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Natural Merodiploidy of the *lux-rib* Operon of *Photobacterium leiognathi* from Coastal Waters of Honshu, Japan[▽]†

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Sequence analysis of the bacterial luminescence (*lux*) genes has proven effective in helping resolve evolutionary relationships among luminous bacteria. Phylogenetic analysis using *lux* genes, however, is based on the assumptions that the *lux* genes are present as single copies on the bacterial chromosome and are vertically inherited. We report here that certain strains of *Photobacterium leiognathi* carry multiple phylogenetically distinct copies of the entire operon that codes for luminescence and riboflavin synthesis genes, *luxCDABEG-ribEBHA*. Merodiploid *lux-rib* strains of *P. leiognathi* were detected during sequence analysis of *luxA*. To define the gene content, organization, and sequence of each *lux-rib* operon, we constructed a fosmid library of genomic DNA from a representative merodiploid strain, *lnuch*.13.1. Sequence analysis of fosmid clones and genomic analysis of *lnuch*.13.1 defined two complete, physically separate, and apparently functional operons, designated *lux-rib*$_1$ and *lux-rib*$_2$. *P. leiognathi* strains *lelon*.2.1 and *lnuch*.21.1 were also found to carry *lux-rib*$_1$ and *lux-rib*$_2$, whereas ATCC 25521^T apparently carries only *lux-rib*$_1$. In *lnuch*.13.1, *lelon*.2.1, *lnuch*.21.1, and ATCC 25521^T, *lux-rib*$_1$ is flanked upstream by *lumQ* and *putA* and downstream by a gene for a hypothetical multidrug efflux pump. In contrast, transposase genes flank *lux-rib*$_2$ of *lnuch*.13.1, and the chromosomal location of *lux-rib*$_2$ apparently differs in *lnuch*.13.1, *lelon*.2.1, and *lnuch*.21.1. Phylogenetic analysis demonstrated that *lux-rib*$_1$ and *lux-rib*$_2$ are more closely related to each other than either one is to the *lux* and *rib* genes of other bacterial species, which rules out interspecies lateral gene transfer as the origin of *lux-rib*$_2$ in *P. leiognathi*; *lux-rib*$_2$ apparently arose within a previously unsampled or extinct *P. leiognathi* lineage. Analysis of 170 additional strains of *P. leiognathi*, for a total of 174 strains examined from coastal waters of Japan, Taiwan, the Philippine Islands, and Thailand, identified 106 strains that carry only a single *lux-rib* operon and 68 that carry multiple *lux-rib* operons. Strains bearing a single *lux-rib* operon were obtained throughout the geographic sampling range, whereas *lux-rib* merodiploid strains were found only in coastal waters of central Honshu. This is the first report of merodiploidy of *lux* or *rib* genes in a luminous bacterium and the first indication that a natural merodiploid state in bacteria can correlate with geography.

Luminescence in *Photobacterium leiognathi* and other luminous bacteria is the product of bacterial luciferase, a mixed-function oxidase that uses oxygen, reduced flavin mononucleotide, and a long-chain fatty aldehyde as substrates to produce blue-green luminescence. The genes for bacterial light production are present as an operon, *luxCDABEG*: *luxA* and *luxB* encode the α and β subunits of luciferase; *luxC*, *luxD*, and *luxE* specify the enzymatic components of a fatty acid reductase complex necessary for synthesis and recycling of the aldehyde substrate; and *luxG* encodes a flavin reductase (14). Most luminous *Photobacterium* species, i.e., *Photobacterium phosphoreum*, *Photobacterium kishitanii*, and *Photobacterium mandapamensis*, also carry *luxF*, which encodes a nonfluorescent flavoprotein, with a *lux* operon gene order of *luxCDABFEG* (2, 3, 4, 23, 28, 37). Linked to the luminescence genes in some *Photobacterium* species, and apparently cotranscribed with them, are genes involved in the synthesis of riboflavin, forming an operon of 10 or 11 genes, *luxCDAB*(*F*)*EG-ribEBHA*, which we refer to here as the *lux-rib* operon (23, 26, 27, 34, 37; this study). Upstream of the *lux-rib* operon in *P. mandapamensis*, a species closely related to *P. leiognathi*, are *lumQ* and *lumP* (encoding proteins of the lumazine operon), and these genes are located adjacent to the *putA* gene (encoding proline dehydrogenase) (29, 30, 31).

Phylogenetic analysis of *lux* and *rib* genes, together with housekeeping genes such as the16S rRNA gene, *gyrB*, *pyrH*, *recA*, *rpoA*, and *rpoD*, has proven helpful in defining evolutionary relationships among luminous bacteria and in the identification of new species (2, 3, 4, 12, 23). Phylogenetic analysis based on *lux* and other genes has also proven effective in providing the bacterial species- and clade-level resolution necessary for testing hypotheses of symbiont-host specificity and evolutionary codivergence in bioluminescent symbioses (16, 23). These studies reveal that the evolutionary divergence of symbiotic luminous bacteria has not followed the evolutionary divergence of their host animals.

Little is known, however, about the evolution of the bacterial luminescence system itself. Based on amino acid sequence identities, a tandem duplication of the ancestral *luxA* gene, followed by sequence divergence in the duplicated gene, is thought to have given rise to *luxB*, leading to the formation of the heterodimeric luciferase present in modern-day luminous bacteria. Similarly, a tandem duplication of *luxB* is thought to have given rise to the *luxF* gene (6, 14, 37). The subsequent loss of *luxF* from the lineage that gave rise to *P. leiognathi* might

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TABLE 1. Bacterial strains used in this study

Species	Strain	Habitat, geographic source, and isolation yr[a]	Reference(s) and/or source
P. leiognathi	ATCC 25521^T	LO, Gulf of Thailand, ca. 1967	8
	lelon.2.1	LO, Sagami Bay, Honshu, Japan, 1983	16; this study
	lnuch.13.1	LO, Wakasa Bay, Honshu, Japan, 2003	This study
	lnuch.21.1	LO, Suruga Bay, Honshu, Japan, 2004	This study
P. kishitanii	ATCC BAA-1194^T	LO, Sagami Bay, Honshu, Japan, 1982	3, 4
P. mandapamensis	ATCC 27561^T	SW, Banda Island, Indonesia, before 1970	21, 43
	PL-721[b]	FS, Sulu Sea, 1975	2, 39
P. phosphoreum	ATCC 11040^T	FS, Delft, The Netherlands, 1934	3
Vibrio orientalis	ATCC 33934^T	SW, Yellow Sea, China, before 1983	53
Vibrio splendidus	ATCC 33125^T	FS, North Sea, before 1955	47

[a] LO, light organ of fish; SW, seawater; FS, skin of marine fish.
[b] Strain PL-721 may be incorrectly designated PL-741 in some records.

reflect the evolutionary divergence of this species from other *Photobacterium* species (2). Recently, strains of *P. mandapamensis* bearing nonsense mutations in *luxF* have been isolated from nature, the first report of naturally occurring mutations in *lux* structural genes (23). The presence of strains bearing *luxF* mutations in nature suggests that *luxF* is less functionally constrained than other *lux* genes and indicates that this gene does not play an essential role in the free-living ecology and symbiosis of this species.

