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Natural replacement of vertically inherited *lux-rib* genes of *Photobacterium aquimaris* by horizontally acquired homologues

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Summary

We report here the first instance of a complete replacement of vertically inherited luminescence genes by horizontally acquired homologues. Different strains of *Photobacterium aquimaris* contain homologues of the *lux-rib* genes that have a different evolutionary history. Strain BS1 from the Black Sea contains a vertically inherited *lux-rib* operon, which presumably arose in the ancestor of this species, whereas the type strain NBRC 104633^T, from Sagami Bay, lacks the vertically inherited *lux-rib* operon and instead carries a complete and functional *lux-rib* operon acquired horizontally from a bacterium related to *Photobacterium mandapamensis*. The results indicate that the horizontal acquisition of the *lux* genes expanded the pan-genome of *P. aquimaris*, but it did not influence the phylogenetic divergence of this species.

Introduction

Bioluminescent bacteria are widespread in the marine environment and play important roles in marine ecosystems (Dunlap and Kita-Tsukamoto, 2006; Dunlap, 2009; Widder, 2010). Most marine luminous bacteria are members of *Vibrionaceae* and can be found in the genera *Vibrio*, *Aliivibrio* and *Photobacterium* (Baumann *et al.*, 1984; Dunlap and Kita-Tsukamoto, 2006; Urbanczyk *et al.*, 2007; 2011a; Ast *et al.*, 2009; Dunlap, 2009). Bacterial luminescence is an activity coded for by the *lux* genes, which in *Photobacterium* are joined with the *rib* genes to form a *lux-rib* operon (Lee *et al.*, 1994; Lin *et al.*, 2001; Ast *et al.*, 2007; Urbanczyk *et al.*, 2011a).

In addition to luminous members of *Vibrionaceae*, certain species of *Enterobacteriaceae* and *Shewanellaceae* are luminous (Jensen *et al.*, 1980; Forst *et al.*, 1997; Makemson *et al.*, 1997; Dunlap and Kita-Tsukamoto, 2006; Waterfield *et al.*, 2009). In most of these bacteria, the *lux* genes have been vertically inherited, whereas some species acquired these genes by horizontal transfer (Ast *et al.*, 2007; Kasai *et al.*, 2007; Urbanczyk *et al.*, 2008; 2011a). The incidence of *lux* gene horizontal transfer appears to be rare, however, and in contrast to widely held views (e.g. Ochman *et al.*, 2000; Gogarten *et al.*, 2002) apparently has not led to speciation of the recipient strain based on current data (Urbanczyk *et al.*, 2008).

The recent description of *Photobacterium aquimaris*, the described strains of which carry an apparently horizontally acquired *luxA* gene (Yoshizawa *et al.*, 2009), provides a possible exception to the view that horizontal transfer of the *lux* genes does not contribute to divergence of the recipient. Analysis of *P. aquimaris* housekeeping genes revealed this species to be closely related to *Photobacterium kishitanii* and *Photobacterium phosphoreum*, whereas the *luxA* gene apparently was acquired from a bacterium related to *Photobacterium mandapamensis* (Yoshizawa *et al.*, 2009). In this study, we examined the evolutionary relationships of different strains of *P. aquimaris* to test the possibility that the phylogenetic divergence (i.e. divergence of lineages inferred from phylogenetic analysis of gene sequences) of this species was influenced by horizontal acquisition of the *lux* genes.

Results and discussion

Two strains of *P. aquimaris*, both thought to carry a horizontally acquired *luxA* gene, have been described (Yoshizawa *et al.*, 2009). To attempt to identify additional strains of this new species for analysis of the evolutionary origin of its *luxA* gene, we surveyed a wide diversity of luminous strains for new *P. aquimaris* isolates. Two luminous strains, BS1 and BS2, isolated from the Black Sea and provisionally identified as *P. phosphoreum* based on hybridization analysis (Wimpee *et al.*, 1991), were

