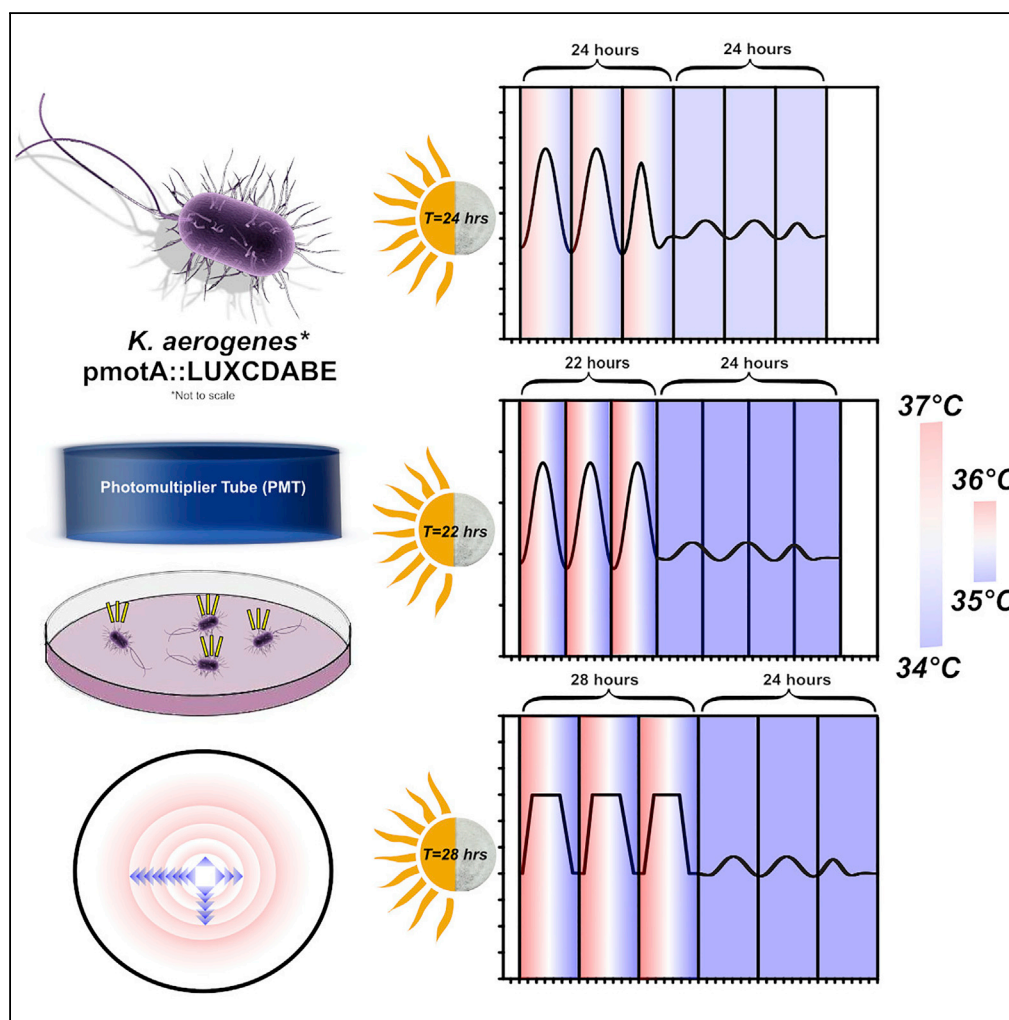**Article**

Entrainment of the Circadian Clock of the Enteric Bacterium *Klebsiella aerogenes* by Temperature Cycles

Jiffin K. Paulose,
Charles V.
Cassone, Kinga B.
Graniczkowska,
Vincent M.
Cassone

vincent.cassone@uky.edu

HIGHLIGHTS

Klebsiella aerogenes, a human gut bacterium, expresses circadian rhythms *in vitro*

The rhythm entrains to temperature cycles of varying periods and amplitudes

The *K. aerogenes* clock can be shifted with heat pulse in a phase-dependent manner

Real-time bioluminescence recording shows a spatiotemporal pattern to the rhythm

Article

Entrainment of the Circadian Clock of the Enteric Bacterium *Klebsiella aerogenes* by Temperature Cycles

Jiffin K. Paulose,[1] Charles V. Cassone,[1] Kinga B. Graniczkowska,[1] and Vincent M. Cassone[1,2,*]

SUMMARY

The gastrointestinal bacterium *Klebsiella (née Enterobacter) aerogenes* expresses an endogenously generated, temperature-compensated circadian rhythm in swarming motility. We hypothesized that this rhythm may be synchronized/entrained *in vivo* by body temperature (T_B). To determine entrainment, cultures expressing bioluminescence were exposed to temperature cycles of 1°C (35°C–36°C) or 3°C (34°C–37°C) in amplitude at periods (T-cycles) of T = 22, T = 24, or T = 28 h. Bacteria entrained to all T-cycles at both amplitudes and with stable phase relationships. A high-amplitude phase response curve (PRC) in response to 1-h pulses of 3°C temperature spike (34°C–37°C) at different circadian phases was constructed, revealing a Type-0 phase resetting paradigm. Furthermore, real-time bioluminescence imaging revealed a spatiotemporal pattern to the circadian rhythm. These data are consistent with the hypothesis that the *K. aerogenes* circadian clock entrains to its host via detection of and phase shifting to the daily pattern of T_B.

INTRODUCTION

Circadian rhythmicity is nearly ubiquitous among eukaryotic organisms and present in at least one prokaryote, the cyanobacterium *Synechococcus elongatus* (Bell-Pedersen et al., 2005). These endogenously generated rhythms of gene expression, physiology, and behavior are expressed in constant environmental conditions and are said to be "free running," expressing an endogenous period of approximately but not exactly 24 h. To be adaptive, endogenous rhythms typically entrain to daily environmental cues or "Zeitgebers," such as the light cycle (LD) or other, non-photic, Zeitgebers to ensure organisms' synchrony with rhythmic environmental conditions. Furthermore, in multicellular organisms, at least, the "biological clock" underlying these rhythms coordinates diverse processes so as to orchestrate advantageous phase relationships among disparate parts of the organisms and among complex physiological processes.

In vertebrate animals, much of the temporal coordination is effected hierarchically by the master pacemaker in the hypothalamic suprachiasmatic nuclei (SCN), which are entrained to LD cycles via the retinohypothalamic tract (Reppert and Weaver, 2002; Moore, 2013). The SCN in turn synchronizes the activities of diverse peripheral oscillators in the brain and elsewhere via a variety of neural and humoral pathways (Earnest et al., 1999; Allen et al., 2001; Schibler et al., 2003; Malloy et al., 2012). These rhythms are the result of a well-described gene network of "positive elements" *clock* and *Bmal1* as well as "negative elements" *period* (*per1, per2, per3*) and *cryptochrome* (*cry1, cry2*) among other clock genes (Reppert and Weaver, 2002; Bell-Pedersen et al., 2005). Among the processes that are orchestrated by this complex web of central and peripheral oscillators are circadian patterns of multiple hormonal signals, such as pineal/enteric melatonin, corticoids, and growth hormone (Moore and Eichler, 1976; Takahashi and Zatz, 1982). Second, autonomic outflow is regulated on a circadian basis as are metabolic rhythms such as oxygen uptake and body temperature (T_B) (Aschoff, 1967).

In addition to the ubiquitous eukaryotic clocks, the cyanobacterium *Synechococcus elongatus* expresses a well-established circadian clock (Cohen and Golden, 2015; Johnson et al., 2017). This single-celled cyanobacterium expresses circadian rhythms in photosynthesis, nitrogen fixation, gene expression, and redox sensing (Cohen and Golden, 2015). The molecular mechanism for the cyanobacterial clock comprises three core clock proteins, KaiA, KaiB, and KaiC (Kondo et al., 1994; Liu et al., 1995). The hexameric KaiC serves as the central component, exhibiting rhythmic autokinase activity followed by autophosphatase activity over the course of the 24-h cycle (Mori et al., 2002; Imai et al., 2004). Phosphorylation is augmented by KaiA (Nishiwaki et al., 2004), whereas phosphatase activity is enhanced by KaiB (Kitayama et al., 2003). Mutation

[1]Department of Biology, University of Kentucky, Lexington, KY 40506, USA

[2]Lead Contact

*Correspondence: vincent.cassone@uky.edu

https://doi.org/10.1016/j.isci.2019.09.007

or knockout of these three proteins abolishes or disrupts circadian rhythms, demonstrating their necessity (Kondo et al., 1994; Ishiura et al., 1998). Furthermore, circadian rhythms of phosphorylation of KaiC can be observed from isolated KaiA, KaiB, and KaiC proteins *in vitro* in the presence of ATP, demonstrating they are sufficient for rhythmicity as well (Nakajima et al., 2005). The KaiABC complex and the circadian rhythm it generates is entrained to the ambient LD cycle through photosynthetic changes in the ATP/ADP ratios (Rust et al., 2011). Although the *S. elongatus* circadian clock remains the only well-characterized prokaryotic circadian clock, the Kai proteins are common in cyanobacterial genomes, suggesting the possibility that other cyanobacteria possess homologous circadian clocks (Dvornyk et al., 2003; Johnson et al., 2017).

