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Relationship of the luminous bacterial symbiont of the Caribbean flashlight fish, *Kryptophanaron alfredi* (family Anomalopidae) to other luminous bacteria based on bacterial luciferase (*luxA*) genes*

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Abstract. Flashlight fishes (family Anomalopidae) have light organs that contain luminous bacterial symbionts. Although the symbionts have not yet been successfully cultured, the luciferase genes have been cloned directly from the light organ of the Caribbean species, *Kryptophanaron alfredi*. The goal of this project was to evaluate the relationship of the symbiont to free-living luminous bacteria by comparison of genes coding for bacterial luciferase (*lux* genes). Hybridization of a *luxAB* probe from the *Kryptophanaron alfredi* symbiont to DNAs from 9 strains (8 species) of luminous bacteria showed that none of the strains tested had *lux* genes highly similar to the symbiont. The most similar were a group consisting of *Vibrio harveyi*, *Vibrio splendidus* and *Vibrio orientalis*. The nucleotide sequence of the luciferase α subunit gene (*luxA*) of the *Kryptophanaron alfredi* symbiont was determined in order to do a more detailed comparison with published *luxA* sequences from *Vibrio harveyi*, *Vibrio fischeri* and *Photobacterium leiognathi*. The hybridization results, sequence comparisons and the mol% G+C of the *Kryptophanaron alfredi* symbiont *luxA* gene suggest that the symbiont may be considered as a new species of luminous *Vibrio* related to *Vibrio harveyi*.

Key words: Bioluminescence – Symbiosis – *Kryptophanaron alfredi*

In fishes, bioluminescence is generally produced by fish tissue in specialized light organs and cells (Herring and Morin 1978). In some families, however, bioluminescence is produced by extracellular symbiotic bacteria that occupy highly developed light organs (Herring 1987). In many cases the bacteria are readily cultured, and have been identified as the well-known free living luminous bacteria *Vibrio fischeri* (host fish family Monocentridae, Fitzgerald 1977; Ruby and Nealson 1976), *Photobacterium leiognathi* (host fish family Leiognathidae and Apogonidae; Reichelt et al. 1977), and *Photobacterium phosphoreum* (several families of deep-living fishes are hosts, Herring 1975; Ruby and Martin 1978). This project has focussed on the association found in the flashlight fishes (family Anomalopidae; Harvey 1912) in which the bacterial symbiont has not yet succumbed to efforts to culture it. Other light organ symbioses in which bacterial luciferase has been detected, but the symbionts have not been cultured, are the ceratioids (midwater angler fishes) and the pyrosomes (colonial tunicates; Leisman et al. 1980). The family Anomalopidae has four genera and five species, all of which are tropical reef dwellers (Johnson and Rosenblatt 1988). The flashlight fishes have relatively large suborbital light organs that communicate with the seawater via pores on the surface. The bacterioids are densely packed in the lumen of the tubules that compose most of the light organ (Kessel 1977). As they grow inside the light organ, the bacterioids are gradually released into the seawater through the pores (Haygood et al. 1984). Leismann et al. (1980) demonstrated high levels of in vitro activity of bacterial luciferase, a light-producing enzyme unique to bacteria, in anomalopid light organ extracts. This result proved unequivocally that the symbiotic bacteria are the source of luminescence in anomalopids, but the luciferase reaction decay kinetics observed were surprising. All of the culturable light organ symbionts (listed above) have luciferases with fast decay kinetics (a measure of the turnover rate of the enzyme; Nealson and Hastings 1979). In contrast, the flashlight fish symbiont luciferases have slow decay kinetics similar to luciferase from *Vibrio harveyi*, a free-living luminous bacterium not known to occur in a light organ symbiosis (Leisman et al. 1980).

