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Diversity and evolution of bacterial bioluminescence genes in the global ocean

Thomas Vannier [1,2,*], Pascal Hingamp[1,2], Floriane Turrel[1], Lisa Tanet[1], Magali Lescot [1,2,*] and Youri Timsit [1,2,*]

[1]Aix Marseille Univ, Université de Toulon, CNRS, IRD, MIO UM110, 13288 Marseille, France and [2]Research Federation for the study of Global Ocean Systems Ecology and Evolution, FR2022/Tara GOSEE, 3 rue Michel-Ange, 75016 Paris, France

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ABSTRACT

Although bioluminescent bacteria are the most abundant and widely distributed of all light-emitting organisms, the biological role and evolutionary history of bacterial luminescence are still shrouded in mystery. Bioluminescence has so far been observed in the genomes of three families of Gammaproteobacteria in the form of canonical *lux* operons that adopt the CDAB(F)E(G) gene order. *LuxA and luxB* encode the two subunits of bacterial luciferase responsible for light-emission. Our deep exploration of public marine environmental databases considerably expands this view by providing a catalog of new *lux* homolog sequences, including 401 previously unknown luciferase-related genes. It also reveals a broader diversity of the *lux* operon organization, which we observed in previously undescribed configurations such as CEDA, CAED and AxxCE. This expanded operon diversity provides clues for deciphering *lux* operon evolution and propagation within the bacterial domain. Leveraging quantitative tracking of marine bacterial genes afforded by planetary scale metagenomic sampling, our study also reveals that the novel *lux* genes and operons described herein are more abundant in the global ocean than the canonical CDAB(F)E(G) operon.

INTRODUCTION

Marine biodiversity and evolution are intimately related with biogeography and ecology (1–3). The *Tara* Oceans expedition recently provided a global picture of the complex interactions between marine micro-organisms and their environment (4–6). Bioluminescence, the chemical emission of visible light, is produced by a remarkable diversity of organisms and is particularly widespread in marine species (7–9). The luciferase enzymes that catalyze the emission of photons have evolved independently over 30 times, by convergence from non-luminescent enzymes (10,11). Although bioluminescent bacteria are the most abundant and widely distributed of all light-emitting organisms (7,12), certain functional and evolutionary aspects of bacterial luminescence still remain enigmatic, such as its biological role which remains a matter of debate (13). Early on bioluminescence was proposed to have evolved from ancient oxygen-detoxifying mechanisms (14–16). It has also been argued that stimulation of DNA repair through the activation of DNA photolyase may confer an advantage to luminous bacteria (17), although this hypothesis is still controversial (18). Yet another hypothesis is that bioluminescence is a visual attractant for zooplankton and fish that both provide ingested bacteria with growth medium and means for dispersal (19). Symbiosis with squid or fish is also an intriguing feature of specific bioluminescent bacteria (20,21).

To date, most of the few culturable light-emitting bacterial species that have been characterized fall within the Gammaproteobacteria class. These bacteria cluster phylogenetically in three families (*Vibrionaceae, Shewanellaceae* and *Enterobacteriaceae*) which all carry a highly conserved *lux* operon (12). Since its first identification 40 years ago, the canonical *luxCDAB(F)E(G)* organization has been systematically observed in all the bacterial bioluminescent genomes (22–24). The *luxA* and *luxB* genes encode the alpha and beta subunits of the luciferase heterodimer that emits light by the oxidation of $FMNH_2$ and a long chain aldehyde. Whereas they both adopt a TIM-barrel fold (25), LuxA specifically displays a disordered loop playing a critical role in light emission (26). LuxC, D and E together form a fatty acid reductase complex responsible for the synthesis of the long chain aldehyde substrate (24,27).

Despite a highly conserved core, some variations have been observed in the *lux* operon organization. Small differences in gene content, for instance the presence of an op-

*To whom correspondence should be addressed. Tel: +33 4 86 09 06 66; Email: magali.lescot@mio.osupytheas.fr
Correspondence may also be addressed to Thomas Vannier. Email: thomas.vannier@mio.osupytheas.fr
Correspondence may also be addressed to Youri Timsit. Email: youri.timsit@mio.osupytheas.fr

tional riboflavin genes or *luxF* in *Photobacterium* species, have been observed (28,29). LuxG, which reduces FMN into FMNH2 is absent in *Photorhabdus spp.* whose operon also contains multiple insertions of ERIC sequences (30). Natural merodiploidy of the *lux-rib* operon has been also noticed in some strains in *Photobacterium leiognathi* (31). While the phylogeny of *lux* genes generally supports a vertical inheritance, multiple examples of instability of the *lux* locus and horizontal gene transfers (HGT) have been reported in different clades, at various taxonomic levels (32–34). Also, mutations or loss of the *lux* operon are frequently observed in non-luminous strains and appear to correlate with some environmental parameters (35–38).

Although bioluminescent bacteria are cosmopolite in the oceans and occupy a great diversity of ecological niches, including surface and deep waters, (39–43), many studies have revealed an intricate relationship between bacterial bioluminescent phenotype, *lux* operon diversity, environmental parameters and life style (29,44–50). Given the apparent ubiquity of bioluminescence in the ocean and the ease with which light emitting bacteria can be isolated from seawater, it has come as a surprise that bacterial bioluminescence has so far escaped detection by previous marine metagenomic studies (51). According to the pioneering authors, the unexpected absence of *lux* genes might have been explained by sampling protocols which filtered out size classes of potential interest, and to sequencing depth which might have been insufficient to catch bioluminescent bacteria if these were of low abundance (51).

In the present report, we surveyed the distribution of bacterial *lux*–related genes in a compilation of publicly available large-scale metagenomic environmental databases (*Tara* Oceans 2009–2013, Malaspina 2010, GOS and OSD2014) giving special care to screen the largest possible variety of organismal size sampling fractions. Spanning a wide spectrum of marine bacteria diversity, including a majority of unculturable species, our study reveals new insights about distribution, diversity and evolution of marine *lux*-related genes and their operon organization at a planetary scale.

MATERIALS AND METHODS

LuxA reference sequence dataset

The coordinates of the *Vibrio harveyi* LuxA/B heterodimeric luciferase (pdbid: 3fgc) were obtained from the Protein Data Bank (PDB) (52) (https://www.rcsb.org/, version 06/22/2019) and used as a reference luciferase structure. The corresponding *V. harveyi* LuxA protein sequence (UniProtKB—P07740) was used to query UniProtKB/Swiss-Prot (53,54) (https://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/swissprot.gz, version 06/14/2019) and Refseq (55) (ftp://ftp.ncbi.nih.gov/refseq/release/release-catalog/RefSeq-release93.catalog.gz) using blastp (56). Ten protein sequences with an *E*-value inferior to 1.0E-128 (threshold above which LuxB proteins are detected) were considered reliable LuxA homologs and formed the seed for the reference LuxA dataset. The ten seed reference LuxA sequences were then aligned with MAFFT with default parameters (57) (http://mafft.cbrc.jp/alignment/software/) and a hidden Markov model (HMM) profile was built using hmmbuild from HMMer v 3.0 with default parameters (58) (http://hmmer.org). The resulting LuxA HMM profile was used to search for additional luciferase homologs using hmmsearch in NR (59) (https://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz, version 06/15/2019) with an E-value threshold of 1.0E-186 set to avoid LuxB homologs. In addition, 55 draft whole genome shotgun (WSG) marine bacterial genomes (https://www.ncbi.nlm.nih.gov/genbank/wgs/, version 07/25/2018) and 334 marine bacterial complete genomes (https://www.ncbi.nlm.nih.gov/genbank/genome/, version 07/25/2018) were screened using the LuxA HMM profile; after removing sequence redundancy, partial and synthetic sequences, we obtained a reference dataset of 129 LuxA protein sequences. This dataset was then used to compute a final LuxA HMM profile (Supplementary Table S1). A similar procedure was used for building the Lux B, C, D, E, G and F reference datasets.

