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Experimental evolution of Vibrio fischeri to squid symbiosis

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EXPERIMENTAL EVOLUTION OF VIBRIO FISCHERI TO SQUID SYMBIOSIS

BY

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THESIS

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ABSTRACT

EXPERIMENTAL EVOLUTION OF VIBRIO FISCHERI TO SQUID SYMBIOSIS

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Background: Co-evolution of *Vibrio fischeri* strains with *Euprymna scolopes* has led to isolates that are superior squid colonists [1, 2]. To better understand how *V. fischeri* adapts to symbiosis with squid we serially transferred a planktonic Hawaiian isolate, H905 [3], and the natural *E. scolopes* symbiont, ES114, in squid. We characterized derived isolates for colonization ability as well as other phenotypic traits that have been implicated as being important to symbiosis.

Results: We hypothesized that as a result of adaptation to symbiosis H905 would become more phenotypically similar to ES114. We see this trend in luminescence and siderophore production of derived isolates; however, biofilm production and motility became more different from the natural squid symbiont.

Conclusions: These findings may indicate that H905 utilizes different mechanisms of colonization than symbiotic isolates, or it could also be the result of differential regulation of phenotypes between H905 and ES114 under squid vs. in-vitro conditions.

CHAPTER I

INTRODUCTION

Symbiosis and Co-Evolution

Nearly all groups of eukaryotic organisms depend on deeply involved partnerships with various bacterial species for success in their environment. Humans, cows, amoebae, ants, and squid [4-8] are just a few of the many organisms that rely on their microbial symbionts to assist them in carrying out normal life functions. Often, the basis for association is the bacterial partner, or symbiont, supplying its host with nutrients or protection that the host cannot itself produce.

The nature of host-symbiont relationships varies greatly, but one element is common throughout, microbial symbionts must be able to bypass natural host defenses. Although symbiotic bacteria pose no threat to a host, there are ample pathogenic species that could cause great harm. To survive, hosts develop ways of blocking invaders, and as a result, commensal bacteria can be blocked as well. Commensal bacteria must develop ways of bypassing host defenses despite having many characteristics in common with pathogenic species the host is defending against. Exactly how symbiotic bacteria are able to regularly colonize hosts, while their pathogenic relatives are not is poorly understood. The process of overcoming these defenses can lead to symbionts that are highly specialized at colonizing particular organisms.

In response to invasion by pathogenic bacteria, hosts survive by evolving new methods of blocking bacteria. This process of co-evolution between host and pathogen has a great impact on commensal bacteria because commensals must be able to overcome new host defenses in addition to the old ones. The co-evolution between host and symbiont can also lead to co-speciation as host groups separate from one another, effectively separating populations of their symbiotic bacteria. This creates species of

hosts that are related to one another by phylogenies similar to those of their commensal bacteria [9].

Squid-*Vibrio* Symbiosis

Vibrio fischeri, the bacterial symbiont of *Euprymna scolopes*, provides its host with camouflage through luminescence. When colonizing a specialized squid "light organ," light produced by *V. fischeri* illuminates the ventral surface of the squid making it indistinguishable from moonlight to nocturnal predators lurking along the sea floor [7]. This camouflage via counterillumination allows the squid to seek their prey at night without becoming prey themselves.

Beyond nighttime camouflage, the relationship between *E. scolopes* and *V. fischeri* is an integral part of the squid's life, and they carry out a daily cycle of *V. fischeri* propagation [7]. At dawn, the squid vent 95-99% of the bacterial contents of their specialized light organs, reserving the rest as a starting culture for the next night. *E. scolopes* grows this culture by providing it with by nutrients and oxygen. With this care, the population becomes dense enough by dusk that quorum-sensing dependent luminescence is activated. The light organ glows throughout the night, protecting the squid. Shortly after dawn, the cycle begins again with a venting event and subsequent re-growth of the bacterial culture [7]. By venting large numbers of *V. fischeri* daily, *E. scolopes* maintains a population of symbiosis-capable *V. fischeri* on the reefs they inhabit which is 24-30 times greater than populations of *V. fischeri* in similar locations not inhabited by the squid [10]. These high concentrations of *V. fischeri* are now available for the offspring of *E. scolopes*. Immediately after hatching, immature squid begin sorting through the seawater for their own bacterial symbionts [7].

In order for *V. fischeri* to colonize *E. scolopes*, there are many barriers the bacterium must overcome. These barriers are the squid's natural defenses against potentially harmful bacteria. Once squid a hatches, it begins pumping seawater through its mantle and past a pair of specialized appendages located directly above the openings to the light organ. When the squid detects bacterial cells in this water, it begins secreting mucous on these appendages and around the pores that open to the light organ. Once in the mucus, *V. fischeri* must be able to aggregate and directionally move toward the pore openings. This requires both motility to swim through the mucous, and the ability to chemotax directly to the pores. Inside the pores are ducts lined with cilia that create a current to push bacteria out of the ducts and back into the mucous. If *V. fischeri* cells are able to overcome these physical barriers, they enter the ducts which contain both oxidative species and macrophages. *V. fischeri* that are able to overcome these challenges are then able to gain entry into the light organ where there is selection for specific characteristics in growth, nutrient utilization, and most importantly, light production [7].

While all wild-type *V. fischeri* are able to colonize *E. scolopes*, some isolates are more competitive than others. Isolates of *V. fischeri* obtained from light organs of wild *E. scolopes* are the best colonizers and out-compete all other wild-type strains[1]. Among environmental strains, ones that are more closely related to the natural symbiont out-compete those that are more distantly related [1]. Similarly, isolates of *V. fischeri* obtained from the light organs of closely related squid species to *E. scolopes* are more competitive than *V. fischeri* isolated from more distantly related squid species [2].

Experimental Evolution

The process of evolution has been studied under laboratory conditions since the late 19th century. At that time, William Dallinger evolved unicellular organisms by gradually increasing the temperature in an incubator over time. In doing so, Dallinger evolved organisms that grew optimally at temperatures more than 80°F higher than their original maximum level of tolerance. In experimental evolution, defined laboratory conditions are used to show the process by which organisms evolve and adapt through natural selection. With their short generation time, and ease of propagating and preserving lineages, bacteria are ideal candidate organisms for performing experimental evolution. The patterns of adaptation discovered in bacteria can be used to explain and predict the evolutionary past, present, and future of a variety of organisms.

One notable experiment continuing today is the Lenski long-term experiment which began in 1988. The Lenski *E. coli* lines have been propagated by serial transfer, which involves growing a culture overnight, and transferring a percentage of that culture into fresh media the following day [11]. During growth, random mutations occur within the population. Through both natural selection by the environment, and random chance of transfer, these mutations lead to lines that are drastically different from their ancestors[12].

Much like Lenski began with isolates not specialized to glucose minimal media, our study began with isolates of *V. fischeri* that are not specialized to symbiosis with *E. scolopes*, and will use a serial transfer style experiment to evolve them in the squid environment. While it is clear that symbiotic isolates of *V. fischeri* are well adapted to the squid environment, it is not clear exactly what traits make them better suited than

free-living environmental isolates of *V. fischeri,* or the evolutionary history that has brought them to this point. Using non-symbiotic isolates and tracing how they evolve to accommodate the squid environment, we can identify traits that are of key importance to initiation and maintenance of the symbiosis.

Previous research has suggested that the current symbiotic strains of *V. fischeri* have evolved over time with *E. scolopes* [9]. During this time, as *E. scolopes'* host defenses changed, they selected for only the *V. fischeri* that were capable of overcoming them; this changed symbiotic *V. fischeri* from their free-living counterparts. A previously characterized visibly luminous environmental isolate, H905, has shown the ability to establish the symbiosis with *E. scolopes*. When colonizing *E. scolopes*, H905 is a poor colonist, and not competitive against strains that are phenotypically similar to the natural symbiont [1]. Due to the phenotypic differences between H905 and ES114, we believe that by passaging H905 through a series of juvenile *E. scolopes*, H905 will adapt to become a better symbiont causing changes in phenotypes known to be associated with symbiosis. Additionally, we hope to identify some of these colonization traits by comparing derived H905 to its ancestor.

Specific Aims

1. Characterize ancestral H905 and ES114
2. Determine evolutionary capacity of H905
3. Evolve H905 and determine the effects of passage

Specific Aim 1: Characterize ancestral H905 and ES114

Characterizing H905 and ES114

We characterized H905 in phenotypes that are known to be associated with colonization of *Euprymna scolopes* including growth, luminescence, siderophore production, motility, and biofilm production. The same traits were measured for ES114 as described in "Methods."

Specific Aim 2: Determine evolutionary capacity of H905

Colonization Characteristics

We determined how many colony forming units per ml (CFU/ml) are needed in an inoculum to colonize 100% of inoculated squid, as well as what inoculum would be sub-optimal, such that we could potentially see improvement as a result of transfer. We also determined how many H905 cells are present in the light organ and how much light each cell produces during an established infection.