In a previous study (Haygood and Cohn 1986) we extracted DNA directly from the light organs of *Kryptophanaron alfredi*, the Caribbean flashlight fish (Silvester and Fowler 1926; Colin et al. 1979). Hybridization of *luxA* (luciferase α subunit) and *luxB* (luciferase β

* The nucleotide sequence reported in this article has been deposited in Genbank under accession number M36597

subunit) probes from *V. harveyi* to Southern blots of *K. alfredi* light organ DNA and *V. fisheri* chromosomal DNA showed that the *K. alfredi* symbiont *lux* genes were more similar to the probes than were *V. fischeri lux* genes, a result consistent with the enzyme kinetics data. Although there is no evidence that the light organ does not contain a pure culture, it is still possible that other bacteria could be present. However, luminous symbiont DNA is the major component of light organ DNA judging from the equal strength of the hybridization signal observed with a *V. harveyi luxA* probe against equal amounts of light organ and *V. harveyi* DNA under low stringency conditions.

The goal of this study was to assess the relationship among the *luxA* genes of the symbiont and those of known luminous bacteria, and by inference, among the bacteria themselves. The approach used was 1) to employ the cloned symbiont *lux* genes as a hybridization probe against DNA from culturable luminous bacteria, and 2) to determine the nucleotide sequence of *luxA* from the *Kryptophanaron alfredi* symbiont and compare it to *luxA* from other luminous bacteria.

Although genetic studies of "unculturable" bacterial symbionts are rare, related approaches have been used in a few other symbiotic systems. Ribosomal RNA sequences have proved useful in the study of hydrothermal vent symbioses (Stahl et al. 1984; Distel et al. 1988). We have chosen to focus on *luxA* genes because 1) published *luxA* sequences are available for comparison with the symbiont (Cohn et al. 1985; Foran and Brown 1988; Illarionov et al. 1988; Baldwin et al. 1989 and 2) *lux* genes would be expected to be much less conserved than rRNA sequences, and thus give better resolution of close relationships. In the long term, such studies may lend insight into the function and regulation of *lux* genes in symbioses, in analogy to studies of *nif* genes in rhizobium symbioses (Halverson and Stacey 1986).

Materials and methods

Plasmids and bacterial strains

Plasmid pDR720 *lux* (Haygood and Cohn 1986) was the source of DNA for the gene probe. M13 subclones and for sequence determination using double stranded DNA. M13mp18 and M13mp19 and *Escherichia coli* host strain JM101 were from Bethesda Research Laboratories (BRL, M13 cloning kit). Strains used in hybridization experiments are listed in Table 1.

Hybridization experiments

DNA from the *Kryptophanaron alfredi* light organ was prepared as in Haygood and Cohn (1986). DNA from other luminous bacteria was prepared according to Ditta et al. (1980). Concentration was determined spectrophotometrically (A_{260}) and confirmed by comparison with standards after agarose gel electrophoresis and ethidium bromide staining. 500 ng of light organ DNA and 1 µg of DNA from other luminous bacteria were bound to a nitrocellulose filter (Schleicher & Schüll BA85 0.45 µm) with a Bio-Rad Bio-dot microfiltration apparatus according to manufacturers instructions.

The probe consisted of a 1.7 kb *HpaI-KpnI* fragment from pDR720*lux*. The *HpaI* site is 107 nucleotides 5′ from the initiation codon of *luxA*, and the *KpnI* site is estimated to be in the middle of the *luxB* gene. Thus the probe encompasses all of *luxA* and about half of *luxB*. The fragment was cut out of a low melting point agarose gel and labeled by random priming with a Pharmacia (Piscataway, New Jersey, USA) kit.

The filter was hybridized in a solution consisting of 6 × SSC, 2.5 mM EDTA, 500 µg/ml denatured salmon sperm DNA and 4% Carnation dried milk at 60° C overnight. Washes were 1 × SSC, 0.1% SDS at hybridization temperature. An autoradiograph was made with Kodak XAR film and two intensifying screens.

DNA sequence determination

M13 clones were constructed using restriction sites from the previously published map and additional sites discovered in the course of sequence determination. Single stranded template DNA was purified according to manufacturer's recommendations (BRL M13 cloning kit), except that in some cases 10-ml cultures were used, and the preparation scaled up accordingly. Some sequence was determined using double stranded pDR720*lux* as the template. These templates were prepared by a modification (L. Bookbinder, personal communication) of a published procedure (Mierendorf and Pfeffer 1987). Briefly, plasmid DNA was purified by CsCl ethidium bromide density gradient ultracentrifugation, 20 µg was precipitated with polyethylene glycol (PEG 8000), denatured with NaOH and used in a standard Sequenase reaction (see below).

