JOURNAL OF BACTERIOLOGY, May 2008, p. 3494–3504 Vol. 190, No. 10
0021-9193/08/$08.00+0 doi:10.1128/JB.00101-08

Phylogenetic Analysis of the Incidence of *lux* Gene Horizontal Transfer in *Vibrionaceae*▽†

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Received 21 January 2008/Accepted 11 March 2008

Horizontal gene transfer (HGT) is thought to occur frequently in bacteria in nature and to play an important role in bacterial evolution, contributing to the formation of new species. To gain insight into the frequency of HGT in *Vibrionaceae* and its possible impact on speciation, we assessed the incidence of interspecies transfer of the *lux* genes (*luxCDABEG*), which encode proteins involved in luminescence, a distinctive phenotype. Three hundred three luminous strains, most of which were recently isolated from nature and which represent 11 *Aliivibrio*, *Photobacterium*, and *Vibrio* species, were screened for incongruence of phylogenies based on a representative housekeeping gene (*gyrB* or *pyrH*) and a representative *lux* gene (*luxA*). Strains exhibiting incongruence were then subjected to detailed phylogenetic analysis of horizontal transfer by using multiple housekeeping genes (*gyrB*, *recA*, and *pyrH*) and multiple *lux* genes (*luxCDABEG*). In nearly all cases, housekeeping gene and *lux* gene phylogenies were congruent, and there was no instance in which the *lux* genes of one luminous species had replaced the *lux* genes of another luminous species. Therefore, the *lux* genes are predominantly vertically inherited in *Vibrionaceae*. The few exceptions to this pattern of congruence were as follows: (i) the *lux* genes of the only known luminous strain of *Vibrio vulnificus*, VVL1 (ATCC 43382), were evolutionarily closely related to the *lux* genes of *Vibrio harveyi*; (ii) the *lux* genes of two luminous strains of *Vibrio chagasii*, 21N-12 and SB-52, were closely related to those of *V. harveyi* and *Vibrio splendidus*, respectively; (iii) the *lux* genes of a luminous strain of *Photobacterium damselae*, BT-6, were closely related to the *lux* genes of the *lux-rib*$_2$ operon of *Photobacterium leiognathi*; and (iv) a strain of the luminous bacterium *Photobacterium mandapamensis* was found to be merodiploid for the *lux* genes, and the second set of *lux* genes was closely related to the *lux* genes of the *lux-rib*$_2$ operon of *P. leiognathi*. In none of these cases of apparent HGT, however, did acquisition of the *lux* genes correlate with phylogenetic divergence of the recipient strain from other members of its species. The results indicate that horizontal transfer of the *lux* genes in nature is rare and that horizontal acquisition of the *lux* genes apparently has not contributed to speciation in recipient taxa.

Horizontal gene transfer (HGT), the acquisition of genes by a member of one bacterial lineage from another lineage, is widely believed to play a major role in bacterial evolutionary divergence and speciation (29, 39, 40, 46). As a possibly common process, HGT also is thought to result in reticulate evolution, precluding or at least seriously complicating reconstruction of bacterial phylogenies (6, 17). The actual frequency of HGT in nature and its influence on bacterial evolution, however, are controversial; many believe that HGT is uncommon, at best has only minor or infrequent effects on the process of bacterial speciation, and does not prevent reconstruction of bacterial phylogenies (23, 24, 36, 38). An intermediate view is that HGT occurs frequently between bacterial lineages, contributing various strain-specific genes and thereby increasing the pan-genome of a bacterial species, but that the bacterium's core genome is inherited vertically and rarely perturbed by HGT (15, 41, 50, 51, 58).

Bacterial luminescence, a distinctive, easily observable phenotype of members of *Vibrionaceae* and certain other bacteria, provides a readily tractable subject for evaluating the frequency of HGT events in nature and the possible impact of those events on bacterial evolution and speciation. The genes for bacterial light production, *luxCDABEG*, are present in bacteria as a conserved, contiguous, and coordinately expressed set of genes, the *lux* operon. The *luxA* and *luxB* genes code for the α and β subunits of the light-emitting enzyme luciferase; *luxC*, *luxD*, and *luxE* specify the enzymatic components of a fatty acid reductase complex necessary for synthesis and recycling of an aldehyde that, along with O_2 and $FMNH_2$, serves as a substrate for luciferase; and *luxG*, present in most luminous bacteria, codes for a flavin reductase (20). Associated with the *lux* operons of certain species of the "*fischeri* group," recently reclassified as members of a new genus, *Aliivibrio* (61), are regulatory genes, *luxR* and *luxI*, specifying a quorum-sensing transcriptional activator and an acyl-homoserine lactone synthase. An additional gene, *luxF*, which codes for a nonfluorescent flavoprotein, is present in the *lux* operons of some luminous bacteria. Also contiguous with the *lux* genes in some

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† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 21 March 2008.

TABLE 1. Bacterial strains used in this study[a]

Strain	Origin	Reference(s) or source
Aliivibrio fischeri ATCC 7744^{Tb}	Seawater	32
Aliivibrio logei ATCC 29985^{Tb}	Gut of arctic mussel	10
Aliivibrio salmonicida ATCC 43839^{Tb}	Farmed Atlantic salmon, Norway	22
Aliivibrio wodanis ATCC BAA-104^{T}	Atlantic salmon with winter ulcer disease, Norway	42
Photobacterium damselae BT-6[b]	Aquarium seawater, Sesoko Island, Okinawa, Japan	This study
Photobacterium leiognathi lnuch.13.1[b]	Light organ of leiognathid fish, Wakasa Bay, Honshu, Japan	3
Photobacterium mandapamensis		
ajapo.4.20[b]	Light organ of acropomatid fish, Saga, Shikoku, Japan	33
ajapo.4.22[b,c]	Light organ of acropomatid fish, Saga, Shikoku, Japan	33
ajapo.5.37[b,c]	Light organ of acropomatid fish, Saga, Shikoku, Japan	33
Vibrio chagasii		
LMG 21353^{T}	Intestine of larval fish, Norway	60
LMG 13219	Rotifer, Greece	60
LMG 13237	Seawater, Greece	60
21N-12[b]	Seawater, 250-m depth at 21°N, Pacific Ocean	This study
SB-52[b]	Coastal seawater, Suruga Bay, Honshu, Japan	This study
Vibrio cholerae		
ATCC 14547[b]	Fish, River Elbe, Germany	54
manz.1.1[b]	Coastal seawater, Manzanita, OR	This study
manz.1.2[b]	Coastal seawater, Manzanita, OR	This study
Vibrio harveyi ATCC 14126^{Tb}	Amphipod (*Talorchestia* sp.), Woods Hole, MA	10
Vibrio orientalis ATCC 33934^{Tb}	Seawater, Yellow Sea, China	62
Vibrio parahaemolyticus ATCC 17802^{T}	Clinical (food poisoning), Japan	9
Vibrio rotiferianus LMG 21460^{T}	Rotifer (*Brachionus plicatilis*), Belgium	31
Vibrio splendidus ATCC 33125^{Tb}	Marine fish, The Netherlands	54
Vibrio vulnificus		
ATCC 27562^{T} (B9629)	Clinical (blood), Florida; E type[d]	8
ATCC 43382 (VVL1)[b]	Clinical (fatal), Florida; E type	48
CMCP6	Clinical, Korea; C type[d]	35, 55
YJ016	Clinical, Taiwan; C type	14, 55
300 1A1	Seawater; E type	55
C7184	Clinical (blood); C type	52, 55
395R	Clinical; E type	55
300 1C1	Seawater; C type	55
SS109B-3B2	Oyster; E type	55
SS108C-5C1	Oyster; E type	55
SPRC10143	Clinical (fatal), California; C type	55