Recently we have shown that the enteric proteobacterium, *Klebsiella* (née *Enterobacter*) *aerogenes*, a human gastrointestinal commensal, expresses a temperature-compensated circadian rhythm (Paulose et al., 2016). These bacteria swarm in the presence of the pineal and enteric hormone melatonin, exhibiting band-like patterns with circadian periodicities. On transformation of the bacteria to express *Photorabdus* luciferase (*luxCDABE*) with the promoter for the flagellar stator MotA (*motA::luxCDABE*), rhythms of bioluminescence were observed that were temperature-compensated with a Q_{10} of 0.96. The presence of melatonin had no effect on the presence or absence of bioluminescence rhythmicity or free-running period, but melatonin did synchronize the rhythm, raising the possibility that this hormone may serve as a Zeitgeber for these organisms. However, technical difficulties concerning reliably delivering melatonin cycles on the semi-solid agar have made these analyses difficult.

Daily patterns of core body temperature (T_B) have been known in the human gastrointestinal system for many years (Aschoff, 1967). These rhythms reliably correlate with many physiological processes, including sleep, metabolism, and feeding (Baehr et al., 2000). Daily patterns of core T_B vary among individuals between 1°C and 1.5°C in amplitude, depending on individuals' "morningness" and "eveningness" (Baehr et al., 2000; Waterhouse et al., 2001). Furthermore, rhythms of temperature in ranges of 1°C–4°C entrain and sustain rhythmic patterns of gene expression in cultured fibroblasts and liver explants (Brown et al., 2002a). The present study demonstrates that the *K. aerogenes* circadian clock shows circadian rhythmicity that can be entrained to changes in ambient temperature (T_A) that correspond to changes in T_B. This entrainment pattern is consistent with the hypothesis that *K. aerogenes* may entrain to its host via non-parametric entrainment to the normal rhythm of T_B.

RESULTS

Entrainment

A summary of amplitude, period, and phase results is provided in Table 1. High-amplitude rhythms of bioluminescence were observed during T-cycles at both 1°C and 3°C amplitudes (Figures 1, 2, and 3). In the absence of melatonin, the amplitude of the bioluminescence rhythm during T = 24 at 1°C was 2.133 counts/s $\pm$ 0.215 counts/s (Figure 1G, bottom). During T = 22 at 1°C, the amplitude was 2.186 $\pm$ 0.165 counts/s (Figure 2G, bottom); during T = 28, it was 3.123 $\pm$ 0.125 counts/s (Figure 3G, bottom). T-cycles at 3°C in the absence of melatonin resulted in bioluminescence rhythms with amplitudes of 4.923 $\pm$ 0.944 counts/s at T = 24 (Figure 1G, top), 1.176 $\pm$ 0.173 counts/s at T = 22 (Figure 2G), and 1.926 $\pm$ 0.563 counts/s at T = 28 (Figure 3G, bottom). In the presence of 1 nM melatonin, the bioluminescence rhythm amplitudes were 5.391 $\pm$ 0.939 counts/s for T = 24 (Figure 1G, top), 1.381 $\pm$ 0.191 counts/s for T = 22 (Figure 2G, top), and 1.635 $\pm$ 0.585 counts/s for T = 28 (Figure 3G, top). Owing to the high variability in amplitude, no systematic changes in amplitude were discernible among T-cycles or owing to the presence or absence of melatonin.

The period of the bioluminescence rhythms during the T-cycles (t) for each set of cultures corresponded to the T-cycles in which they were incubated. In T = 24, the average t was 23.632 $\pm$ 0.161 h in the 3°C control-treated cultures, 24.571 $\pm$ 0.416 h in the 3°C melatonin-treated cultures (Figures 1A and 1E), and 26.000 $\pm$ 0.277 h in the 1°C amplitude (Figures 1B and 1E). In T = 22, the average t was 22.250 $\pm$ 0.164 h in the 3°C control-treated cultures, 22.000 $\pm$ 0.189 h in the 3°C melatonin-treated cultures (Figures 2A and 2E), and 23.439 $\pm$ 0.314 h in the 1°C amplitude (Figures 2B and 2E). In T = 28, the average t was 28.000 $\pm$ 0.000 h in the 3°C control-treated and melatonin-treated cultures (Figures 3A and 3E) and 28.581 $\pm$ 0.127 h in the 1°C amplitude (Figures 3B and 3E).

Free Run

Following release into constant low temperature, the amplitudes of all bioluminescence rhythms decreased 10-fold but persisted (Figures 1, 2, and 3C, and 3G). Following T = 24 at 3°C, the average τ

T-Cycle (h)	Temperature Δ (°C)	Condition		Period (h)	Ψ_{EFR} (h)	Amplitude (Counts)
24	3	Control	Entrainment	23.632 ± 0.161	3.8 ± 0.3	4.923 ± 0.500
			Free Run	24.697 ± 0.532		0.944 ± 0.339
		Melatonin	Entrainment	24.571 ± 0.416	1.6 ± 0.1	5.391 ± 0.939
			Free Run	25.929 ± 0.892		0.436 ± 0.049
	1	Control	Entrainment	26.000 ± 0.277	2.4 ± 0.4	2.133 ± 0.215
			Free Run	24.786 ± 0.921		0.299 ± 0.047
22	3	Control	Entrainment	22.250 ± 0.164	1.2 ± 0.4	1.176 ± 0.173
			Free Run	23.750 ± 0.750		0.128 ± 0.026
		Melatonin	Entrainment	22.000 ± 0.189	1.4 ± 1.2	1.381 ± 0.027
			Free Run	24.286 ± 0.944		0.103 ± 0.027
	1	Control	Entrainment	24.522 ± 0.392	1.1 ± 0.5	2.186 ± 0.165
			Free Run	23.132 ± 0.527		0.088 ± 0.011
28	3	Control	Entrainment	28.000 ± 0.000	0.5 ± 0.4	1.926 ± 0.590
			Free Run	23.667 ± 0.843		0.364 ± 0.239
		Melatonin	Entrainment	28.000 ± 0.000	0.3 ± 0.2	1.635 ± 0.621
			Free Run	24.800 ± 1.114		0.573 ± 0.310
	1	Control	Entrainment	28.581 ± 0.127	0.9 ± 2.9	3.123 ± 0.128
			Free Run	23.912 ± 0.373		0.518 ± 0.0428

Table 1. Summary Results for Entrainment and Free-Running Data from All T-Cycle Experiments
Period, phase angle, and amplitude data are provided across T-cycle, temperature amplitude, treatment, and entrainment versus free-running conditions.

was 24.697 ± 0.532 h in control-treated cultures and 25.929 ± 0.892 h in melatonin-treated cultures (Figures 1C and 1E). Following T = 24 at 1°C amplitude, the average τ was 24.786 ± 0.921 h (Figures 1D and 1E). Following T = 22 at 3°C, the average τ was 23.750 ± 0.750 h in control-treated cultures and 24.286 ± 0.944 h in melatonin-treated cultures (Figures 2C and 2E). Following T = 22 at 1°C amplitude, the average τ was 22.376 ± 0.363 h (Figure 2E). Finally, following T = 28 at 3°C, the average τ was 23.667 ± 0.843 h in control-treated cultures and 24.800 ± 1.114 h in melatonin-treated cultures (Figures 3C and 2E). Following T = 28 at 1°C amplitude, the average τ was 23.912 ± 0.373 h (Figures 3D and 2E).

Phase Angle

The phase (φ) of the bioluminescence rhythms, determined as the half-maximal rise for bioluminescence, of the free-running rhythms (φ_FR) largely corresponded to the φ of the entrained rhythm (φ_E). The phase relationship between φ_E and φ_FR (Ψ_{EFR}) in the 1°C T = 24 cycle was 2.4 ± 0.4 h and in the 3°C T = 24 cycle was 3.8 ± 0.3 h in the absence of melatonin and 1.6 ± 0.1 h in the presence of melatonin (Figure 1F). The Ψ_{EFR} in the 1°C T = 22 cycle was 1.1 ± 0.5 h, and the Ψ_{EFR} in the 3°C T = 22 cycle was 1.2 ± 0.4 h in the absence of melatonin and 1.4 ± 1.2 h in the presence of melatonin (Figure 2F). The Ψ_{EFR} in the 1°C T = 28 cycle was 0.9 ± 2.8 h and in the 3°C T = 28 cycle was 0.5 ± 0.4 h in the absence of melatonin and 0.3 ± 0.2 h in the presence of melatonin (Figure 3F).

