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Circadian Rhythms in Bacteria and Microbiomes

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Dedications

Dedication to Dr. Carol Rae Andersson (May 5, 1965–January 24, 2009) She left us too soon!

Dr. Carol Andersson was the first researcher to join Dr. Susan Golden's lab at Texas A&M University (in 1994) specifically to work on a nascent circadian rhythms project that developed from a collaboration with Golden, Dr. Carl Johnson (Vanderbilt University), and Drs. Takao Kondo and Mashiro Ishiura (initially at the National Institute for Basic Biology in Okazaki). Carol's Ph.D. work at the Australian National University in Canberra had focused on regulation of hemoglobin genes in plants. She enjoyed jumping into the cyanobacterial world for her postdoctoral research and quickly mastered the genetics needed to explore components of the clock. In her words: "I just really like putting together little bits of DNA!" Her understated sense of humor provided daily joy to Dr. Golden. In addition to conducting foundational experiments and developing the circadian monitoring protocols used in the Golden lab, Carol served as an ambassador for the multi-lab project, spending time in the Kondo/Ishiura group with support of a Human Frontier Science Program grant (1996–1999) designed to facilitate interaction among the groups. She tremendously enjoyed interacting with her Japanese colleagues, who

reflected later upon how gracefully she met the cultural challenge of pursuing her vegetarian diet during the visit. When Dr. Kondo visited Texas A&M in early 1995, armed with many clock-modifying clones identified by Dr. Ishiura, Carol quickly performed a beautiful Southern blot that showed all genes mapped to the same locus: the one we now revere as *kaiABC*. Because her efforts were so early in the hunt for the cyanobacterial circadian clock, she laid groundwork for projects that many others benefited from later. After her time in the Golden lab she carried her new love of circadian biology back to the plant science realm, to identify components of the *Arabidopsis* circadian clock in the lab of Dr. Steve Kay. Thereafter, she returned to her native Australia, where she worked in government policy related to food standards. She died sadly young of melanoma, remaining cheerful and finding joy in nature and pets throughout the challenging period of treatment and symptoms. Her memory still brings a smile to the faces of all who knew her.

Contributed by Dr. Susan Golden, University of California, San Diego, CA, USA

Dedication to Dr. Yohko Kitayama (1976–2016)

Yohko Kitayama was born in August 1976, and it was April 1995 when she entered the Faculty of Science at Nagoya University. Our relationship began a few years later when she attended one of my lectures. I remember her enthusiasm and concentrated attention during that lecture. The following year, she chose to join my lab for her graduation project. Needless to say, Ms. Kitayama was a very talented person, and I felt very fortunate to have the opportunity to work with her, especially because she steadfastly maintained her own point of view and devoted herself conscientiously to her research.

Dr. Kitayama was particularly good at molecular biological analysis, and her first research project led to the discovery of SasA by the two-hybrid method. Later, Dr. Kitayama participated in the most important studies of our group, such as the discovery and analysis of the phosphorylation of KaiC, and was a key member of our laboratory. The first research project in which she was the leader was published as a paper in *EMBO J* (2003), which analyzed the dynamics and phosphorylation of Kai proteins in cyanobacterial cells. This work, which led to her Ph.D. degree, was a

precursor to the quantitative analysis of Kai proteins and a stepping stone for the lab to move from molecular biology to biochemistry. Dr. Kitayama's measurement of the absolute amount of Kai proteins in cells and her discovery of the nonlinearity in the regulation of KaiC phosphorylation by KaiB were pivotal to our success in 2005 when we reconstituted the circadian rhythm of KaiC phosphorylation with purified Kai proteins in vitro. After receiving her degree, Dr. Kitayama continued her research as a postdoc, Assistant Professor, and Lecturer, working toward understanding the core mechanism of the circadian clock. Among her many accomplishments, I would particularly like to mention two key studies, namely her study showing that the interaction of the six KaiC monomers within the KaiC hexamer regulates the phosphorylation cycle (Nature Communications, 2013) and her discovery that the circadian rhythm of transcription and translation continues even when the phosphorylation cycle of KaiC is suspended (Genes and Development, 2008). All of these were important discoveries that captured the essence of the circadian clock in cyanobacteria.

Without her contributions, our understanding of the function of the circadian clock in cyanobacteria would not have progressed as far as it has. She was also indispensable to the management of our laboratory. At our laboratory meetings, it was my habit to look at Dr. Kitayama's face first whenever I made a delicate proposal. As long as she was smiling, it was always fine. After she was diagnosed to have stomach cancer in May 2016, I could not do those things anymore. I visited her at the hospital many times after her diagnosis. The treatments must have been very painful, but because of the tender care from her parents, she never stopped smiling. Many people prayed for her to survive as long as possible, but three months later she passed away out of our reach. I would like to dedicate this book to her as a tribute to her dedication to the study of cyanobacterial circadian clocks and my gratitude to her.

Tenderly contributed by Takao Kondo, Dr. Sc.
University Professor, Nagoya University, Nagoya, Japan

To Takao Kondo on the Occasion of His Retirement

It is a pleasure to dedicate this book to Dr. Takao Kondo on the event of his retirement. Because Takao is fundamentally a humble person, I do not think that he considered when he started his scientific training that his career might take him to the heights that he has accomplished. I seriously doubt that Takao ever thought that he would ever be awarded a prize of the stature of the Asahi Prize (in 2006), which is one of the most prestigious prizes a Japanese can attain, as it honors those who have outstanding accomplishments in the fields of arts and academics that greatly contributed to the development and progress of Japanese culture and society at large. Or even grander, that he would be granted a personal audience with the Emperor of Japan to honor (and explain!) his accomplishments. But fortunately, there is some

justice in this world, and Takao has been recognized for the seminal contributions he has made. In particular, his laboratory's discovery of the in vitro KaiABC oscillation (Nakajima et al. 2005) has led to a reevaluation of circadian mechanisms in all organisms, and in particular to more cautious interpretations of the transcriptional and translational feedback loop model for circadian clocks (Tomita et al. 2005; Qin et al. 2010; Johnson 2010).

Takao's first publications were on circadian rhythms of ion fluxes in the duckweed *Lemna* (Kondo 1978; Kondo and Tsuzuki 1978; Kondo 1983). *Lemna* is a particularly interesting organism being a small angiosperm that exhibits photoperiodic flowering responses, and different strains of *Lemna* are short-day plants while other *Lemna* strains are long-day flowerers (Miwa et al. 2006). Therefore, like Colin Pittendrigh, Takao's interest in circadian rhythms was inspired early by an interest in photoperiodism. And apparently he never lost his love for *Lemna* and the puzzle of its photoperiodic responses, since he came back to those research questions after his pioneering work with cyanobacteria (Miwa et al. 2006). However, at some point—probably attracted by the potential of its excellent genetics—Takao developed an interest for the potential of using the unicellular alga *Chlamydomonas* for analyzing circadian mechanisms.

