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Cover illustration: The cover depicts the character in Japanese for "kai", the name of the central circadian clock gene cluster in cyanobacteria. "Kai" means cycle or rotation number in Japanese, and is therefore apropos for a gene cluster that controls circadian cycles in cyanobacteria.

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Preface

Internal biological clock systems exist in nearly all organisms, including humans, rodents, insects, plants, fungi, and bacteria. These biological (circadian) rhythms allow for each system to maintain internal time and likely provide an adaptive advantage to those organisms. The discovery of circadian rhythms in the cyanobacteria was surprising to some who believed that bacteria were too "simple" to possess the machinery necessary for generating these internal rhythms; however, investigations into the basic biology of the temporal separation of oxygen-evolving photosynthesis and oxygen-sensitive nitrogen fixation demonstrated that this diverse group of bacteria was capable of generating and maintaining internal timing.

Since the discovery of a biological clock in cyanobacteria in the 1980s, the field has exploded with new information. The cyanobacterial model system for studying circadian rhythms, *Synechococcus elongatus* PCC 7942, has allowed for a detailed genetic dissection of the bacterial clock due to the methods in molecular biology and biochemistry that are currently available. Although the majority of research has been conducted using *S. elongatus*, work in other cyanobacterial species has been instrumental to our understanding of the bacterial biological clock. In addition, examination of the various, fully sequenced cyanobacterial genomes suggest that there may be several variations upon the same theme for producing internal rhythms in prokaryotes. Through mathematical modeling and generating synthetic oscillators in other bacterial strains, in conjunction with information derived from in vivo and in vitro oscillations, the mechanism for the generation of biological rhythms in a single cell can be better elucidated.

The rapid advancement in our understanding of the bacterial circadian clock is due to many different avenues of discovery and inquiry. The success in understanding bacterial circadian programs is due, in part, to the genetically malleable *S. elongatus* PCC 7942 system and the insightful investigations of geneticists, molecular biologists, evolutionary biologists, and biochemists. What cannot be overlooked when discussing the success of this model system is that the molecular work stands on the shoulders of hundreds of years of circadian insights into the physical, physiological, and chemical basis of rhythms defined by circadian biologists outside the prokaryotic arena. Currently the *S. elongatus* system is arguably one of the best characterized circadian clock systems of any model system, even though it is one of the newest model systems to be investigated.

Thanks to the many advances in our understanding of the bacterial biological clock, this book serves as a timely review of the fundamental process of circadian timing in prokaryotes. It is also organized as a compendium of the most current data on the circadian mechanism in prokaryotes. The chapters in this book are intended to address the history and background of the cyanobacteria and initial investigations and discovery of circadian rhythms in this diverse group of microorganisms (Chaps. 1, 2, 3, 4). The molecular basis and structure of the circadian clock system are reviewed (Chaps. 5, 6, 7), as well as entrainment of the oscillator with the environment (Chap. 8) and the downstream genes and behavioral activities that are controlled by the clock (Chaps. 9, 10, 11). A demonstration of the adaptive significance of the circadian clock in cyanobacteria (Chap. 12) and the prokaryotic clock's remarkable stability are also discussed (Chap. 13). Due to the great diversity of the cyanobacteria as a group, investigations have been conducted to address the evolution of cyanobacterial clock genes and whether those genes are involved in the generation of circadian rhythms in cyanobacterial strains other than the *S. elongatus* model system (Chaps. 2, 14, 15) and mathematical models for *S. elongatus* clock function and synthetic oscillator models are included (Chaps. 16, 17).

Our hope is that this book will serve many audiences, spanning from those who are currently expanding the studies discussed within, to those who are beginning their endeavor into the wonderful world of prokaryotic clock systems. We envision this text as a comprehensive reference of past accomplishments, but hopefully also a stepping stone for future work on this amazing group of microorganisms and timing. We are grateful to each of our colleagues and friends who contributed to this work. It is our hope that you enjoy reading each chapter as much as we enjoyed putting this combined work together.

Jayna L. Ditty
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Contents

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Chapter 1
Classic Circadian Characteristics: Historical Perspective and Properties Relative to the *Synechococcus elongatus* PCC 7942 Model

Jayna L. Ditty and Shannon R. Mackey

Abstract The purpose of this chapter is to introduce the basics of circadian biology relative to the cyanobacterial model system. It is meant to define the terms, characteristics, and rules that pertain to the study of circadian biology in the context of the cyanobacterial systems used to elucidate the mechanisms by which the prokaryotic circadian clock functions. In addition, its purpose is to serve as a conduit to the chapters in this book, which comprehensively review our most recent understanding about each of these canonical characteristics in the *Synechococcus elongatus* PCC 7942 model system as well as other cyanobacterial and prokaryotic systems.

