SYMBIOSIS (2008) 46, 101–108 ISSN 0334-5114

Natural selection on the *lux*A gene of bioluminescent bacteria

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(Received October 19, 2007; Accepted February 13, 2008)

Abstract

Despite a growing literature of *Vibrio, Photobacterium, Shewanella,* and *Photorhabdus* biology, little is known of the function bioluminescence provides to these light-emitting bacteria. Proposed benefits of bioluminescence include evasion of predators or attraction of prey for symbiotic bacterial hosts through a distraction, a method of oxygen consumption to suffocate a host or reduce competition from obligate aerobes, a mechanism that stimulates DNA repair, or as a redox sink. We tested for the presence or absence of destabilizing selection on 31 physicochemical properties of the *lux*A gene of bacterial luciferase in relation to a phylogenetic hypothesis and the location of selection within the protein structure, in an attempt to further understand the evolution of bacterial bioluminescence and its importance to symbiosis. We show that amino acid properties most influenced by destabilizing selection include power to be at the C-terminal, chromatographic index, and isoelectric point. The location of destabilizing selection for isoelectric point within a phylogenetic context indicates that bacterial ecology has had an effect on the evolutionary history of *lux*A, while the presence of destabilizing selection for chromatographic index supports previous findings that bioluminescence in these species is sensitive to environmental osmolarity.

Keywords: Bioluminescence, bacteria, destabilizing selection, evolution, *lux*A, natural selection, *Photorhabdus, Photobacterium, Shewanella,* TreeSAAP, *Vibrio*

1. Introduction

Bioluminescence, the production and emission of light by a living organism as a result of a chemical reaction, occurs in an array of organisms including fish, insects, jellyfish, and bacteria. Production of light by bacteria is unique in that luminous bacteria continuously produce light at a wavelength of 490 nm, while higher organisms (i.e. insects and jellyfish) display only intermittent flashes of light (Haygood, 1993). Many marine fish species are bioluminescent due to the presence of bioluminescent bacterial symbionts that inhabit the fishes light organ. Bioluminescent bacteria are the most abundant and widely distributed of all light-emitting organisms (Meighen, 1994), occupying a wide variety of ecological niches (fish light organs, mammalian gut, nematode gut) and habitats (marine, freshwater, terrestrial, and symbiotic within a host). Currently, only four genera of bacteria are known to naturally bioluminesce: *Vibrio, Photobacterium, Shewanella,* and *Photorhabdus*.

Most luminous *Vibrio cholerae* strains are found in aquatic environments (Colwell et al., 1981; Garay et al., 1985; Falcao et al., 1998) commonly associated with zooplankton (Colwell, 1996). *Vibrio fischeri* is known to form a symbiotic relationship with squid as well as being found in fish in shallow temperate waters (Madigan and Martinko, 2005) and in planktonic environments (Ruby and Nealson, 1978; Ruby and Lee, 1998). In contrast, *Vibrio harveyi*, best known for causing milky ocean, a phenomenon where the ocean glows white at night due to large *V. harveyi* populations, is primarily a free-living bacterium found in the water column of marine environments. Other bioluminescent bacterial genera inhabiting aquatic environments include *Photobacterium* spp., which can be found on the surface of fish, as a symbiont in the light organs of deep water marine fish (Madigan and Martinko, 2005), and in coastal and open-ocean sea water (Ast and Dunlap, 2005), while *Shewanella*

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Presented at the Nematode-Bacterium Symbioses Workshop, April 21–23, 2007, Tucson, AZ, USA

is commonly found free living in freshwater environments (Haygood, 1993). *Photorhabdus* spp., gut endosymbionts in juveniles of entomopathogenic nematodes from the genus *Heterorhabditis,* are the only terrestrial bacteria known to exhibit bioluminescence (Gerrard et al., 2003).

