The Adaptive Value of Circadian Clocks: An Experimental Assessment in Cyanobacteria

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Summary

Circadian clocks are thought to enhance the fitness of organisms by improving their ability to adapt to extrinsic influences, specifically daily changes in environmental factors such as light, temperature, and humidity. Some investigators have proposed that circadian clocks provide an additional "intrinsic adaptive value," that is, the circadian clock that regulates the timing of internal events has evolved to be such an integral part of the temporal regulation that it is useful in all conditions, even in constant environments. There have been practically no rigorous tests of either of these propositions. Using cyanobacterial strains with different clock properties growing in competition with each other, we found that strains with a functioning biological clock defeat clock-disrupted strains in rhythmic environments. In contrast to the expectations of the "intrinsic value model," this competitive advantage disappears in constant environments. In addition, competition experiments using strains with different circadian periods showed that cyanobacterial strains compete most effectively in a rhythmic environment when the frequency of their internal biological oscillator and that of the environmental cycle are similar. Together, these studies demonstrate the adaptive value of circadian temporal programming in cyanobacteria but indicate that this adaptive value is only fulfilled in cyclic environments.

Introduction

Circadian clocks are found in a wide range of organisms from bacteria to mammals. The adaptive value of these clocks has been largely a matter of conjecture based more on plausibility than on rigorous testing [1]. Presumably, the selective force for the evolution of circadian systems was the daily cycles of light, temperature, and humidity present in the natural environment. If so, circadian clocks would be expected to enhance the fitness of an organism by entraining behavioral and physiological processes to occur at optimal phases in the cyclic environment, conferring an "extrinsic" adaptive value [2].

Indeed, several studies suggest that an intact circadian clock does enhance fitness in a variety of organisms in cyclic environments [1–5]. On the other hand, some researchers have proposed that circadian clocks may additionally provide an "intrinsic" adaptive value [6, 7]. That is, circadian pacemakers evolved to become an intrinsic part of internal temporal organization and as such may have become intertwined with other traits that influence reproductive fitness in addition to their original role for adaptation to environmental cycles. Viewed in this way, circadian clocks would be expected to be of adaptive value to an organism in constant conditions as well as in cyclic environments. In support of this hypothesis, populations of *Drosophila melanogaster* raised for hundreds of generations in constant conditions retain the ability to entrain to various light-dark cycles, indicating that even in the absence of environmental selection the components of the circadian system are maintained [7].

We decided to test whether circadian systems confer extrinsic and/or intrinsic adaptive value to fitness in cyanobacteria. These prokaryotes are evolutionarily ancient microorganisms and several species are known to have circadian systems. In *Synechococcus elongatus* PCC 7942, the *kaiABC* gene cluster encodes the cyanobacterial clock proteins that regulate circadian oscillations, and a number of mutations in *kaiA*, *kaiB*, and *kaiC* have been isolated that result in a variety of circadian phenotypes [8]. We used growth in competition between cyanobacterial strains with differing clock properties to experimentally test if circadian clocks enhance reproductive fitness. For bacteria and other asexually reproducing microbes, differential growth of one strain in competition with another is a good measure of reproductive fitness [9, 10]. We show here that strains of cyanobacteria with a functional circadian clock outcompete "clock-disrupted" strains in light-dark cycles (LD) but not in constant light (LL). That result suggests that the circadian clock system enhances reproductive fitness in cyanobacteria, but only in cyclic environments. Our results also show that for strains with a functioning circadian clock, fitness is enhanced when the circadian clock oscillates with a free-running period (FRP) that is similar to that of the environmental cycle.