Specific Aim 3: Evolve H905 and ES114 and determine the effects of passage

Squid Serial Transfer

To begin the transfer process, squid were colonized by the same 100 ml culture of either H905 or ES114. From that point on, six separate populations of both H905 and ES114 were established and passaged through a series of 15 juvenile E. scolopes. The transfer process utilized the natural venting cycle of the squid in order to recover bacteria for infection of the next animal. Samples of vented bacteria, as well as the colonized animals themselves were frozen at -80°C (See figure 1 for transfer diagram).

Data Interpretation/Recovery

Frozen ventate was streaked onto rich media, and isolates with *Vibrio fischeri* characteristics picked for characterization. Recovered isolates were characterized for ability to colonize, as well as growth, luminescence, motility, siderophore production, and biofilm production.

Conclusions/ Predicted Outcomes

The squid host presents many obstacles to bacteria that attempt to colonize its light organ. Establishing the symbiosis means bypassing a gauntlet of stresses that require specific traits to overcome[7]. Even once colonization is established, there are still selective forces at work[13]. The nature of the squid's venting cycle itself may even cause changes to occur in the experimental strain. The growth-venting cycle of *E. scolopes*' colonized population of *V. fischeri* introduces daily bottlenecks in the bacterial populations. If there is selection for determining which colonizers remain in the light organ after venting to start the new population, then the post-venting population may have different traits than the pre-venting population. If there is no selection, and venting happens to a random 95-99% of the population, it is possible that any mutation regardless of whether it is neutral, beneficial, or deleterious, may become a significant portion of the new post-venting population. After being passaged through a series of squid, derived populations were assessed in their ability to colonize squid, and compete against ancestors and the natural symbiont. Through serial passage, we expected to see derived populations of H905 more closely resemble ancestral ES114. The most important effect is increased fitness in the squid environment determined by colonization experiments.

Additionally, because ES114 is so well adapted to squid symbiosis, we do not anticipate significant overall changes in derived ES114 phenotypes.

The process of experimental evolution will give us insight into the relationship between *E. scolopes* and *V. fischeri*. We hope to use this experiment to allow the natural selective process of *E. scolopes* colonization to show what traits are of key importance to establishment and maintenance of the symbiosis. Because many of the defenses used by *E. scolopes* are part of innate immunity in other animals (oxidative stress, macrophages, etc.), these experiments may also show us what traits allow both pathogens and symbionts to bypass host defenses and establish populations in a variety of animals.

CHAPTER II

EXPERIMENTAL EVOLUTION OF VIBRIO FISCHERI TO SQUID SYMBIOSIS

INTRODUCTION

The success of eukaryotic organisms in their niches is dependent upon intimate relationships they share with microbial partners [14, 15]. They rely on their commensal and mutualistic symbionts to assist them in normal life functions ranging from digestion to protection. While the nature of these relationships, and the organisms involved, vary greatly, one commonality is that all microbial symbionts must be able to regularly bypass their host's natural defenses in order to maintain the relationship. Although symbiotic bacteria pose no threat to a host under normal circumstances, there are ample pathogenic species, often closely related to mutualistic ones that could potentially cause great harm to the host [16]. To survive, hosts must block or kill invading bacteria. As a result, beneficial bacteria may also be inhibited. In order to survive, successful symbionts, just like successful pathogens, must develop ways of bypassing established and evolving host defenses [16]. Exactly how symbiotic bacteria are able to regularly colonize hosts, while their pathogenic relatives are not is a poorly understood phenomenon [13]. Additionally, the process of overcoming these defenses as they change over time can lead to symbionts that are highly specialized at colonizing particular host organisms [2]. Understanding this process could give insight into how pathogens and symbionts associate with their hosts and what factors are important in association with and bypass of animal immune defenses.

The ancient association between *E. scolopes* and *V. fischeri* has shaped the evolution of symbiotic *Vibrio* strains. In exchange for a protected, nutrient rich growing environment, *V. fischeri* provides *E. scolopes* with bioluminescence [7]. The squid utilizes this luminescence for counterillumination camouflage to protect itself from

predators when it emerges from hiding in the sand to hunt in the water column [7]. Over time, the co-evolution of *V. fischeri* strains with host *E. scolopes* squid has resulted in isolates of *V. fischeri* that are squid specialists [2]. These specialist strains are adept at colonizing *E. scolopes*, and out-compete other closely related isolates that are not squid-specialists [1]. Although phenotypic differences can be identified between isolates, it is difficult to link an in-vitro phenotype with the increased affinity for squid colonization due to the natural diversity of phenotypes even among squid-specialists [17].

The two strains used in this study, ES114 [17] and H905 [3] were isolated from geographically similar locations, but lead very different lifestyles [1, 17] . ES114 was isolated from the light organ of a wild-caught adult *E. scolopes* squid[17]. In previous studies it has been shown to be a superior squid colonist, and alterations of its phenotypes that are thought to be associated with symbiosis result in attenuated colonization ability. H905 was isolated from Hawaiian seawater, and is a poor colonist both alone (this study), and in competition with other strains [1]. Ancestral ES114 and H905 differ in a number of phenotypes that have been shown, or are suspected to be, important to the symbiosis, including biofilm formation, motility, luminescence, siderophore, and growth [18-22].

In this study, we used the experimental evolution of a non-symbiotic isolate of the luminous marine bacterium, *Vibrio fischeri,* to the sepioloid squid host, *Euprymna scolopes,* in order to glean insight into what bacterial traits are of key importance to establishing and maintaining squid symbiosis. In addition to the non-symbiotic H905, the natural symbiont of *E. scolopes*, ES114 [17], was also passaged to examine the impacts of juvenile squid adaptation without external environmental factors that the natural symbiont may experience in nature. Previously, experimental evolution studies have

been widely used to investigate a range of biological questions [23]. Within the field of host-microbe interactions it has been used to better understand the underlying mechanisms of host-microbe associations and their impact on organisms' evolutionary history [15, 24]. Experimental evolution generally consists of exposing an organism to a given environment over a series of generations in order to determine how the characteristics of the experimental organism are changed as a result of adaptation to the specific pressures of the given conditions. Often this is performed using a serial transfer technique in which batches of the organism (in this case, *V. fischeri*) are grown, and a small portion of that larger culture is passaged to a new cycle (fresh media, etc.). Conveniently, the natural cycle of the squid-Vibrio symbiosis consists of a daily culturing and venting cycle within the squid's light organ; making it an example of natural example of serial transfer and ideal for an experimental evolution study.

Characterization of derived ES114 and H905 gives us new insight into both the squid-*Vibrio* symbiosis and our model system. Symbiosis associated traits (growth, motility, siderophore, biofilm, luminescence) were all quantified before and after squid adaptation for both isolates so we could determine what changes had occurred as a result of squid transfer. Based on the assumption that ES114's superior colonization abilities are linked with its in-vitro phenotypes, we had expected evolved H905 isolates to more closely resemble ancestral ES114 phenotypically as a result of transfer. In the end, we saw alterations in the phenotypes of all H905 populations after passage through squid. Some phenotypes such as luminescence and siderophore production were altered to more closely resemble the natural squid symbiont, ES114. This was an expected result. Others, such as biofilm production and motility, changed so that the squid-passaged H905

was even more different than ES114 than ancestral H905. ES114 populations passaged through our study, however, remained mostly the same as ancestral ES114. These alterations, both expected and unexpected will be studied more closely in the future to help understand the characteristics of the intimate relationship between *E. scolopes* and *V. fischeri*.

METHODS

Bacterial strains and culture conditions: Two strains of *Vibrio fischeri,* both isolated from the same Hawaiian habitat were used including an *Euprymna scolopes* light organ isolate ES114 [17] and a planktonic isolate H905 [3]. Unless otherwise noted, individual colonies of *V. fischeri* were cultured at 28° C in SWT broth [10] containing 70% natural seawater, and 10g/L Bacto-tryptone® (Difco, USA) at 200 RPM or SWT agar plates containing 15% Bacto agar. For storage, strains were grown in LBS liquid medium [25] and frozen at -80°C with 20% glycerol. Artificial sea-water based iron-limited medium supplemented with both 0.3% case amino acids and 0.2% glucose, and buffered with Tris pH (6.8) instead of HEPES [26]

Squid maintenance and serial transfer:

Animal housing conditions:

Adult squid were housed in 40L tanks in a Marine Biotech XR4 Aquaria Rack system maintained at temperatures of 23-26°C. Female squid were housed individually, and male squid were housed either individually or in groups of 2-3. Water used to maintain the system was a combination of Instant Ocean® (Spectrum Brands), and natural seawater obtained from the gulf of Maine (Sachs Aquaculture, St. Augustine FL). All squid were housed in a room with a 12 hour alternating day/night cycle. Egg clutches laid by females were transferred to individual glass bowls and maintained in artificial sea water (Instant Ocean®), rinsed every 48 hours and incubated at room temperature with an air stone for oxygenation until hatching. At hatching, juveniles were promptly removed from the bowl containing the remaining clutch and rinsed in filter sterilized Instant

Ocean® before use. Prior to each colonization experiment, juveniles were held overnight in individual scintillation vials with 4 ml filter sterilized Instant Ocean® and absence of any contaminating *V. fischeri* was confirmed by lack of bioluminescence.