1.1 Introduction

1.1.1 Overview

Our planet rotates about its axis every 24 h, which exposes the majority of plants and animals that inhabit the earth to sidereal fluctuations of light and temperature. This daily change in light and dark was a strong selective force (for those organisms that are subject to it) to devise physiological mechanisms with which to respond to, or better yet predict, when these daily changes were going to occur. As a result of this pressure, organisms have evolved internal timing mechanisms to anticipate the daily variations in light and temperature; this anticipatory behavior provides a selective advantage to the organism (DeCoursey 1961; Ouyang et al. 1998; Michael et al. 2003; Woelfle 2004; Johnson 2005).

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This daily clock phenomenon was termed "circadian" in 1959 by Franz Halberg using the Latin terms *circa* for "about" and *dies* "day". Therefore circadian phenomenon pertain to biological activities with a frequency of one activity cycle every 24 h (Halberg et al. 1977). The purpose of this chapter is to introduce the basics of "circadiana": to define the numerous terms, characteristics, and rules that pertain to the study of circadian biology in the context of the cyanobacterial systems that have been used to elucidate the mechanism by which the prokaryotic circadian clock functions. In addition, its purpose is to serve as a conduit to the chapters in this book, which comprehensively review the most recent understanding about each of these canonical characteristics in the *Synechococcus elongatus* PCC 7942 model system as well as other cyanobacterial and prokaryotic systems.

1.1.2 Historical Perspectives

Investigations into the mechanism that organisms use to relate and respond to diurnal fluctuations in light and temperature have been undertaken at least as early as the 1700s. One of the earliest reports that correlates behavior with specific times of day came from the French astronomer Jean-Jacques d'Ortous deMairan, who made the observation that the leaves of heliotrope plants move in response to changes in light. Even more importantly, he recognized that these leaves would continue to move in the same pattern when kept in constant darkness (DD), generating the first evidence that a behavioral activity could be regulated by an internal mechanism of the plant, and not a result of environmental light and dark cues (deMairan 1729). During the same period, the Swedish botanist Carl Linneaus developed his *horologium florae* or "flower clock," which could be used to tell the time of day based upon when particular plant species would flower (Freer 2003).

The modern field of chronobiology, or the study of biological timing processes in living things, was initiated in the mid-1950s by Colin S. Pittendrigh and Jürgen Aschoff. They were instrumental in defining and organizing the principles of a circadian system that mapped the course for circadian research, and these rules still hold true to the present time (Aschoff 1960, 1981; Pittendrigh 1961, 1981). While the characteristics and principles of circadian biology were being brought to bear by early circadian biologists, a particular question of interest was whether circadian activity was a learned behavior in organisms or had a genetic basis. The work of Erwin Bünning in 1935 alluded to the answer by providing evidence that period length was heritable in bean plants (Bünning 1935); however, it was not until the early 1970s that the first evidence for a genetic basis to circadian activity was brought to light by two independent groups working in fruit flies and fungus. Ronald Konopka and Seymour Benzer isolated *Drosophila melanogaster* mutants that had altered eclosion and activity rhythms. Each of the mutations was complemented by one genetic locus, termed the *period* gene (Konopka and Benzer 1971). Soon after, Jerry Feldman and Marian Hoyle identified the *frequency* gene, which

was shown to be essential for rhythms of asexual spore formation in *Neurospora crassa* (Feldman and Hoyle 1973).

The study of circadian clocks and rhythms was sequestered to eukaryotic models as historical circadian dogma dictated that nuclear structure, intercellular communication, and generation times longer than 24 h were required for rhythmic activity – characteristics that are lacking in prokaryotic cells and, at least in part, in unicellular eukaryotes (Edmunds 1983; Kippert 1987). However, in the 1980s, several lines of evidence were emerging to contradict the "eukaryocentric" circadian requirements. The cyanobacteria are a large and diverse group of microorganisms that are typically photoautotrophic and diazotrophic, and are responsible for a vast majority of the carbon and nitrogen fixation in the environment (see Chap. 2; Garrity 2001). Within several different cyanobacterial species, circadian activity in nitrogen fixation, amino acid uptake, and cell division were identified (see Chap. 3; Grobbelaar et al. 1986; Mitsui et al. 1986; Sweeney and Borgese 1989; Huang et al. 1990; Chen et al. 1991; Grobbelaar and Huang 1992; Schneegurt et al. 1994). While the physiological evidence drastically changed the manner by which scientists thought about circadian biology, a good model system for prokaryotic circadian research was lacking. Ultimately *S. elongatus* PCC 7942 became the model of choice in part because of the vast amount of molecular tools available in this strain (see Chap. 4; Golden 1987; Golden 1988; Kondo et al. 1993, 1994; Ishuira et al. 1998; Andersson 2000).