The general bioluminescent reaction is a complicated process requiring the cooperation of multiple genes. The enzyme luciferase interacts with $FMNH_2$ to form an EFH_2 complex, which subsequently reacts with O_2 to yield an oxygenated enzyme-flavin complex. This complex interacts with aldehyde (RCOH) to form a luciferase-FH_2 O_2 -RCOH complex (Stabb, 2005; Li and Tu, 2005). Decay of this complex goes to completion with the emission of blue-green light at 490 nm (Haygood, 1993; Valkova et al., 1999).

In bacteria, *lux* genes are responsible for the production of light (Kuwabara et al., 1965; Friedland and Hastings, 1967; Baldwin et al., 1989). Bioluminescent bacteria have at least five *lux* genes, each with similar functions across taxa. *lux*AB genes are the genes that code for luciferase, while *lux*CDE genes are the fatty acid reductase complex, and are responsible for synthesizing the fatty aldehyde substrate for the luminescence reaction (O'Kane and Prasher, 1992; Stabb, 2005). While similarity in function of both the *lux*AB and *lux*CDE genes exists across bacterial species, the organization of the *lux* operon varies in each bacterial species (Kasai et al., 2007; Meighen and Szittner, 1992; O'Kane and Prasher, 1992; Fig. 1).

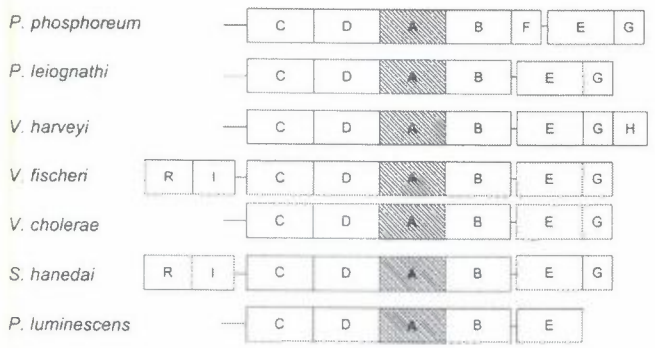


Figure 1. Organization of the *lux* operons of *Vibrio harveyi*, *Vibrio fischeri*, *Vibrio cholerae*, *Photobacterium leiognathi*, *Photobacterium phosphoreum*, *Shewanella hanedai* and *Photorhabdus luminescens*. The arrangement of *lux* A, B, C, and D are conserved across all bioluminescent taxa. *Vibrio fischeri* and *S. hanedai* have two regulatory genes, *lux*I and *lux*R upstream of the *lux*C gene. A significant reduction in the number of *lux* genes can be seen in *Photorhabdus luminescens* in relation to the ancestral states, indicating selection for a decrease in the *lux* operon over time as well as possible selection for an alternative function of bioluminescence in *Photorhabdus* when compared to other bioluminescent bacteria. (Adapted from Kasai et al., 2007; Meighen and Szittner, 1992; and O'Kane and Prasher, 1992).

A great deal is known as to how light is produced in bioluminescent bacteria, though the question of why these bacteria emit light remains unanswered. For bacteria that form a symbiotic relationship with fish, luminescence may provide a distraction that allows the host fish to elude predators or catch prey (Szpilewska et al., 2003), and as such is necessary to maintain a successful relationship. The natural ability of bioluminescent bacteria to reduce molecular oxygen through the oxidation of luciferin led to the proposal by McElroy and Seliger (1962) that light production evolved as a mechanism of oxygen consumption. By consuming oxygen in the surrounding environment, bioluminescent bacteria can out-compete obligate aerobic bacteria (Timmins et al., 2001) as well as slow a host animal's ability to produce toxic oxygen radicals (Stabb, 2005). The production of light by certain bacteria has also been speculated to function as a redox sink, whereby light production acts as a mechanism to reduce excess NADH, which has built up due to growth conditions, to NAD^+ (Stabb, 2005). Stimulation of DNA repair is a more recent idea that has been proposed to explain the evolution of bioluminescence (Czyz et al., 2003). Under this scenario, bioluminescence, even when present at very low levels, activates a photoreactivation reaction, which could act to repair DNA (Czyz et al., 2000). Thus, in some environments the ability to produce even low levels of light could give a luminescent bacterium an advantage over a non-luminescent bacterium (Czyz et al., 2003).