Diversity of *lux* operon

Genbank bacterial genomes containing the reference and marine *lux*-like sequences are download from the NCBI web site (https://www.ncbi.nlm.nih.gov/genbank/). A syntheny graph representing the operon structural organizations was done using Easyfig (60).

OM-RGC Lux homologs search

The *Tara* Oceans OM-RGC dataset (5,61) was screened with each of the Lux HMM profiles obtained above, using hmmsearch with an *E*-value threshold of 1.0E-10 (Supplementary Table S1). Further filtering based on alignment lengths eliminated incomplete Lux sequences. The length thresholds were set to 340, 300, 430, 275, 300 and 200 aa for the LuxA, B, C, D, E and F homologs, respectively.

OM-RGC LuxA homolog structural filtering

Bacterial luciferases and monooxygenases share a highly conserved TIM-barrel fold (25) that renders discrimination from each other difficult from primary sequence alignments alone. We therefore developed a specific procedure to help luciferase/monooxygenase discrimination based on three-dimensional (3D) structure modeling and comparison. The 3D coordinates of close structural homologs of bacterial luciferases were retrieved from PDB (52) and structurally superimposed with PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.) (Supplementary Table S2 and Figure S2a–i). This analysis selected a set of amino acids previously described to be specific of the luciferase active site (62). In a first filtration step, truncated sequences and sequences lacking the catalytic His44 were excluded from our selection. Moreover, a disordered loop from 262–290 (*V. harveyi* LuxA coordinates) that covers the active site is a specific signature of LuxA proteins (63). Thus, we used Disopred v3.1 (64) to systematically calculate the disorder content in all the aligned LuxA homologs. We then filtered out sequences displaying a disorder score below 0.2 in this region (defined by the multiple sequence alignment,

see below) from our final LuxA homolog dataset (Supplementary Table S3).

Phylogenetic analyses of Lux-related sequences

Multiple sequence alignments (MSA) for phylogenetic analyses were obtained with Clustal Omega (65) using default settings. In order to generate a phylogenetic tree that integrates LuxA-related, LuxB and selected monooxygenases (Supplementary Table S2), we first aligned each of these three sequence groups independently, and then processed each of the three MSAs with MaxAlign (66) to remove sequences with excess INDELS that reduce alignment area. TrimAl v1.2 with default setting (67) was then used for automated alignment trimming (removal of poorly aligned positions in the MSAs). The phylogenetic analysis was performed with RAxML (68) (parameters: -f a -# 10000 -m PROTGAMMAAUTO -p 12345 -x 12345). Trees were midpoint rooted and visualized with FigTree (https://github.com/rambaut/figtree/) and Evolview v2 (69). Alignments were visualized and inspected with Jalview (70). A similar procedure was used for the phylogenetic analyses of the other Lux proteins.

Abundance of *lux* genes in the global ocean

We computed the abundance of the canonical and non-canonical *lux* operon genes (Supplementary Table S4 and File S2) by metagenomic read recruitment from large scale marine metagenomic data: *Tara* Oceans 2009–2013 (71–73) (PRJEB402, https://www.ncbi.nlm.nih.gov/; doi:10.1594/PANGAEA.859953, https://doi.pangaea.de/) Malaspina 2010 (74) (Gs0053074, https://img.jgi.doe.gov/), GOS (75,76) (PRJEB8968, https://www.ncbi.nlm.nih.gov/) and OSD 2014 (77) (PRJEB5129, https://www.ncbi.nlm.nih.gov/) (Supplementary Table S5). This procedure, which searches individual reads that align to a query sequence in each of the available raw unassembled metagenomes, is expected to be more sensitive than searching for homologs in the assembled metagenomes (e.g. OM-RGC). Indeed, the raw read mining procedure has the potential to detect even singleton homologous reads, whereas such singletons often don't make it past the assembly stage of reduced datasets such as the OM-RGC. Metagenomic read counts per gene ($RPKM_{MG}$) correspond to the number of mapped reads per query gene divided by the total number of reads sequenced for each sample (in millions) divided by gene length (in kilobases). The reported relative abundance values correspond to: $\frac{\log(1+(RPKM*10^9))}{\log(2)}$. Metagenomic short reads were recruited with Bowtie2 2.1.0 aligner with default parameters (78). We discarded alignments below 80% identity and below 50% of query gene horizontal coverage.

Geographic distribution of specific bacterial genomes

We analyzed the global ocean distribution of *Vibrio Campbellii* ATCC BAA-1116 (NCBI:txid338187) and *Alcanivorax bacterium* GenoA1_TS13_700 (NCBI:txid2072747, Pacific ocean: South China 700m, 19.909 N, 115.243 E) genomes by metagenomic read recruitment from the same marine metagenomes as described above (*Tara* Oceans, Malaspina, GOS and OSD). For each genome, we computed relative genomic abundances as the number of reads mapped onto the genome normalized by the total number of reads sequenced for each sample (Supplementary Table S6) (79). We identified and removed from the read recruitment counts 267 and 528 outlier genes on *V. Campbellii* and *Alcanivorax spp.* respectively, to reduce apparent biases due to atypical genomic regions with low sequence specificity. We generated the world maps with R version 3.2.3 using the packages maps_3.1.1, mapproj_1.2-4 and mapplots_1.5. The 'free-living' organismal size fractions were defined as the 0.22–1.6 μm and 0.22–3 μm filters of *Tara* Oceans, and the 0.2–0.8 μm filters of Malaspina. The 'associated' organismal size fraction was defined as the 0.8–2000 μm filters of *Tara* Oceans, and the 0.8–20 μm filters of Malaspina. Box plots and Mann–Whitney U-test were performed with R.

Biogeography and taxonomic distribution of the OM-RGC *luxA* homologs

We used the Ocean Gene Atlas web service (80) (http://tara-oceans.mio.osupytheas.fr/) to describe the geographic and taxonomic distributions of the 3 groups of OM-RGC LuxA homologs (OLAHs). Abundance and location were visualized using the *Tara* Oceans metagenomic samples corresponding to size fractions 0.22–1.6 and 0.22–3 μm sampled either at surface or mesopelagic depths.