[a] Strains for which DNA sequences were obtained in this study. See also Table 2 for strains from which previously obtained sequences were analyzed in this study.
[b] Bioluminescent; all other strains listed here are nonluminous and apparently lack *lux* genes.
[c] Previously identified incorrectly as *P. leiognathi* based on sequence analysis of *lux-rib*$_2$ genes (33).
[d] E type and C type, environmental and clinical genotypes of *V. vulnificus* strains, respectively (13, 55).

bacteria are genes for synthesis of riboflavin, forming a *lux-rib* operon, *luxCDAB(F)EG-ribEBHA*, which is located adjacent to the chromosomal *putA* gene (coding for proline dehydrogenase) (3). Recently, many strains of *Photobacterium leiognathi* were found to carry two complete, physically separate, and apparently functional *lux-rib* operons, one adjacent to *putA* and the other located elsewhere on the chromosome and flanked by putative transposase genes (3). The conserved gene content and gene order of the luminescence system in bacteria and the high levels of amino acid sequence identities of the Lux proteins among luminous bacteria suggest a common evolutionary origin for the *lux* operon genes.

The presence of the *luxCDABEG* genes is scattered taxonomically in extant bacteria. Most luminous bacteria are members of *Vibrionaceae* (*Gammaproteobacteria*), with luminous species in the genera *Aliivibrio*, *Photobacterium*, and *Vibrio*. Members of two other *Gammaproteobacteria* families, *Shewanella hanedai* and *Shewanella woodyi* (*Shewanellaceae*) and *Photorhabdus* species (*Enterobacteriaceae*), also are luminous. This pattern of incidence suggests that the *lux* genes arose either in the ancestor of the *Vibrionaceae*, followed by horizontal transfer to members of the *Shewanellaceae* and *Enterobacteriaceae*, or earlier, in the ancestor of all three families. Most of the species in these three families, however, lack the *lux* genes, and for many of the luminous species, some or most strains are nonluminous (20). An evolutionary scenario that might account for the scattered incidence of *lux* genes is vertical inheritance with loss of these genes from several lineages leading to extant species of *Vibrionaceae*, *Shewanellaceae*, and *Enterobacteriaceae*. Alternatively, the *lux* genes, regardless of their origin, might have been acquired by many strains and species via horizontal transfer. A combination of loss from many taxa and acquisition by horizontal transfer by other taxa also could account for the scattered incidence of *lux* genes in

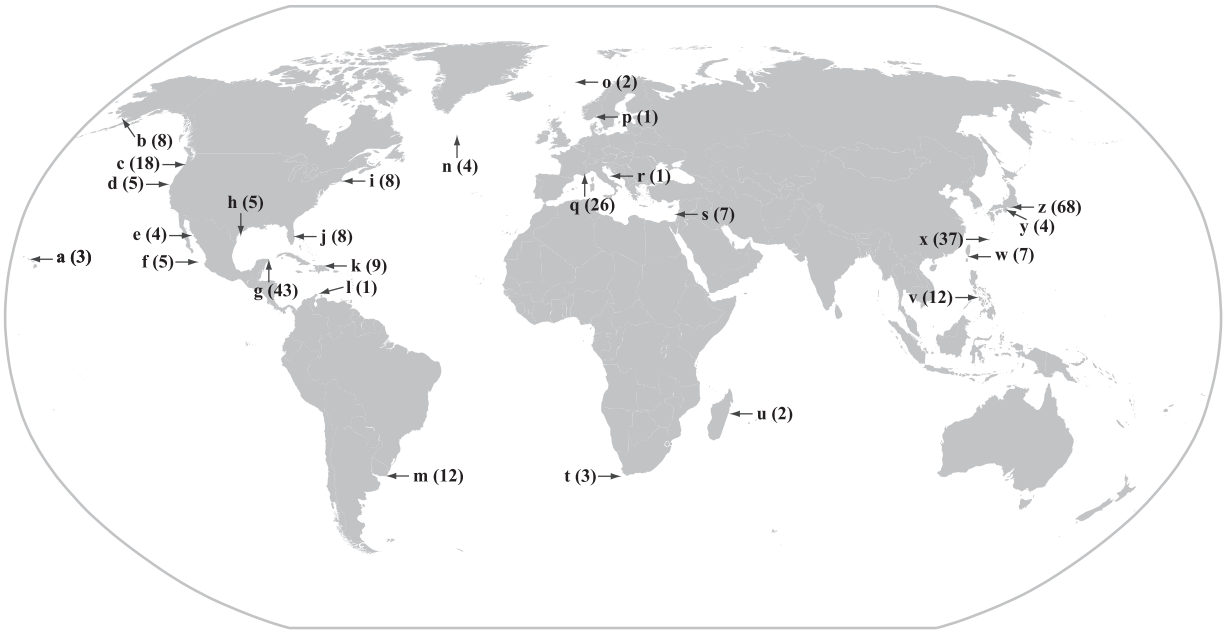

FIG. 1. Collection locations of luminous strains screened for horizontal transfer of *lux* genes. Collection locations are as follows: (a) Hawaii; (b) Alaska; (c) Manzanita, OR; (d) Pacific Ocean off the coast of northern California; (e) Guaymas Basin, Gulf of California; (f) western Pacific Ocean at 21°N; (g) Cancun, Mexico; (h) Galveston, TX; (i) Woods Hole, MA; (j) Fort Lauderdale and Atlantic Ocean off the coast of Florida; (k) Dominican Republic; (l) Caracasbaai, Curacao; (m) José Ignacio, Uruguay; (n) North Atlantic; (o) North Sea; (p) Oslo Harbor, Norway; (q) Il de Bendor, Provence, France; (r) Adriatic Sea; (s) Habonim Beach, Israel; (t) Cape Town, South Africa; (u) Madagascar; (v) Tigbauan, Panay, and Sangat Island, Palawan, Philippines; (w) Tungkang, Taiwan; (x) Nakagusuku Bay and Sesoko Island, Okinawa, Japan; (y) Ago Bay, Honshu, Japan; and (z) Suruga Bay, Honshu, Japan. Species, number of strains, and (in parentheses) letter designations of the locations from which each species was collected are as follows: *A. fischeri*, nine (h, x, z); *Aliivibrio logei*, eight (i); *P. damselae*, one (x); *Photobacterium kishitanii*, 14 (a, e, j, q, t, z); *P. leiognathi*, 13 (j, v, x, y, z); *P. mandapamensis*, 51 (g, j, w, x, z); *Photobacterium phosphoreum*, 20 (b, d, n, o, r); *V. chagasii*, two (f, z); *V. cholerae*, 17 (c); *V. harveyi*, 166 (c, e, f, g, h, k, l, m, p, q, s, u, v, w, x, y, z); *Vibrio orientalis*, two (h). Also, see Table 1 for collection information for *V. vulnificus* (11 strains) and for strains of light-organ symbiotic *P. mandapamensis* (three strains).