While understanding why bacteria bioluminesce is important, it may be equally important to know if luminescence plays a role in the maintenance of symbiosis. In the relationship between *Photorhabdus* and *Heterorhabditis*, it appears that symbiosis does benefit from luminescence. *Photorhabdus* bacteria are known to exhibit two phases: primary phase, characteristic of bacteria with the ability to bioluminesce and produce antibiotics and extracellular enzymes, and secondary phase, which lacks all of the aforementioned characteristics. Phase I *Photorhabdus* variants can support nematode growth and colonize the intestinal tract of *Heterorhabditis* infective juveniles (IJ's) while phase II variants cannot. It has been shown that those traits which differ between the two phases (bioluminescence, production of antibiotics, etc.) represent factors that facilitate symbiosis, termed symbiosis factors (Joyce and Clark, 2003). Joyce and Clark (2003) go on to show that the presence of a *hex*A homologue in phase II *Photorhabdus* represses these symbiosis factors and that insertion into the *hex*A gene of secondary phase *Photorhabdus* restores symbiosis factors, allowing said mutant to support nematode growth and development. This suggests that the lux pathway may be necessary in the maintenance of symbiosis in this system.

As mentioned earlier, *lux*A and *lux*B are the two genes responsible for the production of luciferase, the enzyme that

drives the bioluminescence reaction. *lux*A codes for the alpha subunit of luciferase, which is primarily responsible for the kinetic properties of luciferase (Madvar, et al., 2005). While the high quantum yield bioluminescent reaction requires a heterodimer of both the alpha and beta subunits (*lux*A and *lux*B), the active center of bacterial luciferase is found on the alpha subunit (Noland et al., 1999). Furthermore, the position and presence of the alpha subunit of bacterial luciferase within the *lux* operon appears to be conserved across taxa (Fig. 1), making *lux*A a suitable target to investigate selection across bioluminescent bacterial species.

Traditionally, selection on a protein coding gene was calculated using the ratio of nonsynonymous (d_N) to synonymous (d_S) substitutions, though it has been shown that some of the assumptions made by this method are too conservative (Crandall et al., 1999; Woolley et al., 2003). Furthermore, while the d_N/d_S ratio may indicate the presence of selection on a gene, it does not specify how the selection affects the structure and/or function of the protein (Taylor et al., 2005). By evaluating the presence or absence of selection among particular physicochemical properties of amino acids in relation to a phylogenetic hypothesis and the location of selection within the protein structure, we can more accurately detect the presence of destabilizing selection in an attempt to further understand the evolution of bacterial bioluminescence and its importance to symbiosis. Thus, we tested for the presence or absence of destabilizing selection on 31 physicochemical properties of the *lux*A gene of bacterial luciferase. We then mapped these properties on a phylogenetic tree to determine if selection on specific physicochemical properties could account for differences in the ecology as well as the function of bioluminescence in each of the sampled bacterial species.

