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Multiple *Vibrio fischeri* genes are involved in biofilm formation and host colonization

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Abstract

Biofilms are increasingly recognized as the predominant form for survival in the environment for most bacteria. The successful colonization of *Vibrio fischeri* in its squid host *Euprymna tasmanica,* involves complex microbe-host interactions mediated by specific genes that are essential for biofilm formation and colonization. In the present investigation, structural and regulatory genes were selected to study their role in biofilm formation and host colonization. We have mutated several genes (*pilT, pilU, flgF, motY, ibpA* and *mifB*) by an insertional inactivation strategy. Results demonstrate that structural genes responsible for synthesis of type IV pili and flagella are crucial for biofilm formation and host infection. Moreover, regulatory genes affect colony aggregation by various mechanisms including alteration of synthesis of transcriptional factors and regulation of extracellular polysaccharide production. These results reflect the significance of how genetic alterations influence communal behavior, which is important in understanding symbiotic relationships.

Introduction

In most environments, bacteria are found to be forming sessile communities attached to a surface known as biofilms, which form a major portion of the microbial biomass present in nature (Yoshida & Kuramitsu, 2002; Moorthy & Watnick, 2004; Kievit, 2009). The formation of a biofilm constitutes a common strategy utilized for establishment of symbiotic associations, such as mutualisms (Ariyakumar & Nishiguchi, 2009; Morris & Visick, 2010) and pathogenic interactions (Hoyle & Costerton, 1991).

Vibrio fischeri is a marine bacterium that infects the light organs of sepiolid squids and monocentrid fishes (Nishiguchi *et al.,* 2004), establishing an exclusive partnership beneficial to both host and symbiont (Nyholm & McFall-Ngai, 2004). The association in the Hawaiian bobtail squid (*Euprymna scolopes*) has been used as a model system for more than 20 years. At the onset of the mutualism, free-living bacteria infect juvenile aposymbiotic squids within the first few hours after hatching. Host derived mucus provides a surface that allows bacteria to aggregate (and form a biofilm) prior to colonization (Nyholm *et al.,* 2002), which eventually forms an additional biofilm in the crypts of the squid's light organ complex (Visick & Ruby, 2006). The host provides an appropriate niche for the bacteria to reproduce and form this internal biofilm in the host light organ, allowing an environment where the bacteria produce bioluminescence that is used by the squid to avoid predation in a behavior

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known as counterillumination (Jones & Nishiguchi, 2004). At dawn after the first day of colonization, squids release (or vent) over 90% of the bacteria to the environment to re-populate the bacterioplankton community and infect newly hatched juveniles (Ruby, 1999; Nyholm & McFall-Ngai, 2004). The symbiosis is highly specific and similar to pathogenesis in the dynamics of colonization (Visick & Ruby, 2006).

Successful colonization depends on the activation of numerous genes that lead to the formation of a biofilm. As a result, multiple genes are differentially expressed in biofilms when compared to those in their planktonic counterparts (Eko Niba *et al.*, 2007; Ariyakumar & Nishiguchi, 2009; Chavez-Dozal & Nishiguchi, 2011).

A number of studies have described the genetic basis of biofilm formation of mutualistic vibrios. Some examples include the discovery of hybrid sensor kinases such as *rpoN* (encoding for the σ^{54}; Wolfe *et al.,* 2003), symbiosis polyssacharide cluster (*syp*; Yip *et al.,* 2005) which is either transcriptionally regulated by the Rsc-SypG two-component regulatory system (Morris *et al,* 2011) or by two proteins, SypA and SypE (Morris & Visick, 2010; Morris *et al.,* 2011). Alternatively, the protein RscS has been reported to play an important role in biofilm formation by inducing expression of the Syp polysaccharide (Mandel *et al.,* 2009). Additional studies also emphasize the importance of mannose-sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (*UDPH*) in *Vibrio* biofilm formation (Ariyakuar & Nishiguchi, 2009). Remarkably, we know far less about the genetic basis of biofilm formation in mutualistic associations compared to pathogenic associations. Additionally, biofilms formed by *V. fischeri* and the roles that these play in the *Vibrio*-squid symbiosis are still not fully characterized. Previous studies in other organisms have identified several genes associated with function and formation of bacterial structures that are important for biofilm formation. In *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa* two structural genes, *pilT* and *pilU,* have been described to be important in adhesion and biofilm formation by the production of a hexameric ATPase that is required for the retraction of type IV pilus and “twitching” motility (Whitchurch & Mattick, 1994). Genes such as *flgF* are responsible for flagellum synthesis and have been implicated in biofilm formation, particularly related to the synthesis of a protein that is located between the hook-filament junction and proximal rod (Liu & Ochman, 2007). Flagella synthesis depends upon approximately 50 genes (Aldridge & Hiughes, 2002) and FlgF is considered one of the most important highly soluble proteins for flagellar assembly due to its location in flagellar organization (Saijo-Amano *et al.,* 2004). Another example is *motY,* which encodes one component of the sodium-type flagellar motor pump of certain vibrios (Hossain & Tsuyumu, 2006).