Relevant to both phylogenetic analysis based on *lux* genes and evolution of the luminescence system is the question of the *lux* gene copy number. The *lux* and *rib* genes are tacitly assumed to be present as single copies in *P. leiognathi* and other luminous bacteria. In contradiction of that assumption, we report here that certain strains of *P. leiognathi* carry two complete, physically separate, and apparently functional *lux-rib* operons, one closely associated with *putA* and the other located elsewhere on the chromosome. The presence of multiple copies of each of the *lux* and *rib* genes would presumably provide opportunities for the accumulation of mutations leading to sequence divergence in one or the other copy of each *lux* and *rib* gene and opportunities for recombination between the two operons. Instead, we find that both operons are stably inherited and show little or no evidence of mutation or recombination in different merodiploid strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1 and in the supplemental material. Strains of *P. leiognathi* were isolated from light organs of bacterially luminous marine animals and from seawater collected in various locations in Honshu, Shikoku, and Okinawa, Japan; Taiwan; the Philippine Islands; and Thailand (2, 8, 15, 16, 18; this study) (see the supplemental material for collection details). Bacteria were grown in LSW-70 broth (15), which contained (per liter) 10 g tryptone, 5 g yeast extract, 350 ml double-strength artificial seawater (38), 650 ml deionized water, and, for solid medium, 15 g agar. Genomic DNA was purified from cultures of strains grown overnight in LSW-70 broth using the DNeasy tissue extraction kit (QIAGEN). Strains were identified to the species level by phylogenetic analysis of *lux* and other genes (2, 15, 16, 23; this study).

DNA amplification and sequencing. For DNA amplification by PCR, MasterTaq polymerase (Eppendorf) and the following protocol were used: a 95°C initial denaturing step for 2 min; 35 cycles with a 94°C denaturing step for 20 s, a variable temperature annealing step for 15 s, and an extension step at 68°C for 1 min; a 7-min final extension step at 68°C; and snap cooling to 4°C. PCR primers, annealing temperatures, and exceptions to this protocol can be found at http://www-personal.umich.edu/~pvdunlap/supplementalinfo.html. PCR products were visualized using electrophoresis on 1% agarose gels stained with ethidium bromide and were purified using a QIAquick PCR purification kit (QIAGEN) or a Montage PCR filter kit (Millipore). PCR products were sequenced using the respective PCR primers. Sequencing was carried out by the staff of the University of Michigan Sequencing Core using dye terminator cycle sequencing on a Perkin-Elmer ABI 3730 or 3700 DNA analyzer. Specific primers for species of luminous bacteria other than *P. leiognathi* were designed based on existing publicly available sequences (see http://www-personal.umich.edu/~pvdunlap/supplementalinfo.html). Sequences not obtained in this laboratory were downloaded from public databases.

Cloning of *luxA* and *lux-rib* operons. The *luxA* genes of *P. leiognathi lnuch*.13.1, amplified using primers CWLAPlfor and CWLAPlrev, were cloned using the TOPO 4.0 cloning kit (Invitrogen). A fosmid library of genomic DNA from strain *lnuch*.13.1 was constructed using a CopyControl Fosmid Library Production kit (Epicenter). The library consists of approximately 3,500 clones with inserts of 35 to 40 kb. The library was screened for fosmids containing *lux-rib* sequences by pooling transformant colonies into groups that were subsequently analyzed by PCR using *lux-rib* primers. From these groups, two colonies that each contained a fosmid with a complete *lux-rib* operon were identified, clones B7-25 and C30-24. Fosmids were recovered from colonies using a WizardPlus Miniprep kit (Promega) and sequenced directly using vector-specific and *lux-rib*-specific primers. See http://www-personal.umich.edu/~pvdunlap/supplementalinfo.html for the sequencing strategy. Recovered fosmids were also used as templates for long-range PCR using the TripleMaster PCR system (Eppendorf) to determine the orientation of the inserted DNA and the approximate position of *lux-rib* in the inserted DNA relative to the vector ends.

Plasmid profiling and pulsed-field gel electrophoresis (PFGE). Small plasmids in *P. leiognathi* (15) were purified from strains *lelon*.2.1, *lnuch*.13.1, *lnuch*.21.1, and ATCC 25521^T grown overnight in LSW-70 broth by using a WizardPlus Miniprep kit (Promega). The resulting DNA was electrophoresed through a 0.7% agarose gel (Bio-Rad) and stained with ethidium bromide. Plasmids smaller than 25 kb were present in *lnuch*.13.1, *lelon*.2.1, and *lnuch*.21.1 (data not shown); these plasmids were found to be smaller than the *lux-rib*-containing fosmid clones B7-25 and C30-24.

To visualize plasmids larger than 25 kb, we used PFGE and a Bio-Rad CHEF mapper-chiller system. Genomic DNA in agarose plugs was prepared according to a method described previously by Lucangeli et al. (35), with modifications. Before lysis of the cells embedded in agarose, the plugs were first washed for 1 h at 37°C in buffer (6 mM Tris-HCl [pH 7.5], 100 mM EDTA [pH 8.0], 1 M NaCl, 0.5% Brij 58, 0.2 M sodium deoxycholate, 0.5% *N*-laurosylsarcosine NL-97) with 0.5 mg · ml^{-1} of lysozyme. Genomic DNA was run in 1% SeaKem agarose gels in 1× Tris-acetate-EDTA buffer. Electrophoresis was done for 15 h at 14°C with a gradient of 6 V · cm^{-1}, an angle of 120°, an initial pulse switch time of 5 s, and a final pulse switch time of 15 s, with linear ramping. Two plasmids of approx-

imately 180 to 200 kb, sufficiently large to account for a cosmid clone bearing a *lux-rib* operon, were present in strain *lelon*.2.1, whereas plasmids of 35 to 40 kb or greater were not detected in *lnuch*.13.1 or *lnuch*.21.1.

Similar PFGE procedures were used to identify the chromosomal locations of *lux-rib*$_1$ and *lux-rib*$_2$. Specifically, genomic DNA in agarose plugs was digested for 16 to 18 h with NotI restriction endonuclease (New England Biolabs) at 37°C in the buffer recommended by the manufacturer. PFGE gels were 0.8% SeaKem agarose (Bio Whittaker Molecular Applications) or low-melting-point agarose (Bio-Rad) in 1× Tris-acetate-EDTA buffer. Electrophoresis conditions were as follows: 27 h at 14°C with a gradient of 6 $V \cdot cm^{-1}$, an angle of 120°, an initial pulse switch time of 2.16 s, and a final pulse switch time of 2 min 26.90 s, with linear ramping. Mid Range PFG marker (New England Biolabs) and Yeast Chromosome marker (New England Biolabs) were used as size markers. Gels were stained with ethidium bromide to visualize DNA.

NotI digestion fragments of DNA were recovered using PFGE carried out in gels of low-melting-point agarose. Individual bands were excised, and the agarose was melted (65°C) and digested using β-agarase (New England Biolabs) as recommended by the manufacturer. DNA fragments were purified using phenol-chloroform extraction, recovered by ethanol precipitation, and suspended in distilled water. NotI digestion of *P. leiognathi* strain *lnuch*.13.1 genomic DNA produced seven fragments that ranged in size from approximately 1,500 kb to 220 kb. PCR was performed using the individual DNA fragments as a template and with *lux-rib*$_1$-specific and *lux-rib*$_2$-specific *luxD-luxA* primers.

Phylogenetic analysis. Sequences of the *lux-rib* operon genes of *Photobacterium* strains were aligned to the sequences of *lux* and *rib* genes from other luminous species (Table 2), with the nucleotide alignment based on inferred amino acid sequences; data were analyzed simultaneously with the parsimony criterion using PAUP* (48). Spacer regions between genes in the *lux* and *rib* operons were omitted from the analysis, and inferred deletions were treated as missing data. Jackknife support percentage values (17) were calculated in PAUP* using 1,000 replicates with 10 heuristic searches per replicate, with a jackknife resampling value of 34%, and emulating jacknife resampling, which resamples characters independently. Housekeeping genes (*gapA*, *gyrB*, *recA*, *rpoA*, and *rpoD*) were amplified, sequenced, and analyzed by similar methods to test the relationships within *Photobacterium* determined by *lux-rib* analysis.