It was Takao's venture into the study of *Chlamydomonas* that initiated my interaction and, ultimately, my friendship with Takao in the mid-1980s. At that time, I was a postdoc in Dr. J.W. ("Woody") Hastings' laboratory and I was attempting to continue the development of the *Chlamydomonas* circadian system that had begun with the studies of Dr. Victor Bruce (Bruce 1970). I had inherited Victor Bruce's apparatus for measuring *Chlamydomonas* phototaxis rhythms, but I was not obtaining reproducibly precise rhythms. Takao has a real talent for designing apparatuses and writing computer programs for data acquisition and analysis, and he had transferred his expertise from the *Lemna* system to *Chlamydomonas* phototaxis rhythms. I became aware of the lovely data that Takao was producing from *Chlamydomonas* upon my first visit to Japan in 1984. I came back from that trip to Japan in a very enthusiastic mood about the quality of Takao's phototaxis rhythms from *Chlamydomonas*, so I convinced Woody Hastings to invite Takao to Woody's lab for three months in 1985 to initiate a collaboration on *Chlamydomonas* circadian rhythms. Takao's visit to the USA was followed by my research visit to Takao's lab for three months in 1986 at the National Institute for Basic Biology (NIBB) in Okazaki, Japan (where Takao was a Research Associate, Fig. 1). The primary project accomplished during that 1986 visit was to study the action spectroscopy of light-induced phase resetting of the *Chlamydomonas* clock with Takao, a project that ultimately resulted in three publications about the effects of light and dark on the *Chlamydomonas* clock (Kondo et al.1991; Johnson et al. 1991; Johnson and Kondo 1992).

During that 1986 visit to Okazaki, I learned that Takao is a subtle non-conformist. Some tip-offs to his underlying nature in 1986 were that he liked spicy food (unusual for most Japanese). Also, he was an early adopter of Apple/Macintosh computers in a time when most Japanese scientists used NEC computers (see the Apple computer in Fig. 1). His car was a Subaru when the most popular cars in Japan were Toyota,

Fig. 1 Takao Kondo sitting at his desk at the National Institute for Basic Biology, Okazaki, Japan (1986). Note the Apple computer on his desk behind him

Honda, Mazda, Mitsubishi, or Nissan (Subaru was unusual, but it was 4-wheel drive even then!). Finally, Takao was a mountain climber, not only in the Japan Alps but also in the Himalayas. Not your typical scientist! I think that the ability to think "outside the box" in scientific questions is related to some level of non-conformity. The Nobel Laureate Albert Szent-Györgyi was famously quoted as saying, **"Research is to see what everybody else has seen, and to think what nobody else has thought"** (this quote is thought to be a modification of an earlier statement by Arthur Schopenhauer). In Takao's case, my observations of his atypical preferences and activities correlate with his "out-of-the-box" discoveries and hypotheses, one example being his hypothesis that the ATPase activity is the core pacemaking mechanism of the cyanobacterial clockwork rather than the KaiC phosphorylation and KaiABC complex cycles that his lab also discovered [(Terauchi et al. 2007) and also see the Chapter "Roles of Phosphorylation of KaiC in the Cyanobacterial Circadian Clock" in this volume].

My collaborative visit to Takao's lab was followed by his coming to my lab in 1990–1991 with his family for a 10-month sabbatical (Fig. 2). I had assumed that Takao planned to continue our study of rhythms in *Chlamydomonas* during his sabbatical, so it was a surprise when Takao announced upon his arrival in the USA that he wanted to search for a new model system for studying circadian systems. Apparently, Takao had been conferring with his colleague Masahiro Ishiura, who had convinced Takao that an organism with more molecular genetic tools than *Chlamydomonas* would be better for an intensive circadian investigation. Therefore, Takao came to the USA to explore the possibility that *E. coli* or yeast might have a circadian clock. Takao's interest in testing for rhythms in *E. coli* dovetailed nicely with a long-term interest of mine in the possibility that bacteria might harbor circadian rhythms. In the late 1970s when I was in graduate school, every other chronobiologist appeared to have concluded that prokaryotes were too "simple" to harbor circadian systems, but I became fascinated with the possibility that the environmental selective pressures experienced by many bacteria were as conducive to the evolution of a circadian clock as they were for eukaryotic cells (see

Fig. 2 Takao and Carl (left and middle persons) on a balloon ride in Nashville, USA, in 1991, during Takao's sabbatical visit to Carl's lab

Introductory Chapter in this volume). I was particularly excited at the time by discoveries with *Halobacterium* by Walter Stoeckenius and others (Stoeckenius 1985, 1999) that revealed this bacterium used light energy to pump protons across its plasma membrane to generate a chemiosmotic gradient for synthesizing ATP. I reasoned "here was a bacterium that will really care whether it is light or dark and benefit from a timekeeper to anticipate dawn and dusk." Therefore, as a graduate student in the laboratory of Colin Pittendrigh, I tried to measure daily rhythms of proton pumping in *Halobacterium*. Abysmal failure. Nevertheless, this graduate-student passion prepared my mind to think freely with Takao's mind toward the heretical possibility that bacteria might have clocks.

Takao's sabbatical experiments with *E. coli* and yeast did not bear direct fruit, but our minds were ready for heterodoxy. Part way through Takao's sojourn in my lab, I attended the annual meeting of the American Society for Cell Biology (ASCB) in December of 1990. At this meeting, I presented a poster and by a stroke of luck, the neighboring poster was from the laboratory in Taiwan that had reported circadian rhythms of nitrogen fixation in the cyanobacterium *Synechococcus* RF-1 (Grobbelaar et al. 1986; Huang and Chow 1990). That poster's presenter was Dr. Tsung-Hsien Chen, who was collaborating with Dr. Tan-Chi Huang. As Tsung-Hsien and I started to discuss circadian rhythms in algae as we stood by our posters, I forgot all about the rest of the meeting in my excitement about the

Taiwanese group's research on cyanobacteria, which was the first persuasive demonstration of circadian rhythms in a prokaryote. Note that like *Halobacterium*, photoautotrophic cyanobacteria are also an organism for whom sunlight is the energy source, and therefore, the timing of the daily light/dark cycle would intuitively provide a strong selective pressure for evolution of a clock. Upon my return from the meeting, I convinced Takao that cyanobacteria were the new model system to investigate. Takao and I contacted Drs. Huang and Chen to initiate a collaboration, and they graciously mailed *Synechococcus* RF-1 to my lab. The idea was to clone the promoter for the nitrogenase gene that Dr. Huang had shown to be rhythmically expressed (Huang and Chow 1990), fuse it to a luciferase gene, and introduce it into the organism to create a luminescent organism whose rhythmic data could be automatically collected by the methods pioneered in Woody Hastings' lab for the endogenously luminescent eukaryotic alga *Gonyaulax* (Taylor et al. 1982). A genetically malleable prokaryote whose rhythms could be non-invasively measured by a computerized apparatus for many cycles sounded like a winner!