Phase Response and Transition Curves

Cultures that had been maintained in 34°C for three cycles and then delivered a 1-h pulse of 37°C at different phases of the bioluminescence rhythm initially exhibited a large decrease in bioluminescence, then a very large increase in bioluminescence and a decrease in bioluminescence, all outside the range of detection (Figure 4A). Despite the duration of the temperature shift (1 h), the increase lasted for almost 24 h.
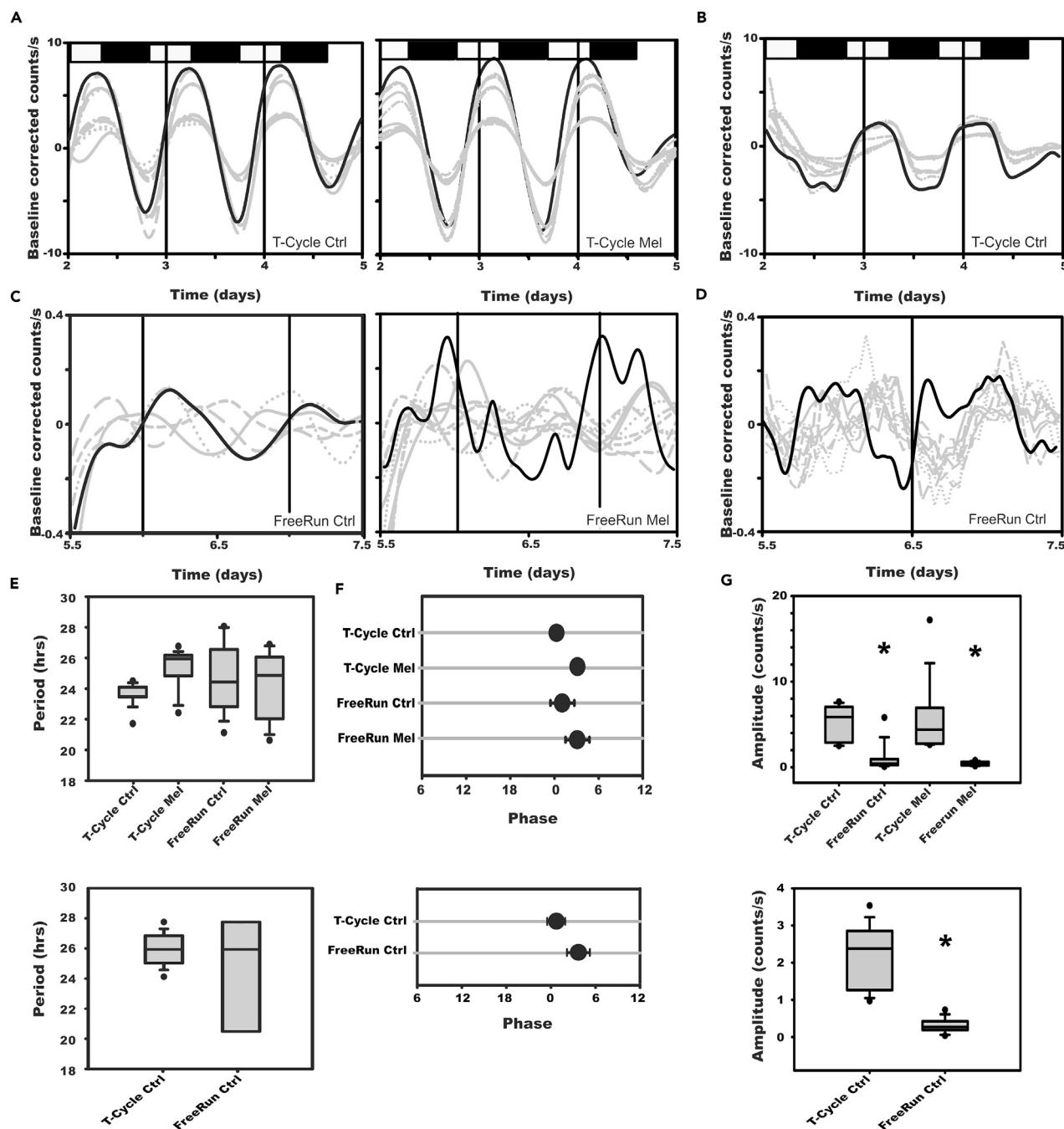
Figure 1. *K. aerogenes motA* Expression Entrains to a 24-h T-Cycle

Bioluminescence from swarming cultures entrained to (A) 3°C or (B) 1°C cycles of 12H:12L. High temperature is indicated by white rectangles and low temperature by black rectangles above entrainment traces in (A) and (B) Representative traces are in black, and other samples determined as rhythmic by MetaCycle are in gray. Rhythms were driven equally well by 3°C in the absence (A, left) or presence (A, right) of 1 nM melatonin. After release into constant low temperature, circadian rhythms persisted in both vehicle-treated (C, left) or melatonin-treated (C, right) cultures. Period analysis (E, top) showed no significant difference in period length in entrained or free-running cultures. Phases of entrained and free-running cultures showed no significant differences (F, top). Amplitudes between entrained and free-running cultures were significantly different in both melatonin-treated and non-treated cultures (G, top). Cycles of temperature of 1°C also entrained cultures (B) and rhythms persisted in constant low temperature (D); however, amplitude was significantly lower than that of 3°C cycling conditions (G, bottom). Period length showed higher variation compared with higher-amplitude temperature cycles (E, bottom), but no significant difference between entrained and free-running cultures was observed. Phases of entrained and free-running cultures under 1°C variation were not significantly different (F, bottom). Amplitudes between entrained and free-running cultures were significantly different under 1°C variation (G, bottom). Asterisks indicate $p < 0.001$ as determined by one-way ANOVA on ranks with Dunn's *post hoc* test.
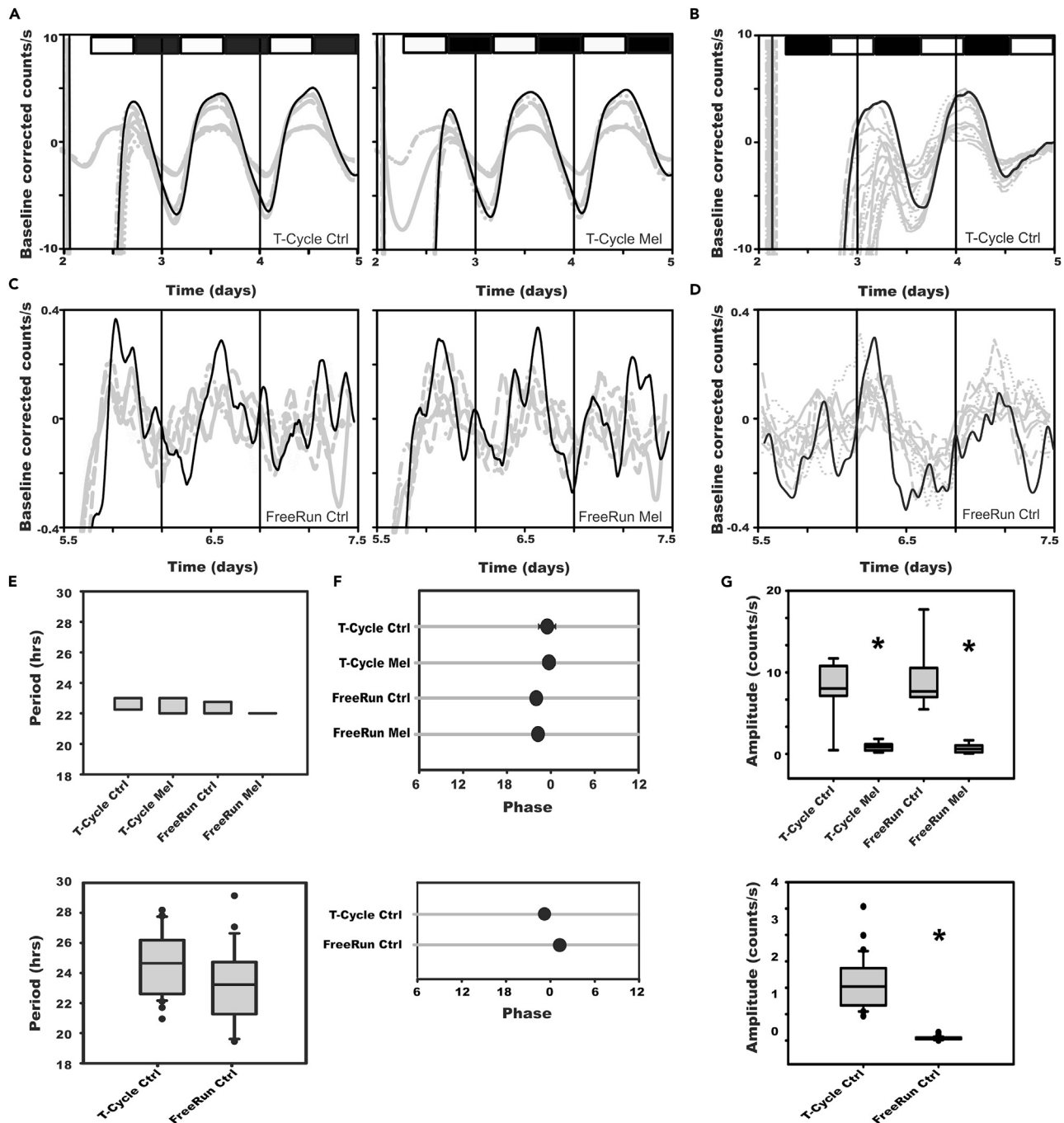
Figure 2. *K. aerogenes motA Expression* Entrains to a 22-h T-Cycle