Analysis of recombination. To test for recombination between the genes of *lux-rib*$_1$ and *lux-rib*$_2$ in *lnuch*.13.1, *lelon*.2.1, and *lnuch*.21.1, we developed a partition congruence analysis, as described here (script to produce PAUP* commands are available on request). Included in the analysis were *lux-rib*$_1$ of *P. leiognathi* ATCC 25521^T, the *lux-rib* operon of *P. mandapamensis* ATCC 27561^T, and, as an outgroup, the *lux-rib* operon of *P. kishitanii* ATCC BAA-1194^T (also known as *pjapo*.1.1^T) (4). Omitted from the analysis were noncoding intergenic spacer regions, the *luxF* gene in *P. mandapamensis* and *P. kishitanii*, and the first 341 bp of the *luxC* sequence, which is not available for the *lux-rib*$_2$ operons of *lelon*.2.1 and *lnuch*.21.1 (Fig. 1). The resulting aligned *lux-rib*$_1$ and *lux-rib*$_2$ coding regions of each strain, 8,732 nucleotides, were analyzed in a progressive 200-nucleotide window that shifted 50 nucleotides with each analytical run (171 200-character partitions and 1 partition of 182 characters). Each partition was subjected to an exhaustive search using PAUP*, with gaps treated as a fifth nucleotide. Each phylogenetic analysis resulted in up to four equally parsimonious trees, yielding a total of 258 trees. The topology of each tree was then compared with that predicted from phylogenetic analysis of entire *lux-rib*$_1$ and *lux-rib*$_2$ operons. Six of the 172 partitions yielded an incongruent tree, and the alignments of these partitions were then visually inspected. Five of the incongruent topologies (four overlapping partitions within the *luxA-luxB* region and one in *luxD*) were ascribed to a lack of character change rather than recombination, whereas one showed a possible recombination event between the *luxB*$_1$ and *luxB*$_2$ genes of strain *lnuch*.21.1 (see Results).

Screening for *lux-rib* merodiploidy. In addition to *lnuch*.13.1, *lelon*.2.1, *lnuch*.21.1, and ATCC 25521^T, 170 strains of *P. leiognathi* isolated from various geographic locations (see Fig. 5; also see the supplemental material) were assayed for the presence of single or multiple *lux-rib* operons using *luxA* PCR amplification and sequencing. The PCR primers used were those that detected multiple *luxA* sequencing chromatogram peaks in *P. leiognathi lnuch*.13.1, *lelon*.2.1, and *lnuch*.21.1. The resulting amplicons were then sequenced, and the resulting chromatograms were examined for single or multiple *luxA* sequence peaks.

GenBank accession numbers and sources of sequence data. GenBank accession numbers for DNA sequence data obtained or used in this study are listed in Table 2. Other sources of DNA sequence data used in the analysis shown in Fig. 1 are *luxCDABEG* (28) and *ribEBHA* (27) of *P. leiognathi* ATCC 25521^T; *lumP*, *luxD* (9), *luxC* (29), *lumQ* (30), *putA* (31), *luxE* (32), *luxG* (33), and *ribEBHA* (34) of *P. mandapamensis* PL-741; *luxABF* (22), *lumP* to *luxC* (unpublished GenBank submission) of *P. mandapamensis* 554; *Photobacterium angustum* SKA34 (http://www.moore.org/microgenome/detail.aspx?id=36); and *P. angustum* S14 (http://www.moore.org/microgenome/detail.aspx?id=8). Annotation of open reading frames (ORFs) for *P. angustum* SKA34 and S14 genome sequences in GenBank as of April 2007, including gene identifying number, cluster of orthologous groups (COG) number, and COG inferred protein group, are as follows: SKA34_07688, VAS14_5429, and COG1566 for orf1 (hypothetical multidrug resistance efflux pump); SKA34_07693, VAS14_15434, and COG3158 for orf2 (hypothetical potassium ion transporter) (COG identification applies to SKA34 only); SKA34_07678 for orf5 (found in SKA34 only) (hypothetical protein); and SKA34_07683, VAS14_15424, and COG2207 for orf6 (hypothetical transcriptional regulator, AraC/XylS family protein). orf3 and orf4 are hypothetical bacterial transposases and are not homologous to any sequences proximal to the *lux-rib* regions of any other strains examined here.

RESULTS

Multiple *luxA* genes in *P. leiognathi*. The sequencing chromatograms of *luxA* PCR amplicons of some *P. leiognathi* strains contained multiple discrete peaks. Exhaustive attempts to eliminate these anomalies through attempts to identify possible sources of DNA contamination were unsuccessful, which suggested that some strains carry multiple *luxA* genes. To test this possibility, we cloned the *luxA* PCR amplicon from *lnuch*.13.1, a strain that showed multiple chromatogram peaks. Individual recovered clones were found to carry one or the other of two different *luxA* sequences, which demonstrated the presence of two *luxA* genes in *lnuch*.13.1.

Identification of two complete *luxCDABEG-ribEBHA* operons. PCR and sequence analysis of *lnuch*.13.1 genomic DNA revealed that other *lux* genes were associated with each *luxA* gene, which suggested that some or all of the *lux* operon *luxCDABEG* might be present in two copies. Despite a concerted effort based on PCR and sequence analysis, however, we were unable to find a close physical linkage between the two putative *lux* operons, as might have resulted from a tandem duplication of all or part of the *lux* operon. This observation suggested that if two operons were present, they were not closely associated. To test this possibility and to define the gene content, organization, and sequence of each putative *lux* operon, we constructed a fosmid library of *lnuch*.13.1 genomic DNA. Sequence-based screening of the recovered fosmid clones identified two clones, B7-25 and C30-24, with different *lux* gene sequences. For both clones, the region containing and immediately flanking the *lux* operon was sequenced (see http://www-personal.umich.edu/~pvdunlap/supplementalinfo.html for PCR primers and sequencing strategy).

Both clones contained a complete *luxCDABEG* operon (Fig. 1). Furthermore, *luxC* was preceded in both clones by the *lumQ* gene. A short region with homology to part of *lumP* also was identified in B7-25 but was absent from C30-24. The *ribEBHA* genes were also present downstream of *luxG* in both clones. The individual *lux* and *rib* genes were of the same lengths in both clones, but the lengths of the individual intergenic spacer regions varied, especially in the AT-rich region between *lumQ* and *luxC* (Fig. 1). These results establish the presence of two distinct *luxCDABEG-ribEBHA* operons in *P. leiognathi lnuch*.13.1, designated here *lux-rib*$_1$ (from fosmid B7-25) and *lux-rib*$_2$ (from fosmid C30-24). Fosmid maps with additional details are presented in the supplemental material. In addition to having the same gene content and organization, the two operons are of similar lengths, approximately 11 and

