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Modeling the Differential Fitness of Cyanobacterial Strains whose Circadian Oscillators have Different Free-running Periods: Comparing the Mutual Inhibition and Substrate Depletion Hypotheses

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In a recent experimental study, Ouyang *et al.* (1998, *Proc. Natl. Acad. Sci. U.S.A.* **95,** 8660–8664) have shown that, in direct competition, cyanobacterial strains whose circadian clocks have free-running periods (FRPs) which match the period of an imposed light/dark (LD) cycle exclude strains whose FRPs are out of resonance with the LD cycle. These differences in competitive fitness are observed despite the lack of measurable differences in monoculture growth rates between the strains. Here we show that the experimental results are consistent with a mathematical model in which cells rhythmically produce a metabolic inhibitor to which they display a sensitivity modulated by their circadian rhythm. We argue that models in which there is a circadian modulation of nutrient uptake kinetics cannot account for the results of these experiments. We discuss possible experiments to further characterize this phenomenon. The experimental protocol we propose can be used to distinguish between mutual inhibition and substrate depletion as underlying causes of the competitive advantage of circadian resonance.

1. Introduction

Circadian oscillators have now been shown to exist in a wide variety of organisms, ranging from multicellular plants (Sweeney, 1987; Kondo & Ishiura, 1999) and animals (Gillette, 1997; Dunlap, 1999) to prokaryotic cyanobacteria (Edmunds, 1988; Kondo *et al.*, 1993; Johnson *et al.*, 1996; Golden *et al.*, 1998). Experiments in which the length of the day/night cycle is varied show that, for an equally wide range of organisms, there is adaptive value not only in having such an oscillator, but in having one whose natural period closely approximates the length of the day. In higher organisms, effects observed when the light/dark (LD) cycles are significantly shorter or longer than the oscillator's natural, free-running period (FRP; measured in constant conditions) include stunted growth (Highkin & Hanson, 1954; Went, 1960), physiological dysfunction (Hillman, 1956), and decreased longevity (Pittendrigh & Minis, 1972; Pittendrigh, 1993; Klarsfeld & Rouyer, 1998). One of the hallmarks of circadian oscillators is that they can be entrained over a range of LD cycle periods. That being the case, why do short or long days decrease fitness?

Recent studies with cyanobacteria have shed new light on this question. The cyanobacterial circadian clock genes have recently been characterized (Ishiura *et al.*, 1998). In a remarkable

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series of experiments, Kondo, Golden, Johnson and co-workers then isolated a number of circadian clock mutants of the cyanobacterium *Synechococcus* which display different free-running periods in continuous light (Kondo *et al*., 1994). A subset of these mutants can be shown by complementation to differ only in their circadian clock genes and display, in monoculture, identical growth rates both in continuous light (hereafter symbolized LL) and in LD cycles of various lengths (Ouyang *et al*., 1998). All strains can apparently be entrained to LD cycles in the experimental range, although there are differences in phase between variables controlled by the circadian clock for strains with different FRPs (Ouyang *et al*., 1998). In direct competition in LL, as would be expected from the monoculture results, two strains can coexist, at least over the time-scale of the experiments. In LD competition experiments however, dramatic differences in competitive fitness emerge: Strains whose circadian clocks have FRPs which match or nearly match the imposed LD rhythm can exclude strains whose FRPs differ significantly from the imposed LD period (Ouyang *et al*., 1998). This somewhat surprising result implies that interaction between the circadian clock and an LD Zeitgeber can confer a competitive advantage on strains whose growth kinetics are indistinguishable under all other growth conditions.

Ouyang *et al*. (1998) have themselves offered a pair of hypotheses to explain this phenomenon: Either differences in fitness are due to competition for a limiting resource, or they are due to the secretion of a diffusible inhibitor of growth. In either case, the activity would be modulated by the circadian rhythm. Both hypotheses can be thought of as special cases of a more general hypothesis, namely that there exist differences in the timing of metabolic events due to the interaction between a strain's circadian clock and the imposed LD cycle. It should be noted that rhythmic metabolism controlled by the circadian clock is likely the rule rather than the exception in cyanobacteria (Liu *et al*., 1995). Moreover, for at least one circadian clock-controlled gene, the phase of the LD cycle at which a maximum in expression occurs differs among strains with different FRPs (Ouyang *et al*., 1998).

In this article, we discuss the formulation of mathematical models based on this general hypothesis. Our aim is to produce minimal, robust models of the phenomena described above. Our treatment of certain aspects of metabolism is highly simplified but the behavior of the resulting models is representative of the behavior of more elaborate models with similar underlying circadian dynamics. We shall, for instance, assume that metabolic activities can depend both on the LD cycle and on the state of the cell's circadian clock. The state of the circadian clock is collapsed onto two states: subjective day and subjective night. For convenience, we introduce the labels sL for subjective day (i.e. a subjective clock phase leading to metabolism normally consistent with light, corresponding to Bünning's photophile phase; Highkin & Hanson, 1954; Pittendrigh, 1993; Thomas & Vince-Prue, 1997) and sD for subjective night (Bünning's skotophile phase). We assume that the durations of light and dark intervals and of the sL and sD phases are all equal. Moreover, we assume that strains differ only by the phase relationship of their sL/sD cycle to the LD rhythm. This is at best a caricature of the true situation. Cells almost certainly retain some capacity for light-phase metabolism when their internal clock tells them it should be dark (and vice versa), but this is a useful starting point to draw out the essential features of models which account for circadian resonance in cyanobacteria.

We begin our study with a model in which it is assumed that cells secrete a growth inhibitor according to a program controlled by the circadian clock and that they are vulnerable to this inhibitor when not secreting it. We show that such a model can successfully account for the experimental observations. We then discuss attempts to construct a model based on circadian modulation of resource exploitation and argue that such models will not in fact lead to a satisfactory explanation of the competitive advantage enjoyed by cyanobacteria whose clocks resonate with the LD cycle. Additional support for our reasoning may be obtained by performing experiments which we describe and whose outcomes differ according to whether competition is governed by mutual inhibition or by resource depletion.

2. Competition based on Secretion of a Growth Inhibitor

In this section, we lay out some general features of our models, describe a particular model based on interaction of the cells via a diffusible inhibitor of growth, and show that this model replicates most of the properties of the experimental system.

For experiments in LD, since the cells are entrained to the imposed rhythm (Ouyang *et al.*, 1998), time is most conveniently measured in LD cycles. Entrainment implies that most phenomena described herein will be periodic with a period of equal length to that of the LD cycle. We will therefore have occasion to refer to particular phases of the LD cycle by the fraction of the cycle elapsed since the beginning of the latest light phase. We will, for instance, denote the time (or phase) of a recurring event occurring after one-fifth of an LD cycle by 0.2LD. Experiments indicate that different strains exhibit different phase relationships between internal circadian oscillations and the external LD cycle (Fig. 4 of Ouyang *et al.*, 1998). The entrainment allows us to describe the phase relationship between the LD and sL/sD cycles of a strain by a phase angle θ (Fig. 1) which is most conveniently represented in the interval $(-0.5, 0.5]$. A positive phase angle indicates a phase advance of the sL/sD cycle with respect to the LD cycle. For instance, a strain with a phase angle of 0.2 has a subjective day that starts at 0.8LD. Conversely, a negative phase angle represents phase retardation. We shall see later that the phase angle can be related to observables under some mild assumptions on the behavior of variables controlled by the circadian clock. We have also undertaken a study of a model based explicitly on a circadian oscillator in which the relationship between the phase angle and LD period is not regarded as a parameter but is itself determined by the model. The results of the latter study will be published elsewhere (Gonze *et al.*, 2000).

We will now describe a model in which cells interact via a diffusible inhibitor. The idea underlying such models is that certain metabolic pathways are incompatible with each other and need to be regulated in such a way as not to be simultaneously active. There are many examples of such

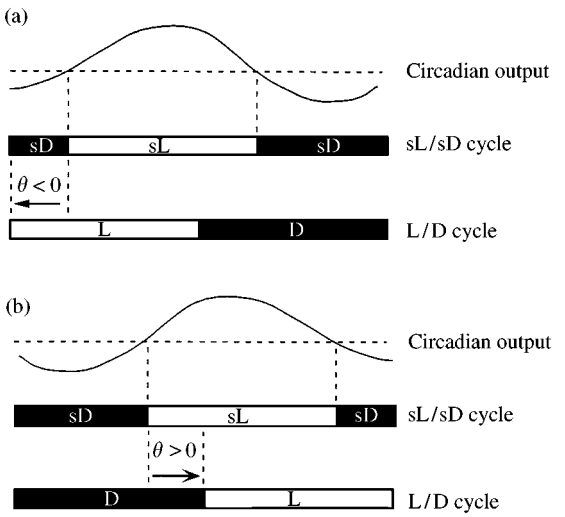

FIG. 1. Definition of the phase angle θ: for cells entrained to an LD cycle, θ is the phase difference between the beginning of subjective day (sL) and the initiation of the illumination as a fraction of the length of the LD cycle, mapped into the interval $(-0.5, 0.5]$. The phase angle may be negative or positive depending on whether sL is delayed [panel (a)] or advanced [panel (b)] with respect to the beginning of L. The metabolic state (subjective day, sL, or subjective night, sD) is itself associated with high or low activity of metabolic pathways controlled by the circadian clock. For instance, elements of the photosynthetic system in cyanobacteria are induced according to a circadian program so the circadian output variable shown could be the maximal photosynthetic activity.

metabolic incompatibilities in photosynthetic organisms such as the cyanobacteria considered here. For instance, it is normally necessary to avoid having photosynthesis and glycolysis occurring simultaneously since this can lead to futile cycling (Lehninger *et al.*, 1993). Nitrogen fixation is also incompatible with photosynthesis since nitrogenase is oxygen-labile. In fact, the photosynthetic and nitrogen fixation systems are under control of the circadian oscillator in non-heterocystous cyanobacteria such as *Synechococcus* to guarantee their temporal separation (Mitsui *et al.*, 1986). It is not inconceivable that some of these effects are mediated by the synthesis of inhibitors according to a circadian program. Cells synthesizing an inhibitor to shut down a particular pathway are using alternative metabolic reactions to support growth so that their growth should be unimpeded by the presence of the inhibitor. On the other hand, if the inhibitor is secreted into the medium, the growth of cells in a different metabolic state might be

halted. Alternatively, a cell might secrete a metabolic poison for its antibiotic effect, production being interrupted when the cell's own metabolism is vulnerable to the inhibitor. Again, in such a scenario, cells of the same species can have a deleterious effect on each other's growth if their metabolic states are not synchronized.