A pivotal event happened shortly thereafter; about a month after receiving the sample of *Synechococcus* RF-1, I happened to be in New York City and decided to "drop in" on Dr. Steve Kay and Andrew Millar, who were working with Dr. Nam-Hai Chua at Rockefeller University. During my visit with Steve and Andrew, I mentioned our plans to make a luminescence reporter strain of *Synechococcus* RF-1. Remarkably, Steve and Andrew had obtained a sample of *Synechococcus* RF-1 and were already underway in the process of making a luminescence reporter strain of this cyanobacterium! This was very depressing news, for at that time neither Takao nor I had much experience with molecular genetic techniques, so it seemed hopeless to compete on the identical approach with Steve and Andrew, who were molecular genetic "jocks." The flaw in both of our plans, however, was that techniques for genetic transformation of *Synechococcus* RF-1 had not been worked out, but we all had hoped that the methods that had been developed for the transformation of other cyanobacterial species would be successful with *Synechococcus* RF-1.

Though discouraged by the news from Steve and Andrew, Takao and I did not give up. We decided to drop further work with *Synechococcus* RF-1 and focus instead upon a cyanobacterial species for which molecular genetic techniques had already been developed. The problem was: what to assay as a circadian output in an uncharacterized strain? In *Synechococcus* RF-1, nitrogen fixation or nitrogenase activity was known to be rhythmic (Grobbelaar et al. 1986; Huang and Chow 1990), but in a new cyanobacterium it was anybody's guess as to what rhythm to assay. Because my lab was doing a lot of 2D gel electrophoresis assays to discover circadian-regulated protein expression in *Chlamydomonas* at that time, we chose to look for rhythmic protein expression in a genetically malleable cyanobacterium. Once found, we reasoned that we could clone that gene's promoter, make a luminescent reporter construct, and transform it into the organism, but we expected to be far behind the "Steve and Andrew team" that was using *Synechococcus* RF-1. In retrospect, this episode is reminiscent of advice to scientists from Dr. Efraim Racker, who wrote a book in 1976 about mitochondrial electron transport that included the

wise statement that "troubles can be good for you" scientifically (as long as you respond to them constructively!) (Racker 1976).

In this case, the reason that these competitive troubles were good for us is that they led us to Dr. Susan Golden (then at Texas A&M University). I was calling cyanobacteriologists for advice about the optimal species/approach and everyone encouraged me to call Susan, who was working on the regulation of gene expression in response to changes of light intensity in the genetically tractable cyanobacterium *Synechococcus elongatus* PCC 7942. When I explained to Susan on the telephone what Takao and I had in mind, she casually mentioned some preliminary data of a postdoc in her lab that suggested the possibility of a daily rhythm in the expression of the key photosynthesis gene, *psbAI*. Even more exciting, Susan's technician had already produced a luminescence reporter strain in which the bacterial luciferase gene set (*luxAB*) was fused to the *psbAI* promoter and transformed into *S. elongatus*. This was a windfall, and it established a collaborative team that was well on its way.

Susan sent the P_{psbAI}::*luxAB* reporter strain of *S. elongatus* to us just before the end of Takao's sabbatical in my lab. On his way back to Japan, Takao and his family visited Woody Hastings in Boston for a few days. As mentioned above, Woody had a custom apparatus that had been designed and built by Dr. Walter Taylor for the specific purpose of long-term, continuous, noninvasive measurements of circadian luminescence from *Gonyaulax* (Taylor et al. 1982). Takao had an opportunity to collect two days of data from the P_{psbAI}::*luxAB* reporter strain in Woody's apparatus before returning to Japan. My remembrance of those data was that only the barest trace of an oscillation could be imagined (Johnson and Xu 2009).

But Takao was not discouraged by the data he had collected in Woody's lab! His talent for designing apparatuses and writing computer programs for data acquisition and analysis was now applied to the cyanobacteria system. After his return to Japan, Takao constructed a clever dual-channel luminometer that automatically closed a lid for a luminescence measurement and reopened the lid for white light irradiation to keep the photosynthetic cyanobacteria happy. Armed with suggestions from Woody about presenting the decanal substrate for bacterial luciferase and his homemade luminometer, Takao was able to measure rhythms that appeared to be entrainable to light/dark cycles. Encouraged by those results, Takao constructed a Japanese version of Woody's multichannel luminescence monitoring system and was able to measure beautiful rhythms of *psbAI* promoter activity as assayed by the *luxAB* luminescence reporter (Kondo et al. 1993). In retrospect, the combination of the *psbAI* promoter fused to *luxAB* and expressed in *S. elongatus* was a happy coincidence, and it remains one of the most robustly rhythmic combinations in cyanobacteria, even after 25 years of extensive research.

Along with the earlier studies on *Synechococcus* RF-1 by Huang and his collaborators (Grobbelaar et al. 1986; Huang and Chow 1990), our first paper on the *S. elongatus* rhythms (Kondo et al. 1993) established that prokaryotic cyanobacteria exhibit circadian rhythms. At that time, I was content to prove that bacteria were also members of the "circadian club," and I was ready to refocus my attention on our studies with *Chlamydomonas*. But Takao had a much larger vision. He recognized that we had established a new model system for circadian studies that could go

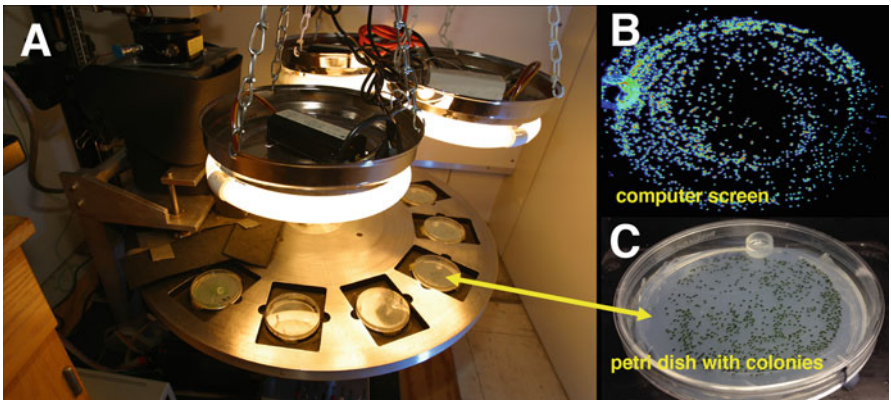

Fig. 3 The "Kondotron." (**a**) Photograph of a Kondotron (this is the Kondotron in Carl's lab; the original Kondotron is in Takao's lab at Nagoya University). The turntable has positions for twelve 100 mm petri dishes. The CCD camera is to the left (and just barely out of view), on top of a felt-encased baffle system to exclude incidental light during the imaging of bioluminescent cyanobacterial colonies on the petri dishes. Three circular fluorescent light fixtures are suspended above the turntable to provide light for photosynthesis to the cyanobacterial plates that are not being imaged. The turntable is rotated by a computer-controlled stepper motor that is underneath the Kondotron and therefore out of view. (**b**) A representative computer screen image of a plate of bioluminescent colonies of *S. elongatus*. Bioluminescent intensity is encoded by pseudocolor with blue (low intensity) to green (medium intensity) to red (high intensity). (**c**) Green *S. elongatus* colonies on an agar plate

