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Regulation of Bioluminescence in *Photobacterium leiognathi* Strain KNH6

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ABSTRACT

Bacterial bioluminescence is taxonomically restricted to certain proteobacteria, many of which belong to the *Vibrionaceae*. In the most well-studied cases, pheromone signaling plays a key role in regulation of light production. However, previous reports have indicated that certain *Photobacterium* strains do not use this regulatory method for controlling luminescence. In this study, we combined genome sequencing with genetic approaches to characterize the regulation of luminescence in *Photobacterium leiognathi* strain KNH6, an extremely bright isolate. Using transposon mutagenesis and screening for decreased luminescence, we identified insertions in genes encoding components necessary for the luciferase reaction (*lux*, *lum*, and *rib* operons) as well as in nine other loci. These additional loci encode gene products predicted to be involved in the tricarboxylic acid (TCA) cycle, DNA and RNA metabolism, transcriptional regulation, and the synthesis of cytochrome *c*, peptidoglycan, and fatty acids. The mutagenesis screen did not identify any mutants with disruptions of predicted pheromone-related loci. Using targeted gene insertional disruptions, we demonstrate that under the growth conditions tested, luminescence levels do not appear to be controlled through canonical pheromone signaling systems in this strain.

IMPORTANCE

Despite the long-standing interest in luminous bacteria, outside a few model organisms, little is known about the regulation and function of luminescence. Light-producing marine bacteria are widely distributed and have diverse lifestyles, suggesting that the control and significance of luminescence may be similarly diverse. In this study, we apply genetic tools to the study of regulation of light production in the extremely bright isolate *Photobacterium leiognathi* KNH6. Our results suggest an unusual lack of canonical pheromone-mediated control of luminescence and contribute to a better understanding of alternative strategies for regulation of a key bacterial behavior. These experiments lay the groundwork for further study of the regulation and role of bioluminescence in *P. leiognathi*.

Bacterial bioluminescence has been the subject of scientific study for over 300 years (1), and light-producing bacteria can be placed phylogenetically into the *Vibrionaceae*, *Shewanellaceae*, and *Enterobacteriaceae* families (reviewed in reference 2). Despite this long-standing interest in luminous bacteria, only in the past few decades has the genetic basis of light production been revealed. These relatively recent studies have focused mainly on a few model organisms, particularly *Vibrio harveyi* (*Vibrio campbellii*) and *Vibrio fischeri* (*Aliivibrio fischeri*), and have contributed immensely to our understanding of bacterial pheromone signaling or quorum sensing (3–5). However, it is known that in marine habitats, luminous bacteria are widely distributed (1) and can be found free living as well as host associated (6), and it has been observed that certain isolates of *Photobacterium leiognathi* do not appear to display cell density-dependent regulation of luminescence (7). Taken together, these observations suggest that the regulation, nature, and ecological functions of bioluminescence are likely diverse.

Light-producing marine bacteria belonging to the genus *Photobacterium* are a good example of this diversity. They have been isolated from seawater samples as well as from eukaryotic hosts (2, 7, 8), and culture-based studies have indicated that *Photobacterium* strains vary in whether cell density-based signaling is used for regulatory control of luminescence (9). However, despite these interesting observations, to our knowledge, regulation of light production in *Photobacterium* species has not been studied genetically without the use of transgenic *Escherichia coli*. In this study, we describe the adaptation of previously developed genetic tools to study the regulation of bioluminescence in *Photobacterium leiognathi* strain KNH6. This strain is among the brightest bioluminescent isolates we have found and is frequently used in teaching laboratories and outreach demonstrations (10). These genetic approaches allowed us not only to identify new and interesting, non-pheromone-based mechanisms of luminescence regulation but also to provide a novel system for studying the regulation and ecological functions of light production and the role of pheromone-based signaling in *Photobacterium* spp.

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TABLE 1 Strains and plasmids used for this study