Squid colonization:

All squid colonizations and experimental transfers were performed with juvenile squid in filter sterilized Instant Ocean® and maintained in either 20ml scintillation vials, glass custard dishes, or 24-well microtiter plates which were well rinsed and allowed to dry completely if re-used. Aside from the initial colonization event, all squid were housed separately when exposed to a colonizing inoculum. Colonization experiments were all performed at room temperature with a 12 hour daily light cycle.

Serial transfer protocol:

Wild-type (Ancestor) ES114 and H905 were streaked for isolation on SWT agar plates and grown at 28°C overnight. Single colonies of each were used to inoculate 2ml of SWT broth, and grown at 28°C with shaking until they reached an OD of 0.4-0.6. The bacteria were diluted to 5,000cfu/ml in 50ml filter sterilized Instant Ocean® and concentration of bacteria confirmed by plating on LBS agar. 10-12 juvenile *E. scolopes* were placed collectively into the inoculum or into uninoculated Instant Ocean® as controls for contamination for 16 hours, and then transferred to scintillation vials containing 3 ml filter sterilized Instant Ocean®. Luminescence was measured to confirm colonization by inoculum, and lack of contamination in no-inoculum control. Animals were then separated into 2ml filter sterilized Instant Ocean® in individual wells of a 24-

well microtiter plate.

In each 24-well plate, four animals were housed including two experimental animals in alternating rows with two un-colonized control animals (See Figure 1) to allow identification of cross-contamination. For two additional days following initial placement in the 24 well plates, individual squid were rinsed and then moved to new wells containing 2 ml sterile Instant Ocean® each morning following venting. After venting on the fourth day, squid were removed and frozen in a 1.7ml microcentrifuge tube at -80°C. Half of the ventate from each individual animal was added to 1.5ml filter sterilized Instant Ocean® in a 20ml scintillation vial and a newly hatched and uncolonized animal was placed in inoculum. The transfer cycle was repeated 15 times with each treatment and included uncolonized controls for every passage. Of the original 10 parallel lines for each strain, a minimum of 6 for each strain were maintained through all sets of 15 transfers.

To control for contamination between lines, uncolonized animals were passaged in rows separating squid containing experimentally evolving bacteria. These animals were checked for luminescence daily. During the course of the experiments no uninoculated control animals became colonized, indicating that the transfers were free of cross contamination. When newly hatched squid were not available on the fourth passage day, the ventate bacteria were frozen to allow restarting at a later date. Specifically, 1ml of ventate from a colonized squid was frozen at -80°C in 15% glycerol. When squid became available, the frozen ventate was thawed at room temperature, the cells pelleted by centrifugation for 2 minutes at maximum speed, the pellet rinsed briefly with 1 ml Instant Ocean® to remove residual glycerol, and the cells were pelleted again. Finally,

the cells were suspended into 1ml filter sterilized Instant Ocean®, and a newly hatched, uncolonized animal was placed in this mixture per normal transfer protocol.

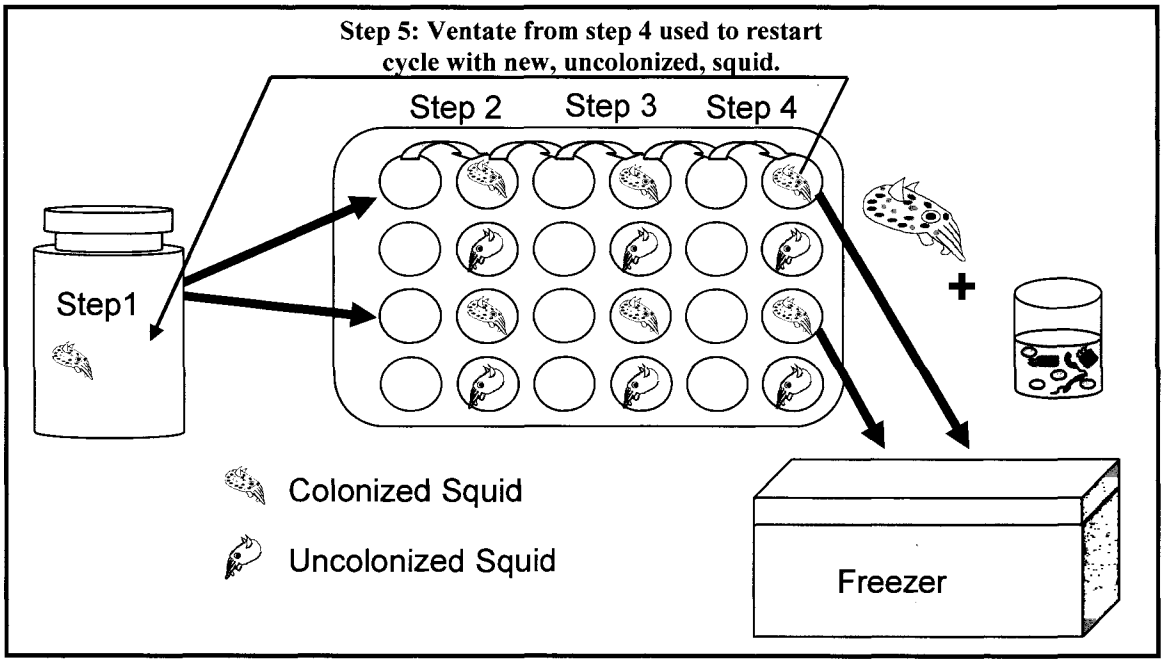

Figure 1: Serial squid transfer: on the first day of each cycle, a single squid is colonized using the bacteria vented on the final day of the previous animal's transfer. In addition, each squid is frozen along with a sample of its ventate at the end of its last transfer cycle. During the 4 days a squid is used for transfer, it is rinsed and transferred to sterile Instant Ocean® daily.

Recovery of evolved isolates:

To recover light-organ evolved strains for characterization, a portion of the frozen ventate was plated and then individual colonies that were morphologically characteristic of *Vibrio fischeri* were streaked for isolation onto SWT agar, grown in LBS liquid media over-night and then frozen. Strains were stored in a rack containing 96-1.5ml tubes, and pin-replicated from these freezer stocks for experiments. See figure 2 for isolation and labeling scheme.

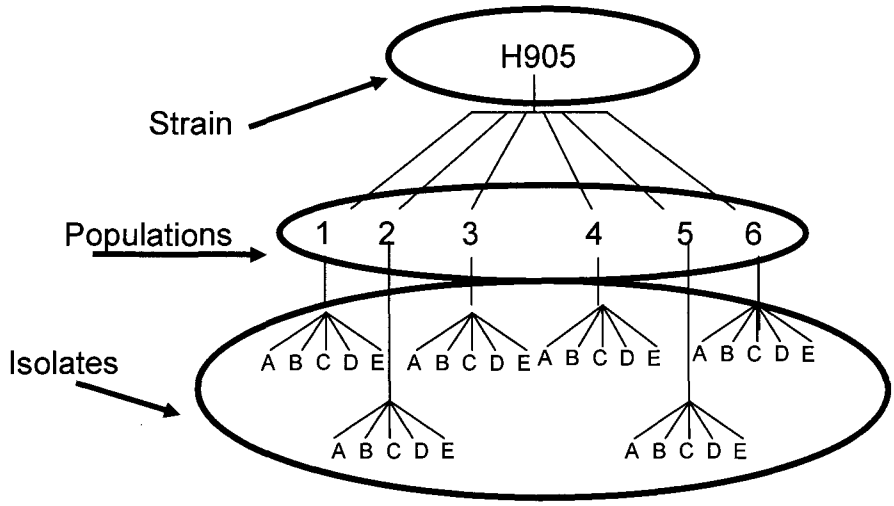

Figure 2: Chart illustrating how strains used in characterization studies were generated. For each strain (H905 and ES114) six (6) populations were passaged serially through squid. From each of those six populations, five isolates were recovered by plating and isolation streaking.

