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BIOLOGICAL PROPERTIES (*IN VITRO*) EXHIBITED BY FREE-LIVING AND SYMBIOTIC *VIBRIO* ISOLATES

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VIBRIONACEAE
SYMBIOSIS
SEPIOLID SQUID
PILI
BIOFILM

ABSTRACT. – Adhesion and biofilm forming ability of symbiotic bacteria play a crucial role in host colonization and tissue infection. Bacteria benefit by adhering to their host in a manner that allows them to successfully maintain contact for the exchange of nutrients, hormones, or other necessary products. This study examined pili morphology, motility, and biofilm formation exhibited by *Vibrio fischeri* strains (free-living and symbiotic). Since these symbiotic factors contribute in some fashion to the interaction between *V. fischeri* and their squid host, variation between strains may be a contributing factor that leads to specificity among different hosts. *V. fischeri* strains examined in this study demonstrated considerable variation in their biological properties when observed *in vitro*. In addition to differences observed between strains isolated from several different host species, we observed variation between strains isolated from the same host species from diverse geographical locations. This study suggests that subtle differences in the biological properties of closely related *V. fischeri* strains may influence the nature of the interaction among *V. fischeri* and their sepiolid hosts.

INTRODUCTION

Bacteria are oftentimes found associated with animals, plants, fungi, and protists, as well as other bacteria. They have been successful in colonizing both extra-cellular and intracellular tissues of their host, enabling some specific function or capability. Likewise, metazoans such as sponges, corals, nematodes, molluscs, and humans can be hosts to symbiotic bacteria that possess different ecological paradigms (mutualistic, pathogenic, or commensal). These hosts obtain their symbionts by a number of mechanisms that deliver the symbionts horizontally (host directly to a new host), vertically (parent to offspring, oftentimes maternally), or environmentally (from the surrounding environment to the host), and can occur prior or subsequent to embryogenesis (Nishiguchi 2001, Stabb & Ruby 2002, Nyholm & Nishiguchi 2008). In particular, environmental transmission has a number of obstacles prior to infection as well as during colonization and persistence that bacteria are constantly subjected to (Stabb & Ruby 2002). Oftentimes, host species develop mechanisms that increase their probability of being colonized by beneficial microorganisms, while discouraging nonspecific ones (Nyholm & McFall-Ngai 2004). This is usually achieved by creating conditions that only their specific symbionts are capable of accommodating (Nyholm *et al*. 2000). To counter these obstacles, traits exhibited by bacteria can vary depending upon the specificity of each association. Symbiotic bacteria develop complex traits such as surface adhesions, chemoattraction to specific polysaccharides, motility towards recognizable receptors, and resistance to specific immune response proteins (*i.e*., catalase), to improve their chances of pioneering host colonization. Most colonizing bacteria are capable of expressing adhesions, and/or pili, and have the ability to form biofilms, making these components key factors in bacterial colonization and symbiosis (Klemm & Schembri 2000, Lee & Schneewind 2001).

Microbial communities have been known to use pili to form biofilms by attaching to both abiotic and biotic surfaces. These pili help form three-dimensional structures through which nutrients diffuse through and waste products diffuse out (Watnick *et al*. 1999). Biofilms formed by gram-negative bacteria are also known to be influenced by environmental conditions (O'Toole *et al*. 2000), and have been known to differentially express extracellular proteins used in biofilm production in response to specific environmental cues (Branda *et al*. 2005).

Pili production has also been affiliated with biofilm production in a number of gram-negative bacteria that respond to both environmental and host cues. *Escherichia coli* are capable of expressing conjugative pili that result in non-specific cell-to-cell and cell-to-surface associations, thereby accelerating initial adhesion and biofilm development (Ghigo 2001, Branda *et al*. 2005). *Pseudomonas aeruginosa* uses type IV pili differentially, based on the external environment it resides in. Type IV pili in these bacteria have been shown to mediate surface attachment and twitching motility. Depending on which carbon source is available, they can differentially use their type IV pili for biofilm formation (Branda *et al*. 2005). In *Vibrio cholerae* El Tor strain, the MshA pili mediated by a

type IV pilus are responsible for attachment to abiotic surfaces in the environment (Watnick & Kolter 1999). Once inside the host intestine, they recruit the toxin-coregulated pilus (TCP), a type IV bundle forming pilus to adhere to the intestinal cells, thereby switching between a benign, environmental niche to a pathogenic one.

Motility and chemotaxis also play key roles in the formation of biofilms (Pratt & Kolter 1998). Mechanisms by which bacteria respond to their symbiotic environment have been found to be similar to those that coordinate regulation of motility and colonization genes in pathogenic associations (Millikan & Ruby 2002). Therefore, bacterial adhesion, biofilm formation, and motility all might be important mechanisms used by symbiotic bacteria to colonize their hosts.

The sepiolid squid – *Vibrio* mutualism represents an environmentally transmitted symbiosis where host embryogenesis is completed in absence of the bacterial symbiont (Ruby & McFall-Ngai 1992, McFall-Ngai & Ruby 1998, Nyholm & McFall-Ngai 2003). Newly hatched juvenile squids obtain their symbiotic bacteria from the surrounding seawater within a few hours, and are then colonized for the rest of their lives (Nyholm & Nishiguchi 2008). These nocturnal squids use bioluminescent *V. fischeri* in a behavior referred to as counterillumination, which eliminates the squid's silhouette in order to evade predators or be inconspicuous to their prey while hunting (Jones & Nishiguchi 2004). At sunrise, squids will bury in the sand, and vent approximately 90-95 % of their bacterial symbiont population. The remaining 5 % of bacteria repopulate the light organ crypts for use the next evening.