M13 universal primer [BRL sequencing kit or United States Biochemical Corp. (USB) Sequenase kit] was used for some reactions; the rest were done with gene specific primers designed from initial sequence data. Some primers were produced by the University of California, San Diego Center for Molecular Genetics DNA synthesis facility, some were purchased from Operon, some were the

Table 1. Species and strains used in hybridizations

Species	Strain	Designation in Fig. 1	Source or reference
Vibrio harveyi	B392	Vh B3	Baumann et al. 1980
Vibrio harveyi	BB7	Vh BB	Belas et al. 1984
Vibrio orientalis	ATCC 33934	Vo	ATCC
Vibrio splendidus	ATCC 33125	Vs	ATCC
Vibrio vulnificus	ATCC 27562	Vv	ATCC
Vibrio fischeri	B61	Vf	Nealson 1977
Vibrio logei	ATCC 15382	Vl	ATCC
Photobacterium leiognathi	PL721	Pl	Nealson and Hastings 1977
Photobacterium phosphoreum	NZ11D	Pp	Ruby and Morin 1978

gift of D. H. Cohn, and some were synthesized in house on an Applied Biosystems 391 DNA synthesizer.

Sequence was determined by the dideoxynucleotide chain termination method (Sanger et al. 1977) using either the Klenow fragment of *E. coli* DNA polymerase I or Sequenase (USB) according to manufacturers instructions.

Sequences were entered into the computer with an International Biotechnologies, Inc. (IBI) gel reader and software. Both strands of the reported sequence (Fig. 2) were determined. Sequence of the region near custom primers and each of the restriction sites used for subcloning was verified by sequence spanning the site on both strands.

Sequence analysis

The published *luxA* sequences from *Vibrio harveyi* (Cohn et al. 1985), *V. fischeri* (Foran and Brown 1988) and *Photobacterium leiognathi* (Illarionov et al. 1988) were downloaded from Genbank via the University of California, San Diego DNA/Protein Sequence Analysis System. Analyses were conducted either by hand or aided by the Pustell Sequence Analysis software (IBI). The PHYLIP protein parsimony algorithm version 3.2 was used for phylogenetic analysis.

Determination of mol% G+C

The thermal denaturation procedure of Herdman et al. (1979) was followed.

Results

Hybridization

The only luminous bacteria that showed positive hybridization under these conditions were *Vibrio harveyi*, *V. splendidus* and *V. orientalis* (Fig. 1). These species are considered to be relatively closely related to each other (Baumann et al. 1980; Yang et al. 1983). Note that there was half as much positive control light organ DNA as other DNA on the filter, and that it is likely that the light organ DNA contains host DNA as well as symbiont DNA. Thus the signal from the experimental DNAs would have to be at least twice as strong as the positive control to be considered equal.

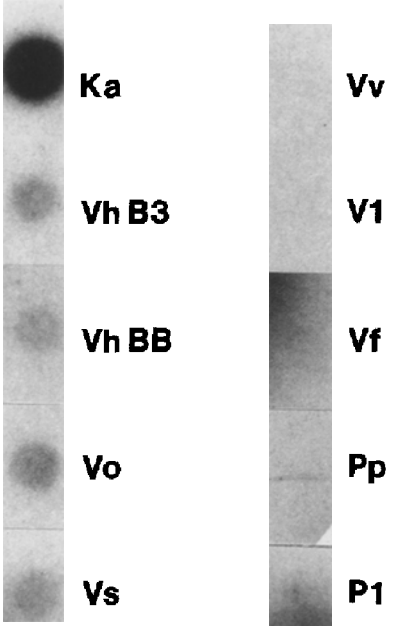

Fig. 1. Hybridization of the *Kryptophanaron alfredi* symbiont *lux* probe to chromosomal DNA of luminous bacteria. See Table 1 for species identification. Ka (*K. alfredi* light organ DNA) positive control is 500 μg of DNA, all others are 1 μg

In order to determine the precise degree of similarity between the *lux* genes of the symbiont and *V. harveyi*, one of the more similar species in the above experiment, direct comparison of the *luxA* nucleotide sequences was done. Comparison of these two sequences with those of *V. fischeri*, a vibrio distantly related to *V. harveyi*, and *Photobacterium leiognathi*, in a different genus, places the degree of sequence similarity within a context useful for determining the relationship of the symbiont to other luminous bacteria.