In-squid phenotype characterization:

After hatching, juvenile *E. scolopes* were maintained overnight in 4 ml of filter sterilized Instant Ocean® in 20ml scintillation vials, and lack of contamination confirmed by an absence of luminescence. Only if entire clutch was confirmed free of contamination were individual squid placed in 4ml of Instant Ocean® containing 1,000cfu/ml of either ancestor or evolved *V. fischeri*. In colonization experiments, individual isolates from within evolved populations were pooled in a single inoculum. For each experiment, animal luminescence was measured at 24 and 48 hours. The colonization of light organs was quantified in a standard protocol which substantially reduces the number of living bacteria residing outside the light organ, but does not significantly reduce the population of *V. fischeri* within the light organ due to the osmoprotective light organ matrix [27]. CFU/light organ were determined from previously frozen light organs that were subsequently homogenized and dilution plated onto LBS agar.

In-vitro phenotype characterization

Growth

Evolved isolate strains were inoculated into a 96 well microtiter plate containing 150µl LBS and grown at 28˚C overnight without shaking. Overnight cultures were pin replicated into sterile 96 well plates containing 150µl LBS and placed in Tecan Infinite®200 plate reader for 20 hours at room temperature. Optical density (595 nm) of each well was measured every 15 minutes with a 5 second shaking step between each measurement. Each growth experiment contained triplicates of each isolate, and data was presented as the average of three of those experiments.

Motility

Motility was assessed using a modification of a previously described motility assay [28]. Isolates were pin replicates into 150µl SWT and grown overnight at 28° without shaking, then spotted (3ul spots, in triplicate for each isolate. onto the surface of a plate containing 70% Natural seawater, 1% Tryptone, and 0.3% agar. Migration distance (circle diameter) was measured every 2-4 hours.

Siderophore

Siderophore production was measured using a modified CAS liquid assay protocol [29]. Briefly, *V. fischeri* ancestral and evolved isolates were grown overnight in a 96 well microtiter plate containing 150µl iron limited artificial seawater-based liquid media, and OD_{595} was measured. Cells were pelleted at 13,000*g and two 50µl aliquots of supernatant were removed, combined with 50µl CAS assay solution, incubated for 15

minutes at room temperature, then the absorbance was measured at an OD_{630} (S). As a reference for no siderophore activity, measurements were also taken for a media blank sample (R). Siderophore activity was calculated as described [29]; and normalized to OD_{595}. Briefly: $\{[(OD_{630\,R}-OD_{630\,S})/OD_{630\,R}]*100\}/OD_{595\,S}$ = % Siderophore units per OD_{595}

Biofilm

Biofilm assay was conducted as previously described (O'Toole) with some modifications. Briefly; individual isolates were grown for 24 hours in 150µl of 0.3% CAA, 0.2% glucose minimal media in 96-well microtiter plate. Absorbance was measured at OD_{595} and plates were inverted to remove unbound cells and media. Wells were then rinsed by gently adding 150µl of sterile water to remove unbound cells. To emptied wells, 200µl 0.5% crystal violet was added and incubated for 30 min at room temperature. Plates were then inverted to remove stain, gently rinsed with Instant Ocean® 4 times, and allowed to dry, inverted, for 15 minutes at 28°C. After drying, wells were de-stained with 200 µl 95% ethanol for 15 minutes at room temperature, and absorbance at OD_{595} was measured after 10 seconds of shaking.

Luminescence

Isolates were grown overnight in LBS broth were replicated into 150µl SWT and grown until they reached an OD of 0.4-0.6. Absorbance at OD_{595} was measured, then, 10µl aliquots were pipetted into 100µl Instant Ocean® in individual wells of a solid white 96-well plate and emitted luminescence was measured for 1 second in a Tecan Infinite®200 plate reader (Switzerland).

Calculations:

We estimated the number of generations during each squid transfer using published data on the dynamics of the squid transfer process[7]. Our calculations are as follows:

Given: Starting inoculum = 20,000 cfu

Founding population (F) = 10 cells

Carrying capacity (K) light organ = 500,000

Population post-venting (P) = 25,000

Day 1 generations = log (K/F)/2 = 15.6

Day 2 and 3 generations = (log (P/F)/2)*2=8.6

Total generations per squid = 24.2

Approximate for 15 squid transfers = 364

It should be noted that these calculations are complicated by conflicting data in regards to the number of cells that establish an infection, and an inability to directly count the number of cells that are withheld at the time of venting. Additionally, the nature of the relationship between H905 and *E. scolopes* has not been specifically calculated (all estimates are based on ES114 colonization.). The number of H905 cells involved in establishing and maintaining symbiosis with *E. scolopes* may also change as the populations adapt to squid symbiosis.

RESULTS

Squid Colonization

Ancestral ES114 is a more efficient squid colonist than ancestral H905. Although both visibly luminous (VL) and non-visibly luminous (NVL) *V. fischeri* naturally occur, squid light organs have limited diversity of predominantly NVL strains of *V. fischeri* [17] and enrich the relative proportion of NVL to VL bacteria in their immediate habitat through their daily culturing and venting [10]. Studies that directly compare VL and NVL planktonic isolates of *V. fishceri* from a squid habitat reveal that VL strains including H905 are poor colonists as evidenced by their comparatively delayed initiation of colonization, their total inability to colonize squid when in direct competition with NVL strains, and the ease at which they are displaced from light organs by NVL strains after establishing and initial population [1]. This data suggests that through squid selection, certain strains of *V. fischeri* have adaptively coevolved to become better symbionts. Because strain H905 has never been directly compared to a light organ isolate such as ES114, we first establish a baseline for its colonization abilities in our system. To do this, we exposed juvenile *E. scolopes* to a standard inoculum density (2000 CFU/ml) of either H905 or ES114, and determined the percent of squid that became colonized as assessed by luminescence emission during the three day period that followed exposure to *V. fischeri*. Colonization was confirmed and cell counts obtained from light organ homogenates at 72 hours. Not only was H905 delayed in initiation as previously shown [1], but also it was less efficient at colonization, and only colonized about half the animals (Figure 3).

Derived populations of H905 were improved in squid colonization. After the first three squid transfer cycles, squid became luminous within 24 hours of exposure to *V. fischeri* (data not shown). Once populations of H905 had been transferred through 15 squid passages, we assessed their ability to establish squid colonization with sub-optimal number of cells. Changes in the ability to colonize at a low inoculum would indicate that derived populations had adapted to better establish symbiosis with the squid host. When exposed to 1000 CFU/ml of ancestral H905, 20% of squid were luminous at 24 hours whereas squid exposed to the same number of cells of derived (passage 15) H905, 100% of the animals became colonized at 24 hours (Table 1). Despite their clearly altered colonization abilities, derived H905 did not differ significantly from ancestral H905 in luminescence per animal; although luminescence per bacterial cell, and CFU/light organ was variable. For example, three out of six derived populations had a colonization level more similar to the ES114 level. Interestingly squid colonized by H905 had a greater number of cells per light organ, as well as more luminescence per bacterial cell (Table 1) as compared to those colonized by ES114.

Due to the very low number of cells at which ancestral ES114 is able to fully colonize 100% of squid, we were not able to reliably quantify similar levels of increased colonization ability for derived ES114 populations.

In-vitro Phenotypes

Growth Characteristics

Growth characteristics of derived ES114 and H905 in rich media are similar to ancestral growth. In order to investigate whether alterations in colonization were not a simple

factor of increased growth, we measured the growth rates of ancestral and derived isolates in rich media. All isolates of ES114 and H905 remained statistically the same in both their growth rate and their growth yield when compared to ancestor (Figure 4). For H905, there were two populations that showed qualitatively higher maximum growth rates than ancestor (Figure 4). Despite their qualitatively faster maximum growth rates, all isolates from both of these populations (3 and 6) end with the same growth yields as the ancestor after 24 hours (data not shown).

Luminescence

Luminescence decreased in derived H905 isolates, and increased in some derived ES114 isolates. Although luminescence is essential to the squid-Vibrio symbiosis [30], isolates that naturally produce visible luminescence in culture are impaired in colonization [1]. Ancestral H905 produces significantly more luminescence in culture than ancestral ES114, so as a result of adaptation to symbiosis we anticipate a reduction in luminescence in derived H905 isolates. Not surprisingly, 26/30 of the H905 derived isolates were less luminous than their ancestor (Figure 5). The dimmed luminescence of these isolates is significantly brighter than ancestral ES114 (Figure 5). When luminescence was averaged across the population, five out of six H905 derived populations had significantly lower luminescence than their ancestor (Figure 5). Surprisingly, derived ES114 isolates also changed in luminescence, with four populations significantly increasing luminescence from the ancestral level (Figure 5). When ES114 derived populations are compared on the same scale as H905's luminescence production derived ES114 luminescence production is an order of magnitude less than that of H905

(Figure 5).