TABLE 2. GenBank accession numbers for DNA sequences used in this study

Species	Strain	Gene(s)[a]	GenBank accession no.
P. angustum	S14		AAOJ01000000[b]
	SKA34		AAOU01000001[b]
P. profundum	SS9		CR378665[b]
P. leiognathi	ATCC 25521^T	*putA-luxC*	EF536344
		luxCDABEG	M63594
		ribEBH	M90094
		ribH-orf1	EF536341
	lelon.2.1	*lux-rib*$_1$	EF536333
		lux-rib$_2$	EF536334
	lnuch.13.1	*lux-rib*$_1$	EF536338[c]
		lux-rib$_2$	EF536332[d]
	lnuch.21.1	*lux-rib*$_1$	EF536335
		lux-rib$_2$	EF536336
P. kishitanii	ATCC BAA-1194^T	*lux-rib*	AY341065
P. mandapamensis	ATCC 27561^T	*putA*	EF536343
		lux-rib	AY341067
		orf1	EF536340
	PL-741	*putA*	U39227
		lumQ	U35231
		lumP	X65611
		luxC	X65156
		luxD	X65612
		luxE	U66407
		luxG	AF053227
		*ribEBH*A	AF364106
		lumP-luxC	X56534
		luxABFE	X08036[e]
P. phosphoreum	ATCC 11040^T	*lux-rib*	AY341063
Photorhabdus luminescens subsp. *laumondii*	TTO1	*luxCDABE*, *ribE*, *ribB*, *ribH*, and *ribA*	NC_005126
Photorhabdus luminescens subsp. *luminescens*	ATCC 29999^T	*luxCDABE*	M90093
Shewanella hanedai	ATCC 33224^T	*luxCDABEG*	AB058949
Shewanella woodyi	ATCC 51908^T	*luxA*	DQ322063
V. cholerae	NCIMB 41^T	*luxCDABE*	AB115761
V. fischeri	ES114	*luxCDABE*	NC_006841
		ribEBH	NC_006840
	MJ-1	*luxCDABE*	AF170104
V. harveyi	B-392	*luxD*	J03950
		luxAB	M10961
		luxE	M28815
		luxG-ribB	M27139[f]
Vibrio logei	ATCC 29985^T	*luxAB*	EF576941
V. orientalis	ATCC 33934^T	*luxDA*	AB058948
V. salmonicida	NCIMB 2262^T	*luxCDABEG*	AF452135
V. splendidus	ATCC 33125^T	*luxAB*	EF536342

[a] The designation *lux-rib* indicates the *luxCDABEG-ribEBHA* genes, and *lux-rib*$_1$ and *lux-rib*$_2$ indicate *luxCDABEG-ribEBHA* operon 1 and operon 2, respectively, of *P. leiognathi* (see the text).
[b] *P. angustum* and *P. profundum* do not have *lux* genes; these sequences were used to demonstrate homology to sequences found flanking the *lux-rib*$_1$ operon.
[c] Sequence from fosmid B7-25.
[d] Sequence from fosmid C30-24.
[e] *luxF* is designated *luxG* in this record.
[f] *ribB* is designated *luxH* in this record.

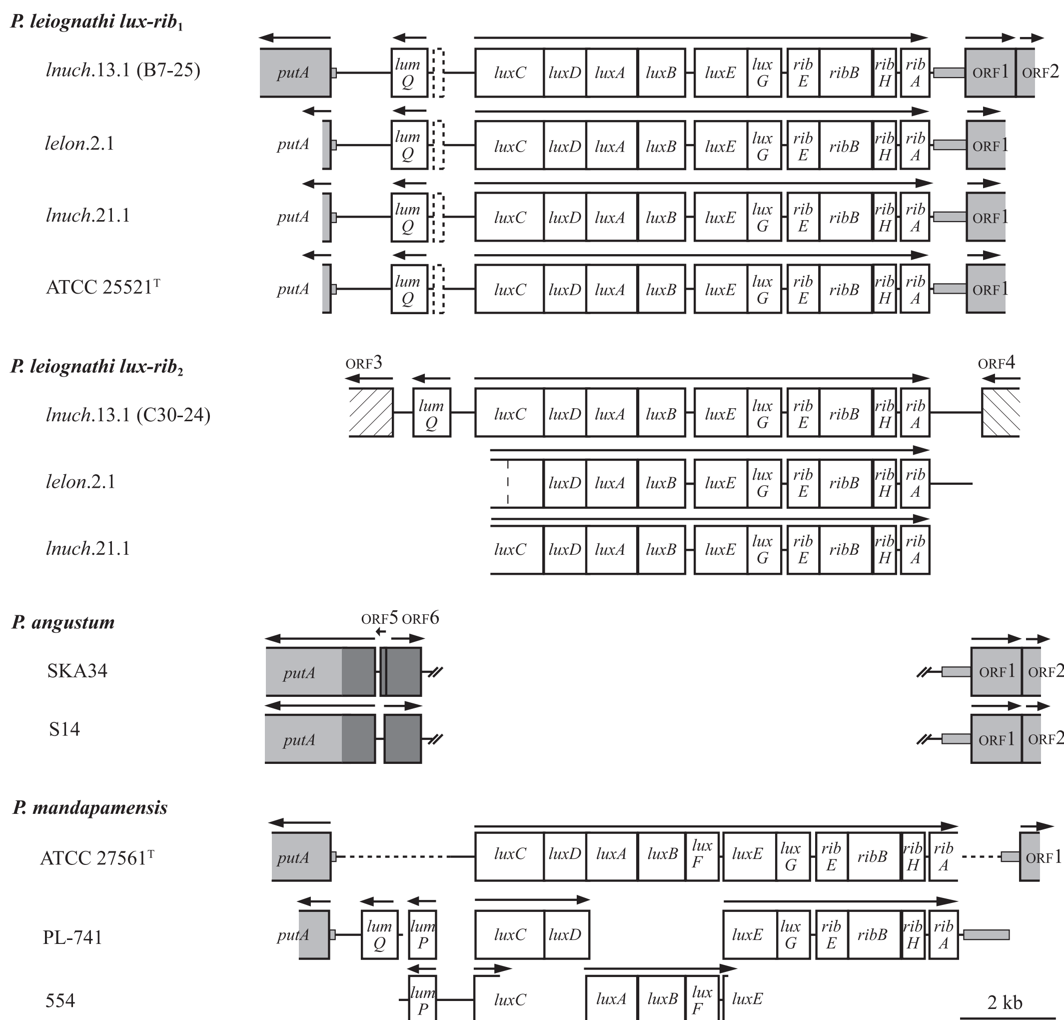

FIG. 1. Gene organization of the *lux-rib*$_1$ and *lux-rib*$_2$ operons of *P. leiognathi*. Genes and spacer regions are drawn to scale. Arrows above genes indicate the direction of transcription. Dashed rectangles between *lumQ* and *luxC* in *P. leiognathi lux-rib*$_1$ sequences indicate an approximately 200-bp region that is alignable to the *lumP* sequence of *P. mandapamensis*. Genes shaded gray have homologs in *P. leiognathi*, *P. mandapamensis*, and *P. angustum*. Small shaded rectangles outside of genes indicate noncoding intergenic sequences of *P. leiognathi* and/or *P. mandapamensis* that are alignable to sequences in *P. angustum* strains SKA34 and S14, including approximately 80 bp before the *P. leiognathi putA* start codon that aligns to a sequence within *putA* in *P. angustum*. Genes shaded dark gray in *P. angustum* indicate regions (orf5, orf6, and 759 bp from the start codon of *putA*) that are not alignable with a gene or intergenic sequence in *P. leiognathi* or *P. mandapamensis*. Double hash marks in the *P. angustum* sequences indicate contiguous sequences that have been separated to indicate the position of the *lux-rib* operon in *P. leiognathi* and *P. mandapamensis*. Blank regions in *P. mandapamensis* strains indicate that the sequence is not available; dashed lines in *P. mandapamensis* ATCC 27561^T indicate regions where the DNA was amplified in this study but not sequenced. The hashed rectangles indicate ORFs (orf3 and orf4) flanking the *lux-rib*$_2$ operon of fosmid C30-24 that are apparently homologous to bacterial transposases (see the text). In *lux-rib*$_2$ of *P. leiognathi lelon*.2.1, a dashed vertical line in the region homologous to *luxC* indicates the site of a single nucleotide deletion that causes a frameshift of *luxC* in this strain, resulting in a stop codon 18 codons later. See the supplemental material for complete maps of the fosmids.

11.5 kb, respectively; their *lux* and *rib* gene coding regions are approximately 90% identical in nucleotide sequence overall, and the genes of both operons are complete and translatable. This is the first example of multiple *lux-rib* operons (or multiple *lux* genes or multiple *rib* genes) in a luminous bacterium.

