RESEARCH ARTICLE

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

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Abstract

Biofilms are increasingly recognized as being the predominant form for survival for most bacteria in the environment. 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. Here, 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. The 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 form 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). Biofilm formation is 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 that is beneficial to both host and symbiont (Nyholm & McFall-Ngai, 2004). Its 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, providing an environment where the bacteria produce bioluminescence that is used by the squid to avoid predation in a behavior 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 with 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

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include the discovery of hybrid sensor kinases such as *rpoN* (encoding for the σ^{54}; Wolfe *et al.*, 2003) and symbiosis polyssacharide cluster (*syp*; Yip *et al.*, 2005), which is transcriptionally regulated either by the RscSypG 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 (Ariyakumar & Nishiguchi, 2009).

Remarkably, we know far less about the genetic basis of biofilm formation in mutualistic associations compared with 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 (Withchurch & 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 as it promotes the synthesis of a DGC (di-guanilate cyclase) that directly controls synthesis of c-di-GMP and (at different concentrations of magnesium) regulates flagellar gene transcription (O'Shea *et al.*, 2006; Wolfe & Visick, 2008). c-di-GMP is a unique novel second messenger that induces extracellular polysaccharide production (Nahamchik *et al.*, 2008), and 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 on the expression of proteinaceous materials that will eventually become the matrix. Most genetic determinants previously described have been reported to be important for a least one aspect of biofilm formation in other Gram-negative bacteria. Therefore, we have chosen to analyse 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, our unpublished data). Our hypothesis predicts that they play a crucial role in forming biofilms and are essential in promoting 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; L^{-1}: 10 g tryptone, 5 g yeast extract and 10 g NaCl) or Luria–Bertani high Salt (LBS; L^{-1}: 10 g tryptone, 5 g yeast extract, 20 g NaCl, 50 mL 1 M Tris, pH 7.5, 3.75 mL 80% glycerol and 950 mL dH_2O) media at 37 and 28 °C. For selection of specific mutant strains, erythromycin (25 μg mL^{-1}) was added to the media.

Mutant construction and complementation

Mutants were constructed by insertion of plasmid pEVS122 as described previously (Ariyakumar & Nishiguchi, 2009). All genes were partially amplified with specific primers designed from the sequenced strain ES114 (NCBI

Table 1. Strains and plasmids used in this study

Strain or plasmids	Description
Plasmids	
pEVS122	R6K Erm^R
pVSV105	pES213 replicon, Cm^R
V. 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.

accession: NC_006840.2; Table 2). PCR products were purified and cloned into 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^{-1}). For complement construction, complete copies of all loci were amplified with specific primers for the entire locus (Table 2), purified and cloned into 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^{-1}) for maintenance of the pEVS122 plasmid and chloramphenicol (10 μg mL^{-1}) for maintenance of pVSV105. Mutants and complemented mutants were verified by Southern blotting.

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 h 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 five wells in each plate per strain (technical replicate) and three plates (biological replicate) for a total of 15 replicates. Results were analysed statistically via Tukey's *post hoc* test.

Scanning electron microscopy (SEM)

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

In vitro chemostat system and confocal-laser scanning microscopy (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^{-1} for 18 h. Chambers were washed with fresh seawater, stained with 1 mL of live/dead stain (SYTO9/propidium iodide, Invitrogen Molecular Probes L3224) for 15 min. Biofilms were subsequently examined by 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*$^-$ strains were examined for any phenotypic changes, such as cell spreading, that leads to colony pattern differentiation. The media consisted of LBS with 0.5% (w/v) Difco bacto-agar, to which glucose

Table 2. Primers used for mutant construction and complementation

Primers	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

(5 g L^{-1}) was added as previously described (Rashid & Kornberg, 2000). Swarm plates were inoculated from an overnight culture in LBS agar (1.5% w/v) using a sterile toothpick. Plates were then incubated at 28 °C for 24 h.

Twitching

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

Indole assays

Extracellular indole detection

Differences in production of extracellular indole were measured according to Kuczynska-Wisnik *et al.* (2010). Overnight cultures of *V. fischeri* ETJB1H wild-type and *ibpA*$^{-}$ were subcultured 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

The effect of indole on biofilm formation was investigated as described by Lee *et al.* (2008). Overnight strains of *V. fischeri* ETJB1H and *ibpA*$^{-}$ were subcultured in 96-well microplates with LBS media (with 0.25, 0.5 and 1.0 mM indole) and incubated at 28 °C for 18 h. The crystal violet assay was used for quantification of biofilm. 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 fresh LBS media until they reached an $OD_{600\ nm}$ of 0.3. Cultures were then diluted to approximately 1×10^3 CFU mL^{-1} in 5 mL of sterile seawater and added to glass scintillation vials where newly hatched juvenile squids were placed (one individual per vial). Seawater was changed with fresh uninoculated seawater every 12 h over a period of 48 h. Animals were

maintained on a light/dark cycle of 12/12 h. After 48 h, animals were sacrificed and homogenized, and the diluted homogenate was plated onto LBS agar plates. Bacteria (CFUs) were counted the next day to determine the colonization efficiency of each strain. Ten animals per strain were used. Results were analysed 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*. *Vibrio 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 and hence host colonization is limited.