1.2 Properties of a Clock-Controlled Rhythm

Regardless of the model system one is using to understand the circadian process, the underlying mechanisms achieve a similar goal: maintain an internal, 24-h time. A circadian clock system is defined as an endogenous mechanism that allows an organism to temporally regulate biological activity as a function of the 24-h day. Such biological activities that are regulated by the circadian clock are therefore coined circadian rhythms (Pittendrigh 1981; Edmunds 1983; Dunlap et al. 2004; Koukkari and Sothern 2006). The rhythmic nature of daily activity can be described by three terms that correspond to the characteristic descriptions of a waveform: period, phase, and amplitude.

1.2.1 Period

The period of a rhythm is defined as the duration of one complete activity cycle (Fig. 1.1). Therefore, a circadian period would be an activity that completed its cycle (with a frequency of approximately 1) over a 24-h period of time (Dunlap et al. 2004; Koukkari and Sothern 2006). When measured under constant conditions (see Sect. 1.3.1) the period is defined as the free-running period (FRP),

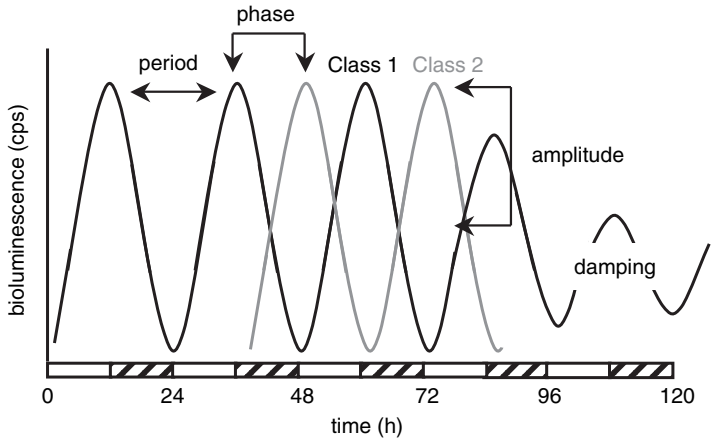

Fig. 1.1 Properties of a circadian activity rhythm as measured in *Synechococcus elongatus* PCC 7942. The traces depict levels of bioluminescence in counts per second (*cps*) over time (*h*) from two representative *S. elongatus* luciferase reporter strains maintained in constant conditions. Alternating *open* and *hatched* bars on the abscissa represent subjective day and subjective night, respectively. Period is defined here by peak-to-peak activity duration over approximately 24 h. Phase is defined here as the time when peak activity is reached. In the *S. elongatus* model, two phases are typically described: Class 1 (*black*) peaks at subjective dusk, while Class 2 (*gray*) peaks at subjective dawn. Amplitude is defined as the magnitude of the oscillation from the mean, where damping is a general trend whereby there is a decrease in rhythmic robustness over time under constant conditions

represented by τ (the Greek symbol *tau*). Observable, and therefore measurable, rhythms of a circadian timing system are not easily measured in bacteria due to their microscopic size and lack of obvious overt behaviors. Therefore, in *S. elongatus* PCC 7942, cyanobacterial promoters were engineered to produce the LuxAB luciferase proteins, as well as their necessary substrates (LuxCDE), from *Vibrio fischeri* and *V. harveyi* respectively, for bioluminescence as an easily measurable and quantifiable output (Kondo et al. 1993). While the average period for circadian rhythms in *S. elongatus* PCC 7942 is approximately 24–25 h (Kondo et al. 1993; Ishiura et al. 1998), the form or shape of the activity rhythm can vary considerably depending upon the promoter used to drive expression of *luxAB*. Waveform patterns of gene expression have been shown to be symmetrical sine curves, asymmetric, saw-tooth, or step-like in form (Liu et al. 1995).