Strain or plasmid	Description[a]	Source or reference
Strains		
Escherichia coli strains		
DH5α λ*pir*	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA*; lysogenized with λ*pir*	12
CC118 λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA*; lysogenized with λ*pir*	8
Vibrio strains		
V. harveyi BB120	Wild-type marine isolate	43
V. fischeri ES114	Wild-type squid symbiotic strain	44
V. fischeri MJ11	Wild-type fish symbiotic strain	45
Photobacterium strains		
KNH6	Bioluminescent seawater isolate	8
KT2c	KNH6 with Tn*5* insertion in VT25_10345 (*sspA*)	This study
KT7c	KNH6 with Tn*5* insertion in VT25_07015 (*mrcA*)	This study
KT8c	KNH6 with Tn*5* insertion in VT25_08820 (*luxF*)	This study
KT10c	KNH6 with Tn*5* insertion in VT25_08835 (*luxD*)	This study
KT16c	KNH6 with Tn*5* insertion in VT25_09400 (*pnp*)	This study
KT17c	KNH6 with Tn*5* insertion in VT25_13875 (*ribD*)	This study
KT19c	KNH6 with Tn*5* insertion in VT25_17790	This study
KT24c	KNH6 with Tn*5* insertion in VT25_08845 (*lumP*)	This study
KT1d	KNH6 with Tn*5* insertion in VT25_00305 (*miaA*)	This study
KT2d	KNH6 with Tn*5* insertion in VT25_09570 (*thyA*)	This study
KT7d	KNH6 with Tn*5* insertion in VT25_05985 (*mdh*)	This study
KT8d	KNH6 with Tn*5* insertion in VT25_03135 (*plsX*)	This study
KT9d	KNH6 with Tn*5* insertion in VT25_08850 (*lumQ*)	This study
KT10d	KNH6 with Tn*5* insertion in VT25_08825 (*luxB*)	This study
KT14d	KNH6 with Tn*5* insertion in VT25_08840 (*luxC*)	This study
KT20d	KNH6 with Tn*5* insertion in VT25_12855 (*sucA*)	This study
KT23d	KNH6 with Tn*5* insertion in VT25_08810 (*luxG*)	This study
AKD920	KNH6 VT25_08840 (*luxC*)::pAKD920	This study
AKD921	KNH6 VT25_13875 (*ribD*)::pAKD921	This study
AKD922	KNH6 VT25_10345 (*sspA*)::pAKD922	This study
AKD923	KNH6 VT25_00305 (*miaA*)::pAKD923	This study
AKD924	KNH6 VT25_09570 (*thyA*)::pAKD924	This study
AKD925	KNH6 VT25_09400 (*pnp*)::pAKD925	This study
AKD926	KNH6 VT25_07015(*mrcA*)::pAKD926	This study
AKD927	KNH6 VT25_17790::pAKD927	This study
AKD928	KNH6 VT25_12855 (*sucA*)::pAKD928	This study
AKD929	KNH6 VT25_05985 (*mdh*)::pAKD929	This study
AKD930	KNH6 VT25_03135 (*plsX*)::pAKD930	This study
AKD931	KNH6 VT25_06970 (*aphA*)::pAKD931	This study
AKD932	KNH6 VT25_15745 (*cqsA*)::pAKD932	This study
AKD933	KNH6 VT25_15750 (*cqsS*)::pAKD933	This study
AKD934	KNH6 VT25_10580 (*hapR*)::pAKD934	This study
AKD935	KNH6 VT25_03485 (*luxO*)::pAKD935	This study
AKD936	KNH6 VT25_00450 (*luxS*)::pAKD936	This study
AKD937	KNH6 VT25_08805 (*ribE*)::pAKD937	This study
AKD938	KNH6 VT25_07845 (*luxR*)::pAKD938	This study
Plasmids		
pEVS104	Conjugation helper plasmid	8
pEVS122	*oriR6K*-based suicide vector; Erm^r	46
pEVS170	Mini-Tn*5*-*erm* delivery vector	15
pAKD601B	*E. coli-V. fischeri* shuttle vector, contains *lacI*^{q} and an IPTG-inducible promoter for overexpression of proteins in *V. fischeri*; Kan^r	17
pAKD701	*E. coli-V. fischeri* shuttle vector with RP4 *oriT*, pES213 and R6K replication origins, and *lacZ* reporter gene; Kan^r	47
pAKD920	pEVS122 containing an 800-bp internal gene fragment of VT25_08840 (*luxC*)	This study
pAKD921	pEVS122 containing an 875-bp internal gene fragment of VT25_13875 (*ribD*)	This study
pAKD922	pEVS122 containing a 319-bp internal gene fragment of VT25_10345 (*sspA*)	This study
pAKD923	pEVS122 containing a 679-bp internal gene fragment of VT25_00305 (*miaA*)	This study
pAKD924	pEVS122 containing an 845-bp internal gene fragment of VT25_09570 (*thyA*)	This study
pAKD925	pEVS122 containing a 994-bp internal gene fragment of VT25_09400 (*pnp*)	This study
pAKD926	pEVS122 containing a 981-bp internal gene fragment of VT25_07015 (*mrcA*)	This study

(Continued on following page)

TABLE 1 (Continued)

Strain or plasmid	Description[a]	Source or reference
pAKD927	pEVS122 containing a 1,400-bp internal gene fragment of VT25_17790	This study
pAKD928	pEVS122 containing a 1,094-bp internal gene fragment of VT25_12855 (*sucA*)	This study
pAKD929	pEVS122 containing a 727-bp internal gene fragment of VT25_05985 (*mdh*)	This study
pAKD930	pEVS122 containing a 782-bp internal gene fragment of VT25_03135 (*plsX*)	This study
pAKD931	pEVS122 containing a 554-bp internal gene fragment of VT25_06970 (*aphA*)	This study
pAKD932	pEVS122 containing a 975-bp internal gene fragment of VT25_15745 (*cqsA*)	This study
pAKD933	pEVS122 containing a 1,314-bp internal gene fragment of VT25_15750 (*cqsS*)	This study
pAKD934	pEVS122 containing a 530-bp internal gene fragment of VT25_10580 (*hapR*)	This study
pAKD935	pEVS122 containing a 1,036-bp internal gene fragment of VT25_03485 (*luxO*)	This study
pAKD936	pEVS122 containing a 502-bp internal gene fragment of VT25_00450 (*luxS*)	This study
pAKD937	pEVS122 containing a 632-bp internal gene fragment of VT25_08805 (*ribE*)	This study
pAKD938	pEVS122 containing a 533-bp internal gene fragment of VT25_07845 (*luxR*)	This study
pAKD939	pAKD701 containing the KNH6 *luxC* (VT25_08840) promoter region	This study
pAKD940	pAKD601B containing the coding sequence of KNH6 VT25_00305 (*miaA*)	This study
pAKD941	pAKD601B containing the coding sequence of KNH6 VT25_10345 (*sspA*)	This study
pAKD942	pAKD601B containing the coding sequence of KNH6 VT25_12715 (*hns*)	This study
pAKD943	pAKD601B containing the coding sequence of KNH6 VT25_09570 (*thyA*)	This study
pAKD944	pAKD601B containing the coding sequence of KNH6 VT25_09400 (*pnp*)	This study
pAKD945	pAKD601B containing the coding sequence of KNH6 VT25_07015 (*mrcA*)	This study

[a] Erm^r, erythromycin resistance; Kan^r, kanamycin resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Photobacterium leiognathi* strain KNH6 was isolated from near-shore waters in Kāne'ohe Bay, O'ahu, HI (8). KNH6 and its constructed derivatives (Table 1) were grown at 25°C in either the rich medium LBS (11) or mineral salts medium (made up of the following, per liter: 378 ml 1 M $NaPO_4$ [pH 7.5], 50 ml 1 M Tris [pH 8.0], 3 mg $FeSO_4{\cdot}7H_2O$, 0.59 g NH_4Cl, 13.6 g $MgSO_4{\cdot}7H_2O$, 0.83 g KCl, 19.5 g NaCl, 1.62 g $CaCl_2{\cdot}2H_2O$, 1.0 g Casamino Acids [vitamin assay; Difco], and 3.6 g glucose). When indicated, 20 μg/ml erythromycin or 100 μg/ml kanamycin was added to cultures for selection of KNH6 derivatives. For complementation assays, 0.1 or 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Research Products International) was added to cultures where indicated. *Escherichia coli* strain DH5α λ*pir* (12) was used for plasmid construction, and DH5α λ*pir* and CC118 λ*pir* (8) were used for conjugative transfer of plasmids into KNH6. *E. coli* strains were grown in LB medium (13), and plasmid-bearing strains were grown in LB containing 20 μg/ml kanamycin or in HiVeg Special infusion broth (Himedia, Mumbai, India) containing 150 μg/ml erythromycin. Plasmids (Table 1) were constructed using standard molecular biology techniques and restriction enzymes from New England BioLabs (Ipswich, MA). Fragments of the KNH6 genome were PCR amplified using Phusion High-Fidelity DNA polymerase (New England BioLabs) with primers from Integrated DNA Technologies (Coralville, IA). Primer sequences are listed in Table S1 in the supplemental material.