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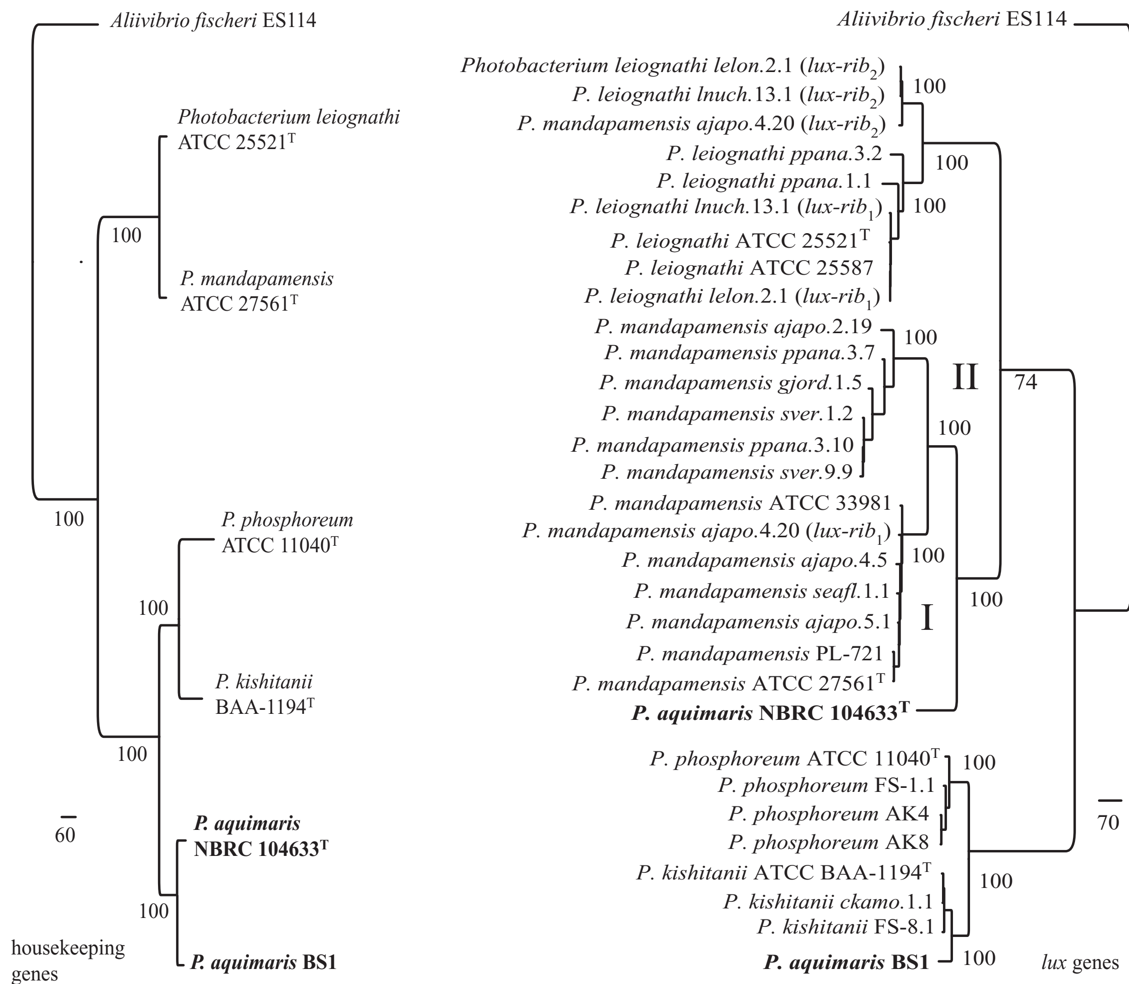

Fig. 1. Relationship between luminous *Vibrionaceae* based on housekeeping genes (left tree) and *luxABFE* genes sequences (right tree). Sequence data were analysed in PAUP* (Swofford, 2003) using the parsimony criterion. Jackknife support percentage values (after 1000 replicates) are shown at the nodes. The housekeeping genes were amplified as described in Ast and colleagues (2007), Urbanczyk and colleagues (2008) and Yoshizawa and colleagues (2009). Strains of *P. aquimaris* are shown in bold. For the housekeeping genes analysis, sequences of six genes, *gyrB*, *pyrH*, 16S, *ftsZ*, *mreB* and *topA* were concatenated and then aligned. The alignment had a total of 5362 characters (631 phylogenetically informative characters); the analysis resulted in a single most parsimonious tree. Analyses based on the individual genes were qualitatively similar to concatenations. Analyses of the concatenated housekeeping genes alignment were also carried out using neighbour-joining and maximum-likelihood algorithms, as done by Yoshizawa and colleagues (2009), and the results were congruent with the parsimony analysis (data not shown). For the *luxABFE* sequences analysis, only protein coding sequences were used. *luxF*, absent in *Photobacterium leiognathi* and *Aliivibrio fischeri*, was treated as missing data. The alignment had a total of 2495 characters (1106 phylogenetically informative characters), and the analysis resulted in two equally parsimonious trees. *Photobacterium aquimaris* strains are shown in bold. Roman numerals I and II refer to *P. mandapamensis* clades I and II respectively (Kaeding *et al*., 2007). Some jackknife support values were omitted for clarity. GenBank accession numbers for the sequences used in both analyses can be found in Table S1.

