Bacterial Bioluminescence Regulates Expression of a Host Cryptochrome Gene in the Squid-Vibrio Symbiosis

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ABSTRACT The symbiosis between the squid Euprymna scolopes and its luminous symbiont, Vibrio fischeri, is characterized by daily transcriptional rhythms in both partners and daily fluctuations in symbiont luminescence. In this study, we sought to determine whether symbionts affect host transcriptional rhythms. We identified two transcripts in host tissues (E. scolopes cry1 [escry1] and cry2) that encode cryptochromes, proteins that influence circadian rhythms in other systems. Both genes cycled daily in the head of the squid, with a pattern similar to that of other animals, in which expression of certain cry genes is entrained by environmental light. In contrast, escry1 expression cycled in the symbiont-colonized light organ with 8-fold upregulation coincident with the rhythms of bacterial luminescence, which are offset from the day/night light regime. Colonization of the juvenile light organ by symbionts was required for induction of escry1 cycling. Further, analysis with a mutant strain defective in light production showed that symbiont luminescence is essential for cycling of escry1; this defect could be complemented by presentation of exogenous blue light. However, blue-light exposure alone did not induce cycling in nonsymbiotic animals, but addition of molecules of the symbiont cell envelope to light-exposed animals did recover significant cycling activity, showing that light acts in synergy with other symbiont features to induce cycling. While symbiont luminescence may be a character specific to rhythms of the squid-vibrio association, resident microbial partners could similarly influence well-documented daily rhythms in other systems, such as the mammalian gut.

IMPORTANCE In mammals, biological rhythms of the intestinal epithelium and the associated mucosal immune system regulate such diverse processes as lipid trafficking and the immune response to pathogens. While these same processes are affected by the diverse resident microbiota, the extent to which these microbial communities control or are controlled by these rhythms has not been addressed. This study provides evidence that the presentation of three bacterial products (lipid A, peptidoglycan monomer, and blue light) is required for cyclic expression of a cryptochrome gene in the symbiotic organ. The finding that bacteria can directly influence the transcription of a gene encoding a protein implicated in the entrainment of circadian rhythms provides the first evidence for the role of bacterial symbionts in influencing, and perhaps driving, peripheral circadian oscillators in the host.
They occur as components of the central oscillator, which resides in the animal brain, and in peripheral oscillators, such as in gut tissues (14). Cryptochromes are evolutionarily derived from the photolyases, which are DNA repair enzymes (Fig. 1D). Whereas all vertebrate cryptochromes arose from the same evolutionary event, invertebrate cryptochromes typically fall into one of two clades, each of which is the product of an independent evolutionary derivation of photolyases (15, 16). Studies of these proteins have demonstrated that members of one clade (Cry1) are light responsive and lead to degradation of repressors of the core clock machinery, and the others (Cry2) are light-independent transcriptional repressors of the core clock genes (17). All crypto-
chromes have the conserved amino acids critical for function, as well as the characteristic domain structure of photolyases (18). However, cryptochromes have a defining C-terminal extension that does not occur in the photolyases. Whereas the role of cryptochromes in circadian rhythms has been well studied for many invertebrate groups (15, 19–24), the identification of cry gene sequences and in one case the expression pattern of a single cryptochrome (25) is the only information available for these genes in the Lophotrochozoa, the superphylum of animals that contains the squid host E. scolopes and its relatives.

V. fischeri occurs as an extracellular symbiont in deep crypt spaces of the light organ of E. scolopes (Fig. 1B). The host animal has strong rhythms in its behavior; as a nocturnal predator, it remains buried in the sand during the day and emerges at night to forage in the water column (Fig. 1C). Host and symbiont cell within the adult light organ have rhythmic patterns of gene expression that underlie day/night activities of the partners in the symbiosis (26). Some behavioral evidence suggests that the night-active host animal uses the luminescence of the bacterial symbiont as an antipredatory camouflage in a process known as counterillumination (27). Studies of the juvenile light organ have shown that the animal has molecular mechanisms by which to detect and respond to the bacterial luminescence (28). Mutant symbionts defective in light emission are incapable of sustaining a symbiosis (29). Such mutants are also defective in inducing full light-organ development (29), which is principally triggered by derivatives of symbiont MAMPs (microbe-associated molecular patterns). MAMPs are a class of molecules specific to microbes that trigger host animal responses. In the development of the squid-vibrio system, the lipid A moiety of lipopolysaccharide (LPS) and the peptidoglycan monomer TCT (or tracheal cytotoxin) are the MAMPs known to be active in inducing host light-organ morphogenesis (30). Further, transcriptomic studies of the juvenile light organ revealed that colonization by luminous V. fischeri cells is required for normal symbiont-induced changes in host gene expression (31). Particularly relevant here is the finding that the luminescence output of the animal is on a daily rhythm (Fig. 1C), which has key features of a circadian rhythm (32). In this rhythm, luminescence peaks at night, when the animal is active. As such, light presentation by symbionts in the organ occurs with timing nearly opposite to that of the exogenous cues of environmental light.

