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Photolyase Confers Resistance to UV Light but Does Not Contribute to the Symbiotic Benefit of Bioluminescence in *Vibrio fischeri* ES114[▽]

Emma L. Walker, Jeffrey L. Bose, and Eric V. Stabb*

Department of Microbiology, University of Georgia, Athens, Georgia 30602

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Recent reports suggest that the selective advantage of bioluminescence for bacteria is mediated by light-dependent stimulation of photolyase to repair DNA lesions. Despite evidence for this model, photolyase mutants have not been characterized in a naturally bioluminescent bacterium, nor has this hypothesis been tested in bioluminescent bacteria under natural conditions. We have now characterized the photolyase encoded by *phr* in the bioluminescent bacterium *Vibrio fischeri* ES114. Consistent with Phr possessing photolyase activity, *phr* conferred light-dependent resistance to UV light. However, upon comparing ES114 to a *phr* mutant and a dark Δ*luxCDABEG* mutant, we found that bioluminescence did not detectably affect photolyase-mediated resistance to UV light. Addition of the light-stimulating autoinducer *N*-3-oxo-hexanoyl homoserine lactone appeared to increase UV resistance, but this was independent of photolyase or bioluminescence. Moreover, although bioluminescence confers an advantage for *V. fischeri* during colonization of its natural host, *Euprymna scolopes*, the *phr* mutant colonized this host to the same level as the wild type. Taken together, our results indicate that at least in *V. fischeri* strain ES114, the benefits of bioluminescence during symbiotic colonization are not mediated by photolyase, and although some UV resistance mechanism may be coregulated with bioluminescence, we found no evidence that light production benefits cells by stimulating photolyase in this strain.

Bacterial bioluminescence is well understood biochemically and genetically (15, 27, 39); however, the functional significance of bioluminescence for bacteria remains uncertain (35). Generating luminescence presumably confers a selective advantage(s) under some circumstances, but this must be weighed against apparently significant energy costs (15, 21). Several hypotheses have been offered to explain how bioluminescence might be advantageous to the growth or survival of light-producing bacteria, and they propose that the value of luminescence might be due to the consumption of reducing equivalents, the reduction of oxygen, or an effect of light itself (29, 35). Recently, it was suggested that bioluminescence benefits bacteria by stimulating the light-dependent photolyase-mediated repair of DNA lesions (7–9, 23, 43). Photolyase is an enzyme that uses light to drive the "photoreactivation" repair of pyrimidine dimers, such as those caused by UV light, in adjacent base pairs on the same strand of DNA (42), and theoretically photolyase could be activated by bioluminescence. This led to the suggestion that bioluminescence may have evolved to stimulate photolyase in an otherwise dark environment (8).

Proponents of the hypothesis that luminescence benefits cells by stimulating photolyase have reported several experiments that support this model. Consistent with a role in repairing pyrimidine dimers, bioluminescence in *Vibrio harveyi* is stimulated by UV irradiation (7, 9). Furthermore, dark *lux* mutants of *V. harveyi* (9) and of other bacteria (23) were more sensitive to UV light than their respective wild-type parents, while *Escherichia coli* clones carrying *V. harveyi* bioluminescence-generating *lux* genes were more UV resistant. Also, in mixed cultures, a dark *luxA* mutant of *V. harveyi* was outcompeted by its bioluminescent parent when the cultures were exposed to UV light (8). Importantly, the relative sensitivity of dark strains in these experiments could be compensated for, at least in part, by exposure to external light, consistent with a benefit mediated by light itself (8, 9, 23). Finally, perhaps the most compelling evidence of a connection between bioluminescence and photolyase is the fact that the UV resistance conferred upon *E. coli* by the *lux* genes is dependent upon *phr*, the gene encoding photolyase (23).

Despite this evidence, it is not yet certain whether bioluminescence plays a significant role in photoreactivation. Some of the experiments described above are open to alternative interpretations. For example, bioluminescence could influence stress resistance and DNA repair through pathways that do not involve photolyase, and experiments such as those involving *lux* expression in *E. coli* may not be relevant to bioluminescent bacteria in the environment. Moreover, photolyase mutants have not been characterized in a naturally bioluminescent bacterium, nor has a connection between photolyase and bioluminescence been tested in bioluminescent bacteria under natural conditions where the bacteria produce light.

We tested the relationship between bioluminescence and photolyase in *Vibrio fischeri* ES114. The genome of this bioluminescent bacterium has been sequenced (34), allowing us to identify *phr* and generate a photolyase mutant, and we were also able to observe the bacterium under conditions where it is naturally luminescent, in the light organ of its host, *Euprymna scolopes*. We found that although bioluminescence enables *V. fischeri* to fully colonize its host (41), this effect is not mediated

* Corresponding author. Mailing address: University of Georgia, Department of Microbiology, 828 Biological Sciences, Athens, GA 30602. Phone: (706) 542-2414. Fax: (706) 542-2674. E-mail: estabb@uga.edu.