2. Materials and Methods

*lux*A cds sequences were obtained from Genbank for seven bacterial species representing four genera, including *Vibrio fischeri* strain ES114 (NC_006841) a mutualistic symbiont from the bobtailed squid (Ruby et al., 2005), *Vibrio harveyi* strain NBRC 15364 (DQ436496), *Vibrio cholerae* strain TP (AY876056) from plankton (Purdy et al., 2005), *Photobacterium leiognathi* strain lleuc.1.1 (AY341070) from the light organ of a leiognathid fish (Ast and Dunlap, 2004), *Photobacterium phosphoreum* strain ATCC 11040 (AY341063) from the skin of a fish (Ast and Dunlap, 2005), *Photorhabdus luminescens* strain TT01 (NC_005126), and *Shewanella hanedai* strain NCIMB 2157 (AB261992). The longest open reading frame for each sequence was determined prior to alignment of the sequences using BioEdit (Hall, 1999). As *lux*A is a coding region, AlignmentHelper 1.2 (http://inbio.byu.edu/faculty/dam83/cdm) was utilized to convert nucleotide sequences into amino acids prior to alignment. Furthermore, AlignmentHelper allows for the fate of each amino acid to be tracked during the alignment process, allowing codon conformations to remain intact following conversion back to nucleotide data. Following multiple alignment of amino acid sequences in MUSCLE 3.3 (Edgar, 2004), sequences were re-input into AlignmentHelper for conversion of the amino acid sequences back into nucleotide data. Phylogenetic relationships of the seven species were inferred from previously published trees as well as a parsimony analysis, conducted in PAUP*4.0b10 (Swofford, 2000), of 16S rRNA for all seven species using 1000 random addition sequences and TBR branch swapping. TreeSAAP v3.0 (Woolley et al., 2003) was utilized to measure selection based on changes in 31 physicochemical amino acid properties. Each property change was classified into one of eight categories based on the magnitude of change, where categories 1–3 indicates a conservative change, with conservative changes representing stabilizing selection, and categories 6–8 signifies a radical change, with radical changes indicating destabilizing selection. TreeSAAP uses inferred evolutionary relationships as well as user provided sequence data to calculate an expected random distribution of possible amino acid changes for each category. Significant deviations are detected by comparing the expected distribution to the observed number of amino acid replacements in the data set given the phylogenetic relationships. A z-score is calculated for each category and significant selection is measured at an alpha of 0.001. Radical changes with a z-score of <0.001 indicate destabilizing selection. TreeSAAP data outputs were mapped onto a linearized and flattened version of the 3-D structure of *lux*A, allowing for visualization of the exact parts of the 3-D structure (i.e. loop, stem, etc.) where selection is taking place, and the effects selection for a particular property has on protein function (Woolley et al., 2003; Taylor et al., 2005).

3. Results and Discussion

Of the 31 amino acid properties tested, 27 exhibited some degree of positive destabilizing selection, including alpha-helical tendency, average number of surrounding residues, beta-structure tendency, buriedness, chromatographic index, coil tendency, composition, equilibrium constant, helical contact area, hydropathy, isoelectric point, long-range nonbonded energy, mean r.m.s. fluctuation displacement, molecular weight, normalized consensus hydrophobicity, partial specific volume, polar requirement, polarity, power to be at the C-terminal, power to be at the middle of the alpha helix, power to be at the N-terminal, refractive index, short-range and medium-range nonbonded energy, solvent accessible reduction ratio, surrounding

hydrophobicity, total nonbonded energy, and turn tendency. Properties that were most influenced by destabilizing selection included power to be at the C-terminal, isoelectric point, and chromatographic index. A few codons showed selection for multiple properties including codons 15, 28, 29, 65, and 145, indicating that certain properties may be correlated.

In the present study, selection for isoelectric point (pI), the pH at which a molecule carries no net electrical charge, occurred most often on the branch separating *Vibrio fischeri,* a symbiotic bacterium living within a light organ, from *Vibrio harveyi* and *Vibrio cholerae,* two aquatic/planktonic bacteria (Fig. 2). From these results one might suggest that difference in environmental pH may be the primary factor that is driving selection for isoelectric point in these bacteria, though this is probably not the case. The products of *lux* operon expression operate within the cytoplasm of the bacterial cell, and most bacteria can maintain an intracellular pH within a range of values (though the range varies for acidophiles, neutrophiles, and alkiphiles, and the intracellular pH values can be considerably different than the pH of the surrounding environment [Booth, 1985; Dilworth and Glenn, 1999]). A typical neutrophile will usually maintain a pH between 7.6 and 7.8 (Booth, 1985; Dilworth and Glenn, 1999), though studies have shown that very few proteins have an isoelectric point close to 7.4 (Kiraga et al., 2007). This can be explained by the fact that proteins are most insoluble, least reactive and unstable in pH close to their isoelectric point (Kiraga et al., 2007). Thus, the maintenance of a fairly homeostatic pH indicates that selection for isoelectric point is not driven by environmental pH. Instead, the presence of significant destabilizing selection on the branch separating a symbiotic bacterium (*V. fischeri*) from two free-living/planktonic bacteria, along with previous data from Kiraga et al. (2007), indicate that selection for isoelectric point is probably driven in part by the ecology of the bacteria.