Genes important for metabolic processes have also been linked to biofilm formation. Heat shock proteins such as *ibpA* are overexpressed during the biofilm state of *Escherichia coli* (Beenken *et al.,* 2004), but their function in biofilm development has not been established. Another example is *mifB,* which is one of the loci responsible for synthesizing bis-(3′-5′)-cyclic-di-guanosine monophosphate (c-di-GMP). Among the multiple genes responsible for c-di-GMP synthesis, *mifB* (magnesium-dependent induction) has recently been identified since it promotes the synthesis of a DGC (Di-Guanilate Cyclase) that directly controls synthesis of c-di-GMP and (under different levels of magnesium) regulates flagellar gene transcription (O’Shea *et al.*, 2004; Wolfe & Visick, 2008). C-di-GMP is a unique novel second messenger that induces extracellular polysaccharide production (Nakhamchik *et al.,* 2008), regulates flagellar biosynthesis, twitching motility and related processes, which also include biofilm formation (Wolfe & Visick, 2008). Formation of biofilms is a complex and dynamic mechanism that relies both on the presence and concentration of bacteria, targeted gene regulation for bacterial aggregation and colony formation, and the expression of proteinaceous materials that will eventually become the matrix. Most genetic determinants

previously described have all been reported to be important for a least one aspect of biofilm formation in other Gram-negative bacteria. Therefore, we have chosen to analyze the role of several structural and regulatory genes (*piU, pilT, flgF, motY, ibpA*, and *mifB*) that are thought to have an important role in *V. fischeri* biofilm formation and host colonization. This study is also important because previous studies have focused on the effects in colonization of the Hawaiian squid host (*Euprymna* scolopes). Here, we focus on the effects of colonization in a different host (*Euprymna tasmanica*) that has been reported to show similar colonization mechanisms (Nishiguchi 2002; Nair & Nishiguchi 2009). Finally, we decided to select and study this set of genes because they have been found to be overexpressed in the biofilm state of *V. fischeri* (RT-PCR studies, unpublished data). Our hypothesis predicts that they play a crucial role in forming biofilms and are essential to promote symbiotic colonization by *V. fischeri* over its *E. tasmanica* host.

Materials and Methods

Strains, plasmids and growth conditions

All strains used are described in Table 1. *Vibrio fischeri* ETJB1H was isolated from the light organ of *Euprymna tasmanica* from Jervis Bay, Australia (Jones *et al.,* 2006). Strains were grown in either Luria Bertani (LB; per litre composition: 10 g tryptone, 5 g yeast extract and 10 g NaCl) or Luria Bertani high Salt (LBS; per litre composition: 10 g tryptone, 5 g yeast extract, 20 g NaCl, 50 mL 1M tris pH 7.5, 3.75 mL 80% glycerol and 950 ml dH_2O) media at 37°C and 28°C. For selection of specific mutant strains, erythromycin (25μg/mL) was added to the media.

Mutant construction and complementation

Mutants were constructed by insertion of the plasmid pEVS122 as described previously (Ariyakumar & Nishiguchi, 2009). All genes were partially amplified with specific primers designed from the sequenced strain ES114 (NCBI accession: NC_006840.2; Table 2). PCR products were purified and cloned into the suicide vector pEVS122, and wild type *V. fischeri* strains were transformed by tri-parental mating via conjugation through a helper strain (Stabb & Ruby, 2002). Strains that had undergone single homologous recombination events with the native gene were selected on LBS plates enriched with erythromycin (25 μg/mL). For complement construction, complete copies of all loci were amplified with specific primers for the entire locus (Table 2), purified, and cloned into the vector pVSV105. This plasmid was introduced into the particular mutant by tri-parental mating. Strains that had been successfully transformed were selected on LBS plates enriched with erythromycin (25 μg/mL) for maintenance of the pEVS122 plasmid and chloramphenicol (10 μg/mL) for maintenance of pVSV105. Mutants and complemented mutants were verified by Southern blot.

Biofilm assays

All bacterial strains (wild-type, mutants and complemented strains) were grown in LBS media and biofilm assays were performed as previously described (Nair & Nishiguchi, 2009). Strains were inoculated in 96 well microplates and incubated for 18 hours at 28°C. After incubation, planktonic cells were removed and the remaining biofilm was stained with crystal violet, which was immediately solubilized with 70% ethanol. Optical density was measured (562 nm), which directly reflects the amount of biofilm formed **(**Ariyakumar & Nishiguchi, 2009). Assays were completed from overnight cultures, using 5 wells in each plate per strain (technical replicate) and three plates (biological replicate) for a total of 15 replicates. Results were statistically analyzed via Tukey Post Hoc test.

Scanning Electron Microscopy

Overnight cultures of all strains were re-inoculated in 5 mL of LBS with an immersed sterile cover slip. Strains were incubated for 18 hours without shaking (Ariyakumar & Nishiguchi, 2009). Coverslips were washed with sterile seawater (32 ppt) and gold coated for scanning electron microscopy (SEM) with a Hitachi S34000-SEM (Hitachi, Schaumburg, IL) as previously described (Greiner *et al.,* 2005). Observations were repeated in triplicate from different overnight cultures.

In vitro chemostat system and CLSM

Biofilm formation was observed under a dynamic environment (continuous-flow) using a modified Kaduri drip-fed chemostat that was assembled and re-designed in our laboratory (Merritt *et al.,* 2005). Overnight cultures were injected with a needle into glass chambers that had been prepared with an inflow (connected to the media reservoir) and outflow (connected to a waste container) siphon. A peristaltic pump supplied fresh LBS media simultaneously to four identical chambers and removed waste at a ratio of 5 mL/min for 18 hours. Chambers were washed with fresh seawater, stained with 1 mL of live/dead stain (SYTO9/Propidium iodide, Invitrogen Molecular Probes L3224) for 15 minutes. Biofilms were subsequently examined by confocal-laser scanning microscopy (CLSM) at the NMSU Fluorescent Imaging Facility (TCS SP5, Leica Microsystems). Samples were measured in triplicate (chemostat was run three different times using different overnight cultures).