further than most other model systems. To take full advantage of a prokaryotic system for analyzing circadian rhythms, a method to facilitate the identification of mutants was necessary. Therefore, with the first publication finished, Takao again drew from his apparatus-designing talent to develop an optimal mutant identification procedure for *S. elongatus* rhythms. Fortunately, single colonies of the P_{psbAI}::*luxAB* reporter strain are luminescent on agar plates so that Takao and Masahiro were able to visualize the rhythms of luminescence emitted by individual colonies using a CCD camera (Kondo and Ishiura 1994). Not content to observe rhythms from just one plate, however, Takao brought his unique talents to bear and designed a Macintosh computer-operated turntable apparatus that could monitor the rhythms of colonies on twelve 100 mm petri dishes simultaneously (Kondo et al. 1994; Johnson and Xu 2009). Up to 12,000 individual colonies could be screened in a single assay. Our laboratory respectfully calls this turntable-screening apparatus the "Kondotron" in honor of its inventor (Fig. 3).

The development of the Kondotron was a breakthrough. It was the first high-throughput screening apparatus for circadian rhythms based on luminescence and enabled molecular genetic expertise to be directed toward developing methods for mutagenesis and complementation of *S. elongatus* that were specifically designed for circadian goals. With the Kondotron, Takao and Masahiro initiated an extensive mutagenesis project. Takao also generously shared the Kondotron technology with me when I was on a sabbatical with Takao in 1994, and we also contributed in a

Fig. 4 A "meeting of the minds" where a group of the collaborators and Japanese students relaxed together at Takao Kondo's mountain home in 1994. Persons in the photo from left to right are Takao Kondo, Setsuyuki Aoki (student), Jim Golden (husband of Susan Golden), Carl Johnson (kneeling), Susan Golden, Yusuke Watanabe (friend of Shinsuke Kutsuna who is pushing Carl), Shinsuke Kutsuna (student), and Masahiro Ishiura

minor way to identifying more clock mutants (98% of the early screening came from the Japanese team). This approach led to the isolation of many interesting mutants exhibiting short periods, long periods, and arrhythmia; the largest range of variation for circadian period mutants for any organism: from 16 h to 60 h (Kondo et al. 1994). The team of collaborators and our students began to grow (Fig. 4).

In the fullness of time, screening for mutants with the Kondotron, complementation of mutants with wild-type DNA, and rescreening for "rescued" clones with the Kondotron again led to the identification of the key cyanobacterial clock genes, *kaiA*, *kaiB*, and *kaiC* (Ishiura et al. 1998). The three *kai* genes are immediately next to each other on the *S. elongatus* chromosome in a master clock gene cluster. Takao and Masahiro named the three-gene cluster "*kai*" for a Japanese word meaning "cycle or rotation number." The identification of the *kai* genes was the key that unlocked the cascade of discoveries summarized in the Introduction and other chapters of this volume, and as they say, "the rest is history" (Johnson and Xu 2009). That cascade culminated in another example of Takao's ability to "think outside the box," which was the mind-boggling discovery of the first in vitro circadian rhythm composed of the proteins KaiA, KaiB, KaiC, and the energy source ATP (Nakajima et al. 2005).

The coincidence of good fortune, "prepared minds," clever ideas, and hard work transformed the *Synechococcus elongatus* system into–arguably–the best understood clockwork at the molecular level in *any* organism, even though it was the

newest comer to clock mechanism analyses. An absolutely indispensable element in that transformation was Takao's unique combination of talents:

1. An excellent scientist by any standards
2. A technical innovator with a talent for designing apparatuses and writing computer programs for data acquisition and analysis
3. A non-conformist who has been willing and able to "think outside the box," and have the courage to pursue and report unconventional hypotheses and results

Takao's papers are not merely of interest in terms of the cyanobacterial clock system; many of his papers should be read by ALL chronobiologists and others with interest in biological clock mechanisms. For me personally the following papers of Takao are especially important in terms of general concepts whose full implications remain unrealized: the likelihood of biochemical oscillators being the core circadian clockwork rather than transcription/translation feedback loops (Tomita et al. 2005; Nakajima et al. 2005), the core of the KaiABC oscillator being its ATPase activity rather than the rhythm of protein interactions and phosphorylation events (Terauchi et al. 2007), and the first truly molecular explanation for a circadian oscillator running with a period of 24 h and not something faster (Abe et al. 2015).

It has been a privilege for me to have been a contributor to the development of a terrific clock-model system with Takao Kondo and our other initiating and ongoing collaborators (Susan Golden and Masahiro Ishiura). The triggering events were Takao's desire to identify a new model system that was genetically malleable and the chance encounter that I had with Tsung-Hsien Chen at the ASCB poster session in 1990. The odyssey has had its frustrations and heartaches coupled with delightfully unexpected twists and turns (Johnson and Xu 2009). As in a statement attributed (perhaps incorrectly) to Albert Einstein, "If we knew what we were doing, it wouldn't be called research, would it?" Compared to me at least, Takao Kondo appeared to know what he was doing.

Contributed by Carl Hirschie Johnson
Vanderbilt University, Nashville, TN, USA

Acknowledgments

Note the fuller treatment of the early development and discoveries with the circadian clock in cyanobacteria in Johnson and Xu (2009). I thank our collaborators and mentors and our present/former lab members, but especially I thank the other three members of the "Quadrumvirate" (Takao Kondo, Susan Golden, and Masahiro Ishiura) for an exciting collaboration that was made possible by good science, good fortune, clever ideas, hard work, and friendship. Finally, I am grateful for research support from the National Institute of General Medical Science (NIH), the National Science Foundation, and the Human Frontiers of Science Program that made this project possible.

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Preface

Many fundamental biochemical and physiological processes were first discovered in bacteria, and after gaining a firm basic understanding in bacteria, the study of these processes was extended to eukaryotic cells and organisms. The phenomenon of circadian rhythmicity is an exception. The first report was of macroscopic observations in plants in 1729 and thereafter studies extended in the 1800s–1900s throughout the eukaryotic phyla to multicellular creatures as diverse as fungi, arthropods, and vertebrates. Starting in the 1950s, eukaryotic unicellular organisms were examined as well, especially algae and protozoa. These timekeepers were not only implicated in adaptive timing on the daily scale but in most cases are also the timing system that measures day length, thereby underlying seasonality and photoperiodism.