Previous work has demonstrated that species in the genus *Euprymna* prefer native *Vibrio fischeri* over non-native *V. fischeri*, even though both types of bacteria colonize juvenile squids equally well by themselves (Nishiguchi *et al*. 1998, Nishiguchi 2002). More recently, different genetic isolates have been found within populations of *Euprymna*, providing additional evidence that subtle differences exist among all competent strains of symbiotic *Vibrio* (Jones *et al*. 2006, Guerrero-Ferreira & Nishiguchi 2007, Mandel *et al*. 2009, Wollenberg & Ruby 2009). Although this general specificity is very common among Indo-West Pacific sepiolids (Nishiguchi *et al*. 1998, Nishiguchi 2002), very little is known about which bacterial traits provide native strains a competitive edge over non-native strains as well as environmental isolates of *V. fischeri* that cannot colonize sepiolids. Previous studies investigating the distribution of native and non-native *V. fischeri* strains in *E. tasmanica* light organs revealed symbiotic bacterial cells expressing at least two different morphological types of pili within the light organ crypts (Nair *et al*. in review). From this observation, we deduced that pili formation and adhesion during light organ colonization is linked to strain differentiation and specificity. Since earlier studies in other gram-negative bacteria suggest the importance of pili in adhesion, biofilm formation, and motility, we decided to investigate and compare these biological properties of both free-living and symbiotic vibrios.

METHODS

Biofilm assay: Biofilm assays were performed to determine whether bacteria could form biofilms *in vitro* (O'Toole & Kolter 1998). All bacteria strains (Table I) used in this study were previously isolated and described in earlier studies (Nishiguchi & Nair 2003). Bacterial strains were grown overnight at 28°C with aeration (250 rpm). The following day strains were re-grown in fresh 32 parts/thousand (ppt) Sea Water Tryptone (SWT) medium to an optical density of 0.1 at 600 nm. OD_{600} was adjusted by using fresh SWT media to ensure that the bacterial strains were in logarithmic growth phase. One mL of each strain was then transferred to fresh sterile test tubes and incubated without shak-

Table I. – *Vibrio fischeri* strains used in this study

Strain designation	Source (host)	Location
Vibrio fischeri ETBB1B	*Euprymna tasmanica*	Australia (Botany Bay)
Vibrio fischeri ET401	*Euprymna tasmanicaI*	Australia (Townsville)
Vibrio fischeri ET301	*Euprymna tasmanica*	Australia (Sydney)
Vibrio fischeri ET101	*Euprymna tasmanica*	Australia (Melbourne)
Vibrio fischeri SR5	*Sepiola robusta*	France (Banyuls-sur-Mer)
Vibrio fischeri SL518	*Sepiola ligulata*	France (Banyuls-sur-Mer)
Vibrio fischeri SA1G	*Sepiola affinis*	France (Banyuls-sur-Mer)
Vibrio fischeri SI1D	*Sepiola intermedia*	France (Banyuls-sur-Mer)
Vibrio fischeri ESP915	*Euprymna scolopes*	Hawaii (Paiko)
Vibrio fischeri ES114	*Euprymna scolopes*	Hawaii (Kaneohe Bay)
Vibrio fischeri EB12	*Euprymna berryi*	Japan (Tokyo Bay)
Vibrio fischeri EM17	*Euprymna morsei*	Japan (Tokyo Bay)
Vibrio fischeri CG101	*Cleidopus gloriamaris*	Australia (Townsville)
Vibrio fischeri MG101	*Monocentris japonicus*	Japan
Vibrio fischeri MDR7	Free-living	USA (Marina del Rey, CA)
Vibrio fischeri WH1	Free-living	USA (Woods Hole, MA)

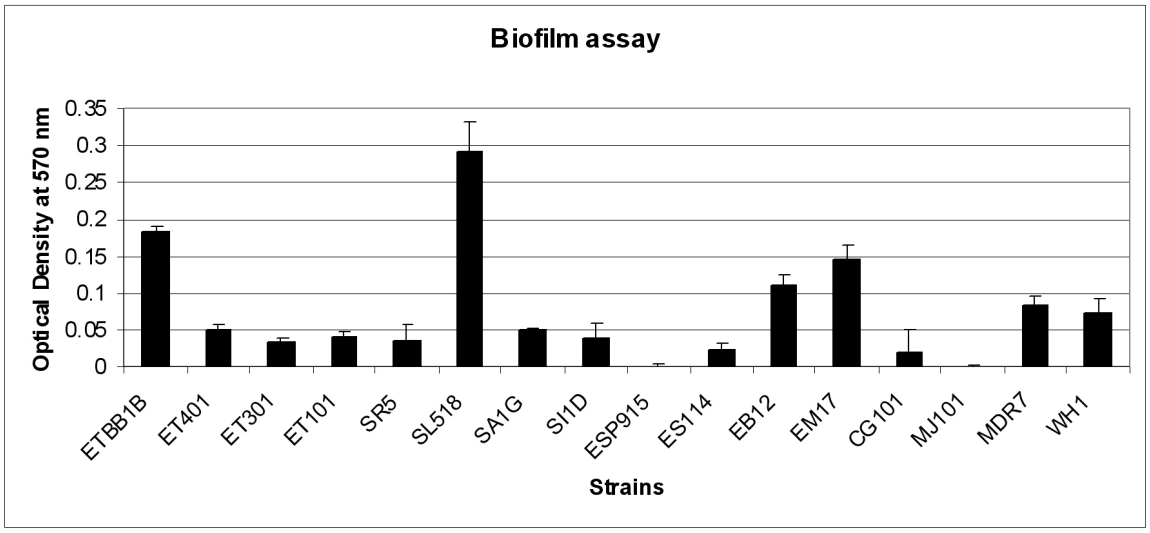

Fig. 1. – Average biofilm produced by closely related *Vibrio fischeri* strains. All measurements were made in triplicate and the average was calculated for each strain (Error bars represent standard error). For strain designation, see Table I.