identified through analysis of housekeeping genes as likely members of *P. aquimaris* (data not shown), and one strain, BS1, was examined in greater detail here.

A multi-gene phylogenetic analysis based on housekeeping genes (16S rRNA gene, *gyrB*, *ftsZ*, *mreB*, *pyrH* and *topA*) revealed that BS1, like the type strain of *P. aquimaris*, NBRC 104633^T, is closely related to but distinct from *P. phosphoreum* and *P. kishitanii* (Fig. 1). However, analysis of the *lux-rib* operon genes (*luxABFE*) of these bacteria revealed a different relationship (Fig. 1). Whereas the *luxABFE* genes of NBRC 104633^T are phylogenetically affiliated with *P. mandapamensis*, as reported for *luxA* by Yoshizawa and colleagues (2009), the placement of the *luxABFE* genes of BS1 was concordant with the *P. aquimaris* housekeeping genes. We interpret this concordance as indicating that the *lux-rib* genes of BS1 were vertically inherited from the ancestor of *P. aquimaris*, which was luminous, whereas those of NBRC 104633^T were horizontally acquired from a bacterium apparently closely related to *P. mandapamensis*.

To gain further insight into this issue, we cloned and sequenced the *lux-rib* operons of BS1 and NBRC 104633^T,

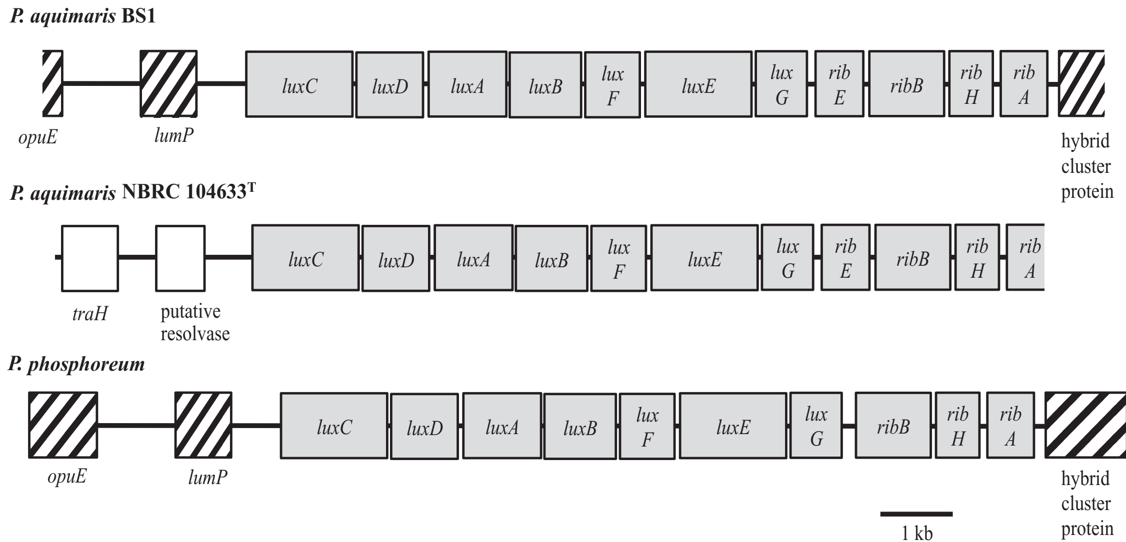

Fig. 2. Gene organization of the *lux-rib* operons of *P. aquimaris* BS1 (upper panel), NBRC 104633^T (middle panel) and *P. phosphoreum* (lower panel). The *P. phosphoreum lux-rib* operon gene organization was determined based on sequences of strains ATCC 11040^T (GenBank accession number DQ988873) and NBRC 13896 (AB104437 and AB065117). Genes shaded grey are homologous, and hashed rectangles indicate sequences homologous between BS1 and *P. phosphoreum*. Cloning and sequencing of the genomic DNA followed the procedure described by Ast and colleagues (2007), except that genomic DNA cloned into pWEB-TNC cosmid (Epicentre) was sheared and the resulting fragments were religated into the pUC118-HincII/BAP (Takara Biosciences) before sequencing.

including flanking DNA (Fig. 2). The genes upstream of the *lux-rib* operon of BS1 are homologous to a lumazine protein gene, *lumP*, and a proline transporter gene, *opuE*, which are also upstream of the *luxC* gene of *P. phosphoreum* (Kasai *et al*., 2007). Downstream of *ribA* in BS1 is a gene homologous to a hybrid cluster protein, which in *P. phosphoreum* is also located downstream of *ribA* (Kasai *et al*., 2007). This apparently conserved pattern of flanking genes suggests that the *lux-rib* operon is in the ancestral genomic position for these genes in the *P. phosphoreum*/*kishitanii*/*aquimaris* clade. This