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Breaking the language barrier: experimental evolution of non-native *Vibrio fischeri* in squid tailors luminescence to the host

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Abstract Although most *Vibrio fischeri* isolates are capable of symbiosis, the coevolution of certain strains with the Hawaiian bobtail squid, *Euprymna scolopes,* has led to specific adaptation to this partnership. For instance, strains from different hosts or from a planktonic environment are ineffective squid colonists. Even though bioluminescence is a symbiotic requirement, curiously, symbionts of *E. scolopes* are dim in culture relative to fish symbionts and free-living isolates. It is unclear whether this dim phenotype is related to the symbiosis or simply coincidental. To further explore the basis of symbiont specificity, we developed an experimental evolution model that utilizes the daily light organ venting behavior of the squid and horizontal acquisition of symbionts for serial passage of cultures. We passaged six populations each derived from the squid-naïve strains of *V. fischeri* MJ11 (a fish symbiont) and WH1 (a free-living isolate) through a series of juvenile squid light organs. After 15 serially colonized squid for each population, or an estimated 290–360 bacterial generations, we isolated representatives of the light organ populations and characterized their bioluminescence. Multiple evolved lines of both strains produced significantly less bioluminescence both in vitro and in vivo. This reduction in bioluminescence did not correlate with reduced quorum sensing for most isolates tested. The remarkable phenotypic convergence with squid symbionts further emphasizes the importance of bioluminescence in this symbiosis, and suggests that reduced light production is a specific adaptation to the squid.

Keywords Bioluminescence · Experimental evolution · Vibrio · Adaptive evolution · Quorum sensing

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1 Introduction

The squid host, *Euprymna scolopes,* and its bioluminescent bacterial partner, *Vibrio fischeri* (currently under consideration for reassignment to the genus *Aliivibrio* [Urbanczyk et al. 2007]) have coevolved to become highly specific in their mutualistic symbiosis (Visick and McFall-Ngai 2000). Due to its experimental tractability, this symbiosis enables the study of the bacterial and host traits that facilitate colonization, many of which parallel those important in pathogenic associations (Ruby 2008). For instance, in response to bacteria including its symbiont, squid secrete mucus, both outside the light organ to trap bacteria, and within the light organ, presumably to maintain homeostasis with the symbiont (Nyholm et al. 2002). They also mount an innate immune response during light organ colonization (Koropatnick et al. 2004; Goodson et al. 2005). These responses are not unlike what is observed during interaction of respiratory or gastrointestinal tract mucosa with invading pathogenic and commensal bacteria respectively (Mason and Huffnagle 2009; Hooper 2009; Patsos and Corfield 2009). Even with the wealth of knowledge we have gained about the colonization process, the exact basis of the specificity is not understood (Nyholm and McFall-Ngai

2004). To date, most studies to identify symbiosis factors have used random or targeted mutagenesis of *V. fischeri* (Graf and Ruby 1998; Visick and Skoufos 2001). Such mutagenic approaches have limits, including the bias towards loss-of-function mutations, the laborious nature of large-scale in vivo screens, and the fact that traits important for symbiosis may not be apparent under the culture conditions used for mutant screening, or outside the host (Yip et al. 2006). We wished to develop a complimentary approach to those currently in use that would reduce some of these biases and better utilize the squid in order to identify and characterize traits that are important for symbiosis. Here we describe the development and validation of a novel squid experimental evolution model, which has the potential to identify important and elusive traits that underlie this mutualism.

Despite the presence of millions of bacteria in natural seawater, only *V. fischeri* is able to colonize the nascent squid light organ (Ruby and Asato 1993). Within hours of hatching, uncolonized squid acquire their symbionts. During colonization, symbionts overcome a gauntlet of host innate-immune defenses, including macrophage-like hemocytes and host-derived oxidative species, as they migrate into the light organ (Koropatnick et al. 2004; Goodson et al. 2006; Davidson et al. 2004; Visick and Ruby 1998; Nyholm et al. 2009). Selection for correct symbionts continues beyond this recruitment. For instance, bacteria rapidly grow within the light organ following initiation but each day this population is curtailed as squid expel (vent) 95% of their symbionts from the light organ (Lee and Ruby 1994a, b; Boettcher et al. 1996). Thus, there are many levels of host selection that occur during the first few days of colonization. It is notable that not all isolates of *V. fischeri* are equally good at squid colonization. Native light-organ symbionts of *E. scolopes* outcompete free-living isolates (Lee and Ruby 1994b) and symbiotic strains from other squid and fish (Nishiguchi 2002; Nishiguchi et al. 1998). These findings suggest that there is much room for improvement among strains that are not squid symbionts and considerable host selection to favor adaptive evolution.

Of all the traits known to contribute to this symbiosis, none is more important than bioluminescence. As the central currency of symbiosis, bioluminescence within squid light organs is essential (Visick et al. 2000), and no dark strains have ever been isolated from squid light organs, indicating the host restricts non-luminous cheating strains by as yet unknown mechanisms that would either require the direct perception of bioluminescence (Tong et al. 2009; Stabb 2005), or the detection of the bioluminescence chemical reaction (such as oxygen consumption) (Ruby and McFall-Ngai 1999). Both light organ symbionts and free-living bacteria produce bioluminescence when in culture, at a conditional cost to maximal growth (Bose et al. 2008). However, light production occurs only at a high cell density, such as would occur in squid light organs (Boettcher and Ruby 1995), but not in a planktonic or free-living environment. Two acylated homoserine lactone (AHL) signal molecules contribute to quorum sensing induction of luminescence (Stabb et al. 2007). The first is N-3-oxohexanoyl-L-homoserine lactone (C6-HSL), the product of the LuxI synthase that is encoded by the first gene in the luminescence (*lux*) operon. Binding of the C6-HSL to its sensor LuxR allows binding to the lux box which activates expression of *luxI* and the entire *lux* operon. The second is N-octanoyl-L-homoserine lactone (C8-HSL), the product of the AinS synthase which cross-talks with the C6-HSL sensor LuxR and with its own sensor pathway to activate a positive regulator of the *lux* operon, LitR (Fidopiastis et al. 2002). Natural symbionts of *E. scolopes* are consistently less luminous in culture than isolates from other *Euprymna* squid, *Loligo* squid, fish, as well as planktonic environments, and this characteristic of dim luminescence also correlates with competitive dominance in the light organ (Nishiguchi et al. 1998). However, when even less than normal luminescence is produced by mutants of the natural symbiotic strain ES114 through altered quorum sensing they are less capable of persistent colonization (Lupp et al. 2003) similar to dark derivatives. The reason for the competitive dominance of naturally dim symbiotic strains, as well as the conservation of functional bioluminescence among non-symbiotic strains remains a mystery.