Motility assays

Swimming—To test for swarming and motility, *V. fischeri* ETJB1H wild-type, *flgF*$^{-}$ and *motY*$^{-}$ were examined for any phenotypic changes, such as cell spreading that leads to colony pattern differentiation. The media consisted of LBS with 0.5% (wt/vol) of Difco bacto-agar, to which 5 g/liter of glucose was added as previously described (Rashid & Kornberg, 2000). Swarm plates were inoculated from an overnight culture in LBS agar (1.5% wt/vol) using a sterile toothpick. Plates were then incubated at 28 °C for 24 hours.

Twitching—To examine twitching motility, *V. fischeri* ETJB1H wild-type, *pilU*$^{-}$ and *pilT*$^{-}$ were chosen for this portion of the study. Media consisted of LBS with 1% (wt/vol) of Difco bacto-agar. Plates were briefly dried and stab inoculated with a sterile toothpick. Strains were placed at the bottom of the each petri plate from an overnight culture in LBS agar (1.5% wt/vol), and incubated at 28 °C for 24 hours (Rashid & Kornberg, 2000).

Indole assays

Extracellular indole detection—To measure differences in production of extracellular indole, a method described by Kuczynska-Wisnik *et al.,* (2010) was used. Overnight cultures of *V. fischeri* ETJB1H wild-type and *ibpA*$^{-}$ were sub-cultured and incubated at 28°C. Indole was measured at different time points of growth by adding 2 mL Kovac's reagent (10 g p-dimethylaminobenzaldehyde, 50 mL 1 M HCl and 150 mL amyl alcohol) to 5 mL of media (after sedimentation of bacteria). This mixture was diluted 1:10 in HCl:amyl alcohol solution and the optical density (540 nm) was measured. Assays were performed in triplicate.

Effect of indole addition—To investigate the effect of indole in biofilm formation, a method described by Lee *et al.* (2008) was used. Overnight strains of *V. fischeri* ETJB1H and *ibpA*$^{-}$ were sub-cultured in 96 well microplates with LBS media (with 0.25, 0.5 and 1.0 mM of indole) and incubated at 28 °C for 18 hours. The crystal violet assay was used for biofilm quatification. All assays were performed in triplicate.

Colonization assays

To determine colonization efficiency, infection assays were performed as previously described (Nishiguchi, 2002). Briefly, overnight cultures of wild-type and mutant strains were regrown in 5 mL of fresh LBS media until they reached an $OD_{600\ nm}$ of 0.3. Cultures were then diluted to approximately 1×10^3 CFU/mL in 5 mL of sterile seawater and added to glass scintillation vials where newly hatched juvenile squids were placed (one individual/vial). Seawater was changed with fresh uninoculated seawater every 12 hours over a period of 48 hours. Animals were maintained on a light/dark cycle of 12/12. After 48 hours, animals were sacrificed and homogenized, and the diluted homogenate was plated onto LBS agar plates. Colony forming units (CFUs) were counted the next day to determine colonization efficiency of each strain. A total of 10 animals/strain were used. Results were analyzed using a Tukey Post Hoc test.

Results and Discussion

Mutational analysis to determine the importance of multiple genes in biofilm formation and host colonization

In this study we examined how mutations in different structural and regulatory genes affect the organization of *V. fischeri* biofilms both *in vitro* and in juvenile *E. tasmanica. V. fischeri* forms biofilms in diverse habitats including the environment and the squid host, which correspond to different ecological lifestyles. In the environment, there are multiple fluctuations of salinity and temperature that have a direct effect upon colonization and persistence (Soto *et al.*, 2009), but our knowledge of the genes that are important for *in vitro* biofilm formation that influence host colonization is very limited.

Since biofilms have been shown to be necessary for successful colonization of sepiolid squids, this study was aimed to understand whether specific structural and regulatory genes were essential for biofilm formation in both abiotic and symbiotic environments. Based on previously reported data, we organized the suite of genes into two categories: (i) those responsible for structural components such as flagella and pili, and (ii) transcriptional regulators of bacterial metabolism that influence synthesis of the components for the formation of the biofilm matrix and backbone.

In order to compare the identity of the genes selected with those of known function, a bioinformatics approach comparing proteins sequences with high similarity patterns was selected. The tools Blastp and MatGat v2 were used to compare sequences with those reported for *Vibrio cholerae* O1 biovar El Tor str N16961 (ID 243277). According to this analysis, identities of sequences with known function are: (a) PilU (*V. fischeri* accession number YP_203815.1) with an identity of 77-78% for twitching motility protein; (b) PilT (*V. fischeri* accession number YP_203814.1) with an identity of 80-82% for twitching motility protein; (c) FlgF (*V. fischeri* accession number YP_205256.1) with an identity of 78-80% to flagellar body basal rod protein; (d) MotY (*V. fischeri* accession number YP_204309.1) with an identity of 65-68% to sodium-type flagellar motor protein; (e) IbpA (*V. fischeri* accession number YP_203396.1) having an identity of 79-81% with a 16 KDa heat-shock protein; and (f) MifB (*V. fischeri* accession number YP_206917.1) with an identity of 40-42% to a diguanilate cyclase with a GGDEF domain. Protein function is conserved for sequence identities equal or higher than 40% (Brenner, 1999). Therefore, the genes (or proteins) described in this study are most likely to share the described functions from other sequenced vibrios.

For this study, we selected an insertional mutagenesis strategy (Ariyakumar & Nishiguchi, 2009) in which the exogenous vector pEVS122 serves as a mutagen and as a molecular tag for identification (Dunn *et al.,* 2005). Complementation was achieved by inserting a

complete copy of the gene contained in the vector pVSV105. Constructs made utilizing these vectors are stable and do not revert (Dunn *et al.,* 2005). This method has been used successfully in our study, enables rapid construction, and has facilitated the screen of defects in the mutated strains, including phenotypic differences (motility, biofilm architecture) and colonization deficiencies. Remarkably, all mutants constructed do not show growth defects when compared to the wild-type (results not shown) and when grown on standard media (LBS).