RESULTS

Diversity of *lux* gene homologs

In order to survey *luxA*, *B*, *C*, *D* and *E* homologs in the global ocean, we first inventoried all Lux sequences deposited in public reference databases (NR, RefSeq, UniProtKB/Swiss-Prot) to produce a set of reference *lux* gene sequences (Supplementary Table S1). As expected for genes forming an operon, a similar number of occurrences of each *lux* genes was found in public reference databases. Surprisingly, this search also revealed a set of *lux* homologs in the reference genomes of six bacteria that had not previously been described as bioluminescent: *Alcanivorax spp., Rhizobacter spp.* (Gammaproteobacteria)*; Enhygromyxa salina, Plesiocystis pacifica* (Deltaproteobacteria)*; Leptospira santarosai* (Spirochaetes)*; Actinomyces spp.* (Actinobacteria) (Supplementary Table S1). These six bacterial reference genomes possess *luxA*, *C*, *D* and *E* of the *lux* operon, with the notable exception of *luxB*. This thus revealed the presence of *lux*-like operons in three bacterial classes never described as capable of producing light: Deltaproteobacteria, Spirochaetes and Actinobacteria. Genes in these *luxB*-less operons will henceforth be referred to as *lux*-like genes.

We then used this set of reference sequences to search for *lux* homologs in the most recent microbial gene catalog of *Tara* Oceans (OM-RGC), which represents assembled genetic material recovered from the free-living mostly bacterial size fractions (0.2–3 μm) collected in the global ocean (Supplementary File S1). Consistent with the current knowledge of bacterial bioluminescence distribution, we detected the presence in the OM-RGC of *lux* genes from

several well-known bioluminescent bacteria such as *Vibrio campbeli, Vibrio cholerae, Aliivibrio fischeri, Shewanella woodyi* and *P. leiognathi* (Figure 1 and Supplementary Table S1).

An initial search for LuxA proteins provided a set of 800 marine candidates having both high similarity score and size close to our reference LuxA sequences (Supplementary Figure S1). Further filtering according to the presence of a correctly positioned structurally disordered region as well as the presence of specific active site residues generated a set of 401 OM-RGC LuxA homologs (OLAHs) (Supplementary Table S3).

By combining LuxA, LuxA-like and LuxB reference sequences together with the 401 marine OLAHs and some selected monooxygenases of known structure, we inferred a phylogenetic tree of LuxA homologs (Figure 1). Phylogeny and disorder content of LuxA sequences are highly congruent (Supplementary Figure S3) as indicated by the clustering of *bona fide* reference LuxA sequences that display a similar disordered loop. The LuxA reference sequences are grouped together in a monophyletic branch well separated from the the LuxB monophyletic branch, both of which are then connected to a group of homologs we annotated as LuxA-like sequences. The bulk of the marine homologs then cluster into three branches, forming OLAH groups 1–3 (Figure 1). However, OLAH groups 1 and 2, as well as LuxA-like sequences all share a similar consensus in the catalytic site with LuxA reference sequences (Supplementary Figure S4). The monooxygenases and OLAH group 3 appear to form a separate branch, coherent with a distinct active site logo signature (Supplementary Figure S4). Interestingly, the few LuxA-like sequences belonging to bacteria that have not been previously described as luminescent organisms form a separate but close branch to the LuxA reference group (bootstrap value of 100). We should note that the LuxA sequences of *Alcanivorax* spp. and *P. pacifica* found in the OM-RGC dataset are grouped with the LuxA-like branch (Figure 1). Two monooxygenase sequences (pdbid: 3i7g and 4uwm) are found within OLAH groups 2 and 3, respectively (Figure 1 and Supplementary Table S2). Phylogenetic analysis of marine LuxB, C, D, E homologs similarly show distinct groups consisting of Lux references, Lux-like and OM-RGC distant relatives (Supplementary Figure S5).

Diversity of *lux* operons

Reference and marine *lux*-like sequences differed not only in their phylogeny, but also in their operon organization. Indeed, we observed operon structural organizations very different from the canonical CDAB(F)E(G) order (Figure 2 and Supplementary Figure S6). In particular, our study revealed a novel CEDA order in which *luxB* is missing, in both Gammaproteobacteria and Deltaproteobacteria. Moreover, the *Leptospira* spp. that belongs to the more distant *Spirochaete* class encodes a CAED operon, while the *Actinomyces* AxxCE *lux*-like operon structure is even more atypical. Importantly, we observed a congruence between the operon organization and the phylogenetic distance in the LuxA sequence tree (Figure 2). Thus, our findings extend the known diversity of the *lux* operon organizations and reveal the existence of non-canonical *lux*-like operons in new taxons.

The similarities between homologous genes in the different operons have been also analyzed (Figure 2 and Supplementary Figure S7). As expected, each *lux* gene is highly similar to its relative in close taxons. Interestingly, the genomes of the two distinct myxobacteria genera *Enhygromyxa* and *Pleisiocistis* (81,82), exhibit full *lux* operons more than 90% identical. High similarities are also found between the *Alcanivorax* and *Rhizobacter lux* like-operons (61.1%). These findings suggest a lateral transfer of the CEDA *lux*-like operon between these species.

Abundance and distribution of *lux*-related genes in the global ocean

In order to investigate the distribution of the *lux-related* genes (in canonical or non-canonical operons), all reference and OLAH sequences were used as queries to recruit raw metagenomic reads from public large scale marine metagenomes (*Tara* Oceans, Malaspina, GOS and OSD) (Supplementary Table S5; see materials and methods). In addition, to differentiate *lux* genes likely harboured in free living bacteria from those likely associated with particles or larger organisms (e.g. through symbiosis), we analyzed their relative abundances in samples obtained by filtering through filters of pore sizes of either 0.22–3 μm (free-living) or 0.8–2000 μm (associated). The abundance of *lux* genes are summarized in Figure 3 and detailed in Supplementary Table S7. We have also estimated the abundance of the canonical *lux* operons of all the well-known bioluminescent bacteria (Figure 2). Rather surprisingly, the *lux*-operon of *V. campbelii* was the only detectable known bioluminescent bacterium, which furthermore was detected in *Tara* Oceans samples only, thus suggesting its higher abundance relative to other bioluminescent bacteria. Reference *lux* genes were not observed in either free-living or associated size fractions collected during the Malaspina expedition (which systematically sampled bathypelagic layers), nor the GOS expedition, nor on OSD2014 solstice day (mostly coastal waters). An important finding was that the reference *V. campbelii luxA* gene was preferentially detected in associated size fractions (i.e. non free-living) collected in just five *Tara* Oceans coastal and surface samples (TARA005, TARA007, TARA008 in the Mediterranean sea, and TARA123, TARA125 close to the Marquise islands) (Supplementary Table S7). This shows that the *V. Campbellii* canonical *lux* operons are mainly associated with particles or larger planktonic species. In contrast, Figure 3 shows that the genes of the *Alcanivorax* non-canonical CEDA operon are abundantly distributed in both *Tara* Oceans and Malaspina samples, in all size fractions (from 0.2 to 2000 μm), both in surface and deep ocean depths.