Transcriptional databases of the light organ (33) have revealed the expression of two genes that encode proteins with high sequence similarity to the known invertebrate cryptochromes. This finding offered the opportunity to investigate and compare the role of cryptochromes in host squid rhythms in response to exogenous (environmental light) and endogenous (bioluminescence) light cues. Of broader significance, the presence of cryptochromes offered the opportunity to determine whether bacterial symbionts and their luminescence can operate as critical features in the elaboration of host rhythms.

Here we characterize phylogenetic relationships of the two cryptochrome genes identified in E. scolopes and activities of these host genes in response to interactions with the bacterial partner. Taken together, these data contribute to our understanding of the extent to which bacterial partners can be integrated into the control of the biological rhythms of their animal hosts.

**RESULTS**

Two cryptochrome genes are expressed in the E. scolopes light organ. We identified two candidate cryptochrome (cry) sequences in existing transcriptional databases produced from the E. scolopes light organ (33). Rapid amplification of cDNA ends (RACE) and subsequent BLAST and alignment analyses showed that the two transcripts are likely homologs of known cryptochromes (Fig. 1D; see also Fig. S1 and S2 in the supplemental material). The derived amino acid sequences of full-length transcripts have the typical structure of cryptochrome (Cry) proteins, with photolyase and flavin adenine dinucleotide (FAD)-binding domains characteristic of members of this protein family (34). In addition, both protein sequences have the conserved tryptophan residues that coordinate flavin binding (18) and conserved serine residues, whose phosphorylation is implicated in protein-protein interactions (35). Phylogenetic analyses placed the E. scolopes Cry proteins, with high confidence, within the two major invertebrate cryptochrome clades (Fig. 1C). The data provide evidence that the light organ expresses the same number of cryptochrome transcripts and that the predicted proteins occur in phylogenetic relationships characteristic of the cryptochromes of most invertebrate species.

**escry1 expression in the light organ is influenced by symbiosis.** To characterize the regulation of expression of the E. scolopes cry (escry) genes in the light organ, we performed real-time quantitative reverse transcriptase PCR (qRT-PCR) with symbiont-colonized juvenile light organs, ~2 days posthatch, at four times over the day/night cycle (Fig. 2). These points were chosen to avoid the daily, noncircadian venting of symbionts that occurs with a dawn light cue (36) and to capture the extremes of the luminescence cycle of the light organs (32). To compare patterns of cry expression in the light organ with those occurring in other invertebrates (37), we also performed qRT-PCR on the heads of the same juvenile animals, which contain tissues that typically have cycling cry expression in animals. Whereas the patterns of message levels for escry1 and escry2 showed statistically significant variation over the day in the head, as observed in other systems (37), i.e., in synchrony with environmental light, only escry1 mRNA levels varied over the day/night cycle in the light organ (Fig. 2A). Further, peak mRNA levels in the light organ were observed in periods of high light-organ luminescence, i.e., shifted ~6 + h from that observed in the head (Fig. 2A) (32). Light organs extracted in the field from mature wild-caught animals show an expression profile similar to that of the lab-raised symbiotic juveniles, providing evidence that the pattern of escry1 expression is neither life stage specific nor due to laboratory conditions (Fig. 2B). To determine whether the induction of rhythms is developmentally regulated by the onset of symbiosis, we characterized diel patterns of mRNA abundance in uncolonized juvenile squid. Animals that lacked symbionts did not show the same diel variation in escry1 mRNA levels observed in symbiotic animals (Fig. 2C), although the light organs did show an intriguing statistically significant decrease in message at the time when luminescence would be increasing if the animals had been colonized. These data provide evidence that escry1 expression cycles in the light organ in a manner consistent with induction by symbiosis.