Bioluminescence from swarming cultures entrained to (A) 3°C or (B) 1°C cycles of 11H:11L. High temperature is indicated by white rectangles and low temperature by black rectangles above entrainment traces in (A) and (B) Representative traces are in black, and other samples determined as rhythmic by MetaCycle are in gray. Rhythms were driven equally well by 3°C in the absence (A, left) or presence (A, right) of 1 nM melatonin. After release into constant low temperature, circadian rhythms persisted in both vehicle-treated (C, left) or melatonin-treated (C, right) cultures. Period analysis (E, top) showed no significant difference in period length in entrained or free-running cultures. Similarly, the phases of entrained and free-running cultures showed no significant differences (F, top). Amplitudes between entrained and free-running cultures were significantly different in both melatonin-treated and non-treated cultures (G, top). Cycles of temperature of 1°C also entrained cultures (B), and rhythms persisted in constant low temperature (D). Period length variability was increased compared with cultures under higher temperature variation (E, bottom), but no significant difference between entrained and free-running cultures was observed. Phases of entrained and free-running cultures under 1°C variation were not significantly different (F, bottom). Amplitudes between entrained and free-running cultures were significantly different under 1°C variation (G, bottom). Asterisks indicate $p < 0.001$ as determined by one-way ANOVA on ranks with Dunn's *post hoc* test.
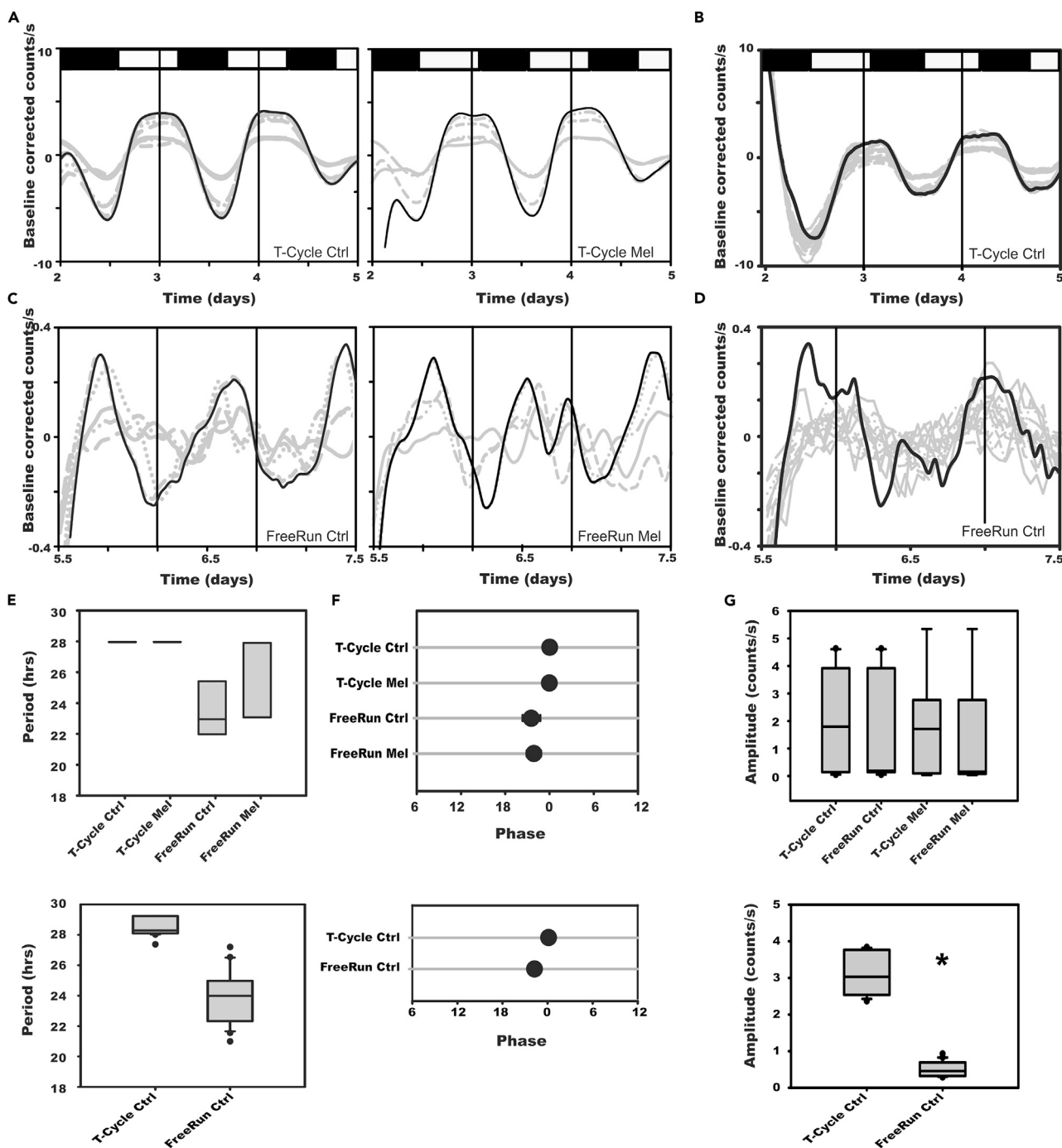
Figure 3. *K. aerogenes motA* Expression Entrains to a 28-h T-Cycle

Bioluminescence from swarming cultures entrained to (A) 3°C or (B) 1°C cycles of 14H:14L. High temperature is indicated by white rectangles and low temperature by black rectangles above entrainment traces in (A) and (B) Representative traces are in black, and other samples determined as rhythmic by MetaCycle are in gray. Rhythms were driven equally well by 3°C in the absence (A, left) or presence (A, right) of 1 nM melatonin. After release into constant low temperature, circadian rhythms persisted in both vehicle-treated (C, left) or melatonin-treated (C, right) cultures. Period analysis (E, top) showed complete entrainment to 28-h T-cycles that decreased upon release into constant low temperature. After aligning peak phase to onset of higher temperature, no significant difference in phase was observed (F, top). Amplitudes between entrained and free-running cultures were not significantly different in either melatonin-treated or non-treated cultures (G, top). Cycles of temperature of 1°C also entrained cultures (B), and rhythms persisted in

Figure 3. *Continued*

constant low temperature (D); however, amplitude was significantly lower than that of 3°C cycling conditions (G, bottom). Period length showed higher variation compared with higher-amplitude temperature cycles (E, bottom), but no significant difference between entrained and free-running cultures was observed. Phases of entrained and free-running cultures under 1°C variation were not significantly different (F, bottom). Asterisks indicate p < 0.001 as determined by one-way ANOVA on ranks with Dunn's *post hoc* test.

Temperature pulses also resulted in phase-dependent changes in φ (Δφ) of the bioluminescence rhythms of these cultures (Figure 4B). In this case, the phase reference (φ$_R$) was the acrophase, or peak, of the bioluminescence rhythm. Calculation of the phase response curve (PRC), in which the φ$_R$ was plotted against the φ$_R$ at which the pulse was delivered (where acrophase corresponded to Circadian Time [CT] 6), revealed a relative insensitivity to the temperature change during the "subjective day" (CT0–CT12). This corresponded to a "dead zone" in the resultant PRC. There was a large delay period (-Δφ) from CT12 to CT18 and a rapid transition to high-amplitude phase advances (+Δφ) from CT18 to CT0.

Calculation of a phase transition curve (PTC), in which the new φ was plotted on the y axis and the old φ was plotted on the x axis, revealed a "Type-0" PTC in that the slope of the resultant linear regression was 0.19 with an r^2 value of 0.11 (Figure 4C).

