Fall 2012

One can't stand on its own: Are non-luminescence traits necessary for V. fischeri colonization of E. scolopes?

Feier Liu
University of New Hampshire - Main Campus, fhd3@wildcats.unh.edu

One can't stand on its own: Are non-luminescence traits necessary for *V. fischeri* colonization of *E. scolopes?*

Feier Liu

Advisor: Cheryl Whistler

Introduction

All higher organisms, including humans, have a constant microbiota associated with them. These interactions are typically assumed harmful as with pathogenic infections, but are often beneficial. One of the most important relationships is the mutualistic symbiosis whereby both the host and symbiont benefit. For example, *Bacteroides* in the intestines of mammals acquires nutrients from their hosts while breaking down complex polysaccharides into short chained fatty acids for the host (**1**). *Ruminococcus* in the cow's rumen digests starch while acquiring nutrients from the cow (**2**). Long-term associations are typically initiated early in life and the host must be able to recognize and select for its symbiont.

The Hawaiian bobtail squid *Euprymna scolopes* and the bioluminescent bacteria *Vibrio fischeri* make up an excellent system for symbiosis studies. *V. fischeri* is the only species that can colonize in the *E. scolopes* light organ, which reduces the chance for contamination and interference when studying the relationship. Also, the symbiotic model is tractable because both *V. fischeri* and the squid are easily manipulated in the lab. The basis of the symbiosis is the ability of *V. fischeri* to provide protective luminescence camouflage to the squid *E. scolopes* which is a target for predators in the ocean; the camouflage eliminates the shadow that the squid may cast from moonlight. At the same time, *E. scolopes* provides growth nutrients including carbon and nitrogen for *V. fischeri.* Hatchling squid do not initially have symbionts in their nascent light organs, and acquire the *V. fischeri* symbionts from sea water, which contains a plethora of bacteria. While *E. scolopes* does not have adaptive immunity, it can discriminate between the millions of bacteria it contacts to select *V. fischeri* as the light organ colonizing species (**3**).

Even though there are aspects of the symbiosis that aren't completely understood, luminescence is well characterized and known to be the main factor that is necessary in the squid-Vibrio relationship. Luminescence is directed by quorum sensing, which is a cell-to cell signaling mechanism typical of interacting bacterial species (**4**). When the bacterial population reaches high density, autoinducer (AI) molecules accumulate to a high enough concentration to activate the *lux* operon which encodes for factors necessary for luminescence. *LuxI* encodes for the AI molecule (3-oxohexanoyl l-homoserine lactone), which continues to stimulate luminescence in the colony; *luxR* codes for the *lux* gene regulator that senses AI, and turns on the rest of the lux operon; *LuxAB* encodes for luciferase, which is responsible for catalyzing luminescence; *luxCDE* encodes for proteins which are necessary for aldehyde production, which yields necessary substrate for luminescence; (**Figure 1**). Using substrates oxygen and aldehyde, luciferase emits light. In the presence of high amounts of oxygen and without luciferase, H_2O_2 may be produced which creates oxidative stress on the bacteria (**5**). Also, when luciferase is present without aldehyde, it can only partially reduce oxygen, producing O_2^- which creates oxidative stress (**Figure 2**).

In addition to luminescence, phenotypes such as resistance to host-generated oxidation (**6**), production of siderophore, sugar and nitrogen metabolism, motility, and host hemocyte binding to invading bacteria also contribute to *V. fischeri* colonization of squid. Siderophore is a soluble iron chelator that helps the bacteria gather iron in low iron level environments. Iron acquisition and utilization is essential for bacterial growth and likely contributes to symbiosis (**7**). Nutrient utilization is important for growth in the light organ (**8**) by *V. fischeri*. Motility and chemotaxis are important properties that allow the bacteria to travel to the light

organ (**9**). Squid hemocytes Squid hemocytes bind better to *V. fischeri* mutants and other bacterial species than they do to wild-type V. fischeri **(10)**, discriminating between symbionts and other bacteria.