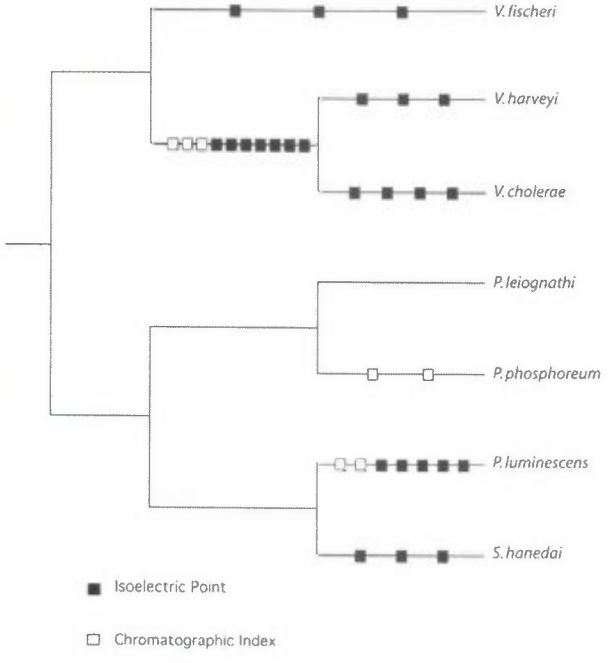


Figure 2. Phylogenetic tree of bioluminescent bacterial relationships with destabilizing selection for isoelectric point and chromatographic index mapped onto corresponding branches. Black and white boxes indicate the number of times destabilizing selection was detected on each lineage for designated properties in the *lux*A gene, with each box representing a single occurrence of statistically significant destabilizing selection. The tree was generated using 16S rRNA sequences.

Photorhabdus bacteria are known to exhibit two phases: primary phase, characteristic of bacteria that are found in insect cadavers where osmolarity and bacterial biomass is high, and secondary phase, characteristic of *Photorhabdus* found in the intestines of infective dauerlarvae where osmolarity and biomass are low. Presence of high osmolarity and rich nutrients, as in the insect cadaver, appears to stabilize the phase I bioluminescent variants of *Photorhabdus* (Krasomil-Osterfeld, 1997). Variation in bioluminescent intensity has also been shown in *Vibrio fischeri* when the bacteria were subjected to high and low osmolarity concentrations, though the limiting factor causing the disparity in light output was revealed to be the aldehyde substrate (Stabb et al., 2004). The present study reveals the presence of significant destabilizing selection for chromatographic index on the evolutionary lineages leading to *Photorhabdus* and *P. phosphoreum* as well as the branch separating *Vibrio fischeri* from *Vibrio harveyi* and *Vibrio cholerae* (Fig. 2). Chromatographic index is defined as the hydropathy of a residue based on interactions of solute, solvent, and hydrophobic absorbent (Prabhakaran, 1990). The typical osmolarity of sea water is 1,000 mosM (Stabb et al., 2004), while the osmolarity in cephalopods is typically greater than sea water (Robertson, 1965; Stabb et al., 2004). Thus, bacterial cells within cephalopod light organs are probably subjected to higher salinities than bacteria that are free living in the ocean. We believe that the increased solute concentration is the reason destabilizing selection for chromatographic index was detected on the branch separating *V. fischeri* (a symbiont of squid) from *V. harveyi* (free living in marine environments) and *V. cholerae* (planktonic). Inversely, teleost fish likely maintain blood osmolarities that are less than the osmolarity of sea water (Fange et al., 1976; Stabb et al., 2004). Thus, in the *Photobacterium* clade we see the presence of destabilizing selection for chromatographic index on the branch that separates the *P. leiognathi* lineage from the *P. phosphoreum* lineage. We attribute the change in chromatographic index to natural selection in response to the difference in osmolarity. Consequently, our data

suggests that osmolarity has had an effect on the evolutionary history of the *lux*A gene of luminescent bacteria.