1.2.2 Phase

The phase of an activity rhythm is defined as the instantaneous state of an oscillation within a period (Fig. 1.1; Dunlap et al. 2004; Koukkari and Sothern 2006). For example, the highest point of any rhythmic activity would be defined as the peak of activity (trough for the lowest). These peaks (or troughs) of activity can be used as

reference points for determining at what point a particular activity occurs the most (or least) during a 24-h day. The majority of genes expressed in a circadian manner, as measured by random promoter:luciferase fusions in *S. elongatus* PCC 7942, were categorized into a number of different classes with the majority of the genes falling into either Class 1, where activity peaked at subjective dusk (the time in constant light, LL, that corresponds to dusk of the entraining light/dark, LD, cycle), or Class 2, where activity peaked at subjective dawn (the time in LL that corresponds to dawn of the entraining LD cycle; Liu et al. 1995).

1.2.3 Amplitude

The amplitude of a rhythm is defined as the magnitude from the mean activity level to either the peak or to the trough of activity (Fig. 1.1; Dunlap et al. 2004; Koukkari and Sothern 2006). Amplitude is an obvious requirement of a cyclic activity, but it is much more difficult to quantify and interpret than the period or phase of a rhythmic behavior, particularly in the cyanobacterial system. Typically, cultures of cyanobacterial cells (in lieu of individual cyanobacterial cells) are measured for circadian activity. Therefore, careful consideration of the number of cells being measured, the innate differences in the particular promoter driving expression of the reporter, and the level of substrate available for luciferase could each affect the measurement of the amplitude (Kondo et al. 1993; Andersson et al. 2000). Additionally, damping, or a decrease in rhythmic activity over time, can confound amplitude measurements; however, this has not been extremely problematic in the *S. elongatus* PCC 7942 model system, as robust rhythms have been measured for over two weeks in constant conditions (Golden and Canales 2003).

1.2.4 Time

The period, phase, and amplitude are all characteristics of activity patterns that are measured over time. When considering time in a circadian system, there are important distinctions that must be noted. Standard clock time is measured by mechanical or atomic clocks that are used to determine time of day with midnight placed in the middle of the dark and noon when the sun is at its highest point. Therefore, when activity is measured under standard conditions, light and dark cycles persist, and activity can be influenced by these light cues. When measured under these cues, activity is measured in zeitgeber time (ZT, "time-giver" in German), as the environmental cues (zeitgebers) of light, dark, and temperature (to name a few) are present to affect behavior (Fig. 1.2; Dunlap et al. 2004; Koukkari and Sothern 2006).

To truly measure endogenously generated circadian activities, rhythms must be measured in the absence of these environmental cues (see Sect. 1.3.1).

In contrast to ZT time, circadian time (CT) is subject only to the internal timing mechanism of the organism and is independent of ZT; as such, CT is measured under constant environmental conditions. Depending on the system, constant conditions can be maintained in either LL or DD. Under LL conditions, the CT "hour" for *S. elongatus* is calculated by dividing the FRP (approximately 25 h) by the 24-h standard time (for an approximate value of 1.04 h). When measuring circadian activity in these unnatural constant conditions, the CT subjective day starts at CT0 (subjective dawn) and refers to the time that corresponds to lights on in ZT. In contrast, subjective night begins at CT12 (subjective dusk), which corresponds to lights off (Fig. 1.2; Daan et al. 2002; Dunlap et al. 2004; Koukkari and Sothern 2006).

1.3 Defining Characteristics of a Circadian Rhythm

An important distinction must be made between whether a particular activity or behavior is merely *responding* to an environmental cue (i.e., lights on), or if the organism is *predicting* the next environmental cue, thereby generating an activity pattern that is regulated by an internal timing mechanism. Therefore, there are three tenets that describe and define a rhythmic activity that is generated internally and is truly circadian in nature. A circadian rhythm: (1) persists in the absence of environmental cues, (2) is entrained by environmental stimuli, and (3) is temperature-compensated (Pittendrigh 1981; Edmunds 1983; Dunlap et al. 2004; Koukkari and Sothern 2006).

1.3.1 Persistence under Constant Conditions

The first intrinsic characteristic of the circadian rhythm is that the pattern of activity continues in the absence of any environmental cue (Fig. 1.2). In the absence of zeitgebers such as light, dark, temperature, or humidity, rhythmic activity continues with the period that is set by the circadian clock. The time needed for one circadian oscillation to occur (either peak-to-peak or trough-to-trough) under these artificial constant conditions is therefore defined as the FRP, as the activity pattern is free from zeitgeber influence and is driven solely by circadian clock control, which demonstrates the endogenous source of the control mechanism. The range of FRPs for organisms is typically 22–25 h (Pittendrigh 1960; Aschoff 1981; Dunlap et al. 2004; Koukkari and Sothern, 2006).