Genome sequencing, assembly, and annotation. Chromosomal DNA was isolated from KNH6 by using an EasyDNA kit (Life Technologies, Grand Island, NY) and was sent to the Oklahoma Medical Research Foundation Genomics Facility for library preparation and sequencing on an Illumina MiSeq sequencer for 250-bp paired-end reads. *De novo* assembly was performed in Ray, version 2.1.0 (14), to generate a 4.6-Mb assembly with 38 contigs of ≥500 nucleotides (nt) and an N_{50} of 637 bp. The number of contigs was reduced to 34 by using PCR and Sanger sequencing (University of Oklahoma Biology Core Molecular Laboratory).

Tn mutagenesis. Transposon (Tn) mutagenesis was carried out using a previously described mini-Tn*5* system (15). Plasmid pEVS170 was introduced into strain KNH6 by conjugative mating (8). Conjugation samples were placed as spots on LBS agar and incubated at 25°C for 24 h. After incubation, spots were resuspended in 0.5 ml of LBS medium, and 100-μl aliquots were plated onto LBS agar containing 20 μg/ml erythromycin. Agar plates were incubated at room temperature for 24 h, and colonies were scanned by eye to identify those with reduced bioluminescence. The mutagenesis procedure was repeated on five separate days, with multiple conjugation spots on each day. In total, approximately 12,000 colonies were screened. Potential dark or dim mutants were tested for kanamycin sensitivity, which indicates a lack of the transposon delivery vector backbone. The location of the transposon insertion in each mutant of interest was determined using rescue cloning based on the R6K replication origin in the transposon, as previously described (15), with the exception of using the restriction enzyme BstUI instead of HhaI. Sanger sequencing of the rescued plasmids containing Tn insertions was performed at the University of Oklahoma Biology Core Molecular Laboratory.

Plate reader-based growth and bioluminescence assays. The growth and bioluminescence of strains were characterized using a BioTek Synergy H1 microplate reader (Winooski, VT). Strains were grown overnight in LBS or, for transposon mutants or strains containing plasmids, in LBS containing antibiotic. Strains were diluted 10-fold in filter-sterilized artificial seawater (Instant Ocean, Blacksburg, VA), and 2 μl was added to each well of a 96-well white microtiter plate (Corning, Corning, NY) containing 200 μl of medium. Plates were incubated with lids at 25°C with double-orbital shaking (slow speed, 282 cpm, 3-mm radius). Growth was monitored by measuring the optical density at 600 nm (OD_{600}), and luminescence was measured for 1 s at a 2.5-mm height, with a gain setting of 100. Luminescence is reported as specific luminescence, which is the luminescence divided by the corresponding OD_{600} value. For each experiment, three independent replicates of each strain were tested, each experiment was repeated at least three times, and data from one representative experiment are shown.

Conditioned medium. To generate conditioned medium, an overnight LBS culture of KNH6 was diluted 250-fold in 10 ml of LBS or mineral salts glucose medium in a 125-ml Erlenmeyer flask. Cultures were grown at 25°C with vigorous shaking in LBS for 3 h (OD_{600} of 0.6) or in mineral salts medium for 4 h (OD_{600} of 0.3). The OD_{600} was measured using a Bio-Rad SmartSpec Plus spectrophotometer (Hercules, CA). Aliquots were centrifuged to remove cells, and the supernatants were filter sterilized. The OD_{600} values for these flask-grown cultures did not directly correspond to the OD_{600} values from the plate reader-based luminescence assays but did represent luminescent cultures in the logarithmic growth phase.

Insertional disruption mutants. To verify growth and luminescence phenotypes associated with disruption of particular genes in the transposon mutants and to generate mutants in loci not identified in the transposon mutant screen, targeted insertional disruption mutant strains were constructed (16). An internal fragment of the target gene was cloned into the suicide vector pEVS122 (12). Plasmids were mobilized into KNH6, and single recombinants were selected by plating on LBS plates containing erythromycin. To reliably obtain single recombinants, it was necessary to clone internal gene fragments of at least 500 bp.

Complementation assays. DNA fragments containing the native ribosome binding site and coding region of the gene of interest were PCR amplified and directionally cloned into plasmid pAKD601B, which allows IPTG-dependent induction of inserts (17). Plasmids were introduced into the respective transposon mutant strains, and cultures were grown as described for the plate reader-based growth and bioluminescence assays, using LBS medium containing kanamycin with 0, 0.1, or 1.0 mM IPTG. Wild-type KNH6 containing pAKD601B was used as a control for the effect of plasmid carriage and/or IPTG addition on growth or bioluminescence. Within an experiment, three independent cultures of each strain were assayed, experiments were repeated three times, and data from one representative experiment are shown.

***lux* operon promoter activity assays.** A *lux* operon promoter reporter plasmid was constructed by PCR amplifying and cloning an approximately 500-bp fragment upstream of the *luxC* start codon into pAKD701 to generate a promoter-*lacZ* fusion (pAKD939). The plasmid was introduced into strains of interest by conjugative mating, with selection on LBS medium containing kanamycin. Strains were grown as described for the plate reader-based growth and bioluminescence assays, using LBS medium containing kanamycin. When the cultures reached an OD_{600} between 0.3 and 0.4, the entire 0.2 ml was harvested and assayed for β-galactosidase activity. Activity was measured using a modified Miller assay as previously described (18). Three independent cultures of each strain were assayed for each experiment, and experiments were repeated twice. Data from one representative experiment are shown. Significance was measured using Student's *t* test.

Nucleotide sequence accession numbers. The sequences from the KNH6 whole-genome shotgun project have been deposited at DDBJ/EMBL/GenBank under accession number LAFZ00000000. The version described here is version LAFZ01000000.

