



pH Adaptation Drives Diverse Phenotypes in a Beneficial Bacterium-Host Mutualism

Neda Nourabadi¹ and Michele K. Nishiguchi^{2*}

¹ Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, MI, United States,

² Department of Molecular and Cell Biology, School of Natural Sciences, University of California, Merced, CA, United States

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*Correspondence:

Michele K. Nishiguchi
nish@ucmerced.edu

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Abiotic variation can influence the evolution of specific phenotypes that contribute to the diversity of bacterial strains observed in the natural environment. Environmentally transmitted symbiotic bacteria are particularly vulnerable to abiotic fluctuations, given that they must accommodate the transition between the free-living state and the host's internal environment. This type of life history strategy can strongly influence the success of a symbiont, and whether adapting to changes outside the host will allow a greater capacity to survive in symbiosis with the host partner. One example of how environmental breadth is advantageous to the symbiosis is the beneficial association between *Vibrio fischeri* and sepiolid squids (Cephalopoda: Sepiolidae). Since *Vibrio* bacteria are environmentally transmitted, they are subject to a wide variety of abiotic variables prior to infecting juvenile squids and must be poised to survive in the host light organ. In order to better understand how a changing abiotic factor (e.g., pH) influences the diversification of symbionts and their eventual symbiotic competence, we used an experimental evolution approach to ascertain how pH adaptation affects symbiont fitness. Results show that low pH adapted *Vibrio* strains have more efficient colonization rates compared to their ancestral strains. In addition, growth rates had significant differences compared to ancestral strains (pH 6.5–6.8, and 7.2). Bioluminescence production (a marker for symbiont competence) of pH evolved strains also improved at pH 6.5–7.2. Results imply that the evolution and diversification of *Vibrio* strains adapted to low pH outside the squid improves fitness inside the squid by allowing a higher success rate for host colonization and symbiotic competence.

Keywords: symbiosis, pH, adaptation, vibrio, beneficial

INTRODUCTION

pH is biologically a potential environmental stress, which can influence the behavior and subsequent physiological adaptations of bacteria to changing environments. For most microorganisms, the ability to change both phenotypically and genetically to abiotic stress and evolve new adaptive mechanisms is crucial (Hoffmann and Sgro, 2011; Cremer et al., 2012; Casacuberta and González, 2013; Lenski et al., 2015; Aussel et al., 2016). Variation in environmental factors such as low pH is likely to induce new physiological changes that impact microbial fitness

and induce mechanisms to cope with this challenge (Casacuberta and González, 2013; Soto and Nishiguchi, 2014; Payne et al., 2016). Most of the time environmental stress provides a trigger for bacterial adaptation and diversification by enhancing their ability to respond quickly and persist throughout those conditions (Lenski et al., 1991; Barrick and Lenski, 2013; Dragosits and Mattanovich, 2013; Brunke and Hube, 2014; Liu et al., 2015). In some cases, adaptation and the subsequent genetic changes can be observed within a few hundred generations, with substantial consequences for fitness of those bacteria. Recently, experimental evolution studies have become a powerful tool to understand functional and structural adaptive changes in bacteria that occur in a short amount of time (Kawecki et al., 2012; Barrick and Lenski, 2013). Microbes are an ideal experimental evolution subject due to their short generation times and large population sizes that can be observed under various conditions simultaneously (Cordero and Polz, 2014). Direct measurements of the competitive fitness of an evolved descendant can be compared to its ancestor *in vivo* and *in vitro* and quantitatively assessed. Additionally, the continual generation of evolved strains are stored frozen (fossil records) and later compared to their respective ancestors— an advantage of using microbes for monitoring evolution progress under various selective pressures that would normally lead to greater diversity in the natural environment (Hindré et al., 2012; Dragosits and Mattanovich, 2013; Wiser et al., 2013).

The beneficial association between sepiolid squids and their *Vibrio* bacteria provides an experimentally tractable model system for the study of the experimental evolution, adaptation, and diversification of these symbionts as they transition between their free-living and symbiotic state (Schuster et al., 2010; Soto et al., 2012, 2014; Soto and Nishiguchi, 2014). During the symbiosis, *Vibrio* bacteria are exposed to periodic acidification while inside the adult host crypts of the light organ during the night cycle due to the high oxygen consumption from bioluminescence activity (Pan et al., 2015; Schwartzman et al., 2015). This change in light organ pH represents a physiological challenge for *V. fischeri* while it resides inside the squid host. *Vibrio* bacteria that can accommodate this change must be able to have a responsive acid-adaptation mechanism for the symbiotic association to be maintained as well as in new *Vibrio* infections. Given the importance of how adaptation to stressful conditions in the surrounding habitat can be co-opted to increase fitness for other cellular functions while in symbiosis, we investigated whether *V. fischeri* that are pre-adapted to low pH outside the squid are better able to colonize and persist in squid light organs. We subjected three different strains of *V. fischeri* for 600 generations at different pH levels and monitored the physiological changes (bioluminescence, production, and growth) in 10 independent evolving lines grown at their respective pH levels. Ancestral strains (control) were compared to evolved strains *in vitro* to determine their fitness prior to animal colonization. These evolved strains were then subjected to juvenile squid colonization assays to determine whether *in vitro* adaptation to low pH affected the ability of *V. fischeri* to perform better when inside squid light organs.