Our natural experimental evolution model of symbiosis capitalizes on the horizontal acquisition of symbionts from the environment and the squid's daily light-organ venting cycle to serially passage populations of *V. fischeri*. Our approach emulates that used by Lenski and colleagues whereby replicate lines of *Escherichia coli* are serially passaged under controlled culture conditions, allowing the observation of bacterial evolution in real time to study its reproducibility and its mechanisms (Riley et al. 2001; Lenski and Travisano 1994). By periodic archiving of samples of the evolving populations we can quantify the dynamics of bacterial adaptation and infer the progression and mechanisms of adaptation. Experimental evolution has been applied to host-microbe models, most notably for viral and bacterial pathogens (Chao et al. 2000; Cooper 2007; Elena et al. 2008; Nilsson et al. 2004; Ebert 1998). In our approach, the squid, not the experimenter, acts as the selective agent on the bacterial populations. Using this approach, we expect that populations of *V. fischeri* that are not squid symbionts would adapt to the squid and become superior symbionts relative to their ancestors. Further, we predicted that the mechanisms by which these non-squid-specific populations adapted would shed new light on how

the host selects bacterial traits to allow this mutualism to function.

We chose as the subjects of our study two strains of *V. fischeri* naïve to squid: WH1 (Lee 1994; Wollenberg and Ruby 2009), a free-living isolate from a region lacking in potential hosts and thus almost certainly not a symbiont, and MJ11 (Ruby and Nealson 1976; Mandel et al. 2009), a fish symbiont. Both strains contrast with the natural symbionts of *E. scolopes* in that they produce in excess of 1,000-fold more luminescence in culture. We predicted that as populations of the naïve strains were passaged in the squid, they would evolve phenotypes that would more closely mirror those of the natural symbionts. If such changes occurred in multiple replicate evolved populations, this phenotypic convergence would be a strong indicator that the traits are adaptive (Harvey and Pagel 1991). Six populations of each strain were serially transferred in parallel through 15 hatchling squid, yielding an estimated 290–360 bacterial generations, at which time the bioluminescence of several individual isolates from each line were observed relative to their ancestor and the native symbiotic strain *V. fischeri* ES114. In each evolved population, we observed a striking reduction in luminescence both in vitro and in vivo that, except for two out of 6 isolates tested, appears un-related to altered quorum sensing. This rapid evolutionary convergence among replicate populations in the trait most tied to the mutualism not only validates the efficacy of our model but also suggests that further characterization of the newly evolved symbionts may reveal previously unknown host and bacterial mechanisms that underlie the function of the mutualism.

2 Materials and methods

2.1 Bacterial strains and culture conditions

Three strains of *V. fischeri* were used in this study: i) the reference strain ES114 (Boettcher and Ruby 1990; Ruby et al. 2005), isolated from the Hawaiian bobtail squid *Euprymna scolopes*, ii) MJ11 (Ruby and Nealson 1976; Mandel et al. 2009), isolated from the Japanese pinecone fish *Monocentrus japonicus*, and iii) WH1, isolated (Lee 1994; Wollenberg and Ruby 2009) from seawater near Woods Hole, MA, where no known animal hosts of *V. fischeri* live. Unless otherwise noted, individual colonies of *V. fischeri* were cultured in liquid seawater-tryptone broth (SWT) containing 0.5% tryptone (Difco, Detroit, MI), 0.3% Bacto™ yeast extract (Difco), and 0.3% glycerol in 70% Artificial Seawater (ASW) (Instant Ocean, Aquarium Systems, Mentor, OH) (Boettcher and Ruby 1990), in SWT containing an additional 150 mM NaCl (SWTO) to enhance luminescence production (Bose et al. 2007), or in LBS medium containing 1% tryptone, 0.5% yeast extract and 2% NaCl (Fisher) in a 20 mM Tris-HCl (Fisher) buffer (pH 7.4) (Graf et al. 1994) at 28°C with shaking at 200 rpm. Solid medium for enumeration of colonies was produced by adding 7.5% agar to either SWT or LBS. For storage, strains were grown in LBS liquid medium and frozen at −80°C with 20% glycerol.

2.2 Squid serial transfer and isolation of evolved representatives

Hatchling squid used in this study were from single clutches produced by adult Hawaiian bobtail squid. Clutches are removed from the adult tanks within 48 h and maintained individually with frequent water changes to dilute contaminating bacteria present in the adult tanks. At hatching, the juvenile squid are promptly removed, triple rinsed by transfer in filter sterilized ASW, and then held overnight in individual vials containing 3 mL ASW. Prior to inoculation, luminescence emmited from each squid was measured to confirm that contaminating bioluminescent bacteria had not colonized.

Single colonies of *V. fischeri* MJ11, WH1 and ES114 were used to inoculate 3 ml of SWT broth, and grown at 28°C with shaking until they reached an optical density OD_{600} of 0.2–0.4. The bacteria were diluted to 3,000 (WH1 and ES114) or 20,000 (MJ11) CFU/mL in 50 mL filter sterilized ASW as confirmed by plating on LBS agar. Failure of MJ11 to colonize squid at this inoculum has been previously reported (Mandel et al. 2009), but here following the initial inoculation, we found that squid which were not luminous at 24 h were colonized based on luminescence at 72 h. Ten juvenile *E. scolopes* were placed collectively into the inoculum or into uninoculated ASW for 16 h, and then transferred to individual scintillation vials containing 3 mL ASW (Fig. 1). Luminescence was measured at 24, 48, and 72 h to confirm that treatment squid were colonized and uninoculated control squid remained uncolonized.