Biofilms formed by the wild-type strain exhibited a flocculent tree dimensional structure (Fig. 1), and mutated strains show deficiencies in biofilm formation in at least one of the assays tested, with all mutants deficient in host colonization. The following results discuss differences among the mutants examined depending on the function/nature of the genes.

FlgF and MotY

Flagellar motility has been demonstrated to be an important factor in bacterial biofilm formation (Houry *et al.,* 2010). To elucidate the role of the flagella in biofilm formation by *V. fischeri*, we used non-flagellated (*flgF*$^{-}$) and non-motile (*motY*$^{-}$) mutants. It is important to recognize that *flgF* forms part of the flagellar operon (composed of flgBCDEFGHIJKL) in vibrios (Merino *et al.,* 2006), and a mutation at this locus can cause polar effects on downstream genes, thereby creating an aflagellate mutant that is the product of a non-functional operon. Results indicate that both non-functional flagella (*motY*$^{-}$) and an aflagellate phenotype (*flgF*$^{-}$) severely impaired biofilm formation (Figs. 2, 3C, 3C1, 3D, and 3D1). Swimming motility was reduced in both mutants (Figs. 4 A, B, and C). Observations from our study demonstrates unequal production of flagella, suggesting that a non-functional flagellum can still partially form biofilms due to its ability to act as an adhesin in a manner that is independent from motility. This behavior has also been observed in the case of enteropathogenic *E. coli* (Giron *et al.*, 2002). Nevertheless, both mutants were equally deficient in colonizing juvenile squids (Fig. 5). Based on these and earlier results (Millikan & Ruby, 2002; 2003; 2004), it is reasonable to propose that flagella are essential for motility and interactions with host cells. How these symbiotic loci are regulated and synchronized during infection still remains an important issue to address in future studies.

PilU and PilT

One of the most renowned roles of pili proteins in biofilm formation is bacterial adherence to surfaces during the initial phases of adhesion (Yildiz & Visick, 2009). *V. fischeri* pili are formed by a number of proteins encoded by genes from the *pil* operon, including *pilA-pilD* (Browne-Silva & Nishiguchi, 2008; Stabb & Ruby, 2003). *pilU* and *pilT* are not part of this particular operon, and their function is related to elongation and retraction, which is important for “twitching” or “gliding” motility (Zolfaghar *et al.,* 2003). These genes produce a hexameric ATPase that has previously been described to be an important virulence factor in *Pseudomonas aeruginosa* (Zolfaghar *et al.,* 2003) and *Neisseria gonorrhoeae* (Firoved & Deretic, 2003), and are important for biofilm formation in these bacteria.

Visual examination of the parental strain, or wild-type *V. fischeri* ETJB1H (Fig. 1A), exhibited a compact flocculent homogeneous organization. At higher magnifications, cells were distinguished by their rod-shaped organization in a mature three-dimensional structure (Figs. 1B, 1C). A dramatic difference in biofilm architecture was observed in all the mutants. For mutants with disruption of their structural pili genes (*pilU, pilT*), biofilm organization remained similar to those observed for the wild-type *V. fischeri* ETJB1H (Figs. 3A, 3A1, 3B, 3B1). These results were consistent with those observed in the microtitre plate biofilm assay.

Pil mutants were significantly impaired in colonizing axenic juvenile squids (Fig. 5). These mutants are defined as accommodation mutants, which do not colonize juvenile squid hosts to the same levels as their wild-type congener (Nyholm & McFall-Ngai, 2004). In addition, a micro-chemostat system was assembled in order to assess the capacity of various wild-type and mutant strains to form biofilms under a dynamic environment. The tested strains were supplied with a constant carbon source with the purpose of examining the growth of the biofilm in real time during 18 hours of incubation. Analysis of the samples by confocal microscopy revealed that biofilms from wild-type *V. fischeri* ETJB1H consisted of dense layers of aggregates of cells (Fig. 6A), whereas biofilms formed from all mutants consisted in non-dense and isolated aggregates (Figs. 6B-F). Differences in community formation were observed in mutants for the *pil* locus when compared to observations using SEM; *pil* mutants did not form biofilms in the chemostat system (Figs. 6B, 6C). All other mutants had similar biofilm formation that was consistent with SEM observations. These contrasting observations suggest that biofilm formation is sensitive to hydrodynamic environments, and twitching motility may be important to overcome the multiple barriers that the bacterium encounters before reaching the host light organ (*i.e.*, ducts and ciliated appendages) in order to form a bacterial community. Twitching motility was assayed and defects were noted in both mutants when compared with the wild-type. In addition, previous observations by transmission electron microscopy indicate that these mutants are hyper-pilated, similar to earlier results in *Pseudomonas aeruginosa* (Bertrand *et al.,* 2010; results not shown). This phenotype was attributed to a defect in depolymerization of the pilin proteins during pilus retraction. The hyper-piliated phenotype in mutated *V. fischeri* may have enhanced bacterial adhesion, leading to an increase in community formation observed in our microtiter plate assay. However, it appears that pilus retraction is essential for colonization and biofilm formation under dynamic conditions.

