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

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vincent.cassone@uky.edu

**HIGHLIGHTS**

*K. 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.

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

Jiffin K. Paulose,1 Charles V. Cassone,1 Kinga B. Graniczewska,1 and Vincent M. Cassone1,2,*

**SUMMARY**

The gastrointestinal bacterium *Klebsiella (nē 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 (TB). 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 TB.

**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 (TB) (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

1Department of Biology, University of Kentucky, Lexington, KY 40506, USA
2Lead Contact
*Correspondence: vincent.cassone@uky.edu
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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., 2003). 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 Q10 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 (Tb) 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 Tb 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 (Tb) that correspond to changes in Tb. 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 Tb.

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 ± 0.215 counts/s (Figure 1G, bottom). During T = 22 at 1°C, the amplitude was 2.138 ± 0.165 counts/s (Figure 2G, bottom); during T = 28, it was 3.123 ± 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 ± 0.944 counts/s at T = 24 (Figure 1G, top), 1.176 ± 0.173 counts/s at T = 22 (Figure 2G), and 1.926 ± 0.563 counts/s at T = 28 (Figure 3G, bottom). In the presence of 1 nM melatonin, the bioluminescence rhythm amplitudes were 5.391 ± 0.939 counts/s for T = 24 (Figure 1G, top), 1.381 ± 0.191 counts/s for T = 22 (Figure 2G), and 1.635 ± 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 (τ) for each set of cultures corresponded to the T-cycles in which they were incubated. In T = 24, the average τ was 23.632 ± 0.173 counts/s at T = 22 (Figure 2G), and 1.926 ± 0.563 counts/s at T = 28 (Figure 3G, bottom). In the presence of 1 nM melatonin, the bioluminescence rhythm amplitudes were 5.391 ± 0.939 counts/s for T = 24 (Figure 1G, top), 1.381 ± 0.191 counts/s for T = 22 (Figure 2G), and 1.635 ± 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.

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
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 amplitude, 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). Finally, following T = 22 at 1°C amplitude, the average τ was 22.376 ± 0.363 h (Figure 2E). Following T = 28 at 3°C amplitude, 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 (φFR) largely corresponded to the φ of the entrained rhythm (φE). The phase relationship between φC and φFR (φFR) 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 φFR in the 1°C T = 22 cycle was 1.1 ± 0.5 h, and the φFR 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 φFR 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.

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**Table 1. Summary Results for Entrainment and Free-Running Data from All T-Cycle Experiments**

| T-Cycle (h) | Temperature Δ (°C) | Condition | Period (h) | ψFR (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 |
Figure 1. *K. aerogenes* motA Expression Entrain 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 Entrain 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. Similar, 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 (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 Entrain 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
Temperature pulses also resulted in phase-dependent changes in $\phi$ ($\Delta\phi$) of the bioluminescence rhythms of these cultures (Figure 4B). In this case, the phase reference ($\phi_0$) was the acrophase, or peak, of the bioluminescence rhythm. Calculation of the phase response curve (PRC), in which the $\phi_0$ was plotted against the $\phi_0$ 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 ($-\Delta\phi$) from CT12 to CT18 and a rapid transition to high-amplitude phase advances ($+\Delta\phi$) from CT18 to CT0.

Calculation of a phase transition curve (PTC), in which the new $\phi$ was plotted on the y axis and the old $\phi$ 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 ($\tau$) was 25.4 ± 0.4 h. This $\tau$ 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 $\tau$ = 24.5 ± 0.5 h; Paulose et al., 2016) and to the average $\tau$ 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 $T_E$. This clock maintains a stable $\Psi$ 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 $\Psi_{EFF}$ is most consistent when temperature onset is used as the $\phi_E$. This is remarkable in view of the fact that the generation time of *K. aerogenes* is 30 ± 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
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 TB. In this regard, one could consider this and other members

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**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.
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 TB 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 TA. These authors suggested that daily changes in TB could serve to synchronize peripheral clocks.

Rhythms of TB 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 (TB), 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) (Polidarova 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. It is not clear whether gastrointestinal melatonin, which is secreted by the intestines themselves (Soderquist 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 (Rath, 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, 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. Timed feeding can independently influence gastrointestinal circadian p (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; Thaisse et al., 2014, 2016). These may be expressed at different p’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 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.
METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.09.007.

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AUTHOR CONTRIBUTIONS
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DECLARATION OF INTERESTS
The authors declare no competing financial interests.

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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 37°C 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 CaCl₂ method and *pmotA::luxCDABE* 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. 1°C 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 1°C or 3°C in the presence or absence of 1 nM melatonin. Group 1 was exposed to a 24 hr cycle (T=24) of 12 hrs. 35°C and 12 hrs. 36°C (1º High Low (HL)12:12) for three days and then released into constant 35°C (Low Low; LL) for 3 days. Group 2 was exposed to a 22 hr cycle (T=22) of 11 hrs. 35°C and 11 hrs. 36°C (1º HL11:11) for three days and then released into constant 35°C (LL) for 3 days. Group 3 was exposed to a 28 hr cycle (T=28) of 14 hrs. 35°C and 14 hrs. 36°C (1º HL14:14) for three days and then released into constant 35°C (LL) for 3 days. Group 4 was exposed to a 24 hr cycle (T=24) of 12 hrs. 34°C and 12 hrs. 37°C (3º HL12:12) for three days and then released into constant 34°C (LL) for 3 days. Group 5 was exposed to a 22 hr cycle (T=22) of 11 hrs. 34°C and 11 hrs. 37°C (3º HL11:11) for three days and then released into constant 34°C (LL) for 3 days. Group 6 was exposed to a 28 hr cycle (T=28) of 14 hrs. 34°C and 14 hrs. 37°C (3º HL14:14) for three days and then released into constant 34°C (LL) for 3 days. These are graphically described in tabular form below.

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

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
The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to Vincent.Cassone@uky.edu or jpaulose@gmail.com.

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