Chromosomal locations of *lux-rib*$_1$ and *lux-rib*$_2$. The absence of a plasmid large enough to account for either fosmid clone B7-25 or C30-24 (Materials and Methods) in *lnuch*.13.1 suggested that both *lux-rib* operons were likely to be chromosomal. To test this possibility, we examined genomic DNA of *lnuch*.13.1 using PFGE and PCR amplification. Analysis of undigested genomic DNA demonstrated the presence of two apparently circular chromosomes in *lnuch*.13.1, one larger than the other (data not shown), as reported previously for *P. leiognathi* and other examined members of the *Vibrionaceae* (40). Digestion of *lnuch*.13.1 genomic DNA with restriction endonuclease NotI yielded seven fragments, which ranged in size from approximately 1,500 kb to 220 kb (Fig. 2). The genome size of *lnuch*.13.1 was therefore estimated to be approximately 5.3 megabases. The seven NotI digestion fragments were purified and used as templates for PCR amplification of each of the two *lux-rib* operons, with primers for a *luxD-luxA* region that is specific in sequence to each operon. The *lux-rib*$_1$-specific primers amplified a product only from fragment

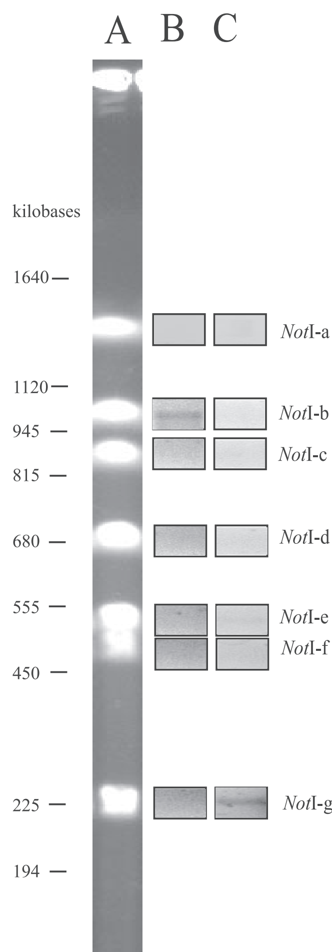

FIG. 2. PFGE analysis of the chromosomal locations of *lux-rib*$_1$ and *lux-rib*$_2$. (A) NotI digestion of *lnuch*.13.1 genomic DNA. (B) PCR amplification using *lux-rib*$_1$-specific primers (luxDfor1 and luxArevsec#3) (see http://www-personal.umich.edu/~pvdunlap/supplementalinfo.html) with genomic fragments NotI-a through NotI-g as templates. (C) PCR amplification using *lux-rib*$_2$-specific primers (luxDfor1 and luxArevprim#3) (see http://www-personal.umich.edu/~pvdunlap/supplementalinfo.html) and genomic fragments NotI-a through NotI-g as templates. Genomic DNA of *lnuch*.13.1 was used as a positive control (data not shown).

NotI-g, whereas the *lux-rib*$_2$-specific primers amplified a product only from fragment NotI-b (Fig. 2). Sequence analysis confirmed that the two amplification products were *luxD-luxA* of *lux-rib*$_1$ and of *lux-rib*$_2$, respectively. These results demonstrate that the two *lux-rib* operons of *lnuch*.13.1 are located on different NotI chromosomal digestion fragments. Given the sizes of the fosmid clones B7-25 and C30-24 and the lack of DNA in common to them, the two *lux-rib* operons are separated at least by 9 kb of DNA.

Consistent with these observations, DNA flanking *lux-rib*$_1$ in *lnuch*.13.1 was distinct from that flanking *lux-rib*$_2$ (Fig. 1). For *lux-rib*$_1$, the *putA* gene was present upstream of *lumQ*, and a gene for a hypothetical multidrug resistance efflux pump (orf1) was present downstream of *ribA*. In *P. angustum* strains SKA34 and S14, which lack *lux* genes, *putA* and the gene for orf1 are present, and they are near each other, separated by less than 2 kb of DNA; this DNA codes for two unidentified ORFs that are unrelated to the *lux* or *rib* genes (Fig. 1). As in *P. leiognathi*, the *lux-rib* genes in *P. mandapamensis* are flanked by *lumP*, *lumQ*, and *putA* and by the gene for orf1 (Fig. 1).

In contrast, the DNA flanking *lux-rib*$_2$ of *lnuch*.13.1 contained neither *putA* upstream of *luxC* nor the gene for orf1 downstream of *ribA*. Instead, homologs of putative bacterial transposase genes flanked *lux-rib*$_2$. Specifically, the region upstream of *luxC* in *lux-rib*$_2$ contained a sequence homologous to a putative transposase gene identified from the genome of *Photobacterium profundum* SS9, and the region downstream of *ribA* in *lux-rib*$_2$ contained a homolog to bacterial transposase genes of the IS*66* family (Fig. 1). These results establish that different, nonhomologous DNA flanks *lux-rib*$_1$ and *lux-rib*$_2$.

Presence and chromosomal locations of multiple *lux-rib* operons in other strains of *P. leiognathi*. Using the information from *lnuch*.13.1, we next asked if other strains of *P. leiognathi* carry the two *lux-rib* operons and if the chromosomal locations of the operons were the same as those in *lnuch*.13.1. We examined in detail two strains, *lelon*.2.1 and *lnuch*.21.1, that, like *lnuch*.13.1, showed multiple *luxA* chromatogram peaks, and one strain, ATCC 25521^T, the sequenced *luxA* amplicon of which consistently exhibited only single peaks. Primers based on *lnuch*.13.1 *lux-rib*$_1$ and *lux-rib*$_2$ were used for the amplification of *lux-rib* sequences from genomic DNA of these strains. Two different *luxA* sequences were identified in *lelon*.2.1 and *lnuch*.21.1, indicating the presence of two *lux-rib* operons, whereas only a single *luxA* sequence was identified in ATCC 25521^T. Complete sequencing of the *lux-rib* operons from *lelon*.2.1 and *lnuch*.21.1 (see http://www-personal.umich.edu/~pvdunlap/supplementalinfo.html for PCR primers and sequencing strategy) demonstrated the presence of two distinct *luxCDABEG-ribEBHA* operons in each of these strains (Fig. 1). As in *lnuch*.13.1, the genes of both operons in *lelon*.2.1 and *lnuch*.21.1 are, with one exception, intact and translatable. The exception is the *luxC* gene in *lux-rib*$_2$ of *lelon*.2.1, which has a single nucleotide deletion; the deletion results in a translational stop codon approximately midway into the gene (Fig. 1). From DNA of ATCC 25521^T, however, no amplicon was generated by any primers based on sequences of *lux-rib*$_2$ of *lnuch*.13.1. These PCR and sequencing chromatogram results demonstrate that both *lux-rib*$_1$ and *lux-rib*$_2$ are present in some strains of *P. leiognathi*, e.g., *lnuch*.13.1, *lelon*.2.1, and *lnuch*.21.1, whereas other strains, e.g., ATCC 25521^T, apparently have only *lux-rib*$_1$. These results also establish that the presence of multiple *luxA* chromatogram peaks, versus single *luxA* chromatogram peaks, provides a reliable indication of the presence of multiple versus single *lux-rib* operons in *P. leiognathi*.