IVIS Imaging

A very-high-amplitude circadian pattern of bioluminescence was observed in *K. aerogenes* cultures (Figure 5A). Detrending data from two example wells (Figure 5B) show that the average period (τ) was 25.4 $\pm$ 0.4 h. This τ was similar to those observed in our previously published data for cultures at 34°C and 37°C in the absence of melatonin employing the LumiCycle system (with τ = 24.5 $\pm$ 0.5 h; Paulose et al., 2016) and to the average τ described earlier. Because the cultures were in the absence of melatonin, they were out of phase with each other (see Video S1). Interestingly, the spatial pattern of the rhythmic bioluminescence unexpectedly emanated from the inoculation site and proceeded to the colonies' periphery (see Video S1).

DISCUSSION

The present study demonstrates that the circadian clock of the enteric bacterium *K. aerogenes*, which had previously been shown to be temperature-compensated (Paulose et al., 2016), entrains to variations of T$_A$ that are comparable with daily changes in T$_B$. This clock maintains a stable Ψ relative to several T-cycles ranging from T = 22 to T = 28 (Figures 1, 2, and 3). These entrainment data are consistent with both the PRC and PTC that show a Type 0 PRC and PTC, capable of entrainment to any environmental temperature cycle (Figure 4). Furthermore, the IVIS imaging data suggest that the circadian rhythm of *K. aerogenes* is expressed in both a temporal and spatial dynamic such that *pmotA::luxCDABE* bioluminescence rhythmically expanded from and withdrew to the site of bacterial inoculation (Figure 5; Video S1). Entrainment appears to be phase locked to the onset of temperature increase, since the Ψ$_{EFR}$ is most consistent when temperature onset is used as the φ$_E$. This is remarkable in view of the fact that the generation time of *K. aerogenes* is 30 $\pm$ 0.3 min (Kelly and Rahn, 1932), much shorter in duration than the period of a single day. This is similar to the situation in *S. elongatus*, whose doubling time is about 10 h (Mori et al., 1996; Nakajima et al., 2005), in the sense that the generation time is shorter than the circadian period. Obviously, *K. aerogenes'* cell division rate is much more rapid than the rate in the cyanobacterium, so the effect is much more dramatic.

It is worth noting that the amplitudes of bioluminescence rhythms during all T-cycles were significantly higher than those during the free run following the T-cycles. The reasons for this decrease may be manifold. First, the decrease in amplitude may reflect damping of the oscillator underlying the overt rhythm. Although this may be partially the case, the free run persists for at least 4 days following release into constant low temperature. Furthermore, if this were the case, we would also expect to see a broadening of the succeeding bioluminescence traces and/or a change in period during the trace. These are not apparent in the data. Second, since these cultures are on semi-solid agar, it is possible that extended time in this culture environment may expend nutrients in the dish. This is clearly possible, but the decrease in amplitude is a square-wave decline following the release into constant temperature. Third, and similarly, the decrease may reflect a decrease in ambient oxygen content in the sealed dishes. O$_2$ necessary for aerobic conditions for this organism, and it is also a required cofactor for the bioluminescence mechanism itself. As with the nutrient argument, we would expect a gradual decline in amplitude rather than the abrupt decrease we
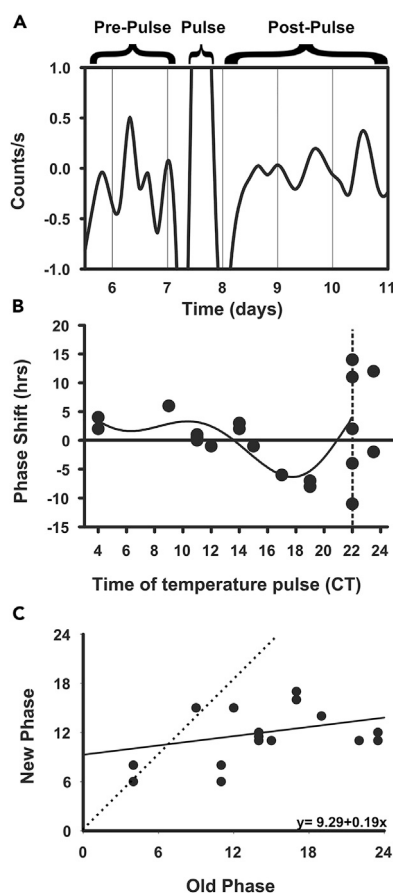
Figure 4. *K. aerogenes* Exhibits "Type 0" Phase Resetting Behavior in Response to Temperature

Schematic (A) describing the experimental design of recording 3 days of free running (after 3 days of 3°C 12H:12L entrainment) followed by a 1-h pulse of 3°C and return to free-running conditions for an additional 3 days. PRC (B) generated by plotting the magnitude of phase shift across 10 phases. Each data point represents a single culture, with multiple experiments comprising the entire dataset. CT6 here is defined as the pre-pulse peak of bioluminescence. A Phase Transition Curve (C) was generated by plotting the pre-pulse phase against the post-pulse phase from the PRC. Linear regression analysis is indicated by the solid line segment. The dashed line segment represents a slope equal to 1.

see. The most likely reason is that entrainment of the oscillator(s) themselves increases the rhythm amplitude of the underlying oscillation. We are currently exploring a different culture method to address these issues.

The host circadian clock is known to affect the composition and luminal location of the intestinal microbiota, of which *K. aerogenes* is a component. Although total microbial activity may not vary over the time of day, individual taxa are rhythmic with different phases over the course of the day (Liang et al., 2014; Thaiss et al., 2014). Disruption of the host's circadian clock by *per1/per2* double knockout (Thaiss et al., 2014), by *Bmal1* knockout (Liang et al., 2015), or by *Clock* mutation (Voigt et al., 2016) alters fecal bacterial composition. In addition to daily changes in microbial composition, rhythms of the biogeography of motile bacteria within the gastrointestinal tract are such that bacteria associate with the intestinal epithelia of mice during the night (Thaiss et al., 2016). Simulated jetlag also disrupts daily patterns of metabolic pathways (Kyoto Encyclopedia of Genes and Genomes; KEGG), such as vitamin metabolism, nucleotide metabolism, and two component/secretion systems (Thaiss et al., 2014), and alters the gut microbiota configuration via increasing Firmicutes bacteria and decreasing Bacteroides (Voigt et al., 2014).

The present data raise the interesting idea that at least this particular member of the human microbiome synchronizes to the gastrointestinal clock (Hoogerwerf et al., 2007; Hoogerwerf, 2010) via a circadian sensitivity to small, daily, and circadian changes in T_B. In this regard, one could consider this and other members
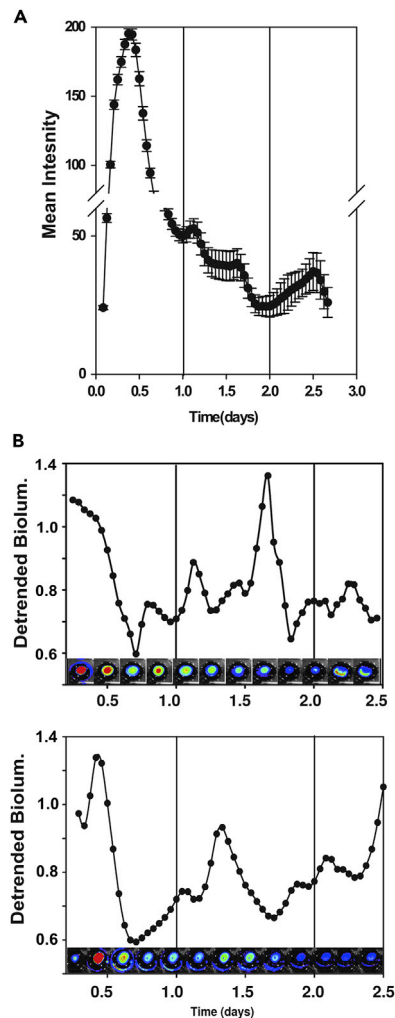
Figure 5. Real-Time Bioluminescence Imaging of *K. aerogenes*

Bioluminescence was measured from a 24-well cell culture plate containing swarming cultures of *K. aerogenes* initially high-amplitude rhythm that damps over time (A). Two representative wells (B) are shown with inset images of the quantified signal at 4-h intervals after baseline correction. The full plate can be seen in Video S1. Average period of bioluminescence from all intact cultures was ~25.0 h. Cultures that cracked during the imaging were not included in the analysis.

of the microbiome as peripheral oscillators, under the control of the mammalian circadian system writ large (Schibler et al., 2003; Brown and Azzi, 2013). In this sense, the idea that T_B may act as an "internal Zeitgeber" in the entrainment of peripheral oscillators is not new. Brown et al. (2002b) have shown that *ex vivo* explants of liver tissue and cultured fibroblasts could be entrained to cycles of T_A. These authors suggested that daily changes in T_B could serve to synchronize peripheral clocks.