MATERIALS AND METHODS

Experimental Evolution

Three strains of symbiotic *V. fischeri* were experimentally evolved under various pH concentrations: *V. fischeri*, ES114 (version: ES114-L; host *Euprymna scolopes*, from Kaneohe Bay, Hawaii), *V. fischeri* ET401 (host *E. tasmanica*, from Townsville, QLD, Australia), and *V. fischeri* EM17 (host *E. morsei* from Tokyo Bay, Japan) at pHs of 6.5, 6.8, 7.2, 7.6, and 8 for 600 generations (Table 1). Based on a pilot study, *V. fischeri* at pH 5.5 and 6 were unable to survive *in vitro* and were not examined further. pH levels for this study were chosen to represent acidic conditions of the light organ (6.5–7.6) and a control pH of 8.0 (seawater pH). For biological significance, 10 independent lines of clonal isolates from each strain were experimentally evolved simultaneously. Chromosomal insertion of the chloramphenicol resistance marker Cam^R into the genomes of *V. fischeri* ET401 and EM17 were made with a mini-Tn7 cassette. The Cam^R cassette was used as a selective agent for discerning between ancestral (WT-control, no Cam^R) and evolved strains (carrying Cam^R). *V. fischeri* JRM200, an isogenic strain of *V. fischeri* ES114-L with the mini-Tn7 Cam^R, was obtained from earlier studies (McCann et al., 2003; Soto et al., 2012). Briefly, *E. coli* strain CC118λpir carrying pEVS104 and another strain of *E. coli* BW23474 carrying pUX-BF13 (Visick and Ruby, 1997; McCann et al., 2003) were combined with the recipient *V. fischeri* strain in a tri-parental mating-bacterial conjugation step and incubated for ~12 h at 28°C. The conjugation mixture was then re-suspended in SWT [seawater tryptone; per liter composition: 700 mL of seawater (30 g of Instant Ocean and 10 g of Marine Mix, 300 mL of dH₂O), 5 g tryptone, 3 g yeast extract, 3.75 mL 80% glycerol] media and spread onto SWT-agar plates (15 g agar/L) with chloramphenicol (20 µg/mL). *V. fischeri* JRM200, ET401 (Tn7 Cam^R), and EM17 (Tn7 Cam^R) were then streaked onto SWT agar plates media with chloramphenicol (20 µg/mL), then incubated at 28°C for 18–24 h. Individual clonal isolates (single colonies) were transferred to 5 mL of SWT with chloramphenicol (20 µg/mL) liquid medium with the selected pH levels and placed in a 28°C shaking incubator set at 225 rpm for 8–12 h. When growth reached OD_{600nm} = 0.5, 10 µL of each culture was transferred (1:500 dilution) to 5 mL of fresh SWT media with chloramphenicol (20 µg/mL) at their respective pH. Experimental evolution was sustained in 18 × 150 mm glass test tubes kept in an Innova[®] 43R incubator shaker (New Brunswick, Enfield, CT) at 28°C, 225 rpm.

Previous studies using experimental evolution on *E. coli* (Wielgoss et al., 2013) illustrated how most mutations under selective pressure occur during the exponential phase of bacterial growth; therefore, all 10 lines of the clonal isolates from the three strains at all pH levels were transferred at the end of log phase. For consistent timing and ease of calculations, culture transfers were completed every 12 h at a 1:500 dilution, correlating to the end of log phase, but prior to stationary phase. Generations of evolving bacteria were determined by calculating the specific generation times of *V. fischeri* JRM200, ET401 (Tn7 Cam^R), EM17 (Tn7 Cam^R). Calculations were completed assuming that bacterial growth is a first-order chemical reaction, where $\mu =$

TABLE 1 | *Vibrio fischeri* strains used in the study.

Strains	Description	Host Squid	Location
Ancestor/Wild-type strains (WT)			
ES114 ^a -WT	Ancestor (control)	<i>Euprymna scolopes</i>	USA (Kaneohe Bay, O'ahu, Hawaii)
ET401-WT	Ancestor (control)	<i>Euprymna tasmanica</i>	Australia (Tasmania)
EM17-WT	Ancestor (control)	<i>Euprymna morsei</i>	Japan (Tokyo Bay)
Evolved strains with selective marker Tn7 and Cam^R			
JRM200 ^b	Mini-Tn7 *Cam ^R	Previously constructed ^c	
ET401-Tn7	Mini-Tn7 *Cam ^R	Previously constructed ^c	
EM17-Tn7	Mini-Tn7 *Cam ^R	Previously constructed ^c	

*Cam^R, Chloramphenicol Resistance.

^aVersion: ES114-L.

^bDerived from ES114-L.

^c(McCann et al., 2003).