In order to further investigate these intriguing findings, we extended this *lux* only metagenomic read recruitment analysis to the distribution of *Vibrio spp.* and *Alcanivorax spp.* full length genomes in the global ocean. Figure 4 summarizes their geographic, water column and free-living/associated distributions (quantitative data are reported in Supplementary Table S6). *Vibrio* genomes are widely distributed in temperate regions, in surface, meso-

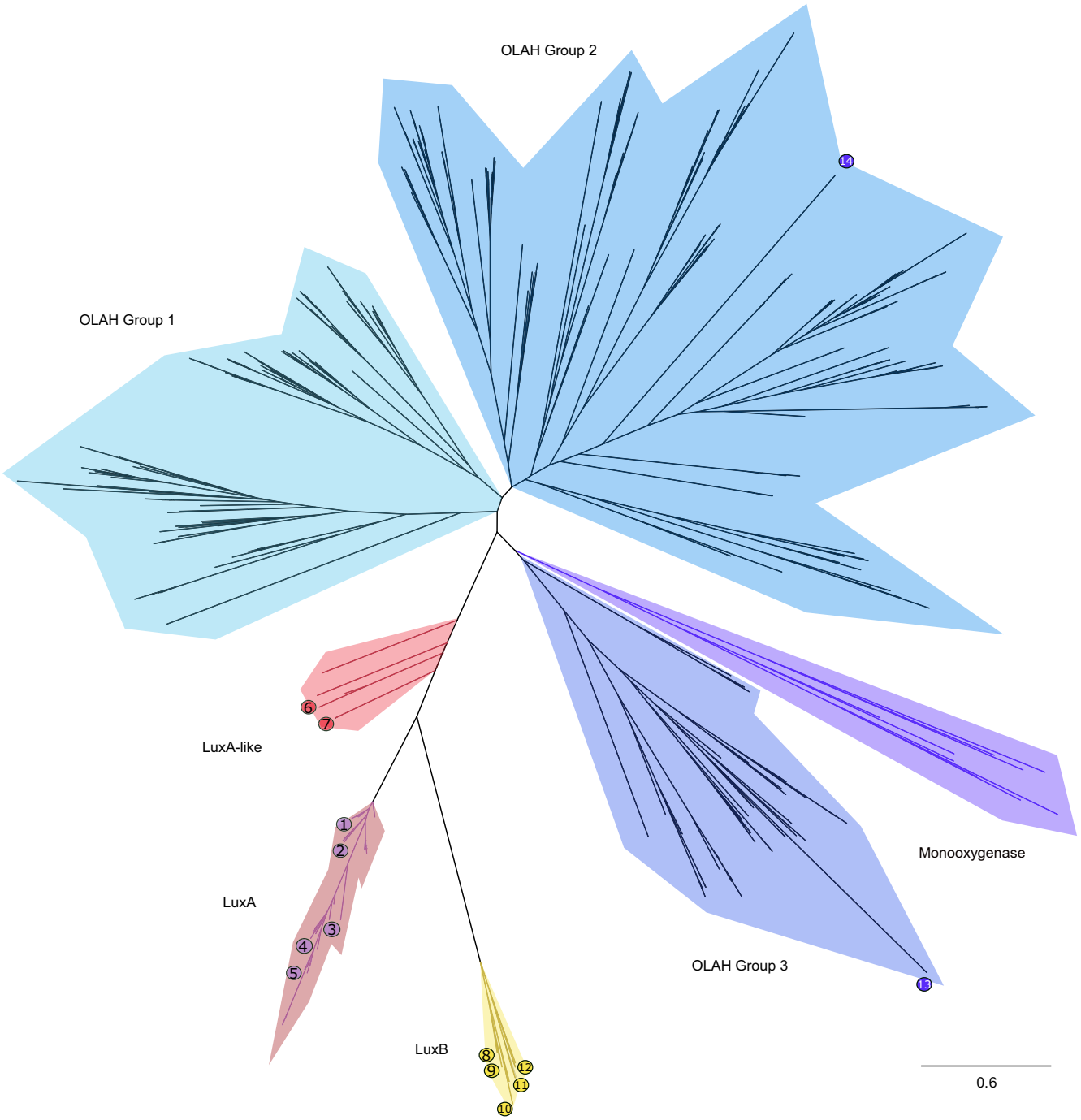

Figure 1. Phylogenetic tree of LuxA and related sequences found in the global ocean. Sequences are annotated as follows: LuxA in brown; LuxB in yellow; LuxA-like in red; OM-RGC LuxA Homologs (OLAH) group 1 (128 sequences), 2 (209 sequences) and 3 (63 sequences) from light to dark blue. Reference monooxygenases clustered together in a separate branch are colored purple; the two purple circles correspond to PDB monooxygenase sequences, 4uwm (13) and 2i7g (14) that branch in OLAH groups 3 and 2, respectively. The pink circles correspond to the OM-RGC assigned as LuxA reference sequences of *Vibrio campbellii* (1), *Vibrio cholerae* (2), *Aliivibrio fischeri* (3), *Shewanella woodyi* (4) and *Photobacterium leiognathi* (5).The red circles correspond to the sequences we annotate as LuxA-like of *Plesiocystis* pacifica(6) and *Alcanivoracaceae* bacterium (7).The yellow circles correspond to the OM-RGC assigned as LuxB reference sequences of *S. woodyi* (8), *A. fischeri* (9), *P. leiognathi* (10), *V. campbellii* (11), *V. cholerae* (12). The origin of the sequences used for tree inference are presented in Supplementary Table S3, and OLAH amino acid sequences are provided in fasta format (Supplementary File S1).