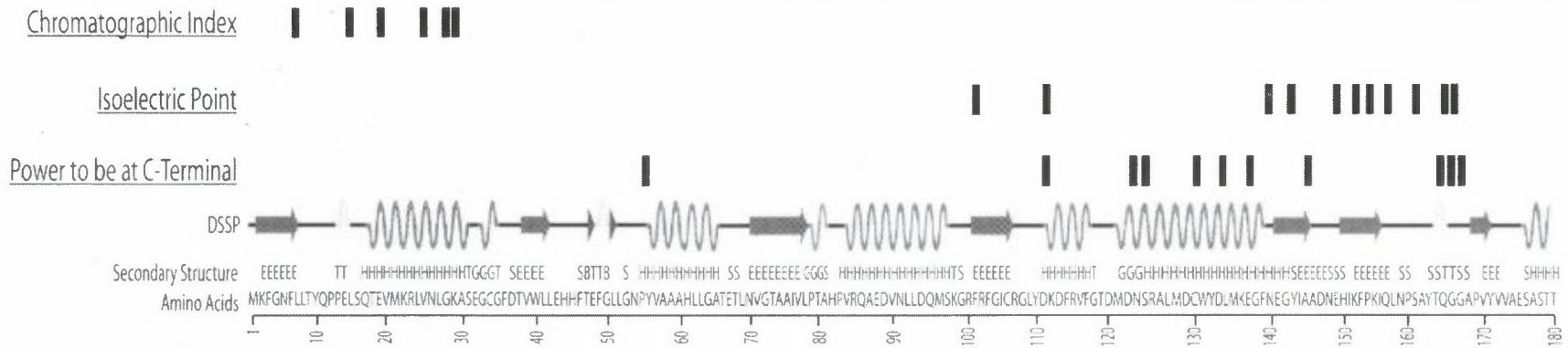
Power to be at the C-terminal is loosely defined as the propensity of the C-terminus of the alpha helix to interact with other residues (Prabhakaran and Ponnuswamy, 1979). While the property 'power to be at the C-terminal' is currently not well understood, selection for this property in the current study is associated with certain features of the secondary structure of the alpha subunit of luciferase. The active site of luciferase is located in a pocket near the C-terminal end of the alpha subunit (Li and Tu, 2005). Adjacent to the opening of the active site lies a 29-residue mobile loop, not present in the beta subunit, from α258 to α286 (Fig. 3). This loop is believed to be important to the gating of the active site and essential to luciferase light-emitting activity (Li and Tu, 2005). Mapping statistically significant destabilizing selection for power to be at the C-terminal onto a linearized version of the 3-D structure of luciferase (Fig. 3), reveals the presence of statistically significant destabilizing selection occurring in the α258 to α286 region. While a better understanding of the property 'power to be at the C-terminal' is needed to elucidate the role that selection on this property plays in the regulation of light emmitance, we reason that the multiple occurrences of significant selection within the α258 to α286 region of luciferase provides further evidence that this mobile loop region may be important to the luciferase light-emitting activity. Additionally, detection of multiple instances of destabilizing selection associated with key features (three turn helix, four turn helix, and hydrogen bonded turns) of the luciferase secondary structure signify that these regions may be interacting with the active site of luciferase and as such may also be critical in the emission of light, though further analyses of these residues are needed to confirm this hypothesis. Further study into selection on other properties such as hydrophobicity, bulkiness, and alpha helical tendencies will reveal information on the importance of the mobile loop region of the alpha subunit of luciferase on the production of light in bacteria.