RESULTS

P. leiognathi KNH6 is a bioluminescent isolate so remarkably bright that we and others have adopted it over other model bacteria for classroom demonstrations of bioluminescence. KNH6 is ~50-fold brighter than *V. harveyi* BB120 and ~250-fold brighter than *V. fischeri* MJ11 (Fig. 1), two commonly used demonstration strains. This bright bioluminescence might reflect an ecological function and/or regulation different from those of the typical model strains, an idea that prompted our genetics-based investigation of the regulation of light production in KNH6.

Genome sequence and strain identification. The draft genome of strain KNH6 consists of 4,552,777 bp in 34 contigs with 4,098 predicted coding sequences. RDP Classifier analysis of 16S rRNA (19) indicated that the strain belongs to the genus *Photobacterium*. Similar to other characterized bioluminescent *Photobacterium* strains (20), the luminescence genes (*luxA* to *-G*) are arranged in an operon and are flanked by genes encoding the riboflavin biosynthesis machinery (*rib* genes) and by the *lumPQ* operon, which encodes the lumazine protein and a predicted regulator (Fig. 2). Strain KNH6 was previously described as *Photobacterium leiognathi* (8). The presence of *luxF* in the luciferase gene operon suggests that KNH6 is closely related to previously characterized "*Photobacterium mandapamensis*" strains (21), an idea supported by the observation that the strain exhibits increased luminescence levels with higher salt levels in the growth medium (22; data not shown). However, whether the species designation is *P. leiognathi* or *P. mandapamensis* is currently unresolved (23). We therefore refer to the strain as *Photobacterium leiognathi* strain KNH6.

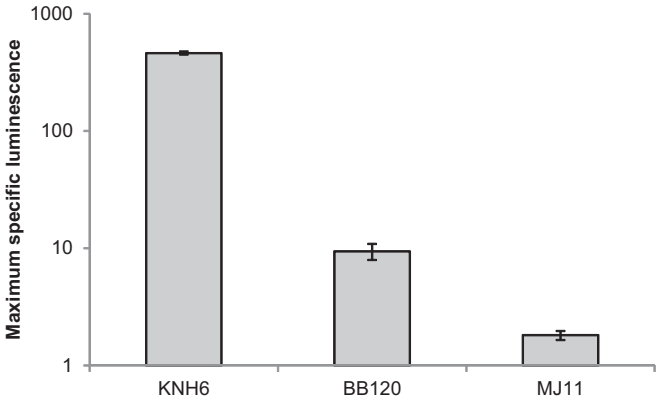

FIG 1 Comparison of maximum specific luminescence values for *P. leiognathi* KNH6, *Vibrio harveyi* BB120, *Vibrio fischeri* MJ11, and *Vibrio fischeri* ES114 during growth in LBS medium. The arbitrary luminescence scale was normalized by setting the maximum luminescence of *V. fischeri* ES114 to 1. Data from one representative experiment are shown, with error bars indicating the standard errors of the means. The experiment was repeated three times with similar results.

KNH6 luminescence patterns during growth lack evidence of high-cell-density-dependent pheromone signaling. The growth and luminescence of KNH6 over time were measured in both rich (LBS) and mineral salts glucose media. In all medium types, luminescence increased rapidly at low cell densities (Fig. 3). In addition, increasing the inoculum dilution 10- and 100-fold did not alter the specific luminescence pattern in LBS medium (data not shown). This is in contrast to previous observations for bioluminescent marine bacteria that regulate light production through pheromone signaling, where light production is not induced at very low cell densities (24). It is interesting that the rapid increase in KNH6 luminescence at low cell density was tempered in LBS medium between OD_{600} values of approximately 0.2 and 0.35 and that this repression was not observed when cells were grown in conditioned medium (Fig. 3A). These results most likely suggest the presence of an inhibitor in the LBS medium, and previous studies of other luminescent bacteria implicated unknown molecules in rich medium that repress light production (25). Because inhibition of light production was not observed in KNH6 during growth in mineral salts glucose medium (Fig. 3B) and the inhibition decreased when cells were grown in LBS medium containing 1/10 the normal amount of tryptone (Fig. 3C), it appears that the effect of conditioned medium on the luminescence curve was due mainly to a reduction in the level of an unknown inhibitor in the rich medium. Taken together, these results suggest that pheromone-based regulation did not play a major role in control of light production in KNH6 under the tested conditions.

Transposon mutagenesis and screening identified 17 loci linked to a reduction in light production. To perform an unbiased search for gene products involved in regulation of luminescence in KNH6, a transposon-based mutagenesis screen was used. Approximately 12,000 mutant colonies were screened on agar

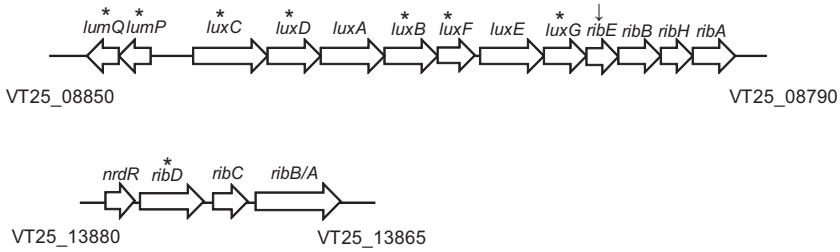

FIG 2 Representation of the *lum*, *lux*, and *rib* operons in KNH6. Open reading frame (ORF) numbers are provided for the first and last genes of each represented region. Asterisks indicate loci identified in the transposon mutant screen. The down arrow indicates that an insertional disruption was targeted to *ribE*.

plates for reduced luminescence, a number that theoretically falls short of a comprehensive screen of nonessential genes (26, 27). However, multiple independent mutations were identified in several loci (Table 2), indicating diminishing returns for further screening efforts. Putative luminescence mutants were further screened in liquid medium for verification of lowered luminescence relative to that of wild-type KNH6. The location of the transposon insertion in each verified mutant was determined through sequencing. Mutants with insertions in predicted luminescence-related genes (*lux*, *rib*, and *lum* genes) were identified, indicating that the screening process was valid (Fig. 2 and Table 2). In addition, transposon insertions were found in unexpected genes. Strains with insertional disruptions of these loci were constructed to verify whether disrupting a particular locus was causal to the luminescence phenotype. Table 2 lists nine loci that, when interrupted via transposon mutagenesis or insertional disruption, resulted in strains with luminescence phenotypes that differed from that of wild-type KNH6 (classified as either dark, dim, or slightly dim) (Fig. 3; see Fig. S2 in the supplemental material). These loci included genes whose products are predicted to be involved in the tricarboxylic acid (TCA) cycle (VT25_05985 [malate dehydrogenase] and VT25_12855 [2-oxogluarate dehydrogenase]), cytochrome *c* synthesis (VT25_17790), fatty acid synthesis (VT25_03135 [*plsX*]), peptidoglycan biosynthesis (VT25_07015 [*mrcA*]), RNA degradation (VT25_09400 [*pnp*]), transcriptional regulation (VT25_10345 [*sspA*]), tRNA modification (VT25_00305 [*miaA*]), and DNA synthesis (*thyA*). It is notable that no putative pheromone signaling loci were identified in the mutagenesis screen.