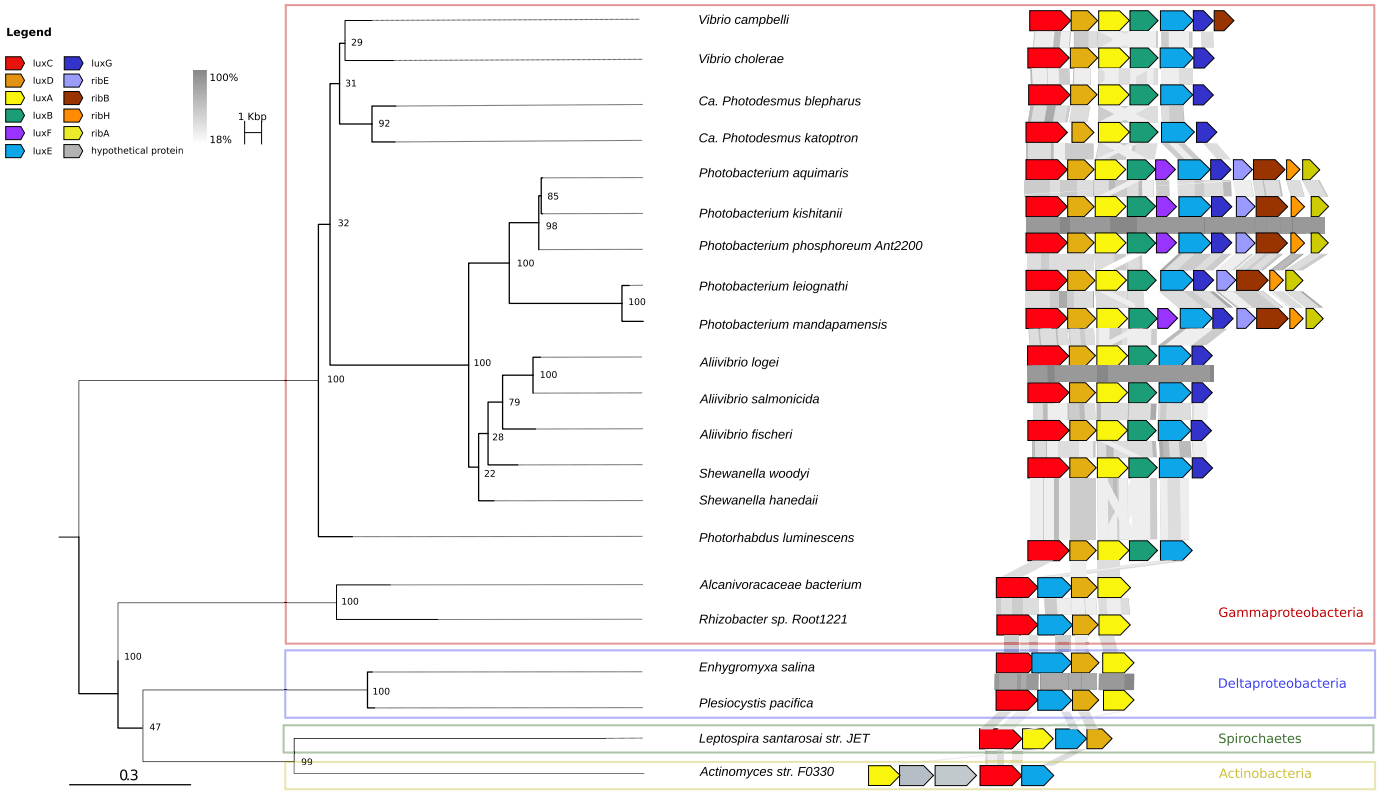

Figure 2. Diversity of lux operons compared to LuxA phylogeny. Bootstrap values are represented for each node of the tree. Percentage similarity between *lux* operon gene products are represented by a color gradient from light gray (low similarity) to dark gray (high similarity). Absence of color indicates no similarity. Colored frames show different groups of bacteria: Gammaproteobacteria in red, Deltaproteobacteria in blue, *Spirochaetes* in green and *Actinobacteria* in yellow. *Rhizobacter* is considered here as Gammaproteobacteria according to the recent standardized bacterial taxonomy (90). Nucleotide sequences of *lux* operon genes are provided in fasta format (Supplementary File S2).

and bathypelagic depths and are not observed in polar-regions (Figure 4A). In contrast to the preference of individual *lux* genes for associated fractions (Figure 3), *Vibrio* full genomes were detected in both free-living and associated fractions, albeit significantly more abundant in the free-living bacterial fractions than in the associated fractions (Mann–Whitney U-test $P < 0.0002$) (Figure 4B). Consequently, our study suggests that free-living *Vibrios* tend to be devoid of the *lux* operon.

Alcanivorax genomes showed a very different distribution (Figure 4C): they were highly abundant (more than 10 times the abundance of *vibrio* genomes), and except for one station (West of the North Atlantic gyre), they were exclusively localized in tropical and sub-tropical zones of the Pacific Ocean. They were present in a wide range of depths from surface to bathypelagic layers. Similarly to *Vibrio*, *Alcanivorax* genomes were present in both the free-living and the associated-size fractions (Figure 4D), which, contrary to observations with *Vibrios*, was consistent with the distribution of the *Alcanivorax lux*-like operon (Figure 3).

To gain further insight in the distribution of sequences corresponding to OLAH group 1 (Figures 5A and B) and OLAH groups 2–3 (Supplementary Figures S6 and 7) of the LuxA phylogenetic tree (Figure 1 and Supplementary Figure S3), we also analyzed their ocean distributions in the *Tara* Oceans samples. Group 1 sequences showed a particularly interesting distribution along the water column. Although they were observed in all oceans and latitudes, they were systematically much more abundant in mesopelagic (about 200–1000 m) than in surface depths (Figure 5C). These sequences clustered into three known groups of bacteria: *Entotheonella* spp., *Actinobacteria* and *Proteobacteria* (Figure 5D). Interestingly, the differences in abundances between surface and mesopelagic depths gradually decreased from group 1 to group 3 (Figure 5C; Supplementary Figures S8c and 9c) and a more diversified taxonomic distribution was also observed in group 3 (Figure 5D; Supplementary Figures S7d and 8d).

DISCUSSION

Many studies have shown that bacterial luminescence is related to environmental parameters and/or life-style, such as symbiotic associations (35–38). Bacterial bioluminescence has so far been observed as a canonical *lux* operon that follows the CDAB(F)E(G) gene order, in genomes of gram-negative bacteria that group phylogenetically in three families of Gammaproteobacteria: the *Vibrionaceae*, *Enterobacteriaceae* and *Shewanellaceae* (12). However, minor variations around this common theme have been observed in various species (12,28–31). Our deep exploration of public genomic and metagenomic marine databases expands the *lux* operon catalog (Figure 2). It also reveals a larger distribution of *lux* operons among bacterial taxons than previously