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TABLE 1. Bacterial strains, plasmids, and oligonucleotides

Strain, plasmid, or oligonucleotide	Relevant characteristics[a]	Source or reference
V. fischeri		
ES114	Wild-type isolate from *Euprymna scolopes*	3
EW1	ES114 Δ*phr* (allele exchanged from pELW1)	This study
EW4	ES114 Δ*phr* Δ*luxCDABEG* (EW1 with *lux* allele exchanged from pEVS153)	This study
EVS102	ES114 Δ*luxCDABEG*	J. L. Bose and E. V. Stabb, unpublished data
JRM100	ES114 mini-Tn*7-ermR* (inserted at intergenic *att* site)	26
E. coli		
CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*; lysogenized with λ*pir*	20
DH5α	F$^-$ *F80dlacZΔM15* Δ(*lacZYA-argF*)*U169 deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	14
DH5αλ*pir*	DH5α lysogenized with λ*pir*	10
Plasmids		
pCR-Blunt II-TOPO	PCR product cloning vector; ColE1 *oriV kanR*	Invitrogen
pALZ1	PCR product (primers JBPHOTO1 and JBPHOTO2; ES114 template) in pCR-Blunt II-TOPO	This study
pALZ5	SpeI-AvrII fragment of pALZ1 containing *phr* and flanking sequences cloned between SpeI-AvrII sites of pEVS118	This study
pALZ6	PCR product (primers JBPHOTO3 and JBPHOTO4; pALZ5 template) SmaI digested and self-ligated	This study
pBluescript KS(+)	ColE1 *oriV ampR*	Stratagene
pELW1	SpeI-digested pALZ6 ligated to SpeI-digested pBluescript	This study
pELW2	PCR product (primers JBPHOTO9 and JBPHOTO10; pALZ1 template) in pCR-Blunt II-TOPO	This study
pELW5	*phr*-containing AvrII-KpnI fragment of pELW2 cloned between SpeI-KpnI sites of pVSV105	This study
pEVS104	R6Kγ *oriV tra trb oriT*$_{RP4}$ *kanR*; conjugative helper	37
pEVS118	R6Kγ *oriV oriT*$_{RP4}$ *chmR*	10
pEVS153	ColE1 *oriV kanR chmR oriT*$_{RP4}$ with Δ*luxCDABEG* and flanking sequence from ES114	Bose and Stabb, unpublished
pVSV105	pES213 *oriV chmR oriT*$_{RP4}$	11
Oligonucleotides		
JBPHOTO1	5′ GCT CTA GAG GTC TGG CAA GGT ACT TAA TAC GGC T 3′	This study
JBPHOTO2	5′ CCG CCT AGG TGG GTG CCG CTG ACA CAA TAA TGA 3′	This study
JBPHOTO3	5′ TCC CCC GGG CAT AGC GTA ATC TTA GCT CTT GTG GGT AAG 3′	This study
JBPHOTO4	5′ TCC CCC GGG TAA CCA CGC TTG AGT ATG ATT AAT ACA TC 3′	This study
JBPHOTO5	5′ CGC ATC GCC TTA GTT GAT TCT AAG GAT TC 3′	This study
JBPHOTO6	5′ CAC CTC AGA AGC AGG CTT TAG CTG AGC TC 3′	This study
JBPHOTO7	5′ GCC CCA CTC TGA AAT ACA AAA TTG CTC AG 3′	This study
JBPHOTO8	5′ GTG TGG GTT GCG GCA CGA GCC ATG 3′	This study
JBPHOTO9	5′ CCG CCC TAG GCA AGA GCT AAG ATT ACG CTA TGG AT 3′	This study
JBPHOTO10	5′ GGG GTA CCG GAT GCC TTG CGA TGG GGT TAT ATC 3′	This study

[a] *ampR*, ampicillin resistance (*bla*); *chmR*, chloramphenicol resistance (*cat*); *ermR*, erythromycin resistance; and *kanR*, kanamycin resistance (*aph*).

by photolyase. Taken together with analyses of cultured cells, our results cast doubt on the importance of bioluminescence in photoreactivation in this bacterium.