We have studied a number of variations of inhibitor-mediated interaction models, many of which have the required behavior. The central difficulty in building these models is to obtain a growth law which produces identical monoculture growth rates but still allow strains whose circadian clocks are nearly resonant with the imposed LD rhythm to have a selective advantage in direct competition. The simplest model we have found with these properties is illustrated in Fig. 2. A metabolic inhibitor is produced during subjective day. It is degraded throughout the day. We do not include a detailed cell division model. Rather, we assume that the number of cells of strain i (N_i) is simply proportional to the biomass and that growth follows a logistic law. The logistic law was chosen to reproduce the existence of an upper density limit which can clearly be seen in the data of Ouyang *et al.* (1998). *Synechococcus* cells can only grow in the light (Johnson *et al.*, 1998). During the coincidence of L and sL, the cells are insensitive to the inhibitor and growth is unconditional. However, in the light during subjective night, cells can grow only if the inhibitor concentration I is below a critical threshold I_c.

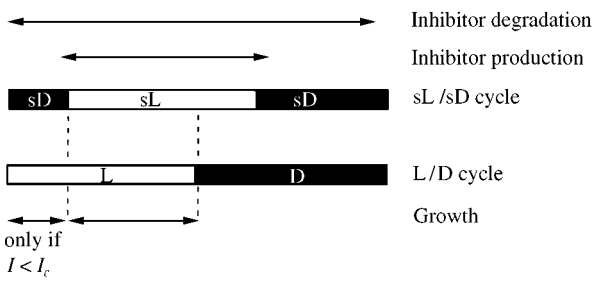

FIG. 2. Sketch of the circadian program in a minimal model for cyanobacterial growth based on rhythmic production of a metabolic inhibitor with time-dependent sensitivity. The inhibitor is produced during subjective day (sL) but degraded constantly. Growth requires light. During subjective day, growth can occur as long as light is available. However, during subjective night (sD), the cells are vulnerable to the inhibitor and can only grow and reproduce if the inhibitor concentration is below a critical value I_c.

Conditional growth in sD both allows pure single-strain cultures to grow at similar rates, regardless of the value of the phase angle, and introduces a difference in the dynamics which will eventually be seen to result in differences in fitness in direct competition.

The evolution equations for n strains coupled through the inhibitor concentration (I) are

$$\frac{dN_i}{dt} = k_i(t)N_i\left(1 - \sum_{j=1}^{n} N_j\right) - \gamma N_i, \quad (1a)$$

$$\frac{dI}{dt} = \sum_{i=1}^{n} N_i\left(p_i(t) - \frac{V_{max}I}{K_M + I}\right) - \gamma I, \quad (1b)$$

where γ is the dilution rate in continuous culture ($\gamma = 0$ in batch culture), and

$$k_i(t) = \begin{cases} k & \text{in } L \text{ and strain } i \text{ is in sL or } I < I_c, \\ 0 & \text{otherwise;} \end{cases} \quad (1c)$$

$$p_i(t) = \begin{cases} p & \text{when strain } i \text{ is in sL,} \\ 0 & \text{otherwise.} \end{cases} \quad (1d)$$

In the following simulations, as in the experiments of Ouyang *et al.* (1998), we shall consider only the cases $n = 1$ and 2 but our treatment holds for any number of strains. We have written the logistic growth law in eqn (1a) in symmetric form with respect to each of the strains since we assume that cells of different strains are nearly identical. We have also normalized the cell numbers so that the limiting cell density or concentration is 1. Equations (1c) and (1d) express the circadian dependence of growth and inhibitor production in the model. The parameter k is the maximum (low-density) specific growth rate and p is the specific inhibitor production rate during subjective day. We choose a standard Michaelis–Menten form for the degradation term on the basis of biochemical realism. However, neither the form of the production term nor the form of the degradation term is crucial, essentially identical results being obtained in appropriate parameter ranges with other functional forms

(production inhibited by I, degradation linear in I, etc.). Also note that, in the discussion to follow, when discussing the growth characteristics of pure cultures, we have omitted subscripts which serve to distinguish strains in mixed culture. Finally, the differential equations of this and other models for cyanobacterial competition are extremely stiff and somewhat ill-behaved for some sets of parameters. Our integration strategy has therefore been to prefer relatively straightforward constant-step-size integration methods such as Runge–Kutta methods and also to verify all results reported here with at least one other independently written integrator.

As seen experimentally, the growth curves of individually batch-cultured strains of our model cyanobacteria can be made essentially identical both in LD cycles and in LL by a judicious choice of parameters of our model (Fig. 3). There is just one property of real cyanobacterial cultures which we cannot at this time adequately mimic: our model does not quantitatively reproduce the circadian gating of cell division observed in *Synechococcus* grown in LL (Mori *et al.*, 1996). There is a brief pause at the transition from subjective day to subjective night, but the inhibitor is cleared from the system so fast that this is not detectable on the scale of Fig. 3(a). We attempted to lengthen the duration of the reproductive pause in LL by slowing removal of the inhibitor or decreasing the critical inhibitor concentration. However, this resulted in significantly different growth curves for the different model strains. Since our model does display a reproductive pause, it would no doubt be possible to reproduce circadian gating with a more elaborate metabolic model drawn up along the lines of this one.

Coexistence is an automatic property of this model for mixed cultures grown in continuous

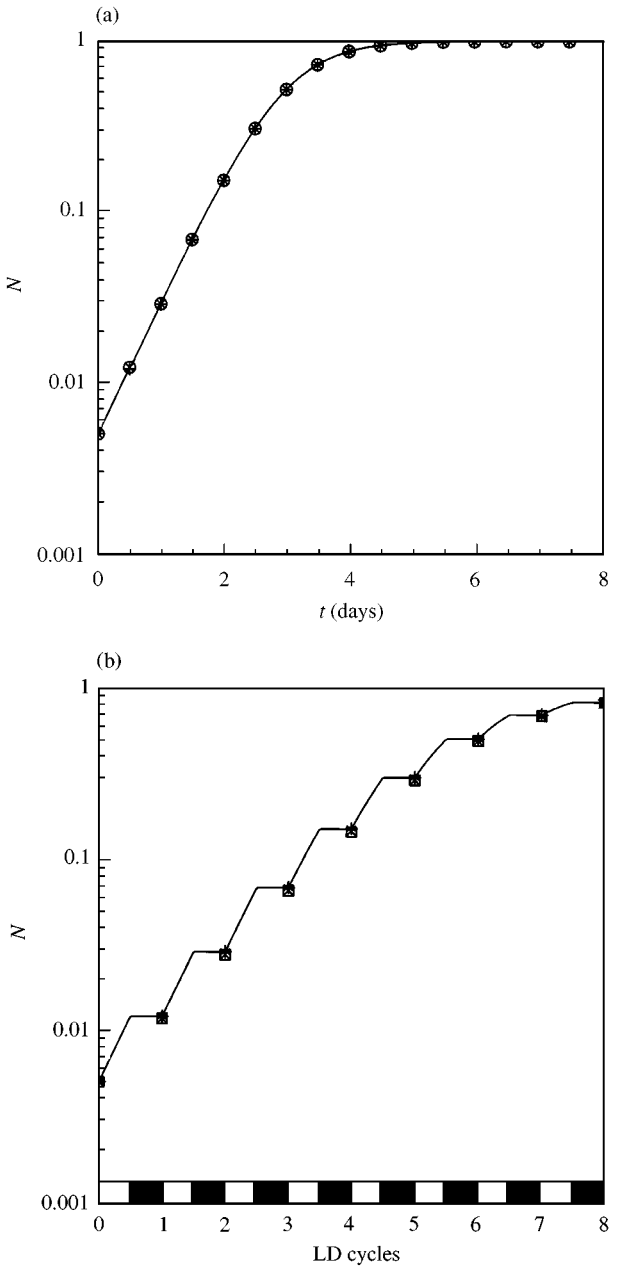

FIG. 3. Growth curves for batch culture of single strains of model cyanobacteria with dynamics given by eqns (1) and parameters $k = 1.8$, $I_c = 0.01$, $p = 500$, $V_{max} = 1000$ and $K_M = 0.05$, (a) in LL [(+) FRP = 0.9; (—) FRP = 1; (○) FRP = 1.1; (×) FRP = 1.2] and (b) in LD [(+) $\theta = -0.4$; (∗) $\theta = -0.2$; (—) $\theta = 0$; (×) $\theta = 0.2$; (□) $\theta = 0.4$]. These parameters were chosen to fit the LL growth curves and to produce LD results compatible with those of Ouyang *et al.* (1998). The units (except for the time-scales) are arbitrary. We show a detailed growth curve for one strain in each case and sample the other growth curves more sparsely to facilitate comparisons of these simulation results to the experimental results shown in Fig. 1 of Ouyang *et al.* (1998). Simulations in LL correspond to a cell culture which has been synchronized by LD cycles prior to passage in LL (i.e. all cells are initially in phase). The results of simulations with a distribution of circadian phases (i.e. a distribution of values of θ) are indistinguishable from those of panel (a) on the scale of this figure. In LL, there is no externally imposed time-scale so we label the time axis in laboratory days. On the other hand, LD cycles provide a natural time-scale both for measuring elapsed time and for measuring the strain phase angles θ. Note that the agreement between the growth curves obtained at different FRPs in LL or at different phase angles in LD is excellent.

light (data not shown), in accord with the behavior of experimental mixed cultures (Ouyang *et al.*, 1998). In LL, unless the cells are presynchronized, the cells will be distributed more or less uniformly over the circadian cycle. Accordingly, about half the cells of a strain will be in subjective day, and the other half in subjective night at any given time. Thus, the rate of production of the inhibitor will be about half-maximal for that number of cells and the steady-state concentration of inhibitor will be roughly steady at half the highest level reached in synchronized monocultures. There are two possibilities: Either this level is above the threshold for growth during subjective night, or it is not. If the inhibitor level is above I_c only cells in sL will grow, but half the cells of each strain being in sL at any given time, neither strain gains an advantage and the relative population levels are maintained. If the inhibitor level is below I_c, none of the cells are prevented from reproducing so that the specific reproduction rates ($\dot{N}_i/N_i$) for both strains are again equal and both populations are maintained. Note that coexistence in LL of unsynchronized strains does not depend on the initial numbers of cells of each strain.