$(\ln N - \ln N_0) / (t - t_0)$ and $g = \ln 2 / \mu$, where μ represents the growth rate, N is the number of cells at time t , N_0 is the number of cells at t_0 , and g is the generation time (Soto et al., 2009). Using the previously calculated generation time of each strain for all five pH levels, evolutionary “frozen fossil records” were generated by freezing down cultures with 20% glycerol final volume every 100 generations, and storing each in 2 mL cryo-vials (Wheaton, NJ) at -80°C . To check for contamination and experimental progress, 50 μL of each culture that was frozen was also spread on SWT-Cam agar plates and examined for the viability of strains through growth studies the following day. All cultures were continually transferred to fresh SWT-Cam (20 $\mu\text{g}/\text{mL}$) at their respective pH levels. Frozen fossil records were collected at 0, 100, 200, 300, 400, 500, and 600 generations. For the experimental calculations we used three replicates from each of the 10 lines of frozen fossil records of the evolved strains from each 100th generation. Each pH treatment had 90 samples (three replicates \times 10 lines \times three strains).

Growth and Bioluminescence Assays

All ten lines of evolved bacterial strains at 100, 200, 300, 400, 500, and 600 generations and the ancestral (control) strains were grown in SWT liquid media with pH levels adjusted to of 6.5, 6.8, 7.2, 7.6 and 8 by acetic acid (99.7+% A.C.S reagent, Sigma-Aldrich, St. Louis, MO) and measured with an Orion PerpHecT pH Meter (Model 330—Thermo Scientific, Waltham, MA). pH had no negative affect on the antibiotic used throughout this study for all evolved isolates.

Cultures were shaken at 225 rpm at 28°C overnight, then sub-cultured at a 1:500 dilution into new SWT media for all strains at all pH levels measured. All samples were grown to $\text{OD}_{600\text{nm}}$ of 0.3 at 28°C and shaken at 225 rpm in an Innova[®] 43R incubator shaker (New Brunswick, Enfield, CT). Cells were inoculated in 5 mL SWT media at 1:10 dilution in triplicate (3 independent bacteriological test tubes (glass, 18 \times 150 mm) per each time point/pH levels) for a 12-h growth study. Reading of all triplicate samples were taken every hour by Spectramax spectrophotometer (Molecular Devices, Sunnyvale, CA). The

term Relative Light Unit-RLU for bioluminescence measurement is used since luminometers typically do not yield a measurement directly in units of photons. During the measurements, the Luminoskan Ascent luminometer (Thermo Scientific, Waltham, MA) was set to one light unit and all other measurements were made relative to the set value. Bioluminescence (Relative light units, RLUs) was measured simultaneously every hour with a Luminoskan Ascent luminometer (Thermo Scientific, Waltham, MA). Growth rates of samples were determined by calculating the generation time of each bacterial strain during each growth study (Soto and Nishiguchi, 2014). The factors of Relative Light Unit (RLU) measurement per generation at specific pH levels for each strain were used in a two-way ANOVA analysis for statistical analysis of the bioluminescence assay. GraphPad Prism (8.0.0) software was used for all analysis ($p < 0.001$, $N = 10$).

For growth assays, the two-way ANOVA analysis (GraphPad Prism, 8.0.0) were conducted using values of the growth rate per strains (evolved and ancestral bacteria) in various pH levels at different generations ($p < 0.001$, $N = 10$).

Host Co-ionization Assays

To determine whether pre-adaptation to low pH impacts animal host colonization compared to non-evolved strains, infection assays were performed as previously described (Nishiguchi, 2000; Chavez-Dozal et al., 2014). For single and competition infection experiments, overnight cultures of the 600 generation wild-type (control) ES114, EM17, ET401 and evolved JRM200, ET401-Tn7, EM17-Tn7 strains were regrown in 5 mL of fresh SWT medium at their respective evolved pH until they reached an $\text{OD}_{600\text{nm}}$ of 0.3. Cultures were then diluted to $\sim 1 \times 10^3$ CFU/mL in 5 mL of sterile seawater and added to glass scintillation vials where newly hatched juvenile squids were placed (one individual/vial). Three independent plates of the same inoculation seawater with each respective strain were plated for all samples, and CFUs were counted the next day for the accuracy of the initial inoculum. Seawater (pH = 8) was changed with fresh non-sterile seawater (without bacteria) every 12 h over a period of 48 h. Animals were maintained on a light/dark cycle of 12/12. After 48 h colonization (48 h = standard colonization assay time), animals were sacrificed and homogenized, and the diluted homogenate was plated onto SWT agar plates for both wild-type and evolved *V. fischeri*, and SWT with chloramphenicol (20 $\mu\text{g}/\text{mL}$) for the *V. fischeri* evolved strains. A second set of animals were selected for competition studies, where juvenile squids were co-infected with the ancestral strain (WT-control) and their respective evolved strain, sacrificed after 48 h, homogenized, and the diluted homogenate plated onto SWT and SWT with chloramphenicol (20 $\mu\text{g}/\text{mL}$) agar plates. The concentration of CFUs was the same as the prior set of studies as well as in the competition studies, with the combined total being the same CFUs as the single infections (5000 CFUs total). Colony forming units (CFUs) were counted the next day to determine colonization efficiency of each strain. For calculation accuracy, the plates containing SWT with chloramphenicol (20 $\mu\text{g}/\text{mL}$) were subtracted to quantify evolved strains colonies from wild-type (control). This was due to the fact that only the evolved strains have the capacity to grow on agar plates containing chloramphenicol (20 $\mu\text{g}/\text{mL}$).

whereas both ancestral and evolved will grow on SWT without chloramphenicol. A total of 15 animals/strain were used for each competition assay, and five non-infected (aposymbiotic) juveniles were used as negative controls for each sample run. We chose evolved strains at pH 7.2 since these had the most significant change compared to all other pH concentrations measured, and due to our limited supply of animals for the study. Results of single colonization and competition study were analyzed using two-way ANOVA analysis with GraphPad Prism (8.0.0). The factors of analysis for this study are calculated CFU/Light Organ values per strains for both single colonization and competition studies ($p < 0.001$ and $N = 15$).