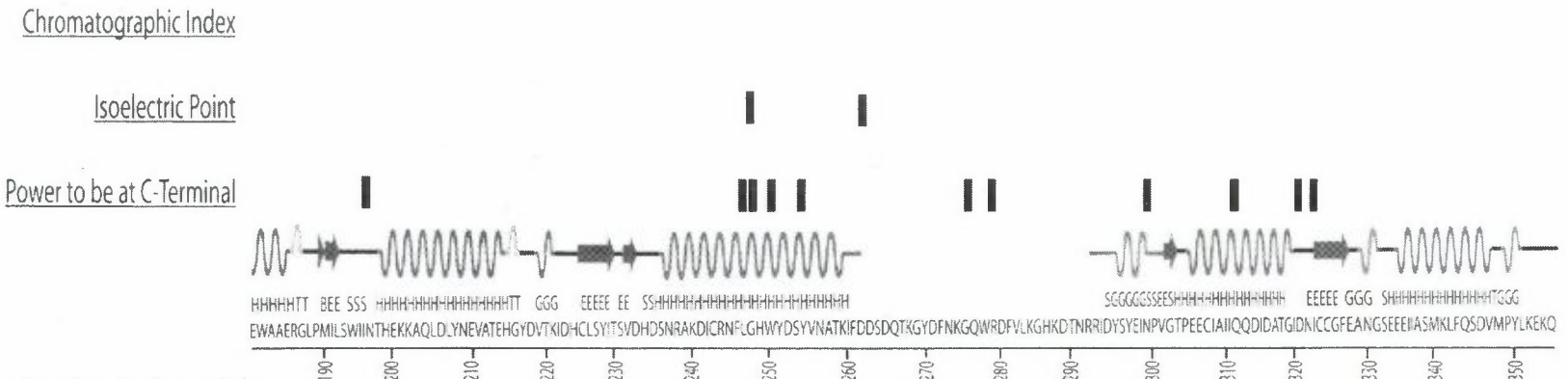
The unique ability of certain bacteria to bioluminesce compels many observers to generate scenarios for the origin and maintenance of light production in these bacteria. Each time a new explanation/hypothesis is proposed, it is assumed that bioluminescence confers some type of fitness benefit to bioluminescent bacteria, and often precludes the idea that bioluminescence might not have a direct function. In their critique of the adaptationist program, Gould and Lewontin (1979) note that evolutionary biologists tend to focus exclusively on immediate adaptations while ignoring phylogenetic legacies and constraints. As with the exemplar spandrels of St. Mark's Cathedral in Venice, bioluminescence, particularly as it exists in symbiotic relationships, provides a design "so elaborate, harmonious and purposeful that we are tempted to view it as the starting point of any analysis, as the cause in some sense to the surrounding architecture (Gould and Lewontin, 1979)." Instead, it may well be that bioluminescence in some of these species is analogous to an architectural constraint, a necessary secondary effect which originated from some other purpose or function.

Using the preceding two scenarios (bioluminescence confers some benefit to its possessor; bioluminescence as a byproduct), one can effectively evaluate numerous scenarios surrounding the evolutionary origin and maintenance of bioluminescence. If we evaluate the hypothesis that bioluminescence is used as an attractant or deterrent, we see that this hypothesis seems logical for those symbiotic bacteria that inhabit light organs of fish, but fails to account for luminescence in *Photorhabdus*. *Photorhabdus*, the bacterial symbiont of the nematode *Heterorhabditis* is typically confined to the gut of its host and the hemocoel of larval insects, with both hosts inhabiting soil environments. As the phase of *Photorhabdus* that glows is typically only found in insect cadavers, there is no intuitive benefit of glowing to attract prey or distract a predator, as all necessary resources for survival and reproduction are present in the insect cadaver.