ing at 28°C for 24 hours. Three replicate samples were measured for each strain. SWT media with no inoculum were used as a negative control for this experiment. After incubation, tubes were washed three times with 32 ppt seawater. Tubes were left upside down for 5 minutes to drain excess seawater. Subsequently, one mL of a 0.2 % aqueous crystal violet solution was added to each tube and incubated at room temperature for one hour. The crystal violet was poured out, and tubes were rinsed three times with 32 ppt seawater. During the last two washes, test tubes were vortexed briefly to ensure complete removal of crystal violet that remained on the sides of each test tube and away from the region of biofilm formation. Tubes were left inverted for 10-15 minutes to drain excess water. Crystal violet bound to the biofilm was then eluted by adding one mL of 80:20 (vol/vol) mixtures of ethyl alcohol and acetone. The tubes were vortexed till all the crystal violet attached to the glass was eluted. Using a UVIKON XL Spectrophotometer (Research instruments Inc.) the amount of biofilm formed was then quantified based on the OD reading at 570 nm, which is directly proportional to the amount of crystal violet binding to the bacterial biofilm produced in each test tube. Standard error was calculated for each of the strains tested.

Motility assay: Bacterial strains were grown overnight at 28°C with rapid shaking (250 rpm). The following day strains were re-grown in fresh 32 ppt SWT medium to 0.1 OD_{600}. Strains that exceeded 0.1 OD_{600} were adjusted by using fresh SWT media. 10 µL of each bacterial culture was plated as spots on a 32 ppt SWT plate using 0.5 % motility agar. Motility was determined by measuring the diameter of each spot after 24 hours incubation at 28°C. Motility assays for every strain were completed in triplicate and photographs of each plate were taken using BioRad phosphorimager (BioRad, Hercules, CA). Distance measurements for each strain were computed and an average of each strain (from the three samples) was calculated along with the standard error.

Transmission electron microscopy of pili: Bacteria were grown overnight at 28°C without shaking. A 200 mesh formvar coated nickel grid was floated on 50 µL of each bacterial culture for 5 minutes. Grids were rinsed for 30 seconds in distilled water and stained with 2 % uranyl acetate for two minutes. Excess uranyl acetate was blotted from the grid, and grids were then rinsed for 30 seconds and air dried. Samples were observed using a Hitachi H-7000 Transmission electron microscope (TEM) at an accelerating voltage of 75kv. Photographs were taken on 3.25 x 4 Kodak EM film and scanned on an Epson flatbed scanner.

RESULTS

Biofilm data

Biofilm formation was measured based on the optical density (570 nm) of the crystal violet solution eluted from the dissolved biofilm formed on each glass test tube. Biofilm concentrations were calculated by subtracting the OD_{570} measured from each of the control tubes (sterile SWT media) from the OD_{570} measurement of the eluted crystal violet/biofilm solution. This value was averaged from the three replicates and then used to determine whether differences existed among the 16 *Vibrio* strains isolated from different host species and habitats (Fig. 1).

Both free-living *V. fischeri* MDR7 and WH1 strains had similar concentrations of biofilm formed, with OD_{570} of approximately 0.07-0.08 (Fig. 1). For symbiotic strains, *V. fischeri* ETBB1B (*E. tasmanica*, Australia), SL518 (*S. ligulata*, France) EM 17 (*Euprymna morsei*, Japan) and EB12 (*E. berryi*, Japan) isolates had the highest levels of biofilm formation, ranging from 0.11 to nearly 0.3 OD_{570}. All *E. tasmanica* (ET) *V. fischeri* strains, with the exception of ETBB1B, had similar biofilm production,

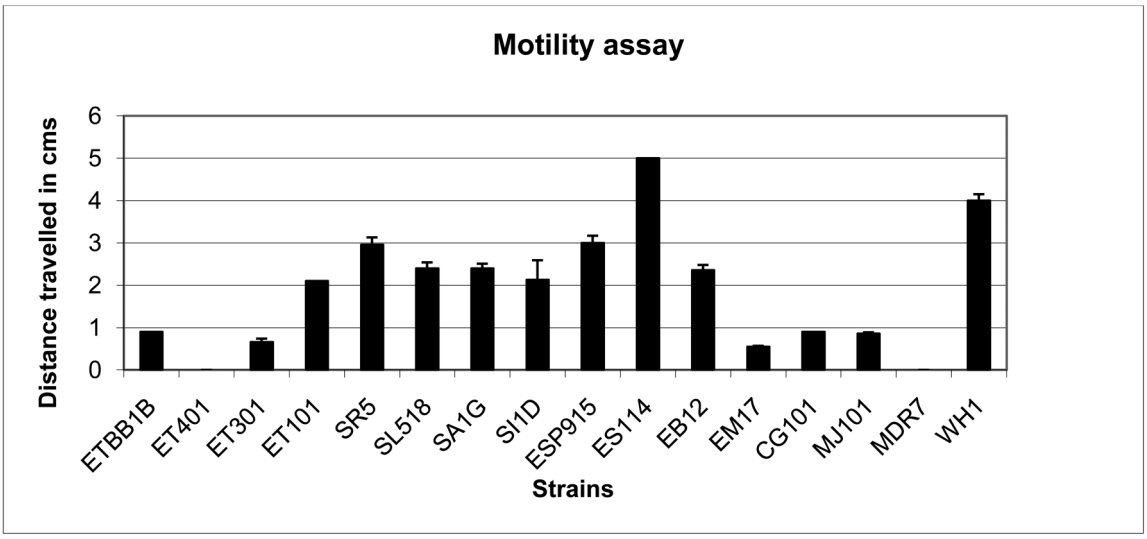

Fig. 2. – Motility measurements (in centimeters) for all 16 *Vibrio fischeri* strains. 10 µL of culture (0.1 OD_{600}) was placed in the center of each agar Petri dish and allowed to grow for 24 hours at 28°C. Measurements were made in triplicate, and the average was made for each strain. Error bars represent standard error. For strain designation, see Table I.