RESULTS

Growth Rate of Ancestor and Evolved Strains Under Various pH Conditions

Evolved *V. fischeri* strains JRM200, ET401-Tn7, and EM17-Tn7 at 600 generations demonstrated significantly different growth rates at pH 6.5, 6.8, and 7.2 when compared to their respective ancestral strains (T = 0) ES114-WT, ET401-WT, and EM17-WT at the same pH (Figures 1A–F). The two-way *post hoc* ANOVA analysis demonstrated significant effects for pH levels 6.5, 6.8, and 7.2 among all bacterial strains. The *post-hoc* ANOVA interaction analysis between various pH levels for different generations of bacterial strain shown significant differences ($p < 0.001$, $N = 10$). pH 7.2 shown significant differences at 400, 500, and 600 generations in comparison to pH 6.5, 6.8, 7.2, and 8. pH 7.6 and pH 8 shown no significant differences among each other, but there were significantly different with other pH levels. pH 6.5 and 6.8 were shown no significant differences among each other, but there were significantly differ among other pH levels at different generations ($p < 0.001$, $N = 10$).

Bioluminescence

Bioluminescence increased from 300 to 600 generations with significantly higher luminescence at 600 generations in relative light units for evolved *V. fischeri* strains JRM200, ET401-Tn7, EM17-Tn7 at pH 6.5, 6.8, and 7.2 when compared to their ancestral strains at the same pH (Figures 2A–C). The two-way *post hoc* ANOVA analysis demonstrated significant effects for all pH levels among all bacterial strains with respect to the Relative Light Unit (RLU) of bioluminescence. The *post-hoc* ANOVA interaction analysis between various pH levels for different RLU measurements per generation of each bacterial strains showed significant differences. pH 7.2 showed significant differences at 400, 500, and 600 generations in comparison to pH 6.5, 6.8, 7.2, and pH 8. pH 7.6 and 8 showed no significant difference among each other, but there were significantly different with other pH levels. pH 6.5 and 6.8 had no significant difference among each other, but they were significantly different among other pH levels at different generations ($p < 0.001$, $N = 10$).

Animal Studies

Single Infection Assays

To examine the effect of pH adaptation prior to symbiosis, colonization studies between ancestral *V. fischeri* ES114-WT,