Since *Photorhabdus* probably does not utilize bioluminescence as an attractant, an adaptationist might propose that the alternative idea of a redox sink provides a more logical explanation as to why *Photorhabdus* bioluminesces. Furthermore, one might reference the reduction in the *lux* operon of *Photorhabdus* (Fig. 1) for support of an alternative hypothesis. A reduction in the genes utilized by *Photorhabdus* to produce light could indicate that lack of need for one function (i.e. attraction or repulsion) has caused a reduction of genes in the operon through evolutionary time, and a transition of these genes to a novel function (i.e. redox sink). To test the idea of operon reduction leading to an alternative function, more taxa are needed in the present analysis beyond those bacteria that possess *lux* genes, yet do not bioluminesce. Furthermore, from a biomass perspective, the redox sink hypothesis gains credence, as greater biomass of bacteria and nutrients exist in the insect cadaver than is found in the nematode gut. Subsequent reduction of excess NADH (which has accumulated due to high biomass) to NAD^+ would cause an excess production of light, leading to the increased bioluminescence that is generally observed with *Photorhabdus* when inside the insect cadaver.

Finally, it should also be considered that bioluminescence in *Photorhabdus* has no primary function, and that the bacteria bioluminesce only because they possess the genes that allow for luminescence. Two points lend support to this idea. First is the generally accepted view that *Photorhabdus,* like the luminescent bacteria *Shewanella*, acquired its *lux* operon through horizontal gene transfer from *Vibrio* (Kasai et al., 2007). Thus, if this is a non-functional trait that has been recently acquired via





Key to Secondary Structure Codes

G = 3-turn helix
H = 4-turn helix
I = 5-turn helix
T = hydrogen bonded turn
E = beta sheet
B = residue in isolated beta bridge
S = bend

horizontal gene transfer rather than a trait that has been passed vertically over millions of years, it may not have had sufficient time, evolutionarily speaking, to have been completely lost, and as such light is still emitted without providing any real advantage to the organism. If this is the case, the intensity of light emitted might be expected to decrease over time in *Photorhabdus*, when compared to its luminescent counterparts. Experiments by Meighen (1999) lend support to this idea, finding that *Photorhabdus* emits a light intensity that is considerably lower than *Photobacterium leiognathi, Photobacterium phosphoreum, Vibrio fischeri,* and *Vibrio harveyi.*

Second is the fact that the closely related taxon *Xenorhabdus,* a bacterium which inhabits an almost identical niche (the gut of the nematode *Steinernema,* and the insect hemocoel), does not possess a *lux* operon. So if *Xenorhabdus*, which encounters similar environmental conditions within its nematode and insect hosts thrive without bioluminescing, then why would *Photorhabdus* need to bioluminesce? In this case a compensatory mechanism in *Xenorhabdus* (i.e. a redox sink analogue) could support the idea that bioluminescence as a redox sink is beneficial to *Photorhabdus.* Alternatively, absence of an analog in *Xenorhabdus* would lend support to the non-functional hypothesis. While the lack of function scenario assumes that negative selection pressure has been absent throughout the evolution of this bioluminescent bacterium, further tests evaluating the energetic costs of light production on *Photorhabdus* fitness need to be conducted to resolve this notion.

Acknowledgements

We gratefully acknowledge David McClellan for assistance with TreeSAAP and Nicole Lewis-Rogers for helpful discussion on TreeSAAP and data visualization. This work was supported in part by the United States Department of Agriculture (CSREES NRI 2005-35302-16089), and a Mentored Environment Grant from Brigham Young University.

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Figure 3. Location of statistically significant destabilizing selection (depicted as black bars) for the properties chromatographic index, isoelectric point, and power to be at the C-terminal, in relation to the amino acid sequence and secondary structure of bacterial luciferase. Destabilizing selection was detected at two distinct codon positions within residues 258 to 286, the mobile loop region (indicated by a gap in the secondary structure diagram) adjacent to the proposed active site of *lux*A. Multiple instances of destabilizing selection for power to be at the C-terminal were also found to be associated with key features of the luciferase secondary structure. Amino acid, secondary structure sequence (Kabasch and Sander, 1983), and secondary structure diagram (labeled DSSP) were obtained from the RCSB Protein Databank (Berman et al., 2000).

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