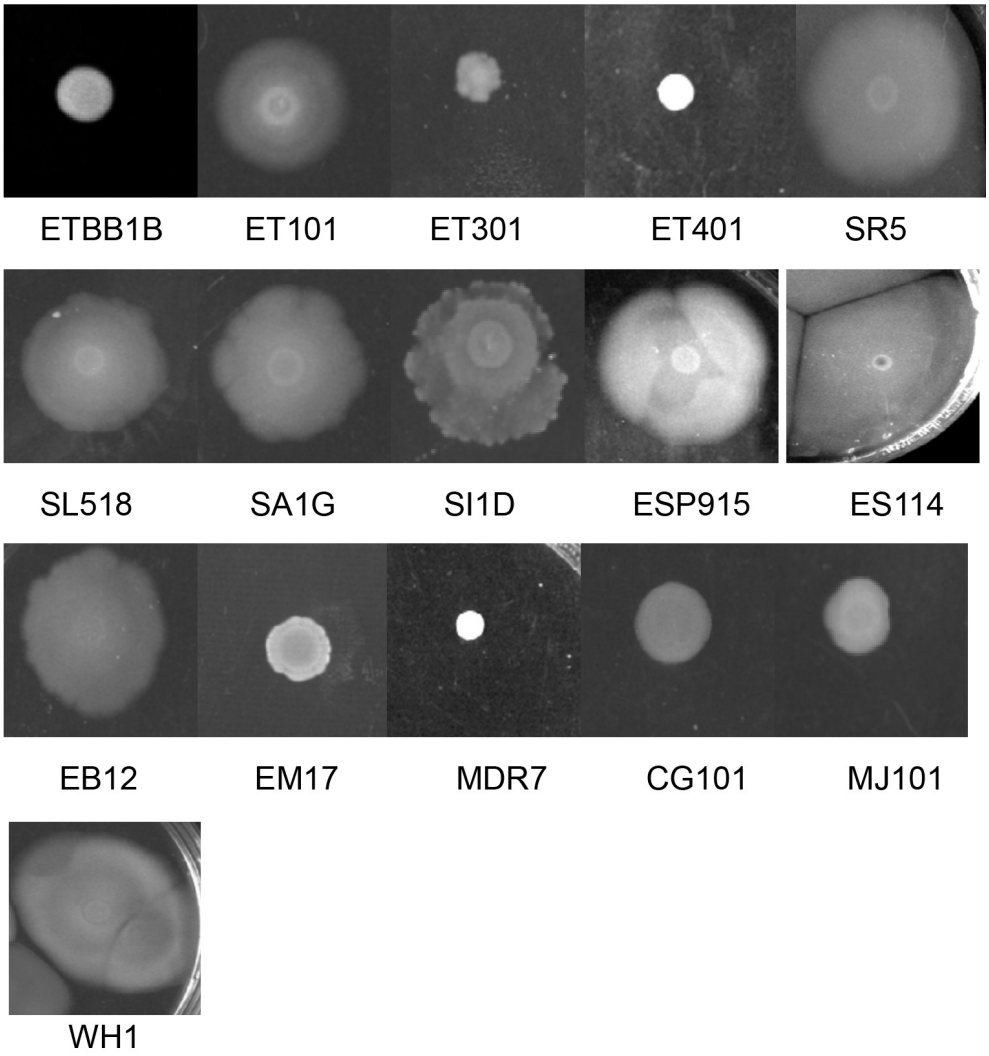

Fig. 3. – 24 hr motility time point on 0.5 % SWT agar plates for free-living and symbiotic *Vibrio fischeri* strains. For strain designation, see Table I.

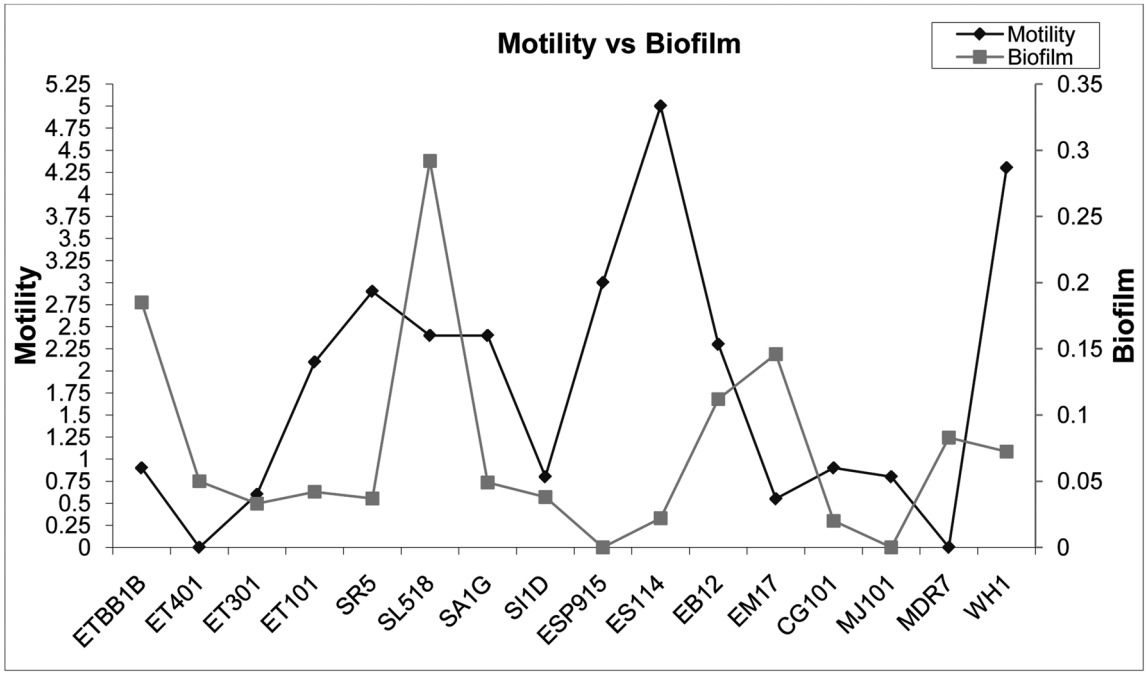

Fig. 4. – Motility vs biofilm formation for all *Vibrio fischeri* strains examined in this study. For strain designation, see Table I.