Previous studies show that more than any other trait, luminescence is required for the symbiosis. For example, dark bacteria are never isolated from light organs of wild-caught squid. Bacteria that have the *luxA* gene knocked out, and therefore produce no light, initially colonize squid normally; but by 48 hours, the population is 2 to 4-fold reduced when compared to luminous bacteria (**Figure 3**). When dark mutants are mixed with wild-type *V. fischeri*, the population of dark mutants drop even more (**11**). Furthermore, when *luxA* mutants are the only light organ colonists, the squid continues to recruit symbionts (**12**), a behavior that is not observed in squid colonized with luminous symbionts. While there are often cheaters in other cooperative systems, the *E. scolopes* and *V. fischeri* relationship doesn't allow them; the selectivity in an organism without specific immunity is especially interesting. To date, no study has shown why dark bacteria are unfit in light organs.

There are several proposals attempting to explain bioluminescent *V. fischeri* selection. It could be a direct effect by bioluminescence, as recognized by cytochrome photoreceptors in the squid light organ. When light is absent, the squid acts to eliminate non-bioluminescent bacteria and continues to search for the appropriate symbionts to ensure that it has light for protection**(12)**. However, if this was true, then other bioluminescent bacteria could also survive in the light organ since the squid doesn't have specific immunity mechanisms. Therefore, it could also be due to other factors linked with light production that affects selection. One possibility is the reduction of oxidative stress. The luminescence process

reduces oxygen and therefore decreases the concentration of oxygen in the light organ. This protects *V. fischeri* from damage of oxygen-sensitive enzymes while the low oxygen level also making the light organ more susceptible to colonization(**11**).

Other than oxidative stress, which is part of light production, there are also other factors that affect colonization and survival of *V. fischeri*. These factors, including hemocyte binding, chitinase activity, auxotrophy, biofilm formation and siderophore production, are not directly related to luminescence process, however, it is possible that they are linked to and therefore affected by the *lux* gene. To date, there has been no definitive explanation on *V. fischeri* selection and little study on other phenotypes linked to bioluminescence.

Hypothesis: While luminescence is essential for colonization, there are other traits, such as metabolism, oxidative response, siderophore production, or hemocyte binding, necessary for survival linked to *lux* gene expression or bioluminescence that promotes symbiosis.

Objectives:

1. To identify mutations that influence luminescence
2. To identify other traits associated with those mutations
3. To develop specific hypotheses about how traits linked to light production influence decreased survival of LuxA mutants in squid.

Methods:

Objective 1. To identify mutations that influence luminescence: An existing transposon library of 2064 mutants of *V. fisheri* was pin-replicated into a clear 96 well plate and incubated until the cells reach consistent luminescence induction at 0.16-0.33OD (approximately 4-6 hours), at which time, 100 μ L of the culture was transferred into a white

96 well plate and luminescence was measured and normalized to the OD_{600} reading. Any strain that differed significantly from the wild-type for luminescence were further characterized. The genes were identified from an existing database to allow further hypothesis generation. For instance, a strain with 4-fold luminescence increase was found to have a mutation at VF_2504, encoding for putative cytoplasmic protein.

Objective 2. To identify other traits associated with identified mutations: 107 mutants were identified as having altered luminescence and were further analyzed for associated phenotypes including:

A. Oxidative resistance: *V. fischeri* was pin-replicated into LBS and growth was be monitored for four hours. The culture was then pin-replicated into one 0.03% H_2O_2 plate and three 0.05% H_2O_2 plate and the cultures were incubated overnight. Viability of cells indicated efficient response to oxidative stress.

B. Morphology: *V. fischeri* was pin-replicated onto LBS and SWT agar plates. Changes in colony morphology were noted from appearance on agar.

C. Biofilm: *V. fischeri* was incubated in microtiter plates overnight and growth determined by OD_{600}. After expelling planktonic cells, the remaining biofilm was stained with 1% crystal violet and OD_{600} was measured to estimate biofilm densities.

D. Siderophore: *V. fischeri* was pin-replicated into iron-free medium in clear 96-well plates and incubated overnight. The cells were pelleted and siderophore was determined from supernatant in a colorimetric assay of sequestration, normalized to OD_{600}.