Location and size of the lux genes

The coding region of the *Kryptophanaron alfredi* symbiont *luxA* gene is 1074 nucleotides long and codes for a protein of 357 amino acids (Fig. 2). The intergenic region between *luxA* and *luxB* structural genes is 33 nucleotides, compared with 29 in *V. harveyi*, 30 in *V. fischeri* (reported as 39 in ATCC 7744; Baldwin et al. 1989) and 48 in *P. leiognathi*.

Codon usage

Overall codon bias is greater in both *V. fischeri* and the *K. alfredi* symbiont than in *V. harveyi* (data not shown). However, just on the basis of the low mol% G+C of the *V. fischeri* and the *K. alfredi* symbiont *luxA* genes, one would predict greater codon bias as a result of the lack of G and C containing codons. The mol% G+C of nucleotides at third positions in codons is 31.4% for the *K. alfredi* symbiont, 26.8% for *V. fischeri*, 41.5% for *P. leiognathi* and 48.5 in *V. harveyi*. Indeed, in contrast to the sequence homologies, the codon dialects of the *V. fischeri* and the *K. alfredi* symbiont genes are much more similar to each other than to *V. harveyi*. Because the differences in codon dialect between the *K. alfredi* symbiont and *V. harveyi* presumably reflect significant differences in tRNA populations, which would be expected to drive the genes to diverge, the high level of nucleotide sequence conservation between the two genes is all the more striking (see below).