MATERIALS AND METHODS

Bacteria and culturing techniques. The bacterial strains, plasmids, and oligonucleotides used in this study are described in Table 1. Plasmid constructs were transformed and maintained in *E. coli* strain DH5α or DH5αλ*pir*, with the latter used specifically for plasmids containing the R6Kγ replication origin. The conjugative helper plasmid pEVS104 was maintained in *E. coli* strain CC118λ*pir*. *E. coli* was grown in LB medium (28) or brain heart infusion medium, and *V. fischeri* was grown in either SWT medium, which contained 5 g of tryptone, 3 g of yeast extract, 3 ml of glycerol, and 700 ml of Instant Ocean (Aquarium Systems, Mentor, Ohio) per liter, or in LBS medium, which contained 10 g of tryptone, 5 g of yeast extract, 20 g of NaCl, and 20 mM Tris-hydrochloride (Tris, pH 7.5) per liter of water. Agar (15 mg ml^{-1}) was added to solidify the media for plating experiments. Culture density was measured as the absorbance at 595 nm (A_{595}) using a BioPhotometer (Brinkman Instruments, Westbury, N.Y.).

Chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). When added to LB medium for selection of *E. coli*, ampicillin, chloramphenicol, and kanamycin were used at concentrations of 100, 20, and 40 μg ml^{-1}, respectively. When added to brain heart infusion medium for selection of *E. coli*, erythromycin was used at a concentration of 150 μg ml^{-1}. When added to LBS medium for selection of *V. fischeri*, chloramphenicol, erythromycin, and kanamycin were used at concentrations of 2, 5, and 100 μg ml^{-1}, respectively. To add *N*-3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL), a 200-μg ml^{-1} stock solution was prepared in ethyl acetate, an appropriate volume was dispensed into flasks or tubes, the ethyl acetate was allowed to evaporate, and then the medium was added so that the final concentration of 3-oxo-C6-HSL was 200 ng ml^{-1}.

Genetic techniques and analyses. We generated plasmids using standard cloning and DNA manipulation techniques. DNA ligase and restriction enzymes were

obtained from New England Biolabs (Beverly, Mass.), and oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa). Plasmids were purified using QIAGEN Mini-prep kits (QIAGEN Inc., Valencia, Calif.), and DNA was recovered from restriction and ligation reactions with the DNA Clean and Concentrator-5 Kit (Zymo Research, Orange, Calif.). We used the Zero-Blunt-TOPO PCR Cloning Kit (Invitrogen, Carlsbad, Calif.) to clone PCR products into pCR-Blunt II-TOPO. PCR was performed with an iCycler (Bio-Rad Laboratories, Hercules, CA) using KOD HiFi DNA Polymerase (Novagen, Madison, Wis.). In addition to using a high-fidelity polymerase, we sequenced the cloned PCR products to ensure that unintended base pair alterations were not incorporated into our constructs. DNA sequencing was conducted at the University of Michigan DNA Sequencing Core Facility, and sequences were analyzed using Sequencher 4.1.2 (Gene Codes Corp., Ann Arbor, Mich.). Sequencing was completed using the primer oligonucleotides JBPHOTO5, JBPHOTO6, JBPHOTO7, and JBPHOTO8 (Table 1). Protein sequence comparisons were generated with BLAST (1), and similarities were calculated using the BLOSUM62 scoring matrix (19).

Details of plasmid construction are included in Table 1. To generate the Δ*phr* mutant allele, we PCR amplified *phr* and flanking sequences from *V. fischeri* ES114 using primers JBPHOTO1 and JBPHOTO2, cloned this PCR product into pCR-Blunt II-TOPO, and then subcloned the *phr*-containing fragment into the small, mobilizable vector pEVS118 (10). We then used primers JBPHOTO3 and JBPHOTO4, which are directed outward from the start and stop codons of *phr* and contain SmaI recognition sites near their 5′ ends, to PCR amplify the vector and sequences flanking *phr*. The template was then destroyed by DpnI treatment, and the PCR product was digested with SmaI and self-ligated to generate pALZ6, which contains the genomic region surrounding *phr* but with the sequences between the *phr* start and stop codons replaced by 5′-CCCGGG-3′. pALZ6 was ligated to pBluescript KS(+) to generate pEW1, a mobilizable ColE1-containing vector for allelic exchange of the in-frame *phr* deletion. To generate the *phr* complementation vector pEW5, we specifically PCR amplified *phr* using primers JBPHOTO9 and JBPHOTO10, cloned this PCR product into pCR-Blunt II-TOPO, and subcloned a *phr*-containing fragment into shuttle vector pVSV105 (11).

Mutant alleles and the stable vectors for complementation were transferred to *V. fischeri* from *E. coli* by triparental mating using the conjugative helper plasmid pEVS104 (37). For mutant construction, recombinational marker exchange was scored by screening for the appropriate antibiotic resistance phenotype, and putative double recombinants were screened by PCR to determine which had reverted to wild type and which had incorporated the Δ*phr* or Δ*luxCDABEG* mutant allele. The nonbioluminescent phenotype of the Δ*luxCDABEG* mutant also confirmed the replacement of the wild-type *lux* allele.