E. Chitinase activity: *V.fischeri* was inoculated on standard shrimp chitin slurry agar and incubated for several days. Clearing of the agar indicated chitinase activity.

F. Motility: the ability of *V. fischeri* evolved strains was observed as the migration of bacteria in a low concentration of swim agar (0.5%).

G. Auxotrophy: *V. fischeri* was be replicated onto 1) glycerol minimal medium, 2) minimal medium supplemented with casamino acids, 3) minimal medium supplemented with NAG, and 4) LBS rich medium. Direct growth was observed.

Incompetency in these phenotypes marked the bacterial strain for further study on linkage between luminescence and non-luminescence traits.

Objective 3. To develop hypotheses about how traits linked to light production influence decreased survival of LuxA mutants in squid. Specific traits studies were be determined after identification of possible phenotypic linkage. Examples are:

- If siderophore was determined as an associated phenotype (e.g. if siderophore and luminescence were inversely co-regulated), I might hypothesize that less light could lead to more siderophore, introducing toxic amounts of excess iron, therefore, restoring normal siderophore production to the mutant may improve colonization. A *luxA* mutant with reduced siderophore production will be generated to observe for both luminescence and survival abilities. If the mutant performed better in colonization without increase in luminescence, then the trait was the direct effector in selection that is linked to bioluminescence.
- If biofilm formation was determined an associated phenotype (e.g. if biofilm and lunminescence were directly co-regulated), I might hypothesize that greater biofilm production could lead to greater luminescence since luminescence is increased through quorum sensing. *V. fischeri* living in a biofilm could increase the amount of

AI molecules since there will be a larger number of them together and therefore further activating the lux operon.

Results and Discussion

The current *V. fisheri* mutants library of 2064 mutants was screened using the luminescence assay. Of the current library, 107 of the mutants were found to deviate from wild-type luminescence level. Ten of these mutants exhibited lower luminescence while 97 exhibited higher luminescence. The selected 107 mutants were then analyzed for other phenotypes, including oxidative resistance, morphology, biofilm production, siderophore production, chitin utilization, motility, and nutrient utilization using various assays; and of these mutants, 58 were found to have no other defects or variations in other phenotypes. **Figure 5** shows the list of mutants with respective luminescence and genes.

Table 1. Mutants with oxidative resistance defects.

VFS	LUM	Siderophore	H_2O_2	Chitin	Motility
VFS009-E7	3.45	BLNK	50	WT	WT
VFS015-C5	0.14	LO	50	no halo	low
VFS015-D3	0.14	WT	75	WT	low

Three mutants were found to have deficiency in oxidative resistance(**Table 1**). The two mutants exhibiting lower luminescence have mutations in genes corresponding to flagellar proteins and were also deficient in motility(**Figure 5**). Since the genes matched more with the motility defect, it is more likely the bacteria's lack of movement is making it more susceptible to oxidants in its environment. Although *V. fischeri* utilizes oxygen through the light production process, which reduces oxidative threats, there is insufficient evidence to suggest that hydrogen peroxide resistance is linked to luminescence.

Table 2. Mutants with only morphology variations

VFS Plate	LUM	SWTO Morph	LBS Morph
VFS006-A1	0.17	white	striated,wrinkled
VFS009-H10	7.28	white	WT
VFS017-H9	5.51	WT	dark yellow,transparent, striated
VFS002-H9	2.87	WT	striated,wrinkled
VFS009-G2	5.87	WT	transparent
VFS018-A1	5.15	WT	transparent
VFS002-H10	2.44	WT	wrinkled
VFS009-B1	6.48	WT	wrinkled
VFS009-H7	16.06	WT	wrinkled
VFS014-H8	6.72	WT	wrinkled

One mutant, with mutation at VF_A0919, exhibited morphology differences on both SWT and LBS agar; it appeared white on SWT and striated and wrinkled on LBS agar(**Table 2**). The affected gene is long-chain-fatty-acid ligase luxE(**Figure 5**). Since this mutant exhibited lower luminescence at 17% that of the wild-type, the gene might have altered the fatty acid chains present in the cell membrane and vacuoles to affect substrate transport and luminescence transduction. However, the other mutants with altered morphologies exhibited high luminescence compared to the wild-type(**Table 2**). Many of these mutants had altered genes for transporters or protein synthesis. This could be due to higher sensitivity to auto-inducers to promote luminescence production.