Rhythms of T_B are not the only Zeitgeber for either peripheral oscillators or the microbiome. Certainly, in the case of peripheral tissues, endocrine signals such as melatonin or glucocorticoids (Cassone, 1990; Challet, 2015; Cuesta et al., 2015) are direct outputs of the central pacemaker in the SCN and synchronize peripheral rhythms. Furthermore, it is known that circadian regulation of sympathetic activity influences and/or affects circadian rhythms in pineal melatonin (Klein et al., 1997), cardiovascular function (Warren et al., 1994; Agarwal, 2010), hepatic gene expression (Cailotto et al., 2005; Vujović et al., 2008), and gastrointestinal activity (Malloy et al., 2012).

In the case of the gastrointestinal system, all three of these pathways, metabolic (T_B), endocrine, and sympathetic, may play roles in synchronizing the intestinal microbiome. The gastrointestinal system comprises

a circadian clock, expressing circadian rhythms in gene expression (Davidson et al., 2003; Hoogerwerf et al., 2007, 2008; Sladek et al., 2007), intestinal motility (Hoogerwerf et al., 2007; Hoogerwerf, 2010; Malloy et al., 2012), and electrolyte absorption (Soták et al., 2011) *in vivo* and *in vitro*. Although these rhythms persist *in vitro*, they are influenced by input from the circadian system, since *per1/per2* double knockout (Hoogerwerf et al., 2010), SCN lesion (Malloy et al., 2012), and constant light (LL) (Polidarová et al., 2011) abolish them when food is available *ad libitum* but are restored in both cases by timed feeding.

It is interesting to note that, although the circadian clock in *K. aerogenes* was originally identified because of the bacterium's sensitivity to melatonin and although nanomolar concentrations of melatonin synchronize circadian patterns of bioluminescence (Paulose et al., 2016), we could find no evidence that melatonin influences the bacterium's sensitivity to T_A. It is not clear whether gastrointestinal melatonin, which is secreted by the intestines themselves (Söderquist et al., 2015), serves as an additional Zeitgeber.

It is also unclear how swarming, both as a physiological state and a population-dependent behavior, is related to the circadian rhythm in *K. aerogenes*. The entrained and free-running rhythms observed here are being generated by tens of millions of bacteria that are coordinating the activation of motor flagella. The pattern of swarming, resembling a "bulls-eye" pattern, has been extensively reported in *Proteus mirabilis*. In this species, two distinct populations of cells exist; swarming cells that rely on environmental conditions and cell-cell contact move away from the initial inoculation point in a coordinated fashion and then dedifferentiate into swimming cells that undergo cell division (Rather, 2005; Copeland and Weibel, 2009). This process is repeated at the swarm edge, resulting in the formation of the concentric rings that form the bullseye. *K. aerogenes*, according to our bioluminescence data, maintains a population of cells at the inoculation point. This appears to be the origin of each round of swarming, and previous generations are overlapped by the succeeding generation. This would explain the pattern of bioluminescence observed in real-time imaging of swarming colonies, where each wave of bioluminescence originates at the inoculum site and radiates outward to the edge of the wells (Figure 5B and Video S1).

The picture that emerges is a complex series of nested circadian clocks, each dependent on the other. The SCN is a master pacemaker that entrains to the LD cycle and in turn entrains multiple peripheral oscillators throughout the body through multiple pathways, including changes in T_B, endocrine signals, and neural outputs (Moore, 2013). Among these peripheral oscillators is the gastrointestinal system, which houses a semi-independent circadian system that controls many gastrointestinal functions (Hoogerwerf et al., 2007; Sladek et al., 2007). These are modified by system inputs directly from the SCN via sympathetic activity or endocrine signals and indirectly via changes in T_B. Timed feeding can independently influence gastrointestinal circadian φ (Malloy et al., 2012). Nested within the gastrointestinal clock, the microbiome differentially expresses multiple rhythms in KEGG pathways as well as changes in the spatial distribution of bacteria between luminal and surface components (Liang et al., 2014; Thaiss et al., 2014, 2016). These may be expressed at different φ's within the circadian cycle. Finally, nested within the intestinal microbiome, at least one species of bacteria, *K. aerogenes*, expresses its own independent circadian clock (Paulose and Cassone, 2016), which can synchronize rhythms of motility and gene expression to the host's rhythm on T_B and perhaps other signals such as melatonin. The questions that arise are manifold. Does the rhythm we see in *K. aerogenes in vitro* persist in the lumen of the gut *in vivo*? Are the mechanisms by which *K. aerogenes* keep time homologous to cyanobacterial circadian clocks? Preliminary data described in Paulose et al. (2016) suggest at least similarity of *kaiABC* genes to genes within the *K. aerogenes* genome; however, no orthologous functions were found. If this is the case, it points to the remarkable conclusion that the emergence of circadian clocks predates oxygen-evolving photosynthesis! Finally, how does the rhythmic microbiome influence the host's circadian clock? These are all questions that are at least now addressable with the identification of a single component of the microbiome as part of this meta-biological clock.

Limitations of the Study

We have shown that the human gut commensal bacterium *K. aerogenes* is capable of entraining to T-cycles of 24, 22, and 28 h at temperature differentials of 1°C and 3°C and have described the dynamics of this entrainment by way of PRC. Some of the bioluminescence rhythms (i.e., those in free-running conditions) are low in relative amplitude and absolute quantity. This is likely due to nutrient depletion and could be remedied by using liquid cultures, protocols for which we are currently exploring.

REFERENCES

Agarwal, R. (2010). Regulation of circadian blood pressure: from mice to astronauts. Curr. Opin. Nephrol. Hypertens. *19*, 51–58.

Allen, G., Rappe, J., Earnest, D.J., and Cassone, V.M. (2001). Oscillating on borrowed time: diffusible signals from immortalized suprachiasmatic nucleus cells regulate circadian rhythmicity in cultured fibroblasts. J. Neurosci. *21*, 7937–7943.

Aschoff, J. (1967). Human circadian rhythms in activity, body temperature and other functions. Life Sci. Space Res. *5*, 159–173.

Baehr, E.K., Revelle, W., and Eastman, C.I. (2000). Individual differences in the phase and amplitude of the human circadian temperature rhythm: with an emphasis on morningness-eveningness. J. Sleep Res. *9*, 117–127.

Bell-Pedersen, D., Cassone, V.M., Earnest, D.J., Golden, S.S., Hardin, P.E., Thomas, T.L., and Zoran, M.J. (2005). Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat. Rev. Genet. *6*, 544–556.

Brown, S.A., Zumbrunn, G., Fleury-Olela, F., Preitner, N., and Schibler, U. (2002a). Rhythms of mammalian body temperature can sustain peripheral circadian clocks. Curr. Biol. *12*, 1574–1583.

Brown, S.A., Zumbrunn, G., Fleury-Olela, F., Preitner, N., and Schibler, U. (2002b). Rhythms of mammalian body temperature can sustain peripheral circadian clocks. Curr. Biol. *12*, 1574–1583.

Brown, S.A., and Azzi, A. (2013). Peripheral circadian oscillators in mammals. Handb. Exp. Pharmacol. *217*, 45–66.

Cailotto, C., La Fleur, S.E., Van Heijningen, C., Wortel, J., Kalsbeek, A., Feenstra, M., Pévet, P., and Buijs, R.M. (2005). The suprachiasmatic nucleus controls the daily variation of plasma glucose via the autonomic output to the liver: are the clock genes involved? Eur. J. Neurosci. *22*, 2531–2540.

Cassone, V.M. (1990). Effects of melatonin on vertebrate circadian systems. Trends Neurosci. *13*, 457–464.

Challet, E. (2015). Keeping circadian time with hormones. Diabetes Obes. Metab. *17*, 76–83.

Cohen, S.E., and Golden, S.S. (2015). Circadian rhythms in cyanobacteria. Microbiol. Mol. Biol. Rev. *79*, 373–385.

Copeland, M.F., and Weibel, D.B. (2009). Bacterial swarming: a model system for studying dynamic self-assembly. Soft Matter *5*, 1174–1187.

Cuesta, M., Cermakian, N., and Boivin, D.B. (2015). Glucocorticoids entrain molecular clock components in human peripheral cells. FASEB J. *29*, 1360–1370.

Davidson, A.J., Poole, A.S., Yamazaki, S., and Menaker, M. (2003). Is the food-entrainable circadian oscillator in the digestive system? Genes Brain Behav. *2*, 32–39.

Dvornyk, V., Vinogradova, O., and Nevo, E. (2003). Origin and evolution of circadian clock genes in prokaryotes. Proc. Natl. Acad. Sci. U S A *100*, 2495–2500.

Earnest, D.J., Liang, F.Q., Ratcliff, M., and Cassone, V.M. (1999). Immortal time: circadian clock properties of rat suprachiasmatic cell lines. Science *283*, 693–695.