Measurement of UV resistance. *V. fischeri* cells were grown in 3 ml of LBS in 18-mm tubes shaken at 28°C until the A_{595} was between 0.2 and 0.5. The cells were then pelleted by centrifugation, resuspended in filter-sterilized Instant Ocean, and diluted 10-fold in Instant Ocean, and 500-μl aliquots were placed into wells in two 24-well microtiter plates. This pelleting and resuspension did not detectably affect the bioluminescence (data not shown). One microtiter plate was exposed, with the lid removed, to a preset intensity (in μJ/cm^2) of UV light in a UV-Stratalinker 1800 (Stratagene, La Jolla, Calif.), while the other plate was left unexposed. The cells were allowed to recover for 2 h before they were dilution plated onto LBS agar to determine the UV survival frequency, which was defined as the percentage of CFU ml^{-1} in the UV-treated sample relative to the number of CFU ml^{-1} in the untreated sample. To determine the influence of light on UV survival, cell suspensions were placed in four parallel microtiter plates, and two of these microtiter plates were exposed to UV light in a UV-Stratalinker that had been placed in a darkroom. Following exposure, one UV-treated plate and one untreated plate were allowed to recover for 2 h under white fluorescent laboratory lights, while the remaining plates for the "dark" treatment were kept in the darkroom during recovery and were dilution plated under a dim red light.

***Euprymna scolopes* colonization assays.** *E. scolopes* hatchlings were infected with *V. fischeri* using previously described inoculation procedures (32, 36) and overnight exposures of the squid to Instant Ocean containing *V. fischeri*. Within each experiment, similar numbers of mutant or wild-type cells were present in the respective inocula. Inoculant strains were pregrown unshaken in 5 ml of SWT in 50-ml conical tubes at 28°C so that the A_{595} was between 0.3 and 0.8, the cultures were diluted in Instant Ocean to between 1,000 and 3,000 total CFU ml^{-1}, and *E. scolopes* juveniles were exposed to the inocula for between 12 and 14 h before being rinsed in *V. fischeri*-free Instant Ocean. The squid were homogenized at 48 h postinoculation, and the homogenates were serially diluted and plated onto LBS. Following overnight incubation, the colonies were counted to determine the number of CFU per animal. To generate mixed infections with both strains EW1 and JRM100, *E. scolopes* hatchlings were exposed to mixed inocula with roughly equal numbers of EW1 and JRM100 cells for 18 h before being rinsed in inoculum-free Instant Ocean. Ratios of EW1 to JRM100 were determined after dilution plating the cells on LBS by patching 50 colonies on LBS containing erythromycin. Relative competitive indices (RCIs) were calculated by dividing the ratio of EW1 to JRM100 in the squid after 48 h by the ratio of these strains in the inoculum.

ORF	#aa	homolog/description	% identity / similarity (#aa aligned)
VFA0751	515	TyrR	49 / 67 (514)
VFA0752	314	*E.coli* YbgA, upstream of *phr*	34 / 65 (158)
VFA0753	479	Phr, photolyase	44 / 60 (474)
VFA0754	617	hypothetical	-
VFA0755	45	hypothetical	-
VFA0756	315	*E.coli* CjrA	26 / 47 (94)

FIG. 1. Schematic representation of the genetic organization near *phr* in *V. fischeri* ES114. ORF designations (e.g., VFA0753) are taken from the *V. fischeri* genome project (34). The arrows represent ORFs and indicate the direction of gene transcription, as well as the gene size, which is presented relative to the scale bar. "#aa" indicates the number of amino acids encoded by each ORF. Protein similarities were identified using BLAST (1) and the BLOSUM62 scoring matrix (19). The identity and similarity to *E. coli* K12 ORFs are presented, although closer matches with the same gene designation were found for less well-characterized ORFs (e.g., from vibrios). Several vibrios contain closer matches to ORF VFA0756, and most of these have been designated hypothetical.

RESULTS

The goal of this study was to characterize photolyase in *Vibrio fischeri* strain ES114, which is naturally bioluminescent and was originally isolated from the light organ of the Hawaiian bobtail squid, *E. scolopes*. Specifically, we sought to test whether there is a relationship between photolyase and bioluminescence both in culture and during symbiotic colonization.