Table 3. Mutatns with only biofilm production abnormalities.

VFS Plate	LUM	biofilm
VFS017-F1	2.71	high
VFS017-F7	4.83	high
VFS017-G9	3.66	high
VFS018-D6	5.29	high
VFS018-E10	3.72	high
VFS018-H8	5.86	high

Six mutants were found to be solely deviant in biofilm production and all were over-producers compared to the wild-type(**Table 3**). Most of the mutated genes were responsible for protein production(**Figure 5**). Greater biofilm production could lead to greater luminescence since luminescence is increased through quorum sensing. The density of bacteria in biofilm increases auto-inducer production and recognition, and therefore would increase luminescence production. Biofilm could also assist with colonization as it reduces the chance of ejection from the light organ.

Table 4. Mutatns with siderophore production variations.

VFS Plate	LUM	Siderophore	Motility	biofilm
VFS009-H5	3.59	HI	WT	high
VFS003-G9	5.16	HI	WT	WT
VFS021-B5	0.47	HI	WT	WT
VFS008-D7	2.48	LO	WT	WT
VFS008-F11	2.44	LO	WT	WT

There were 3 mutants with only higher siderophore production and 2 mutants with only lower siderophore production(**Table 4**). All the mutated genes were responsible for various protein productions(**Figure 5**). All the low siderophore producers also were more luminous. This could be due to that greater luminescence requires a lot of energy and proteins that are similarly needed in siderophore production. The lack of components then leads to lowered siderophore production. The 2 mutants with only higher siderophore production had completely opposite luminescence production compared to each other. This could be due to there is another phenotype that has been affected, but was not included in the phenotypic tests in this experiment.

Table 5. Mutants with deficiency in chitin utilization.

VFS Plate	LUM	Chitin
VFS009-F8	3.25	no halo
VFS013-E9	1.83	no halo

Two mutants were deficient in only in chitin utilization. Both of these mutants produced greater luminescence(**Table 5**). Similar to the siderophore case, it is possible that luminescence production requires a large amount of energy and components in protein production. The lack of amino acids then prevents chitinase production, which is the essential component in chitin utilization. There were three other mutants that were deficient in chitin utilization and other phenotypes. The only one that showed lower luminescence is a mutant which was also deficient in motility, oxidative resistance, and siderophore production; and it's the combination of all of these traits that make up the cause for luminescence deficiency.

Table 6. Mutants with altered mobility.

VFS Plate	LUM	Motility
VFS008-G2	2.85	high
VFS008-H1	2.35	high
VFS009-G1	4.64	high
VFS011-C5	7.20	high
VFS020-H7	20.50	high
VFS009-G4	2.21	low
VFS009-H8	8.34	low
VFS014-H6	6.18	low

There were five mutants with greater motility and higher luminescence and three mutants with less motility and higher luminescence(**Table 6**). This shows completely opposite correlation, therefore, no significant conclusion can be drawn from between motility and luminescence.

Table 7. Mutants with growth deficiency in minimal media.

VFS Plate	LUM	Siderophore	Motility	biofilm	CAA	NAG	glycerol
VFS003-F10	10.57	BLK	low	high	no growth	WT	no growth
VFS014-C10	10.91	WT	low	high	no growth	WT	WT

All the mutants grew in minimal media with NAG. Two mutants didn't grow in minimal media with CAA and one mutant didn't grow in minimal media with glycerol(**Table 7**). All three of these mutants were also associated with altered motility and biofilm phenotypes, therefore, there is also insufficient evidence to conclude the association between nutrient utilization and luminescence.