Motility

Motility was altered in derived H905 isolates but was not in ES114 derived isolates. When colonizing *E. scolopes*, *V. fischeri* must swim through host mucus and colonize crypt spaces [7, 25, 31]. However, increased motility mutants of ES114 less effectively colonize squid [28]. It is not surprising that the squid colonist ES114 is more motile than ancestral H905 (Figure 6). What is surprising is that all derived isolates of H905 have significantly decreased motility when compared to ancestor (Figure 6). In addition to this quantitative shift in motility, derived isolates of H905 also show altered migration patterns in soft agar (Figure 7). During the first 18 hours after being spotted onto soft agar, ancestral H905 as well as both ancestral and derived ES114 spread from their origin making a diffuse circle in the agar (Figure 7). Derived H905 isolates, however, create a densely populated center and then slowly spread in the agar leaving apparent "microcolonies" instead of a diffuse ring, (Figure 7) similar to what has been observed with AinS quorum sensing mutants[25]. After 18 hours, the derived isolates of H905 will form a second ring outside of the area containing "microcolonies" where cells appear evenly distributed, much like the ancestor (data not shown). It should also be noted that ancestral H905 can sometimes form a less dramatic version of this phenotype, with apparent "microcolonies" forming towards the center of its diffuse ring of motility (data not shown). Derived isolates of ES114 have maintained ancestral-type motility (Figure 7).

Siderophore Production

Siderophore production by derived H905 and ES114 isolates is altered. Siderophores are iron sequestering molecules that have been shown to be important in other models of host-microbe interactions [32] as well as in the squid-Vibrio symbiosis[19]. Ancestral ES114 produces qualitatively more siderophore than ancestral H905 (Figure 8). As H905 transitions from to a symbiotic lifestyle, we predicted it would shift towards increased siderophore production, similar to ES114. Derived isolates of both ES114 and H905 had altered siderophore production (Figure 8). At passage 15, all derived H905 populations had increased siderophore production, with four out of six showing quantitatively more siderophore production in all isolates. Populations of ES114 that had also been passaged through *E. scolopes* had variable siderophore production amongst populations and isolates (Figure 8). Despite the variability seen in derived ES114 isolates, none were significantly different in siderophore production compared to ancestral ES114.

Biofilm Production

Biofilm production increased in derived H905 isolates, but did not change in derived ES114 isolates. During colonization of *E. scolopes*, *V. fischeri* forms aggregates in the squid's mucous before migrating to the light organ [33]. Before squid passage, ancestral H905 produced significantly more biofilm than ES114 (Figure 9), although neither strain produced large amounts of biofilm in culture. All derived populations of H905 increased in biofilm production. Biofilm production by ancestral ES114 is very low, and derived isolates maintain biofilm production that is much lower than even ancestral H905 (Figure 9). Derived ES114 biofilm production was highly variable, and as such it was difficult to

determine whether any patterns in biofilm alteration occurred as a result of transfer (Figure 9).

FIGURES

ANCESTRAL SQUID COLONIZATION

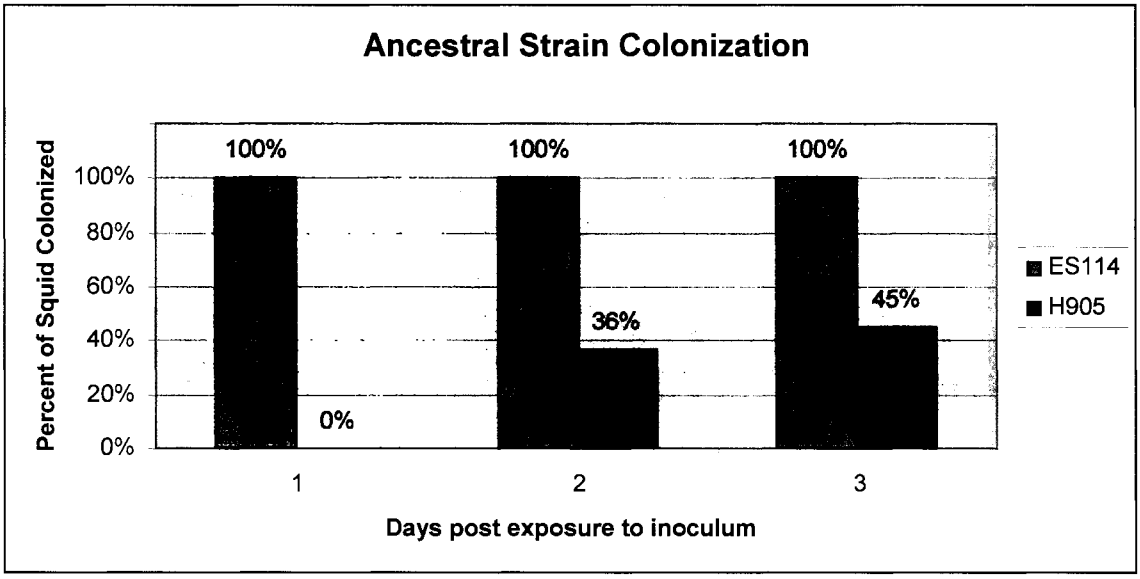

Figure 3: Ability of ancestral ES114 and H905 to colonize juvenile *E. scolopes* at 2200cfu/ml. Each squid cohort contained 9 animals that were communally exposed for 3 hours to either ES114 or H906 and separated into individual vials containing FSW.

DERIVED SQUID COLONIZATION

Strain	Inoculum (CFU/ml)	24 Hours		48 Hours	
		Percent Luminous	Percent Luminous	Relative Luminescence	CFU/Light organ(thousands)
Ancestor ES114	955	50	80	0.13	5.6
Ancestor H905	1125	20	20	0.85	11.9
Evolved Passage 15 H905 Populations:					
1	1100	100	100	0.97	10.0
2	900	100	100	0.78	11.4
3	1050	100	100	0.40	11.4
4	1005	100	100	1.02	6.2
5	1024	100	100	0.69	6.5
6	950	100	100	0.84	5.8

Table 1: Quantification of colonization abilities of derived H905 isolates after 15 squid passages. Cohorts of 5 animals were exposed to1000cfu/ml +/- 10% for 3 hours then transferred to individual vials of filter sterilized Instant Ocean. Luminescence was measured at 24 and 48 hours, at which time squid were sacrificed and plated to determine colonization by plating as well as luminescence. CFU/Light organ was measured for each experimental animal, and shown is the luminescence per cell based on whole animal luminescence and light organ plate counts. Relative luminescence was calculated as the total light emitted from a light organ divided by the number of bacterial cells (*1000).

GROWTH CHARACTERISTICS

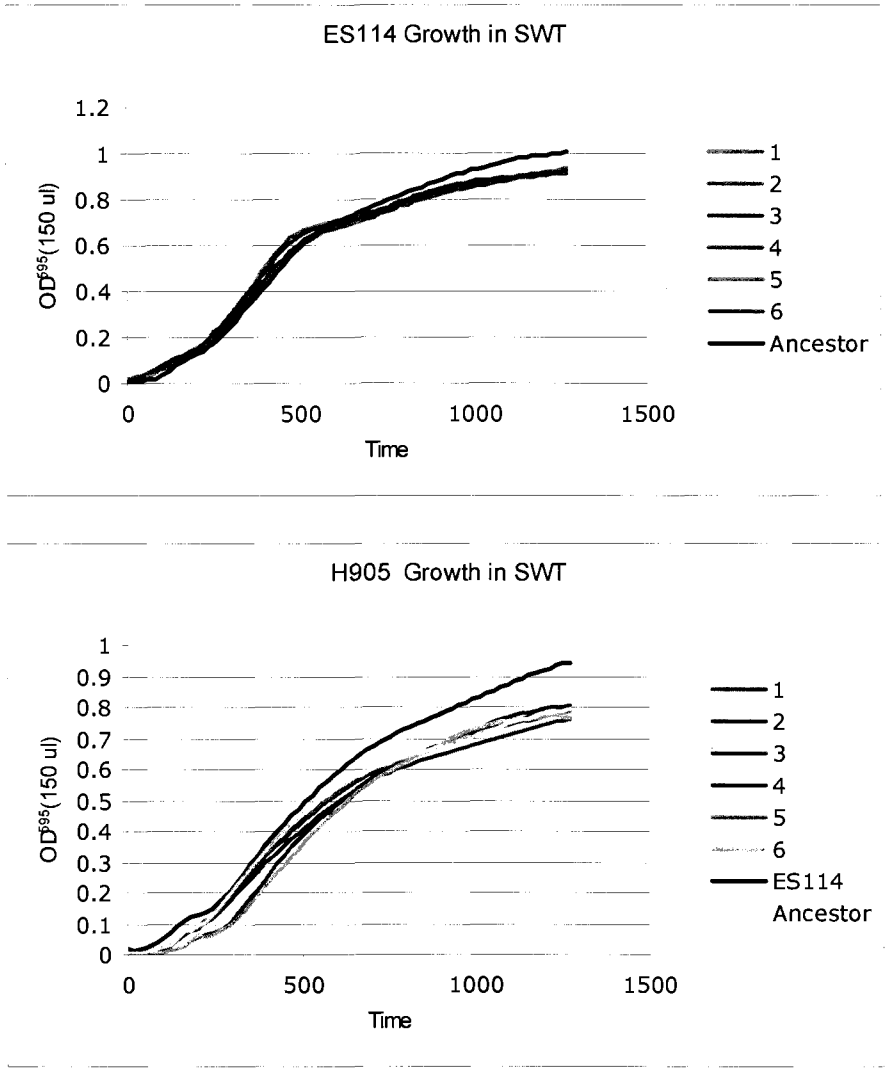

Figure 4: Growth characteristics of H905 (A) and ES114 (B) in SWT measured by reading OD_{595} of cultures in a 96 well plate every 15 minutes. Error bars represent 95% confidence intervals, and each bar represents the average of 5 isolates per population in triplicate.