In LD, competition experiments have been carried out both in a fed-batch system (batch growth for a number of days with dilution at fixed intervals with fresh medium) and in continuous (chemostat) culture (Ouyang *et al.*, 1998). We show the results of simulated fed-batch competition experiments in Figs 4 and 5. In these simulations, the cell and inhibitor concentrations are reduced by a predetermined factor at fixed time intervals. Recall that strains grown in LD are entrained to the imposed rhythm and that each strain can be characterized by a phase angle θ_i under these conditions. Each curve in these figures corresponds to a different experiment between two strains of different phase angles. The results for competition in the chemostat are qualitatively identical. In direct competition in LD, exclusion of one strain normally occurs in our model (Fig. 4). For approximately balanced initial conditions [i.e. $N_1(0) \approx N_2(0)$], the favored strain is generally the one with the smallest phase

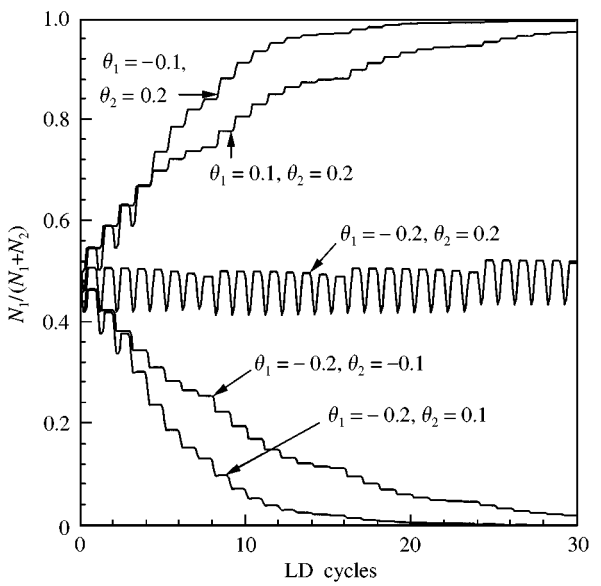

FIG. 4. Competition between pairs of strains of model cyanobacteria in a fed-batch reactor with dynamics given by eqns (1) for the parameters of Fig. 3, with dilution by a factor of 100 every eight LD cycles. The initial conditions for each simulation are $N_1(0) = N_2(0) = 0.0025$, $I(0) = 0$. Competitive exclusion is the rule, the size of the phase being the main factor determining which strain will survive. When the phase angles are of approximately equal magnitudes and opposite signs however, exclusion is slow and may be mistaken for coexistence.

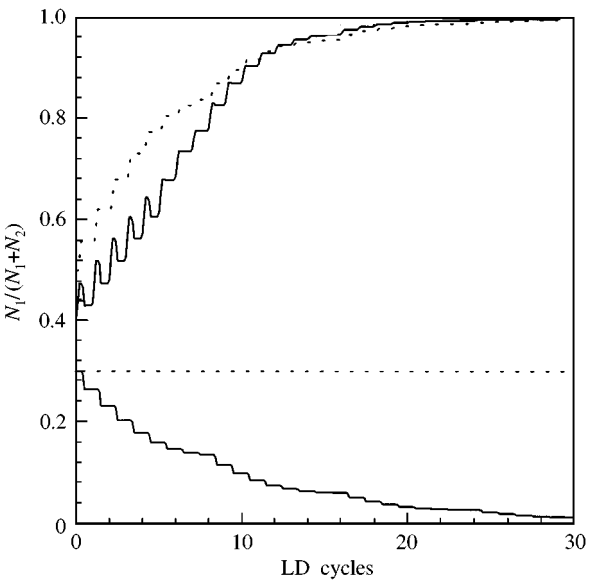

FIG. 5. Competition between pairs of strains of model cyanobacteria in a fed-batch reactor for the same parameters and operating conditions as Fig. 4, varying the initial conditions while keeping $N_1(0) + N_2(0) = 0.005$. The behavior as we vary the initial ratio depends on the signs of the phase angles. If the phase angles of the two strains are of opposite signs, there exists a threshold below which the strain with the smaller phase angle is eliminated. If the phase angles are both positive, the strain with the larger phase angle is always eliminated (not shown). (———) $\theta_1 = 0.1$, $\theta_2 = -0.2$; (- - - - -) $\theta_1 = -0.15$, $\theta_2 = -0.3$.

angle (in absolute value), i.e. the cell line whose circadian rhythm is most nearly synchronous with the driving LD cycle. Small phase angles should be associated with near-resonance of the circadian oscillator with the period of the LD cycle. When the phase angles are of approximately equal magnitude, one or the other strain can win out, depending on the initial conditions, dilution schedule, and so on. However, in these cases, the time to exclusion is always much longer that in a case where the phase angles are substantially different.

To understand why the strain of smallest absolute phase angle generally excludes its competitor in this model, consider the diagrams given in panels (a) of Figs 6 and 7. These show the growth phases of a pair of competing strains along with the subjective circadian cycles and a simplified representation of the inhibitor dynamics as a function of time for a few representative cycles. Figure 6(a) compares competing strains whose phase angles are $\theta_1 = 0.1$ and $\theta_2 = -0.2$. The first strain is already in subjective day at the beginning of illumination and remains in subjective day for most of the L phase. It continues to grow until it enters sD phase, at which point inhibitor production by strain 2 halts its growth. The second strain's growth is inhibited at the start of illumination because it is in sD and strain 1 is producing the inhibitor. It begins to grow immediately on entering sL phase and continues to grow until the culture enters darkness. With phase angles of opposite sign and populations of similar sizes, each strain grows during the coincidence of L and sL. The strain of smallest phase has the longest overlap of L and sL so strain 1 in Fig. 6(a) outgrows its competitor.

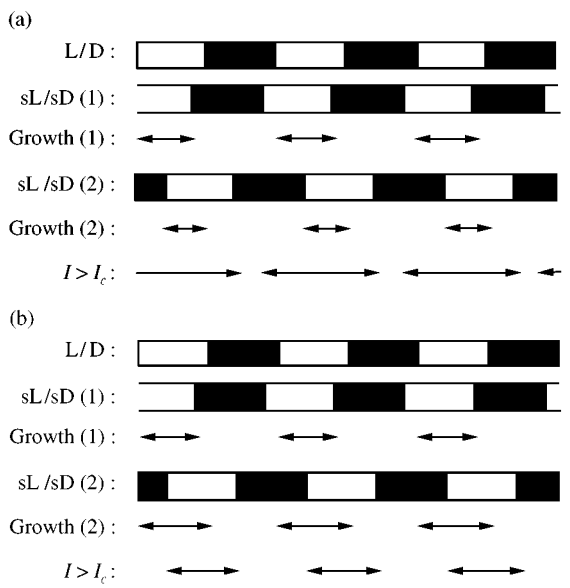

FIG. 6. Dynamical diagram for the competition experiments illustrated as solid curves in Fig. 5; $\theta_1 = 0.1$, $\theta_2 = -0.2$. Panel (a) $N_1(0) = N_2(0) = 0.0025$; (b) $N_1(0) = 0.0015$, $N_2(0) = 0.0035$. Each panel shows, from top to bottom, the illumination, the subjective day/night cycle of strain 1 and its growth periods, the corresponding variables for strain 2, and the periods during which the inhibitor's concentration is above threshold. When the signs of the phase angles are opposite and the strain with the larger phase angle is not too numerous [panel (a)], the strain with a smaller phase angle (1) experiences a longer growth period, which explains why it normally excludes its competitor. Sufficient numerical superiority however allows the strain with the larger phase angle (2) to eliminate the inhibitor during its vulnerable phase and thus to extend its growth period to the full L phase [panel (b)]. This allows a strain with a large phase angle to eliminate a strain with a smaller phase angle.

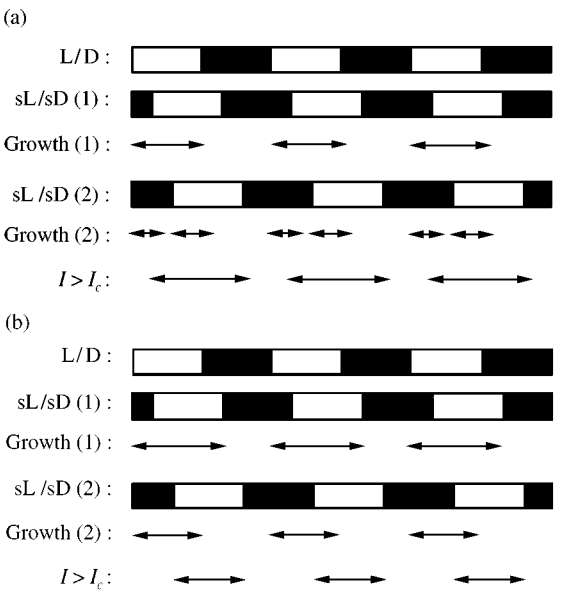

FIG. 7. Dynamical diagram for the competition experiments illustrated as dotted curves in Fig. 5; $\theta_1 = -0.15$, $\theta_2 = -0.3$. Panel (a) $N_1(0) = N_2(0) = 0.0025$; (b) $N_1(0) = 0.0015$, $N_1(0) = 0.0035$. The notation follows the conventions established in Fig. 6. When strains whose phase angles are both negative compete, the strain with the smallest phase angle (1) grows throughout the L phase. If the initial populations are approximately even [panel (a)], growth of a strain with a large phase angle (2) is interrupted for a time, which causes its overall growth to be slower. If the large phase-angle strain has a population sufficiently larger than its competitor's [panel(b)], it can degrade the inhibitor sufficiently quickly to avoid interruption of its growth and both strains grow throughout L phase.

We can analyse the case of phase angles of the same sign analogously. In Fig. 7(a), we consider the case in which $\theta_1 = -0.15$ and $\theta_2 = -0.3$. Each strain grows in coincidence of L and sL, but both strains also grow when they are simultaneously in sD phase because neither is then producing the inhibitor and the latter's concentration then falls to a very low level. The strain with the smallest absolute phase angle (strain 1) can then grow throughout the period of illumination whereas the other strain exhibits a pause in growth and is thus eventually eliminated from culture by dilution.

