapid light-induced increase, the NRA decreases more rapidly in LL than previously in DD. T = 25 °C.

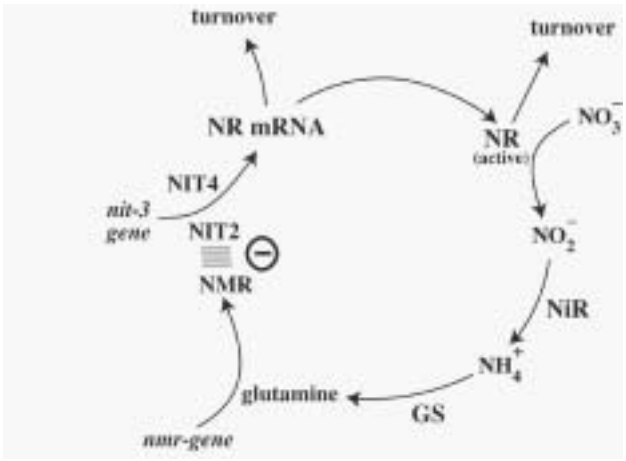

Figure 4. A simplified scheme of nitrate assimilation and the nitrate reductase (NR) negative feedback loop in *Neurospora crassa*. NIT2 and NIT4 are positive regulators, which are required for the transcription and regulation of the *nit-3* gene (the structural gene of NR). Active NR catalyzes the reduction of nitrate to nitrite, which is reduced to ammonium (catalyzed by nitrite reductase [NiR]) and further incorporated into glutamine (catalyzed by glutamine synthetase [GS]). Due to a not completely understood mechanism, glutamine activates the negative regulator NMR (nitrogen metabolite regulation), which binds directly to NIT2 and inhibits transcription of *nit-3* (Pan et al., 1997; Marzluf, 1997).

reductase pathway, the NRA oscillations can be considered as an indicator for *Neurospora*'s metabolic (nitrogen) activity. Although a yet unknown *frq*-independent oscillator that drives the NRA oscillations could always be postulated, it seems equally reasonable to consider that NR is part of an autonomous oscillator defined partly or wholly by the NR negative feedback loop (Fig. 4). Such an oscillator would regulate nitrate uptake and assimilation to ensure an increased metabolic/transcriptional activity during late night to early morning (Correa et al., 2003; Nowrousian et al., 2003). It is tempting to speculate that other control processes—for example, the sulfur regulatory circuit (Kumar and Paietta, 1995; Perkins et al., 2001)—could be regulated in a similar circadian-type fashion, where positive and negative elements (CYS3 and SCON1/SCON2, respectively) may play similar dynamic roles as the positive and negative regulators in the *frq* or *nit-3* negative feedback loops.

Whether *Neurospora*'s nitrate reductase loop (Fig. 4) can autonomously generate true circadian oscillations, including temperature compensation, or may be part of a network of multiple oscillators (Correa et al., 2003) will be the subject of subsequent work.

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