Conclusion

Of all the phenotypes tested for the luminescence mutants, oxidative resistance, motility, and nutrient utilization showed no direct correlation to luminescence production. Morphology could have possible associations, however, there are several details in morpohology that could be ultimately linked to colonization. Siderophore and chitin utilization are two phenotypes that are best fit for further study. High luminescence production could have utilized large amounts of energy and essential nutrients, preventing the synthesis of siderophore or chitinase. The siderophore and chitinase level then alters the mutants' behavior, ultimately affecting *V. fischeri*'s colonization abilities in *E. scolopes.*

Appendices

Figure 1. *a)* In quorum sensing, bacteria secrete autoinducers which instigate activation of *lux* gene in *V. fischeri*. b) Larger population would have stronger luminescence (13).

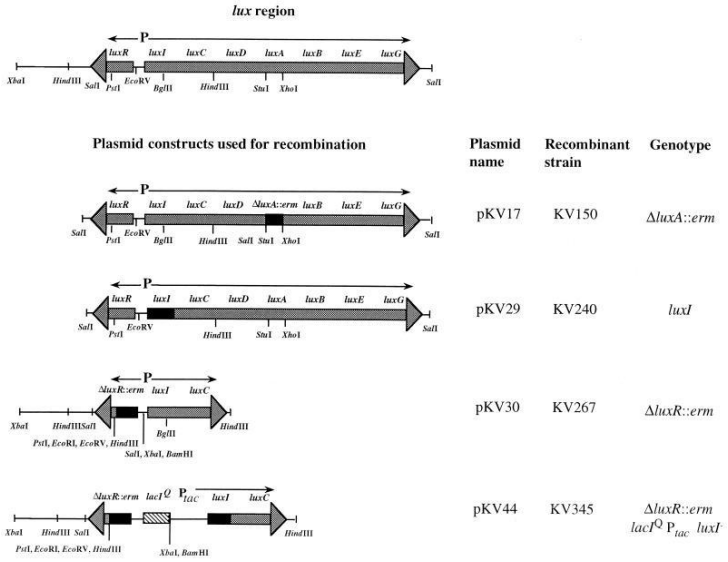

Figure 2. Black bars represent the population after 24 hours and striped bars represent the population after 48 hours. There is no significant drop in *V. fischeri* population in wild-type colonization after 48 hours. *luxA, luxI, and luxR* , all of which are dark mutants, dropped in population significantly after 48 hours (2).

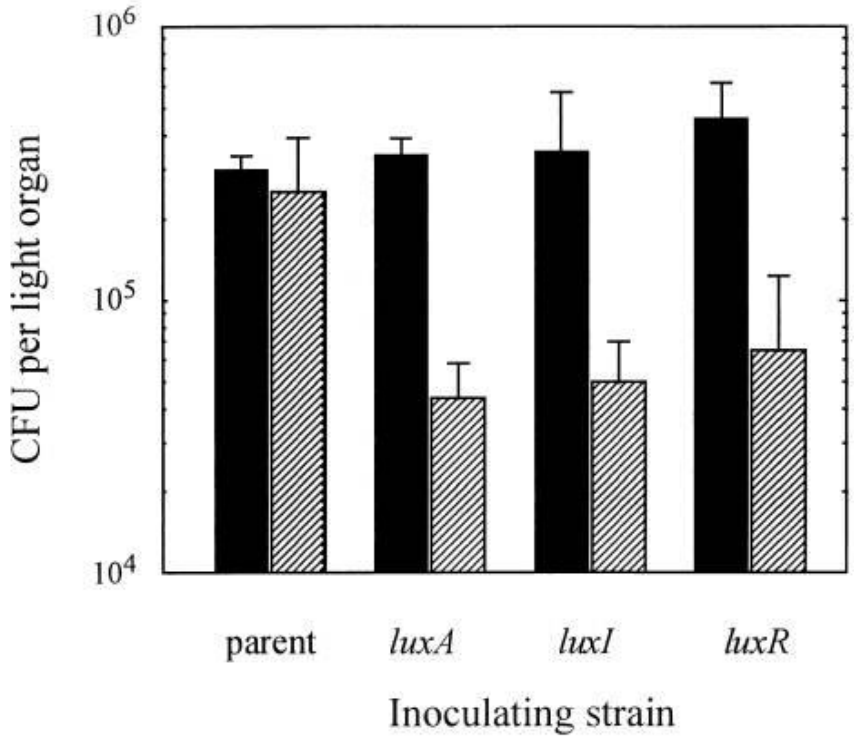

Figure 3. With aldehyde, luciferase enzyme reduces oxygen and produces light. Without aldehyde, luciferase only partially reduces oxygen to O_2^- and does not generate light (12).