Surprisingly, however, bacteria in the form of cyanobacteria (aka "blue-green algae") did not join the organismal pantheon of Circadiana until the 1980s, and the story of how that happened is described in this volume in the Introduction and in the Dedication to Takao Kondo. And even though cyanobacteria are now well established to have genuine circadian timekeepers, the evidence for circadian clocks in bacteria other than the cyanobacteria remains murky.

This book is "timely" (pun intended) because we now stand at a watershed moment in the investigation of circadian phenomena in prokaryotes. The study of circadian properties in cyanobacteria has been tremendously productive, for even though cyanobacteria are the latest of the major model systems to enter the field of chronobiology (see Introduction), we now know more about circadian mechanism and adaptive significance in cyanobacteria than in any other system, which is a testament to the vigor of methods that can be applied to bacteria. Once initiated with the luminescence reporter strain of *Synechococcus elongatus*, progress on understanding the clockwork mechanism has proceeded at breakneck speed, culminating in the discovery that the KaiA/B/C clock proteins could be used to reconstitute a circadian oscillator *in vitro*, the only known system where circadian rhythms can be studied outside of a living organism. Moreover, the Kai proteins proved to be readily crystallizable, thereby enabling the full power of structural and biochemical/

biophysical techniques to be deployed in determining the post-translational oscillator mechanism and its relationship to the transcriptional/translational feedback loop.

A further advantage of the cyanobacterial system is that it has allowed direct access to questions about the adaptive significance of biological clocks. Testing even simple hypotheses about fitness can be challenging in higher organisms where many processes may become interdependent over evolutionary time so that the adaptive value of a specific trait divorced from the whole is difficult to ascertain. Cyanobacteria have yielded unambiguous answers to assessments of the relative role of various environmental factors in the natural selection of clocks by introducing the "competition assay" as a rigorous test of fitness. In so doing, the fitness experiments in cyanobacteria subsequently inspired similar fitness tests in plants and animals.

As successful as the study of the circadian system in cyanobacteria has been, however, we believe that elucidation of cyanobacterial clocks is merely the overture to a large opera. In other words, this watershed moment marks the blossoming of the study of prokaryotic clocks from cyanobacteria to the much larger arena of all prokaryotes and in some cases the relationships of bacteria with eukaryotic hosts. Homologs of the *kaiB* and *kaiC* genes discovered in cyanobacteria that encode the core clock components KaiB and KaiC are found widely among Eubacteria and Archaea. While it is possible that *kaiB* and *kaiC* homologs perform different functions in other species of prokaryotes, it seems likely that the analogy of the role of these genes in the cyanobacterial timekeeping clock along with the fact that many bacteria exist in strongly rhythmic environments is indicative of a conservation of function as well as of sequence. Moreover, it is exciting to consider that the fundamental properties of biological timekeeping in other prokaryotes may be much more varied than those found among eukaryotes. There is already a hint of this diversity in that some prokaryotes such as *Prochlorococcus* and *Rhodopseudomonas* (both of which harbor *kaiB* and *kaiC* but not functional *kaiA*) may be exhibiting damped-oscillator or even hourglass-timer behavior rather than canonical self-sustained circadian rhythms. Even more titillating are the observations of possible rhythms in the gut bacterium *Klebsiella (née Enterobacter) aerogenes*, which together with the fascinating observations of rhythms in the gut microbiome opens a new realm of timekeeping interactions between eukaryotic hosts and prokaryotic residents. Because of the diversity of timing functions that appear to exist within the *kai* gene family, and because the selective pressures on microbial populations are often intense, we may be on the verge of obtaining precise answers to fundamental questions that have lingered since the beginnings of the study of biological rhythms. Namely, why are circadian clocks found in some organisms but not in others? Why do clocks have the properties that they do? How does a clock evolve from a non-oscillating ancestor? In that context, this book is a harbinger of topics that we foresee as the next great wave of discoveries in chronobiology:

1. Mechanism: what is the clockwork mechanism in cyanobacteria and what is the diversity of possible mechanisms that can adaptively keep time (including modeling)? (Chapters in this volume by: Miwa, Golden and LiWang, Rust, Iwasaki, Mori and Uchihashi, Axmann, Kim and Kim, Akiyama, Ito, Nishiwaki-Ohkawa, Byrne)

2. Evolution and adaptiveness of clocks; how do they enhance fitness? (Chapters in this volume by: Jabbur, Rust, Ito)
3. Harnessing clock properties to enhance bioproduction (Chapter in this volume by: Wang)
4. Ecology of clocks in nature; interactions within communities of clocks in nature (Chapters in this volume by: Hörnlein and Bohuis, Hevroni and Philosof)
5. Interactions among clocks, e.g., microbiomes and their hosts, in some cases with health implications (Chapters in this volume by: Heinemann, Tran Graniczkowska and Cassone)

On a more personal level, this book marks another turning point. The scientists who initiated the study of circadian rhythms in cyanobacteria are reaching the stage of their careers and lives to "pass the baton" to younger researchers. This book is dedicated to three different researchers of cyanobacterial clocks, but primarily to Takao Kondo on the event of his retirement. And the other pioneers will soon follow (the pioneers of rhythms in *Synechococcus* RF-1, Drs. Tan-Chi Huang and Tsung-Hsien Chen, have already retired 15 years ago). At this opportune moment, we are looking backward and looking forward. The cycle of investigators and investigations reinitiates, in a fashion reminiscent of a poem by Theodor Seuss Geisel (aka "Dr. Seuss"):

"How did it get so late so soon? It's night before it's afternoon. December is here before it's June. My goodness how the time has flewn. How did it get so late so soon?"

Nashville, TN, USA — Carl Hirschie Johnson
Chicago, IL, USA — Michael Joseph Rust

Contents

The Bacterial Perspective on Circadian Clocks

Carl Hirschie Johnson and Michael Joseph Rust

Abstract Prokaryotes were long thought to be incapable of expressing circadian (daily) rhythms. Research on nitrogen-fixing cyanobacteria in the 1980s squashed that dogma and showed that these bacteria could fulfill the criteria for circadian rhythmicity. Development of a luminescence reporter strain of *Synechococcus elongatus* PCC 7942 established a model system that ultimately led to the best characterized circadian clockwork at a molecular level. The conclusion of this chapter lists references to the seminal discoveries that have come from the study of cyanobacterial circadian clocks.