with an average of 0.04 OD_{570}. Interestingly, *V. fischeri* ETBB1B exhibited four-times more biofilm production when compared to other ET strains isolated from *E. tasmanica* squid hosts from different populations (Fig. 1, Table I). In contrast, both *V. fischeri* ES114 and ESP915 (*E. scolopes*, Hawaii) exhibited minimal biofilm formation, even though both strains were isolated from two different squid populations. Only one fish symbiont (*V. fischeri* CG101) examined was capable of producing biofilm, but was comparably less than other strains tested (0.01 OD_{570}). *V. fischeri* MJ101, a fish symbiont of *Monocentrus japonicus*, did not produce any detectable biofilm.

Motility assays

Motility for all *V. fischeri* strains was measured by calculating the distance traveled by each bacterial strain on low agar (0.5 %) concentration SWT plates (Figs. 2, 3). Symbiotic *V. fischeri* ES114 (from *E. scolopes*) exhibited the highest motility (5 cm) followed by the free-living strain *V. fischeri* WH1 (3.9 cm). Among all the *E. tasmanica* strains studied, *V. fischeri* ET101 was observed to exhibit the highest motility (1.7 cm). Both fish symbionts *V. fischeri* CG101 and *V. fischeri* MJ101 demonstrated similar motility capabilities, between 0.1 and 0.2 cm. Unlike the other symbiotic strains tested for motility *V. fischeri* ET301 (*E. tasmanica*, Australia) and *V. fischeri* SI1D (*S. intermedia*, France) demonstrated a more globular/non-uniform edge for each spot after incubation (Fig. 3). Interestingly, all ET strains from Australia differed in their motility rates (slow to fast) during *in vitro* growth (Figs. 3, 4), despite the fact that they infect the same species of *Euprymna*. Figure 4 demonstrates the relative biofilm formation and motility of each of the strains tested.

Pili morphology

Results from the transmission electron microscopical (TEM) observations made evident that closely related *V. fischeri* strains exhibited a number of different pili morphotypes (Fig. 5). *V. fischeri* ES114 was observed to have the smallest pili, and individual cells exhibited bifurcation among those pili (Fig. 5A). This was morphologically different from pili expressed by another Hawaiian symbiotic *V. fischeri* strain, ESP915, found in the same host species (Fig. 5B). Both *V. fischeri* strains colonize *E. scolopes*, but are from different populations (ES114 is from Kaneohe Bay, and ESP915 from Paiko, see Table I). Aggregated bacterial strains were observed to produce more pili than solitary bacteria, oftentimes with the pili interacting between each cell (Fig. 5D). Strains such as *V. fischeri* SL518, which was one of the strongest biofilm producers, also produced dense pili (Fig. 5D).

Both *V. fischeri* ETB1B and *V. fischeri* EM17 (*E. tasmanica* and *E. morsei* hosts) exhibited longer pili (Figs. 5C, 5G), and were noted to be less dense than *V. fischeri* SR5 and *V. fischeri* SL518, to both of which were strains from Mediterranean squid hosts (*S. robusta* and *S. ligulata*). It was also noted that *V. fischeri* EB12 (*E. berryi*, Japan), had long, continuous pili forming between two