bacteria. However, neither scenario has been tested phylogenetically. The only available evidence on these questions, which suggests that *Photorhabdus* and luminous *Shewanella* species acquired their *lux* genes by horizontal transfer from a member of *Vibrionaceae* (20, 27, 34), is based on analysis of nucleotide and amino acid sequence similarities, criteria that lack a rigorous evolutionary basis and therefore do not provide definitive evidence for or against HGT.

To address these issues and gain insight into the frequency of horizontal transfer of the *lux* genes and its impact on speciation, we used an evolutionary approach to test for and characterize instances of *lux* gene horizontal transfer in *Vibrionaceae*. Over 300 luminous strains, most of which were newly isolated from nature, were examined for incongruence of phylogenies based on multiple housekeeping genes and multiple *lux* genes. The results, which indicate that horizontal transfer of the *lux* genes in nature apparently is rare and has not contributed to speciation in recipient taxa, support the scenario of vertical inheritance with multiple loss.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains examined in detail in this study are listed in Table 1. For routine culture, strains were grown at room temperature (20 to 22°C) with aeration in LSW-70 (21), which contained 10 g tryptone, 5 g yeast extract, 350 ml double-strength artificial seawater (44), 650 ml deionized water, and, for solid medium, 15 g · liter^{-1} agar.

Isolation and screening of luminous bacteria. Bacterial strains newly isolated in this study were taken primarily from coastal and open-ocean seawater at various locations worldwide (Fig. 1). Samples of aseptically collected seawater were spread on LSW-70 agar plates made with 40 g · liter^{-1} agar (7), incubated overnight at 18 to 25°C, and examined for luminous colonies, which were then picked and purified on the same medium. Purified strains were stored in cryoprotective medium at −75°C (20).

For photometric detection of luminescence, cells were taken from early-stationary-phase broth cultures or overnight cultures on plates of LSW-70 agar (15 g · liter^{-1} agar), and their luminescence was monitored with a Turner BioSystems (Sunnyvale, CA) 20/20 luminometer operated at high sensitivity.

Genomic DNA extraction, PCR, and sequencing. Extraction of genomic DNA from overnight broth cultures and amplification of housekeeping genes *gyrB*, *recA*, and *pyrH* (specifying gyrase subunit B, recombinase A, and uridylate kinase, respectively) and of the *luxCDABEG* genes followed previously described protocols (2, 59). Primer sequences and amplification conditions for each gene and strain are provided in the supplemental material. PCR products were visualized using electrophoresis on 1% agarose gels stained with ethidium bromide and were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA). PCR products were sequenced using the respective PCR primers, and sequencing was carried out by the staff of the University of Michigan Sequencing Core using dye terminator cycle sequencing on a Perkin-Elmer (Waltham, MA) ABI 3730 or 3700 DNA analyzer.

Phylogenetic analysis. Provisional species identifications were based on sequence analysis of *gyrB* or *pyrH*, and instances of *lux* gene horizontal transfer were identified provisionally by incongruence between phylogenies based on *gyrB* or *pyrH* and *luxA*. Nucleotide and amino acid identities were calculated using PAUP* (57). For detailed phylogenetic analyses based on multiple housekeeping genes and multiple *lux* genes, concatenated sequences of *gyrB*, *recA*, and *pyrH* and of *luxCDABEG* genes were analyzed (Table 2). DNA sequences were aligned by reference to the inferred amino acid sequences. Characters from genes unavailable for analysis were treated as missing data, and spacer regions between *lux* genes were omitted from the analysis. The *luxF* sequence was treated as missing data in taxa lacking this gene. Sequence data were analyzed simultaneously with TNT (30). Outgroup sequences were from *Photorhabdus lumine-*