Figure 5 shows the effect of unbalanced initial conditions in this model. Different effects are observed depending on the signs of the phase angles. If the phase angles of the competing strains are of opposite signs (Fig. 5, solid curves), there is a threshold in the initial cell number ratio beyond which the numerically superior cell line excludes its competitor, even if the former has a larger absolute phase angle. The dynamical diagram for this case is shown in Fig. 6(b). Comparison of panels (a) and (b) of this figure show that the growth period of the strain with the smaller phase angle is essentially unaffected. However, the strain with the larger phase angle now grows throughout the L phase because it is able to keep the concentration of the inhibitor below threshold during its sD phase due to its numerical superiority. Accordingly, it outgrows and eventually excludes the strain with the smaller phase angle.

If the phase angles are both negative, for sufficiently unbalanced initial conditions in favor of the strain with the larger phase angle, true coexistence is observed (Fig. 5). Compare the dynamical diagrams, panels (a) and (b) of Fig. 7. Again, strain 2 can, given sufficient numerical superiority and despite its larger phase angle, degrade the inhibitor sufficiently quickly to avoid having its growth interrupted. In this case however, this only makes the growth periods equal in length so that the initial ratio is maintained.

Finally, if the phase angles are both positive, the strain with the smallest phase angle always drives the other to extinction, regardless of the initial cell numbers. The dynamical diagram in Fig. 8 shows why. Even with strongly unbalanced initial populations, the strain with the larger phase angle always pauses briefly as it switches from daytime to nighttime metabolism. Exclusion is accordingly a very slow process in this case.

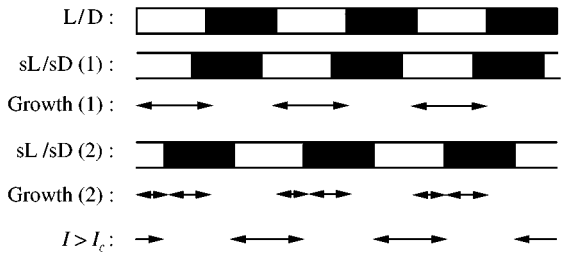

FIG. 8. Dynamical diagram for a competition experiment between strains with $\theta_1 = 0.1, \theta_2 = 0.3$ and initial conditions $N_1(0) = 0.001$, $N_2(0) = 0.004$. See Fig. 6 for notation. Regardless of the imbalance in initial conditions, the strain with the smallest phase angle (1) always eliminates its competitor (2) if the phase angles are both positive. Exclusion occurs because the growth of the strain with a larger phase angle is necessarily interrupted at this strain's sL/sD transition when it becomes vulnerable to the inhibitor. When there is a large imbalance in the initial populations, the pause in the growth of the strain with the larger phase angle can be extremely short (as shown here) so that exclusion occurs very slowly and may be mistaken for coexistence.

The model behaviour is not particularly sensitive to the parameters although dramatic changes in any one parameter can destroy some of the properties described above. The inhibitor clearance kinetics is particularly important. As a case in point, we mention the effect of the critical inhibitor concentration I_c on the dynamics. The coincidence of the LD single-strain growth curves shown in Fig. 3(b) is not exact. Strains with positive phase angles actually grow slightly more slowly than strains exhibiting a negative or zero θ because the former must pause during L as their metabolism switches from sL to sD to clear the inhibitor from solution. (Note that this is the effect responsible for the qualitatively correct but quantitatively incorrect circadian gating in LL in our model.) It takes longer to reduce I below I_c the smaller the inhibition threshold is. This effect thus becomes significant when I_c is very small. This parameter should therefore not be too small if we are to correctly reproduce the growth behavior of single strains in LD. In competition experiments, I_c mainly affects the value of the critical initial fraction above which the strain

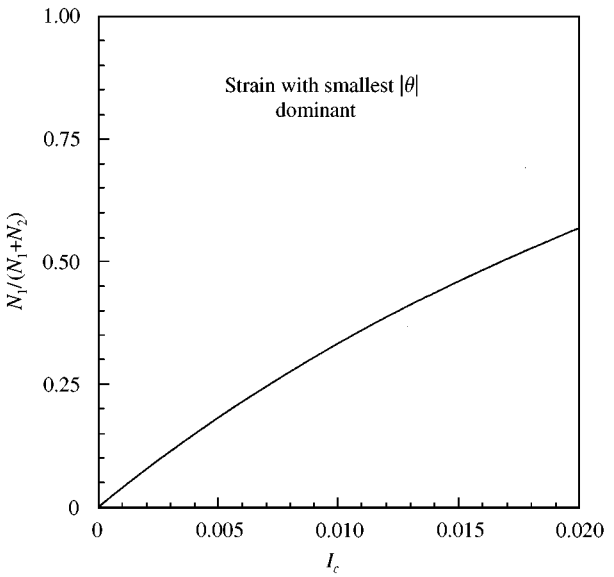

FIG. 9. Graph of eqn (A.3) which delimits the region in which the strain with the smallest absolute phase angle unconditionally eliminates its competitor for $p = 500$, $V_{max} = 1000$ and $K_M = 0.05$. If the phase angles are of opposite sign, the competitive exclusion relationship is reversed below the curve. If both phase angles are negative, coexistence is observed for initial conditions below the curve. If both phase angle are positive, the strain with the smaller value of θ always eliminates its competitor, although the second strain persists much longer for initial conditions below the curve than above.

with the smaller phase angle eliminates its competitor if at least one of the strains has a negative θ. The details are given in Appendix A. The curve giving the critical fraction as a function of I_c is shown in Fig. 9. At small values of I_c, the strain with the smallest $|\theta|$ excludes its competitor over a wider range of initial conditions than at higher values of this parameter. While Ouyang *et al.* (1998) did not directly explore the effect of varying the initial conditions, the robustness of their results suggests that a 1 : 1 initial mixture is reasonably far from the separatix (A.3). This would favor a relatively low value of I_c, assuming that a growth inhibitor is implicated in the competitive dynamics. Values as much as two or three orders of magnitude lower than the one we chose for our simulations can still produce reasonable results for the single-strain growth curves while maximizing the range of initial conditions over which strains with large phase angles are excluded.

As noted earlier in this section, we have studied a number of variations to the basic model. We have, for instance, tried different synthesis and degradation laws. We have also examined models in which inhibitor synthesis requires light and others in which inhibitor degradation is limited to subjective night. The qualitative behavior of these models is similar to that of the basic model.

3. Competition for a limiting substrate

Much of the metabolism of *Synechococcus* appears to be under circadian control (Mitsui *et al.*, 1986; Chow & Tabita, 1994; Liu *et al.*, 1995). Thus, we can imagine that such processes as substrate uptake and utilization are at least partly under circadian control. We therefore attempt to construct a model of the differential fitness of cyanobacterial strains based on circadian modulation of substrate utilization. It now seems to us that this is not possible, for reasons which we explain in this section.

Consider first a Monod-type cell growth model in which the state of the system is described by n biomass (or cell number) variables N_i and one (limiting) substrate concentration S (Monod, 1942):

$$\frac{dN_i}{dt} = N_i f_i(S, t) - \gamma N_i, \tag{2a}$$

$$\frac{dS}{dt} = -\mu \sum_{i=1}^{n} N_i f_i(S, t) + \gamma(S_0 - S), \tag{2b}$$

where μ is the amount of substrate required to produce one unit of biomass, S_0 is the concentration of substrate in the feed solution, and $f_i(S, t)$ is the function which expresses the dependence of growth on the substrate concentration for a given strain. In such a model, substrate uptake and growth are very strongly coupled so that there is only one point in the model where a circadian program can intervene, namely in the rate of growth (the function f_i), which is directly proportional to the substrate uptake rate. Recall that in *Synechococcus*, growth only occurs in the light (Mori *et al.*, 1996; Johnson *et al.*, 1998) and that strains can be selected which display identical monoculture growth curves in LD cycles (Ouyang *et al.*, 1998). Only trivial circadian programs in which uptake does not depend on the

sL/sD cycles have both of these properties for Monod models. In the absence of other effects (such as the inhibitor secretion postulated in the previous section), trivial circadian programs cannot lead to differences in fitness among strains in mixed cultures. Programs in which, for instance, there is a decreased efficiency of uptake during subjective night always lead to differences in growth rates among strains because they lead to differences in efficiency of exploitation of the substrate during the exponential phase. The direct coupling between uptake and growth causes an automatic translation of differences in uptake kinetics into differences in growth rates. The introduction of delay into the growth law (Cooney & McDonald, 1995) does not alter this property of Monod models.

Adding variables only helps if uptake and growth can be temporally dissociated. We might, for instance, consider a model in which we keep track of the internal concentration of the limiting substrate. Consider, for instance, the following equations which slightly generalize a form studied by Oyarzun & Lange (1994):

$$\frac{\mathrm{d}N_i}{\mathrm{d}t} = N_i g_i(Q_i, t) - \gamma N_i, \tag{3a}$$

$$\frac{\mathrm{d}Q_i}{\mathrm{d}t} = f_i(S, t) - g_i(Q_i, t)(Q_i + \mu), \tag{3b}$$

$$\frac{\mathrm{d}S}{\mathrm{d}t} = -\sum_{i=1}^{n} N_i f_i(S, t) + \gamma(S_0 - S). \tag{3c}$$

In these equations, Q_i is the amount of substrate stored per unit biomass (the "cell quota"). Equation (3b) includes terms both for substrate utilization ($-\mu g_i(Q_i, t)$) and for dilution by growth ($-Q_i g_i(Q_i, t)$). For batch cultures, $\gamma = 0$. For simulations of fed-batch processes, dilutions by fresh medium affect the cell numbers and the substrate concentration, but not the cell quotas (Q_i) since the substrate is assumed to be irreversibly transported into cells.

If there is uptake in the light (the growth period), we can obtain models based on eqns (3) which display identical growth curves for different strains in monoculture, or models which show differential fitness between strains in competition, but not models which have both properties. The reason is essentially the same as for the Monod models: the coupling between uptake and growth is too direct. If both processes occur simultaneously, an increase in the rate of uptake is directly reflected in an increase in the rate of growth.

We tried to weaken the coupling of uptake to growth in a model with uptake during L by using a growth function of the Hill type with a high Hill coefficient and a low cell quota threshold for growth. Our reasoning was that, if the dependence of the rate of growth on the internal substrate concentration was weak across most of the range (i.e. if $\partial g_i/\partial Q_i$ is small everywhere except in a narrow range at low Q_i), the timing of uptake relative to growth might become less important and it might be possible to obtain similar individual growth curves for our simulated cyanobacterial strains while still allowing uptake in the light. However, this proved unsuccessful.