1 The "No Clocks in Proks" Dogma

Circadian (daily) rhythms are fundamental control systems that regulate a wide variety of biological phenomena, such as behavior, development, metabolism, and gene expression (Dunlap et al. 2004). These "biological clocks" help organisms adapt to the dramatic daily transformation of their environment, particularly daily changes in light, temperature, humidity, and so on. Circadian rhythms are defined by three major phenomenological criteria that are well established (see Box 1). The first criterion is that circadian rhythms persist in constant conditions (i.e., constant temperature and either constant light or constant darkness) with a period of approximately 24 h. The second criterion is that these rhythms are temperature compensated, so that they proceed at almost the same rate (= same period) within a permissive range of constant ambient temperature. Finally, the third criterion is that these endogenous rhythms of approximately 24 h can be entrained to exactly

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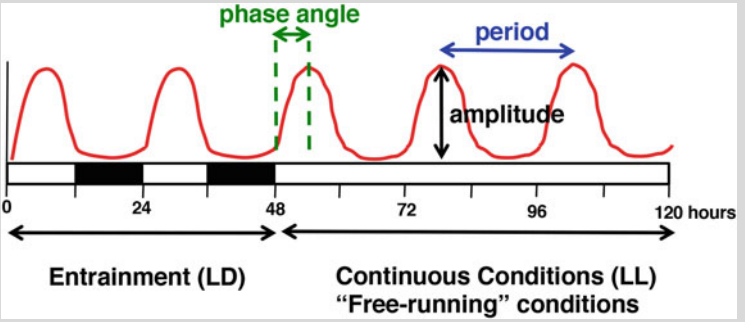
24 h by daily cues in the environment, such as light/dark cycles, temperature cycles, social cues, etc.

Box 1 Three Criteria Define Circadian Rhythmicity

Three major phenomenological criteria, rather than molecular mechanisms, define circadian biological clocks:

1. Circadian clocks display free-running rhythms that oscillate with a self-sustained period that is near, but not exactly, 24 h when environmental conditions such as temperature and either light or darkness are constant.
2. Circadian clocks exhibit "temperature compensation" which ensures that the period of the free-running rhythm remains nearly constant at different constant ambient temperatures; thus, such timing systems remain relatively accurate by not running too fast on warm days or too slowly on cold days.
3. Circadian rhythms entrain to relevant environmental 24-h cycles, e.g., when an organism is exposed to a particular 24-h light-dark cycle, its rhythm will align with a specific phase relationship to that environmental cycle and take on a period of exactly 24 h.



Prior to the 1980s, chronobiologists had decided that there were "no clocks in proks" (aka prokaryotes), so the dogma became that circadian rhythms were exclusively expressed by eukaryotic organisms. A few dissenting reports (Halberg and Conner 1961; Sturtevant 1973) had not been convincing. For example, a suggestion by Franz Halberg that *E. coli* might have a circadian clock (Halberg and Conner 1961), was based on a statistical analysis of an old study by Rogers and Greenbank (1930) in which *E. coli* cells were grown in rich nutrient medium in an apparatus whose temperature control was almost certainly poor. Therefore, the possible daily trends extracted by the statistical analyses (Halberg and Conner 1961) were likely to be merely a result of a diurnal cycle of temperature, to which the growth rate of *E. coli* is exquisitely sensitive. This and other unconvincing studies led chronobiologists to conclude that prokaryotic cells, either unicellular or multicellular, were too

"simple" to express circadian behavior (Ehret and Wille 1970; Schweiger and Schweiger 1980; Kippert 1986), despite the fact that there were almost no published reports of rigorous tests of the proposition (One exception was that of Taylor [1979] who did perform a rigorous test). Some models of the circadian mechanism in eukaryotic cells relied on this "no clocks in proks" dogma as a necessary factor so that the models implicated eukaryotic-specific features (e.g., organelles, eukaryotic-specific transcription) as intrinsic parts of the circadian clockwork (Ehret and Trucco 1967; Schweiger and Schweiger 1977; Kippert 1986). Kippert argued as late as 1991 that the evidence for circadian rhythms in prokaryotic cyanobacteria was equivocal (Kippert 1991); his concerns were based on the possible influence of an unidentified exogenous factor. However, that possibility was ruled out by experiments in which rhythms were measured simultaneously from cyanobacterial cultures that had been entrained to light/dark cycles that were 180° out of phase, and that continued to free-run out of phase when released into LL (Kondo et al. 1993; Aoki et al. 1995). There is no doubt at this time that at least cyanobacteria among bacteria have a *bona fide* circadian clockwork mechanism.

While the adaptive significance of a daily clock to the photosynthetic cyanobacteria may now seem self-evident in retrospect (see next section), it might still be argued that a 24-h clock is inconsistent with the rapid-growth lifestyle of many non-photosynthetic prokaryotes. Is it possible that other prokaryotes have circadian clocks? This is a challenging question, with momentous implications for understanding the early evolution of circadian rhythmicity. Many bacteria experience substantial environmental changes during the day and night. For example, most free-living bacteria are exposed to daily cycles of light and darkness and/or temperature (Fig. 1). Even the gut microbiota is exposed to daily changes in the intestinal environment, as most animal hosts eat on a daily schedule—usually during the day for diurnal animals and during the night for nocturnal animals—which creates a daily rhythm of feast and fast in the digestive tract (Johnson et al. 2017; Jabbur et al. 2021). Anticipating daily changes might also facilitate protective responses in bacteria that are exposed to the deleterious effects of sunlight (Fig. 1). The ultraviolet (UV) component of sunlight damages DNA, but even visible components of sunlight are absorbed by cytochromes in the electron-transport chain and affect metabolism (Robertson et al. 2013). The effects of sunlight inspired the "Escape From Light" hypothesis for the original evolution of circadian clocks, which proposed that the predominant selective pressure was the daily cycles of light and darkness in which light impaired growth and metabolism, damaged nucleic acids, etc. (Pittendrigh 1965, 1993). Therefore, the fact that many bacteria experience strongly rhythmic environments means that many bacteria might have evolved a circadian system in respond to these selective forces.

Compared with clocks in eukaryotes, bacterial clocks may be more similar to the ancestral clocks that first evolved on Earth, and therefore the most informative in terms of understanding the evolution of circadian systems (Johnson et al. 2017; Jabbur et al. 2021). We think that there is an excellent chance that circadian clocks will be found in bacteria other than cyanobacteria. One line of evidence is that the *kaiB* and *kaiC* genes of the cyanobacterial *kaiABC* clock gene cluster (Ishiura et al.