Bioinformatic characterization of *phr* in *V. fischeri* ES114. Analysis of the ES114 genome (34) revealed one putative photolyase (*phr*) gene. This open reading frame (ORF), which was designated VFA0753 by the genome project, is located on chromosome II and encodes a protein that is 44% identical and 60% similar to the photolyase of *E. coli* K12 (Fig. 1). The ORF was annotated by the genome project as photolyase, and we refer to it here as *phr*. The genetic context of VFA0753 further supports its *phr* designation, as the gene immediately upstream of it, VFA0752, encodes a protein that is homologous to YpgA, which is encoded by the ORF immediately upstream of *phr* in *E. coli* (Fig. 1). Moreover, ORFs encoding proteins similar to YbgA are also upstream of putative photolyase genes in *Vibrio cholerae* (17), *Vibrio vulnificus* (6), and *Vibrio parahaemolyticus* (25), although in each of these vibrios a putative transcriptional regulator is encoded in between *ybgA* and *phr*. In each of these four vibrios, the ORFs encoding proteins similar to Phr and YbgA are also found on chromosome II, although homologs of VFA0751 (TyrR), VFA0754, and VFA0756 (Fig. 1) are on chromosome I in these vibrios.

The genetic organization near *V. fischeri phr* indicates that it

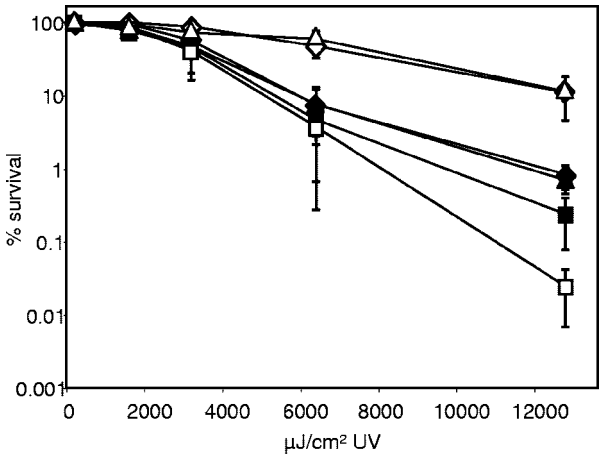

FIG. 2. Effects of UV dose and subsequent recovery in the light (open symbols) or dark (filled symbols) on survival of *V. fischeri* ES114 (diamonds), the Δ*luxCDABEG* mutant EVS102 (triangles), or the Δ*phr* mutant EW1 (squares). Percent survival was calculated relative to non-UV-exposed cells. The data represent means with standard errors for three independent cultures of each strain.

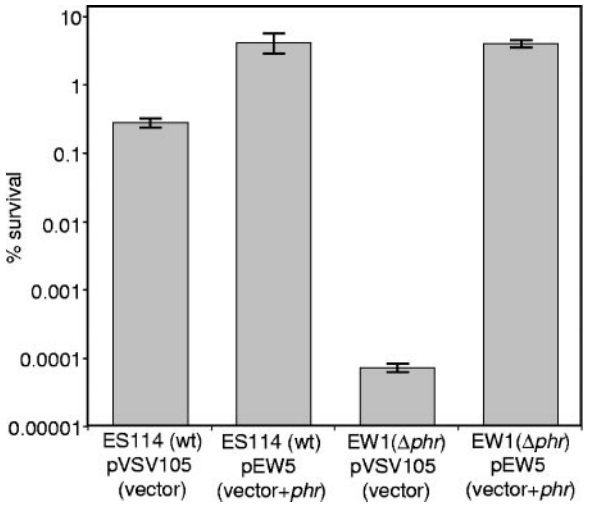

FIG. 3. Complementation of UV sensivity in Δ*phr* mutant EW1. Wild-type or EW1 (Δ*phr*) cells carrying either pVSV105 or pEW1 (*phr* cloned in pVSV105) were exposed to 12,000 μJ/cm^2 UV and allowed to recover under fluorescent white lights for 2 h prior to being plated. Percent survival was calculated relative to non-UV-exposed cells. The data represent means with standard errors for three independent cultures.

may be expressed on a polycistronic transcript (Fig. 1). *phr* overlaps VFA0752 by 7 bp, and it is separated by only 59 bp from VFA0754, strongly suggesting that it is cotranscribed with the former and possibly with the latter. VFA0754 was annotated by the genome project as encoding methionine sulfoxide reductase, an enzyme that is similar to photolyase in that it repairs damaged macromolecules. However, we found no significant similarity between the protein encoded by VFA0754 and characterized methionine sulfoxide reductases, and we have annotated this ORF as encoding a hypothetical protein (Fig. 1).