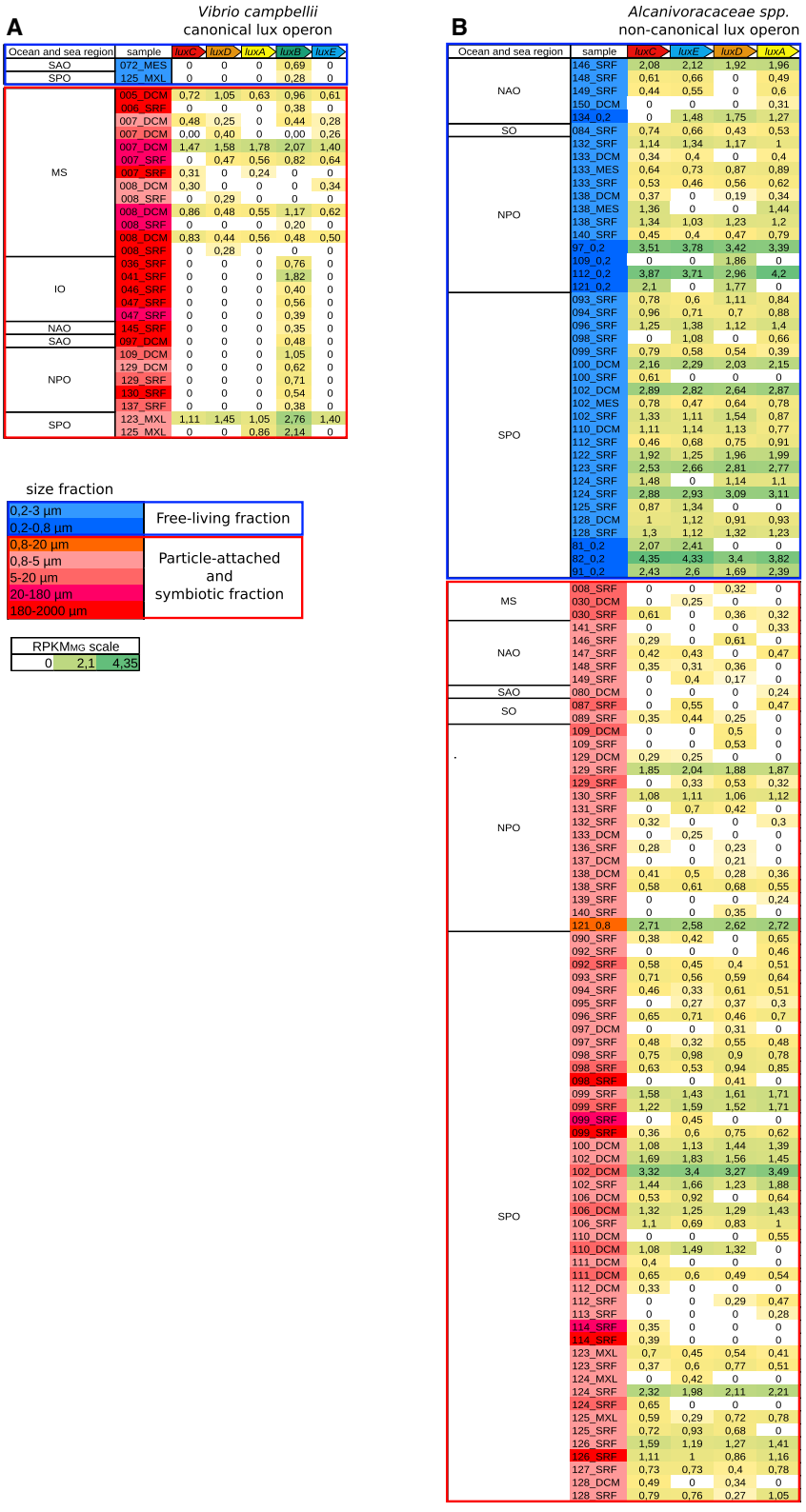

Figure 3. Abundance of *lux* genes in the global ocean. $RPKM_{MG}$ values of canonical and non-canonical lux genes are listed when at least one gene of the operon has significant matches with reads of the sample (Supplementary Table S7). (**A**) $RPKM_{MG}$ of the *Vibrio campbellii* canonical lux operon. (**B**) $RPKM_{MG}$ of the *Alcanivoracaceae bacterium* non-canonical *lux*-like operon. $RPKM_{MG}$ cells are color coded with increasing abundace (white, yellow, green). Size fractions are indicated with blue squares for free-living fractions and red squares for particle-attached and symbiotic fractions. Samples are organized geographically: South Atlantic Ocean (SAO), South Pacific Ocean (SPO), Mediterranean Sea (MS), Indian Ocean (IO), North Atlantic Ocean (NAO) and Southern Ocean (SO). Nucleotide sequences of *lux* operon genes are provided in fasta format (Supplementary File S2). Samples used for the metagenomic read recruitment are listed in Supplementary Table S5.

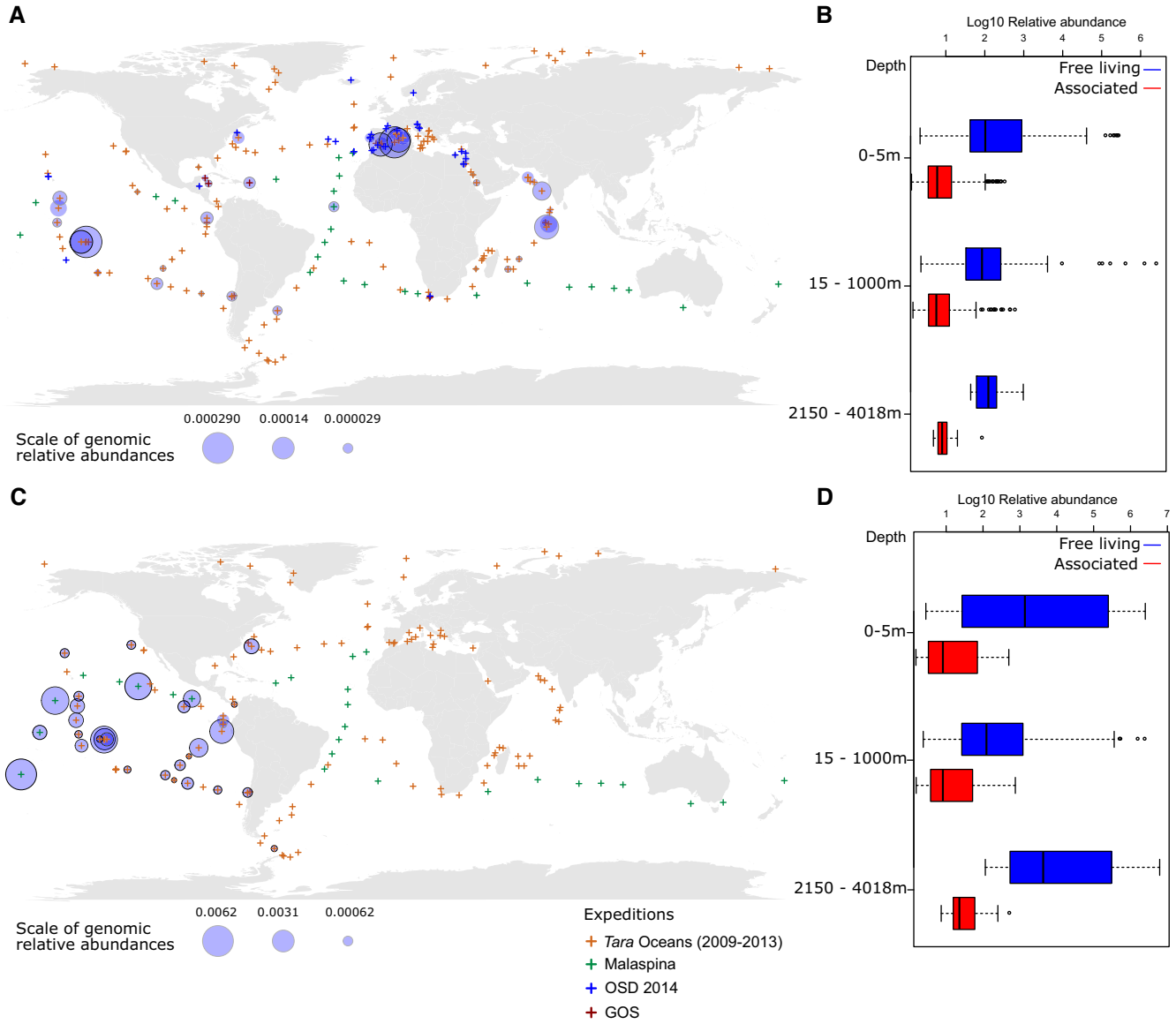

Figure 4. Geographic, water column and free-living or associated distribution of *Vibrio spp.* and *Alcanivoracacae* genomes. Geographic distribution of (**A**) *Vibrio spp.* and (**C**) *Alcanivoracacae* in *Tara* Oceans 2009–2013 (orange crosses), Malaspina (green crosses), OSD 2014 (blue crosses) and GOS (brown crosses) sampling stations. The size of the circles indicates the relative genomic abundances of these genomes measured combining samples at different depths and size fractions available at the station (Supplementary Table S6). Stations with at least two distinct lux genes are circled in black. Boxplots showing the Log10 of the relative abundance of (**B**) *Vibrio spp.* and (**D**) *Alcanivoracacae* at different depths. Blue boxes represents free living size fractions and red boxes the associated size fractions. Genomes and samples used for metagenomic read recruitment are listed in Supplementary Tables S4 and 5.

known, as well as a highly variable *lux* operon organization repertoire (Figure 2). We describe the existence of a new *lux*-like CEDA operon, lacking the *lux*B gene, in both Gammaproteobacteria and Deltaproteobacteria (83).