**Abundant EsCry1 localizes to the apical surfaces of light-organ epithelial cells that are adjacent to the symbiont.** To determine if the EsCry1 protein was produced in close proximity to
of the predicted full length of EsCry1 (Fig. 3B) and similar to that of Cry1 proteins in other invertebrates (25, 26); no cross-reactivity was detected in the membrane fraction (M). The antibody also detected another protein at a molecular mass of ~42 kDa, which is consistent with a common breakdown product of invertebrate Cry1 proteins detected in a Western blot (see, e.g., reference 38).

In analyses of light-organ tissues examined with confocal immunocytochemistry, the EsCry1 antibody showed cross-reactivity in the cells of the crypt epithelia that surround the symbiotic partner (Fig. 3C). The labeling occurred throughout these cells but often showed concentration at the apical surfaces (Fig. 3C and D). Comparisons of immune cross-reactivity revealed no detectable differences in protein abundance or localization among uncolonized animals and those colonized by wild-type or Δlux V. fisheri (Fig. 3D).

**Peak expression of es cry 1 requires symbiont luminescence.** Because the es cry1 mRNA levels reflected diel patterns of symbiont luminescence and the Cry protein localized near the site where symbionts reside in the light organ, we used V. fisheri mutants (Δlux) defective in light production (Fig. 4A) to determine whether symbiont luminescence is critical for the entrainment of es cry1 mRNA cycling (37). At the time of highest es cry1 expression in symbiotic animals, i.e., 14 h past “dawn” (see Fig. 2A), expression of this gene in animals colonized by the Δlux mutants was not significantly different from that in uncolonized animals (Fig. 4C). Genetic complementation of lux genes has been shown to restore normal host responses (29), but here we sought to isolate the effect of light exposure from other potential effects of luminescence, particularly influences on the oxygen environment. Thus, to complement the light defect phenotypically, we used exposure to exogenous blue light (Fig. 4B). mRNA levels of Δlux mutant-colonized animals complemented with exogenous blue light had a fold change in es cry1 mRNA levels similar to that of animals colonized with wild-type V. fisheri (Fig. 4D). At 2 days postcolonization, the density of Δlux bacteria in the light organ was about 10% of that of the wild-type strains, similar to values previously reported (Fig. 4F) (29). A lysine auxotroph (lysA::TnKan) that colonizes the light organ to the same extent as the Δlux mutant (39) but exhibits per-cell luminescence similar to that of the wild type also induced significantly higher es cry1 expression than the Δlux bacteria (Fig. 4D), providing further evidence that the presence of bacterial light, not wild-type bacterial density, increases es cry1 expression. Finally, we characterized expression of es cry1 in the head and determined that it was not affected by colonization state or strain (Fig. 4E), suggesting that the symbionts do not induce a systemic host response that influences the behavior of the genes in the head.

**Symbiont MAMPs enable light to induce cry1 cycling in the light organ.** Because the data showed that bacterial luminescence is essential for peak cry expression in the organ, we sought to determine whether light alone was sufficient to induce the cycling of es cry1 expression. When we exposed the light organs of non-symbiotic animals to a cycle of exogenous blue light of a wavelength similar to that emitted by wild-type bacterial symbionts, es cry1 expression did not cycle (Fig. 5A). Exposure to exogenous blue light and derivatives of symbiont MAMPs, specifically the lipid A component of lipopolysaccharide (LPS) and the peptidoglycan monomer (tracheal cytotoxin [TCT]), however, did induce cycling (Fig. 5B). However, treatment with only TCT or lipid