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

Strain	GenBank accession no. or reference for sequence of:			
	recA	*gyrB*	*pyrH*	*lux* operon genes[c]
Aliivibrio fischeri ATCC 7744^T	AJ842417	AY455874	EF415528	AY341062 (*luxCDABE*)
Aliivibrio fischeri ES114[a]	VF0535	VF0012	VF1960	VFA0923 to VFA0918 (*luxCDABEG*)
Aliivibrio logei ATCC 29985^T	AJ842457	EF380255	EF380234	EF576941 (*luxAB*)
Aliivibrio salmonicida ATCC 43839^T	EF380243	EF380256	EU118245	AF452135 (*luxCDABE*)
Aliivibrio wodanis ATCC BAA-104^T	EF380244	EF380257	EU118246	NA
Photobacterium angustum ATCC 25915^T	AJ842354	AY455890	EF380235	NA
Photobacterium damselae subsp. *damselae* ATCC 33539^T	AJ842357	AY455889	EF380236	NA
Photobacterium damselae BT-6	EU118224	EU118205	EU118247	EU122290 (*luxDABE*)
Photobacterium iliopiscarium ATCC 51760^T	AJ842362	AY455878	EF380237	NA
Photobacterium kishitanii ATCC BAA-1194^T	EF415552	AY455877	EF415536	DQ988874 (*luxCDABFEG*)
Photobacterium leiognathi ATCC 25521^T	AJ842364	AY455879	EF380238	M63594 (*luxCDABEG*)
Photobacterium leiognathi lnuch.13.1	EU118227	EU118211	EU118253	EF536338 (*luxCDABEG*$_1$) EF536332 (*luxCDABEG*$_2$)
Photobacterium mandapamensis ATCC 27561^T	EF415548	AY455883	EF415532	DQ988878 (*luxCDABFEG*)
Photobacterium mandapamensis ajapo.4.20	EU118221	EU118202	EU118237	EU122285 (luxCDABFEG$_1$) EU122286 (*luxCDABFEG*$_2$)
Photobacterium phosphoreum ATCC 11040^T	EF415550	AY455875	EF380239	DQ988873 (*luxCDABFEG*)
Photorhabdus luminescens subsp. *laumondii* TTO1[a]	plu1249	plu0004	plu0674	plu2079 to plu2083 (*luxCDABE*)
Shewanella hanedai ATCC 33224^T	NA	AF005693	NA	AB058949 (*luxCDABEG*)
Shewanella woodyi ATCC 51908^T[a]	SWooDRAFT_4188	SWooDRAFT_0158	SWooDRAFT_4084	SWooDRAFT_2999 to SWooDRAFT_3004 (*luxCDABEG*)
Vibrio aestuarianus ATCC 35048^T	AJ842369	NA	59[b]	NA
Vibrio alginolyticus ATCC 17749^T	AJ842373	AF007288	59	NA
Vibrio anguillarum ATCC 19264^T	AJ842375	NA	59	NA
Vibrio brasiliensis LMG 20546^T	AJ842376	NA	59	NA
Vibrio campbellii ATCC 25920^T	AJ842377	AB014950	EF596641	NA
Vibrio chagasii LMG 21353^T	AJ842385	AJ577820	EU118252	NA
Vibrio chagasii 21N-12	EU118217	EU118198	EU118233	EU122293 (*luxDABE*)
Vibrio chagasii SB52	EU118230	EU118214	EU118256	EU122294 (*luxDABE*)
Vibrio chagasii LMG 13219	AJ842383	EU118208	EU118250	NA
Vibrio chagasii LMG 13237	AJ842384	EU118209	EU118251	NA
Vibrio cholerae ATCC 14035^T	AJ842386	NA	59	NA
Vibrio cholerae ATCC 14547	EU118222	EU118203	EU118239	AB115761 (*luxCDABEG*)
Vibrio cholerae manz.1.1	EU118228	EU118212	EU118254	EU122291 (*luxDABE*)
Vibrio cholerae manz.1.2	EU118229	EU118213	EU118255	EU122292 (*luxDABE*)
Vibrio cholerae N16961[a]	VC_0543	VC_0015	VC_2258	NA
Vibrio coralliilyticus ATCC BAA-450^T	AJ842402	NA	59	NA
Vibrio cyclitrophicus ATCC 700982^T	AJ842405	AM162562	NA	NA
Vibrio diabolicus LMG 19805^T	AJ842407	NA	59	NA
Vibrio diazotrophicus ATCC 33466^T	AJ842411	AB014951	59	NA
Vibrio fluvialis ATCC 33809^T	AJ842419	NA	59	NA
Vibrio fortis LMG 21557^T	AJ842422	NA	59	NA
Vibrio furnissii ATCC 35016^T	AJ842427	NA	59	NA
Vibrio harveyi ATCC 14126^T	DQ648369	DQ648280	EU118238	EU122288 (*luxCDABEG*)
Vibrio hispanicus LMG 13240^T	AJ842445	NA	59	NA
Vibrio ichthyoenteri ATCC 700023^T	AJ842446	NA	59	NA
Vibrio kanaloae LMG 20539^T	AJ842450	AJ577821	59	NA
Vibrio lentus ATCC BAA-539^T	AJ842452	AM162564	59	NA
Vibrio mediterranei ATCC 43341^T	AJ842459	EF380258	59	NA
Vibrio mimicus ATCC 33653^T	AJ842468	EF380259	EU118242	NA

Continued on following page

TABLE 2—*Continued*

Strain	GenBank accession no. or reference for sequence of:			
	recA	*gyrB*	*pyrH*	*lux* operon genes[c]
Vibrio mytili ATCC 51288^T	AJ842472	NA	59	NA
Vibrio natriegens ATCC 14048^T	AJ842473	NA	59	NA
Vibrio navarrensis ATCC 51183^T	AJ842474	NA	59	NA
Vibrio neptunius LMG 20536^T	AJ842478	NA	59	NA
Vibrio nereis ATCC 25917^T	AJ842479	NA	59	NA
Vibrio ordalii ATCC 33509^T	AJ842482	NA	59	NA
Vibrio orientalis ATCC 33934^T	AJ842485	EF380260	EU118243	EU122287, AB058948 (*luxCDABE*)
Vibrio pacinii LMG 19999^T	AJ842486	NA	59	NA
Vibrio parahaemolyticus ATCC 17802^T	AJ842490	AF007287	EU118240	NA
Vibrio pelagius ATCC 25916^T	AJ842495	AB014954	59	NA
Vibrio pomeroyi LMG 20537^T	AJ842497	AJ577822	59	NA
Vibrio proteolyticus ATCC 15338^T	AJ842499	NA	59	NA
Vibrio rotiferianus LMG 21460^T	AJ842501	EU118210	EF596722	NA
Vibrio shilonii ATCC BAA-91^T	AJ842507	NA	59	NA
Vibrio splendidus ATCC 33125^T	AJ842511	EF380261	EU118241	EF536342 (*luxAB*)
Vibrio tasmaniensis LMG 20012^T	AJ842515	AJ577823	59	NA
Vibrio tubiashii ATCC 19109^T	AJ842518	NA	59	NA
Vibrio vulnificus ATCC 27562^T	AJ842523	AY705491	59	NA
Vibrio vulnificus VVL1 (ATCC 43382)	EU118223	EU118204	EU118244	EU122289 (*luxCDABEG*)
Vibrio vulnificus 300 1C1	EU118218	EU118200	EU118235	NA
Vibrio vulnificus SS108C 5C1	EU118232	EU118216	EU118258	NA
Vibrio vulnificus 395R	EU118220	EU118199	EU118234	NA
Vibrio vulnificus C71840	EU118225	EU118206	EU118248	NA
Vibrio vulnificus 301 1A1	EU118219	EU118201	EU118236	NA
Vibrio vulnificus ENV1	EU118226	EU118207	EU118249	NA
Vibrio vulnificus SPRC 10143	EU118231	EU118215	EU118257	NA
Vibrio vulnificus CMCP6[a]	VV1_1591	VV1_0996	VV1_1861	NA
Vibrio vulnificus YJ016[a]	VV2805	VV0014	VV2555	NA
Vibrio xuii LMG 21346^T	AJ842529	NA	59	NA
Vibrio sp. strain BB120[a]	VIBHAR_03513	VIBHAR_00445	VIBHAR_03235	VIBHAR_06244-06339
Uncultured symbiont of *Kryptophanaron alfredi*	NA	NA	NA	M36597 (*luxAB*)

[a] For *A. fischeri* ES114, *P. luminescens* subsp. *laumondii* TTO1, *S. woodyi* ATCC 51908^T, *V. cholerae* N16961, *V. vulnificus* CMCP6, *V. vulnificus* YJ016, and *Vibrio* sp. strain BB120, locus tags from genome sequencing projects are given.

[b] Some *pyrH* sequences were reported previously (59) and can be obtained from the multilocus sequence analysis identification page for *Vibrionaceae* at http://www.taxvibrio.lncc.br.