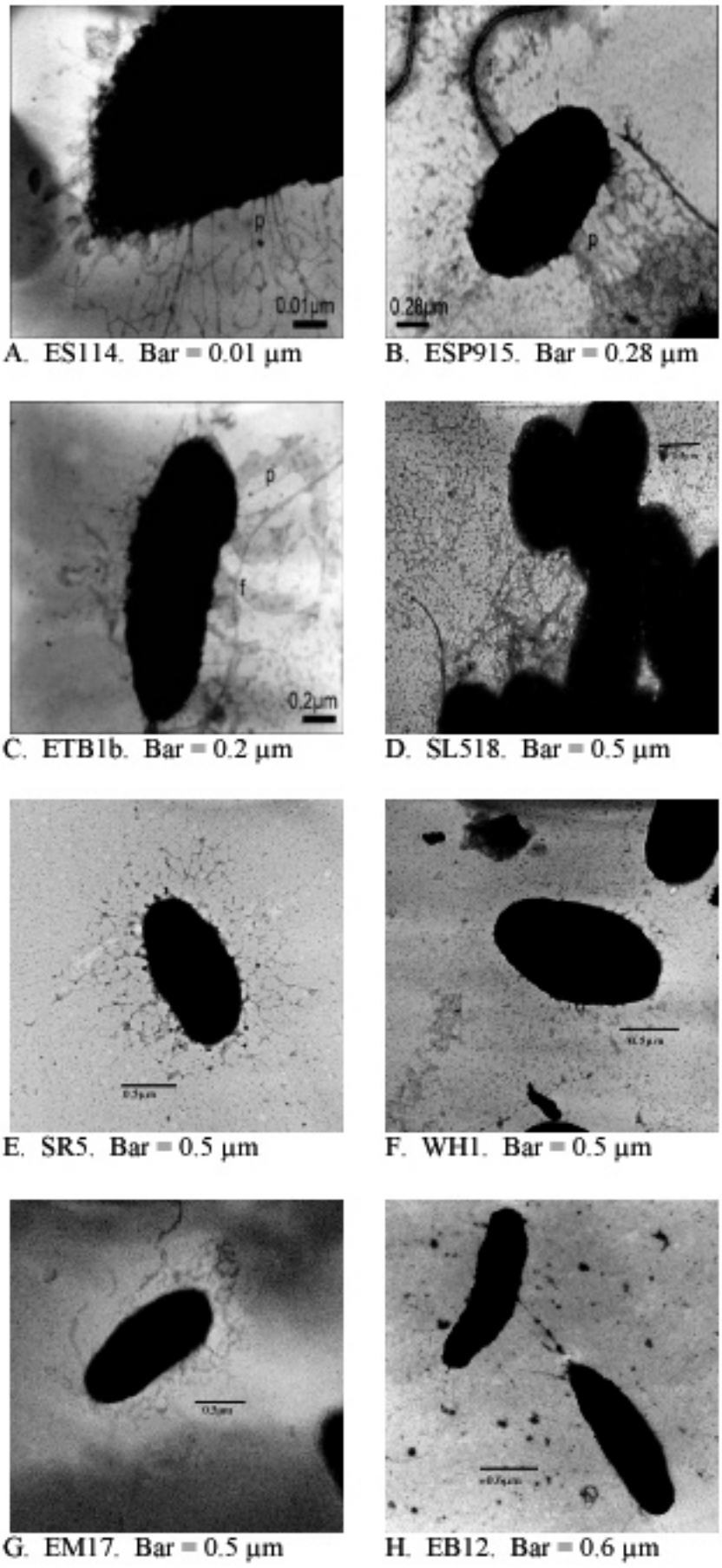

Fig. 5. – Negatively stained *Vibrio fischeri* cells exhibiting different pili morphologies in culture. For strain designation, see Table I.

cells (Fig. 5H). Finally, *V. fischeri* WH1, a free-living isolate, had short, stubby pili along the cell membrane (Fig. 5F). Other strains of *V. fischeri* screened did not reveal any pili (data not included).

DISCUSSION

The goal of this study was to determine whether symbiotic and free-living, *V. fischeri* strains differed in their motility, ability to form biofilm, and produce pili *in vitro*. Our interests were to examine possible differences between wild type strains that may affect infection and colonization capabilities before and after invading squid host light organs. Results from this study revealed that (1) both free-living and symbiotic *V. fischeri* strains have different biofilm forming capabilities; (2) most *V. fischeri* strains demonstrated their capacity to twitch and swim during *in vitro* motility assays; (3) there was heterogeneity in pili morphology among closely related *V. fischeri*.

With the exception of symbiotic *V. fischeri* CG101 and *V. fischeri* MJ101 fish strains, the majority of *V. fischeri* strains studied were isolated from squid light organs. *V. fischeri* MDR7 and *V. fischeri* WH1 were the only two free-living vibrios that were closely related to the other symbiotic *V. fischeri* (Nishiguchi & Nair 2003). Since all squid host species obtain their bacterial symbionts from the surrounding seawater within a few hours after hatching (McFall-Ngai & Ruby 1991, Montgomery 1993), it would be of particular interest if differences in symbiont behavior affect this event. Environmental persistence and host colonization pose very different challenges for symbiotic bacteria (Nishiguchi 2001, Hsiao *et al*. 2006, Jones *et al*. 2006), since vibrios have developed the ability to not only survive outside the host as free-living organisms, but within the host as a symbiotic mutualist. Each niche necessitates *V. fischeri* to develop, express, or modify specific structures like flagella and/or pili, allowing the accommodation of both habitats when the symbiont encounters a different situation. Previous work has demonstrated that genes specific for motility are particularly necessary for the initial infection of *V. fischeri* into the *E. scolopes* light organ; without this structure, they are incapable of efficiently colonizing its squid host (Millikan & Ruby 2002, Millikan & Ruby 2003). Additionally, specific genes that are only expressed during the free-living stage prior to infection demonstrate that chemotaxis (*che* loci), pili assembly (*pilM*), and precursors to biofilm formation (*mshA*) are present during this portion of the life history of *V. fischeri*, and not when inside the light organ of its squid host (Ariyakumar & Nishiguchi 2009, Jones & Nishiguchi 2006). This exemplifies the breadth that *V. fischeri* exhibits and is capable of achieving between periods of symbiosis and host venting. Whether subtle changes exhibited by each strain determines host specificity, or is driven by environmental constraints that may be dictated by each particular habitat, such factors may be correlated to the differences observed in closely related *V. fischeri* strains in this study.

Environmental signals and cellular structures have also been shown to be required for adhesion to the intestinal epithelium, and are controlled under various regulatory pathways leading to biofilm formation (Watnick *et al*. 1999). For example, nutrient dependence is not the only factor that induces formation of biofilms, and that numerous bacterial species can form biofilms even in nutrient deficient environments (Pratt & Kolter 1998). In addition, quorum sensing has also been linked to the formation of biofilm, which may play an important role in regulating inter-microbial communication within and between species in the biofilm matrix (Hooper & Gordon 2001). For example, *V. cholerae*, acyl homoserine lactone CAI-1 (cholera autoinducer 1) plays a significant role in biofilm formation, and has also been shown to control LuxO activity (Hsiao *et al*. 2006). Similarly, *V. fischeri* ES114 has a conserved cluster of genes (*syp* operon) that promotes symbiotic colonization as well as biofilm formation (Yip *et al*. 2005, Yip *et al*. 2006). Both examples suggest the possibility that autoinducers and regulatory elements may have an additional role in biofilm formation as well as colonization and luminescence within squid hosts.