Results and Discussion

The Circadian Clock in Cyanobacteria Has Adaptive Value Only in Rhythmic Environments

For all competition experiments, we used strains of cyanobacteria that were isogenic except for point mutations in the essential cyanobacterial clock genes *kaiA*, *kaiB*, and *kaiC* [11]. The strains CLAb and CLAc harbor different point mutations in *kaiC* that give rise to apparent arrhythmicity or damped rhythms, respectively (Figure 1A) [8, 11]. In pure cultures, there was no detectable difference in the growth of these strains in either LL or LD12:12 as compared with wild-type cultures (Figure

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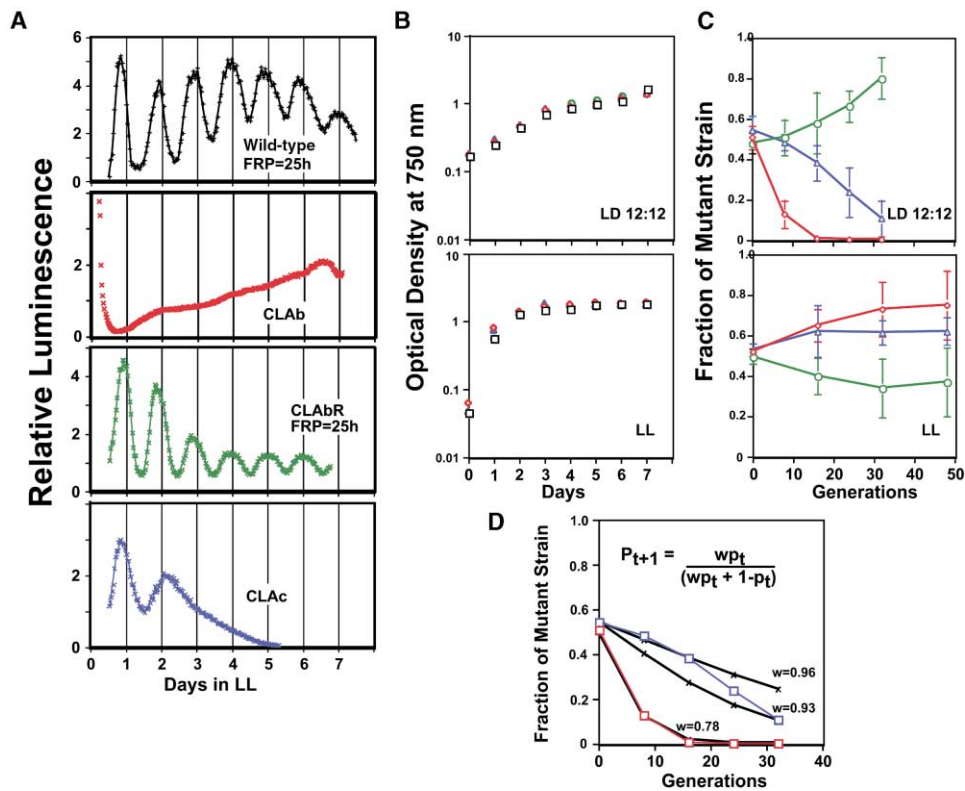
Figure 1. Competition of Clock-Disrupted Strains with Wild-Type

(A) The circadian phenotypes of luminescence emission from wild-type (AMC 343), the *kaiC* mutants CLAb and CLAc, and the rescued mutant strain CLAbR. Wild-type and CLAbR have a FRP of ~25 hr; the *kaiC* mutant CLAb appears to be arrhythmic, and the *kaiC* mutant CLAc displays a rapidly damped oscillation. All strains have a luciferase construct that reports the promoter activity of the *psbA1* gene [19] assayed with a CCD camera/turntable device [8].

(B) Growth of these strains in pure cultures in constant light (LL) and in LD12:12; in LL, strains complete ~2 generations/day and in LD12:12, strains complete ~1 generation/day. In (A) and (B), black, wild-type; red, CLAb; green, CLAbR; and blue, CLAc.

(C) Competition between wild-type and mutant strains in LD12:12 (top) and LL (bottom) plotted as the fraction of mutant in the mixed culture versus the estimated number of generations (mean ± SD; n = 4); red, CLAb; green, CLAbR; and blue, CLAc.

(D) Comparison of the raw data plotted in (C) for CLAb (red) and CLAc (blue) in LD12:12 with the results of modeling (black) using the equation shown, where w = relative fitness of the indicated strain and p = fraction of a given strain in mixed population (p_t for generation t, p_{t+1} for generation t+1) [10] (Experimental Procedures).