[c] The specific *lux* genes analyzed are listed in parentheses. NA, not available.

scens subsp. *laumondii* TTO1 (18). Jackknife resampling values (25) were calculated in TNT with 10,000 resampling replicates.

Nucleotide sequence accession numbers. GenBank accession numbers for sequences obtained in this study are EU118198 to EU118258 and EU122285 to EU122294. A complete list of accession numbers for sequences used in this study is given in Table 2.

RESULTS

Predominantly vertical inheritance of the *lux* genes in *Vibrionaceae*. To distinguish between multiple losses and frequent horizontal acquisition as evolutionary scenarios accounting for the scattered incidence of *lux* genes, we screened luminous bacterial strains for congruence between phylogenies based on *gyrB* or *pyrH*, representative housekeeping genes, and *luxA*, a representative *lux* gene. Strains exhibiting incongruence were then subjected to detailed phylogenetic analysis based on multiple housekeeping genes and multiple *lux* genes to more rigorously test and characterize putative horizontal transfer events. Three hundred three luminous strains, representing 11 species of *Aliivibrio*, *Photobacterium*, and *Vibrio*, were examined; these strains were isolated from coastal and open-ocean seawater and skin and intestines of fish from many locations worldwide (Fig. 1). Based on sequence analysis of the *gyrB* or *pyrH* gene, most of the strains were identified as members of species previously identified as luminous or as having luminous representatives. For nearly all of the strains, phylogenies based on *gyrB* or *pyrH* and *luxA* were found to be congruent (Fig. 2). Furthermore, no instance in which the *lux* genes of a luminous species had replaced the *lux* genes of another luminous species was identified, i.e., *lux* gene sequences of strains identified using housekeeping genes as belonging to known luminous species were typical of that species. These results indicate that the *lux* genes are predominantly vertically inherited and are stable in most extant luminous taxa. Therefore, multiple losses instead of frequent acquisition by horizontal transfer most likely account for the scattered incidence of *lux* genes in *Vibrionaceae*. Notable in this regard is *Vibrio cholerae*, a species in which most strains lack the *lux* genes (16, 20, 49, 53). For luminous strains of *V. cholerae*, phylogenies based on *gyrB* and

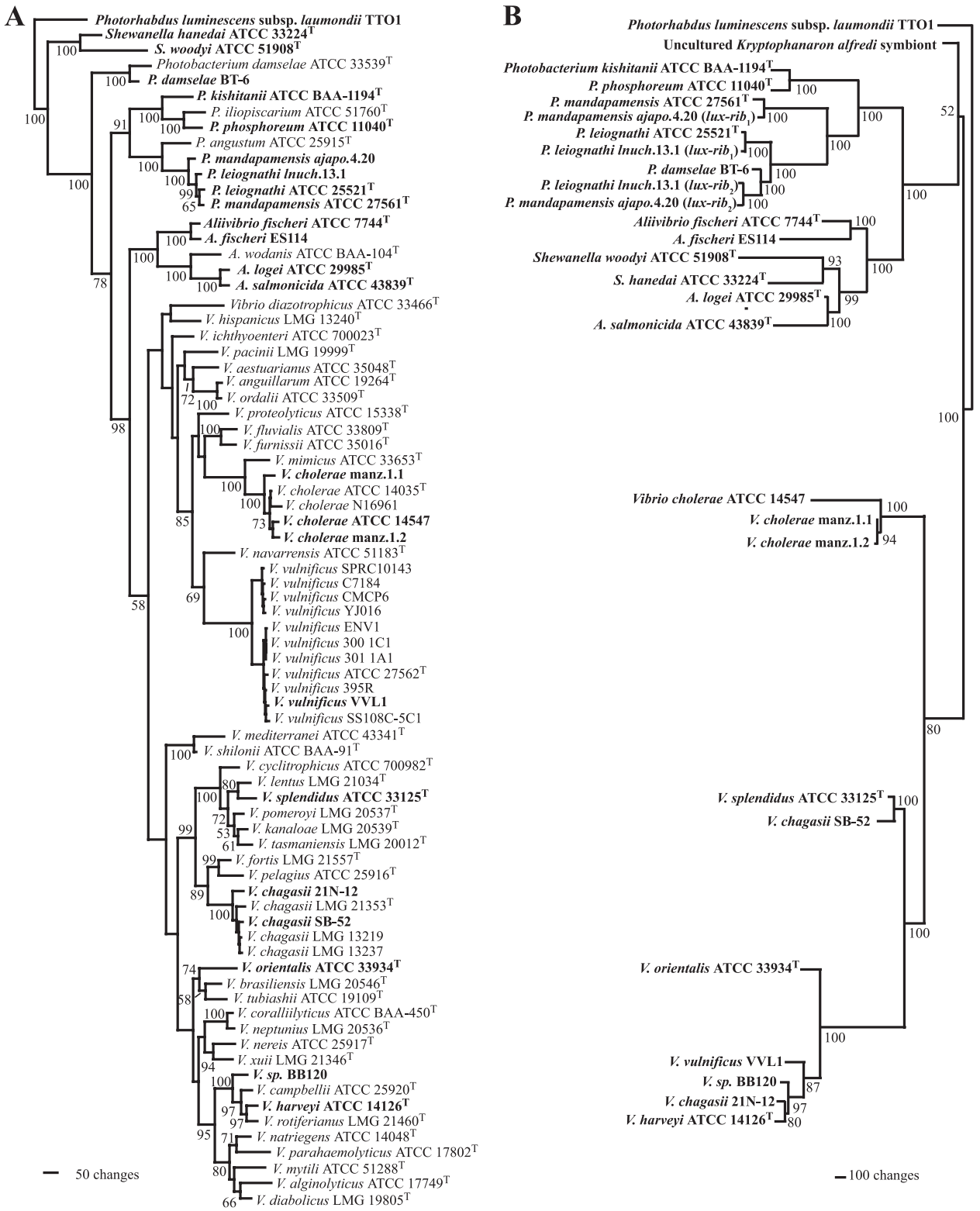

FIG. 2. Phylogenetic trees of the three housekeeping genes *gyrB*, *recA*, and *pyrH* (A) and the *lux* operon genes (B); luminescent taxa are shown in bold. The housekeeping gene analysis resulted in 36 equally parsimonious trees. Tree length, 8327; consistency index (CI), 0.247; retention index (RI), 0.675. The jackknife values are shown at the nodes; some values for *V. vulnificus*, *V. cholerae*, and *V. chagasii* clades were omitted for clarity. Parsimony analysis of the *lux* operon genes sequences resulted in a single tree: tree length, 14311; CI, 0.546; RI, 0.732. Each of the housekeeping genes separately yielded the same relationship of VVL1 to the other *V. vulnificus* strains (data not shown).