As 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: (1) those responsible for structural components such as flagella and pili, and (2) transcriptional regulators of bacterial metabolism that influence synthesis of the components for the formation of the biofilm matrix and backbone.

To compare the identity of the genes selected with those of known function, a bioinformatics approach comparing protein sequences with high similarity was used. The BLASTP and MATGAT v2 programs were used to compare sequences with those reported for *Vibrio cholerae* O1 biovar El Tor str N16961 (ID 243277). According to this analysis, sequences with known function were: (1) PilU (*V. fischeri* accession number YP_203815.1) with an identity of 77–78% to twitching motility protein; (b) PilT (*V. fischeri* accession number YP_203814.1) with an identity of 80–82% to 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 > 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 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 with the wild-type (results not shown) and when grown on standard media (LBS).

Biofilms formed by the wild-type strain exhibited a flocculent three-dimensional structure (Fig. 1), and mutated strains showed deficiencies in biofilm formation in at least one of the assays tested, with all mutants deficient in host colonization. The following results detail 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 and 3c, c1, d and d1). Swimming motility was reduced in both mutants (Fig. 4a–c). Observations from our study demonstrate unequal production of flagella, suggesting that a nonfunctional flagellum can still partially form biofilms due to its ability to act as an adhesin in a manner that is independent of motility. This behavior has also been observed for enteropathogenic *E. coli* (Giron *et al.*, 2002). Nevertheless, both mutants were equally deficient in colonizing juvenile squids

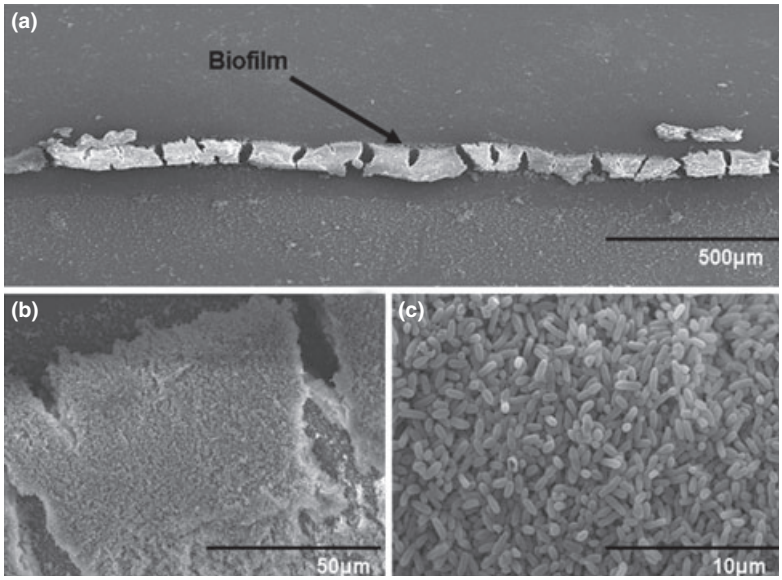

Fig. 1. Scanning electron micrographs of biofilm formed by the wild-type strain (*Vibrio fischeri* ETJB1H) on the liquid/air phase of a coverslip: (a) scale bar = 500 μm, 10× magnification; (b) scale bar = 50 μm, 50× magnification; (c) scale bar = 10 μm, 3000× magnification.