LUMINESCENCE

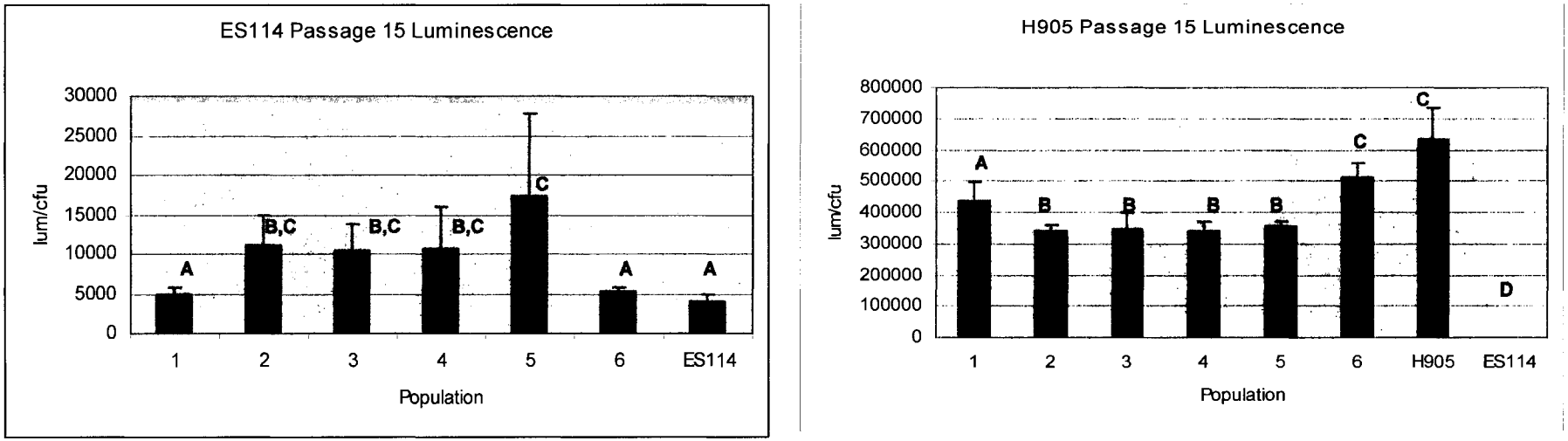

Figure 5: Luminescence production of evolved isolates of H905 and ES114. Luminescence of growing populations was measured by adding aliquots of cells into white 96 well plates and reading for luminescence in a Tecan plate reader. Measurements were made in quadruplicate, and the data shown is from a representative experiment that was repeated with the same result. Error bars represent 95% confidence intervals, and letters above columns show groupings as determined by a p value of </= .05 in an ANOVA.

MOTILITY

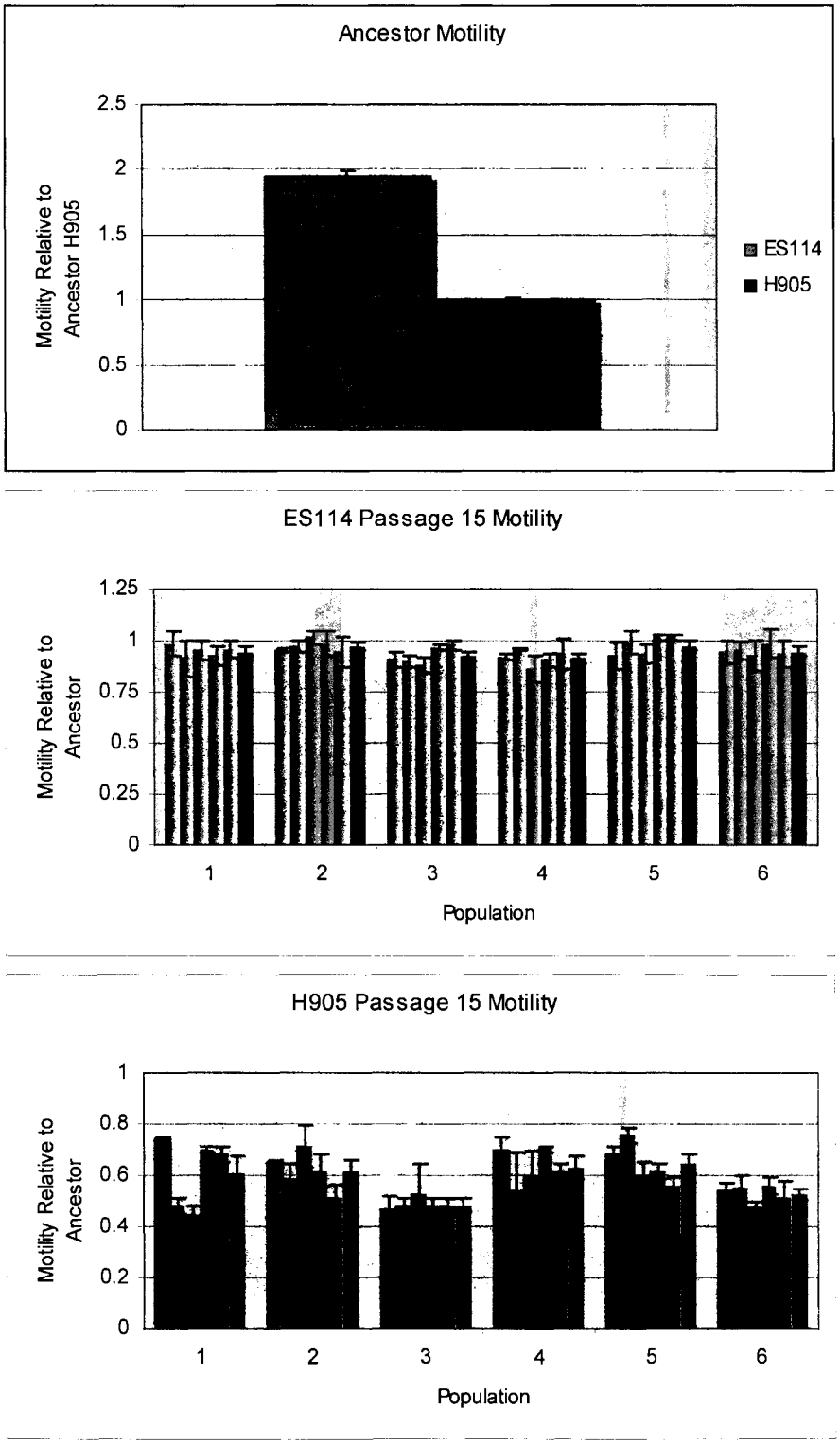

Figure 6: Motility phenotypes of ancestral and derived (15 squid passages) H905 and ES114 (A). Growth rates of H905 (B) and ES114 (C) populations at passage 15 are shown with error bars representing 95% confidence intervals. Each bar represents the average of 5 isolates per population (each in triplicate)