Hoogerwerf, W.A., Hellmich, H.L., Cornélissen, G., Halberg, F., Shahinian, V.B., Bostwick, J., Savidge, T.C., and Cassone, V.M. (2007). Clock gene expression in the murine gastrointestinal tract: endogenous rhythmicity and effects of a feeding regimen. Gastroenterology *133*, 1250–1260.

Hoogerwerf, W.A., Sinha, M., Conesa, A., Luxon, B.A., Shahinian, V.B., Cornélissen, G., Halberg, F., Bostwick, J., Timm, J., and Cassone, V.M. (2008). Transcriptional profiling of mRNA expression in the mouse distal colon. Gastroenterology *135*, 2019–2029.

Hoogerwerf, W.A., Shahinian, V.B., Cornélissen, G., Halberg, F., Bostwick, J., Timm, J., Bartell, P.A., and Cassone, V.M. (2010). Rhythmic changes in colonic motility are regulated by period genes. Am. J. Physiol. Gastrointest. Liver Physiol. *298*, G143–G150.

Hoogerwerf, W.A. (2010). Role of clock genes in gastrointestinal motility. Am. J. Physiol. Gastrointest. Liver Physiol. *299*, G549–G555.

Imai, K., Nishiwaki, T., Kondo, T., and Iwasaki, H. (2004). Circadian rhythms in the synthesis and degradation of a master clock protein KaiC in Cyanobacteria. J. Biol. Chem. *279*, 36534–36539.

Ishiura, M., Kutsuna, S., Aoki, S., Iwasaki, H., Andersson, C.R., Tanabe, A., Golden, S.S., Johnson, C.H., and Kondo, T. (1998). Expression of a gene cluster kaiABC as a circadian feedback process in cyanobacteria. Science 281, 1519–1523.

Johnson, C.H., Zhao, C., Xu, Y., and Mori, T. (2017). Timing the day: what makes bacterial clocks tick? Nat. Rev. Microbiol. 15, 232–242.

Kelly, C.D., and Rahn, O. (1932). The growth rate of individual bacterial cells. J. Bacteriol. 23, 147–153.

Kitayama, Y., Iwasaki, H., Nishiwaki, T., and Kondo, T. (2003). KaiB functions as an attenuator of KaiC phosphorylation in the cyanobacterial circadian clock system. EMBO J. 22, 2127–2134.

Klein, D.C., Coon, S.L., Roseboom, P.H., Weller, J.L., Bernard, M., Gastel, J.A., Zatz, M., Iuvone, P.M., Rodriguez, I.R., Bégay, V., et al. (1997). The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. Recent Prog. Horm. Res. 52, 307–357.

Kondo, T., Tsinoremas, N.F., Golden, S.S., Johnson, C.H., Kutsuna, S., and Ishiura, M. (1994). Circadian clock mutants of cyanobacteria. Science 266, 1233–1236.

Liang, X., Bushman, F.D., and FitzGerald, G.A. (2014). Time in motion: the molecular clock meets the microbiome. Cell 159, 469–470.

Liang, X., Bushman, F.D., and FitzGerald, G.A. (2015). Rhythmicity of the intestinal microbiota is regulated by gender and the host circadian clock. Proc. Natl. Acad. Sci. U S A 112, 10479–10484.

Liu, Y., Golden, S.S., Kondo, T., Ishiura, M., and Johnson, C.H. (1995). Bacterial luciferase as a reporter of circadian gene expression in cyanobacteria. J. Bacteriol. 177, 2080–2086.

Malloy, J.N., Paulose, J.K., Li, Y., and Cassone, V.M. (2012). Circadian rhythms of gastrointestinal function are regulated by both central and peripheral oscillators. Am. J. Physiol. Gastrointest. Liver Physiol. 303, G461–G473.

Moore, R.Y. (2013). The suprachiasmatic nucleus and the circadian timing system. Prog. Mol. Biol. Transl. Sci. 119, 1–28.

Moore, R.Y., and Eichler, V.B. (1976). Central neural mechanisms in diurnal rhythm regulation and neuroendocrine responses to light. Psychoneuroendocrinology 1, 265–279.

Mori, T., Saveliev, S.V., Xu, Y., Stafford, W.F., Cox, M.M., Inman, R.B., and Johnson, C.H. (2002). Circadian clock protein KaiC forms ATP-dependent hexameric rings and binds DNA. Proc. Natl. Acad. Sci. U S A 99, 17203–17208.

Mori, T., Binder, B., and 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.

Nakajima, M., Imai, K., Ito, H., Nishiwaki, T., Murayama, Y., Iwasaki, H., Oyama, T., and Kondo, T. (2005). Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. Science 308, 414–415.

Nishiwaki, T., Satomi, Y., Nakajima, M., Lee, C., Kiyohara, R., Kageyama, H., Kitayama, Y., Temamoto, M., Yamaguchi, A., Hijikata, A., et al. (2004). Role of KaiC phosphorylation in the circadian clock system of Synechococcus elongatus PCC 7942. Proc. Natl. Acad. Sci. U S A 101, 13927–13932.

Paulose, J.K., Wright, J.M., Patel, A.G., and Cassone, V.M. (2016). Human gut bacteria are sensitive to melatonin and express endogenous circadian rhythmicity. PLoS One 11, e0146643.

Paulose, J.K., and Cassone, V.M. (2016). The melatonin-sensitive circadian clock of the enteric bacterium Enterobacter aerogenes. Gut Microbes 7, 424–427.

Polidarová, L., Sládek, M., Soták, M., Pácha, J., and Sumová, A. (2011). Hepatic, duodenal, and colonic circadian clocks differ in their persistence under conditions of constant light and in their entrainment by restricted feeding. Chronobiol. Int. 28, 204–215.

Rather, P.N. (2005). Swarmer cell differentiation in Proteus mirabilis. Environ. Microbiol. 7, 1065–1073.

Reppert, S.M., and Weaver, D.R. (2002). Coordination of circadian timing in mammals. Nature 418, 935–941.

Rust, M.J., Golden, S.S., and OShea, E.K. (2011). Light-driven changes in energy metabolism directly entrain the cyanobacterial circadian oscillator. Science 331, 220–223.

Schibler, U., Ripperger, J., and Brown, S.A. (2003). Peripheral circadian oscillators in mammals: time and food. J. Biol. Rhythms 18, 250–260.

Sladek, M., Rybová, M., Jindráková, Z., Zemanová, Z., Polidarová, L., Mrnka, L., O'Neill, J., Pácha, J., and Sumová, A. (2007). Insight into the circadian clock within rat colonic epithelial cells. Gastroenterology 133, 1240–1249.

Söderquist, F., Hellström, P.M., and Cunningham, J.L. (2015). Human gastroenteropancreatic expression of melatonin and its receptors MT1 and MT2. PLoS One 10, e0120195.

Soták, M., Polidarová, L., Musílková, J., Hock, M., Sumová, A., and Pácha, J. (2011). Circadian regulation of electrolyte absorption in the rat colon. Am. J. Physiol. Gastrointest. Liver Physiol. 301, G1066–G1074.

Takahashi, J.S., and Zatz, M. (1982). Regulation of circadian rhythmicity. Science 217, 1104–1111.

Thaiss, C.A., Zeevi, D., Levy, M., Zilberman-Schapira, G., Suez, J., Tengeler, A.C., Abramson, L., Katz, M.N., Korem, T., Zmora, N., et al. (2014). Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. Cell 159, 514–529.

Thaiss, C.A., Levy, M., Korem, T., Dohnalová, L., Shapiro, H., Jaitin, D.A., David, E., Winter, D.R., Gury-BenAri, M., Tatirovsky, E., et al. (2016). Microbiota diurnal rhythmicity programs host transcriptome oscillations. Cell 167, 1495–1510.e12.

Voigt, R.M., Forsyth, C.B., Green, S.J., Mutlu, E., Engen, P., Vitaterna, M.H., Turek, F.W., and Keshavarzian, A. (2014). Circadian disorganization alters intestinal microbiota. PLoS One 9, e97500.

Voigt, R.M., Summa, K.C., Forsyth, C.B., Green, S.J., Engen, P., Naqib, A., Vitaterna, M.H., Turek, F.W., and Keshavarzian, A. (2016). The circadian clock mutation promotes intestinal dysbiosis. Alcohol. Clin. Exp. Res. 40, 335–347.

Vujović, N., Davidson, A.J., and Menaker, M. (2008). Sympathetic input modulates, but does not determine, phase of peripheral circadian oscillators. Am. J. Physiol. Regul. Integr. Comp. Physiol. 295, R355–R360.

Warren, W.S., Champney, T.H., and Cassone, V.M. (1994). The suprachiasmatic nucleus controls the circadian rhythm of heart rate via the sympathetic nervous system. Physiol. Behav. 55, 1091–1099.