TABLE 3. Nucleotide and inferred amino acid sequence identities of *lux* genes

Genes and recipient strain	Comparison strain[a]	% Identity	
		Nucleotide	Amino acid
luxDAB			
Vibrio vulnificus VVL1	*Vibrio harveyi* ATCC 14126^T	94.9	97.1
Vibrio chagasii 21N-12	*Vibrio harveyi* ATCC 14126^T	96.6	97.9
Vibrio chagasii SB-52	*Vibrio splendidus* ATCC 33125^T	91.5	97.5
Photobacterium damselae BT-6	*Photobacterium leiognathi lnuch*.13.1 (*lux-rib*$_2$)	95.4	95.9
Photobacterium mandapamensis ajapo.4.20	*Photobacterium leiognathi lnuch*.13.1 (*lux-rib*$_2$)	99.3	99.4
Shewanella hanedai ATCC 33224^T	*Aliivibrio fischeri* ATCC 7744^T	73.6	77.2
	Aliivibrio fischeri ES114	72.9	74.0
	Aliivibrio logei ATCC 29985^T	77.5	81.6
	Aliivibrio salmonicida ATCC 43839^T	79.1	84.9
Shewanella woodyi ATCC 51908^T	*Aliivibrio fischeri* ATCC 7744^T	73.3	79.7
	Aliivibrio fischeri ES114	71.1	75.7
	Aliivibrio logei ATCC 29985^T	75.9	83.0
	Aliivibrio salmonicida ATCC 43839^T	76.9	82.9
luxCDABE[b]			
Photorhabdus luminescens TTO1	*Aliivibrio fischeri* ATCC 7744^T	63.4	62.3 (*luxDABE*)
	Aliivibrio fischeri ES114	63.9	61.1
	Aliivibrio logei ATCC 29985^T	64.5	62.0 (*luxAB*)
	Aliivibrio salmonicida ATCC 43839^T	65.2	64.0
	Photobacterium kishitanii ATCC BAA-1194^T	63.8	59.7
	Photobacterium leiognathi ATCC 25521^T	61.4	58.8
	Photobacterium mandapamensis ATCC 27561^T	63.4	61.3
	Photobacterium phosphoreum ATCC 11040^T	63.9	59.3
	Shewanella hanedai ATCC 33224^T	64.6	63.2
	Shewanella woodyi ATCC 51908^T	62.5	61.6
	Vibrio cholerae ATCC 14547^T	65.2	66.8
	Vibrio harveyi ATCC 14126^T	65.0	67.4
	Vibrio orientalis ATCC 33934^T	65.4	67.5
	Vibrio splendidus ATCC 33125^T	70.4	75.4 (*luxAB*)

[a] The listed comparison strains are representative of a species.
[b] Unless noted otherwise in parentheses in final column.

luxA were congruent, and phylogenetic analysis of multiple housekeeping gene sequences did not separate luminous and nonluminous strains (Fig. 2). Therefore, the *lux* genes apparently have been lost from many strains of this species. Exceptions to the general pattern of phylogenetic congruence were found, however, as described below.

Horizontal acquisition of *lux* genes by *Vibrio vulnificus* VVL1. *Vibrio vulnificus* VVL1 (ATCC 43382), a human pathogen (48, 55) (Table 1), differs from other strains of *V. vulnificus* in being bioluminescent, and it is the only luminous member of *Vibrionaceae* documented to have caused a fatal human infection. To determine if other strains of *V. vulnificus* might be luminous but cryptically so, 10 strains representing environmental and clinical genotypes of this species (Table 1) (55) were tested for the presence of the *lux* genes. When grown under conditions in which VVL1 produces a high level of luminescence, none of the additional strains was visibly luminous, and none produced light that could be detected with a sensitive photometer. Furthermore, none yielded a *luxA* or *luxB* amplicon when tested with PCR primers effective for amplifying these genes from VVL1 and other luminous bacteria. The genomes of two of these strains, YJ016 and CMCP6, have been fully sequenced (14), and neither contains *lux* genes. These results establish that VVL1, of the 11 strains of *V. vulnificus* examined, is unique in producing luminescence and carrying *lux* genes. Preliminary sequence analysis revealed VVL1 *lux* genes to be similar in nucleotide and inferred amino acid sequences to *Vibrio harveyi lux* genes (Table 3). This similarity, the uniqueness of luminescence in VVL1 among examined *V. vulnificus* strains, and the lack of *gyrB-luxA* congruence suggested that VVL1 acquired its *lux* genes by horizontal transfer.

To test this possibility phylogenetically, we reconstructed the evolutionary relationships of VVL1 with other luminous and nonluminous members of *Vibrionaceae*. A phylogeny based on the housekeeping genes *gyrB*, *recA*, and *pyrH*, analysis of which robustly resolves members of *Vibrionaceae* (2, 59), was constructed and compared with a phylogeny based on the *luxCD ABEG* genes for luminous species (Fig. 2; Table 2). The analysis of housekeeping genes placed VVL1 within the clade formed by the other *V. vulnificus* strains, confirming its species identification by other criteria (48). Phylogenetic placement of VVL1 based on the *lux* genes, however, did not match that based on the housekeeping genes (Fig. 2). The *lux* genes of VVL1 instead were evolutionarily very closely related to those of *V. harveyi*, a species that, based on the divergence in sequences of *gyrB*, *recA*, and *pyrH*, is phylogenetically distinct from *V. vulnificus*. The discordance between housekeeping and *lux* gene phylogenies supports the hypothesis that VVL1 acquired its *lux* genes by horizontal transfer, and the data indicate that *V. harveyi* was the likely donor of those genes. These results are the first demonstration based on phylogenetic criteria of an apparent interspecies horizontal transfer of the *lux* genes from a luminous to a nonluminous bacterium.

Horizontal acquisition of *lux* genes in *Vibrio chagasii*. *Vibrio chagasii* is a species for which luminous strains had not previ-

ously been reported (60). Luminous strains 21N-12 and SB-52, isolated from seawater off the coast of Mexico and from Suruga Bay, Japan, respectively (Fig. 1; Table 1), were provisionally identified by *gyrB* sequence analysis as members of *V. chagasii*. Previously characterized strains of *V. chagasii*, LMG 21353^T, LMG 13219, and LMG 13237, were tested and found not to produce luminescence and to not carry *luxA* or *luxB* genes. Preliminary sequence analysis revealed similarity of the *lux* genes of 21N-12 and SB-52 to *lux* genes of *V. harveyi* and *Vibrio splendidus*, respectively (Table 3). This similarity, the apparently atypical luminescence of 21N-12 and SB-52, and the lack of *gyrB-luxA* congruence suggested that these strains acquired their *lux* genes by horizontal transfer. Phylogenetic analysis based on multiple housekeeping genes (*gyrB*, *recA*, and *pyrH*) confirmed the identification of 21N-12 and SB-52 as *V. chagasii* (Fig. 2), but phylogenetic placement of 21N-12 and SB-52 based on the housekeeping genes did not match that based on the *lux* genes. Instead, the *lux* genes of 21N-12 were closely related to those of *V. harveyi*, and the *lux* genes of SB-52 were closely related to those of *V. splendidus* (Fig. 2). These results support the hypothesis that 21N-12 and SB-52 acquired their *lux* genes horizontally, with *V. harveyi* and *V. splendidus*, respectively, as the likely donors.