The Δ*phr* mutant lacks light-dependent UV resistance "photoreactivation" activity. To test whether *phr* encodes a functional photolyase, we generated and characterized mutant EW1, which contains an in-frame deletion of the gene. Both EW1 and its wild-type parent, ES114, displayed a dose-dependent sensitivity to UV light; however, ES114 possessed a light-dependent mechanism of UV resistance that was absent in the mutant (Fig. 2). ES114 also appeared slightly more resistant to UV light than the mutant, even when recovery was in the dark, possibly due to minor photoreactivation stimulated by the darkroom red light or to Phr-assisted excision repair, which has been observed in *E. coli* (31). Restoring the *phr* gene in *trans* to the Δ*phr* mutant complemented its UV sensitivity (Fig. 3), indicating that this phenotype was not due to effects on VFA072, VFA0754, or other genes. Taken together, our data indicate that *V. fischeri phr* encodes the bacterium's photoreactivating photolyase.

Bioluminescence does not appear to stimulate photolyase in *V. fischeri* ES114. Over a range of UV doses, we saw no difference in the UV sensitivity, or the photoreactivation capacity, of ES114 or the dark Δ*luxCDABEG* mutant EVS102 (Fig. 2), suggesting that bioluminescence does not play a role in photoreactivation. On the other hand, the cells used in these experiments had been grown to low cell density and were relatively dim. It has been reported that exposure to UV light increases expression of the *lux* genes in some bacteria even at low cell density (7, 9); however, we considered the possibility that this was not the case in ES114 and that the similarity between ES114 and EVS102 might reflect the relatively dim luminescence ES114 generates in culture (3). We therefore amended the cultures with 200 ng ml^{-1} of the autoinducer 3-oxo-C6-HSL, an amount similar to that found in the light organ (4), which induced luminescence ~500-fold to levels comparable to that seen in the symbiosis. Although addition of 3-oxo-C6-HSL stimulated luminescence in ES114, there was still no detectable difference between ES114 and the Δ*luxCDABEG* mutant EVS102 with regard to UV sensitivity or photoreactivation (Fig. 4).

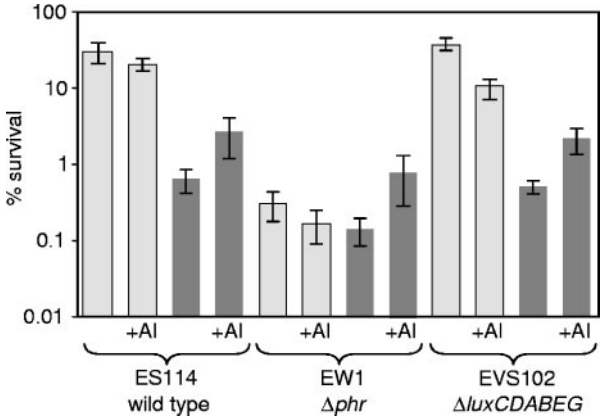

FIG. 4. Effects of pregrowth in 200 ng ml^{-1} 3-oxo-C6-HSL (indicated by "+AI") and outgrowth in the light (light bars) or dark (dark bars) on survival of *V. fischeri* ES114, the Δ*luxCDABEG* mutant EVS102, or the Δ*phr* mutant EW1 following exposure to 12,000 μJ/cm^2 UV. Percent survival was calculated relative to non-UV-exposed cells. The data represent means with standard errors for three independent cultures of each strain.

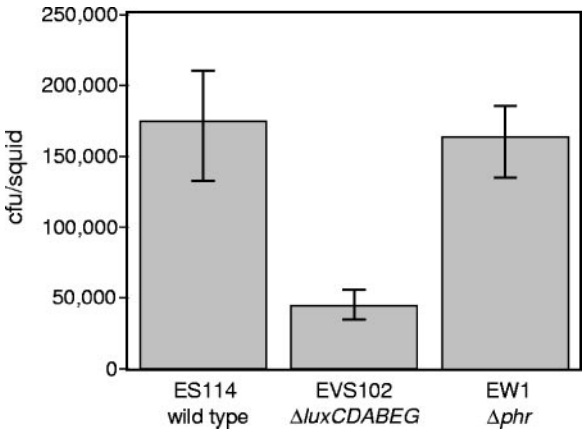

FIG. 5. Symbiotic colonization of *E. scolopes* hatchlings by the wild type, Δ*luxCDABEG* mutant EVS102, or Δ*phr* mutant EW1. Average colonization levels 48 h after inoculation with the indicated strain are shown, with standard errors ($n = 13$ or 14).