IbpA

IbpA is a heat shock protein that is synthesized from an operon controlled by the σ^{54} subunit of the RNA polymerase, and induced under heat and other stress conditions (Kuczynska-Wisnik *et al.,* 2010). *IbpA* has been detected among stress-response genes that are overexpressed in biofilm populations; however, little is known about its function during biofilm formation. Recent results in *E. coli* demonstrate that lack of IbpAB proteins inhibit formation of biofilm at the air-liquid interface. In absence of these proteins, cells experience oxidative stress and overproduce extracellular indole (Kuczynska-Wisnik *et al.,* 2010), which is known to be a transcriptional regulator of many genes, including those involved in polysaccharide production (the biofilm matrix) and the quorum sensing cascade (bacterial communication, implicated in community formation). In order to test differences in indole, we performed an assay that measures production of indole over time and its effect on biofilm formation. The mutant *ibpA*$^{-}$ produces significantly more indole when compared with wild-type and complemented strains after 5 hours of incubation (Fig. 7A); alternatively, indole produces a significant reduction in biofilm formation (Fig. 7B). IbpA is classified as a small heat shock protein (Kuczynska-Wisnik *et al.,* 2010) and few studies have elucidated the importance of small heat shock proteins (sHsps) in host infection. Deficiencies in biofilm production were more apparent in regulatory mutants (*ibpA*$^{-}$, *mifB*$^{-}$), which appeared less complex and dense (Figs. 2, 3E, 3E1, 3F, 3F1). Our findings demonstrate that the *ibpA*$^{-}$ mutant does not infect the host squid as efficiently as the wild-type (Fig. 5), suggesting that IbpA may also be necessary for colonization of host squid tissues; however, we cannot determine the specific role of this particular protein in colonization. Future studies will examine the specific role of IbpA in adhesion and colonization of the squid light organ.

MifB

The second regulatory gene analyzed in this study was *mifB,* which is responsible for the synthesis of bis-(3′-5′)-cyclic-di-guanosine monophosphate (c-di-GMP; Visick & Wolfe, 2008). MifB is a small molecule that acts as a second messenger and regulates many distinct processes in bacteria, including synthesis of virulence factors and cellulose production (Cotter & Stibitz, 2007). Our findings indicate that *mifB*$^{-}$ mutants are neither able to produce biofilms nor impaired in infecting juvenile squid hosts (Figs. 2, 5), which corroborates earlier studies examining *mifB* function related to bioluminescence, motility, and colonization (O'Shea *et al*., 2005; 2006; Visick *et al*., 2007). The lack of biofilm formation in these mutants may be due to the absence of exopolyssaccharide production in this mutant. A recent study determined that c-di-GMP in *V. vulnificus* regulates extracellular polysaccharide production, which is an important component of the biofilm matrix (Nahamchik *et al.,* 2008). Similar to other symbiotic associations, c-di-GMP is proposed to have an important role in regulating changes in gene expression in *V. cholerae* during host infection; specifically regulating transcription of numerous virulence genes (Tamayo *et al.,* 2008). A proposed model suggests that *mifB* catalyzes the production of the c-di-GMP pathway to inhibit flagellar synthesis (Visick *et al.,* 2007). Mutation of this gene affects migration, which is directly related with disruption of biofilm formation. Additionally, Visick *et al.* (2007) reported that mutants lacking the *mif* genes are able to synthesize flagella in the presence of abundant Mg^{+2} (present in the seawater). This phenomenon may be related to dependent induction of biofilms, and future studies will address the effect of seawater components (such as Mg^{+2} concentration) on biofilm formation.

Biofilm formation appears to be under regulation of multiple genes. This study complements previous investigations that describe the roles of numerous genes in community biofilm formation in the *Vibrio-Euprymna* association. Earlier studies have focused on the *syp* operon and its regulation (Yip *et al*., 2005; Morris & Visick, 2010; Morris *et al*., 2011) and the role of isolated genes (not part of an operon) such as *mif* (O'Shea *et al*., 2004), *mshA*, and UDPH (Ariyakumar & Nishiguchi, 2009). Elucidation of the genetic mechanisms studied here provides another avenue for understanding the control of biofilm formation and consequently host colonization.

Conclusions

This study focused on deciphering the importance of several structural and regulatory genes in biofilm formation and host colonization. Results from our experiments indicate that all genes in our study are involved in the formation of mature biofilms, which is also important for the successful establishment and persistence of the mutualism between *V. fischeri* and *E. tasmanica.* Furthermore, we demonstrated that there is a difference in biofilm formation in static cultures and hydrodynamic environments when some of the structural genes are mutated (*pilU, pilT*), suggesting that there are special requirements for initial attachment prior to biofilm formation (twitching motility). Further research will be focused on the regulatory mechanisms of these genes and other various pathways that control biofilm formation and host colonization in order to interpret the mechanisms of symbiotic associations.

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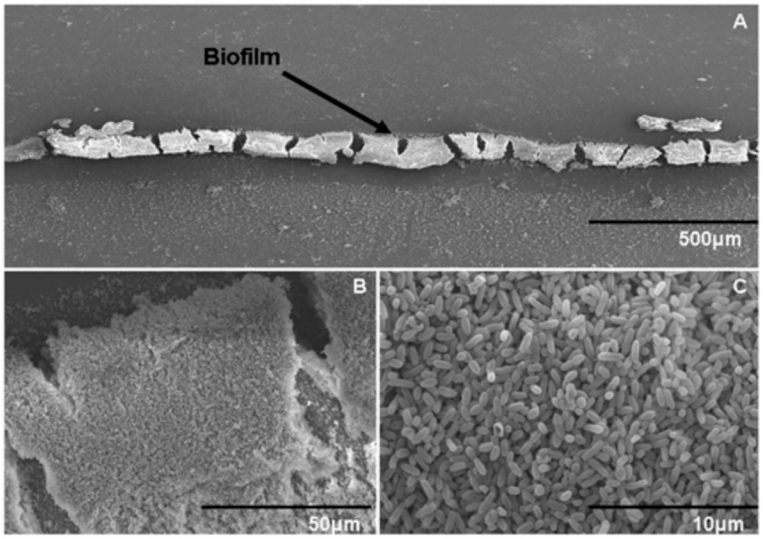

Figure 1.