One might consider trying to dissociate growth from uptake by restricting growth to the coincidence of L and sL and having uptake occur in sD. Leaving aside the fact that the experimental data on *Synechococcus* growth kinetics in LL does not support these hypotheses (Mori *et al.*, 1996), this shortens the growth phase for strains with larger absolute phase angles which, in LD, leads to significant differences in monoculture growth rates.

Since *Synechococcus* cells grow in the light, only models in which uptake occurs in the dark (i.e. is perfectly photoinhibited) can reproduce both monoculture and competition results in LD. Figure 10 illustrates such a model based on eqns (3). We used growth and uptake functions of classical hyperbolic form:

$$f_i(S, t) = \begin{cases} \dfrac{f_{\max} S}{S + K_f} & \text{in } D \text{ when strain } i \text{ is in sD,} \\ 0 & \text{otherwise;} \end{cases} \tag{4a}$$

$$g_i(Q_i, t) = \begin{cases} \dfrac{g_{\max} Q_i}{Q_i + K_g} & \text{in } L, \\ 0 & \text{in D.} \end{cases} \tag{4b}$$

As seen in Fig. 11, this model accounts for the known LD behavior of both single-strain and mixed *Synechococcus* cultures rather well. The

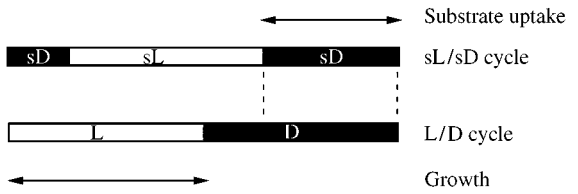

FIG. 10. Sketch of the circadian program in a model for cyanobacterial growth based on competition for a limiting substrate. Growth occurs in the light and is dependent on the intracellular concentration of the limiting substrate. Uptake occurs during the coincidence of darkness and subjective night. This appears to be the simplest uptake and growth schedule which allows reproduction of the experimental results of Ouyang *et al.* (1998) in LD by guaranteeing disjunction of uptake and growth while introducing a non-trivial kinetic difference between the strains. However, because it requires a period a darkness, it has no obvious extension to growth in LL.

rule governing the exclusion relationships is quite different in this model than in our model based on growth inhibition: in the model defined by eqns (3) and (4), the first strain to start transporting the substrate once it becomes available excludes the other. If they both start at the same time (i.e. both strains have positive phase angles so that uptake starts for both at the onset of darkness), there may be coexistence [as shown in Fig. 11(b)] or not, depending on the uptake kinetics. The underlying logic of this exclusion relationship implies a sensitivity to the time of dilution [Fig. 11(c)] not seen in the simple model based on inhibition studied in Section 2. In models of exclusion based on circadian-modulated substrate uptake, it is possible to choose the time of dilution (i.e. of substrate resupply) in such a way that one or the other strain has exclusive access to the substrate for a time. The effect is most dramatic for strains with phase angles of opposite signs. Consider Fig. 11(c): for most dilution schedules, strain 1 (which has a positive phase angle, $\theta_1 = 0.3$) excludes strain 2 ($\theta_2 = -0.1$). However, if dilution occurs between 0.7 and (roughly) 0.9LD, strain 2 excludes strain 1 because the former has exclusive access to the substrate from 0.7LD to the end of the dark phase. If dilution occurs too late in the LD cycle, strain 2 may not deplete the substrate sufficiently to deny its competitor access to this resource. Marked oscillations in the cell number ratio may be observed, as seen in Fig. 11(c). The strain with the positive phase angle regains its dominance if dilutions occur even later in the cycle. By way of contrast, in models of exclusion by mutual inhibition, while it may be possible to temporarily favor a strain by reducing the inhibitor concentration upon dilution during the strain's vulnerable phase, this advantage is short-lived: when the two strains are next simultaneously in sL and producing the inhibitor, all memory of the dilution is lost. Accordingly, the circadian phase of the dilutions has very little effect on the long-term fate of the culture.

In the substrate competition model which is the main focus of this section, coexistence can be observed under a variety of circumstances. As noted above, if the phase angles are both positive, the two strains will coexist unless their phase angles are significantly different in size and the dilution phase is chosen from a rather narrow range. If the two strains have negative phase angles, the strain whose phase angle is nearest to zero generally excludes the other, but the two may coexist if the circadian phase of the dilutions falls just before or during the uptake period of the second strain.

The uptake dynamics also implies that the exclusion relationships for the substrate-competition model in the chemostat are somewhat different than in fed-batch. For instance, strains with phase angles of opposite signs can coexist in a chemostat (but not in fed batch) because they each have exclusive access to the substrate for a period of time and are thus guaranteed a period during which they can build up their internal reserves without direct competition.

Despite its ability to reproduce the behavior of cyanobacterial cultures in LD, the model described above cannot account for the experimental results in LL because it has no clear extension to these conditions, there being no uptake at all in the light. Using this model as a starting point, we attempted to create a model which could also work in LL by having only partial photoinhibition of uptake by light. Accordingly, we replaced the uptake function (4a) by

$$f_i(S, t) = \begin{cases} \dfrac{f_{max}(1 - \varphi L(t))S}{S + K_f} & \text{when strain } i \text{ is in sD,} \\ 0 & \text{otherwise;} \end{cases} \tag{5}$$

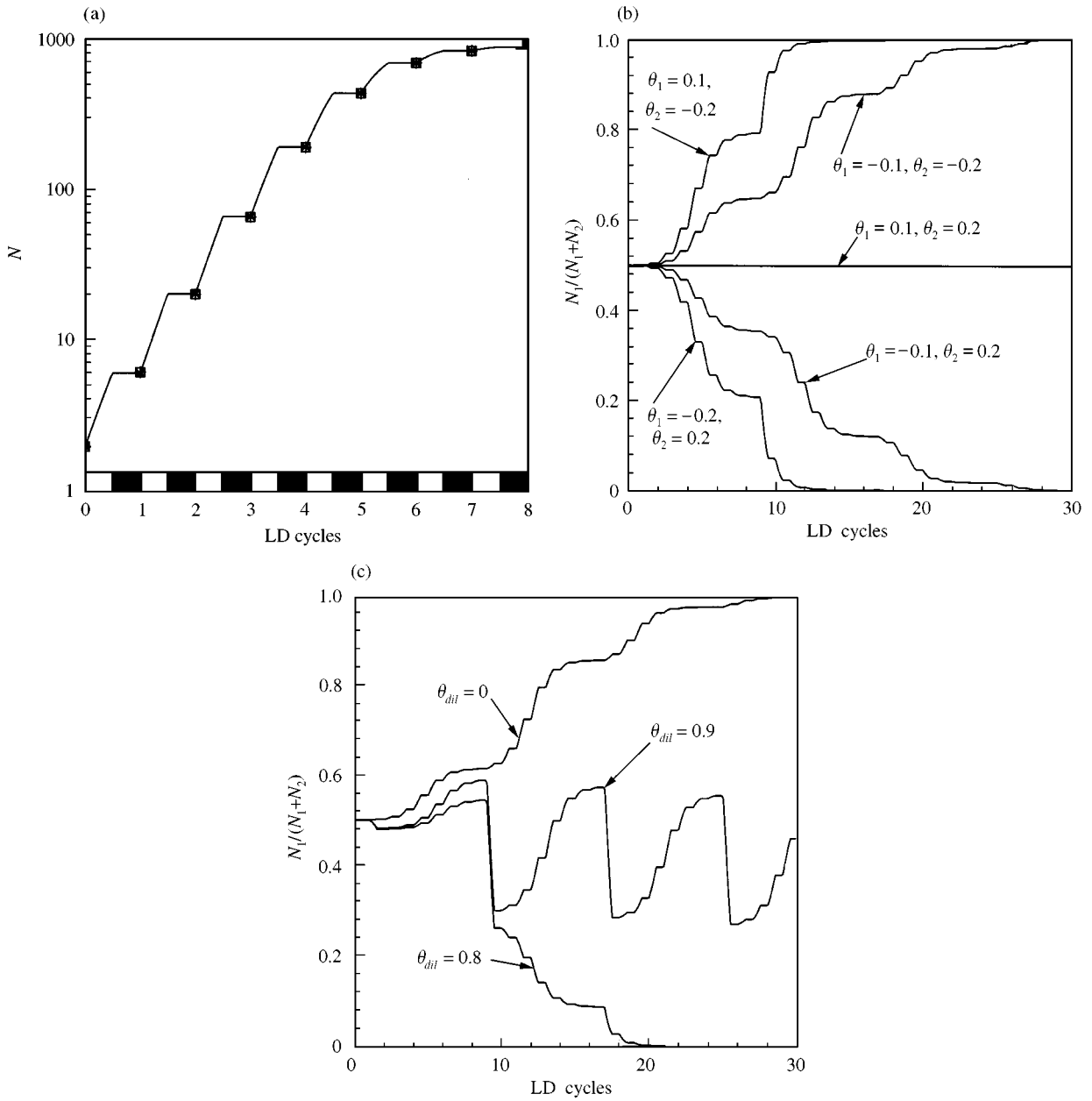

FIG. 11. (a) Monoculture and (b,c) competition simulations in LD for the model with growth on a limiting substrate defined by eqns (3) and (4) with $f_{max} = 1$, $K_f = 0.01$, $g_{max} = 2.5$, $K_g = 0.001$ and $\mu = 0.001$. The initial substrate concentration was 1 (in arbitrary units). The monoculture simulations correspond to batch growth. [(+) $\theta = -0.4$; (*) $\theta = -0.2$; (—) $\theta = 0$; (×) $\theta = 0.2$; (□) $\theta = 0.4$]. The competition simulations were performed for fed-batch culture with dilution using a solution of unit substrate concentration by a factor of 100 every eight LD cycles. The exclusion relationships depend on the time at which uptake begins, the first strain to exploit the resource after its replenishment excluding the other. For instance, if dilutions occur at a circadian phase of 0LD (panel b), strains with positive phase angles are dominant over strains with negative phase angles because the former start to take in the substrate immediately after the onset of darkness. In panel (c), we show the effect of varying the circadian phase of dilution (θ_{dil}) on the competition between two strains with opposite phase angles ($\theta_1 = 0.3$, $\theta_2 = -0.1$). Strain 1 accesses the substrate first and excludes strain 2 if dilutions occur much before the former stops taking in the substrate (at 0.7LD). This is the case when dilutions are timed to coincide with the beginning of an LD cycle (i.e. $\theta_{dil} = 0$). If the dilutions occur after strain 1 stops taking in the substrate or perhaps slightly earlier, but not too late in the cycle, strain 2 may remove enough substrate from solution to prevent strain 1 from accessing significant amounts of nutrient at the next onset of darkness. Accordingly, strain 1 is eventually starved out (e.g. $\theta_{dil} = 0.8$). If the dilutions occur very late in the cycle, strain 1 remains dominant because strain 2 does not have time to significantly reduce the amount of substrate available before photoinhibition halts uptake (data not shown). For intermediate circadian dilution phases (e.g. $\theta_{dil} = 0.9$) marked oscillations in the cell number ratio are observed. These oscillations generally lead to extinction of one or the other strain, but on a very long time-scale.

where $L(t)$ has the value 1 in the light and 0 in the dark, and $\varphi \in [0, 1]$ is the efficiency of photoinhibition. However, this model is very badly behaved: in LD competition, strains with large positive phase angles eliminate any competing strain, including a perfectly resonant strain with $\theta = 0$! This occurs because cells start to pump in the substrate as soon as they enter subjective night so that cells with a phase advance, even at high degrees of photoinhibition, substantially reduce the amount of substrate available to competing strains. Again we conclude that the LD growth curves of Ouyang *et al.* (1998) are inconsistent with a model in which differences in the circadian program which controls the utilization of a limiting substrate account for differences in fitness of the strains.