conclusion was supported by phylogenetic analysis of complete *lux-rib* operons from representative luminous *Photobacterium* strains, including sequences of *lux-rib* operons of BS1 and NBRC 104633^T (Fig. S1). In contrast, the genes upstream of the *lux-rib* operon of NBRC 10463^T are a putative resolvase and a plasmid transport protein (*traH*) (genes downstream of *ribA* were not recovered). The resolvase and *traH* genes have no homology to sequences found in *P. mandapamensis* strain *svers*.1.1 genome (Urbanczyk *et al*., 2011b), or sequences flanking luminescence genes in other *Vibrionaceae*, and they have no role in light production. Instead, they apparently function in horizontal transfer of genes via superintegrons or plasmids (Hazen *et al*., 2010). The presence of these two genes upstream of *luxC* in NBRC 104633^T is further support for horizontal acquisition of the *lux-rib* genes in NBRC 104633^T.

The results obtained here (Fig. 1) and previously (Yoshizawa *et al*., 2009) indicate that the source of the *lux-rib* genes of NBRC 104633^T was likely to be a bacterium closely related to *P. mandapamensis*. Two phylogenetically distinct clades, I and II, however, comprise this species (Wada *et al*., 2006; Kaeding *et al*., 2007), and certain strains of this species acquired a second *lux-rib* operon, *lux-rib*$_2$, by horizontal transfer (Ast *et al*., 2007; Urbanczyk *et al*., 2008). Therefore, the likely source of the NBRC 104633^T *lux-rib* genes is not obvious. To attempt to identify which of these lineages might have been the source of these genes, we carried out a detailed sequence analysis of the *luxABFE* genes in luminous members of *Vibrionaceae*, including the horizontally transferred *lux-rib*$_2$ operon of *P. mandapamensis ajapo*.4.20 (Fig. 1). The results confirm that the *lux-rib* operon of NBRC 104633^T originated in bacterium closely related to extant members of *P. mandapamensis*, but the donor bacterium apparently belongs to neither clade I nor clade II. Furthermore, the analysis revealed that the horizontal transfer of *lux-rib* genes of NBRC 104633^T was not a second example of the horizontal gene transfer (HGT) event that gave rise to the *lux-rib* merodiploidy of *P. mandapamensis ajapo*.4.20. These results indicate that the donor of the *lux-rib* operon of NBRC 104633^T could belong to a previously unrecognized clade of *P. mandapamensis* that either has gone extinct or has not yet been sampled. Alternatively, sequence divergence observed in the analysis shown in Fig. 1 is a result of rapid *lux-rib* operon evolution in recipient *P. aquimaris* since the HGT event.