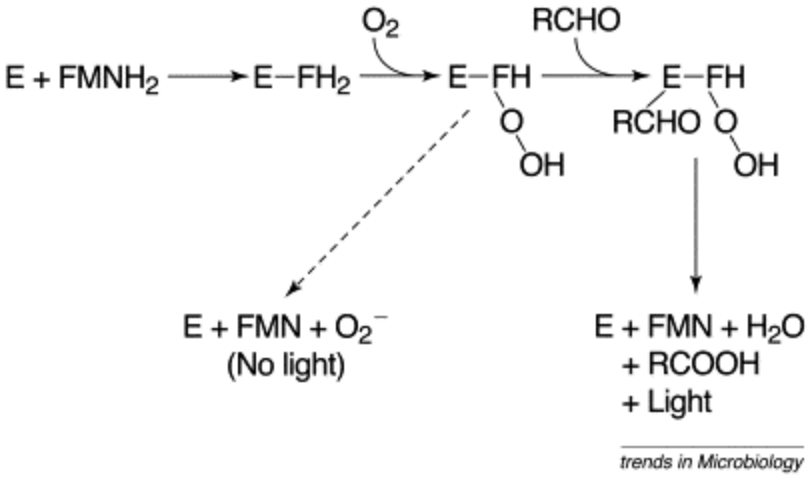

Figure 5. List of mutants with their respective luminescence level and mutated gene.

VFS Plate	LUM	Gene
VFS002-H10	2.44	methyl-accepting chemotaxis protein
VFS002-H9	2.87	dTDP-glucose 4,6-dehydratase
VFS003-C8	11.18	RNA polymerase factor sigma-54
VFS003-F10	10.57	3-phosphoshikimate 1-carboxyvinyltransferase
VFS003-G9	5.16	dTDP-glucose-4,6-dehydratase
VFS004-C1	3.30	acetolactate synthase 2 catalytic subunit
VFS006-A1	0.17	long-chain-fatty-acid ligase LuxE
VFS007-H9	10.23	cystathionine gamma-synthase
VFS008-B1	3.04	homoserine O-succinyltransferase
VFS008-B10	3.70	6-phosphofructokinase
VFS008-D7	2.48	hypothetical protein
VFS008-F11	2.44	bipartite nuclear localization signal containing protein
VFS008-G2	2.85	pilin subunit PilA
VFS008-G4	2.50	hypothetical protein
VFS008-G7	2.89	sulfate adenylyltransferase subunit 1
VFS008-H1	2.35	peptidase
VFS009-B1	6.48	tagatose-bisphosphate aldolase
VFS009-E7	3.45	n/a
VFS009-F8	3.25	DNA-damage-inducible SOS response protein
VFS009-G1	4.64	hypothetical protein
VFS009-G2	5.87	3-hydroxylacyl-(acyl carrier protein) dehydratase