Four squid were housed in each 24-well microtiter plate (Costar). Two animals were experimental and received evolving *V. fischeri* populations; these were in alternating rows with two aposymbiotic (non-colonized) control animals to allow detection of cross-contamination (Fig. 1). Over the course of the experiment, no aposymbiotic control animals became colonized with luminescent *V. fischeri*. Following venting of their light organs on two subsequent days after their initial placement in the 24 well plates, squid were transferred into an adjacent well to rinse and then moved to new well containing 2 mL ASW. After venting on the fourth day, squid were rinsed, removed and frozen at −80°C. Half of the ventate (1 mL) from each individual animal was added to 2 mL ASW in a 20 ml scintillation vial and a newly-hatched uncolonized animal was placed in

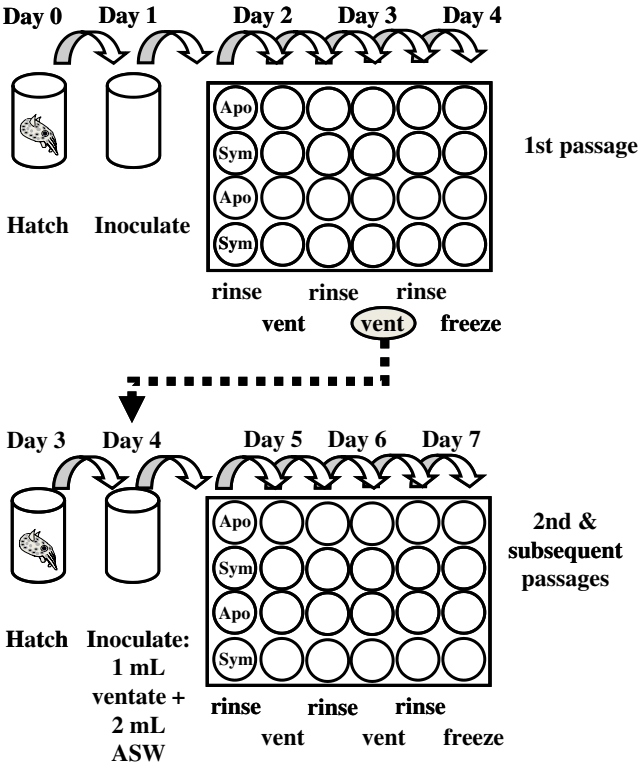

Fig. 1 Schematic of the squid transfer process. The transfer process at initial colonization and subsequent squid passages using ventate is shown for one experimental plate housing two treatment animals and two aposymbiotic control animals. See text for details

inoculum derived entirely from the light organ of the previously passaged squid ventate. The other half of the ventate was frozen in 15% glycerol at −80°C to archive the bacterial population. The transfer cycle was repeated 15 times for each bacterial treatment. Although some bacterial lines went extinct during the course of the experiment due to premature squid death or unavailability of juvenile squid, a minimum of six lines for each strain were propagated in parallel for all 15 squid passages.

On a few occasions, it was necessary to stop the evolution experiment when squid were not available on the third passage day. In these instances, the ventate was frozen in 15% glycerol to allow restarting at a later date. When squid again became available, the frozen ventate was thawed, the cells pelleted by centrifugation for 2 min at maximum speed, the pellet rinsed briefly with 1 mL ASW to remove residual glycerol, and the cells were re-pelleted. Finally, the cells were suspended into a final volume of 3 ml ASW, and a newly hatched, uncolonized squid was placed in this mixture per normal transfer protocol.

To isolate evolved representatives, 10 μl of each frozen ventate was plated onto SWT agar, and isolated colonies that were phenotypically distinguishable as *V. fischeri* based on distinctive colony size, color, and morphology were re-streaked for isolation. Only two other colony morphotypes arose from plating squid ventate: a small colorless clear colony type, and a wrinkly opaque white colony type. These colony types have never been isolated from light organs and are easily distinguishable from *V. fischeri*. All colonies we identified as *V. fischeri* were confirmed bioluminescent in culture. Some strain identities were also confirmed by sequencing of the *gapA* gene, which has been used for within-species phylogeny of *V. fischeri* (Nishiguchi et al. 1998), or by PCR using *gapA* primers that we designed specifically to strain type. A total of five isolates per line were stored as representatives of the population.

2.3 Calculation of populations sizes

We estimated the number of generations during each squid transfer using published data on the dynamics of the squid colonization process (Nyholm and McFall-Ngai 2003; Wollenberg and Ruby 2009). Nyholm and colleagues observed that 1% of the bacterial inoculum accumulates as an aggregate outside the light organ, and within 2–3 h this entire aggregate migrates into the three pores that lead to three crypts on each side of the bi-lobed light organ (Nyholm and McFall-Ngai 2003). Based on these observations, with a typical inoculum size of 15,000 CFU/5 mL per squid in our system, we might expect that 150 bacteria would initiate colonization of each side of the light organ (for a total of 300 bacteria). Wollenberg and colleagues studied the frequency with which light crypts were colonized by a single type or both types from a mixture of bacteria harboring two different fluorescent labels, and concluded that colonization of each of the six crypts was initiated by as few as 1–2 bacteria regardless of the size of the inoculum (for a total of 6–12 bacteria per squid) (Wollenberg and Ruby 2009). Following colonization by ES114, the population size in light organs reach an estimated 10^5 CFU by 12 h, prior to venting 95% of its population at dawn (Boettcher et al. 1996; Lee and Ruby 1994a, b). Within several hours, this population recovers to its maximum size.