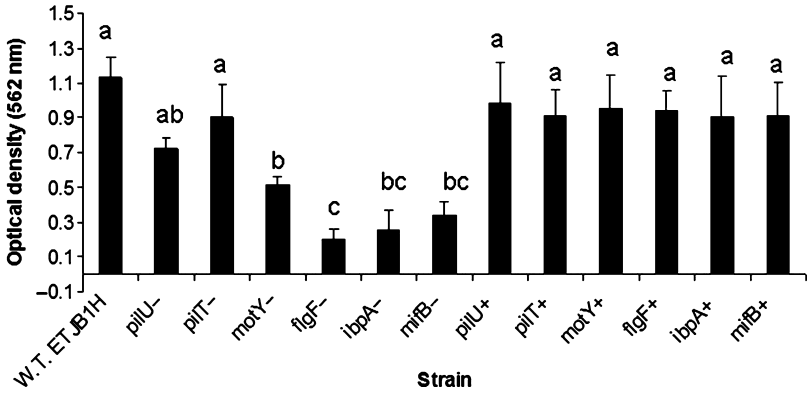

Fig. 2. *In vitro* biofilm formation for the various strains of *Vibrio fischeri* ETJB1H. Data are plotted as the mean $OD_{562\ nm}$ ± SD, with multiple comparisons calculated between groups. Different lower case letters on the abscissa indicate significant differences ($P < 0.05$) between groups, according to the Tukey *post hoc* comparison. According to these data, mutants *pilU*$^-$ and *pilT*$^-$ do not significantly differ from the wild-type (labeled 'a'), *pilU*$^-$ is labeled 'ab', i.e. is not different from *motY*$^-$, mutants *motY*$^-$, *flgF*$^-$, *ibpA*$^-$ and *mifB*$^-$ are significantly different from the wild-type (labeled 'b', 'c' or 'bc'), and *ibpA*$^-$ and *mifB*$^-$ (labeled 'bc') are not different from *flgF*$^-$.

(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). *Vibrio fischeri* pili are formed by a number of proteins encoded by genes from the *pil* operon, including *pilA–pilD* (Stabb & Ruby, 2003; Browne-Silva & Nishiguchi, 2008). The *pilU* and *pilT* genes 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 *P. aeruginosa* (Zolfaghar *et al.*, 2003) and *N. gonorrhoeae* (Firoved & Deretic,

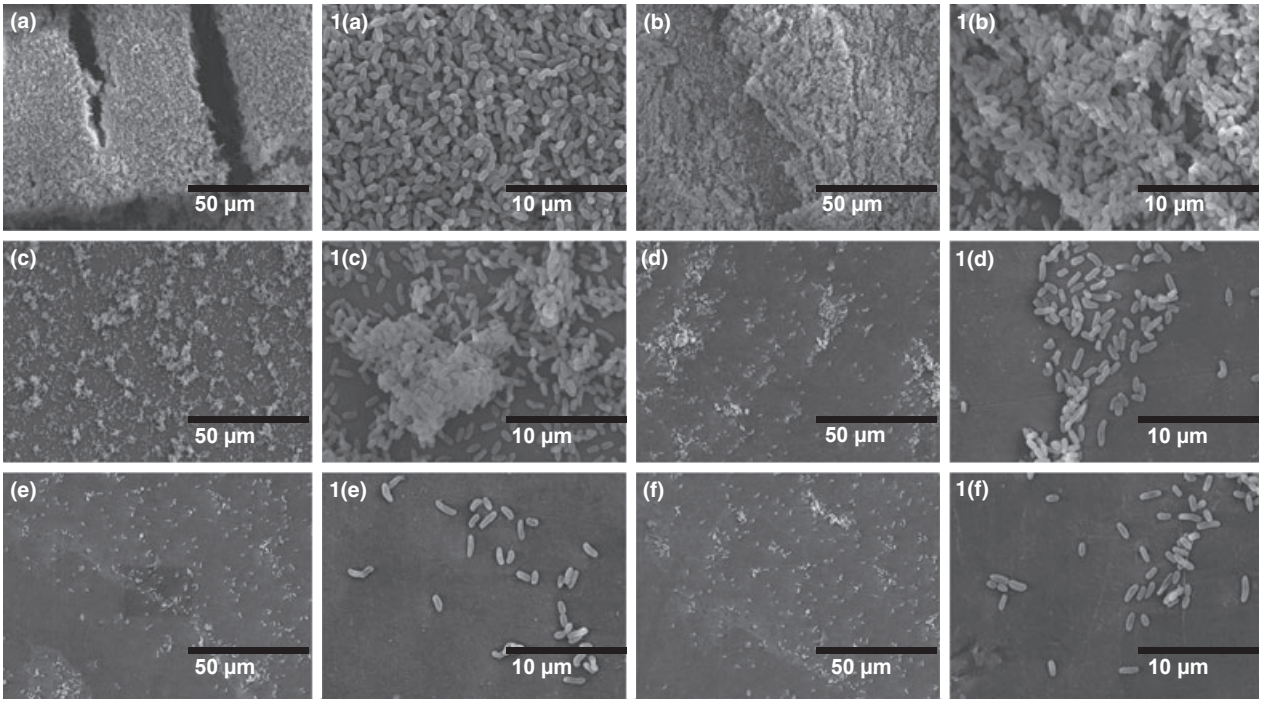

Fig. 3. Scanning electron micrographs of biofilms formed by mutant strains *pilU*$^{-}$ (a, a1), *pilT*$^{-}$ (b, b1), *motY*$^{-}$ (c, c1), *flgF*$^{-}$ (d, d1), *ibpA*$^{-}$ (e, e1), *mifB*$^{-}$ (f, f1); scale bars = 50 μm at 50× magnification and 10 μm at 3000× magnification.