VFS Plate	LUM	Gene
VFS009-G4	2.21	CheW/CheY hybrid protein CheV1
VFS009-H10	7.28	cysteine synthase
VFS009-H5	3.59	beta-D-GlcNAc beta-1,3-galactosyltransferase
VFS009-H7	16.06	dihydroxy-acid dehydratase
VFS009-H8	8.34	flagellar basal body-associate protein
VFS011-C5	7.20	DNA mismatch repair protein MutS
VFS013-E9	1.83	n/a
VFS013-G11	18.35	phosphoadenosine phosphosulfate reductase
VFS014-C10	10.91	erythronate-4-phosphate dehydrogenase
VFS014-H6	6.18	lipid A biosynthesis lauroyl acyltransferase
VFS014-H8	6.72	TadG-like protein
VFS015-C5	0.14	flagellar hook protein FlgE
VFS015-D3	0.14	flagellar biosynthesis protein FlhA
VFS017-F1	2.71	dihydroorotase
VFS017-F7	4.83	immunogenic protein
VFS017-G1	7.99	DNA-binding transcriptional regulator HexR
VFS017-G9	3.66	multidrug resistance protein A
VFS017-H3	2.89	methyl-accepting chemotaxis protein
VFS017-H6	3.69	zinc-carboxypeptidase precursor
VFS017-H9	5.51	3,4-dihydroxy-2-butanone-4-phosphate synthase
VFS018-A1	5.15	zinc ABC transporter periplasmic substrate-binding protein
VFS018-D6	5.29	methyl-accepting chemotaxis protein
VFS018-E10	3.72	n/a
VFS018-H8	5.86	hypothetical protein
VFS020-A10	7.60	transcriptional regulator PdhR
VFS020-H7	20.50	integral membrane protein involved in stabilizing FtsZ ring during cell division
VFS021-B5	0.47	C8-HSL autoinducer synthesis protein AinS

References

1. Comstock, L.E. 2009. Importance of Glycans to the Host-Bacteroides Mutualism in the Mammalian Intestine. Cell Host & Microbe, **5**:522-526

2. Klieve A., M. O'Leary , L. McMillen, and D. Ouwerkerk. 2007. Ruminococcus bromii, identification and isolation as a dominant community member in the rumen of cattle fed a barley diet. Journal Of Applied Microbiology **103**: 2065-2073.

3. Nyholm, S. and M. McFall-Ngai. 2004. The Winnowing: establishing the squid–vibrio symbiosis. **2**: 632-642

4. Visick, K. L. 2005. Layers of Signaling in a Bacterium-Host Association. J. Bacteriol. **187**:3603-3606.

5. Tu, S. Activity coupling and complex formation between bacterial luciferase and flavin reduction. 2008. Photochem. Photobiol. Sci.

6. Visick, K. L. and E. G. Ruby. 1998. The periplamic group III catalase of *Vibrio fischeri* is required for normal symbiotic competence and is induced both by oxidative stress and by approach to stationary phase. J. Bacteriol. **180**:2087-2092.

7. Graf, J. and E. G. Ruby. 2000. Novel effects of a transposon insertion in the *Vibrio fischeri glnD* gene: defects in iron uptake and symbiotic persistence in addition to nitrogen utilization. Mol. Microbiol. **37**:168-179.

8. Graf, J. and E. G. Ruby. 1998. Host-derived amino acids support the proliferation of symbiotic bacteria. Proc. Nat. Acad. Sci. U.S.A. **95**:1818-1822.

9. Millikan, D. S. and E. G. Ruby. 2004. Vibrio fischeri Flagellin A Is Essential for Normal Motility and for Symbiotic Competence during Initial Squid Light Organ Colonization. J.

Bacteriol. **186**:4315-4325.

10. **Nyholm, S., J. Stewart, E. Ruby, M. McFall-Ngai.** 2009. Recognition between symbiotic Vibrio fischeri and the haemocytes of Euprymna scolopes. Environmental Microbiology. **11**:483-493.
11. **Stabb E.** Personal Communication.
12. **Visick, K.L., J. Foster, J. Doino, M. McFall-Ngai, and E.G. Ruby.** 2000. *Vibrio fischeri lux* Genes Play an Important Role in Colonization and Development of the Host Light Organ. J Bacteriol. **182**: 4578-4586.
13. **Ruby, E. G. and M. J. McFall-Ngai**. 1999. Oxygen-utilizing reactions and symbiotic colonization of the squid light organ by *Vibrio fischeri*. Trends. Microbiol. **10**:414-20.
14. **Schuster, B., L.A. Perry, V.S. Cooper, and C.A. Whistler.** 2010. Breaking the language barrier: and experimental evolution of non-native *Vibrio fischeri* in squid tailors luminescence to the host. Symbiosis.
15. **Perez, P.D. and S.J. Hagen.** 2004. Heterogeneous Response to a Quorum-Sensing Signal in the Luminescence of Individual *Vibrio fischeri*. Nature Reviews Microbiology. 2:632-642.