Given that we can produce models based on competition for a substrate which have the desired properties in LD, there is another possibility to consider, namely that cells have altogether different metabolism in LL than they do in LD cycles. Our inability to reproduce the LL results would then simply be due to our insisting on using a single model to cover both situations. However, we do not believe that there are significant metabolic differences in LD and in LL. When cultures are moved from LD to LL, the overall pattern of gene expression and metabolic activity is remarkably unchanged, even after a number of cycles in continuous light (see, for instance, Mitsui *et al.*, 1986; Liu *et al.*, 1995; Golden *et al.*, 1998). In particular, it appears that circadian patterns are relatively undisturbed on moving to continuous light. While there is no doubt that some adaptations are necessary for efficient growth in continuous light, these do not seem to involve the major changes that would justify an altogether different metabolic model in LL than is used in LD.

We mention one final possibility: if the substrate S is pumped into the cells and then converted into biomass in two steps rather than one, it might just be possible to reproduce most of the experimental results if each step of the conversion occurs in roughly alternating phases of the circadian cycle (e.g. pumping in sL, conversion of the internal substrate to a usable form in sD, utilization for growth in L). However, there is no obvious experimental evidence in support of a model of this form. Starved cells placed in fresh medium would experience growth delays of about one day if such a model accurately represented the kinetics of cell reproduction. We know of no evidence of delays of this magnitude in the literature on microorganisms culture. Furthermore, such delays would almost certainly be harmful to an organism's ability to compete for resources. We therefore reject this possibility as being too speculative.

To summarize: if the monoculture and competition experiments of Ouyang *et al.* (1998) in both LL and LD are to be explained by a model in which circadian modulation of uptake is the central feature, it is necessary for the growth rate to be independent of the uptake rate. Simultaneous uptake and growth create such a dependency. Models which achieve the required independence of the rates by temporal separation of these processes would generally do so at the expense of not being obviously applicable in continuous light. Other alternatives, such as metabolic adaptations leading to altogether different dynamics in continuous light than in LD cycles, strike us as being highly artificial.

4. Proposal for Experimental Validation

Although we believe our theoretical arguments excluding substrate competition models for the differential fitness of *Synechococcus* strains in competition to be sound, it would be preferable to eliminate this possibility by an experimental test. There is one major difference in behavior between the two classes of models which persists across all model variations we have examined: the sign of the phase angle is much more significant in models with a limiting substrate than in models with an inhibitor. This difference is due to a kind of temporal asymmetry in the kinetics of substrate depletion: once a molecule of substrate has been removed from solution by a cell, other cells neither have access to that molecule, nor is there any action they can take to gain access to it. Accordingly, a cell which starts transporting a substrate early gains an advantage over its competitors. On the other hand, an inhibitor molecule synthesized and released into the medium by one cell can be degraded by another so that the temporal relationships "early" and "late"

are much less significant in models based on mutual inhibition. This observation on the differences in the temporal symmetry of models of the two classes is relatively independent of model details.

To obtain means of experimentally discriminating between mutual inhibition and substrate depletion, it will be necessary to characterize the relationship of the phase angle to the LD period for the various strains. This is possible, provided the following two hypotheses hold:

1. The phase angle of one strain relative to another is approximately the same for any circadian output variable.
2. The phase (as a fraction of the LD cycle length) at which a maximum (or minimum, or other reproducible metabolic event) in a particular circadian output variable occurs is approximately the same for any strain in an LD cycle of duration equal to the FRP of that strain.

Both of these hypotheses are suggested by currently available data (Ouyang *et al.*, 1998) and are experimentally verifiable.

If hypothesis 1 holds, one can choose any convenient circadian output variable to obtain the relationship of the phase angle to the LD period. One performs a series of experiments in which each strain is entrained to LD cycles of various lengths. For each LD period studied, one measures the time at which a reproducible features of the dynamics (maximum or minimum of the variable, crossing of a certain threshold, etc.) occurs after the beginning of illumination. Let us call this time $\Delta t_i(T)$, where the subscript labels the strain and T is the period of the LD cycle. Measurements are made at a number of different LD cycle lengths. Hypothesis 2 then allow us to compute an experimental phase angle by

$$\vartheta_i(T) = \frac{\Delta t_i(T_i)}{T_i} - \frac{\Delta t_i(T)}{T}, \tag{6}$$

where T_i is the FRP of strain i. This definition is based on the idea that the circadian oscillator and its outputs are all entrained to the LD period T. Thus, if the start of circadian day is shifted by some fractional amount θ on moving to an LD period T, every circadian output variable, each of which is entrained to the rhythm of the underlying circadian oscillator, should shift by a similar amount. The fractional shift ϑ_i calculated using eqn (6) from any convenient circadian variable should therefore roughly equal the phase angle θ_i defined in our Fig. 1. The two definitions do differ by a small term which depends linearly on the delay between the circadian oscillator state variables and the circadian output variables. This small term vanishes in the critical region near $T = T_i$. The details are given in Appendix B.

Once the relationship between phase angle and LD period has been established for two or more strains, one performs a set of competition experiments at different LD cycle lengths T. The results of these experiments are correlated with the previously determined $\vartheta_i(T)$ relationships. If the dominant strain is always the one with the smaller $|\vartheta_i|$, the circadian resonance phenomenon is probably due to mutual inhibition [Fig. 12(a)]. The resource depletion case differs from mutual inhibition in two ways [Fig. 12(b)]: first, there should be a range of LD periods in which both phase angles are positive and coexistence is observed. Second, there will be a range of values of T in which one strain has a relatively large positive phase angle while the other has a small negative phase angle, but the former [strain 1 in Fig. 12(b)] excludes the latter.

To put it in another way, data analysis should seek to establish whether or not there exists a symmetry between positive and negative phase angles with respect to competitive fitness. If the fitness of a strain can be predicted from the magnitude of ϑ_i alone, smaller $|\vartheta_i|$ at a particular T implying greater fitness, it is highly likely that a diffusible inhibitor is involved [Figure 12(a)] and unlikely that resource competition plays a major role. On the other hand, if the sign of ϑ_i is more significant than its size for strains which, over some range of values of T, have phase angles of opposite sign, resource competition is strongly indicated [Fig. 12(b)], the behavior of models with inhibition being essentially inconsistent with this result.

It is tempting to attempt to use the different sensitivities to the circadian phase of dilution for the two classes of models considered here to

distinguish between these possibilities. However, experimental manipulations based on this principle are unlikely to exclude definitively one or the other possibility. The difficulty is that, unless the phase of the dilutions falls in a fairly narrow range and the phase angles are reasonably different, there is no (or very little) effect. Moreover, dilution schedule effects are very dependent on substrate uptake kinetics. The results of such experiments are thus likely to be difficult to interpret, although they might provide support for conclusions reached by other means.

5. Discussion and Conclusions

We have shown that a model of circadian resonance based on mutual inhibition can account for the main experimental observations of Ouyang *et al.* (1998). These observations are as follows: Strains which are genetically identical except for components of their circadian clocks can have identical growth rates in monoculture both in LL and in LD. In competition in LL, no strain has a clear advantage. In LD however, strains with an FRP which nearly matches the period of the imposed LD cycles are able to exclude their competitors. Models based on substrate depletion on the other hand are only able to explain the experimental results in LD if growth and uptake are temporally dissociated. Since growth occurs in L, uptake must be confined to D. Models which are so constructed however lack an obvious interpretation in LL. It thus appears that simple substrate-depletion models cannot account for all observations in the *Synechococcus* experiments. However, we recognize that it would be hubris to claim that we have definitively excluded all models based on substrate depletion. We have therefore proposed an experimental protocol which may be able to discriminate between the two classes of models considered here and whose results should in any event provide additional information useful for modeling.