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**FIG 2** Day/night cycle variation in E. scolopes cryptochrome expression. (A) The expression of es cry1 and es cry2 in the squid light organ and head over four points in the day/night cycle. Graphs indicate the relative expression of es cry1 and es cry2 as measured by qRT-PCR. Yellow and black bars denote the cycle of exogenous light, and the blue and black bars show the cycle of bacterial light in the light organ (Fig. 3A). We made an antibody to a peptide sequence unique to EsCry1. In extracts of the light organ, the antibody cross-reacted with a low-abundance protein species in the soluble fraction (S) at a molecular mass of ~63 kDa, the size of the predicted full length of EsCry1 (Fig. 3B) and similar to that of Cry1 proteins in other invertebrates (25, 26); no cross-reactivity was detected in the membrane fraction (M). The antibody also detected another protein at a molecular mass of ~42 kDa, which is consistent with a common breakdown product of invertebrate Cry1 proteins detected in a Western blot (see, e.g., reference 38). In analyses of light-organ tissues examined with confocal immunocytochemistry, the EsCry1 antibody showed cross-reactivity in the cells of the crypt epithelia that surround the symbiotic partner (Fig. 3C). The labeling occurred throughout these cells but often showed concentration at the apical surfaces (Fig. 3C and D). Comparisons of immune cross-reactivity revealed no detectable differences in protein abundance or localization among uncolonized animals and those colonized by wild-type or Δlux V. fisheri (Fig. 3D).

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A did not induce cycling of escry1 expression (see Fig. S3 in the supplemental material).

**DISCUSSION**

The data presented in this study provide evidence that bacterial symbionts in the *E. scolopes* light organ influence the expression of a single cryptochrome gene and that luminescence of the symbionts may therefore provide input to a circadian oscillator in the host. In the larger context, these data suggest the possibility that EsCry1 localizes specifically to the apical surfaces of cells interacting directly with symbionts and that presentation of symbiont MAMPs enables *cry* responses to luminescence. The mechanism by which MAMP presentation primes the light-organ crypt cells to interact with light remains to be determined, but the system apparently ensures that the crypt cells respond solely to light presented in the context of the bacterial symbiont and not to environmental light presented on the day/night cycle.

The data presented here suggest a number of areas for future research efforts in the squid-vibrio system. A likely fruitful area will be to determine the extent to which *escry1* influences the various daily rhythms that have been described. In addition to the early studies of rhythms of bioluminescence (32), recent analyses of the transcriptomes of the symbiont and its supporting host epithelium at several points over the day/night cycle revealed a profound daily rhythm of gene expression in both partners (26). The data showed that 9.6% of the total available host transcriptome is regulated over the day-night cycle, similar to the proportion (~8%) of the total transcriptome controlled by the circadian clock in the tissues of other animals (1, 44). The transcriptomic

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rhythms but, significantly, to imbalances in the microbiota (52). An emerging hypothesis is that the host and its microbiota work together to develop and maintain biological rhythms that are essential to the homeostasis of the symbiosis. The complexity of the mammalian systems presents a significant challenge to the study of their rhythms. The study of simpler systems, such as the squid-vibrio system and the Drosophila gut community, may provide valuable insight into the rules governing symbiont influence on host rhythms.

**MATERIALS AND METHODS**

**General methods.** Adult *Euprymna scolopes* squid were collected and maintained as previously described (53). Juveniles from this breeding colony were collected within 15 min of hatching and placed in filtered-stabilized Instant Ocean solution (FSIO) (Aquarium Systems, Mentor, OH). For all experiments, animals were maintained on a 12-h light-dark cycle. Uncolonized juveniles were maintained in FSIO. Symbiotic juveniles were exposed to 5,000 *V. fischeri* cells per ml of FSIO overnight. Colonization of the host animals by the wild-type strain was monitored by taking luminescence readings using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA); uncolonized animals and animals colonized with the light-deficient mutant EVS102 (55) (∆lux) were also checked with the luminometer to ensure that the light organs had not been colonized by wild-type strains. To determine CFU per light organ, tissues were homogenized in FSIO and determine CFU per light organ, tissues were homogenized in FSIO and plated on LBS medium (LB agar containing 2% [wt/vol] NaCl) (35). Strains that were used include the wild-type strain ES114 (54), the light-deficient mutant EVS102 (55) (∆lux), and the lysine auxotroph VCW3F6 (39). All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

**Exogenous blue-light and MAMP stimuli.** To determine whether the decrease in _esr1_ expression seen in _Δlux_-colonized animals was due to the lack of bacterial luminescence and not another consequence of delet-
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A

Light only

2

1

PG monomer, lipid A, and light (†)

3

2

1

2 8 14 20

Time (h)