1B). Therefore, these mutations in *kaiC*, which have a major impact upon the circadian system, do not drastically affect the growth rate of these strains in pure cultures [11]. When grown in competition, both CLAb and CLAc were out-competed by wild-type cells in LD12:12 cycles (Figure 1C). In comparison, CLAbR, a "rescued" strain in which the wild-type *kaiC* gene was restored in the mutant, is not out-competed in mixed populations with wild-type cells in LD12:12 cycles (Figure 1C). This indicates that the mutation in the clock-specific gene *kaiC*, rather than a putative secondary mutation, is responsible for these phenomena. There is a significant difference in the kinetics of CLAb versus CLAc in competition with wild-type cells in LD12:12 (Figure 1C, two-sample t test, p = 0.002). CLAb is more rapidly eliminated (within ~16 generations) in mixed cultures with wild-type than is CLAc (~24–32 generations) in LD12:12. Unlike CLAb, CLAc is not initially arrhythmic, but shows a damped oscillation (Figure 1A) that is probably restimulated each day on exposure to light when in an LD cycle.

In contrast to the results obtained in a LD12:12 environment, the apparent arrhythmic (CLAb) and damped (CLAc) strains compete effectively with wild-type in constant light; both strains were maintained in the mixed population with wild-type cells at levels close to their starting proportions (Figure 1C). Interestingly, the proportion of CLAb increased significantly from its starting proportion in mixed populations with wild-type (repeated measures ANOVA, p = 0.0119), implying that a normally functioning circadian clock may actually be a disadvantage in a constant environment. The damped CLAc strain in mixed cultures with wild-type showed no significant difference from the starting proportion in LL (p = 0.1957).

Estimation of the Fitness Advantage Conferred by the Circadian Clock in Rhythmic Environments

To estimate the reproductive fitness of CLAb and CLAc relative to wild-type in LD12:12, we used a simple model that assumes that there is constant selective pressure throughout all phases of growth and at all population

densities (Figure 1D, Experimental Procedures). Using this model, we estimate that the fitness of the arrhythmic strain CLAb relative to wild-type is ∼0.78 in LD12:12, while that of the damped strain CLAc is between 0.93 and 0.96 (Figure 1D). Since the growth rate of CLAb in pure culture (Figure 1B) does not vary by the ∼20% fitness advantage seen, this is most likely an example of soft selection where the reduced fitness of one genotype is seen only under competition [12]. The "rescued" strain CLAbR shows an unexplained fitness advantage of ∼5% over wild-type cells, but only in LD12:12 (w for wild-type is ∼0.95).

In our previous work, we suggested that optimally phased cyanobacterial strains may secrete a diffusible factor that inhibits the growth of nonoptimally phased strains [10]. Our hypothesis provided the basis for a mathematical model that posited the rhythmic secretion of and rhythmic sensitivity to a growth inhibitor [13, 14]. The model made the prediction that less fit strains could compete effectively with more fit strains if the less fit strain were in a large enough excess (composing ∼60%–70%) in the starting populations [13, 14]. We tested this prediction with mixtures of the arrhythmic CLAb strain and wild-type; mixed populations were composed of ∼75% (Figure 2A) or ∼90% (Figure 2B) CLAb cells. These proportions were selected because they were significantly larger than those predicted by the mathematical model to be at a bifurcating fraction (∼60%–70% [14]). The mixed cultures were placed in either LL or LD12:12 conditions, and the composition of the populations was monitored for 25–40 generations. As expected, CLAb is maintained at or slightly above these high starting levels in mixed cultures in LL; however, the fraction of the arrhythmic strain in the mixed populations drops dramatically in LD12:12 cycles (Figure 2). Under these conditions, it appears that the reduction in fitness suffered by CLAb cannot be completely overcome simply by starting with more individuals in the population as predicted by the model [13, 14]. There appears to be no statistically significant association in the rate at which CLAb declines in the population and the initial starting proportion of the less fit strain (repeated measures ANOVA, p = 0.345 for 90:10 versus 50:50), suggesting that the relative fitness of the weaker strain does not change in a frequency-dependent manner.