The founding population (F) is estimated to be either 12 or 300 cells, the carrying capacity (K) of the juvenile light organ is 500,000 cells (derived from data herein) and the population post-venting 95% of its light organ (P) is 25,000 cells. We calculated the total number of bacterial generations for each squid transfer as:

$$\text{Day 1 generations} = \log_2(K/F)$$

$$\text{Day 2 and 3 generations} = \log_2(K/P)$$

We estimated the effective population size (N_e) as the harmonic mean of the population sizes at each step in the

selection cycle. Given the number of generations (i) and the population size at each generation (N_i), effective population size was calculated as:

$$N_e = i/(\{1/N_1\} + \{1/N_2\} + \ldots + \{1/N_i\})$$

2.4 Serial passage in culture

A mass action culture evolution in seawater based defined media was developed as a control to the squid evolution. The regime replicates the number of generations during each growth cycle in the squid following colonization and after each venting event (as determined empirically by direct plating of the cultures) and introduces similar bottlenecking seen in the squid during venting and colonization initiation of each new squid. *V. fischeri* MJ11, WH1 and ES114 were conditioned at 28°C overnight in 1X ASW supplemented with 10 mM Hepes, 0.333 mM K_2HPO_4, 18.7 mM NH_4Cl, 0.0144% Case Amino Acids, and 0.53 mM Glucose. Six replicate lines for each ancestor were serially passaged at 6, 12 and 24 h for 15 days by transferring 2 μl of culture into 2 mL of fresh culture evolution media. The culture evolution was incubated at 28°C with shaking. After the 15th day of culture evolution, each line was plated on SWT plates and colonies were randomly selected as population representatives. There were no colony morphology variants observed. Relative luminescence of the culture evolved population representatives was determined in SWTO liquid broth using the Tecan Infinite M200 plate reader equipped with luminescence detection (Tecan, Durham NC).

2.5 Luminescence quantification

Luminescence was quantified using a Turner 20/20 luminometer (Turner Designs, Sunnyvale CA). Quantitative luminescence was measured in squid (n=3–5), 48 h post-colonization, first directly on squid housed in individual vials and then following homogenization of squid in 100 μl ASW which releases the bacteria from the light organ and oxygenates the bacteria, which would increase luminescence if the reaction were oxygen limited. The number of bacteria within the squid was enumerated by colony counts of dilutions plated onto LBS agar plates, following overnight incubation. The data from three experiments were examined for block effects by a one-way ANOVA and once we determined there were none, the data combined, presented as the mean, and analyzed by independent sample T-tests with 95% confidence intervals (SPSS Statistics V. 17.0).

To quantify luminescence induction in vitro, overnight cultures of *V. fischeri* were inoculated into SWTO broth at 0.5% and grown with shaking, and aliquots were removed at 30 min intervals to determine the luminescence and OD_{600}. For some experiments N-3-oxohexanoyl-L-homoserine lactone (C6-HSL) (Sigma) and N-octanoyl-L-homoserine lactone (C8-HSL) (Sigma) were added to SWTO at a final concentration of 120 nM. The experiment was repeated with similar results, and one representative experiment is presented.

2.6 Induction of luminescence by AHLs in conditioned broth

To assess the production of AHLs by the various strains, we used luminescence produced by *V. fischeri* ES114 following growth in broth conditioned with culture supernatant from the ancestral and evolved strains as an indication of AHLs present in the culture supernatant. Conditioned broth was prepared by growing bacterial cultures to a final OD_{600} of 3.0 in SWTO, pelleting cells by centrifugation at 12,000 × *g*, and filter-sterilizing the cleared supernatant by passage through a 0.2 μm filter. The supernatant was combined 1:1 with fresh SWTO, and 200 μL of an overnight culture of *V. fischeri* ES114 was inoculated in 15 mL in 125 mL flasks. Luminescence and OD_{600} was measured from aliquots at 30 min intervals. As controls, luminescence produced by ES114 in unconditioned SWTO media, and broth conditioned with supernatant from either *V. fischeri* ES114 or CL24 ($AinS^-$ $LuxI^-$) (Lupp and Ruby 2005). The experiment was repeated with similar results, and the data from one representative experiment is presented.

2.7 Competitions in squid

The squid inocula were made as previously described but in 1:1 ratio of a single AVL and a DVL strain from population 6 confirmed by direct plating. Seven squid for each replicate competition were placed in inoculum for 12 h, and then removed and rinsed in fresh ASW. Light organ homogenates were plated as previously described, and then 50 colonies from each squid were patched on SWTO media to determine their visual luminescence phenotype. The relative competiveness index RCI was calculated as the (final ratio, wild-type CFU/mutant CFU)/(initial ratio, wild-type CFU/mutant CFU).

3 Results

3.1 Development of a squid experimental evolution model

Our experimental evolution design approximates the natural acquisition of the symbiont from the environment, its growth within the light organ, and its re-colonization of newly-hatched squid at each generation. Notably, beyond

preparation of the initial inoculum, culture-based methods were not used during evolution in the squid. This model incorporates natural selection on the bacterial population at several stages, including but not limited to i) the initiation of colonization of juvenile squid in competition with other bacteria, ii) growth within the light organ, iii) persistence in light organs following venting, iv) survival in seawater following venting (the planktonic lifestyle), and v) initiation of a new round of colonization following adaptation to a squid light organ. Thus, as strains that are not squid symbionts must colonize the juvenile squid and persist throughout the squid venting behavior, our model favors increased competitiveness in several traits relevant to the symbiosis. Moreover, evolved traits are likely not artifacts of culture methods as nearly all population growth has occurred in the context of the squid host.