MOTILITY Continued

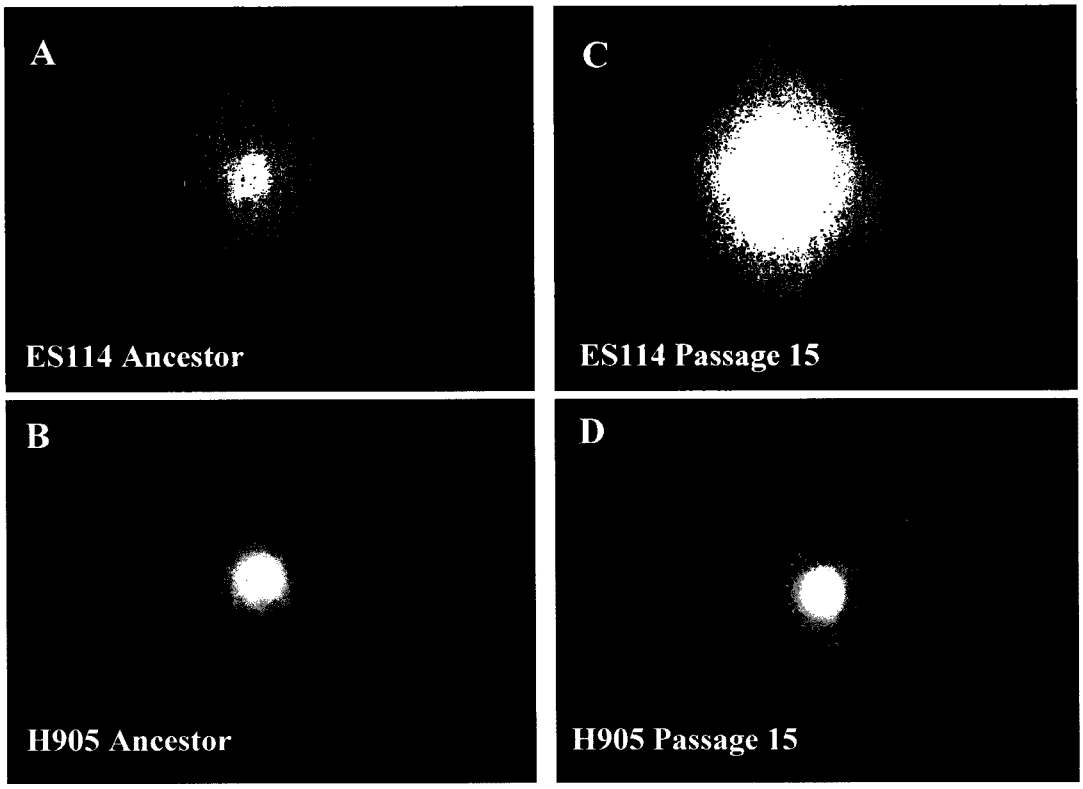

Figure 7: Pictures of typical morphologies of ES114 (A) and H905 (B) ancestors as well as ES114 (C) and H905 (D) passage 15 evolved isolates.

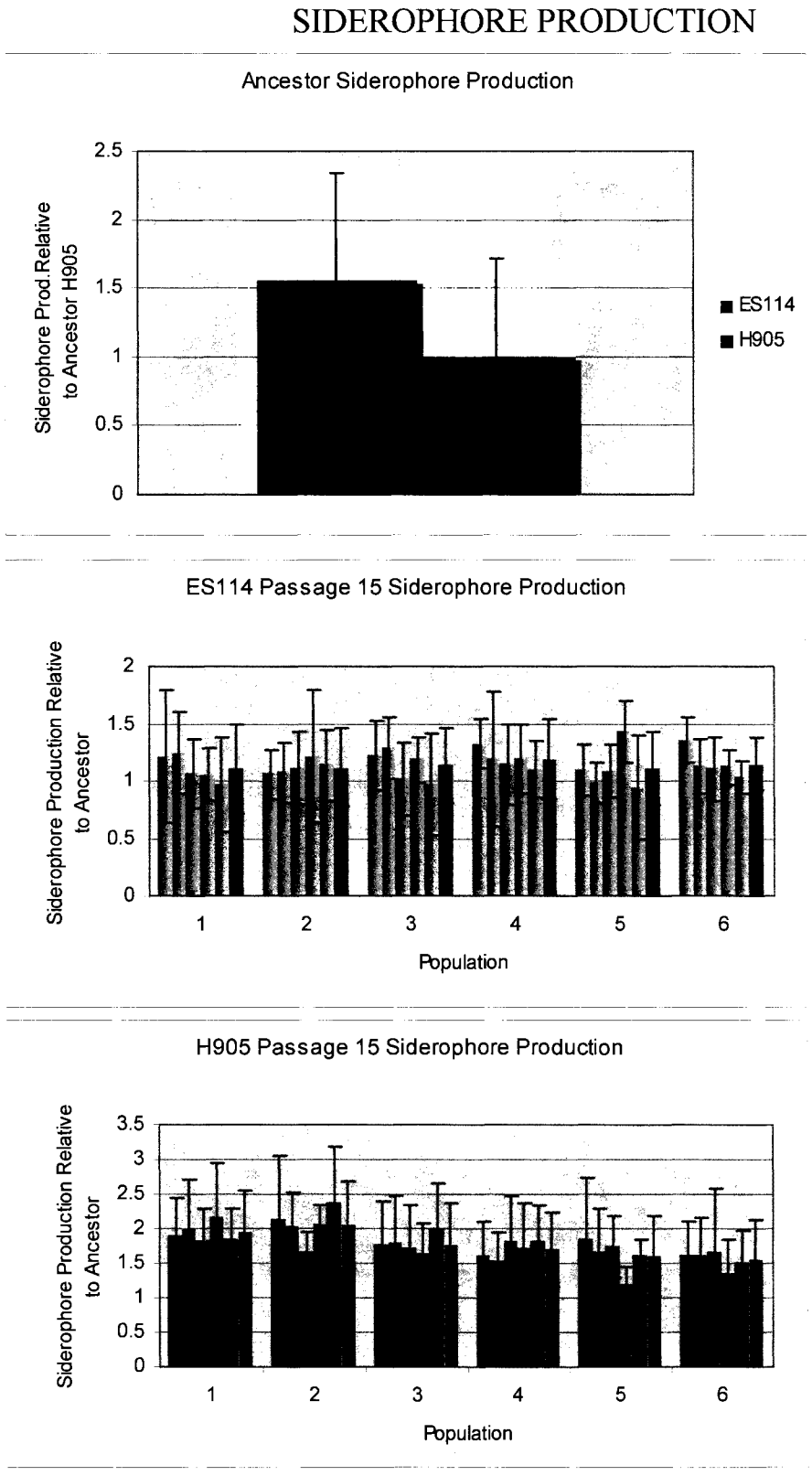

Figure 8: Quantification of Siderophore production of individual isolates as well as pooled populations at passage 15. Siderophore was measured using a modified liquid CAS assay [29] (A) Direct comparison of ES114 and H905 ancestor shows production relative to H905's siderophore production. (B, C)Results for individual isolates and populations are shown as siderophore production relative to ancestral isolate, either ES114 or H905. In all cases, error bars represent 95% confidence intervals.