Waterhouse, J., Folkard, S., Van Dongen, H., Minors, D., Owens, D., Kerkhof, G., Weinert, D., Nevill, A., Macdonald, I., Sytnik, N., et al. (2001). Temperature profiles, and the effect of sleep on them, in relation to morningness-eveningness in healthy female subjects. Chronobiol. Int. 18, 227–247.

Supplemental Information

Entrainment of the Circadian Clock

of the Enteric Bacterium *Klebsiella aerogenes*

by Temperature Cycles

Jiffin K. Paulose, Charles V. Cassone, Kinga B. Graniczkowska, and Vincent M. Cassone

Transparent Methods

Experimental Model and Subject Details

Bacterial Strains

 K. aerogenes and *E. coli* clinical isolates (gift from Dr. John Seabolt, U. of Kentucky) and DH5α with *luxCDABE* driven by the promoter region of *motA* (gift from Brian Ahmer, Ohio State University), were initially cultured in LB Broth at 37ºC in a shaking incubator. All chemicals were purchased from Sigma (St. Louis, MO) and diluted in water. Transformation of *pmotA::luxCDABE* into K. aerogenes: *K. aerogenes* were made competent by $CaCl_2$ method and *pmotA::luxCDBAE* plasmid extracted from the host strain was transformed into *K. aerogenes* by heat shock. Transformants were selected for on tetracycline-supplemented medium and stored as glycerol stocks for future studies. Details of this transformation are available in Paulose et al. (Paulose *et al.*, 2016).

Method Details

Bioluminescence Recording

 Bacterial bioluminescence has been used as an accurate measure of prokaryotic rhythmicity for some time (Liu *et al.*, 1995). For our Lumicycle (Actimetrics, Il) data, 2µl of overnight cultures were stabbed and released into the center of 35mm culture dishes containing 5 mls of semi-solid EMB agar with or without 1nM melatonin. 1ºC entrainment studies were performed in the absence of melatonin. Plates were sealed with 40mm cover glass by sterile vacuum grease and placed into an automated photomultiplier-based bioluminescence recorder. Each sample was counted for 70 seconds on a rotating platform. Raw bioluminescence baselines were subtracted using a 24-hour running average via Lumicycle Analysis software (Actimetrics, Chicago, IL).

 To confirm bioluminescence rhythms, cultures were inoculated on a 24-well cell culture plate containing semi-solid EMB agar as described above in the absence of melatonin and placed in a Perkin Elmer *In Vivo* Imaging System Spectrum (IVIS) (Perkin Elmer, Waltham, MA), residing in the Department of Laboratory Animal Resources facilities at the University of Kentucky Lee Todd BioPharm Building. The

IVIS system was set to bioluminescence acquisition with default medium binning settings on the camera. A total of 6 cultures were analyzed simultaneously at a 5 second exposure time every 60 mins for 66 hours.

Temperature Cycles

The Lumicycle was placed in a light-tight incubator, while temperature was controlled by a Proportion, Integral, Derivative (PID) controller (Automation Direct SOLO SL4896 with accompanying software v.1.1.0.5; Cumming GA). Cultures were exposed to cycles of high and low temperature of varying periods (T-cycles) and amplitudes of either 1ºC or 3ºC in the presence or absence of 1 nM melatonin. Group 1 was exposed to a 24 hr cycle (T=24) of 12 hrs. 35ºC and 12 hrs. 36ºC (1º High Low (HL)12:12) for three days and then released into constant 35ºC (Low Low; LL) for 3 days. Group 2 was exposed to a 22 hr cycle (T=22) of 11 hrs. 35ºC and 11 hrs. 36ºC (1º HL11:11) for three days and then released into constant 35ºC (LL) for 3 days. Group 3 was exposed to a 28 hr cycle (T=28) of 14 hrs. 35ºC and 14 hrs. 36ºC (1º HL14:14) for three days and then released into constant 35ºC (LL) for 3 days. Group 4 was exposed to a 24 hr cycle (T=24) of 12 hrs. 34ºC and 12 hrs. 37ºC (3º HL12:12) for three days and then released into constant 34ºC (LL) for 3 days. Group 5 was exposed to a 22 hr cycle (T=22) of 11 hrs. 34ºC and 11 hrs. 37ºC (3º HL11:11) for three days and then released into constant 34ºC (LL) for 3 days. Group 6 was exposed to a 28 hr cycle (T=28) of 14 hrs. 34ºC and 14 hrs. 37ºC (3º HL14:14) for three days and then released into constant 34ºC (LL) for 3 days. These are graphically described in tabular form below.

	T-Cycle, T=	Δ temp.	3 cycles of:		Then:
Group 1	24	1°C	12hr 35°C	12hr 36°C	72hr 35°C
Group 2	22	1°C	11hr 35°C	11hr 36°C	72hr 35°C
Group 3	28	1°C	14hr 35°C	14hr 36°C	72hr 35°C
Group 4	24	3°C	12hr 34°C	12hr 37°C	72hr 34°C
Group 5	22	3°C	11hr 34°C	11hr 37°C	72hr 34°C
Group 6	28	3°C	14hr 34°C	14hr 37°C	72hr 34°C

Phase Response Curve

Cultures were maintained in the Lumicycle in a 3ºC HL12:12 for 3 days and then released into 34ºC LL for 3 days. At 6 different phases of the bioluminescence rhythm, cultures were exposed to a 1-hr pulse of 37ºC. Cultures were allowed to free-run for an additional 3 days at 34ºC.

Quantification and Statistical Analysis

IVIS images were imported into ImageJ (Schneider, Rasband and Eliceiri, 2012) as a virtual stack for baseline subtraction. Mean intensity was measured in cultures that remained intact throughout the imaging period by drawing a circle encompassing each well to be analyzed. Linear detrending of damping luminescence was accomplished using the *forecast* statistical package in R, which resulted in the removal of 2 timepoints at the beginning and end of the dataset. Baseline-corrected Lumicycle data and IVIS data were further analyzed for rhythmicity using MetaCycle (Wu *et al.*, 2016), an R-based statistical package for analyzing large time series datasets with individual information (for each culture). This program identified cultures that expressed statistically significant rhythmic profiles that exhibited BHQ false discovery statistic – otherwise known as FDR – of <0.05 and a p-value of <0.01 to reject files from further consideration. Using these criteria, 8-12 cultures/condition were analyzed for each experiment. Circadian phase, period and amplitude of bioluminescence rhythms from cultures that passed these criteria were defined by an integration of three algorithms, ARSER analysis of circadian data by harmonic integration (Yang and Su, 2010), JTK_CYCLE, a non-parametric test for rhythmicity in large datasets (Hughes, Hogenesch and Kornacker, 2010), and the Lomb-Scargle method for detection of rhythmic signals from time series datasets (Glynn, Chen and Mushegian, 2006). Period and phase data (with phase-correction based on predicted periods during each respective T-cycle) were obtained by the integration function included in the Metacycle package using only those cultures that passed p-value and FDR thresholds in all three statistical tests mentioned above. Periods, amplitudes and phases were compared by 1- or 2-way ANOVA, where appropriate. All analyses were performed using SigmaStat software (Systat, CA).

Declaration of interests

References

Glynn, E. F., Chen, J. and Mushegian, A. R. (2006) 'Detecting periodic patterns in unevenly spaced gene expression time series using Lomb-Scargle periodograms.', *Bioinformatics (Oxford, England)*, 22(3), pp. 310–6. doi: 10.1093/bioinformatics/bti789.

Hughes, M. E., Hogenesch, J. B. and Kornacker, K. (2010) 'JTK_CYCLE: an efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets.', *Journal of biological rhythms*, 25(5), pp. 372–80. doi: 10.1177/0748730410379711.

Liu, Y. *et al.* (1995) 'Bacterial luciferase as a reporter of circadian gene expression in cyanobacteria.', *Journal of bacteriology*, 177(8), pp. 2080–6.

Paulose, J. K. *et al.* (2016) 'Human gut bacteria are sensitive to melatonin and express endogenous circadian rhythmicity', *PLoS ONE*. Edited by S. Yamazaki, 11(1), p. e0146643. doi: 10.1371/journal.pone.0146643.

Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. (2012) 'NIH Image to ImageJ: 25 years of image analysis', *Nature Methods*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 9(7), pp. 671–675. doi: 10.1038/nmeth.2089.

Wu, G. *et al.* (2016) 'MetaCycle: an integrated R package to evaluate periodicity in large scale data.', *Bioinformatics (Oxford, England)*, 32(21), pp. 3351–3353. doi: 10.1093/bioinformatics/btw405.

Yang, R. and Su, Z. (2010) 'Analyzing circadian expression data by harmonic regression based on autoregressive spectral estimation.', *Bioinformatics (Oxford, England)*, 26(12), pp. i168-74. doi: 10.1093/bioinformatics/btq189.