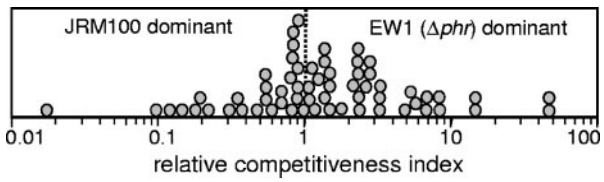

FIG. 6. Competition during mixed infection between *V. fischeri* EW1 (Δ*phr*) and JRM100. JRM100 is a derivative of ES114 marked with an intergenic mini-Tn*7-ermR* insertion that does not affect competitiveness (26). Juvenile squid were exposed to an ~1:1 mixture of the strains at a total concentration of 2,500 CFU ml^{-1} for 14 h, and the RCI of EW1, defined as the ratio of EW1 to JRM100 in the squid divided by the ratio of these strains in the inoculum, was determined for each coinfected animal 48 h after inoculation. The RCI is plotted as a circle. A dotted line marks the RCI of 1, where the strain ratio in the squid matches that in the inoculum and there is no difference in strain competitiveness.

Interestingly, the addition of 3-oxo-C6-HSL appeared to increase resistance to UV light when the cells recovered from UV exposure in the dark; however, this trend was seen in the wild type, the dark mutant EVS102, and the Δ*phr* mutant EW1 (Fig. 4), indicating that this effect is not directly related to bioluminescence or to photolyase. For each of the three strains, pregrowth in 3-oxo-C6-HSL significantly ($P < 0.05$) enhanced the survival of cells incubated in the dark following UV exposure in two out of three independent experiments, and when data for the three strains were pooled, the effect of 3-oxo-C6-HSL was significant ($P < 0.05$) in all three experiments.

We also examined ES114, the dark Δ*luxCDABEG* mutant EVS102, and the *phr* mutant EW1 under conditions where bioluminescence is naturally induced and is beneficial to the bacteria, during symbiotic colonization of the *E. scolopes* light organ. Consistent with other studies (41; J. L. Bose, C. S. Rosenberg, and E. V. Stabb, unpublished data), we found that populations of the *lux* mutant in squid light organs 48 h after inoculation were three- to fourfold lower than the symbiont populations in ES114-infected animals (Fig. 5). However, colonization by the *phr* mutant EW1 was indistinguishable from that of ES114 (Fig. 5). In some instances, symbiotic defects can be detected only if strains are forced to compete for colonization; however, as shown in Fig. 6, EW1 was not outcompeted by JRM100, a marked derivative of ES114 with competitiveness similar to that of the wild type (26). Thus, in contrast to the benefit of bioluminescence in this environment, we found no evidence of an important role for photoreactivation during symbiotic colonization.

DISCUSSION

We have shown that the *V. fischeri* ES114 *phr* gene (ORF VFA0753) encodes the photolyase enzyme of ES114 and that the benefit of bioluminescence for this bacterium during host colonization is not mediated by stimulation of Phr. ORF VFA0753 encoded the only photolyase (Phr) homolog in the ES114 genome, an in-frame deletion mutant of *phr* lacked photoreactivation (Fig. 2 and 4), and this phenotype could be complemented with the *phr* gene in *trans* (Fig. 3). However, although bioluminescence enabled the bacteria to fully colonize the *E. scolopes* light organ, as reported elsewhere (41), the *phr* mutant was unaffected in host colonization (Fig. 5 and 6). Thus, in the light organ environment where the bacteria are naturally bioluminescent and where bioluminescence is advantageous for the bacteria, the benefits of bioluminescence are not mediated by photolyase. Moreover, bioluminescence did not detectably stimulate photoreactivation in culture, even when bioluminescence was stimulated by the addition of the autoinducer 3-oxo-C6-HSL (Fig. 4). Taken together, our data do not support the model that bioluminescence functions to stimulate photolyase-mediated DNA repair in *V. fischeri* ES114.

We cannot rule out the possibility that bioluminescence does benefit ES114 or other bacteria by stimulating photolyase-mediated DNA repair in some situations. It is worth noting that even with the addition of 3-oxo-C6-HSL, strain ES114 produces only about 1/10 the luminescence of *V. fischeri* strain MJ1 (Bose et al., submitted), and perhaps luminescence functions to stimulate photolyase only in very bright strains. Our characterization of photolyase in *V. fischeri* ES1114 should facilitate future studies of this enzyme in more highly luminescent strains, such as MJ1. It should also be noted that most of the evidence supporting a connection between photolyase and bioluminescence has been generated using *V. harveyi* or the *V. harveyi lux* genes cloned in *E. coli* (8, 9, 23), and our experiments do not address the possibility that luminescence in *V. harveyi* is tied to photolyase.