For *lelon*.2.1, *lnuch*.21.1, and ATCC 25521^T, the order and orientation of genes flanking *lux-rib*$_1$ were found to be the same as those for *lnuch*.13.1. Specifically, the DNA upstream of *luxC* contained a short region with some homology to *lumP*, followed by *lumQ* and *putA*, and the region downstream of *ribA* contained the gene for orf1 (Fig. 1). These results demonstrate positional homology of *lux-rib*$_1$ in the four strains of *P. leiognathi* tested. In contrast, positional homology of the *lux-rib*$_2$ operons in *lnuch*.13.1, *lelon*.2.1, and *lnuch*.21.1 could not be demonstrated. Attempts to amplify the regions upstream of *luxC* and downstream of *ribA* in *lelon*.2.1 and *lnuch*.21.1 using primers based on the sequences from fosmid clone C30-24

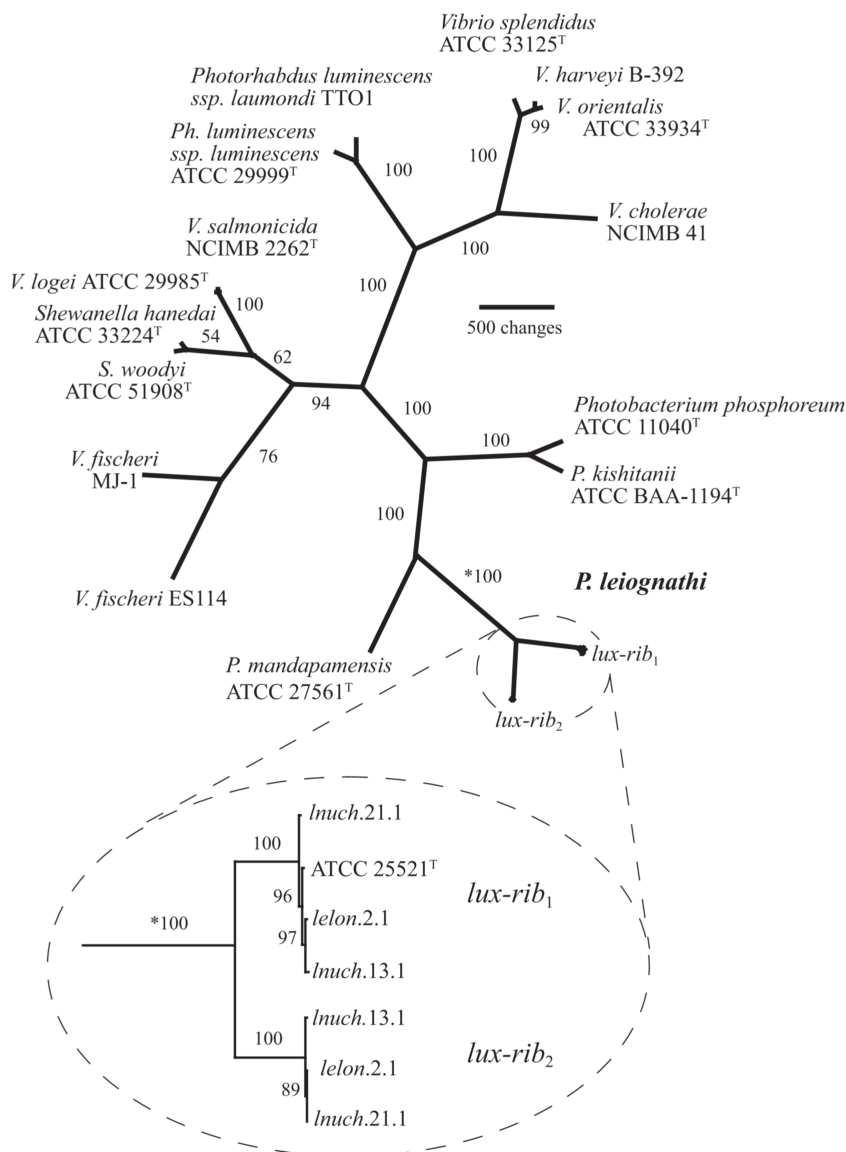

FIG. 3. Phylogenetic hypothesis of relationships among luminous bacteria based on *lux* and *rib* gene sequences. The tree is unrooted because bacterial *lux* genes are present only in members of the *Vibrionaceae*, *Shewanellaceae*, and *Enterobacteriaceae*; no outgroup bearing the *lux* genes is known. The total number of aligned nucleotide positions in the data set is 11,021; exclusion of noncoding spacer regions and parsimony-uninformative characters resulted in 4,745 nucleotides for analysis. The single most parsimonious hypothesis is shown (length, 13,739; consistency index, 0.615; retention index, 0.712). Numbers at nodes are jackknife resampling values. The two *lux-rib* operons from *P. leiognathi* strains *lnuch*.13.1, *lelon*.2.1, and *lnuch*.21.1 (circled with dashed line) have distinct sequences but are each other's closest relatives (circled inset below the main figure; the asterisk indicates that the jackknife value is for the same branch on each part of the figure). The primary *lux-rib* operon (*lux-rib*$_1$) is proximal to *putA* in all strains, whereas the secondary *lux-rib* operon (*lux-rib*$_2$) is flanked by putative bacterial transposases in strain *lnuch*.13.1. Phylogenetic analysis based on housekeeping genes (i.e., *gapA*, *gyrB*, *recA*, *rpoA*, and *rpoD*) of *Photobacterium* species yielded trees consistent with the *lux-rib* hypothesis shown here.

(*lnuch*.13.1; *lux-rib*$_2$) failed to produce the predicted products. Therefore, either the chromosomal locations of *lux-rib*$_2$ or the sequences flanking *lux-rib*$_2$ differ in these three strains.

Evolutionary origin of *lux-rib*$_2$. With the complete sequences of *lux-rib*$_1$ from *lnuch*.13.1, *lelon*.2.1, *lnuch*.21.1, and ATCC 25521^T and of *lux-rib*$_2$ from *lnuch*.13.1, *lelon*.2.1, and *lnuch*.21.1, we were in a position to define the extent of phylogenetic divergence between genes of the two operons. We hypothesized that *lux-rib*$_2$, which is flanked by putative bacterial transposase genes in *lnuch*.13.1, was acquired by lateral gene transfer from another species of luminous bacteria.

To test this hypothesis and gain insight into the evolutionary origin of *lux-rib*$_2$, we carried out a parsimony analysis of *lux-rib*$_1$, *lux-rib*$_2$, and *lux* and *rib* genes of other species of luminous bacteria in *Vibrionaceae*, *Shewanellaceae*, and *Enterobacteriaceae*. Instead of a close relationship between *lux-rib*$_2$ and the *lux* and *rib* genes of one of these other species of bacteria, we found that *lux-rib*$_2$ was most closely related to *lux-rib*$_1$ of *P. leiognathi* (Fig. 3). These results rule out lateral transfer from another bacterial species as the origin of *lux-rib*$_2$ in *P. leiognathi*. Instead, the results indicate that *lux-rib*$_2$ originated from within *P. leiognathi*. Separate phy-

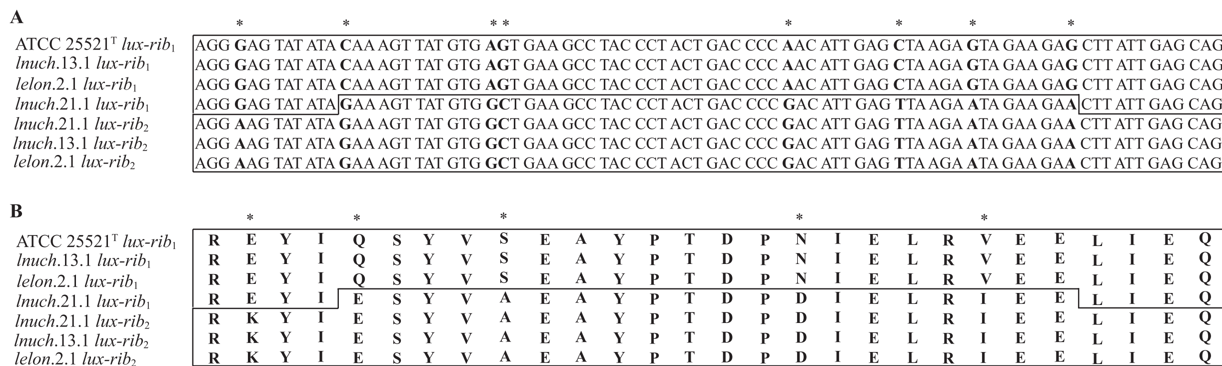

FIG. 4. Alignment of a region of *luxB* from *lux-rib*$_1$ and *lux-rib*$_2$ of *P. leiognathi lnuch*.21.1. (A) Nucleotide alignment. (B) Amino acid alignment. Asterisks highlight differences between the sequences; variable nucleotides are shown in boldface type.

logenetic analysis of each *lux* and *rib* gene resolved the same relationships as those shown in Fig. 1 (see the supplemental material).