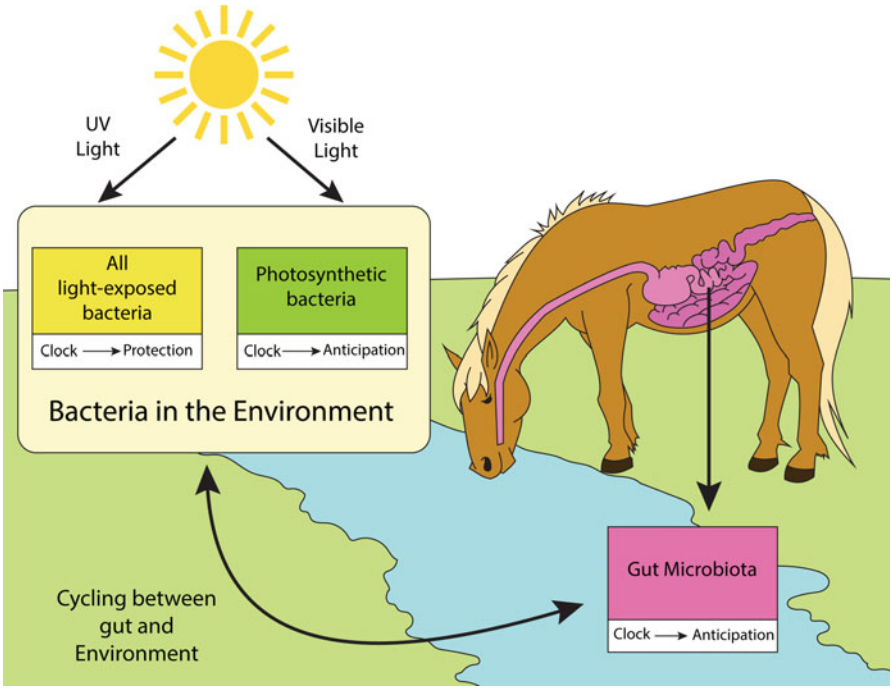


Fig. 1 Many bacteria are exposed to daily selective pressures. Free-living bacteria can be exposed to daily cycles of light and temperature that affect the viability (for example, exposure to UV light) and/or provide energy (for example, through photosynthesis). Even the gut microbiota is often exposed to daily cycles of nutrients, owing to the rhythmic eating habits of the host and temperature, as most animal hosts have daily rhythms of body temperature that are metabolically controlled in endotherms and behaviorally controlled in ectotherms. These same bacteria may be exposed to daily environmental cycles of light and temperature following excretion from the gut (Modified from Johnson et al. 2017)

1998) have homologs among many other eubacterial and Archaeal species, including playing important roles in signal transduction among Archaea (Makarova et al. 2017). It is also quite reasonable that circadian systems may have evolved in non-cyanobacterial prokaryotic species independently of the *kai* system; therefore, the absence of *kaiBC* in any given bacterial species should not be taken to be evidence of the absence of a circadian clock. The key to discover circadian clocks in non-cyanobacterial prokaryotes will be to find the proper conditions and to discover an appropriate parameter to measure. For example, *E. coli* cells may not express a circadian rhythm under optimal growth conditions, such as those encountered in the rich intestinal environment or in the laboratory. But *E. coli* cells can live under very different conditions in nature, for example, in soil or water after excretion from a host, barely surviving until they are again ingested (Fig. 1). In these sub-optimal conditions, a daily clock could again become adaptive. A final evolutionary aspect to consider is whether the ancestral relationship of cyanobacteria to the progenitor of

the chloroplast of higher plants (Sánchez-Baracaldo et al. 2017) means that the clock mechanism of a cyanobacterium-like prokaryote was passed by endosymbiosis into its host? While this remains an open question, at this time there is no evidence for such a postulate since no *kai* gene homologs have been found in the nuclear or chloroplast genomes of higher plants.

2 Data Dethroned the Dogma: Circadian Rhythms in Cyanobacteria

In the mid-1980s, several papers were published that began to unseat the "no clocks in proks" dogma (Stal and Krumbein 1985; Mitsui et al. 1986; Grobbelaar et al. 1986). Those researchers reported that diazotrophic cyanobacteria (unicellular *Synechococcus* sp. Miami BG 43511 and 43522; unicellular *Synechococcus* sp. RF-1, and filamentous but non-heterocystous *Oscillatoria*) display daily rhythms of nitrogen fixation in light/dark cycles and in constant light. One of these studies showed that the cyanobacteria also express a daily rhythm of photosynthesis which was 180° out of phase from the nitrogen-fixation rhythm; photosynthesis peaked at midday, while nitrogen fixation occurred at night (Mitsui et al. 1986).

These reversed-phase relationships are particularly interesting from the perspective of adaptive significance. The nitrogen-fixing enzyme nitrogenase is inactive in the presence of even tiny amounts of oxygen, which creates a major design problem for photosynthetic diazotrophs like nitrogen-fixing cyanobacteria whose photosynthesis releases oxygen (Gallon 1992). Nitrogen-fixing organisms have contrived differing solutions to this problem, notably the spatial separation of photosynthesis and nitrogen fixation in filamentous cyanobacteria that develop specialized non-photosynthesizing cells (heterocysts) for fixing nitrogen. Spatial segregation may not be a practical solution in tiny unicellular bacteria, however. As one of several tactics to accomplish mutually incompatible tasks, some unicellular cyanobacterial strains separate photosynthesis and nitrogen fixation temporally (Gallon 1992; Mitsui et al. 1986; Grobbelaar et al. 1987; Schneegurt et al. 1994). Rhythms of both photosynthesis and nitrogen fixation continued in constant light and were entrained by prior light/dark cycles (Stal and Krumbein 1985; Mitsui et al. 1986; Grobbelaar et al. 1986; Huang and Grobbelaar 1995), so two of the crucial properties of circadian rhythmicity were satisfied.

The third crucial circadian characteristic—temperature compensation—was initially demonstrated in several strains within the genus *Synechococcus*. In the marine *Synechococcus* WH7803, Sweeney and Borgese (1989) found temperature-compensated rhythms of cell division. In the freshwater *Synechococcus* RF-1 isolated from rice fields, the Taiwanese group reported temperature-compensated rhythms of nitrogen fixation and amino acid uptake (Huang et al. 1990; Chen et al. 1991). Using bacterial luciferase reporters of gene expression (Liu et al. 1995a; Andersson et al. 2000), temperature compensation and the other salient properties of circadian

rhythms were demonstrated in *Synechococcus elongatus* PCC 7942 (Kondo et al. 1993), *Synechocystis* PCC 6803 (Aoki et al. 1995), *Anabaena* PCC 7120 (Kushige et al. 2013), and *Thermosynechococcus elongatus* (Onai et al. 2004). The temperature compensation exhibited by the thermophilic *Thermosynechococcus* is particularly spectacular: a Q_{10} of 1.08 over a range of more than 30 °C, from 30 °C to 60 °C (Onai et al. 2004)!