Scanning electron micrograph (SEM) of biofilm formed by the wild-type strain (*V. fischeri* ETJB1H) on the liquid/air phase of a coverslip. A. Biofilm formed on coverslip glass, scale bar corresponds 500µm, 10X magnification, B. Biofilm formed on coverslip glass, scale bar corresponds 50 µm, 50X magnification, C. Biofilm formed in coverslip glass, scale bar corresponds 10 µm, 3000X magnification.

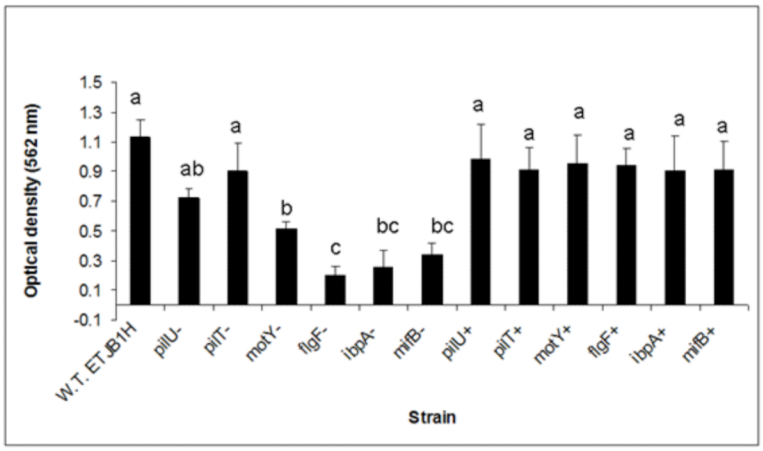

Figure 2.

In vitro biofilm formation for the various strains of *V. fischeri* ETJB1H. Data are plotted as the mean OD_{562} ± 1SD, with multiple comparisons calculated between groups. Different lower case letters on the abscissa indicate significant differences ($p<0.05$) between groups, according to the Tukey PostHoc comparison. According to these data, mutants *pilU*$^{-}$ and *pilT*$^{-}$ do not significantly differ from the wild-type (labeled with "a"), *pilU*$^{-}$ is labeled "ab" which means it is not different from *motY*$^{-}$, mutants *motY*$^{-}$, *flgF*$^{-}$, *ibpA*$^{-}$ and *mifB*$^{-}$ are significantly different from the wild-type (labeled with "b", "c" or "bc"), *ibpA*$^{-}$ and *mifB*$^{-}$ (labeled "bc") are not different from *flgF*$^{-}$.

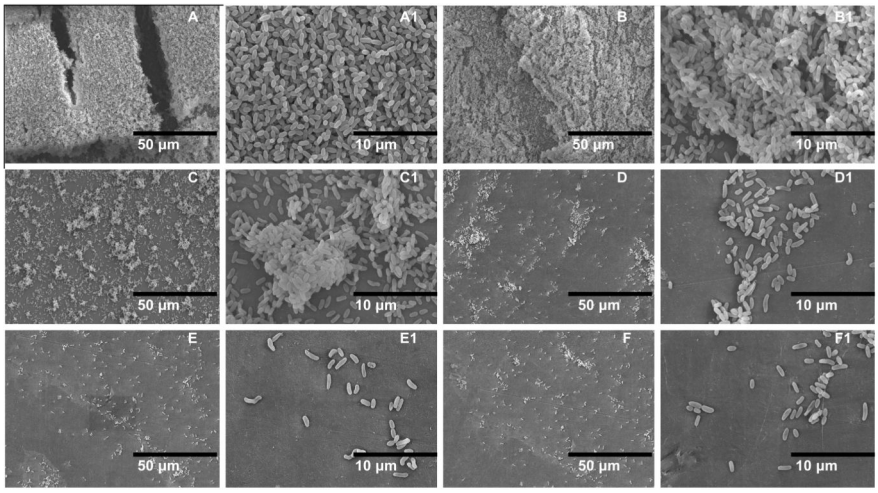

Figure 3.
Scanning electron micrograph (SEM) of biofilms formed by mutant strains. *pilU*$^{-}$ (A,A1), *pilT*$^{-}$ (B,B1), *motY*$^{-}$ (C,C1), *flgF*$^{-}$ (D,D1), *ibpA*$^{-}$ (E,E1), *mifB*$^{-}$ (F,F1), scale bar corresponds to 50µm at 50X magnification and 10µm at 3000X magnification.

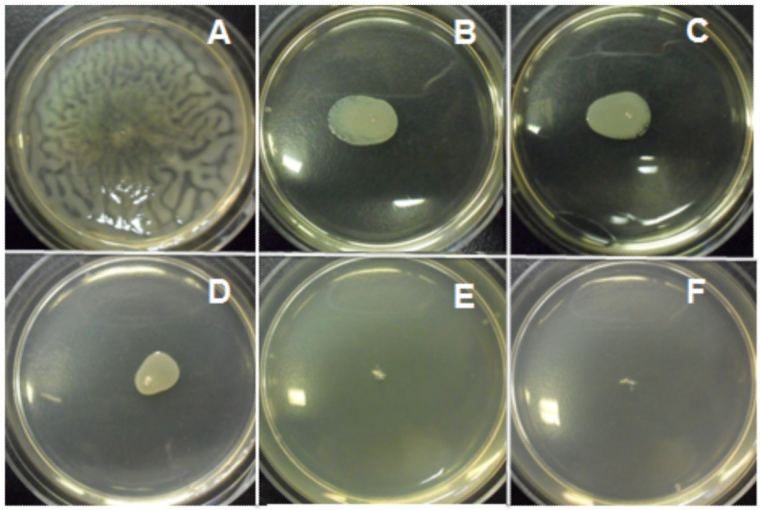

Figure 4.
Motility assays. Swimming for strains A. *Wild-type* ETJB1H, B. *flgF-* strain and C. *motY-* strain. Twitching for strains D. *Wild-type* ETJB1H, E. *pilT-* strain and F. *pilU-* strain. Plates were photographed after 24 hours of incubation.