In Gammaproteobacteria, the CEDA *lux*-like operon configuration is observed in *Pseudomoniales* (*Rhizobacter*) and *Oceanospirales* (*Alcanivorax*) (84). Since the canonical CDAB(F)E(G) form of the *lux* operon is highly conserved in three Gammaproteobacteria families (7,12), it is somewhat surprising to find a distinct *lux* gene order among the Gammaproteobacteria. Remarkably, the finding of two different *lux* operon organizations within the Gammaproteobacteria appears to be congruent with the phylogeny of this clade. Indeed, according to Gammaproteobacteria species phylogeny (83), the CDAB(F)E(G) and CEDA *lux*-like operon configuration correspond to the divergence of two distinct groups, the PO (Pseudomonadales and Oceanospirillales) and VAAP/Entero (Vibrionales, Alteromonadales, Aeromonadales and Pasteurellales/Enterobacterials), respectively (Figure 2). Despite an absence of homology around the operon in the genomes of the two Gammaproteobacteria families harbouring the CEDA *lux-like* operon, the *lux*-like genes display a high similarity, suggesting a lateral transfer of the *lux* operon between the two remote *Rhizobacter* and *Alcanivorax* genera (Supplementary Figure S7). Such

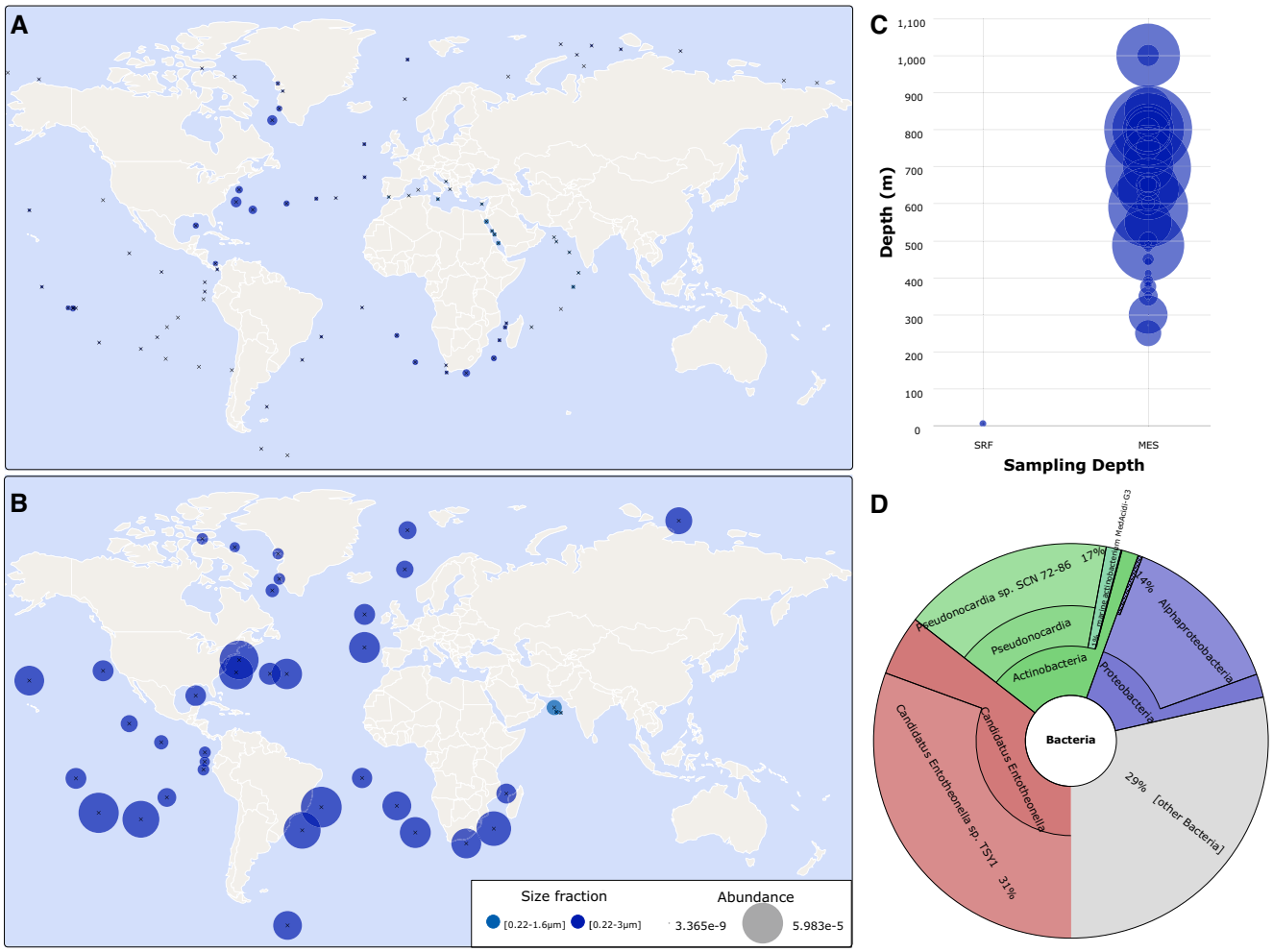

Figure 5. The OM-RGC LuxA homolog (OLAH) group 1 sequences are observed in all oceans and are more abundant in the mesopelagic layer. Geographic distribution and abundance of OLAH group 1 sequences in the *Tara* Oceans samples for the 0.22–1.6 μm and 0.22–3 μm size fractions at (**A**) surface and (**B**) mesopelagic depths. Gray crosses indicates *Tara* Oceans sampling stations. No mesopelagic sampling was done in the Mediterranean sea and in the Indian Ocean. (**C**) Abundance according to sampling depths. The size of the circles indicate the relative genomic abundances of the OLAH group 1 sequences. (**D**) Pie chart depicting percent abundance of OLAH group 1 sequences according to taxonomic classification. The list of OLAH group 1 identifiers used to retrieve biogeography from the Ocean Gene Atlas web service (http://tara-oceans.mio.osupytheas.fr/) are provided in Supplementary Table S3.

lateral transfer might have occured in rhizosphere microbiomes shared by these two species (85). In addition to marine environments (84) *alcanivorax spp.* has been indeed observed in coastal areas where it thrives in oil-polluted soils and rhizospheres (85).