Amino acid sequence comparisons

Figure 3 shows an alignment of the predicted amino acid sequences of the luciferase α subunit from the *K. alfredi* symbiont, *V. harvey*, *V. fischeri* and *P. leiognathi*. The proteins differ in size; the *V. fischeri* and *P. leiognathi*

```
                                                                50
ACTAGCTGTCACCTGATACGCTAACTTATTGTCATTCACCGTTTGATGGAATAATAAAGGACGCTAAA ATG AAA TTT GGA AAT
                                                                                Met Lys Phe Gly Asn

                        100
TTT TTA TTG ACA TAT CAA CCA CCA CAA CTT GAT CAA AAA GAG GTT ATT AAG CGG TTA GTC AAC TTA
Phe Leu Leu Thr Tyr Gln Pro Pro Gln Leu Asp Gln Lys Glu Val Ile Lys Arg Leu Val Asn Leu
150                                                                 200
GGA CAA GCT TCT GAG TCC TGT GGT TTT GAT ACT GCT TGG TTG TTA GAG CAT CAC TTT ACG GAA TTT
Gly Gln Ala Ser Glu Ser Cys Gly Phe Asp Thr Ala Trp Leu Leu Glu His His Phe Thr Glu Phe
                                                    250
GGA TTA CTT GGT AAC CCT TAT GTT GCT GCC GCA AAC CTA CTT GGC GCA ACA AAA AAA CTT CAT GTG
Gly Leu Leu Gly Asn Pro Tyr Val Ala Ala Ala Asn Leu Leu Gly Ala Thr Lys Lys Leu His Val
                            300
GGT ACT GCT GCG GTT GTA TTG CCT ACG GCC CAT CCT GTT CGC CAA CTG GAG GAT GTG AAT CTC CTT
Gly Thr Ala Ala Val Val Leu Pro Thr Ala His Pro Val Arg Gln Leu Glu Asp Val Asn Leu Leu
350                                                                 400
GAC CAA ATG TCA AAG GGT CGC TTT AAA TTT GGT ATT TGT CGA GGA TTA TAC GAT AAA GAT TTT CGC
Asp Gln Met Ser Lys Gly Arg Phe Lys Phe Gly Ile Cys Arg Gly Leu Tyr Asp Lys Asp Phe Arg
                                                    450
GTA TTC GGT ACG GAT ATG AGT AAC AGC AGA GAA TTA ATG AAT TCC TGG TAT GAT ATA ATG ACT AAG
Val Phe Gly Thr Asp Met Ser Asn Ser Arg Glu Leu Met Asn Ser Trp Tyr Asp Ile Met Thr Lys
                            500
GGT ATG ATA GAG GGG CAT GTA TCA TCA GAT AAC GAA CAT ATT AAA TTT CCT AAA GTT AAA GTT TCG
Gly Met Ile Glu Gly His Val Ser Ser Asp Asn Glu His Ile Lys Phe Pro Lys Val Lys Val Ser
    550                                                             600
CCA AAT TCT TAT ACG CAA AGA GGT GCT CCT GTC TAT GTT GTT GCA GAA TCA GCG TCA ACC ACT GAA
Pro Asn Ser Tyr Thr Gln Arg Gly Ala Pro Val Tyr Val Val Ala Glu Ser Ala Ser Thr Thr Glu
                                                    650
TGG GCT GCA GAG AGA GGT CTA CCA ATA ATA CTA AGC TGG ATT ATT AAC AAT AAT GAA AAA AAA TCA
Trp Ala Ala Glu Arg Gly Leu Pro Ile Ile Leu Ser Trp Ile Ile Asn Asn Asn Glu Lys Lys Ser
                                700
CAG CTT GAT CTT TAC AAC GAA ATT GCA CTT GAG CAT GGA CAC GAT GTT TCC AAT ATA GAT CAC TGT
Gln Leu Asp Leu Tyr Asn Glu Ile Ala Leu Glu His Gly His Asp Val Ser Asn Ile Asp His Cys
        750                                                             800
ATG TCT TAT ATC ACC TCC GTT GAT CAT AAT AGC AAT AAA GCA AAA GAT ATT TGC AGG GAC TTT TTA
Met Ser Tyr Ile Thr Ser Val Asp His Asn Ser Asn Lys Ala Lys Asp Ile Cys Arg Asp Phe Leu
                                                    850
GCT CAC TGG TAT GAC TCT TAT TTC AAT GCT ACC AGT ATC TTT GAC GAT TCA AAT CAA ACC AAA GGT
Ala His Trp Tyr Asp Ser Tyr Leu Asn Ala Thr Ser Ile Phe Asp Asp Ser Asn Gln Thr Lys Gly
                            900
TAT GAT TTC AAC AAA GGT CAA TGG CGT AAT TTT GTT TTA AAA GGA CAT AAA GAT ACT AAT CGC CGG
Tyr Asp Phe Asn Lys Gly Gln Trp Arg Asn Phe Val Leu Lys Gly His Lys Asp Thr Asn Arg Arg
        950                                                             1000