Reproductive Fitness Is Enhanced when the Circadian Clock Oscillates with a Frequency Similar to the Environmental Cycle

Two cyanobacterial strains with mutations in *kaiC* were previously shown to out-compete wild-type strains in mixed cultures when their FRP was similar to that of the LD cycle [10]. That result is not limited to *kaiC* mutants; similar results are seen in competition when any of the clock genes *kaiA*, *kaiB*, and *kaiC* are mutated (Figure 3). The FRPs of the various strains are shown in Figure 3A. The growth rates of these strains are indistinguishable in pure culture in LD11:11 and LD15:15 cycles (Figure 3B) and when the strains were grown in either constant light or LD12:12 cycles [15]. Thus, there does not appear to be a detectable difference in the growth rates of these strains when grown separately under the conditions used for competition.
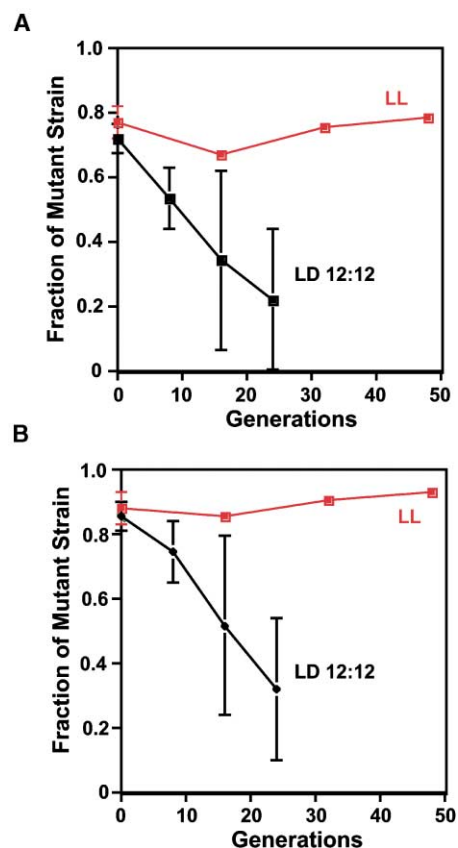

Figure 2. Competition between the Clock-Disrupted Mutant CLAb and Wild-Type when CLAb Has a Starting Advantage

(A) Kinetics of competition between wild-type (AMC343) and CLAb with the CLAb strain at ∼75% of the initial mixed population.
(B) Kinetics of competition between wild-type and CLAb with CLAb at ∼90% of the starting population. For both (A) and (B), the fraction of CLAb is plotted versus the estimated number of generations for competition in LL (red) and LD12:12 (black); n = 4 trials for both experiments (±SD).

The strains with altered FRPs were competed against wild-type cells in LD cycles that had equal amounts of light and darkness, but in which the frequency of the cycle differed: a 22 hr cycle (LD11:11) and a 30 hr cycle (LD15:15). When wild-type cells compete against the short period *kaiB* mutant, B22a in LD15:15, the wild-type cells rapidly out-compete B22a cells (Figure 3C), but in LD11:11, B22a defeats wild-type. Conversely, a long period *kaiA* mutant, A30a, "wins" against wild-type cells in a 30 hr LD cycle but is defeated by wild-type cells in the 22 hr LD cycle (Figure 3C). The results with B22a and A30a are similar to those obtained with the *kaiC* mutants C22a and C28a that were previously tested [10]. Figure 3C depicts a direct comparison of all four strains competing with wild-type in new experiments conducted simultaneously. The same combinations of clock mutants and wild-type strains grown together in constant light (LL) show that all are maintained in the mixed cultures at densities that are close to their original starting proportions (data not shown), suggesting that there is little selection against these genotypes in the absence of an LD cycle.
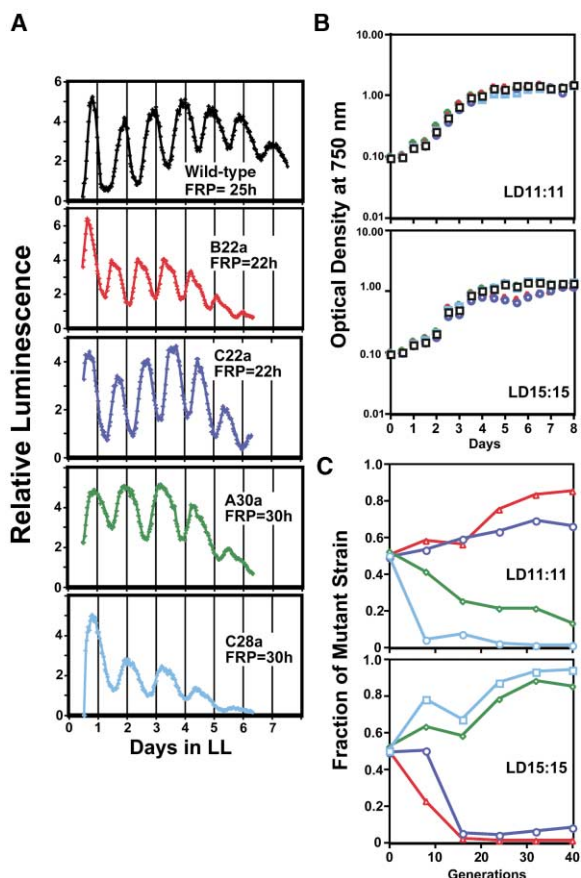
Figure 3. Competition of Mixed Cultures in LD11:11 and LD15:15 Cycles