To interpret the observed evolutionary patterns in this system, we estimated both the total number of generations and the effective population size during each cycle. It should be noted that estimates of these values are complicated in our model by natural fluctuations in the size of the founding population, the proportion of vented bacteria, as well as the capacity of the light organ of each squid. Based on two reports that have specifically approximated the number of bacteria that initiate each colonization event (Nyholm and McFall-Ngai 2003; Wollenberg and Ruby 2009), both using the native symbiont strain ES114, we have estimated that in each squid passage there are between 19 and 24 generations, with an effective population size between 84 (if the founding population were 12) and 1928 (if the founding population were 300). The total number of generations that occurred in each population following 15 transfers is therefore approximately 290–360 generations. Since our design models the natural symbiosis cycle with ES114 and no estimations have been made with strains that are not squid symbionts, the actual number of bacterial generations that occurred with these strains is ambiguous, and our calculations are only meant to serve as a general approximation.

3.2 Adaptation of squid-naïve strains to the squid light organ resulted in reduced luminescence in culture

To study adaptation by *V. fischeri* to symbiosis with squid, we evolved a fish symbiotic strain, MJ11, and a free-living strain, WH1, and as a control the natural symbiont, ES114, through exclusive serial squid passage. These ancestral non-native strains differ phenotypically from the natural squid symbiont, ES114, perhaps most notably by their much greater luminescence in vitro. Following transfer through 15 squid, we observed remarkable convergent evolution towards reduced luminescence in evolved populations. Five isolates from each of six independent experimental lines of MJ11 and WH1 (a total of 60 isolates) were visually inspected for luminescence on SWTO agar and designated as either Ancestral-level Visibly Luminous (AVL), Decreased but Visible Luminescence relative to ancestor (DVL) and Not-Visibly Luminous (NVL). Within each of the six evolved MJ11 populations, NVL colonies arose, and in four lineages all isolates were NVL (Table 1). For four evolved WH1 populations, a DVL colony type arose. MJ11 population 6 was passaged through two additional squid for 17 passages total and the DVL phenotype arose and fixed in the population.

Although the rapid rise and convergence of mutants with decreased luminescence in multiple populations suggests hard selection of squid symbiosis for reduced luminescence as an adaptive trait (Harvey and Pagel 1991), preferential venting of less fit symbionts (Millikan and Ruby 2004) could also lead to the fixation of less fit strains with reduced luminescence during ventate to squid transfer due to their high proportion in ventate. Although this is unlikely due to the fact that less competent symbionts can be outcompeted even when presented at 100-fold higher concentration in starting inoculum (Millikan and Ruby 2004), there remains the possibility that the DVL and NVL types are less fit than AVL. To test the fitness directly, we competed in 1:1 ratio a DVL and AVL isolate from the same population (population 6). If the DVL isolate was less fit it would be outcompeted by the AVL isolate, which was not the case (AVL RCI = 0.103; 95% confidence ± 0.116).

To further characterize the reduced luminescence of the evolved strains, we quantified the in vitro luminescence of three squid-evolved isolates from each strain relative to their ancestral strains and the wild-type squid symbiont, ES114 (Fig. 2). We selected three isolates from different populations for WH1 and MJ11 evolved lines that had DVL

Table 1 Percentage of luminescent colony types from experimentally evolved lines

Ancestor	Line	%AVL	%DVL	%NVL
MJ11	1			100
	2			100
	3			100
	4			100
	5		20	80
	6	40	60	
WH1	1		100	
	2		100	
	3		100	
	4	100		
	5	100		
	6	80	20	

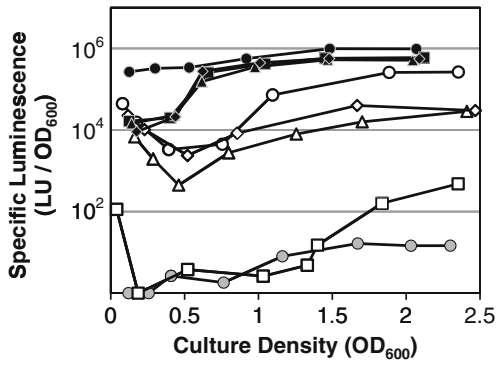

Fig. 2 In vitro Luminescence of wild-type and evolved isolates. The luminescence of ES114 (●), ancestral MJ11 (○), MJ11:1-1 (□), MJ11:4-1 (Δ), MJ11:6-1 (◊), ancestor WH1 (●), WH1:1-3 (■), WH1:2-2 (▲), and WH1:3-2 (♦) grown in SWTO was determined throughout the growth cycle

or NVL phenotypes where they represented the majority of reduced luminescent colonies. The fish symbiont, MJ11, is 10,000-fold more luminous in culture than ES114 (t=6.136, p<0.001). In contrast, evolved isolate MJ11:1-1 was nearly 1,000-fold less luminous than its ancestor (t=6.134, p< 0.001). Isolates MJ11:4-1 and MJ11:6-1 evolved 10-fold decreases in luminescence (t=5.823, p<0.001; t=5.346, p< 0.01). Free-living ancestral strain WH1 is even brighter than MJ11, over 50,000-fold more luminous in culture than ES114 (t=3,465, p<0.001). Three isolates from independent lines derived from WH1 (WH1:1-3, WH1:2-2, and WH1:3-2) evolved a more subtle ~ two-fold reduction of luminescence in vitro (t=12.782, p<0.001; t=12.532, p< 0.001; t=12.263, p<0.001). For ES114, the luminescence of populations either did not differ from that of ancestor ore increased subtly, but significantly by ~2–3.5 fold (four populations).

The average maximal luminescence levels of MJ11, WH1, and ES114 evolved in mass action cultures as a control did not change from ancestral levels. Only one isolate from the MJ11 culture evolution had a significant change in luminescence level, and was ~7-fold brighter than the ancestor (t=6.092; p=0.001). Two ES114-derived isolates evolved in culture became significantly brighter, one isolate was ~17-fold brighter (t=9.666; p<0.05), and the other was ~8-fold brighter (t=19.98; p<0.001). No individual WH1-derived isolates evolved in culture differed significantly from its ancestor in luminescence.