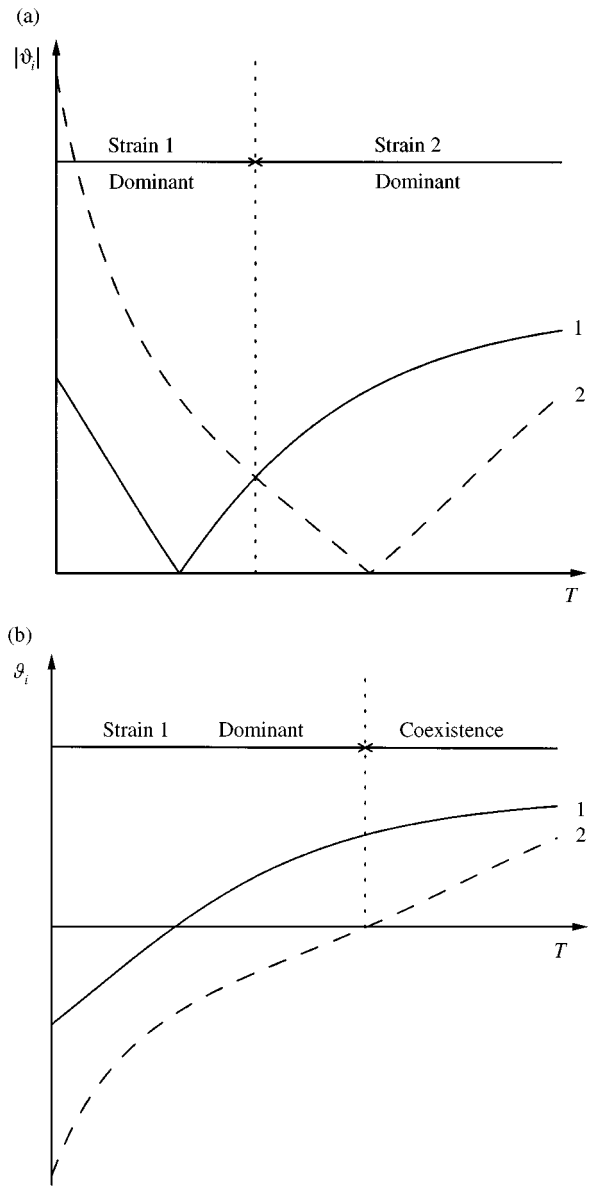

FIG. 12. Schematic representation of the exclusion relationships in the two classes of models. If circadian resonance is due to mutual inhibition, then the absolute value of the experimental phase angle ϑ_i can be used to predict which strain will be excluded. Panel (a) shows sketches of some possible phase angle to LD period (T) relationships (not computed from any model or data) for two strains. Strain 1 would exclude strain 2 to the left of the dotted line, while strain 2 would exclude strain 1 to the right. On the other hand, if substrate depletion is the mechanism for circadian resonance in *Synechococcus*, the signs of the two phase angles are significant. If dilutions are carried out at the beginning of an LD cycle and the phase angles of the two competing strains are as shown in panel (b), strain 1 excludes strain 2 to the left of the dotted line, and the two strains coexist to the right. A transition from exclusion to coexistence when both phase angles become positive [corresponding to the substrate depletion case, panel (b)] should be experimentally distinguishable from a change in relative fitness near the LD cycle length at which $|\vartheta_1| = |\vartheta_2|$ [the mutual inhibition hypothesis, panel (a)].

The modeling results presented here are reasonably robust. For our model with a diffusible inhibitor, we have already commented on the possibility of changing the forms of eqns (1) and even of considering other temporal relationships between the LD and subjective day/night cycles and the inhibitor dynamics. For instance, we get very similar results if the inhibitor synthesis and degradation alternate in time (e.g. synthesis during subjective day and degradation only during subjective night). What is important is the existence of a phase of vulnerability to the inhibitor during growth whose duration depends on the phase relationship between the LD and sL/sD cycles. The general features of the solutions for our models based on substrate depletion are also robust to changes in the kinetic laws. Both models display some parametric sensitivity so that not every feature of the growth and competition experiments can be reproduced with randomly chosen parameters, but suitable parameter sets are seasonably easy to find. This suggests that the region of parameter space in which the experimental behavior is reproduced is reasonably large.

Our results suggest that mutual inhibition is a more likely mechanism for circadian resonance than resource competition. Supposing that cyanobacteria do secrete a metabolic inhibitor according to a circadian program, what might that inhibitor be? Speculation at this stage might seem premature, but there is reason to believe that the inhibitor, if there is one, might be either a peroxide or a free radical. It is known that *Synechococcus* cells are sensitive to peroxides and to free radicals, their sensitivity varying with metabolic state (Conter 1987; Sakamoto *et al.*, 1998). Respiration and photosynthesis, both processes carried out by cyanobacteria, tend to produce peroxides and free radicals. Furthermore, these substances diffuse through the bacterial membrane, as hypothesized in our model (Tichy & Vermaas, 1999). If compounds of these classes are responsible for the competitive advantage observed in the experiments of Ouyang *et al.* (1998), it might be possible to reduce or eliminate the selective pressure for strains with nearly resonant FRPs by adding either catalase, superoxide dismutase or other peroxide and free radical detoxification enzymes to the growth medium.

Given that resonance of a strain's FRP with the imposed LD cycle enhances fitness in a competitive situation, one might consider using this phenomenon to select mutants of a given FRP (C. H. Johnson, pers. comm.). Unfortunately, as our modeling results show (Fig. 5), this will not necessarily work. The wild-type strain, which will be numerically superior to any mutant strains in a typical sample, may under some circumstances be able to eliminate a less numerous mutant population, even if the latter is better adapted to the imposed LD cycle. Under such conditions, a near-resonant mutant's population can stagnate or even decline. Because these phenomena are sensitive to the signs of the phase angles, it should in principle be possible to provide a prescription for the selection of circadian clock mutants based on circadian resonance. This will require the experimental characterization of the phase angle to LD period relationship for wild-type cells, as described in Section 4 of this work. Furthermore, protocols for enhancement of small populations are model-dependent so that experimental input on the mechanism of exclusion (mutual inhibition vs substrate depletion) will be required before such a protocol can be rationally designed.

In addition to the study of Ouyang *et al.* (1998), others have tried to show that a circadian clock which resonates with the environmental day/night rhythm can confer a selective advantage on organisms so endowed. The results are perhaps clearest in studies performed with plants (Hillman, 1956; Went, 1960). Temperature compensation of the circadian rhythm FRP is not particularly good in some higher plants so the FRP can be varied by adjusting the growth temperature, thus alleviating the need for the development of a set of clock mutants to study circadian resonance (although such mutants are now available in *Arabidopsis*; Millar *et al.*, 1995). Went (1960) has shown that growth of higher plants is optimal in LD cycles which roughly match the circadian oscillator FRP. Went's observations also suggest that the high sensitivity of plant growth to temperature is itself due to circadian resonance: Outside a relatively narrow range of temperatures, the 24 h day becomes mismatched to the plant's circadian oscillator FRP. This suggests a different kind of circadian resonance

phenomenon than observed by Ouyang *et al.* (1998) since in the latter case, differences in growth rates only appear in a competitive context. The detailed mechanisms of circadian resonance in these two cases are thus likely to be different although the effect, namely evolutionary pressure for a wild-type circadian clock with a period of approximately 24 h at typical ambient temperatures, is the same.

Insects provide the best animal models of effects associated with circadian resonance. Aschoff and Pittendrigh have independently shown that wild-type insects live longest in 24 h LD cycles (reviewed by Pittendrigh, 1993) although, oddly, *Drosophila melanogaster* lives longest in continuous darkness (Allemand *et al.*, 1973). Mutants with fast or slow circadian clocks may also live longer in LD cycles which resonate with their circadian clocks (Pittendrigh & Minis, 1972; Klarsfeld & Rouyer, 1998).

Cyanobacteria have several advantages over multicellular organisms for the experimental study of circadian rhythms: they can be cultured in tightly controlled conditions. They grow and reproduce rapidly so that experiments spanning dozens of generations can be completed in a reasonable time. Finally, being relatively simple organisms, the design and interpretation of genetic and physiological studies is more straightforward in bacteria than in multicellular organisms. Thus, despite the fact that cyanobacteria were only recently recognized as having true circadian rhythms (Mitsui *et al.*, 1986; Huang *et al.*, 1990; Kondo *et al.*, 1993; Johnson *et al.*, 1996), it should be possible to make rapid progress using these microorganisms as an experimental system in tracing the causes of circadian resonance phenomena from the molecular, through the physiological, to the population level. It is to be hoped that these efforts will in time lead to broadly applicable principles with respect to the coupling of metabolism to circadian rhythms and thus to the evolutionary pressures brought to bear on the circadian oscillator.

Proceeding from the experimental observation that cyanobacterial strains with different FRPs display different phase relationships of circadian output variables to an imposed LD cycle, we have produced models of circadian resonance which do not depend on an explicit oscillator model. Models of the circadian oscillator for a few organisms are available. While a specific model for *Synechococcus* does not yet exist, and while some doubt persists as to the relationship of the cyanobacterial oscillator to that of higher organisms (Golden *et al.*, 1998; Kondo & Ishiura, 1999), it should be highly instructive to study a model of circadian resonance which does incorporate a description, however abstract, of the circadian oscillator. How does the phase angle vary with length of the LD cycle? What happens if we fix the length of the LD cycle and vary the lengths of the L and D phases? What happens if stable entrainment is not obtained? Are arrhythmic mutants at a competitive disadvantage with respect to strains with functional circadian oscillators? We are currently studying these and related questions and will report the results in a subsequent publication (Gonze *et al.*, 2000).

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REFERENCES

ALLEMAND, R., COHET, Y. & DAVID, J. (1973). Increase in the longevity of adult *Drosophila melanogaster* kept in permanent darkness. *Exp. Gerontol.* **8,** 279–283.

CHOW, T.-J. & TABITA, F. R. (1994). Reciprocal light-dark transcriptional control of *nif* and *rbc* expression and light-dependent posttranslational control of nitrogenase activity in *Synechococcus* sp. strain RF-1. *J. Bacteriol.* **176,** 6281–6285.

CONTER, A. (1987). Preirradiation of medium induces a subsequent stimulation or inhibition of growth according to the physiological state in *Synechococcus lividus* in culture. *Radiat. Res.* **109,** 342–346.

COONEY, M. J. & MCDONALD, K. A. (1995). Optimal dynamic experiments for bioreactor model discrimination. *Appl. Microbiol. Biotechnol.* **43,** 826–837.

DUNLAP, J. C. (1999). Molecular bases for circadian clocks. *Cell* **96,** 271–290.

EDMUNDS, L. N. (1988). *Cellular and Molecular Bases of Biological Clocks.* New York: Springer.

GILLETTE, M. U. (1997). Cellular and biochemical mechanisms underlying circadian rhythms in vertebrates. *Curr. Opin. Neurobiol.* **7,** 797–804.

GOLDEN, S. S., JOHNSON, C. H. & KONDO, T. (1998). The cyanobacterial circadian system: a clock apart. *Curr. Opin. Microbiol.* **1,** 669–673.

GONZE, D., ROUSSEL, M. R. & GOLDBETER, A. (2000). A model for the enhancement of fitness in cyanobacteria based on resonance of a circadian oscillator with the external light-dark cycle (in preparation).

HIGHKIN, H. R. & HANSON, J. B. (1954). Possible interaction between light–dark cycles and endogenous daily rhythms on the growth of tomato plants. *Plant Physiol.* **29,** 301–302.