(A) The circadian phenotypes of wild-type (AMC343, FRP ~25 hr) and mutants (mutations in *kaiB* [B22a, FRP ~22 hr], *kaiA* [A30a, FRP ~30 hr], and *kaiC* [C22a, FRP ~22 hr; C28a, FRP ~30 hr]).
(B) Growth of the strains in pure cultures in LD11:11 (upper) and LD15:15 (lower).
(C) Kinetics of competition in mixed cultures between wild-type and the mutant strains in LD11:11 (upper) and LD15:15 (lower). Data are plotted as the fraction of the mutant strain in the mixed culture versus the estimated number of generations. A representative trial is plotted for each of the four strains (n = 2). In (A) and (B), black, wild-type; in (A), (B), and (C), red, B22a; dark blue, C22a; green, A30a; and light blue, C28a.

Therefore, the fitness advantage conferred by consonance between the circadian clock of a strain and the environmental cycle appears to be independent of the specific cyanobacterial clock gene (*kaiA, kaiB,* or *kaiC*) that is mutated to produce the altered FRP (Figure 3C). The fitness of the B22a and A30a strains relative to wild-type under the different growth conditions suggests that the relative fitness of the less successful strain can be as low as 0.85 (Table 1). Consequently, in rhythmic environments a circadian system with an appropriate FRP can significantly enhance the reproductive fitness of cyanobacteria.

Enhanced Reproductive Fitness Conferred by the Circadian Clock Provides Evidence for Extrinsic Adaptive Value in Cyanobacteria

Our competition studies show that the circadian pacemaker in cyanobacteria confers a significant reproduc-

Table 1. Relative Fitness, w, of *kaiA* and *kaiB* Mutants in Competition with Wild-Type in Various Light/Dark Cycles

Strains in Competition	Relative Fitness, w, of B22a or A30a
B22a versus AMC 343 in LD11:11	1.05
B22a versus AMC 343 in LD15:15	0.85
A30a versus AMC 343 in LD11:11	0.93
A30a versus AMC 343 in LD15:15	1.05

tive advantage in light/dark cycles when the period of the biological clock is similar to that of the environmental cycle. This fitness advantage is observed regardless of which of the cyanobacterial clock genes, *kaiA, kaiB,* or *kaiC*, is mutated (Figure 3). Furthermore, these experiments show that strains lacking a functional circadian pacemaker have a low reproductive fitness when grown in a rhythmic environment (Figure 1). This disadvantage cannot be completely overcome even when these "clockless" cells greatly outnumber cells with a functioning circadian clock in the initial population (Figure 2). The observation that a strain which shows a damped oscillation (CLAc) is able to compete more effectively than an apparently arrhythmic strain (CLAb) in LD cycles implies that a damped oscillator that is restimulated daily by the LD cycle is better than a disrupted oscillator (Figure 1). Perhaps a damped oscillator can be entrained so that cellular events can be phased moderately well. Nevertheless, the damped oscillator is out-competed by wild-type in LD, which underscores why evolution appears to have favored sustained circadian oscillators over damped or hourglass oscillators for programming biological processes over the day.