Nonetheless, we believe that several theoretical considerations make photoreactivation an unlikely natural role for bioluminescence. For example, photoreactivation in bacteria is optimally stimulated by 380-nm- to 440-nm-wavelength light (12, 22), but bioluminescent bacteria usually emit blue (~500-nm-wavelength) light, with different strains emitting light with wavelengths ranging from 476 nm to 540 nm (33, 39). To our knowledge, photolyase has not been closely studied in a bioluminescent strain, but it has been examined in *V. parahaemolyticus*, a species that includes bioluminescent strains and is a close relative of *V. harveyi* and *V. fischeri*. In nonluminescent *V. parahaemolyticus* WP28, photolyase is maximally stimulated by light in the 375-nm to 425-nm range, 475-nm light is less than 10% as effective for photoreactivation, and light at >500 nm is

ineffective (30). Thus, unless the photolyases of bioluminescent *V. parahaemolyticus* strains are highly diverged from that of WP28, there will be relatively little overlap in the bioluminescence and photoreactivation spectra in these strains.

The possibility that bioluminescence is not well attuned to the absorption requirements of photolyase raises the issues of efficiency and energetics. Hastings and Nealson calculated that the energy of each photon emitted by bioluminescence is equivalent to the hydrolysis of six ATP molecules (15). Even more energy would be required if the bioluminescence efficiency is <100%, as is likely the case, and this does not even take into account the considerable energy devoted to Lux protein synthesis (16, 21). Moreover, it seems likely that most of the photons generated will not be absorbed and utilized by photolyase, and this is supported by our observation that there was no detectable difference in the intensities of bioluminescence emitted from cultures of the wild type and the photolyase mutant (data not shown). Thus, energy devoted to bioluminescence-mediated photoreactivation could conceivably be equivalent to the hydrolysis of tens, hundreds, or possibly more ATP molecules per lesion repaired. This seems a remarkably inefficient process, although the energy cost might be outweighed by the importance of maintaining genetic integrity.

Perhaps more problematic is the question of whether there is a dark environment where bioluminescent bacteria would benefit from light-mediated activation of photolyase. *Vibrio* spp. are common near the ocean surface (13), where they are exposed to UV light. However, in this setting, the bacteria would also be exposed to light that could support photoreactivation, and at increased depths, UV light is filtered out to a greater degree than the visible light that would support photoreactivation (2, 18). Although photolyase also binds to DNA lesions other than pyrimidine dimers, and in *E. coli* it stimulates excision repair of such lesions (31), these examples do not constitute photoreactivation and do not require light. Until a natural condition is found under which photoreactivation is beneficial in an environment lacking light, the evidence suggesting that bioluminescence functions to activate photolyase arguably reflects artificial conditions.

One unexpected finding of our study was that *phr* is located on chromosome II in *V. fischeri*, *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* (6, 17, 25, 34). In members of the family *Vibrionaceae*, chromosome II contains a higher relative representation of unusual and presumably niche-specific genes than does the larger chromosome I, which has a higher representation of housekeeping genes. Although photolyase might be considered one of the housekeeping genes found on chromosome II, it could also reflect the fact that the benefit of having *phr* is specific to certain environments, such as those exposed to UV light. In this regard, it is interesting to note that *Photobacterium profundum* strain SS9, a member of the *Vibrionaceae* that is adapted to deep, dark marine environments, lacks a photolyase gene (40).

Another interesting and unexpected finding was that the addition of 3-oxo-C6-HSL to stimulate bioluminescence also appeared to increase the survival of *V. fischeri* exposed to UV light and then kept in the dark but that this UV resistance was mediated by a mechanism independent of bioluminescence or photolyase (Fig. 4). This phenomenon was statistically significant ($P < 0.05$) for each strain in two out of three experiments, and it could provide an alternative explanation for the UV sensitivity of undefined (e.g., chemically induced) dark mutants. If bioluminescence and some mechanism(s) of UV resistance are coregulated, then some regulatory mutants might be both dark and UV sensitive without a direct causal relationship between bioluminescence and UV resistance.

The functional significance of bioluminescence for light-producing bacteria remains uncertain, and the observation that bioluminescence allows *V. fischeri* ES114 to fully colonize its host is equally mysterious. Several plausible explanations have been offered (35), suggesting that bioluminescence acts as a sink for excess reductant (5), as an antioxidant (24, 38, 41), or as a symbiotic signal to the host that is required for light organ tissue to develop into an environment receptive to colonization (M. McFall-Ngai and C. Whistler, personal communication). Given our results and the arguments above, we are skeptical of the idea that the driving force behind the evolution of bioluminescence was that it played a significant role in stimulating photolyase. There may not be a single, simple role for bioluminescence, and it seems likely that bioluminescence may serve different purposes depending on the bacterium and the environment. Our laboratory and others are actively investigating these multiple possible functions of bioluminescence.

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