HILLMAN, W. S. (1956). Injury of tomato plants by continuous light and unfavorable photoperiodic cycles. *Am. J. Bot.* **43,** 89–96.

HUANG, T.-C., TU, J., CHOW, T.-J. & CHEN, T.-H. (1990). Circadian rhythm of the prokaryote *Synechococcus* sp. RF-1. *Plant Physiol.* **92,** 531–533.

JOHNSON, C. H., GOLDEN, S. S., ISHIURA, M. & KONDO, T. (1996). Circadian clocks in prokaryotes. *Mol. Microbiol.* **21,** 5–11.

JOHNSON, C. H., GOLDEN, S. S. & KONDO, T. (1998). Adaptive significance of circadian programs in cyanobacteria. *Trends Microbiol.* **6,** 407–410.

KLARSFELD, A. & ROUYER, F. (1998) Effects of circadian mutations and LD periodicity on the life span of *Drosophila melanogaster*. *J. Biol. Rhythms* **13,** 471–478.

KONDO, T. & ISHIURA, M. (1999). The circadian clocks of plants and cyanobacteria. *Trends Plant Sci.* **4,** 171–176.

KONDO, T., STRAYER, C. A., KULKARNI, R. D., TAYLOR, W., ISHIURA, M., GOLDEN, S. S. & JOHNSON, C. H. (1993). Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 5672–5676.

KONDO, T., TSINOREMAS, N. F., GOLDEN, S. S., JOHNSON, C. H., KUTSUNA, S. & ISHIURA, M. (1994). Circadian clock mutants of cyanobacteria. *Science* **266,** 1233–1236.

ISHIURA, M., KUTSUNA, S., AOKI, T., S., IWASAKI, H., ANDERSSON, C. R., TANABE, A. GOLDEN, S. S., JOHNSON, C. H. & KONDO, T. (1998). Expression of a gene cluster *kaiABC* as a circadian feedback process in cyanobacteria. *Science* **281,** 1519–1523.

LEHNINGER, A. L., NELSON, D. L. & COX, M. M. (1993). *Principles of Biochemistry*, 2nd Edn, pp. 631–632. New York: Worth.

LIU, Y., TSINOREMAS, N. F., JOHNSON, C. H., LEBEDEVA, N. V., GOLDEN, S. S., ISHIURA, M. & KONDO, T. (1995). Circadian orchestration of gene expression in cyanobacteria. *Genes Dev.* **9,** 1469–1478.

MILLAR, A. J., CARRÉ, I. A., STRAYER, C. A., CHUA, N.-H. & KAY, S. A. (1995). Circadian clock mutants in *Arabidopsis* identified by luciferase imaging. *Science* **267,** 1161–1163.

MITSUI, A., KUMAZAWA, S., TAKAHASHI, A., IKEMOTO, H., CAO, S. & ARAI, T. (1986). Strategy by which nitrogen-fixing unicellular cyanobacteria grow photoautotrophically. *Nature* **323,** 720–722.

MONOD, J. (1942). *Recherches sur la Croissance des Cultures Bactériennes*. Paris: Hermann.

MORI, T., BINDER, B. & JOHNSON, C. H. (1996). Circadian gating of cell division in cyanobacteria growing with average doubling times of less than 24 hours. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 10183–10188.

OUYANG, Y., ANDERSSON, C. R., KONDO, T., GOLDEN, S. S. & JOHNSON, C. H. (1998). Resonating circadian clocks enhance fitness in cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 8660–8664.

OYARZUN, F. J. & LANGE, K. (1994). The attractiveness of the Droop equations II. Generic uptake and growth functions. *Math. Biosci.* **121,** 127–139.

PITTENDRIGH, C. S. (1993). Temporal organization: reflections of a Darwinian clock-watcher. *Annu. Rev. Physiol.* **55,** 17–54.

PITTENDRIGH, C. S. & MINIS, D. H. (1972). Circadian systems: longevity as a function of circadian resonance in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* **69,** 1537–1539.

SAKAMOTO, T., DELGAIZO, V. B. & BRYANT, D. A. (1998). Growth on urea can trigger death and peroxidation of the cyanobacterium *Synechococcus* sp. strain PCC 7002. *Appl. Environ. Microbiol.* **64,** 2361–2366.

SWEENEY, B. M. (1987). *Rhythmic Phenomena in Plants*, 2nd Edn. San Diego: Academic.

THOMAS, B. & VINCE-PRUE, D. (1997). *Photoperiodism in Plants*, 2nd Edn. San Diego: Academic.

TICHY, M. & VERMAAS, W. (1999). In vivo role of catalase-peroxidase in *Synechocystis* sp. strain PCC 6803. *J. Bacteriol.* **181,** 1875–1882.

WENT, F. W. (1960). Photo- and thermoperiodic effects in plant growth. *Cold Spring Harbor Symp. Quant. Biol.* **25,** 221–230.

APPENDIX A

Exclusion Boundary for the Mutual inhibition Model

In the model with a growth inhibitor, if at least one of the competing strains has a negative phase angle, then the strain with the larger phase angle can maintain itself or, under suitable conditions, eliminate the strain with a smaller phase angle if the former enjoys sufficient numerical superiority (Fig. 5). The fundamental reason for this is the same, whether the strains have phase angles of opposite sign and exclusion always results or the strains both have negative phase angles and coexistence can be observed: When the strain with the smaller absolute phase angle (strain 1) switches from the sD to the sL metabolic state and starts producing the inhibitor, its competitor (strain 2) will be able to keep the inhibitor concentration below the critical value I_c if cells of the latter strain are sufficiently numerous (Figs 6 and 7). The curve in the space of initial conditions which separates one basin of attraction (strain 1 eliminates strain 2, strain 2 coexists with or eliminates strain 1) from the

other can be determined by a relatively straightforward argument.

The success of strain 1 in eliminating strain 2 is determined by the events which occur when strain 1 enters sL while strain 2 is in sD. Under these conditions, strain 1 begins to synthesize the inhibitor while strain 2 is metabolically vulnerable. The evolution equation for I is [eqns (1b) and (1d)]

$$\frac{dI}{dt} = N_1\left(p - \frac{V_{max}I}{K_M + I}\right) - N_2\frac{V_{max}I}{K_M + I}. \quad \text{(A.1)}$$

If this occurs in L, both strains continue to grow at the same specific growth rate as long as $I < I_c$. The ratio of N_1 to N_2 therefore remains constant while the inhibitor concentration remains low since the strains have identical specific growth rates. Accordingly, provided strain 2 does not enter sL during the transient evolution, I will increase to the steady-state solution I^* of eqn (A.1) given by

$$I^* = \frac{K_M p f_1}{V_{max} - p f_1}, \quad \text{(A.2)}$$

where $f_1 = N_1/(N_1 + N_2)$ is the fraction of cells of strain 1 in the culture. If $I^* < I_c$, the concentration of I never rises to a value sufficient to inhibit the growth of strain 2 and the ratio of the two populations remains constant during this period. If, additionally, the phase angles are opposite in sign, there is a period during L in which conditions are unfavorable to the growth of strain 1 and the latter is eliminated. In the $f_1 \times I_c$ plane (Fig. 9), the curve which separates the two basins of attraction is therefore given by $I^* = I_c$ or, from eqn (A.2),

$$f_1 = \frac{I_c V_{max}}{p(K_M + I_c)}. \quad \text{(A.3)}$$

Note that, for fixed values of the parameters, the final state of the system is uniquely determined by the proportions of the two populations and not by their actual sizes.

APPENDIX B

Comparison of the Two Definitions of the Phase Angle

This paper presents two definitions of the phase angle, embodied respectively in Fig. 1 and in eqn (6). The purpose of this appendix is to demonstrate that the two definitions should be in rough agreement given a few entirely reasonable assumptions about the dynamics of the circadian oscillator and the coupling of circadian output variables to the oscillator.

Since the circadian oscillator is T-periodic, any of its state variables can be expanded in a Fourier series. Suppose that the circadian output variables are coupled to a particular oscillator state variables x. The phase angle shifts the time origin of each term of the Fourier series of x:

$$x(t;T) = \sum_{n=0}^{\infty}\left[a_n \cos\left(2\pi n\left(\frac{t}{T} + \theta(T)\right)\right) + b_n \sin\left(2\pi n\left(\frac{t}{T} + \theta(T)\right)\right)\right]. \quad \text{(B.1)}$$

Our reduction of the circadian states of cells to sL and sD phases which occupy a constant fraction of the LD cycle requires the Fourier coefficients a_n and b_n to be at least roughly independent of T. If this is the case then (for instance) a maximum in x is advanced by $\theta(T)$ relative to its position in the LD cycle when $T = T_i$.

If the circadian output variables are to express the same periodicity as the oscillator, only a limited class of transformations from x to an output variable y are possible. Since non-monotonic transformations allow period multiplication, it seems sensible to restrict our attention to monotonic relations. The relationship between x and y can also in general involve delays due to intracellular signal relay. Thus, we assume that $y(t;T) = Y(x(t-\zeta;T))$ where $Y(\cdot)$ is a monotonic function of its argument and ζ is a fixed delay. It is a straightforward exercise in calculus to show that, given these restrictions, each extremum in x produces a corresponding extremum in y delayed by ζ time units.

Now suppose that we want to measure $\vartheta(T)$ by tracking the motion of the maximum in y as the LD cycle length is varied. The motion of this maximum is a direct reflection of the motion of the maximum in x. Suppose that, when $T = T_i$, the maximum in x occurs at some time t_0, corresponding to a fractional circadian time of t_0/T_i. At a general LD cycle length T, due to the assumed homogeneity of the Fourier expansion of x, the maximum occurs at $t_0/T_i - \theta(T)$. The corresponding maxima in y at the two LD periods occur at $\Delta t_i(T_i)/T_i = (t_0 + \zeta)/T_i$ and $\Delta t_i(T)/T = t_0/T_i - \theta(T) + \zeta/T$ so that, according to eqn (6),

$$\vartheta(T) = \theta(T) + \zeta\left(\frac{1}{T_i} - \frac{1}{T}\right). \tag{B.2}$$

We would normally expect the trailing term of eqn (B.2) to be small since it involves a difference of similar quantities and since the delay ζ would normally be small compared to the LD period. Note also that θ is small when $T \approx T_i$ so that the difference between the two definitions of the phase angle should be relatively unimportant even at small phase angles.