Prior work with the *V. fischeri* genome validates that 10 separate pilus gene clusters are contained within the genome, including mannose sensitive hemagglutinin (*mshA*) as one of the eight type IV pilus loci (Ariyakumar & Nishiguchi 2009, Ruby *et al*. 2004). In addition, both *V. fischeri* ESP915 (another ES strain) and *V. fischeri* ETJB (an ET strain) express these genes either in their free-living or symbiotic state (Jones & Nishiguchi 2006). Vibrio bacteria have been shown to be capable of recruiting different types of pili, depending on whether they are in association with their host (Watnick & Kolter 1999). Employing different pili morphotypes as needed or at different times may increase chances of survival in seawater as well as increasing successful initiation of infection and colonization of a new host. Based on this, it was expected that symbiotic *V. fischeri* strains would exhibit different biofilm formation when compared to free-living *V. fischeri* strains that are not able to colonize animal hosts. The hypothesis that differences in biofilm production exist between strains of *V. fischeri* isolated from distinct host species was confirmed by our studies (Fig. 1). Furthermore, strains isolated from the same squid host but different populations (*i.e*., *E. tasmanica*) displayed different levels of biofilm formation, even though all strains can equally colonize *E. tasmanica* hosts (Nishiguchi 2002). Previous work has demonstrated that *V. fischeri* strains are commonly shared across a network of *Euprymna* hosts, and occupy niches that may contain huge biogeographical distances (Jones *et al*. 2006). Therefore, squid specificity is not what is driving morphological differences such as pili and flagellar formation, but the combina-

tion of environmental survival and host infection prior to colonization.

This study suggests that *V. fischeri* symbionts contain a number of mechanisms that are phenotypically plastic, and have the ability to contribute to host specificity. Recently, we have begun to investigate the role of symbiotic genes in the formation of biofilm during symbiosis. Particularly, genes such as UDP-glucose dehydrogenase, which is a precursor for biofilm formation, is solely expressed by bacteria during symbiosis (Allegrucci *et al*. 2006, Jones & Nishiguchi 2006). The fact that this gene is solely expressed in *V. fischeri* present in the squid light organ strongly suggests bacterial biofilm is being formed in the light organ crypts. Preliminary evidence has demonstrated that bacteria deficient at this locus are unable to adhere to the epithelial lined crypts of the squid light organ, which does not allow them to successfully colonize the light organ to the level of the parental wild type strain (Ariyakumar & Nishiguchi 2009).

Given that most chronic infections are initiated through biofilms (Stewart & Costerton 2001) and that pili play a crucial role in biofilm formation (Watnick & Kolter 1999), it is also likely that biofilm formation in squid light organs is pili driven and not just *mshA* or UDP-glucose dehydrogenase dependent. Based on this assumption, it was expected that pili morphotypes expressed by the bacteria as well as their biofilm forming ability would assist colonization at different stages of this mutualism. The initial step would be entering the light organ of the squids. Our results from motility assays demonstrate a huge amount of variation that *Vibrio* bacteria have in their motility rates *in vitro*. Since colonization of newly hatched *Euprymna* requires the bacteria to locate the external pores (Nyholm *et al* 2000, Wolfe *et al*. 2004), higher motility rates may confer a selective advantage by allowing them to out-compete other symbiotic vibrios prior to entering the squid light organ. Once the bacteria are inside the light organ, the ability to adhere (pili) and form matrices (biofilm) with other bacteria or squid epithelia may provide yet another selective advantage for vibrio bacteria to be better at infection and colonization. Our observations strongly suggest that vibrios are highly plastic in their abilities to accommodate their immediate surroundings, which supports the hypothesis that squid host specificity may not be the only factor that drives specificity in this closely-knit mutualism.

The goal of this study was to illustrate whether symbiotic and free-living *V. fischeri* strains varied in qualities that are important for colonization. Biofilm formation, motility and twitching, and pili morphotypes were all found to be different among both symbiotic and free-living strains, with no correlation to geographical location or squid host species. Such implications suggest that specificity among *Vibrio* bacteria is not a simple, straightforward matter; and that the evolution of such environmentally transmitted associations are molded by both abiotic factors surrounding the free-living stage, as well as the importance of the light organ (biotic) during the symbiosis stage. Future mutational studies on biofilm forming and pili synthesis genes, followed by the ability of these mutated strains to colonize their host would provide more insight on the roles each of these factors have in host colonization.

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REFERENCES

Allegrucci M, Hu FZ, Shen K, Hayes J, Ehrlich GD, Post JC, Sauer K 2006. Phenotypic characterization of *Streptococcus pneumoniae* biofilm development. *J Bacteriol* 188: 2325-2335.

Ariyakumar DS, Nishiguchi MK 2009. Characterization of two host specific genes, mannose sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (UDPDH) that are involved in the *Vibrio fischeri-Euprymna tasmanica* mutualism. *FEMS Microbiol Lett* 299: 65-73.

Branda SS, Vik S, Friedman L, Kolter R 2005. Biofilms: The matrix revisited. *Trends Microbiol* 13: 20-26.

Ghigo JM 2001. Natural conjugative plasmids induce bacterial biofilm development. *Nature* 412: 442-445

Guerrero-Ferreira RC, Nishiguchi MK 2007. Identification of light organ symbionts from the genera *Uroteuthis, Loliolus*, and *Euprymna*. *Cladistics* 23: 1-10.

Hooper LV, Gordon JI 2001. Commensal host-bacterial relationships in the gut. *Science* 292: 1115-1118.

Hsiao A, Liu Z, Joelsson A, Zhu J 2006. *Vibrio cholerae* virulence regulator-coordinated evasion of host immunity. *Proc Natl Acad Sci USA* 103: 14542-14547.

Jones BW, Huttenberg J, Lopez JE, Nishiguchi MK 2006. Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. *Mol Ecol* 15: 4317-4329.

Jones BW, Nishiguchi MK 2004. Counterillumination in the bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Mar Biol* 144: 1151-1155.

Jones BW, Nishiguchi MK 2006. Differentially expressed genes reveal adaptations between free-living and symbiotic niches of *Vibrio fischeri* in a fully established mutualism. *Can J Microbiol* 52: 1218-1227.