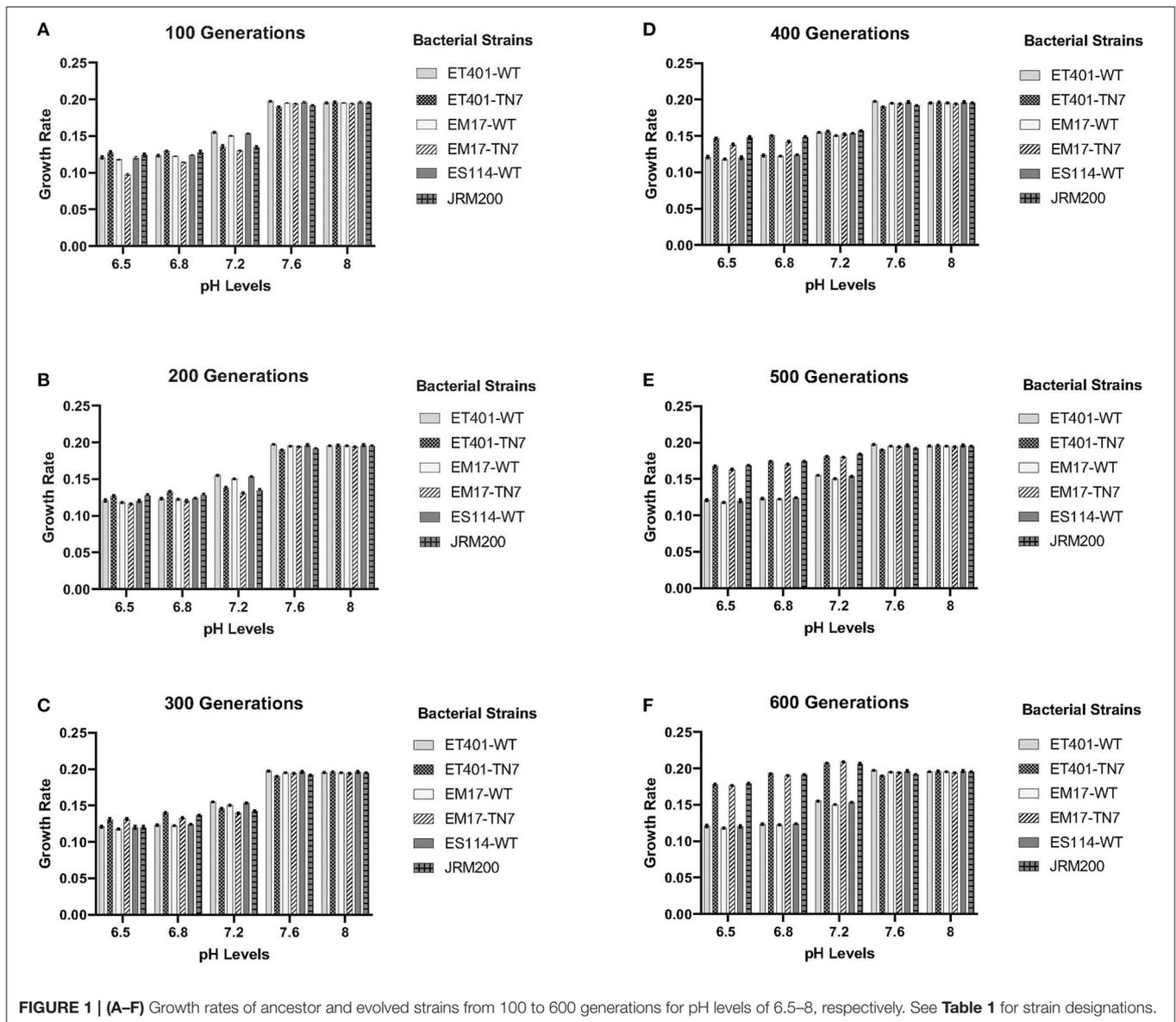
ET401-WT, and EM17-WT and evolved JRM200, ET401-Tn7 (Cam^R), and EM17-Tn7 (Cam^R) strains at 600 generations at pH 7.2 were completed in the Hawaiian bobtail squid *E. scolopes*. All strains demonstrated significant differences between the ancestor and evolved strain ($p < 0.001$; Figure 3A). Native Hawaiian *V. fischeri* ES114 exhibited higher colonization rates than non-native strains ET401 and EM17 in *E. scolopes* for both ancestral and evolved strains. Evolved strains ET401-Tn7 and EM17-Tn7 at 600 generations have the ability to colonize non-native *E. scolopes* light organs, but are significantly lower than either ancestor or evolved native ES114 strains (Figure 3A). Overall, single strain infections indicate that native strains (both ancestor and evolved) colonize their specific squid host preferentially, but non-native *V. fischeri* strains (EM17 and ET401 ancestor and evolved) are also capable of colonizing a non-native squid host (Figure 3A). All aposymbiotic Hawaiian bobtail squid *E. scolopes* had zero CFUs during colonization study. For ancestral and evolved strains, two-way *post hoc* analysis of variance (ANOVA) for factors of CFU/light organ and strains (evolved and ancestral at 600 generations/pH 7.2) showed significant effect of strain ($p < 0.001$, $N = 15$).

Animal Competition Assays

Competition studies of ancestral *V. fischeri* ES114-WT, ET401-WT, and EM17-WT and evolved JRM200, ET401-Tn7 (Cam^R), and EM17-Tn7 (Cam^R) of *V. fischeri* at 600 generations (pH 7.2) showed significant differences between all three strains examined (Figure 3B). Evolved native Hawaiian strain JRM200 and ET401-Tn7 (Cam^R) at 600 generations had higher colonization rates than non-native evolved strain EM17-Tn7 (Cam^R) at 600 generations in *E. scolopes*. Additionally, each of the evolved strains outcompeted their ancestral strain (JRM 200, ET401-WT, and EM17-WT; Figure 3B). *V. fischeri* EM17-WT had the lowest colonization rate of all strains examined during competition with its ancestor (Figure 3B). All aposymbiotic Hawaiian bobtail squid *E. scolopes* had zero CFUs during colonization study. For all ancestral and evolved strains, a two-way analysis of variance (ANOVA) for factors of CFU/light organ and bacterial strains (evolved and ancestral at 600 generations/pH 7.2) showed significant effects of strain type ($p < 0.001$, $N = 15$).