Horizontal acquisition of *lux* genes in *Photobacterium damselae*. *Photobacterium damselae*, like *V. chagasii*, is a species for which luminous strains had not previously been reported (28). A luminous strain, BT-6, isolated from seawater in Okinawa (Fig. 1; Table 1), was provisionally identified by *gyrB* sequence analysis as *P. damselae*. Preliminary analysis revealed nucleotide and amino acid sequence similarities of the BT-6 *lux* genes to *lux* genes of the *lux-rib*$_2$ operon of *P. leiognathi* (3) (Table 3). Phylogenetic analysis based on multiple housekeeping genes, including the 16S rRNA gene, and *lux* genes confirmed the identification of BT-6 as *P. damselae* and indicated that the *lux-rib*$_2$ operon of *P. leiognathi* was the likely source of the BT-6 *lux* genes (Fig. 2). This is the first reported instance of a likely transfer of the *lux* genes of the *lux-rib*$_2$ operon of *P. leiognathi* to another species.

Merodiploidy and horizontal acquisition of *lux* genes in *Photobacterium mandapamensis*. *Photobacterium mandapamensis* is a luminous species that is closely related to *P. leiognathi*; these two species can be distinguished by molecular phylogenetic criteria, by some phenotypic traits, and by their different host species ranges (1, 33). Strain *ajapo*.4.20, isolated from the light organ of an acropomatid fish (Table 1), the primary symbiont of which is *P. mandapamensis* (33), was found here to carry two *lux-rib* operons. One operon, *lux-rib*$_1$, was located adjacent to *putA*, the common and putatively ancestral chromosomal location for *lux-rib* genes in *Photobacterium* (3). Sequence analysis of genes of this operon confirmed the identification of this strain as *P. mandapamensis*. The *lux* genes of the second *lux-rib* operon, located elsewhere in the genome, were found however to be closely related to those of the *P. leiognathi* *lux-rib*$_2$ operon (Fig. 2). These results provide a third example of natural merodiploidy of the *lux-rib* operon in luminous bacteria and the second instance of apparent horizontal transfer of the *lux* genes of the *lux-rib*$_2$ operon of *P. leiognathi* to another species. Two other strains of *P. mandapamensis*, *ajapo*.4.22 and *ajapo*.5.37, also were found here to be merodiploid for the *lux-rib* operon; as with *ajapo*.4.20, the second *lux-rib* operons of these strains were closely related to *lux-rib*$_2$ of *P. leiognathi*. In a previous report (33), these two strains had been identified incorrectly as *P. leiognathi* based on phylogenetic analysis of the *lux-rib*$_2$ operon.

Interfamily horizontal transfer of *lux* genes. Similarities in the inferred amino acid sequences of *lux* genes in members of other families of *Gammaproteobacteria*, i.e., *Shewanella hanedai* and *Shewanella woodyi* (*Shewanellaceae*) and *Photorhabdus* species (*Enterobacteriaceae*) (Table 3), have been interpreted as evidence of horizontal acquisition of *lux* genes to these bacteria from species of *Vibrionaceae* (20, 27, 34). To test these proposed instances of interfamily HGT using evolutionary criteria, we compared phylogenies based on housekeeping and *lux* genes for *Shewanella*, *Photorhabdus*, and luminous members of *Vibrionaceae*. The *lux* genes of *S. hanedai* and *S. woodyi* were found to be closely related to those of members of *Aliivibrio* (Fig. 2). These results are consistent with horizontal transfer of the *lux* genes from a member of the *Aliivibrio* lineage to *S. hanedai* and *S. woodyi*. Analysis of the sequences of the *luxR* and *luxI* genes of *S. hanedai*, *Allivibrio fischeri*, and *Allivibrio salmonicida* (45), which revealed a close relationship among these genes (data not shown), further supports this conclusion. In contrast, phylogenies based on housekeeping and *lux* genes for *Photorhabdus* were congruent (Fig. 2). Whether *Photorhabdus* acquired *lux* genes by horizontal transfer therefore remains unresolved. Consistent with this result, nucleotide and amino acid identities of the *luxCDABE* genes of *P. luminescens* TTO1 with those of other species of luminous bacteria are relatively low (Table 3).

DISCUSSION

The results of this study indicate that acquisition of the bacterial *lux* genes by HGT in nature apparently is rare. HGT is thought to occur very commonly in bacteria (29, 39, 40, 46), and luminescence, a distinctive, easily observed phenotype, provides direct access to bacteria from nature that bear functional *lux* genes. However, despite an extensive sampling of luminous members of *Vibrionaceae* isolated from a variety of habitats and locations worldwide, we found only four instances consistent with HGT, two in *Vibrio*, with transfer of the *lux* genes from luminous to nonluminous species, and two in *Photobacterium*, with transfer of genes of the *lux-rib*$_2$ operon of *P. leiognathi* to other *Photobacterium* species. No instance in which the *lux* genes of one luminous species had replaced those of another luminous species was found, so homologous gene replacement may be even more rare than horizontal gene acquisition (11, 47). Phylogenetic analysis also supported a hypothesized interfamily transfer of the *lux* genes from members of a third *Vibrionaceae* genus, *Aliivibrio*, to species of *Shewanella* (*Shewanellaceae*) but did not confirm a postulated horizontal acquisition of *lux* genes by *Photorhabdus* (*Enterobacteriaceae*). Strains that had acquired *lux* genes horizontally in all cases were otherwise phylogenetically indistinguishable from nonluminous strains of that species; thus, none of the identified instances of apparent horizontal acquisition of *lux* genes has led to evolutionary divergence of the recipient lineage. These results indicate that the bacterial *lux* genes are transferred primarily by vertical inheritance and that the rare instances of apparent horizontal acquisition, although contrib-

uting to the pan-genome of recipient species, have not played an obvious role in their speciation. If the same is true for other genes, HGT may be uncommon in and have no major impact on the evolution of *Vibrionaceae*.

Previously proposed instances of *lux* gene HGT (20, 27, 34) were based primarily on inferred amino acid sequence similarity comparisons. Such comparisons, which identify the best similarity matches for genes from different organisms, provide a rapid screen for possible HGT events (Table 3), but they are not reliable for evolutionary inference (5, 23). We therefore used phylogenetic reconstruction, i.e., character state methods, to more rigorously test and characterize putative horizontal transfer events. Incongruent housekeeping gene and *lux* gene phylogenetic trees provide strong evidence that the bacterial *lux* genes have been acquired horizontally in the few cases described here. Although events other than HGT can give rise to incongruent trees (5), they are unlikely to account for the instances of apparent *lux* HGT described here.