In Deltaproteobacteria, we have also found the non-canonical CEDA *lux* operon form in two *myxoccocale* marine bacteria (86), *Enhygromyxa salina* (81) and *P. pacifica* (82). The close homology between the CEDA operon of these two distant species that share the same ecological niche strongly supports a lateral transfer origin (81,82) (Supplementary Figure S7). Remarkably, two distant bacterial classes share the CEDA configuration: the Gammaproteobacteria and the Deltaproteobacteria. Our findings cannot yet distinguish between an HGT or/and a vertical inheritance of this operon configuration from a common ancestor of these two classes. In the latter case, the CEDA configuration would correspond to an ancestral *lux* operon that predated the *luxA*/*luxB* duplication, congruent with the LuxA inferred phylogenetic tree. On the another hand, an HGT scenario is not uncommon between distant taxons when two species share a common niche (87).

We also show that *Leptospira* spp. belonging to a bacterial taxon phylogenetically distant from proteobacteria, the *Spirochaetes,* also carries an even more distinctive *lux* operon configuration: the CAED form. *Leptospira* genera occupy a great variety of habitats (88) and display highly plastic genomes consistent with frequent HGT events (89). A yet more distant form AxxCE is observed in *Actinomyces,* a gram-positive *Actinobacteria* (90). Interestingly, it has been reported that some *Mycobacteria* spp. contain a locus, *mel2*, very similar to the *lux* operon (91–93). Strikingly, the *mel2* locus organization follows a similar AxxCE order as found here in *Actinomyces*. Consistent with the absence of the *luxB* gene from the *Actinobacter lux* operon, the analysis of the relatedness of *MelF* to *luxA* and *luxB* placed *MelF* on an independent branch equally related to both of them (91). Our results suggest that the non-canonical *lux*-

like operon of gram-positive bacteria may correspond to an ancestral form and support the hypothesis of Kasai *et al*. suggesting that the *lux* operon originated in gram-positive bacteria (32).

In order to better understand the link between the genetic diversity, evolution and the ecology of the *lux* operon, we have searched independently each *lux* genes in the global ocean and collected information about their geographic distribution, their localization in the water column and their presence in either free-living or associated size fractions. Two distinct scanning procedures of the metagenomic data provided complementary results.

First, we investigated the presence of *lux* genes in the assembled metagenomic data (OM-RGC) by protein homology search (Figure 1), thus providing a qualitative view of the presence of *lux* genes in the global ocean. Previous analyses did not recover *lux* sequences from assembled metagenomic DNA of planktonic marine microorganisms (51), thus suggesting that luminous *Photobacterium, Aliivibrio* and *Vibrio* were present at abundances so low they went undetected with afforded sequencing efforts (29). In the present study however, the higher sequencing deepness detected several well-known bioluminescent bacteria such as *V. campbellii, V. cholera, A. fischeri, S. woodyi* and *P. leiognathi*. We infer that the other well-known marine bioluminescent bacteria that are not detected here are either not represented in the *Tara* Oceans sampling locations, or in abundances too low to allow their assembly. Interestingly, the OM-RGC assembly does contain *lux*-like genes belonging to the non-canonical CEDA *lux*-like operon of *Alcanivorax spp.* (84) and *P. pacifica* (82), two species that have been recently characterized as marine bacteria.

Second, we quantitatively explored the biogeographic abundances of known reference *lux* operons by metagenomic read recruitment, a method that provides a measure of geographic abundance of *lux* operons in the global ocean. Only *V. campbellii* and *Alcanivorax* spp. *lux* genes were detected, indicating either that they have a higher abundance than other known bacteria containing *lux* or the *lux*-like genes, or that the niche habitats of other bioluminescent bacteria were not sampled by *Tara* Oceans, Malaspina, GOS and OSD. The comparison of their full genome distributions in the global ocean shows contrasting stability of the *lux* operon in *V. campbellii* compared to *Alcanivorax spp.*. The *Vibrios* appear to contain an accessory *lux* operon preferentially found in symbiotic or particle associated strains localized in coastal environments (Figures 3 and 4). This is consistent with previous studies showing that non-luminescent *Vibrios* lacking the *lux* operon are free-living (35,36,38). In contrast, most of the detected *Alcanivorax* strains stably possess the CEDA-*lux* operon in their genomes and display a homogenous distribution restricted to the Pacific Ocean. *Alcanivorax* spp. are ∼10× time more abundant than *V. campbellli*, and are slightly more frequently observed in free living fractions (Figures 3 and 4). The two distinct patterns of bacterial/*lux* operon relationships suggest that the different *lux* operon configuration may have different functions and genomic stability. Although several *Alcanivorax* spp. have been characterized as hydrocarbon degrading bacteria, to our knowledge, their bioluminescent phenotypes are not documented.

To obtain finer grain resolution of bacterial bioluminescence evolution and biogeography, we carried out a comprehensible analysis of LuxA homologs, carefully selected on the basis of both sequence homology, structural and disorder properties. Interestingly, disorder content is congruent with phylogeny and shows distinct patterns in the three well separated OLAH groups (Figure 1 and Supplementary Figure S3). We believe that this set of sequences may provide new insights to decipher luciferase evolution pathways. LuxA belongs to the group C family of two component monooxygenases, which adopt a very similar TIM-barrel fold and probably share a common ancestor (94–96). However, a comparison of LuxA and the closest non-luminous monooxygenase structures (pdb_id: 4us5 and 4uwm) (97,98) revealed that their active sites have both different sequences and structures (Supplementary Table S2 and Figure S2a–i). A first interesting result is the discovery of two OLAH groups (1 and 2) that share many common features with reference LuxA that seem to distinguish them from monooxygenases (Supplementary Figures S3 and 4). Remarkably, the phylogeny and the ocean distribution of OLAH sequences also show a congruent pattern. Indeed, sequences of the cosmopolite OLAH group 1 are systematically much more abundant in mesopelagic layers (Figure 5A–C). In contrast, the two OLAH groups 2 and 3 that include known reference monooxygenases are uniformly distributed along the water column (Supplementary Figures S8a–d and S9a–d). The fact that the group 1 is preferentially found in dark environments is coherent with the hypothesis that they could represent a new class of luciferases. An alternative hypothesis explaining higher abundance deep in the ocean could be a specific adaptation to high-pressure of luminescent or non-luminescent proteins. Interestingly, more than one third of OLAH group 1 sequences are assigned to *Entotheonella*. These bacteria have been recently described to form symbiotic associations with sponges and are characterized by an over-representation of FMN-monooxygenase genes (99), that are closely related to luciferases. Such reservoirs of FMN-monooxygenase genes may have contributed to the evolution of luciferases.

How and why bacterial luciferases have evolved to catalyze light-emission is still an open question. To date, the diversity of bioluminescent bacteria is predominantly based on the study of culturable Gammaproteobacteria harbouring a highly conserved *lux* operon organization. Our metagenomic study provides new insights about *lux* operon diversity and distribution in the global ocean, as well as clues for future exploration on luciferase evolution and functions in marine bacteria including unknown and uncultivable species.

SUPPLEMENTARY DATA

Supplementary Data are available at NARGAB Online.

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