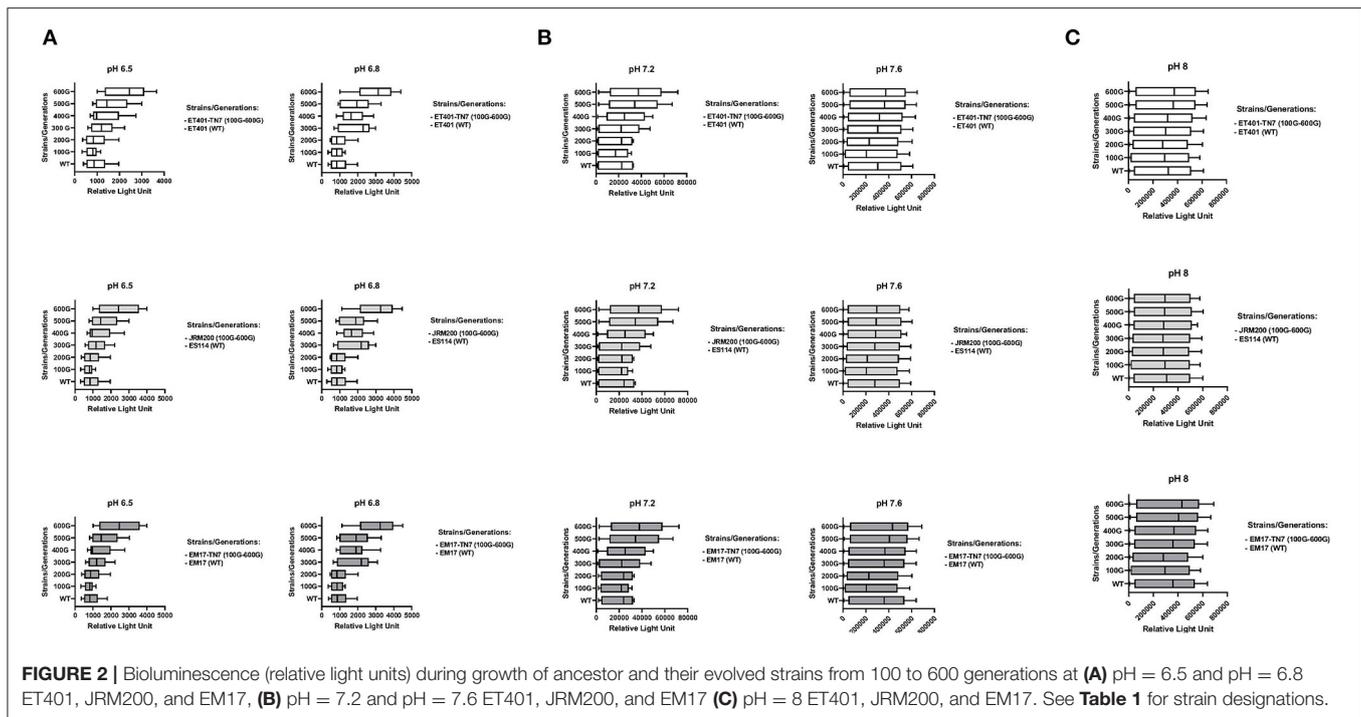
DISCUSSION

In this study, pH adapted strains of *V. fischeri* were evolved to 600 generations in order to examine how pH adaptation would influence colonization and subsequently symbiotic fitness. Adaptation of *V. fischeri* JRM200, ET401-Tn7, and EM17-Tn7 was not significant until 400 and up to 600 generations for all evolved strains. Since there were no specific measurements of acid resistance to the mildly acidic environment during the study, these results only represent adaptation under various pH growth conditions *in vitro* and subsequently when these evolved isolates were challenged inside the squid light organ *in vivo*. Our results suggest that *V. fischeri* strains that are evolved under low pH conditions *in vitro* gained the ability to better colonize juvenile squids *in vivo*, increasing their overall fitness for successful host colonization, indicating that increased symbiont diversity



offers new avenues for symbiotic associations to exploit. Previous studies using evolved *V. fischeri in situ* demonstrate that they are amenable to experimental evolution in a novel squid host, and were observed to be just as competitive as their ancestral strain as early as 400 generations (Soto et al., 2014). Such symbiont driven benefits can be influenced by environmental factors (Hussa and Goodrich-Blair, 2013), and in some cases abiotic variables may alter factors that enable *V. fischeri* to not only adapt and survive under those conditions, but indirectly provide advantages for host colonization (Riggins et al., 2013). Perhaps increased growth rate and bioluminescence activity, two influential factors important for symbioses, are influenced by both physiological and genetic changes that regulate squid-host selectivity during experimental evolution and provide a competitive advantage during colonization. Earlier work using experimentally evolved bacteria has shown that specific phenotypic characteristics give

one bacterial genotype a better competitive advantage under certain environmental conditions (Lenski et al., 1991; Barrick and Lenski, 2013; Zaman et al., 2014). This adaptability may be translated into specific genetic mutations to create a novel strain that is more fit than its predecessor (Lenski et al., 2015; Ribeck and Lenski, 2015). For example, resilience to low pH in various host enteric environments by *Escherichia coli*, *Shigella sonnei*, *Salmonella enterica*, and *Bifidobacterium animalis* has provided evidence that these bacteria have evolved mechanisms to combat acid stress such as the acid-inducible tolerance response (Wielgoss et al., 2013) and expression of acid stress proteins to accommodate low pH (ASPs; (Sanchez et al., 2007; Ramos-Morales, 2012; González-Rodríguez et al., 2013; Lund et al., 2014)). Oftentimes, the ability of mutualistic and pathogenic *Vibrio* species to persist in aquatic environments is highly correlated with the diversity of populations (Johnson,