Although circadian rhythms persist under constant conditions, the FRP is still sensitive to changes or differences in various zeitgebers. The FRP in LL has been shown to vary in response to changing light intensities. First described by Jürgen Aschoff and now affectionately referred to as Aschoff's rule, diurnal organisms typically display a shorter FRP, or a "faster" clock, under high light intensities

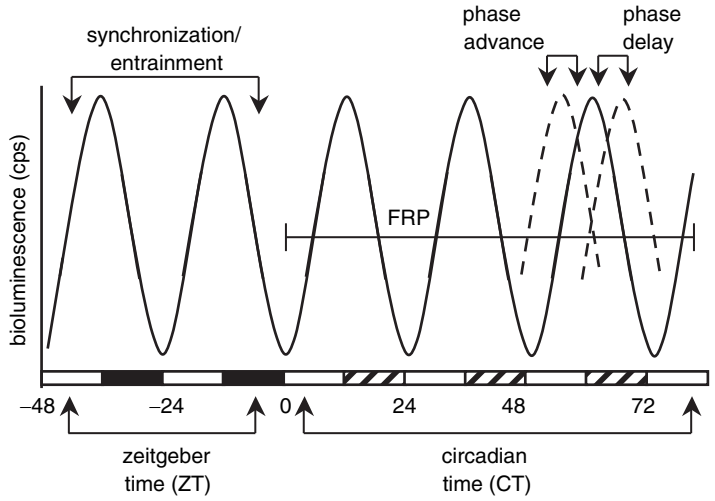

Fig. 1.2 General characteristics of a circadian rhythm. The abscissa designates time (*h*). Negative values represent zeitgeber time, in which *S. elongatus* cultures are exposed to LD cycles in order to entrain their internal clocks. Alternating *open* and *black* bars represent when cells are exposed to light and darkness, respectively. Positive values represent circadian time, when cells are in constant light (LL). Alternating *open* and *hatched* bars represent subjective day and subjective night, respectively. *FRP* is the free-running period, which is the measured persistence of the rhythm in the absence of an environmental cue. In response to zeitgeber cues provided during LL, phase shifting occurs to ensure that activity coincides with the correct time of day. The resulting phase of the activity pattern is shifted either earlier (phase advance) or later (phase delay), while the period is not altered

than at low light intensities. Conversely, nocturnal organisms exhibit the opposite response with a longer FRP under constant high light intensities than under constant low light (Aschoff 1981).

1.3.2 Entrainment by Environmental Cues

Under natural conditions, where organisms are subject to daily changes in light and dark, circadian rhythms are not free running; rather, they are entrained to local environmental cues (Fig. 1.2). Because FRPs are close to (but very rarely) 24 h in length, the circadian system must be able to reset its rhythm by zeitgebers each day to avoid falling out of phase with local standard time. If this were not the case, an organism with an FRP of 22 h would become active approximately 2 h earlier each day, such that after six days, the activity rhythm would occur a full 12 h out of phase from the natural environmental cycle. Therefore, the circadian system must be cognizant and responsive to these environmental cues such that the circadian clock, and therefore activity, is entrained to local time.

A synchronizer is an agent or signal that promotes the synchrony of multiple clocks within a population. Typically in *S. elongatus* experiments, two LD cycles are used to synchronize all clocks within the culture population. Entrainment results in the internal biological rhythm having a period that matches that of the environmental stimulus, and entrainment to a particular cue (such as light or dark) ensures two things: (1) the period of the activity rhythm is equal to that of the LD cycle, (2) the phase of the activity rhythm is appropriately stable and occurring at the correct time of day. The difference (in hours) between the phase of the clock-driven rhythm and the rhythm of the entraining stimulus is the phase angle (Aschoff 1960; Moore-Ede et al. 1982; Dunlap et al. 2004; Koukkari and Sothern 2006).

When the circadian mechanism responds to a zeitgeber, the ultimate goal is to maintain the proper phase of activity or inactivity, which ensures that daily behaviors are occurring at the proper time within a cycle. This process, known as phase shifting, is the change in the timing of the phase of a rhythm in relation to the zeitgeber information of the previous phase. Again, to ensure that activity phases are occurring at the correct time of day, activity phases can be shifted to occur earlier (advance) or later (delay) in the day (Fig. 1.2; Aschoff 1960; Johnson 1999; Dunlap et al. 2004; Koukkari and Sothern 2006).