Fig 5 The effect of MAMPs on esr1y expression. (A) Expression of esr1y in the light organs of uncolonized animals exposed to exogenous blue light at four time points over the day/night cycle. (B) esr1y light-organ expression in animals exposed to exogenous blue light, 10 μM peptidoglycan monomer, and 10 ng/ml V. fischeri lipid A in seawater. Graphs indicate the relative expression of esr1y as measured by qRT-PCR. Yellow and black bars denote the cycle of exogenous white (overhead) light, and the blue and black bars show the schedule of blue LED light presentation. All data were normalized to the time point of lowest expression in each graph. Error bars represent the standard errors of the means; n = 3 to 6 biological replicates and 2 technical replicates per condition. †, ANOVA P value < 0.05; *, pairwise comparison, P < 0.05; **, pairwise comparison, P < 0.01.
Quantitative reverse transcriptase PCR. All qRT-PCR assays were performed in compliance with the MIQE guidelines (61). Gene-specific primers were designed for escry1 and escry2, and the *Euprymna scolopes* 40S ribosomal RNA sequence was used as a control for equal well loading (see Table S2 in the supplemental material). For each experiment, negative controls were run without a template and with cDNA reactions run with no reverse transcriptase to ensure the absence of chromosomal DNA in the reaction wells. The efficiencies of all qRT-PCR primer sets were between 95 and 100%. Data were analyzed using the Comparative Cq (ΔΔCq) method (62). qRT-PCR was performed on *E. scolopes* cDNA using IQSYBR green supermix or SsoAdvanced SYBR green supermix (Bio-Rad, Hercules, CA) in an iCycler thermal cycler or a CFX Connect real-time system (Bio-Rad, Hercules, CA). Amplification was performed under the following conditions: 95°C for 5 min, followed by 45 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. Each reaction was carried out in duplicate, and each reaction mixture contained 0.2 μM primers and 10.4 ng cDNA. To determine whether a single amplicon resulted from the PCR reactions, the presence of one optimal dissociation temperature for each PCR reaction was assayed by incrementally increasing the temperature every 10 s from 60 to 89.5°C. Each reaction in this study had a single dissociation peak. Standard curves were created using a 10-fold dilution series of the PCR product with each primer set.

**Western blotting.** A polyclonal antibody to EsCry1 was produced in rabbit (GenScript, Piscataway, NJ) to two unique peptides within the EsCry1 sequence (CFGIEPECEEQKKPI and CGSCLPNHQENPELL), chosen for their predicted antigenicity and lack of similarity to other *E. scolopes* or *V. fischeri* fisheri proteins. Protein samples for Western blotting were prepared as described previously (63). Protein concentrations of the samples were then determined using a Qubit 2.0 fluorometer (Life Technologies, Grand Island, NY). The proteins were separated on a 10% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA) in an iCycler thermal cycler or a CFX Connect real-time system (Bio-Rad, Hercules, CA). Amplification was performed under the following conditions: 95°C for 5 min, followed by 45 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. Each reaction was carried out in duplicate, and each reaction mixture contained 0.2 μM primers and 10.4 ng cDNA. To determine whether a single amplicon resulted from the PCR reactions, the presence of one optimal dissociation temperature for each PCR reaction was assayed by incrementally increasing the temperature every 10 s from 60 to 89.5°C. Each reaction in this study had a single dissociation peak. Standard curves were created using a 10-fold dilution series of the PCR product with each primer set.

**Immunocytochemistry.** Light organs were fixed, permeabilized, and stained with rhodamine or Alexa 633 phalloidin (Life Technologies, Grand Island, NY). The fixed light organs were then exposed to secondary antibody, washed, and developed as previously described (63). The antibody was diluted 1:250 in blocking solution and incubated overnight. Samples were then counterstained with rhodamine or Alexa 633 phalloidin (Life Technologies, Grand Island, NY). The proteins were separated on a 10% SDS-PAGE gel with 40 μg of protein per lane and then transferred to a nitrocellulose membrane with a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA) per the manufacturer’s instructions. The membrane was blocked overnight at room temperature as previously described (63). The antibody was diluted 1:250 in blocking solution and incubated with the membrane for 3 h at room temperature. The blot was then exposed to secondary antibody, washed, and developed as previously described (63).

**Nucleotide sequence accession numbers.** Nucleotide sequence accession numbers are as follows: for escry1, KC261598; for escry2, KC261599 (NCBI GenBank).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00167-13/-/DCSupplemental.

Figure S3, EPS file, 0.2 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.

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