BIOFILM PRODUTCION

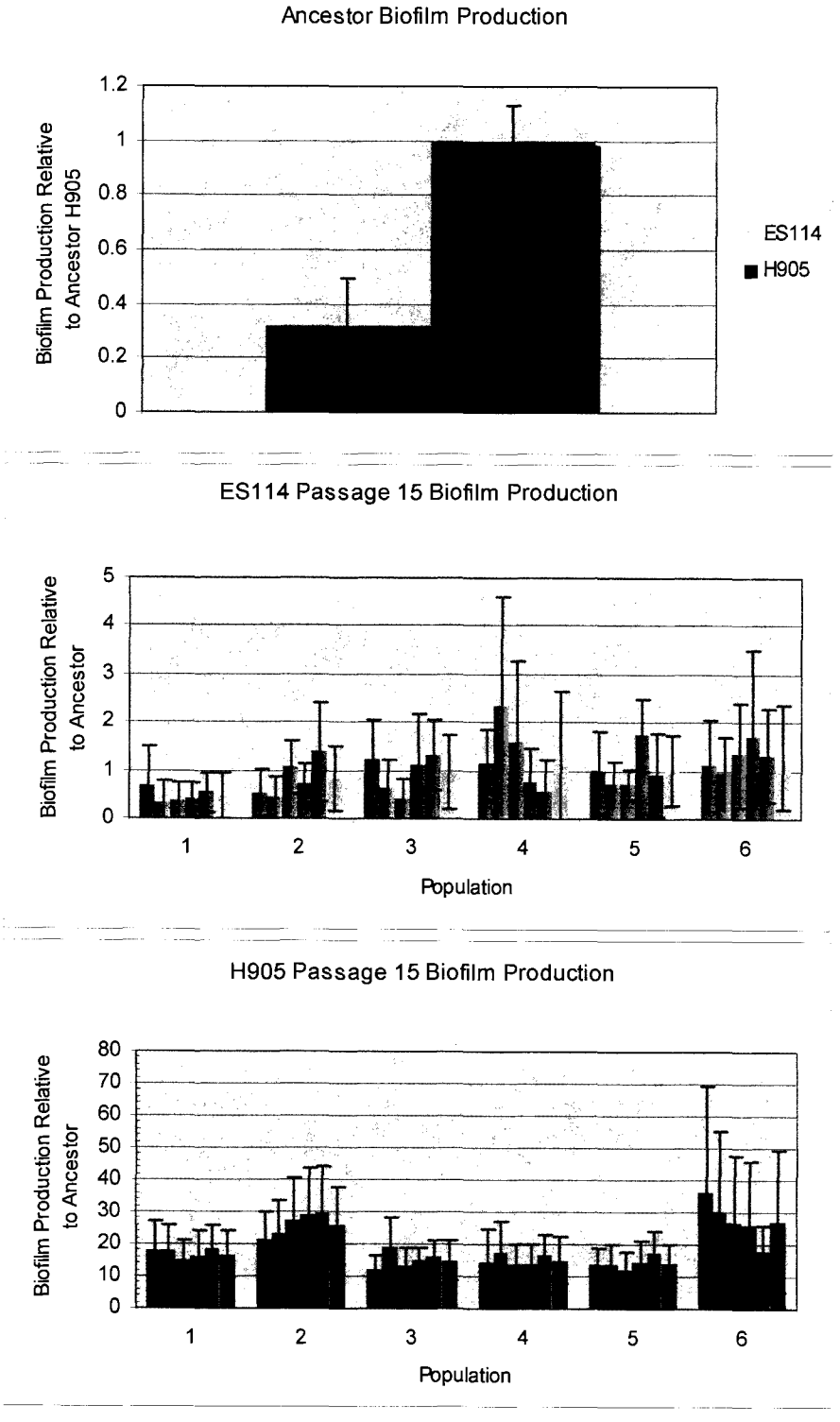

Figure 9: Biofilm production for both derived and ancestral populations of H905 and ES114 as measured using a 96 well plate crystal violet biofilm assay [34]. (A) Biofilm production of ancestral ES114 and H905 shown as percentages of ancestral H905 biofilm production (A,B) H905 and ES114 passage 15 biofilm production represented as percentages of their respective ancestors' biofilm production. In both cases, 1-6 represent populations derived from H905 or ES114 and the bars within these populations are individual isolates. For each graph, error bars show 95% confidence intervals.

DISCUSSION

Although the ubiquity of host-microbe interactions is widely known and accepted as being essential to species' development and survival [15, 24, 35, 36], the basis of and mechanisms for these relationships are poorly understood. Researchers have sought to understand these relationships by investigating microbial traits thought to be associated with these important interactions. Often, studies involve altering the characteristic presumed necessary for symbiotic competence, and quantifying the alteration's impact on the organism's ability to interact with its host [22, 37]. This study both compliments and expands upon this type of directed, one at a time, gene or trait based research by allowing the dynamics of colonization and host environment provide the selective environment for symbiotic adaptation and then investigating the impacts on "symbiotic" traits in a non-symbiotic bacterium. As a proof of concept with the model we predicted that as naïve planktonic bacteria were subjected to exclusive serial transfer in squid, not only would they become better symbionts, but some traits would converge with the symbiont ES114. As a result of the method in this study, we have confirmed the importance of some known symbiotic traits, and also made a step towards understanding key differences between isolates of the same bacterial species as they relate to symbiosis. Additionally, we have shown that our model system is capable of being used to study evolutionary processes under more natural conditions than many laboratory models allow.

Passage of H905 through *E. scolopes* resulted in vastly improved colonization (Figure 4) indicative of adaptive evolution and altered in-vitro phenotypes; however, our expectations that derived ES114 would remain phenotypically identical to its ancestor

which is already highly evolved to the squid symbiosis [1]and that H905 would become more similar to ES114 in all symbiotic phenotypes were not met. After 15 squid-transfer cycles, all populations of H905 were improved at colonizing *E. scolopes* (figure 3), and were also phenotypically distinct from their ancestor. Some predicted changes in symbiosis-associated phenotypes were correlated with increased colonization ability. In the short time they were passaged through the squid (about 350 generations), most H905 evolved isolates decreased in luminescence and increased in siderophore production which is a phenotypic shift toward the known symbiont's traits. With biofilm production (which has not yet been shown to contribute to symbiosis) and motility, however, evolved H905 diverged even more from ES114. Interestingly, it is these two traits, motility and biofilm production, which changed most dramatically and consistently (Figures 6&9). The consistency of decreased motility and increased biofilm production across all derived H905 populations indicates that these traits may be partly responsible for the increased squid colonization, however other explanations, such as mutation hitchhiking, are also possible. Siderophore production is implicated in many host associations, and natural symbionts typically produce low amounts of luminescence relative to H905 [17-19], so changes we saw in these traits in our evolved lines may also have contributed to improved squid colonization, but not all populations of derived H905 are distinct from their ancestor in these traits. Regardless of their role, the fact that not all populations are altered in the same one symbiotic characteristic, it is unlikely that any one of them is responsible for the improved squid colonization.

The general maintenance of some ancestral phenotypes in passaged ES114 populations suggests that, for these phenotypes, ES114 was already well adapted to squid colonization. It also indicates that the differences between natural symbiosis and our model were not sufficient to lead to conserved significant phenotypic changes during the natural symbiont's passage. There were, however, some traits where derived ES114 populations were altered from their ancestor. Some derived populations have decreased motility and/or increased luminescence relative to ancestor. There is, however, no phenotype that is significantly altered in all populations of ES114 making it inconclusive whether they were the result of selection from the system or a result of genetic drift exacerbated by the daily bottlenecking events involved in squid transfer. This indicates that in large part our model was an accurate representation of the natural conditions of squid association to which ES114 had adapted in nature, but that it is possible there are some evolutionary pressures in our system that are not in nature, or vice versa. There are a couple reasons why this may be the case. First, ES114 was isolated from an adult squid light organ. This means that ES114 had potentially been serially passaged by that adult animal for several months, and specifically adapted to adult symbiosis. Second, in nature, *Vibrio fischeri* experiences cycles of squid colonization and time spent in the water between colonizing squid. Our model all but eliminates this time spent in the open ocean, and the most time spent in a non-squid environment for our passaged lines would be several hours-not days or months as may occur in nature. The pressures from transfer through squid were, however, sufficient to warrant phenotypic changes in H905 in addition to improvements in squid colonization. This not only confirms that our model system led to adaptive evolution in passaged strains, but also suggests that some of the

phenotypes we chose to study were ones that convey significant colonization advantages.

One of the most significant findings in this study is the maintained phenotypic difference between derived H905 and ancestral ES114. These results may lead to gleaning insight into some of the yet-unknown differences between H905 and ES114 and how they approach squid colonization. One possible explanation for this difference is that ancestral ES114's low biofilm production is a product of gene regulation. In-vitro biofilm production, which is what our assay, measured may be low but when colonizing the squid ES114 up-regulates biofilm production. H905, not having adapted to the squid for a long period of time, may not have the same gene regulatory mechanisms in place, and therefore showed increased biofilm production in in-vitro assays because it was selected for during colonization and it had no down-regulation mechanism in place. This can be said for any of the phenotypes we examined, as the in-Vitro assays we performed would be unable to identify changes in either strain involving regulatory mechanisms active during squid colonization. Alternatively, evolved isolates of H905 may have altered mechanisms of colonizing *E. scolopes* when compared to ES114. It is possible that as a result of their long-term co-evolution, ES114 is able to bypass *E. scolopes'* natural immune responses either undetected, or detected but unharmed. In order to overcome this deficit of history, H905 must utilize an alternative method for colonizing. It is possible that the increased biofilm production is a protective mechanism, as it has been shown to be in many pathogenic bacteria, which provides H905 isolates a better chance of colonizing despite increased killing by the host defenses.

What is clear as a result of this study is that it is possible for two strains of closely related bacteria to effectively adapt to the same conditions in very different ways. While the traits that we characterized in this study are likely relevant to symbiosis, there is still a wide range of other traits that we did not measure. It is likely that there is a wealth of knowledge awaiting recovery within the genomes of these derived populations of both H905 and ES114. Future studies will examine the differences between ES114 and other strains that are not natural *E. scolopes* colonists using experimental evolution and more detailed characterization of the derived lines. It is not possible to easily and cost-effectively sequence individual isolates using a reference genome, which exists for *Vibrio fischeri*. This technology will allow us to more accurately pinpoint changes that occurred as a result of squid adaptation.

In our study, effective adaptation happened rapidly and colonization efficiency increased even at early stages of the passage. This indicates that under the selective pressure of host immune response, maladapted colonists can quickly overcome challenges presented by a susceptible host. This has implications in how emerging pathogens may be changing in order to better adapt to human and other relevant hosts. By understanding the mechanisms and dynamics of H905's adaptation to *E. scolopes*, we may be able to better understand and approach emerging environmental pathogens. This experiment differs from much of the work done in experimental evolution because the squid host is a more dynamic and natural environment for the bacteria to adapt to than commonly used defined media environments. This presents, we believe, a more realistic picture of how evolution happens in nature, and in this case we have discovered that

adaptation in nature may happen much more quickly than we would anticipate based on studies of simple laboratory conditions, such as single carbon source environments. These studies are similar in that they show major improvements in fitness during the early stages of adaptation and smaller changes thereafter, however our study shows that an organism with a severe deficit in an environment can become nearly as fit in that environment after a very short period of time; for bacteria perhaps days or months rather than years or millennia.

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