ATT GAT TAC AGT TAT GAA ATC AAT CCA CTT GGT ACT CCT CAA GAG TGT ATT GAA ATT ATT CAA TCA
Ile Asp Tyr Ser Tyr Glu Ile Asn Pro Val Gly Thr Pro Gln Glu Cys Ile Glu Ile Ile Gln Ser
                                                    1050
GAT ATC GAT GCA ACT GGT ATC CAT AAT ATC TGT TGT GGT TTT GAG GCC AAT GGT TCT GAG ACT GAG
Asp Ile Asp Ala Thr Gly Ile His Asn Ile Cys Cys Gly Phe Glu Ala Asn Gly Ser Glu Thr Glu
                                1100
ATC ATA GCA TCA ATG AAG CTT TTC CAA TCA GAC GTT ATG CCT TAC TTG AAA GAA AAA AGT AAC TGT
Ile Ile Ala Ser Met Lys Leu Phe Gln Ser Asp Val Met Pro Tyr Leu Lys Glu Lys Ser Asn Cys
        1150                                                            1200
TAG TTTACACGATACTCAGATAAAAAGGAATTAACT ATG AAA TTC GGA CTA TTT TTT CAA AAT TTT CTT TCT GAA
End                                     Met Lys Phe Gly Leu Phe Phe Gln Asn Phe Leu Ser Glu
AAT CAA TCA TCA GAA A
Asn Gln Ser Ser Glu
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Fig. 2. Nucleotide sequence and predicted amino acid sequence of *luxA* from the *K. alfredi* symbiont. The gene beginning immediately downstream of *luxA* is *luxB*. Possible ribosome binding sites are underlined

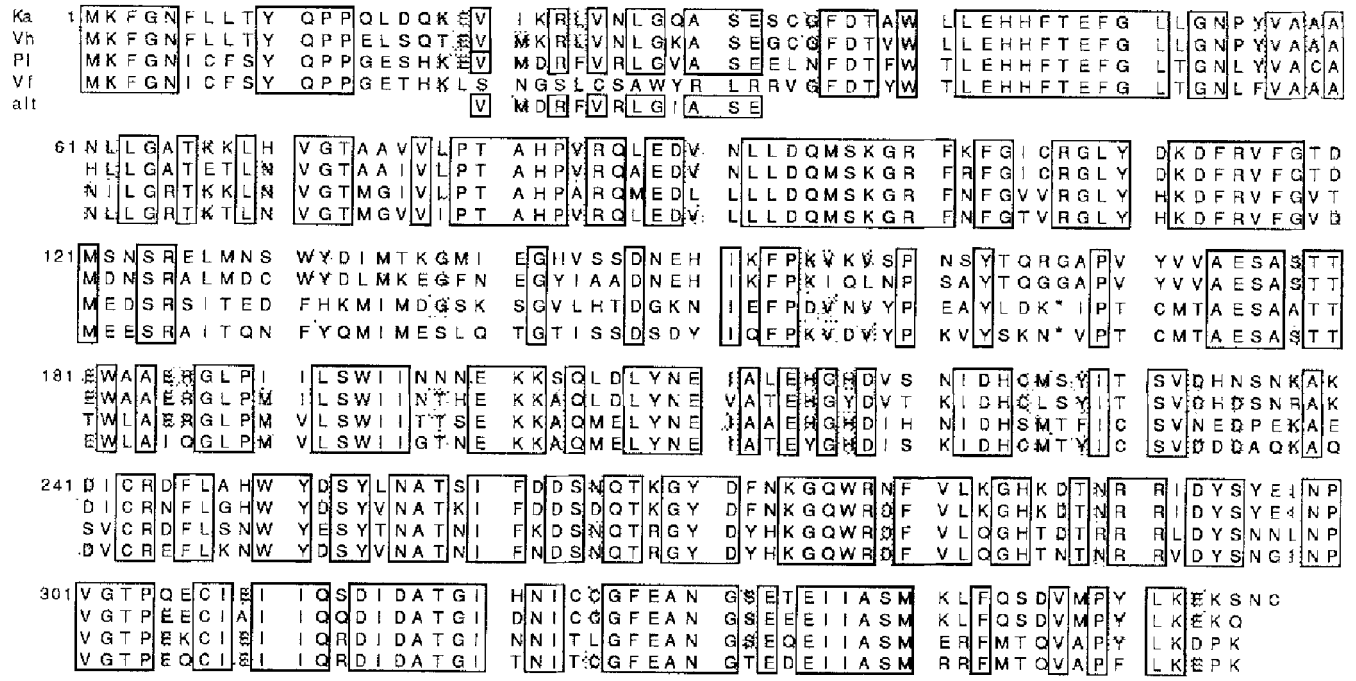

Fig. 3. Comparison of predicted amino acid sequences of luciferase α subunit from the *K. alfredi* symbiont (Ka), *Vibrio harveyi* (Vh), *Photobacterium leiognathi* (Pl), and *V. fischeri* (Vf). Translation of a minus one frame shift of the *V. fischeri* sequence yielded a region of high similarity (alt) which is shown below the *V. fischeri* sequence. Boxed nucleotides are shared between four sequences, shaded nucleotides are shared between three sequences

proteins are 354 amino acids (not 355 as previously reported for *V. fischeri*, Foran and Brown 1988) and the *V. harveyi* protein is 355 amino acids. The *V. fischeri* and *P. leiognathi* luciferase α subunits are one amino acid shorter than that of *V. harveyi* apparently due to a loss of a single codon relative to the other two genes, probably between the conserved tyrosine (position 163) and proline (position 169). The gap shown in Fig. 3 was located to optimize similarity among the four nucleotide sequences. Comparison with additional luciferase α subunit sequences in the future may locate the gap with greater certainty. The *K. alfredi* symbiont has the largest luciferase α subunit at 357 amino acids. The greater length of the *K. alfredi* symbiont protein is due to deletion of a nucleotide in the codon prior to the TAA stop codon found in the other three genes resulting in a −1 frame shift and consequent loss of the stop codon. Two additional amino acids are added to the carboxyl terminus of the protein before another stop codon is encountered (Fig. 2, 3).

It is interesting to note that the cysteinyl residue at position 106 in the *V. harveyi* and the *K. alfredi* symbiont sequences is not conserved in *V. fischeri* and *P. leiognathi* (it is replaced by valine). This residue is postulated to reside in or near the flavin-binding site of the enzyme (Cohn et al. 1985; Johnston et al. 1986), and thus the lack of conservation is surprising.