This analysis also revealed that the *lux-rib*$_2$ operons of *lnuch*.13.1, *lelon*.2.1, and *lnuch*.21.1 are very closely related to each other, as are the *lux-rib*$_1$ operons of these strains and of ATCC 25521^T (Fig. 3). Therefore, although *lux-rib*$_1$ and *lux-rib*$_2$ are phylogenetically distinct from each other, each has diverged very little among the different strains carrying them. It should be noted in this regard that the merodiploid strains were collected from different geographic locations and as much as 20 years apart (Table 1).

Recombination between *lux-rib*$_1$ and *lux-rib*$_2$. The presence of two complete *lux-rib* operons with a high level of sequence identity in strains of *P. leiognathi* would presumably provide opportunities for recombination. We therefore tested for recombination between *lux-rib*$_1$ and *lux-rib*$_2$ by carrying out a partition congruence analysis on the sequences of the aligned *lux-rib*$_1$ and *lux-rib*$_2$ coding regions of *lnuch*.13.1, *lelon*.2.1, and *lnuch*.21.1. Analysis of the partition trees revealed that nearly all of them, 166 of 172, were topologically congruent, indicating no detectable recombination between the two operons. Six partitions did exhibit incongruence, but for five of these partitions, the incongruence could be ascribed to a lack of character change rather than recombination. One partition, however, for the *luxB*$_1$ and *luxB*$_2$ genes in strain *lnuch*.21.1 showed evidence consistent with recombination, with a portion of *luxB*$_1$ apparently exchanged for *luxB*$_2$ sequence (Fig. 4). These results indicate that despite the extensive homology, recombination between genes of the *lux-rib*$_1$ and *lux-rib*$_2$ operons of merodiploid strains of *P. leiognathi* apparently occurs only rarely.

Nonrandom geographic distribution of merodiploid *P. leiognathi* strains. We noted that the collection locations of *lux-rib* merodiploid strains, *lnuch*.13.1, *lelon*.2.1, and *lnuch*.21.1, all from Honshu, Japan, differed from the collection location, the Gulf of Thailand, of ATCC 25521^T, which carries only *lux-rib*$_1$. This difference suggested that strains bearing multiple *lux-rib* operons might have a nonrandom geographic distribution. To test this possibility, we examined 170 additional strains of *P. leiognathi* collected from various locations from Honshu, Shikoku, and Okinawa, Japan; Taiwan the Philippine Islands; and the Gulf of Thailand (Fig. 5; see also the supplemental material). These strains were tested for single or multiple *lux-rib* operons using the primers that revealed multiple *luxA* chromatogram peaks for *lnuch*.13.1, *lelon*.2.1, and *lnuch*.21.1. One hundred five of the additional strains were found to carry a

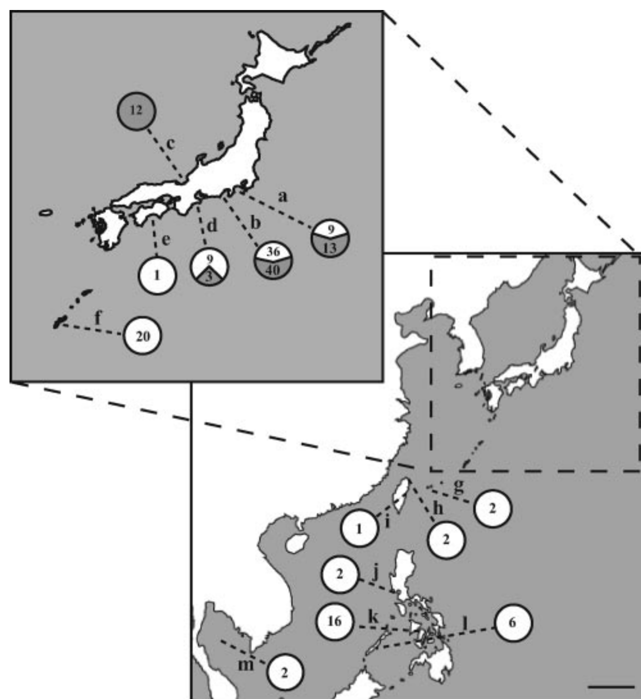

FIG. 5. Map of Japan and Southeast Asia showing the geographic origins of *P. leiognathi* strains bearing single or multiple *lux-rib* operons. The scale bar is approximately 500 km. The inset shows an enlarged map of the main islands of Japan (some landmasses were omitted for clarity). Locations: a, Sagami Bay, Kanagawa Prefecture, Honshu, Japan; b, Suruga Bay, Shizuoka Prefecture, Honshu, Japan; c, Wakasa Bay, Fukui Prefecture, Honshu, Japan; d, Ago Bay, Mie Prefecture, Honshu, Japan; e, Tosa Bay, Kochi Prefecture, Shikoku, Japan; f, Nakagusuku Bay, Okinawa-honto, Okinawa Prefecture, Japan; g, Funauki Bay, Iriomote Island, Okinawa Prefecture, Japan; h, Taipei, Taiwan; i, Dahsi, Taiwan; j, Manila Bay, Luzon, Philippine Islands; k, Iloilo, Panay, Philippine Islands; l, Palawan, Philippine Islands; m, Gulf of Thailand. Numbers next to each location indicate the number of strains identified as bearing single (white area in circle) or multiple (gray area in circle) *lux-rib* operons.

single *lux-rib* operon, whereas 65 of the additional strains carried multiple *lux-rib* operons. Strains bearing a single *lux-rib* operon were obtained throughout the geographic sampling range, but strains bearing multiple *lux-rib* operons were found only in coastal waters of central Honshu (Fig. 5). Thus, despite extensive sampling, we were unable to find *P. leiognathi* strains bearing multiple *lux-rib* operons south of Honshu, Japan. Therefore, *lux-rib* merodiploid strains have a geographic distribution that apparently is limited to coastal waters of Honshu. This is the first indication that a natural merodiploid state in a bacterial species can correlate with geography.

DISCUSSION

Certain strains of *P. leiognathi*, a coastal marine luminous bacterium, carry two intact and apparently functional *luxCDABEG-ribEBHA* operons. Merodiploidy extends to an 11th gene, *lumQ*, which is closely associated with but transcribed divergently from the *lux-rib* operons. The two *lux-rib* operons are distinct in sequence and chromosomal location. One operon, *lux-rib*$_1$, is flanked by *putA* and the gene for a hypothetical multidrug resistance efflux pump (orf1). Positional homologies of *lux-rib*$_1$ in strains of *P. leiognathi*, of the *lux-rib* operon of *P. mandapamensis*, and of flanking DNA in *P. angustum* suggest that the site between *putA* and the orf1 gene is the ancestral chromosomal location of the *lux-rib* operon in *P. leiognathi* and *P. mandapamensis*. The second operon, *lux-rib*$_2$, is present in many but not all strains of *P. leiognathi*, and it is flanked by genes specifying transposases but not by *putA* and the orf1 gene.