One of the most surprising results of our study is that in constant conditions, the circadian clock system is of no benefit and might even be deleterious to cyanobacteria (Figure 1C); this is contrary to predictions made earlier on the basis of internal temporal organization [6, 7, 16, 17]. This probably means that an intact circadian system regulates processes in a manner that is not optimal in a constant environment. For example, perhaps the clock rhythmically represses processes such as photosynthesis; this regulation could be adaptive at the onset of the daily dark phase in LD, but maladaptive in constant light. Strains with a damped oscillator seem not to do better than wild-type in constant light, suggesting that their residual oscillator does not allow them to take full advantage of the continuously available illumination. Taken together, our results show that an intact clock system whose FRP is consonant with the environment significantly enhances the reproductive fitness of cyanobacteria in rhythmic environments; however, this same clock system provides no adaptive advantage in constant environments and may even be somewhat detrimental to the organism.

Conclusions

We show here that cyanobacterial strains with a functioning biological clock have a reproductive advantage over clock-disrupted strains in rhythmic environments, but not in constant environments. Furthermore, cyanobacterial strains with different circadian periods compete most effectively in a rhythmic environment when

the frequency of their internal biological oscillator matches that of the environmental cycle; this fitness advantage is independent of the cyanobacterial clock gene (*kaiA, kaiB,* or *kaiC*) mutated to produce the altered FRP. Together, these results demonstrate the adaptive value of the cyanobacterial circadian clock but suggest this value is realized only in rhythmic environments.

Experimental Procedures

Strains
Cyanobacterial strains were selected based on equivalent growth rates in LL, plating efficiencies, and stability of FRP in all phases of growth [8, 10, 15, 18]. Strains that showed wild-type FRPs ($\sim$25 hr at 30°C) were AMC149 (spectinomycin resistance) and AMC343 (chloramphenicol resistance); they were genetically engineered from *Synechococcus* sp. PCC7942 by targeting a *luxAB* reporter under control of the *psbAI* promoter to a neutral site in the chromosome [19]. From AMC149, strains B22a (FRP $\sim$22 hr), A30a (FRP $\sim$30 hr), C22a (FRP $\sim$22 hr), C28a (FRP $\sim$30 hr), CLAb (arrhythmic), and CLAc (damped rhythm) were derived by ethyl methanesulfonate mutagenesis; all mutant strains are spectinomycin resistant [8, 10]. Strains A30a and B22a have point mutations in *kaiA* and *kaiB*, respectively, while C22a, C28a, CLAb, and CLAc have point mutations in *kaiC*; all mutations result in single amino acid changes [11]. Plating efficiencies of these strains are equivalent [15, 18]. Circadian phenotypes were determined by luminescence from a luciferase reporter [8, 19].

Culture Conditions
Modified BG-11 medium [20] was used for both liquid and solid media. Cell density was estimated by measurement of OD_{750} for both pure and mixed cultures (early stationary phase of $OD_{750} \approx 1.0$). Cell number was determined from mixed and pure cultures by plating on three dishes of BG-11 supplemented with the appropriate antibiotic (20 μg/ml spectinomycin for AMC149, A30a, B22a, C22a, C28a, CLAb, and CLAc or 7.5 μg/ml chloramphenicol for AMC343) followed by growth in LL for 5–8 days at 28°C.

Competition Experiments
All experiments were performed in batch liquid cultures and were incubated at 30°C with aeration under white fluorescent light (90–100 μM·m^{-2}·s^{-1}, measured with a planar light detector). Strains were grown in pure culture to an OD_{750} of approximately 1.0 and were then diluted 1:1000, and equal volumes of the two strains were then mixed. Samples from this mixed population were then immediately plated so that the initial composition of the population could be measured. For mixed cultures to be placed in LD cycles, the onset of the light phase was initiated immediately after this first plating. Mixed cultures in LL were diluted 1:30 and the composition of the population sampled every 4 days; the cultures in LD cycles were diluted 1:30 and sampled in the illuminated period of the cycle every 8 days. For experiments in which one strain was given a starting advantage in the population, pure cultures were grown and diluted as described above, but the starting population was established by mixing a proportionally greater volume of one diluted strain over the other.