In addition, complementation assays were performed with mini-Tn*5* mutant strains displaying dark or dim luminescence phenotypes (KT2c, *sspA* mutant; KT7c, *mrcA* mutant; KT1d, *miaA* mutant; KT2d, *thyA* mutant; and KT16c, *pnp* mutant). The growth and luminescence phenotypes of KT2c (*sspA*) were complemented by providing in *trans* either *sspA* (Fig. 4A) or *sspA* plus *sspB*, the downstream gene that appears to be cotranscribed with *sspA* (data not shown). Controlled expression of *mrcA* did not complement the luminescence phenotype in KT7c (Fig. 4B). This gene does not appear to be part of an operon. Partial complementation was observed when *miaA* was expressed in KT1d (Fig. 4C). Because *miaA* is predicted to be the last gene in a three-gene operon, the operon was cloned and expressed in *trans*, but no further complementation was observed (data not shown). Finally, complementation was observed when *thyA* was expressed in KT2d (Fig. 4D) and when *pnp* was expressed in KT16c (Fig. 4E).

***lux* operon promoter activity is significantly reduced in strains with transposon insertions in *sspA* and *pnp*.** To investi-

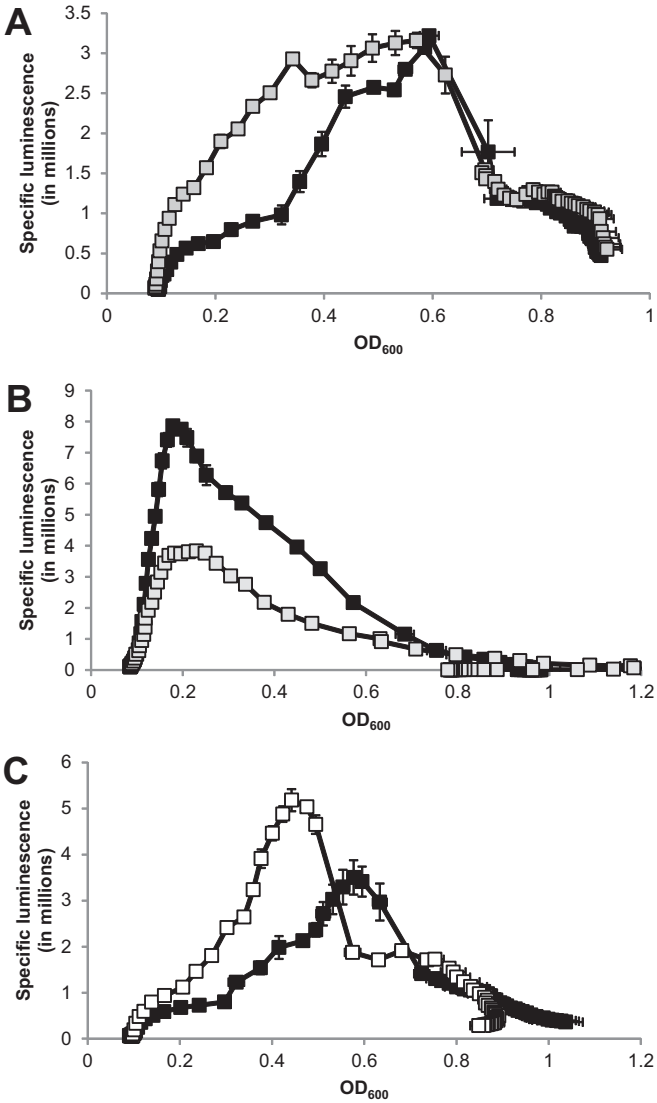

FIG 3 Specific luminescence of KNH6 during growth in regular and conditioned media. (A) Luminescence during growth in LBS medium (black squares) and conditioned LBS medium (gray squares). (B) Luminescence during growth in mineral salts glucose medium (black squares) and conditioned mineral salts glucose medium (gray squares). (C) Luminescence during growth in LBS medium (black squares) and LBS medium containing 0.1× tryptone (white squares). Data are from one representative experiment, with error bars indicating standard errors of the means. Corresponding growth curves are presented in Fig. S1 in the supplemental material.

TABLE 2 Luminescence-related loci identified in the transposon mutagenesis screen

Gene category and ORF no.	Annotation (protein, gene)	Representative Tn*5* mutant strain[a]	Phenotype in liquid medium[b]
Predicted luminescence-related genes			
VT25_08845	Lumazine protein, *lumP*	KT24c (1)	Dim
VT25_08850	AraC family transcriptional regulator, *lumQ*	KT9d (1)	Dim
VT25_08840	Acyl-coenzyme reductase, *luxC*	KT14d (4)	Dark
VT25_08835	Acyl transferase, *luxD*	KT10c (2)	Dark
VT25_08825	Alkanal monooxygenase, *luxB*	KT10d (2)	Dark
VT25_08820	Flavin-utilizing monooxygenase, *luxF*	KT8c (2)	Dark
VT25_08810	FMN reductase, *luxG*	KT23d (1)	Dim
VT25_13875	5-Amino-6-(5-phosphoribosylamino)uracil reductase, *ribD*	KT17c (1)	Dim
Other genes			
VT25_10345	Stringent starvation protein A, *sspA*	KT2c (1)	Dark
VT25_00305	tRNA dimethylallyltransferase, *miaA*	KT1d (2)	Dark
VT25_09570	Thymidylate synthase, *thyA*	KT2d (1)	Dim
VT25_09400	Polynucleotide phosphorylase/polyadenylase, *pnp*	KT16c (1)	Dim
VT25_07015	Penicillin-sensitive transpeptidase, *mrcA*	KT7c (1)	Dim
VT25_17790	Cytochrome *c* biogenesis protein	KT19c (1)	Slightly dim
VT25_12855	2-Oxoglutarate dehydrogenase, *sucA*	KT20d (1)	Slightly dim
VT25_05985	Malate dehydrogenase, *mdh*	KT7d (1)	Slightly dim
VT25_03135	Phosphate acyltransferase, *plsX*	KT8d (1)	Slightly dim