Alignment of the predicted amino acid sequences revealed a region of surprisingly low similarity (positions 19–32) in the *V. fischeri* luciferase α subunit sequence (Fig. 3). Translation and alignment of a −1 frame shift of the nucleotide sequence restored similarity to the region from position 20–32. Likewise, the nucleotide sequence shows enhanced similarity if a single base is removed at position 54 or 55 and a base added between base 97 and 102. Thus it appears that there is either a major frame shift mutation in *V. fischeri* strain MJ1 *luxA*, or an error in the published sequence. The published sequence and the Genbank file agree in this region, so it is not an error in data entry into Genbank. A *luxA* sequence from a different strain of *V. fischeri* (ATCC 7744, not in Genbank at the time of this writing, Baldwin et al. 1989) does not show this frame shift.

The percentage of amino acids in the luciferase α subunit identical to the *K. alfredi* symbiont protein is 83% for *V. harveyi*, 63% for *V. fischeri* (65% if the frame shift is restored) and 62% for *P. leiognathi*.

Nucleotide sequence comparisons

The percentage of nucleotides identical to the *K. alfredi* symbiont *luxA* coding region is 75% for *V. harveyi luxA*, 63% for *V. fischeri luxA* (64% if the frame shift is restored) and that of *P. leiognathi* is 63%. The intensity of the signal in Fig. 1 from *V. orientalis* and *V. splendidus* shows that they are about as similar to the symbiont genes as is *V. harveyi*.

Phylogenetic analysis

luxB is the result of a gene duplication of *luxA* (Baldwin et al. 1989). All luminous bacteria that have been examined

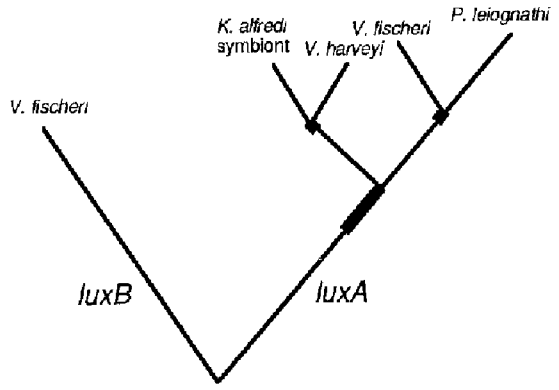

Fig. 4. Phylogenetic tree based on amino acid sequences of *luxA* and *luxB*. Branch lengths are proportional amino acid replacements deduced by the PHYLIP protein parsimony algorithm. Heavy bars show range of minimum and maximum possible replacements

Table 2. Genomic and *luxA* coding region mol% G+C of luminous bacteria

Species	Genome	*luxA* Coding region
K. alfredi symbiont	NA	37.4
V. fischeri	38.9[a]	37.0
P. leiognathi	42.9[a]	43.1
V. harveyi	46.4[a]	45.1

NA, not available
[a] From Baumann et al. 1980

have both α and β subunits of luciferase, therefore the duplication must have occurred before the divergence of the luminous bacteria. Therefore, *V. fischeri luxB* was used as the outgroup for the comparison of the genes. The region aligned was position 1 to 240 of the *Kryptophanaron alfredi* symbiont and *V. harveyi luxA*s, position 239 of the *V. fischeri* and *P. leiognathi luxA*s and 242 of the *luxB*. As shown in Fig. 4, based on relative similarity of the *luxA* region, the *K. alfredi* symbiont is more closely related to *V. harveyi* than to *V. fischeri* and *P. leiognathi*.

These relationships can be further analyzed by comparison of mol% G+C of the *luxA* coding region. Table 2 shows that the mol% G+C of the *luxA* coding region correlates well with the mol% G+C of the whole genome in *V. harveyi*, *V. fischeri* and *P. leiognathi*. Miyamoto et al. (1988) found this correlation to be true for *V. harveyi lux B* and *D* as well as *luxA*, although not for *luxC*.

Mol% G+C of light organ DNA

Direct measurement of mol% G+C in light organ samples is difficult because of the very limited amounts of DNA available and the fact that the light organ DNA consists of a mixture of DNA from the luminous symbiont and the host fish. A preliminary attempt to measure the mol% G+C of light organ DNA by thermal denaturation gave an approximate mol% G+C of 35%.

Discussion

The hybridization results suggest that the *Kryptophanaron alfredi* symbiont is not closely allied to any of the major marine luminous bacteria. Luminous bacteria that were not tested are *Xenorhabdus luminescens* (Thomas and Poinar 1979), *Alteromonas hanedai* (Jensen et al. 1980) and *Vibrio cholerae* (Hada et al. 1985). Although it is conceivable that these bacteria are more closely related to the symbiont than those tested, it is unlikely, because *X. luminescens* is a terrestrial organism, *A. hanedai* is a polar species adapted to low temperatures and *V. cholerae* is found in freshwater and estuarine environments.