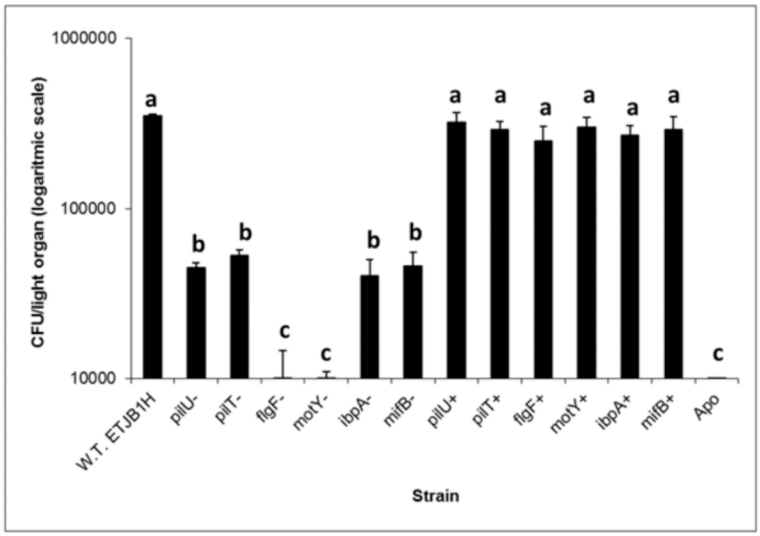

Figure 5.

Colonization assay 48-hour post-infection of juvenile *E. tasmanica* by wild-type and mutant strains of *Vibrio fischeri* ETJB1H. Mutant strains exhibited significant differences when compared with wild-type and complemented strains. Apo= aposymbiotic or non-infected juvenile squids. Data are plotted as the mean OD_{562} ± 1SD. Multiple comparisons were calculated between groups using the Tukey PostHoc comparison. Different letters indicate significant differences ($p<0.05$) between groups. According to these data *pilU-, pilT-, ibpA-* and *mifB-* are labeled "b" which indicates that are significantly different from the wild-type (labeled as "a"); *flgF-* and *motY-* (labeled as "c") are also different from the wild-type ("a") and from those one labeled as "b". Number of squid tested n=10 per strain.

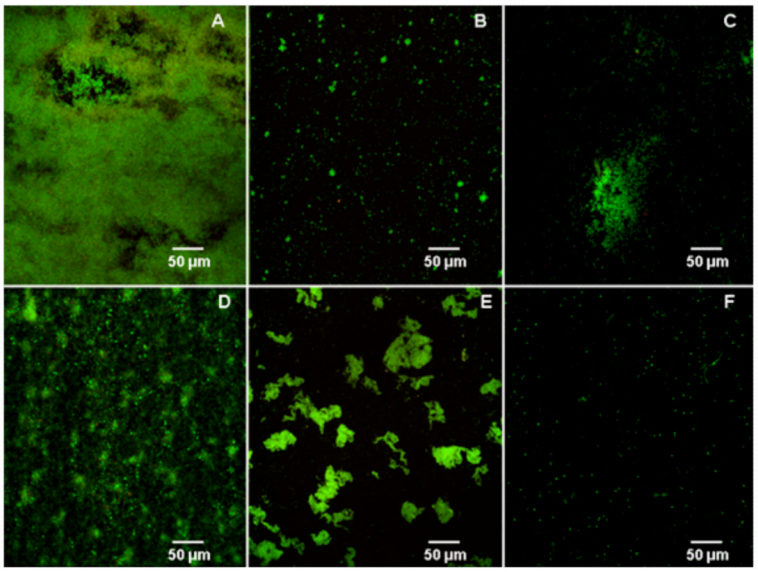

Figure 6.

Confocal Scanning Laser Microscopy (CSLM) of different *V. fischeri* strains. A. Wild-type *Vibrio fischeri* ETJB1H (thickness 19.35 ± 3.30 µm), B. *pilU*$^{-}$ (thickness 3.45 ± 0.18 µm), C. *pilT*$^{-}$ (thickness 3.66 ± 0.12 µm), D. *motY*$^{-}$ (thickness 4.84 ± 0.59 µm), E. *flgF*$^{-}$ (thickness 2.14 ± 0.67 µm), F. *ibpA*$^{-}$ (thickness 0.93 ± 0.27 µm) . Strain *mifB*$^{-}$ is not shown since it looks the same as *ibpA*$^{-}$ (thickness 0.88 ± 0.42 µm). Scale bar corresponds to 50 µm at 10X magnification. Mean thickness was calculated from 5 different image stacks (or "z" stack).