[a] Numbers in parentheses indicate the number of independent Tn*5* mutants for each locus.
[b] Compared to wild-type KNH6 grown in LBS medium.

gate whether the decrease in luminescence in transposon mutant strains to dim or dark levels was due to regulation at the level of transcription initiation, a *lacZ*-based reporter assay was performed. The putative promoter region upstream of *luxC* was cloned into the *lacZ* reporter plasmid pAKD701, and the resultant plasmid was mobilized into wild-type KNH6 and the nine KNH6 Tn*5* mutant strains. Cultures were sampled in the log phase, and cell density, luminescence, and promoter activity were measured (Table 3). In the strains with transposon insertions in *sspA* (KT2c) and *pnp* (KT16c), significantly lower levels of promoter activity than that in the wild type were observed, suggesting that, in these backgrounds, the lower luminescence levels could be explained by a reduction in *lux* gene expression. In KT1d (*miaA*), depending on the experiment, the *lux* operon promoter activity was either not significantly different from that of the wild type or significantly higher. In the remainder of the mutants, with Tn insertions in

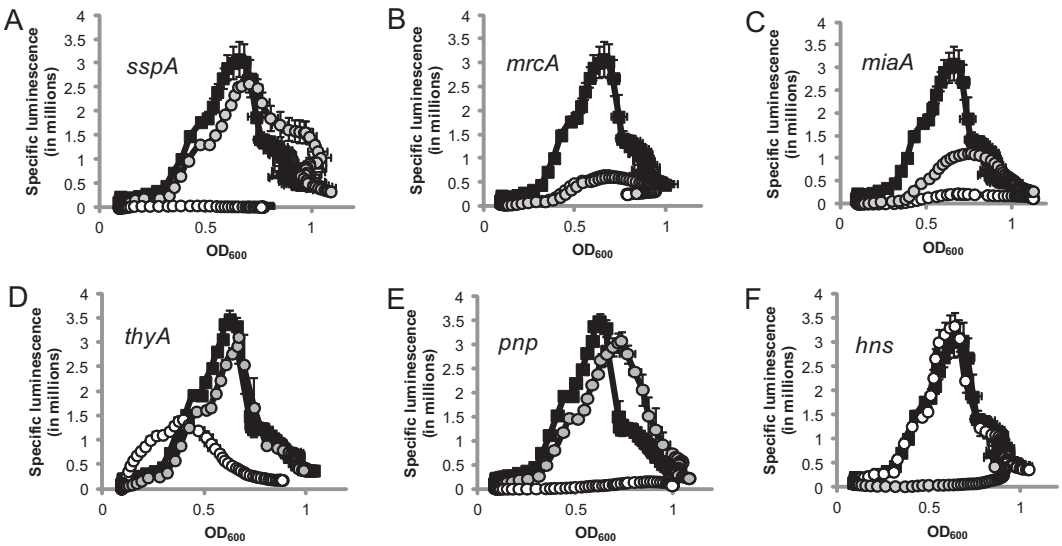

FIG 4 Specific luminescence curves from complementation assays. For all panels, the black squares indicate wild-type KNH6 containing pAKD601B grown in rich (LBS) medium and exposed to 1.0 mM (A to C) or 0.1 mM (D to F) IPTG. (A) KT2c (*sspA*) containing pAKD941 grown in LBS (white circles) and LBS containing 1.0 mM IPTG (gray circles). (B) KT7c (*mrcA*) containing pAKD945 grown in LBS (white circles) and LBS containing 1.0 mM IPTG (gray circles). (C) KT1d (*miaA*) containing pAKD940 grown in LBS (white circles) and LBS containing 1.0 mM IPTG (gray circles). (D) KT2d (*thyA*) containing pAKD943 grown in LBS (white circles) and LBS containing 0.1 mM IPTG (gray circles). (E) KT16c (*pnp*) containing pAKD944 grown in LBS (white circles) and LBS containing 0.1 mM IPTG (gray circles). (F) KNH6 containing pAKD942 (H-NS expression plasmid) grown in LBS (white circles) and LBS containing 0.1 mM IPTG (gray circles). Error bars represent standard errors of the means. Data shown are from one representative experiment.

TABLE 3 β-Galactosidase assays of *lux* operon promoter activity in wild-type KNH6 and selected transposon mutant strains grown in LBS medium

Strain (ORF, gene where Tn*5* inserted)	Avg (SE) β-galactosidase activity (Miller units)[a]	Fold difference in specific luminescence (mean [SE])[b]
KNH6	34.3 (0.9)	1
KT2c (VT25_10345, *sspA*)	4.3 (0.2)[d]	0.012 (0.006)[d]
KT1d (VT25_00305, *miaA*)	48.9 (0.8)[d]	0.0073 (0.0012)[d]
KT2d (VT25_09570, *thyA*)	35.9 (2.6)	0.71 (0.029)
KT16c (VT25_09400, *pnp*)	14.1 (1.6)[d]	0.0037 (0.0025)[d]
KT7c (VT25_07015, *mrcA*)	29.7 (1.8)	0.15 (0.015)[d]
KT19c (VT25_17790)	35.4 (1.1)	0.47 (0.055)[c]
KT20d (VT25_12855, *sucA*)	36.5 (0.1)	0.55 (0.011)[c]
KT7d (VT25_05985, *mdh*)	34.5 (3.0)	0.53 (0.1)[c]
KT8d (VT25_03135, *plsX*)	35.0 (3.0)	0.36 (0.069)[c]

[a] The average β-galactosidase activity for the empty plasmid (pAKD701) in KNH6 was 0.1 Miller unit.
[b] Values shown are relative to the specific luminescence of KNH6 ($1.66 \times 10^6 \pm 2.11 \times 10^5$).
[c] Significantly different from KNH6 as determined using Student's *t* test ($P \leq 0.05$).
[d] Significantly different from KNH6 as determined using Student's *t* test ($P \leq 0.01$).

thyA, *mrcA*, VT_2517790, *sucA*, *mdh*, and *plsX*, promoter activity levels were similar to that in the wild type despite lower luminescence levels in these strains. In addition, *lux* operon promoter activity was tested in strain KT9d (Tn insertion in *lumQ*, encoding a predicted regulatory protein) and was not significantly different from that in the wild type (data not shown).