The *lux-rib* merodiploidy reported here is the first example of multiple copies of *lux* or *rib* genes in a luminous bacterium. Natural merodiploidy is not uncommon in bacteria, but it usually involves individual genes or small genetic regions and is thought to arise typically by tandem duplication (see, e.g., references 1, 19, 45, and 54). The actinomycete *Actinomadura* sp., for example, carries two *rpoB* genes that are 93% identical on the same 3-kb genomic DNA fragment; both copies are stably inherited without recombination (50). It is also known that many bacteria carry multiple copies of the *rrn* operon, with the number of operons apparently corresponding to the rate of response to resource availability (24). The situation in *P. leiognathi*, however, differs markedly and apparently is unique: the two *lux-rib* operons are well separated, each operon contains 11 genes, and merodiploidy apparently did not arise by a tandem duplication of one of the operons. Furthermore, *lux-rib* merodiploidy is common in *P. leiognathi*, having been detected in 68 of 174 tested strains, but the merodiploid state is not found in all strains of this species.

The presence of transposase genes flanking *lux-rib*$_2$ indicates a potential for this operon to transfer among *P. leiognathi* strains and to other species. Despite the potential for mobility, *lux-rib*$_2$ apparently was not acquired by *P. leiognathi* by lateral transfer from another species of luminous bacteria (Fig. 3). The two operons are phylogenetically distinct but are more closely related to each other than either one is to the *lux* or *rib* genes of any other known species of luminous bacteria. The evolutionary divergence between *lux-rib*$_1$ and *lux-rib*$_2$, which is evident both at the level of the whole operon and at the level of individual genes (Fig. 3; also see the supplemental material), also demonstrates that *lux-rib*$_2$ is not likely to have arisen by a recent duplication of *lux-rib*$_1$. Furthermore, the merodiploid state appears to be stable; more than 20 years separate the collection of merodiploid strains of *P. leiognathi* from the environment (Table 1; also see the supplemental material), with little sequence divergence for either operon over that time. We also found that an extended period of growth of *lnuch*.13.1 in continuous culture (approximately 400 generations) did not lead to a loss or altered chromosomal location of *lux-rib*$_2$ (data not shown). It is possible, therefore, that *lux-rib*$_2$ arose in the distant past within, and was acquired by transposon-mediated transfer from, a lineage of *P. leiognathi* that either has not yet been sampled or has gone extinct. If borne out by future studies, this scenario would represent the first documented instance of intraspecific transfer of the *lux-rib* genes in luminous bacteria. With respect to phylogenetic analysis based on *lux* and *rib* genes, the presence of two distinct *lux-rib* operons in certain strains of *P. leiognathi* adds complexity to analyses based on these genes, but it does not prevent the discrimination of this species from other luminous bacteria.

The putative transposases flanking *lux-rib*$_2$ show similarity to insertion sequence elements characteristic of some plasmids (10) and pathogenic islands (20) in the *Vibrionaceae*. In this regard, Rajanna et al. (42) previously proposed that the *Vibrio cholerae* pathogenicity island is excised from the chromosome and transferred between cells as an extrachromosomal circular molecule, thereby spreading *V. cholerae* pathogenicity island-encoded genes among strains. One possibility, therefore, is that *lux-rib*$_2$ was transferred among some strains of *P. leiognathi*, possibly during light organ symbiosis with fishes and squids, where bacterial population densities are high, by a mechanism similar to that involved in the transfer of pathogenic islands. However, determining the frequency, mechanism, and conditions under which *lux-rib*$_2$ is transferred will require much additional work. Given the potential for mobility of *lux-rib*$_2$, we also cannot exclude the possibility that some strains of *P. leiognathi* carry more than two *lux-rib* operons.

The presence of multiple copies of the *lux-rib* operon in a cell presumably provides opportunities for recombination and for the accumulation of nonsense mutations in the duplicate genes. These events could presumably lead to changes in the gene content and gene order of the *lux* operon of *P. leiognathi* and possibly lead to the evolution of novel functions in the duplicate genes. Recombination between the genes of *lux-rib*$_1$ and *lux-rib*$_2$, however, is apparently rare. Only one apparent instance of possible recombination, between the *luxB*$_1$ and *luxB*$_2$ genes in strain *lnuch*.21.1, was identified (Fig. 5). Similarly, mutations leading to a loss of function in the genes of either *lux-rib* operon are remarkably infrequent. With a single exception, all the genes of both operons in *lnuch*.13.1, *lelon*.2.1, and *lnuch*.21.1 are intact and translatable. This retention of functionality suggests the possibility of selection for the physiological function of the genes of both *lux-rib* operons. In this regard, it is known that selection can protect both copies of duplicated genes under conditions in which expression benefits the cell (25) and that selection for increased levels of gene products can lead to the retention of duplicated genes (see, e.g., reference 54). The number of genes involved and the substantial energetic costs associated with *lux* gene expression

and the activity of Lux and Rib proteins (see, e.g., reference 13) argue for this selection hypothesis.

Increases in gene copy number would be expected to increase the cellular levels of the protein products of those genes (45). The presence of two functional *lux-rib* operons in merodiploid *P. leiognathi* would therefore presumably result in higher cellular levels of the Lux and Rib proteins than in strains carrying a single operon. Possibly reflecting *lux-rib* merodiploidy, two different luciferases, one soluble and the other apparently associated with the cytoplasmic membrane, were reported many years ago for *P. leiognathi* strain S-1 (5, 7). Whether retention of the *lux-rib* merodiploid state is subject to selection, however, and what the possible physiological benefits may be for cells producing higher levels of Lux and Rib proteins are not obvious at this time. The limited geographic distribution of *lux-rib* merodiploid strains (see below) is intriguing in this regard and suggests a possible connection with environmental conditions in coastal waters of Honshu, Japan, possibly the cold winter water temperatures there (49), compared to warmer coastal areas south of Honshu. Molecular genetic and biochemical analyses will be needed to determine whether both operons are actually expressed, whether their expression may be regulated differently, and what the effects on cellular levels of Lux and Rib proteins may be. At this time, essentially nothing is known about the regulation of luminescence in *P. leiognathi* except that the strains tested exhibit a population density-responsive induction of luminescence and luciferase synthesis that does not appear to be dependent on acyl-homoserine lactones (11). The demonstration here of multiple *lux-rib* operons in certain strains of *P. leiognathi* provides the genetic foundation for an analysis of these issues.

The nonrandom geographic distribution of merodiploid *P. leiognathi* strains (Fig. 5) is unexpected. Bacterial distributions in nature are widely considered to be cosmopolitan, although nonrandom geographic patterns, especially in the presence of physical barriers to dispersal, have been identified (see, e.g., references 41, 44, 46, 51, and 52). In the case of *P. leiognathi*, which exists in the fluid marine environment, no physical barrier to dispersal is evident, yet our extensive sampling has found merodiploid strains only in coastal waters of central Honshu. One explanation for the nonrandom distribution of merodiploid strains is simple historical contingency (36); the merodiploid state, assuming that it arose relatively recently in cells in coastal waters of central Honshu, may not have had sufficient time to disperse to the south. Northward flow of the Kuroshio Current (49) might delay or prevent the southerly dispersal of merodipliod strains. Alternatively, strains carrying multiple *lux-rib* operons might have a physiological advantage in coastal waters of Honshu, as mentioned above, thereby allowing them to persist despite the presence of strains that carry only *lux-rib*$_1$. Much additional work will be needed to test these notions and determine the possible environmental conditions involved in the maintenance and limited geographic distribution of *lux-rib* merodiploid strains of *P. leiognathi*.

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