Only a single *lux-rib* operon was found in NBRC 104633^T and BS1 in the amplification and sequencing work carried out here. Furthermore, attempts to PCR-

amplify *lux-rib* sequences from NBRC 104633^T genomic DNA with primers based on BS1 or from BS1 genomic DNA with primers based on NBRC 104633^T were unsuccessful (data not shown). We also attempted to amplify *lux-rib* genes of NBRC 104633^T and BS1 using specific primers designed for amplification of *P. phosphoreum*/*P. kishitanii* or *P. mandapamensis lux-rib* operons (Ast *et al*., 2007), and all amplicons had the same sequence as the cloned *lux-rib* operons of each strain (data not shown). We also designed universal primers that can amplify *luxA* sequence in NBRC 104633^T, BS1, as well as in *P. phosphoreum* and *P. mandapamensis*. The product of PCR amplification with the universal primers always resulted in a single sequence of *luxA* in all strains used. Cloning and analysis of seven random fragments of NBRC 104633^T *luxA* amplified using universal primers also resulted in the same sequence (data not shown). These observations and the results presented above indicate that the horizontally acquired *lux-rib* genes completely replaced the vertically inherited luminescence genes in NBRC 104633^T. Whether this acquisition preceded, coincided with or followed loss of the vertically inherited genes is unknown.

In order to better understand the loss of the vertically inherited *lux-rib* operon from the NBRC 104633^T, we attempted to amplify sequences flanking the *lux-rib* operon of BS1 using the NBRC 104633^T genomic DNA as a template. Only sequences located adjacent to *ribA* were amplified, but no sequences from the *luxC* side of the BS1 *lux-rib* operon were amplified (data not shown). Therefore, the mechanism under which the vertically inherited *lux-rib* operon of the NBRC 104633^T was lost remains unknown. In this regard, however, gene replacement by homologues horizontally acquired from a distantly related species can reduce fitness of the recipient (Lind *et al*., 2010), so loss of the vertically inherited *lux* genes might have preceded their horizontal acquisition. Furthermore, there are several instances of strains lacking *lux* genes in otherwise luminous species of *Vibrionaceae* (Kaeding *et al*., 2007; O'Grady and Wimpee, 2008; Wollenberg *et al*., 2012). Therefore, it is possible that along with NBRC 104633^T, both non-luminous strains of *P. aquimaris* lacking the *lux* genes and luminous strains of this species that carry the vertically inherited *lux* genes exist. Regardless of those issues, we note that here as well as in previously described instances (Urbanczyk *et al*., 2008), the horizontal acquisition of the *lux* genes has expanded the pan-genome of *P. aquimaris* but has not led to phylogenetic divergence of this species.

Conclusion

The results presented here provide the first observation of a complete replacement of vertically inherited luminescence genes by horizontally acquired homologues. Different strains of *P. aquimaris* contain homologues of the *lux-rib* genes that have a different evolutionary history. Strain BS1 contains the vertically inherited *lux-rib* operon, which presumably arose in the ancestor of this species, whereas the type strain NBRC 104633^T lacks the vertically inherited *lux-rib* operon and instead carries a complete and functional *lux-rib* operon acquired horizontally from a bacterium related to *P. mandapamensis*.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Relationship between luminous *Vibrionaceae* based on housekeeping genes (left tree) and *luxCDABFEG-ribEBHA* genes sequences (right tree). The housekeeping tree was repeated from the Fig. 1 for reference. For the *lux-rib* sequences analysis, only protein coding sequences were used. Sequence data were analysed in PAUP* (Swofford, 2003) using the parsimony criterion. Treated as missing data were: *luxF*, absent in *P. leiognathi* and *A. fischeri*; *ribE*, absent in *P. phosphoreum*; and *ribEBHA*, absent in *A. fischeri*. The *lux-rib* sequence alignment had a total of 9453 characters, of which 2539 were phylogenetically informative. The analysis resulted in a single, most parsimonious tree, and analyses based on the individual genes gave the same phylogenetic placement. The trees were visualized using FigTree v. 1.3.1. Analyses of the housekeeping genes were also carried out using neighbour-joining and maximum-likelihood algorithms, as done by Yoshizawa and colleagues (2009), and the results were congruent with the parsimony analysis (data not shown). GenBank accession numbers for the sequences used in both analyses can be found in Table S1.

Table S1. GenBank accession numbers for sequences used in phylogenetic analyses. For *A. fischeri* ES114 gene locus tags from whole genome sequencing project (CP000020) were used.

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