Disrupting *sspA* may alter H-NS levels. As described above, the KNH6 Tn*5* mutant strain KT2c (*sspA*) was almost completely dark, the growth and luminescence phenotypes could be complemented, and β-galactosidase assays indicated that *lux* operon promoter activity was reduced in this strain. These characteristics suggested that SspA either directly or indirectly plays a role in regulation of luminescence in KNH6. In *E. coli*, SspA is involved in decreasing expression levels of the global regulator H-NS (28). Analysis of the KNH6 *lux* promoter region by using Virtual Footprint (29) and the *E. coli* H-NS consensus binding sequence indicated three potential binding sites, approximately 220, 300, and 440 bp upstream of the *luxC* start codon (data not shown). To determine whether H-NS could play a role in influencing luminescence levels in KNH6, an inducible *hns* expression plasmid was constructed (pAKD942) and introduced into the wild-type strain. Growth and luminescence assays performed under inducing and noninducing conditions indicated that inducing *hns* transcription with IPTG resulted in lower luminescence levels (Fig. 4F), suggesting that H-NS plays a direct or indirect role in influencing luminescence in this strain. Although H-NS is a global regulator, growth of the strain in the presence of IPTG was similar to that in the absence of IPTG, and quantitative PCR indicated that gene expression is not globally decreased when *hns* expression is induced (see Fig. S3 in the supplemental material).

Disruption of putative pheromone signaling loci does not affect light production. The transposon-based screen did not reveal a role for pheromone signaling loci in the luminescence phenotype. Analysis of the genome sequence of KNH6 identified genes similar to known pheromone signaling genes in other organisms, including *cqsA*, *cqsS*, *hapR*/*luxR* from *V. harveyi* (not homologous to *luxR* from *V. fischeri* [30]), *luxS*, *aphA*, *luxU*, and *luxO* (Table 4). However, genes with similarity to *luxM*, *luxN*, *luxP*, *luxQ*, *luxR*, or *luxI* from *Vibrio fischeri* were not found. Each of the putative pheromone signaling genes was targeted using insertional disruption, with the exception of *luxU*, a 360-bp gene that was not successfully targeted due to current limitations of the system (see Materials and Methods). Insertional disruption strains were assayed for differences in growth and luminescence compared to wild-type KNH6. With the exception of a reduction in repression of luminescence early in growth in the *aphA* mutant (AKD931) (see Fig. S2 in the supplemental material), little difference was found in the growth and luminescence of these mutants relative to the wild type. These results are consistent with the prediction, based on the observed wild-type luminescence phenotypes (Fig. 3), that regulation of luminescence in KNH6 is not directly tied to previously described pheromone signaling systems under these growth conditions.

DISCUSSION

We first became interested in *P. leiognathi* strain KNH6 because it produces copious amounts of light during laboratory growth, much more so than other commonly studied strains. This observation led to the question of why this strain produces so much light, a process that is considered energetically expensive. To begin to address this question, we used genetic approaches to investigate the regulation of light production in KNH6 to determine whether it was similar to that in other, less bright isolates.

Measurement of luminescence during laboratory growth in various medium types suggested that KNH6 was not using pheromone-based signaling as a major regulatory mechanism (Fig. 3). It was previously reported that certain *Photobacterium* isolates did not display cell density-dependent regulation of luminescence (7, 9); however, study of the regulation of light production in these and other luminescent *Photobacterium* strains at the genetic level was hampered by a lack of tools. In the present study, we adapted several genetic tools, previously developed for use in *V. fischeri*, for use in KNH6. With minor modifications, as described in Materials

TABLE 4 Predicted KNH6 pheromone signaling-related proteins

Predicted protein (ORF no.)	BLASTx similarity to known pheromone signaling-related protein (accession no.)
AphA (VT25_06970)	67% identical (80% positive over 178 amino acids) to *Vibrio cholerae* AphA (YP_002811314)
CqsA (VT25_15745)	58% identical (76% positive over 329 amino acids) to *Vibrio harveyi* BB120 (*Vibrio campbellii* BAA-1116) CqsA (A7N6R9)
CqsS (VT25_15750)	37% identical (61% positive over 689 amino acids) to *V. harveyi* BB120 CqsS (A7N6S2)
HapR (VT25_10580)	48% identical (68% positive over 193 amino acids) to *V. cholerae* HapR (YP_002809317)
LuxO (VT25_03485)	68% identical (81% positive over 467 amino acids) to *V. harveyi* BB120 LuxO (A7MVC2.2)
LuxU (VT25_03485)	40% identical (59% positive over 82 amino acids) to *V. harveyi* BB120 LuxU (A7MVC1.1)
LuxR (VT25_07845)	77% identical (90% positive over 208 amino acids) to *V. harveyi* BB120 LuxR (YP_001443432)
LuxS (VT25_00450)	81% identical (90% positive over 171 amino acids) to *V. harveyi* BB120 LuxS (Q9Z5X1)

and Methods, these transposon and plasmid tools were successfully used to investigate the regulation of light production in KNH6.

To perform an unbiased screen for gene products involved in light production in KNH6, a transposon-based approach was used and resulted in the identification of mutants that displayed reduced luminescence levels on agar plates and in liquid medium. In our screen, a majority of the *lux* and *lum* operon genes were identified, and multiple independent mutants in several loci were recovered (Fig. 2 and Table 2), suggesting that many of the loci linked to light production are represented in this study. Besides the expected mutants in the *lux*, *lum*, and *rib* operons, nine additional loci were identified in the screen.

The mutants representing these nine loci were grouped into three general categories based on their luminescence phenotypes: slightly dim, dim, and dark. The slightly dim mutants had transposon insertions in genes whose products are presumably involved in the TCA cycle, cytochrome *c* synthesis, and fatty acid synthesis. Because of the link between these genes and core metabolic cellular processes and/or molecules consumed during luciferase activity (NADH and long-chain aliphatic aldehyde) (reviewed in references 2 and 6), it is not surprising that these disruptions could lead to changes in overall light production but not influence *lux* operon promoter activity.