3.3 Changes in quorum sensing are not the predominant mechanism for convergent reduction of luminescence in evolved lines

Because variation in AHL production or perception can influence luminescence levels in the laboratory (Visick et al. 2000; Lupp and Ruby 2005) and among natural isolates of *V. fischeri* (Gray and Greenberg 1992), we reasoned that changes quorum sensing could lead to rapid adaptation and optimization of light production for life in the squid light organ. To evaluate changes in AHL production by evolved lines, we monitored the timing and level of luminescence induction by ES114 grown in broth conditioned by the various ancestral and evolved strains (Fig. 3). Ancestral MJ11 and WH1 produce higher levels of AHL than ES114 as indicated by the 1,000-fold greater luminescence induction of the reporter ES114 in broth conditioned by MJ11 or WH1 compared to ES114. Altered AHL production does not appear to be the predominant mechanism of reduced luminescence for isolates evolved in the light organ. Only MJ11:1-1 induced luminescence at a level similar to ES114. Isolate MJ11:4-1 also appears to have a delay in its ability to induce ES114. In contrast, isolate MJ11:6-1 and all lineages derived from WH1 induce ES114 luminescence equally well as their ancestors. This suggests that evolved reduction in luminescence is not due to AHL reduction in most evolved isolates; therefore, our data indicates that adaptation to the squid in this system occurred by one or more mechanisms other than AHL production.

Next we inspected changes in the response to AHL by monitoring luminescence induction with the addition of synthetic C6-HSL (Fig. 4). Isolate MJ11:1-1 produced less luminescence in response to the addition of C6-HSL than its ancestor, indicative of a reduced ability to sense or respond to AHLs. Isolates MJ11:4-1 and MJ11:6-1 responded similarly to the ancestor. All WH1 evolved isolates were unchanged in their response to exogenous AHLs. The response of all evolved strains to synthetic C8-HSL, which is a less potent inducer of luminescence (Lupp et al. 2003), was indistinguishable from that of the ancestor (data not shown).

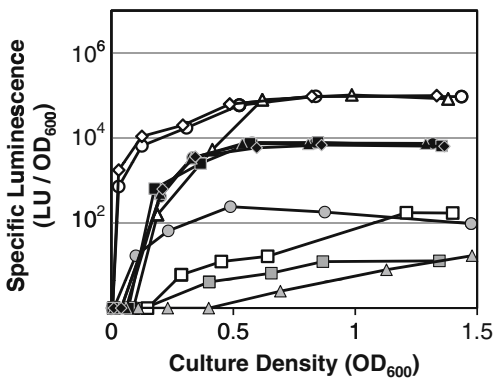

Fig. 3 Luminescence induction of *V. fischeri* ES114 by AHL-conditioned broth from wild-type and evolved isolates. Specific luminescence of ES114 in unconditioned SWTO broth (▲), and in broth conditioned by ES114 (●), CL24 (an *ainS*, *luxI* double mutant) (■), ancestor MJ11 (○), MJ11:1-1 (□), MJ11:4-1 (Δ), MJ11:6-1 (◊), ancestor WH1 (●), WH1:1-3 (■), WH1:2-2 (▲), and WH1:3-2 (♦)

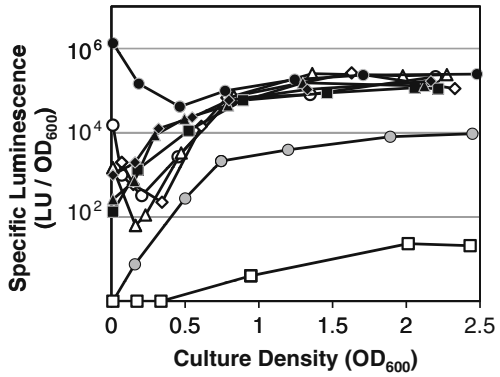

Fig. 4 Luminescence response of wild-type *V. fischeri* and evolved isolates to synthetic C6-HSL. The luminescence of wild-type ES114 (●), ancestor MJ11 (○), MJ11:1-1 (□), MJ11:4-1 (Δ), MJ11:6-1 (◊), ancestor WH1 (●), WH1:1-3 (■), WH1:2-2 (▲), and WH1:3-2 (♦) grown in SWTO supplemented with 120 nM C6-HSL was determined throughout the growth cycle

3.4 Evolved populations produced significantly less luminescence in vivo

Although changes in luminescence in vitro were readily apparent and quantifiable, a truer measure of adaptation to the squid is luminescence in vivo. Therefore, we compared the luminescence per bacterial cell of each isolate and its ancestor in culture to that produced in colonized squid (Fig. 5). A notable characteristic of ES114 is that it produces significantly more light per cell within squid light organs than in culture (Boettcher and Ruby 1990). Ancestral MJ11 also produces more light in squid than in culture, and while isolates derived from MJ11 produced less luminescence in vivo (MJ11:1-1 t=9.013, p<0.001; MJ11:4-1 t=6.647, p< 0.001; MJ11:6-1 t=6.768, p<0.001), the differences were less dramatic than those in vitro. In contrast, luminescence per cell of the WH1 ancestor was similar in culture to that in colonized squid, but evolved WH1 lineages all significantly decreased luminescence in vivo (WH1:1-3 t=3.847, p< 0.001; WH1:2-2 t=3.481, p<0.001; WH1:3-2 t=3.763, p< 0.001). In fact, for isolates evolved from WH1, the reduction of light per bacterial cell was even greater in squid (~50 fold) than in culture (~2 fold). All of the WH1 evolved lines produced similar levels of luminescence after squid homogenization, which introduces oxygen to the already induced culture, suggesting that even the very bright ancestors are not limited for oxygen in the host, and that the additional reduction of light by WH1 in light organs is not caused by the squid restricting oxygen in the light organ.