The results of the phylogenetic analysis are consistent with the enzyme kinetics and hybridization data. Since the *K. alfredi* symbiont and *V. harveyi* genes have diverged almost as much as those of *V. fischeri* and *Photobacterium leiognathi*, which are distinct species currently assigned to different genera (although both were earlier classified as *Photobacterium*; Baumann et al. 1980), that degree of difference suggests that the symbiont is probably not *V. harveyi*, but a different species of *Vibrio*. Indeed, the *luxA* relationships shown in Fig. 4 do not support the assignment of *V. fischeri* to the genus *Vibrio*. Baldwin et al. (1989) reported a similar conclusion based on comparison of three *luxA* sequences.

If the correlation between genomic and *luxA* coding region mol% G+C (Table 2) holds for the *K. alfredi* symbiont, one would predict that the genomic mol% G+C of the symbiont would be about 39%. The direct measurement was significantly lower, but due to the low amplitude of the signal and the fact that light organ DNA is not a pure sample, this is only a rough estimate. Both estimates agree that the mol% G+C of the symbiont is likely to be at the low end of the range found in luminous bacteria. Among all the species in the genus *Vibrio* only *V. fischeri* (38.9%) and its close relative *Vibrio logei* (41.2%) have values close to that inferred for the *K. alfredi* symbiont (Baumann et al. 1980, Yang et al. 1983). The lack of nucleotide and amino acid sequence similarity with *V. fischeri luxA*, the lack of hybridization of the symbiont probe to *V. fischeri* and *V. logei* DNA (Fig. 1), as well as the difference in luciferase decay kinetics between the *K. alfredi* symbiont luciferase and *V. fischeri* luciferases, weigh heavily against identification of the symbiont as *V. fischeri* or *V. logei*. Thus it seems most probable that the *K. alfredi* symbiont is a new species of luminous marine vibrio.

The major uncertainty in this assessment lies in the hypothesis that the evolution of the *lux* genes reflects the evolutionary history of the symbiont as a whole. For instance, it could be postulated that the *lux* genes evolved anomalously relative to the rest of the genome. In addition, these data do not exclude the possibility that the *lux* genes were laterally transferred to the symbiont. Analysis of other genes, such as ribosomal RNA genes or enzyme structural genes not involved in bioluminescence could test these possibilities.

This work provides the foundation for studies of the evolution of the bacterial symbionts in the family

Anomalopidae as a whole, since it should be possible to obtain the corresponding sequences from the symbionts of most members of the family. If the results support the hypothesis that the symbiosis has driven the evolutionary divergence of the flashlight symbionts, it will be possible to infer actual rates of evolution of the symbiont genes from the evolutionary history of the host fish family as suggested by Ochman and Wilson (1987). This period encompasses the occurrence of divergence as long ago as the Cretaceous (144–66 million years ago) and as recent as the Pliocene (5 million years ago, Johnson and Rosenblatt 1988).

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