The most important zeitgebers for the circadian clock are the daily cycles in light and dark. While the clock mechanism must be sensitive to these cues, it does not mean that the circadian clock is sensitive, or responsive, to these cues at all times of day. As has been shown for many organisms that use light as an entraining signal, light exposure in the early subjective night produces phase delay shifts whereas light in the late subjective night produce phase advance shifts; light during the subjective day produces a very small, if any, phase shift. A phase response curve (PRC) for an organism in response to a particular zeitgeber can be generated by plotting the time at which the zeitgeber signal is provided to the organism on the *x*-axis, and the magnitude (in hours) and direction of the shift (advance or delay) to that signal are plotted on the *y*-axis (advance on the positive and delay on the negative *y*-axis; Aschoff 1960; Johnson 1999; Dunlap et al. 2004; Koukkari and Sothern 2006). In general, an organism's clock is less responsive to pulses of darkness during the subjective night and pulses of light during the subjective day.

1.3.3 Temperature Compensation

For a circadian clock to be accurate in the environment, it must maintain its periodicity despite changes in daily temperature. This property, named temperature compensation, is a result of the observation that the value of the FRP changes very little over different temperatures within the physiological range of the organism. One could imagine this as an important facet of the circadian mechanism, as it would be detrimental to an organism to have its circadian clock run faster or slower on a warm or cold day, respectively (Aschoff 1960; Sweeny and Hastings 1960; Dunlap et al. 2004; Koukkari and Sothern 2006). In a typical enzymatic reaction,

temperature directly influences the rate at which that reaction proceeds. The Q_{10} temperature coefficient is the measure of this phenomenon, whereby the rate of a reaction tends to increase by a factor of two or three with every 10°C increase. The Q_{10} value for the period of a circadian rhythm is typically 0.8–1.2, indicating that the rhythmic activity is insulated from changes in temperature. Temperature insulation of the circadian mechanism should not be misconstrued as temperature insensitivity. Temperature has been demonstrated to be a strong zeitgeber for many circadian systems and can be used to entrain the circadian clock to adjust the phase of the activity rhythm (Aschoff 1960; Liu et al. 1998; Dunlap et al. 2004; Koukkari and Sothern 2006). While LD cycles are the strongest environmental cues for entraining the *S. elongatus* clock, temperature has been shown to be an effective zeitgeber as well (Lin et al. 1999; Schmitz et al. 2000; Ditty et al. 2003).

1.4 Introduction to the Cyanobacterial Circadian Clock Mechanism

Underlying the properties characteristic of the circadian rhythm is the circadian mechanism itself. It is comprised of internal molecules that drive rhythmic gene expression and therefore regulate cellular processes on a 24-h time scale (Aschoff 1960; Pittendrigh 1981; Dunlap et al. 2004; Koukkari and Sothern 2006). The individual molecular players of a circadian clock are very complex, but the overall game in which they play is easily modeled with discrete, but interacting, units. This simple organization divides the circadian mechanism into three basic elements: the oscillator, an input pathway and an output pathway (Fig. 1.3).

Central to the circadian mechanism is the oscillator (also known as the pacemaker) that is responsible for maintaining and disseminating the 24-h time information. As dictated by the characteristics of the circadian rhythms it controls, it is entrainable by environmental cues. The ability of the oscillator to communicate

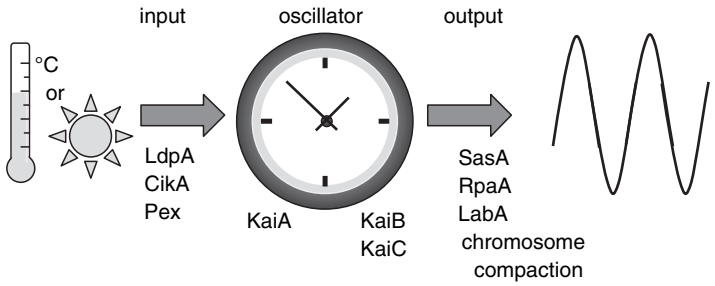

Fig. 1.3 Simple model for the *S. elongatus* PCC 7942 circadian clock, showing the three conceptual designations for the circadian clock (*input*, *oscillator*, *output*), along with known *S. elongatus* PCC 7942 genes involved in each unit. See text for brief descriptions of each gene and directives to chapters within this book that fully describe each of these processes