The more interesting mutants are the dim and dark mutants, which seem to fall into three main groups: those potentially affected in transcriptional regulation, those potentially affected in nucleic acid metabolism and modification, and those potentially affected in peptidoglycan synthesis. Although it was initially expected that several regulatory proteins would be identified in the transposon screen, instead only one mutant was identified that appears to have a link to the global regulatory protein H-NS. Our studies of KT2c indicate that SspA could play a role in regulation of bioluminescence through alteration of H-NS levels. These results are supported by the known link between SspA and H-NS in *E. coli* (28) and by previous results demonstrating a role for H-NS in repression of *V. fischeri* luminescence gene expression in native and heterologous hosts (27, 31). The latter studies involved a bacterium that uses pheromone signaling to regulate light production. The finding suggesting a similar regulatory approach in KNH6 indicates a possible common regulatory mechanism among luminescent bacteria that lies outside the previously described pheromone signaling systems. However, further work is needed to determine how H-NS levels may influence light production in KNH6.

Three dim and dark mutants appear to be affected in nucleic acid metabolism or modification: KT16c (RNA metabolism), KT1d (RNA modification), and KT2d (DNA metabolism). In strain KT16c, the transposon inserted into *pnp*, which is predicted to encode a polynucleotide phosphorylase. In *E. coli*, Pnp has been linked to mRNA and small RNA (sRNA) stability (32, 33). Interestingly, *lux* operon promoter activity was significantly decreased in this mutant background, suggesting a link between RNA stability and regulation of luciferase production. Further investigation will be needed to determine whether specific RNAs contribute to regulation of *lux* operon expression. A role for sRNAs in regulation of bioluminescence in other bacteria is not without precedence (34, 35), although the mechanism involves pheromone sensing, which does not appear to be relevant in our study. The second RNA-related mutant (KT1d) contained a transposon insertion in *miaA*, which is predicted to encode a tRNA dimethylallyltransferase. A lack of this enzyme in *E. coli* results in pleiotropic phenotypes, including increased mutation rates (36) and altered steady-state RpoS levels (37). In this mutant background, *lux* operon promoter activity was not lowered (Table 3), and it is an intriguing consideration that tRNA modification could be involved in translational fidelity of the *lux* operon gene products, although this remains to be tested experimentally.

The third nucleic acid-related mutant (KT2d) is predicted to be affected in DNA metabolism, with a transposon insertion in *thyA*. In *E. coli*, *thyA* encodes thymidylate synthase, which plays an important role in DNA synthesis through conversion of dUMP to dTMP (38). It is currently not clear why disruption of *thyA* leads to a decrease in luminescence in KNH6, but the effect does not appear to be at the level of *lux* operon transcription initiation (Table 3).

The final mutant, KT7c, is presumably affected in peptidoglycan synthesis. In this strain, the transposon inserted into *mrcA*, which is predicted to encode PBP1A, a peptidoglycan glycosyltransferase. It has been reported previously that isolated dim mutants of various *Vibrio* and *Photobacterium* spp. have altered colony morphologies and sensitivities to antimicrobial agents (6, 39, 40). Although these mutants were never characterized at the genetic level and the mutations are likely linked to different genes and gene products, KT7c may represent an example of a link between cell structure and luminescence. However, although both the *mrcA* transposon mutant and targeted insertional disruption mutant displayed decreased luminescence compared to that of the wild type (Fig. 3; see Fig. S2 in the supplemental material), expression of *mrcA* in *trans* in the transposon mutant background did not complement the luminescence phenotype, suggesting the possibility of a secondary mutation(s) that influences the phenotype and making conclusions about the link between MrcA and light production currently difficult. However, these results suggest that further exploration of the possible link between altered surface characteristics and luminescence levels is warranted.

One interesting outcome of the transposon mutagenesis screen is that no predicted pheromone signaling loci were identified as being important for the regulation of luminescence under the conditions tested. Growth and luminescence assays indicated that KNH6 does not use this method as a main control of light production (Fig. 3). To investigate this possibility, targeted insertional disruption strains with disruptions in predicted pheromone signaling loci were constructed (Table 4). However, these mutant strains did not display increased or decreased light production levels compared to that of the wild type. Based on our analysis of the KNH6 genome, this strain is predicted to be capable of producing two different types of pheromone, i.e., autoinducer 2 (AI-2; produced by LuxS) and a cholera autoinducer type 1 molecule(s) (CAI-1; produced by CqsA). Similar to a nonluminescent *Photobacterium angustum* strain (41) and a luminescent *Photobacterium mandapamensis* strain (42), KNH6 appears to be capable of producing AI-2 but not of sensing this molecule, due to the lack of *luxP* and *luxQ* in the genome. Therefore, an insertional disruption in *luxS* would not necessarily be expected to result in phenotypic differences from the wild type. In addition, similar to *P. angustum* and other *Photobacterium* spp., KNH6 also appears to carry a frameshifted *cqsA* gene (41). In *P. angustum*, CAI-1 molecules are not produced under normal laboratory growth conditions in rich medium, but under certain limiting growth conditions, it is pre-

dicted that ribosome slippage overcomes this frameshift and that CqsA and the CAI-1 molecules are produced (41). Based on these results, it is expected that CAI-1 molecules would not be produced in KNH6 under the growth conditions tested, and insertional disruption of *cqsA* would also not result in phenotypic differences from the wild type. The study of this interesting frameshifted *cqsA* gene and its role in *Photobacterium* biology has been limited by a lack of genetic tools for *P. angustum*. Although there does not appear to be a connection between CqsA-linked pheromone signaling and induction of bioluminescence in KNH6, the ability to genetically manipulate this strain will allow future study of this interesting signaling pathway in *Photobacterium* spp.

At the most basic level, the results of this study identify gene products related to bioluminescence in *Photobacterium* spp. that do not appear to employ pheromone-based signaling as a main regulatory mechanism for light production under the conditions tested. More broadly, these results lay a foundation for a greater understanding of the role of bioluminescence in marine bacteria, and for such studies the ability to apply genetic tools is key. Further study of KNH6, combined with the potential application of these genetic tools for use in other *Photobacterium* strains, may strongly contribute to deciphering the role of luminescence and pheromone signaling in the physiology and ecology of these interesting bacteria.

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