A possible criticism of our results is that analysis of *lux* genes underestimates the occurrence of HGT, because the *lux* genes may be subject to special selection. A strong counterpoint to this view, however, is that the bacterial *lux* genes do not appear to have evolved differently from housekeeping genes. There is no evidence for a separate evolutionary history for the *lux* genes, other than their apparent loss from many species and strains of *Vibrionaceae*. Sequence divergence of the *lux* genes, individually and collectively, resolves the same evolutionary relationships as housekeeping genes, although sometimes with a better delimitation of closely related species and clades (1, 2, 4, 33; also this study). Therefore, the bacterial *lux* genes do not appear to be subject to special selection or to have undergone genetic drift different from that experienced by core housekeeping genes. We nonetheless cannot exclude the possibility that horizontal transfer of other genes in *Vibrionaceae* is more common than that seen for the *lux* genes.

In this regard, horizontal acquisition of *lux* genes may be more common than the four instances identified here. Detection of bacteria bearing the *lux* genes was based on visual screening for luminescence, a phenotype that requires the presence and a high level of expression of the *luxCDABEG* genes. Several classes of *lux* HGT events that would not be detected by this screening are possible. Bacteria that acquired only some of the *lux* genes, e.g., *luxCDA* or *luxAB*, or those in which the *luxCDABEG* genes had mutations would be dark or would produce too low a level of luminescence to be detected visually, as would those strains unable to transcribe the *lux* genes at a high level in culture, such as those lacking necessary regulatory genes or regulatory factors, and those in which the activities of the Lux proteins do not lead to high levels of luminescence in laboratory culture (12, 26, 49, 53). PCR and hybridization screening methods therefore may reveal additional instances of *lux* horizontal transfer, as presumably will whole-genome sequence analysis of members of *Vibrionaceae* and other bacterial families. Regardless, the work reported here establishes with evolutionary criteria that horizontal transfer of the *lux* genes apparently has occurred but that the transfer of functional *lux* genes is rare.

Many factors likely influence and possibly limit the horizontal acquisition of *lux* genes and their stable, functional retention by nonluminous bacteria. The examples reported here suggest that HGT may be more common among closely related bacteria. Other factors include the availability of *lux* DNA in the recipient's environment, the susceptibility of acquired *lux* DNA to degradation by restriction endonucleases of the recipient cell, constraints on integration of *lux* DNA into the recipient genome, and the extent of physiological compatibility of the luminescence proteins in the recipient cell (11, 37, 56). Once acquired, stable retention of the *lux* genes in a functional state might be curtailed in the absence of strong positive selection for the activities of the *luxCDABEG* gene products by the accumulation of mutations, by excision, and by reduced fitness of progeny of the recipient cell (11, 37). Even if levels of *lux* DNA in the recipient's environment are high, this multistep winnowing process could sharply limit the incidence of *lux* HGT. Furthermore, given the costs associated with replication and expression of the bacterial *lux* genes (19) and given that the metabolic and physiological benefits of the luminescence system for bacteria are not well understood (20), it is difficult to envision the selective pressures that would lead to the fixation of *lux* genes in progeny of recipient cells and the subsequent spread of these genes from isolated individuals and patchy populations to the global population of a recipient species (11). We therefore speculate that the examples of apparent *lux* HGT shown here are the rare stable instances of an otherwise highly transient process of infrequent *lux* gene acquisition that typically is followed by rapid loss.

A possible exception to this "infrequent acquisition-rapid loss" scenario is the situation with the *lux-rib*$_2$ operon of *P. leiognathi*. In at least one *P. leiognathi* strain, the *lux-rib*$_2$ operon is flanked by putative transposase genes, suggesting mobility of *lux-rib*$_2$ via transposon-mediated transfer (3). Here, we identified a strain of *P. damselae*, a nonluminous bacterium, and strains of *P. mandapamensis*, a luminous species, that bear the *P. leiognathi lux-rib*$_2$ genes. The implication of these instances of apparent *lux* HGT is that the *lux-rib*$_2$ genes might be highly mobile and therefore might be transferred frequently to nonluminous and other luminous bacteria. Furthermore, at least in *P. leiognathi*, the merodiploid state appears to be stable over ecological time (3).

In contrast to the situation for other bacteria examined here, the origin of *lux* genes in *Photorhabdus* remains obscure. The conserved gene order, *luxCDABE*, and inferred amino acid sequence similarities of the *luxA* and *luxB* genes with those of *V. harveyi* and other bacteria suggest horizontal acquisition of the *lux* genes from a member of *Vibrionaceae* (27). Because the chromosomal locations of *lux* genes in ecologically distinct strains of *P. luminescens* apparently differ (43), horizontal transfer might have occurred more than once (27), although genomic rearrangements also could account for the apparently different chromosomal locations. A recent report, based on similarity analysis of inferred amino acid sequences, presents a more complicated scenario, in which the *luxCDABE* genes were transferred from a gram-positive bacterium to *V. cholerae* and then later were transferred from *V. cholerae* to *P. luminescens* (34). The lack of evolutionary analysis, the invoking of an unknown gram-positive bacterium as the source of the *P. luminescens lux* genes, and the multiple steps involved weaken the possible validity of this scenario. Furthermore, we demonstrate here that *lux* and housekeeping gene phylogenies for *P. luminescens* are congruent (Fig. 2). A more likely scenario,

therefore, is that the *lux* genes were acquired by *Photorhabdus* from a member of *Vibrionaceae* in the distant past, followed by substantial sequence divergence of the *Photorhabdus lux* genes. This scenario is consistent with the generally low *lux* nucleotide and amino acid sequence similarities of *Photorhabdus* and *Aliivibrio*, *Photobacterium*, and *Vibrio* species (Table 3) and with the observed congruence of *lux* and housekeeping gene phylogenies.

ACKNOWLEDGMENTS

We thank Claudine Vereecke (BCCM/LMG Bacteria Collection) for providing the LMG strains of *V. chagasii*, Thomas Rosche for assistance with strains of *V. vulnificus*, and Elizabeth Warner for typing strain VVL1. The following individuals facilitated seawater sampling or provided seawater samples: Holger Jannasch (deceased) and captain and crew of the R/V Atlantis II, Celia Lavilla-Torres, Seishi Kimura, Tetsuo Yoshino, Atsushi Fukui, Prosanta Chakrabarty, John Sparks, Julian Adams, Heather Adams, and Karen Beeri. DNA sequencing was carried out by the staff of the University of Michigan Sequencing Core.

This work was supported by grant DEB 0413441 from the National Science Foundation.

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