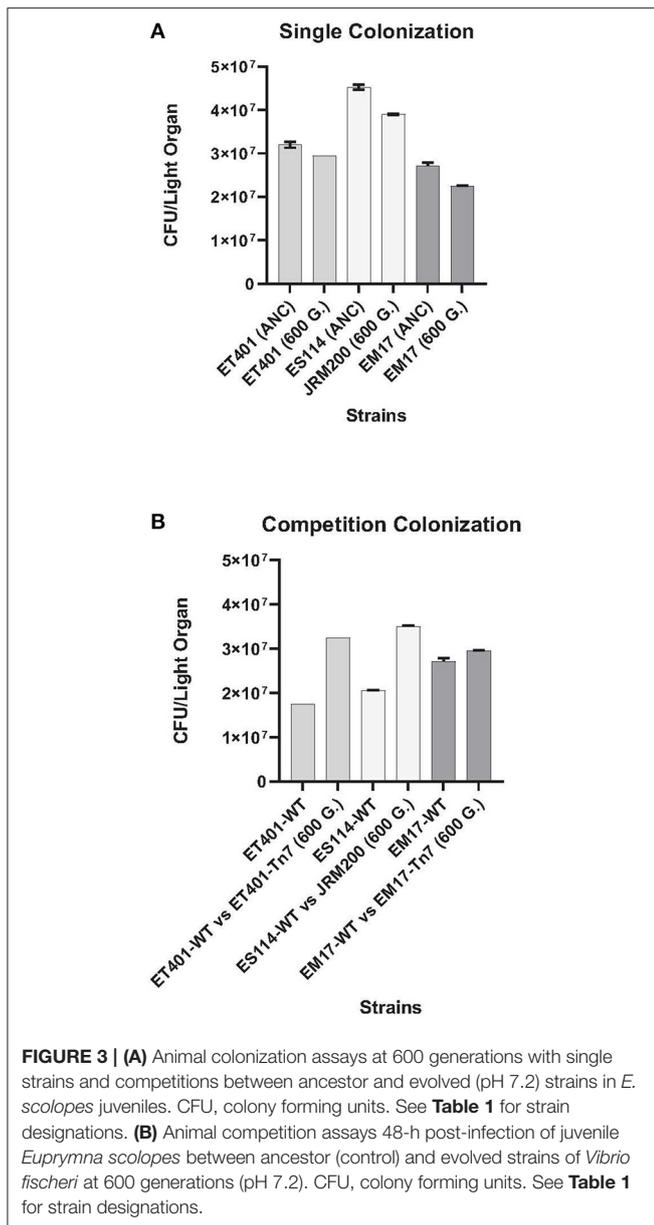


2013; Ceccarelli and Colwell, 2014; Haley et al., 2014; Shaw et al., 2014). Both mutualistic and pathogenic vibrios have developed complex regulatory networks that ensure successful colonization and expression of their colonization/virulence genes while exposed to low pH (de la Fuente-Núñez et al., 2013; Otto, 2014; Saleem, 2015). Bacteria such as *V. cholerae* can subsist in very diverse and different ecological niches when not in their host, yet maintain the ability to colonize the human intestine when the opportunity arises (Runft et al., 2014). Our study demonstrates that infection, colonization, and persistence by both native and non-native experimentally evolved *V. fischeri* strains (ES114-JRM200, ET401-Tn7, and EM17-Tn7) was enhanced when these strains were evolved to low pH prior to exposure to juvenile squids. The potential of *V. fischeri* variants to quickly evolve to environmental fluctuations may explain how genetically related strains are found in multiple hosts across large geographic distances (Jones and Nishiguchi, 2006; Soto et al., 2012; Coryell et al., 2018). Recent studies demonstrate how well one strain of *V. fischeri* (EM17) can adapt to pH stress with respect to their growth, bioluminescence, and host colonization through an *in-vitro* evolution process up to 2,000 generations, where significant differences were observed between acid (pH 6.0) and alkaline (pH 10.) stress (Cohen et al., 2020). Given this wide ecological breadth, *V. fischeri* is a perfect example of how environmental adaptation can influence both host specificity and symbiont biogeography.

Bioluminescence

Bioluminescence results show that relative light units (RLUs) of low pH adapted *V. fischeri* strains ES114, ET401, and EM17 at pH 6.5, 6.8, and 7.2 increased from 400 to 600 generations

(Figure 2). These results suggest that adaptation to lower pH may also enhance the bioluminescence activity of evolved *V. fischeri* strains, which would entail changes in *lux* operon functionality and alternations to the metabolic changes that influence input/output from associated light producing proteins (Wier et al., 2010; Wilson, 2013). Bacteriogenic bioluminescent animals such as squid and fish have evolved organs specifically dedicated to host luminescent bacteria (e.g., *V. fischeri*, *V. logei*), whose bioluminescence is used during certain behaviors such as hunting and counterillumination (Jones and Nishiguchi, 2004; Haddock et al., 2010; Bose et al., 2011). The observed increase in bioluminescence activity of evolved strains in this study suggests that maintenance of this important symbiotic feature gives the cell an evolutionary advantage over ancestral strains (Stabb and Visick, 2013). Given that luminescence is important in establishing and maintaining the symbiosis, it is not unlikely that biofilm production is also affected by this phenomenon. Recent proteomic studies on biofilm production between free-living and symbiotic states in *V. fischeri* have detected a bioluminescence regulatory protein which is responsible for regulating the quorum sensing cascade and subsequently increasing exopolysaccharide production in the biofilm matrix (Chavez-Dozal et al., 2015). Although differences in biofilm formation between ancestral and evolved strains were not examined in this study, biofilm production may be affected similarly to bioluminescence and other symbiotic loci when *V. fischeri* is evolved at low pH. Previous research has demonstrated that the day-night oscillation of *V. fischeri*'s metabolic pathways shift from aerobic respiration to fermentation, and the concomitant expression of genes from catabolic cycles suggest variation from a neutral to an acidic pH level in the



light organ crypt (Wier et al., 2010; McFall-Ngai, 2014). Thus, enhanced bioluminescence activity that is dependent upon high bacterial cell density in the light organ can be impacted when *V. fischeri* is resistant to low pH (Jones and Nishiguchi, 2006; Guerrero-Ferreira and Nishiguchi, 2010). Future studies will focus on the regulation of bioluminescence and biofilm related proteins both in ancestral and evolved strains to determine how adaptation to low pH influences these specific symbiotic loci. Additionally, understanding the synergism of low pH adapted genes to other regulatory networks responsible for bacterial physiological behavior during infection and colonization will further our knowledge on the genetic diversity of *V. fischeri* to such conditions in the wild.