Determination of Composition of Populations under Competition
The difference in antibiotic resistance between the mutant strains (spectinomycin) and the wild-type AMC343 (chloroamphenicol) was used to score the composition of mixed cultures. At regular intervals, each mixed culture was plated onto six Petri dishes, with three dishes of BG-11 containing spectinomycin (20 μg/ml) and three dishes of BG-11 containing chloramphenicol (7.5 μg/ml) for growth in LL for 5–8 days. The number of colonies was then counted using a CCD camera apparatus (Alpha Innotech, San Leandro, CA). As an independent confirmatory method, samples from mixed cultures were also plated on BG-11 lacking any antibiotics, and the circadian phenotypes of the resulting colonies were determined by measuring luminescence using the CCD camera/turntable device [8]. The proportion of mutant versus wild-type cells in mixed cultures deter-

mined by this method was then compared to the results determined by plating on BG-11 medium containing either spectinomycin or chloramphenicol.

Comparison of Growth Rates in Pure Cultures
Batch liquid cultures of individual strains growing in LL or in LD cycles at 30°C were used to compare growth rates of the strains used in competition. Pure cultures of each strain were grown as described, samples were taken, and the OD_{750} was measured approximately every 4 hr in LL and every 8–12 hr in LD cycles for 8 days or until the cultures reached stationary phase. OD_{750} was then plotted against time for a comparison of growth in pure culture between the strains.

Estimation of Relative Fitness
To estimate the relative fitness of the different strains in competition, we used a simple model that assumes that the selective pressure is constant throughout all phases of growth and at all population densities. In this model, the proportion of a strain in a subsequent generation, P_{t+1}, is related to the relative fitness of a strain, w, and the proportion of that strain in the previous generation P_t such that:

$$P_{t+1} = \frac{wP_t}{(wP_t + 1 - P_t)}$$

This is an iterative model that calculates the proportion for each generation based on a given relative fitness. In Figure 1D, results are plotted every 10 generations. Competition results were fitted by eye to the best curve(s) of relative fitness generated by the model.

Statistical Analysis of Competition Data
To estimate the significance of the difference in the kinetics of competition between the two arrhythmic strains and wild-type in LD12:12, a two-sample t test was performed comparing the proportion of CLAb at generation 16 to the proportion of CLAc at generation 16 using the statistical software package, JMP In version 4.04. To estimate the significance in the variation among the repeated trials of competition between the strains CLAb and CLAc versus wild-type (AMC343) in constant light, a repeated measures ANOVA was performed using the statistical software SAS version 8.02. The results of competition between each strain and wild-type were compared over time with the starting proportion of the competition.

Construction of CLAbR from CLAb
The mutant *kaiC* allele in CLAb was replaced with the wild-type allele carried on the plasmid pAM1736 in a two-step "hit and run" procedure resulting in the rescued CLAbR (as described in [10]). The plasmid pAM1736 was made by inserting a wild-type *kaiC* sequence into the vector pRL278, which carries a kanamycin resistance gene and the *sacB* gene [21]. CLAb cells were washed with 10 mM NaCl, resuspended in 1 ml BG-11 with $\sim$200 ng pAM1736, and incubated at 30°C for 3–4 hr in darkness with gentle shaking. 150 μl of this culture was plated on BG-11 with kanamycin (20 μg/ml) to select for integration of pAM1736 by single crossover homologous recombination. Several kanamycin-resistant colonies were chosen at random, incubated in liquid BG-11, and then plated on BG-11 containing 5% sucrose without kanamycin to select for "loopout" of the plasmid, the reverse of the integration event. Kanamycin-sensitive colonies were screened to confirm the rhythmic expression of the *luxAB* reporter in the rescued CLAbR.

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