3 Establishing the *Synechococcus elongatus* PCC 7942 Model System

The aforementioned studies on the species *Synechococcus* RF-1, *Synechococcus* WH7803, *Synechococcus* 43511/43522, and *Oscillatoria* demonstrated that the canonical properties of circadian clocks (Box 1) were demonstrable in prokaryotic cyanobacteria. Strangely, the paradigm-shifting significance of those studies was not widely appreciated among the Chronobiology community. It was at that time that Takao Kondo, Carl Johnson, Susan Golden, and Masahiro Ishiura entered the study of cyanobacterial clocks. Other chronobiologists started to take seriously the idea that cyanobacteria were ready to join the menagerie of circadian organisms, probably because Carl Johnson and Takao Kondo were already "card-carrying" chronobiologists who were well known in the field (which is a sad reflection on the predominance of personalities over evidence in persuasion). Kondo/Johnson/Golden/Ishiura recognized that rapid future progress would depend upon using an organism with excellent genetic techniques and automatable data collection. The previously used strains (*Synechococcus* RF-1 et al.) did not have well-established genetic techniques, and moreover, the rhythms that were measured (nitrogen fixation, amino acid uptake, cell division, et al.) required laborious manual measurements. Consequently, while the earlier studies demonstrated the existence of circadian clocks in prokaryotes, they did not identify an optimal organism for an extensive molecular/genetic analysis of the clock mechanism.

To reap the technical benefits that prokaryotes potentially offer for an in-depth analysis of clock mechanisms and evolution, Kondo/Johnson/Golden/Ishiura identified a cyanobacterium that was amenable to molecular/genetic analyses, and that could be engineered to express circadian rhythms of a parameter that can be assayed continuously for many cycles by an automated system. Susan Golden provided an optimal cyanobacterium and genetic techniques, and Kondo/Johnson provided the clock background and technical expertise for rhythm collection (Johnson and Xu 2009; Dedication to Takao Kondo in this volume). The "optimum cyanobacterium" that Susan Golden contributed was the genetically tractable *Synechococcus elongatus* PCC 7942 with the *Vibrio harveyi* luciferase gene cassette (*luxAB*) expressed under control by the promoter for the photosystem II gene, *psbAI* (Kondo et al. 1993; Andersson et al. 2000). The luminescence rhythm expressed by this reporter strain in liquid cultures or from single colonies on agar medium was

assayable by an automated monitoring system such that the luminescence rises during the day and falls during the night (Johnson and Xu 2009; Dedication to Takao Kondo in this volume). The luminescent glow rhythm was an accurate reporter of gene expression, confirming the hypothesis that this glow rhythm reflects circadian control over the promoter of the *psbAI* gene (Liu et al. 1995a).

While it could be argued that the first paper on circadian rhythms in *Synechococcus elongatus* 7942 (Kondo et al. 1993) did not add very much conceptually to the demonstration of circadian clocks in prokaryotes that had been accomplished by the aforementioned studies in *Synechococcus* RF-1, *Synechococcus* WH7803, *Synechococcus* 43511/43522, and *Oscillatoria* (Hall and Rosbash 1993), several key characteristics of the *Synechococcus elongatus* system were the harbinger of a terrific new model system:

1. The *luxAB* luciferase reporter enabled the automated recording of rhythms and relatively high throughput screening of mutant strains.
2. *S. elongatus* 7942 has only one copy of the *kaiABC* cluster, which simplified circadian genotype/phenotype relationships (many other cyanobacterial species have several copies of *kaiB* and/or *kaiC*, which would have complicated the genetic analyses of the clockwork if they had been the first system to be tested).
3. Genetic tools (developed by Susan Golden's lab and other laboratories):

   S. elongatus undergoes homologous recombination, which allows precise genetic targeting and complementation.
   S. elongatus is naturally competent and therefore is easily transformed.
   Both transformation and conjugation techniques had been developed for *S. elongatus* when the circadian analyses started.
   S. elongatus was optimal for a saturational mutagenesis approach because it has a small genome (smaller than *E. coli* !) for which the complete genome sequence became available early in the circadian analyses.

4. *S. elongatus* is easy to grow in liquid cultures or on agar plates and exhibits "simple" prokaryotic genetic organization.

The coincidence of good fortune, clever ideas, "prepared minds," and hard work has transformed the *Synechococcus elongatus* system into the best-understood clockwork at the molecular level in *any* organism. Jacques Monod, in his Nobel Prize acceptance speech, said, "The ambition of molecular biology is to interpret the essential properties of organisms in terms of molecular structures" (Monod 1966). That is an ambitious goal. But, in the case of the circadian clockwork in cyanobacteria, we are VERY close to attaining Monod's goal. With complete 3-D structures of the key clock proteins individually and in complexes, an in vitro oscillator, and molecular explanations for why the oscillator has a period close to ~24 h (and not, for example, 8 h), we are on the threshold of a truly molecular explanation for the ticking of a circadian clock.

The conclusion of this chapter is a listing of some references to the key discoveries that have come from the study of the (predominantly) *S. elongatus* circadian clock system with a special emphasis on the seminal studies that initiated that

approach/topic (this is an incomplete and subjective listing and some key references may have been inadvertently neglected; if so, please forgive us).

Topics of Bacterial Clocks and Selected Key References

Demonstration of Circadian Clocks in Cyanobacteria. Cyanobacteria were the first and still the only prokaryotes definitively known to express *bona fide* circadian rhythms.

This volume: Introduction; Dedication to Takao Kondo
Grobbelaar et al. (1986)
Kondo et al. (1993)
Johnson et al. (1996)
Johnson and Xu (2009)

Possible Circadian Clocks in Other Bacteria. Other prokaryotes besides cyanobacteria have been suggested to harbor circadian clocks (or "proto" clocks), but this conclusion is not yet widely accepted.

This volume: Introduction; chapters by Jabbur et al.; Axmann; Cassone
Min et al. (2005)
Ma et al. (2016)
Paulose et al. (2016)
Johnson et al. (2017)
Sartor et al. (2019)
Eelderink-Chen et al. (2021)

Communities: The interface between biological clocks in communities, such as within the microbiome and in the natural environment. The temporal relationships among different species living in communities is an under-studied but fascinating topic. Can clock information be passed to cells/organisms of other species? Beyond entrainment, how do circadian clocks in microbes interact with natural environments?

This volume: chapters by Jabbur et al.; Elinav; Keshavarzian; Hörnlein et al.; Hevroni and Philosof
Thaiss et al. (2014)
Voigt et al. (2014)
Leone et al. (2015)
Sartor et al. (2019)
Hellweger et al. (2020)

Clocks in Cyanobacteria; Establishing the *Synechococcus elongatus* System. As discussed in this Chapter, the first circadian clocks discovered in prokaryotes were in cyanobacteria, but not in *S. elongatus*. Nevertheless, once the luciferase reporter system was established in *S. elongatus,* its advantageous characteristics enabled rapid progress in understanding the clock system of cyanobacteria.

This volume: Introduction; Dedication to Takao Kondo pp. XXX
Kondo and Ishiura (1994)
Kondo et al. (1993)