Animal Studies

Symbiotically competent *V. fischeri* that are free-living in the environment have evolved to form a close and persistent association with host squids (McFall-Ngai, 2014; Schwartzman and Ruby, 2016). Likewise, these symbiotic *V. fischeri* must be able to coexist with the host and be recognized as “beneficial” by the squid immune system, while maintaining features that allow a successful colonization (Sachs et al., 2011). The evolution of specific recognition, colonization, and persistence mechanisms both in the symbiont and host are therefore a combination of environmental and host specific factors that enable the success of such beneficial associations (Sachs et al., 2011; Soto et al., 2012, 2014; Guerrero-Ferreira et al., 2013). For example, cooperation between *Rhizobia* and leguminous plants occurred due to the benefit of nitrogen fixation, which is a very costly process (Sachs and Simms, 2008). In the squid-*Vibrio* model system, similar phenomena occur when the bacteria produce bioluminescence for the host, while in return the host provides a nutrient rich environment for growth (Wier et al., 2010; Wernegreen, 2013; McFall-Ngai, 2014; Soto and Nishiguchi, 2014; Pepper et al., 2015). The squid host maintains the symbiosis and controls cell density by venting 95% of its *Vibrio* symbiont to the surrounding environment at dawn. Thus, the host has evolved a mechanism to cull and maintain only the “best fit” bacteria that can produce luminescence for counterillumination (Jones and Nishiguchi, 2004; Wier et al., 2010; Wernegreen, 2013; McFall-Ngai, 2014; Soto and Nishiguchi, 2014; Pepper et al., 2015). *V. fischeri* that have evolved to outcompete and survive this diel cycle of venting have been “selected” through multiple generations as in an experimental evolution scenario. Since light production is a costly metabolic process, oxygen levels in the light organ slowly decrease throughout the night causing this drop in pH (Schwartzman et al., 2015; Schwartzman and Ruby, 2016). Whether pH adapted vibrios are better able to accommodate this change, thereby impacting the repopulation efficiency to positively influence the number of pH adapted symbionts may explain how environmental conditions complement *V. fischeri*’s success as a beneficial microbe. Since our pH adapted *V. fischeri* strains were shown to be more fit than their ancestral lines both *in vitro* and *in vivo*, this may be indicative of specific loci that are beneficial for both pH accommodation and host colonization and persistence. Additionally, symbiotic loci in *V. fischeri* have been found to be more conserved than homologs in free-living strains (Howard et al., 2015). One example is the mannose sensitive hemagglutinin proteins (MshA) that are often induced by environmental factors. MshA is important for light organ colonization (Llorca, 2008; Ariyakumar and Nishiguchi, 2009; Chavez-Dozal et al., 2014) but also for inducing cell-cell communication at low pH (Llorca, 2008). Perhaps proteins such as MshA from evolved *V. fischeri* strains have been impacted due to experimental evolution at low pH, thereby changing the colonization efficiency such that evolved strains outcompete ancestral strains during host competition studies. Future studies will examine *msh* loci and other symbiotic loci of both ancestral and evolved strains to determine whether subtle genetic changes are correlated to the evolution at low pH.

CONCLUSIONS

This study emphasized the broad view of whether *V. fischeri* can respond to selective pressures “outside the light organ of the host” (e.g., pH) and subsequently increase their fitness in their symbiotic state. During symbiosis, *V. fischeri* constantly experience shifts between the light organ milieu and the surrounding aquatic environment. Given that *V. fischeri* can adapt quickly to changing pH stress concentrations under laboratory conditions indicates that this bacterium has the plasticity and breadth that extends far beyond its life history in the ocean (Cohen et al., 2020). Since *V. fischeri* is cosmopolitan in nature, the subtle differences between free-living strains (those that cannot infect sepiolid squids) and symbiotic isolates can offer clues to how host selection and environmental can influence the dynamics between these two life history strategies, and render the pathway toward bacterial speciation among closely related vibrios.

Future research work will expand on examining the transcriptomes of both ancestral and evolved lines in order to determine whether pH adaptation does indeed have pleiotrophic effects in symbiotic *V. fischeri*. Examining changes in biofilm formation of evolved strains may also elude to whether loci regulating structure/matrix proteins and luminescence production are also affected by low pH, and whether such mutations effect luciferase activity. Future proteomic studies on biofilm production between evolved and ancestral strains of *V. fischeri* can eventually lead us to identify unique regulatory protein(s) related to pH stress and their function. This may help to better understand the evolutionary impact

on fitness and successful symbiosis under low pH stress at the molecular level.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by New Mexico State University Institutional Animal Care and Use Program.

AUTHOR CONTRIBUTIONS

MN conceived of the project idea. NN ran all the experiments and analyzed the data. NN and MN wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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