4 Discussion

Little is known of the traits that distinguish squid symbionts from closely related non-symbiotic strains, but phylogenetic and phenotypic studies of *V. fischeri* and their hosts strongly suggest a history of coevolution (Nishiguchi et al. 1998; Nishiguchi 2002). To aid in the discovery of factors that contribute to specificity, we developed a natural model of experimental evolution in the squid. We then used this model to test whether two strains that are not squid symbionts and also differ substantially from symbiotic strains in luminescence, could evolve adaptations to this host. Six populations derived from each of the two strains were exclusively passaged through 15 juvenile squid for an estimated 290–360 bacterial generations, a number that pales in comparison to the time that natural symbionts coevolved with the host. Even so, evolved isolates from multiple lineages commonly produced less luminescence than their non-symbiont ancestors, suggesting that this phenotype is a direct and rapid adaptation to the squid symbiosis lifestyle (Harvey and Pagel 1991).

Arguably, our utilization of the natural squid transfer process relatively free from experimenter manipulation makes the resulting adaptation more relevant to the symbiosis and could have reduced the number of mutations arising as experimental artifacts, but we also recognize there are some limitations to our model. Firstly, we chose to use ventate for each round of colonization rather than culture symbionts from light organs because it mirrors the natural colonization process and reduces experimenter and

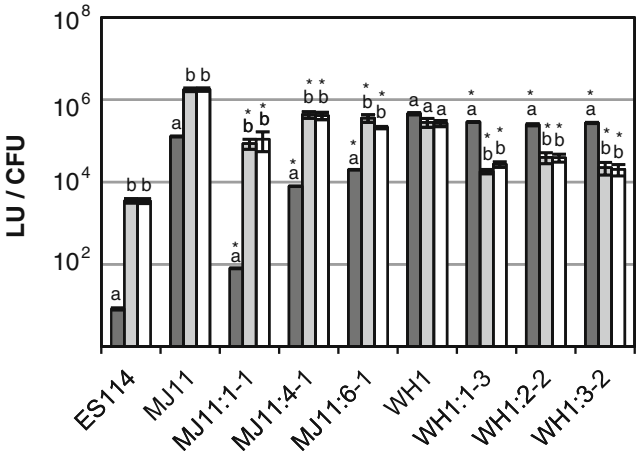

Fig. 5 Specific luminescence in vitro and in situ of wild-type and evolved lines. The maximum luminescence normalized to CFU of isolates in culture (*dark grey bars*) was determined in SWTO at full induction. The luminescence of isolates in live squid (*light grey bars*) and from the same squid following homogenization (*white bars*) was determined at 48 h post-colonization and was normalized to CFU determined from direct colony counts of serially diluted and cultured homogenate. The mean from three replicate experiments is presented. *Error bars* represent SE. *Different letters* represent significant statistical differences of luminescence in culture versus in light organs for each isolate. *Same letters* indicate there is no significant difference based on independent sample T-tests (95% confidence intervals). *Asterisks* represent significant differences (p<0.05) from the specific ancestor

culture selection. This ventate contains 95% of the light organ culture that has adapted over a 3 day period, but it may not represent all light organ symbionts equally well. For instance, some beneficial mutations arising late in the transfer cycle could be lost from the population through retention of certain symbionts (Sycuro et al. 2006; Millikan and Ruby 2004). However, it is important to note that such preferential venting during the first 2 days would lead to a sweep resulting in fixation of the beneficial mutation in the light organ population, and a majority or even all of the vented bacteria thereafter would contain this light organ adapted symbiont. Because we are interested not only in isolates that are steadfast light organ residents, but also those who survive in seawater and successfully reinitiate colonization, our design balances these different abilities that may affect adaptation to the squid. But, relying on a squid-generated inoculum also introduces uncertainty to estimates of the effective population size and complicates our analysis of the evolution process. Nevertheless, this uncertainty would not be resolved even if the inoculum size were controlled by the researcher because the exact relationship between inoculum and founding population size is under debate (Nyholm and McFall-Ngai 2003; Wollenberg and Ruby 2009). Additionally, the founding population size may vary by strain and even change within a lineage as it evolves. We also chose not to mark strains prior to evolution despite their utility for head-to-head competitions because markers deemed neutral at the onset of the experiment may not stay neutral throughout the evolution process (Riley et al. 2001; Cooper 2007). We reasoned that any mutation arising during the process of adaptation could be used as a molecular signature for later competition, or if necessary the strains could be marked at the conclusion of evolution. We recognize that some mutations or phenotypes that arise may not be adaptive, as would occur if some traits linked genetically to different beneficial traits hitchhiked to high frequency, or if the beneficial mutations themselves tended to be pleiotropic. Careful and detailed confirmation of the role of any trait in the symbiosis will be necessary. Fortuitously, in this first experiment, both sets of populations converged with each other and with the natural symbiont in their level of luminescence, and the fact that luminescence is the best known symbiosis trait provides important validation. Through direct competitions with isolates from the same population, we show here that strains with reduced luminescence are more fit than those with ancestral luminescence levels. We believe that the minor limitations especially concerning the dynamics of the adaptive process are balanced by the unbiased power of the model to identify important mechanisms of symbiosis.

Although the dynamics of the bacterial population remain under study, we can nevertheless infer the magnitude of the adaptation by each lineage relative to their non-symbiont ancestors. The rate of adaptation depends upon the availability of the rarer fraction of mutations that are beneficial and ultimately depends on the mutation rate of the organism (in *V. fischeri* we estimate this at $\sim 10^{-9}$ per base pair based on spontaneous resistance to rifampicin [Whistler and Schuster, unpublished]), the population size, and the number of generations. Further, the probability that a mutation will become fixed is influenced by the relative selective value of the mutation and the degree of bottlenecking of the populations, which strongly influences the effective population size. Populations that periodically cycle through small population bottlenecks, such as *V. fischeri* colonizing squid or pathogens founding new infections, are effectively nearer the size of the bottleneck than the population maximum between bottlenecks. The most probable adaptive phenotype is the reduced luminescence of multiple lines from each evolved population. The fact that several lines converged on the luminescence level of the native symbiont suggests either that mutations enabling this phenotype are extremely common or that their relative advantage is substantial. We first consider their probable relative advantage. Given the approximate number of generations that occurred in each evolved population (290–350) and two reasonable estimates of the number of bacteria that colonize hatchling squid (12 or 300), we can infer s, the selective value of the adaptive mutations. The number of generations required for a mutation to rise to a frequency of 95%, g, is estimated as:

$$g = 2/s^{*}\ln(\mathrm{N_e})$$

where $\mathrm{N_e}$ is the effective population size, estimated as between 83 and 1,928 cells (Lenski 1991). It follows that mutations (either singularly or as combinations of mutations) of at least 2.5 to 5% benefit would account for their rise from one cell to 95% of the population by 15 passages. However, this estimate presumes that such mutations are immediately available in each population, which is doubtful given the very small estimated $\mathrm{N_e}$. Small populations are unlikely to contain beneficial variation, especially when the per-genome rate of all mutations is $\sim 10^{-3}$ (mutations per bp, $10^{-9,}$ × genome size, 4×10^{6} bp). Further, continued severe population bottlenecks will likely purge variation, even when beneficial, and produce heterogeneity among different populations because of the stochastic effects of bottlenecks and mutation. In light of these potential constraints on adaptation and especially on the parallelism of the evolved response, and the fact that lines of MJ11, WH1, and ES114 evolved in seawater based culture conditions did not reduce in luminescence, it seems more likely that adaptive traits rise to fixation nearly immediately following their appearance in the population

owing to selective advantages much greater than 5%. If this were the case, we would expect that the timing of the evolution of reduced luminescence would vary among populations, and our preliminary assessment of isolates from earlier transfers supports this inference (Schuster and Whistler, unpublished data). To resolve the important questions of how rapidly squid symbiosis may evolve within *V. fischeri* and the strength of selection imparted by the squid, we are characterizing the adaptive dynamics within each population in greater detail and plan to directly measure the fitness advantage of evolved populations over their ancestors in the light organ.

To examine a potential physiological mechanism of increased luminescence both in vitro and in vivo, we explored altered quorum sensing. Although other mechanisms have been identified such as ArcA (Bose et al. 2007), few mechanisms discovered in culture contribute substantially to altered luminescence in vivo. In contrast, altered signal production or signal perception could rapidly decrease luminescence levels in vivo (Visick et al. 2000; Lupp et al. 2003). We found that some but not all isolates were altered in quorum sensing ability (Figs. 3 and 4). Specifically, for the evolved WH1 isolates signal production did not change and only one evolved MJ11 line, MJ11:1-1, had a lower maximum induction than the ancestor indicative of reduced signal production. This same one isolate (MJ11:1-1) appears to have reduced response to synthetic AHL, and thus also may be deficient in perceiving the signal. If reduced signal production alone were the cause of the reduced luminescence, we would expect exogenous AHL to fully restore this strain to ancestral levels (Visick et al. 2000), which it did not. Conceivably a defect in LuxR or its binding site upstream of the *luxI* promoter (the lux box) could lead to both phenotypes (Visick et al. 2000) as LuxR is a positive regulator of C6-HSL production, but these data are also consistent with the production of a soluble co-repressor or inhibitor of luminescence. All other isolates were fully induced by exogenous AHL. We also investigated luminescence production of each isolate in squid light organs, and confirmed that each isolate that had reduced luminescence in culture was also less luminous in squid relative to the ancestor (Fig. 5). All three WH1-evolved isolates that were only modestly reduced in luminescence in culture compared to ancestor (~2 fold), were even further reduced in luminescence in light organs relative to ancestor (~50 fold), reinforcing the importance of the squid host for providing appropriate cues for luminescence regulation that were not emulated in culture. Luminescence of the MJ11 ancestor was brighter per cell in squid than in culture, and although overall luminescence decreased, this dynamic remained the same for evolved isolates. Notably, the mechanism for luminescence reduction of MJ11 derived lines is likely due to changes that are not dependent on the squid host environment, whereas the most significant reduction of luminescence in WH1-evolved lines is in response to the squid. Although we have only begun to characterize the underlying mechanisms for these phenotypes, our results reveal that the lineages evolved diverse mechanisms of reduced luminescence that are not limited to changes in quorum sensing regulation. Because there are multiple mechanisms at play, the phenotype is likely not a pleiotropic artifact of another beneficial phenotype, but rather a direct adaptation resulting in improved squid symbiosis.

The rapid adaptive evolution of reduced luminescence through serial squid passage reveals that it is not simply the ability to produce enough light but also not too much light that is important in this symbiosis. It is widely held that the role of bioluminescence is to counter-illuminate and camouflage the squid, providing protection from predation during its nocturnal behavior, a premise which is supported by the fact that the squid host matches the luminescence output to ambient moonlight (Jones and Nishiguchi 2004). Thus, the evolution of the mutualism would depend upon the presence of predators that provided natural selection for luminous squid. Even if predators selected for this association, at this stage in their highly evolved association, squid-*Vibrio* interactions select directly for the optimized luminescence. This is certainly supported by the fact that non-luminous strains are compromised in their ability to maintain their host colonization, even when mixed with luminous strains in squid reared in the laboratory without predators (Visick et al. 2000). It would appear that squid not only restrict the growth of dark strains that 'cheat' by not producing light but also select for those that do not produce too much light. By characterizing the mutations that underlie these phenotypes, we hope not only to identify the mechanisms by which the bacterium becomes a symbiont, but also to measure the selective forces of the squid light organ that favor these adaptations. Although our focus has been to understand the molecular and biological mechanisms that underlie this symbiosis, ours as well as other experimental models should prove useful for the study of ecological questions such as host range expansion, competitive exclusion, and invasion. Ultimately, understanding how microbes rapidly expand their host range in this model system could help enlighten the mechanisms that pathogenic Vibrios and even other bacterial species employ to infect a host.

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