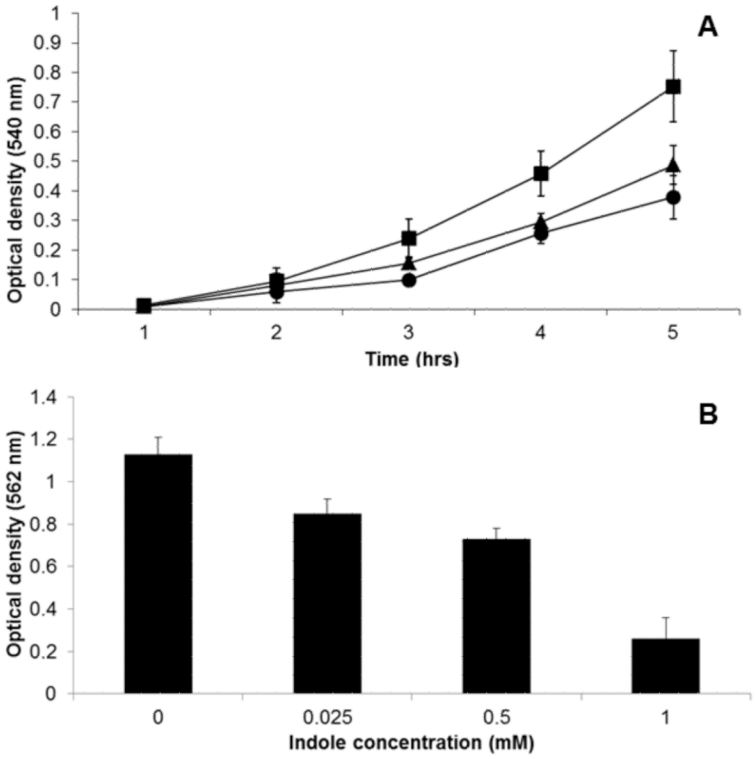

Figure 7.

Indole assays. A. Indole production test, squares represent the mean for *ibpA*$^{-}$, triangles represent the mean for the wild-type, and circles represent the mean for the complement (*ibpA*$^{+}$) strain. Indole production was calculated in triplicate (three different clones from the same strain). Production of indole from the *ibpA*$^{-}$ strain was significantly different from the other two strains. B. Effect of indole on biofilm formation on biofilms formed from the ETJB1H wild-type strain. Different concentrations of indole were added and biofilm mass was reduced significantly when concentration increased 2 fold or higher.

Table 1

Strains and plasmids used in this study.

Strains or Plasmids	Description
Plasmids:	
pEVS122	R6K $^{*}Erm^{R}$
pVSV105	pES213 replicon, $^{*}Cm^{R}$
Vibrio fischeri strains:	
ETJB1H	Wild-type from Jervis Bay, New South Wales, Australia
pilT−	ETJB1H (*pilT*::pEVS122). PilT insertion mutant
pilU−	ETJB1H (*pilU*::pEVS122). PilU insertion mutant
motY−	ETJB1H (*motY*::pEVS122). MotY insertion mutant
flgF−	ETJB1H (*flgF*::pEVS122). FlgF insertion mutant
ibpA−	ETJB1H (*ibpA*::pEVS122). IbpA insertion mutant
mifB−	ETJB1H (*mifB*::pEVS122). MifB insertion mutant
pilT+	ETJB1H (*pilT*::pEVS122) complemented with pVSV105::*pilT*
pilU+	ETJB1H (*pilU*::pEVS122) complemented with pVSV105::*pilU*
motY+	ETJB1H (*motY*::pEVS122) complemented with pVSV105::*motY*
flgF+	ETJB1H (*flgF*::pEVS122) complemented with pVSV105::*flgF*
ibpA+	ETJB1H (*ibpA*::pEVS122) complemented with pVSV105::*ibpA*
mifB+	ETJB1H (*mifB*::pEVS122) complemented with pVSV105::*mifB*

$^{*}Erm^{R}$(Erythromycin resistance), Cm^{R} (Chloramphenicol resistance)

Table 2

Primers used for mutant construction and complementation.

Primers	Primer sequence 5′-3′	PCR product size (bp)
pilT− Forward	GGATCCCTCGTGGCTGTGCTGCTGTGTT	316
pilT− Reverse	TCTAGACGCAAAGCTGAGCGAAGTGCT	316
pilU− Forward	GGATCCGGCTGCGATGACGGGGTATCG	450
pilU− Reverse	TCTAGAGTCTGCAACGCGTGGCGTGT	450
motY− Forward	GGATCCGCCCAATGGGTGAAACTCGTGC	172
motY− Reverse	TCTAGACTGCACCATCACCCGGCATCC	172
flgF− Forward	GGATCCAGCCATGAGTGGCGCAAAGC	456
flgF− Reverse	TCTAGAGCCATCGCTTCAGCTGGTGC	456
ibpA− Forward	GGATCCATGGCGGTAGCTGGCTTTGCT	187
ibpA− Reverse	TCTAGACCATCGTTGCGCCCACCACTT	187
mifB− Forward	GGATCCTGGCTATGGGGATTACCCGTTGGA	920
mifB− Reverse	TCTAGACAACTGAAGGCACTCACCTTGCGT	920
pilT+ Forward	ATATCTAGATACTAGTCATAGAAACGATTACCGAGGAAA	1038
pilT+ Reverse	TTACCCGGGTGATCATTTGTTTCAGTATTTGATCC	1038
pilU+ Forward	ATATCTAGAACTGTGTCATTCAGCCTGACACAAAGGAGTT	1101
pilU+ Reverse	TTACCCGGGAATGCCCACCAAACAAATCGCAA	1101
motY+ Forward	ATATCTAGAATCGTCAACGGCATGACCCATAGATTTAGG	879
motY+ Reverse	TTACCCGGGCGTCCTAGAGAAATGACAACACGACGG	879
flgF+ Forward	ATATCTAGATATTATAACTTCAGATAGATTATTGGAGTTC	750
flgF+ Reverse	TTACCCGGGACCCATAATGCTGGATTCATT	750
ibpA+ Forward	ATATCTAGATACTCACTATTGCTTAAATTAAAGGATAGT	440
ibpA+ Reverse	TTACCCGGGACAGCGCCTTTATGTTCAAT	440
mifB+ Forward	ATATCTAGAATGGTGATTACTCCCCCTAATGCCCAGGAGC	1971
mifB+ Reverse	TTACCCGGGTGCCGGGCCGATATGTGGCT	1971