Klemm P, Schembri MA 2000. Bacterial adhesins: Function and structure. *Int J Med Microbiol* 290: 27-35.

Lee VT, Schneewind O 2001. Protein secretion and the pathogenesis of bacterial infections. *Genes Dev* 15: 1725-1752.

Mandel MJ, Wollenberg MS, Stabb EV, Visick KL, Ruby EG 2009. A single regulatory gene is sufficient to alter bacterial host range. *Nature* 458: 215-220.

McFall-Ngai MJ, Ruby EG 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial symbiosis. *Science* 254: 1491-1494.

McFall-Ngai MJ, Ruby EG 1998. Sepiolids and Vibrios: When first they meet. *Bioscience* 48: 257-265.

Millikan DS, Ruby EG 2002. Alterations in *Vibrio fischeri* motility correlate with a delay in symbiosis initiation and are associated with additional symbiotic colonization defects. *Appl Environ Microbiol* 68: 2519-2528.

Millikan DS, Ruby EG 2003. FlrA, a sigma^{54}-dependent transcriptional activator in *Vibrio fischeri*, is required for mobility and symbiotic light organ colonization. *J Bacteriol* 185: 3547-3557.

Montgomery MK, M. J. McFall-Ngai 1993. Embryonic development of the light organ of the sepiolid squid *Euprymna scolopes* Berry. *Biol Bull* 184: 296-308.

Nair VS, Ghoshroy S, Nishiguchi MK 2009. Distribution and localization of native and non-native vibrios in the bobtail squid, *Euprymna tasmanica* (Mollusca: Cephalopoda). *Microbiol Ecol* in review.

Nishiguchi MK 2001. Co-evolution of symbionts and hosts: The sepiolid-*Vibrio* model. *In* Seckbach J ed, Symbiosis: Mechanisms and Model Systems, Cole-Kluwer Academic, Dordrecht, The Netherlands: 757-774.

Nishiguchi MK 2002. Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. *Microbiol Ecol* 44: 10-18.

Nishiguchi MK, Nair VA 2003. Evolution of pathogenicity and symbioses in Vibrionaceae: An combined approach using molecules and physiology. *Int J Syst Evol Bacteriol* 53: 2019-2026.

Nishiguchi MK, Ruby EG, McFall-Ngai MJ 1998. Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-*Vibrio* symbioses. *Appl Environ Microbiol* 64: 3209-3213.

Nyholm SV, Deplancke B, Gaskins HR, Apicella MA, McFall-Ngai MJ 2002. Roles of *Vibrio fischeri* and nonsymbiotic bacteria in the dynamics of mucus secretion during symbiont colonization of the *Euprymna scolopes* light organ. *Appl Environ Microbiol* 68: 5113-5122.

Nyholm SV, McFall-Ngai MJ 2003. Dominance of *Vibrio fischeri* in secreted mucus outside the light organ of *Euprymna scolopes*: the first site of symbiont specificity. *Appl Environ Microbiol* 69: 3932-3937.

Nyholm SV, McFall-Ngai MJ 2004. The winnowing: Establishing the squid-*Vibrio* symbiosis. *Nat Rev Microbiol* 2: 632-642.

Nyholm SV, Nishiguchi MK 2008. The evolutionary ecology of a sepiolid squid-*Vibrio* association: From cell to environment. *Vie Milieu* 58: 175-184.

Nyholm SV, Stabb EV, Ruby EG, McFall-Ngai MJ 2000. Establishment of an animal-bacterial association: Recruiting symbiotic vibrios from the environment. *Proc Natl Acad Sci USA* 97: 10231-10235.

O'Toole G, Kolter R 1998. Flagellar and twitching motility are necessary for *Psuedomonas aeruginosa* biofilm development. *Mol Microbiol* 30: 295-304

O'Toole G, Kaplan HB, Kolter R 2000. Biofilm formation as microbial development. *Annu Rev Microbiol* 54: 49-79.

Pratt LA, Kolter R 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* 30: 285-293.

Ruby E, Henderson B, McFall-Ngai M 2004. Microbiology. We get by with a little help from our (little) friends. *Science* 303: 1305-1307.

Ruby EG, McFall-Ngai MJ 1992. A squid that glows in the night: Development of an animal-bacterial mutualism. *J Bacteriol* 174: 4865-4870.

Stabb EV, Ruby EG 2002. RP4-based plasmids for conjugation between *Escherichia coli* and members of the Vibrionaceae. *Methods Enzymol* 358: 413-426.

Stewart PS, Costerton WJ 2001. Antibiotic resistance of biofim. *The Lancet* 358: 135-138.

Watnick PI, Fullner KJ, Kolter R 1999. A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. *J Bacteriol* 181: 3606-3609.

Watnick PI, Kolter R 1999. Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Mol Microbiol* 34: 586-595.

Wolfe AJ, Millikan DS, Campbell JM, Visick KL 2004. *Vibrio fischeri* s^{54} controls motility, biofilm formation, luminescence, and colonization. *Appl Environ Microbiol* 70: 2520-2524.

Wollenberg MS, Ruby EG 2009. Population structure of *Vibrio fischeri* within the light organs of *Euprymna scolopes* from two Oahu populations. *Appl Environ Microbiol* 75: 193-202.

Yip ES, Geszvain K, DeLoney-Marino CR, Visick KL 2006. The symbiosis regulator RscS controls the *syp* gene locus, biofilm formation and symbiotic aggregation by *Vibrio fischeri*. *Mol Microbiol* 62: 1586-1600.

Yip ES, Grublesky BT, Hussa EA, Visick KL 2005. A novel, conserved cluster of genes promotes symbiotic colonization and s54-dependent biofilm formation by *Vibrio fischeri*. *Mol Microbiol* 57: 1485-1498.

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