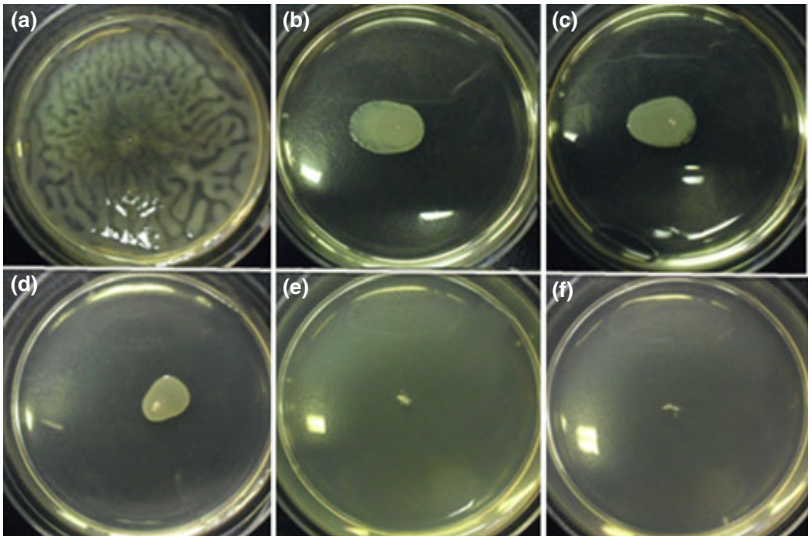

Fig. 4. Motility assays. Swimming for (a) wild-type ETJB1H, (b) *flgF*$^{-}$and (c) *motY*$^{-}$ strains. Twitching for (a) wild-type ETJB1H, (e) *pilT*$^{-}$and (f) *pilU*$^{-}$ strain. Plates were photographed after 24 h incubation.

2003), and are important for biofilm formation in these bacteria.

Visual examination of the parental strain, or wild-type *V. fischeri* ETJB1H (Fig. 1a), revealed a compact flocculent homogeneous organization. At higher magnifications, cells were distinguished by their rod-shaped organization in a mature three-dimensional structure (Fig. 1b, c). 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 (Fig. 3a, a1, b, b1). These results were

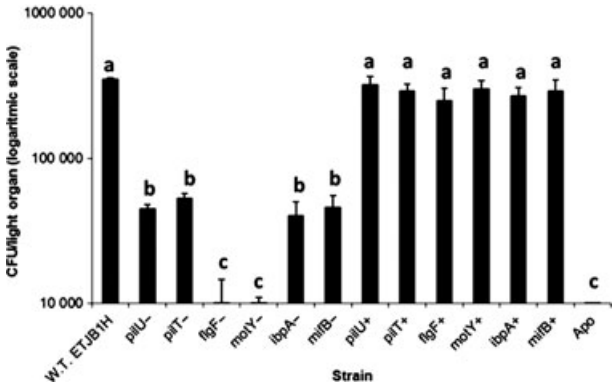

Fig. 5. Colonization assay 48 h post-infection of juvenile *Euprymna tasmanica* by wild-type and mutant strains of *Vibrio fischeri* ETJB1H. Mutant strains exhibited significant differences when compared with wild-type and complemented strains. Apo, aposymbiotic or noninfected juvenile squids. Data are plotted as the mean $OD_{562\ nm} \pm SD$. Multiple comparisons were calculated between groups using the Tukey *post hoc* 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 'a'); *flgF*$^-$ and *motY*$^-$ (labeled 'c') are also different from the wild-type ('a') and from those labeled 'b'. Ten squid were tested per strain.

consistent with those observed in the microtiter 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 microchemostat system was assembled 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 in order to examine growth of the biofilm in real time over 18 h 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 (Fig. 6b–f). Differences in community formation were observed in mutants for the *pil* locus when compared with SEM observations; *pil* mutants did not form biofilms in the chemostat system (Fig. 6b, c). 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 hyperpilated, similar to earlier results in *P. 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 hyperpiliated 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 the 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). To test differences in indole production, we performed an assay that measures production of indole over time and its effect on biofilm formation. The mutant *ibpA*$^-$ produced significantly more indole when compared with wild-type and complemented strains after 5 h of incubation (Fig. 7a); however, indole significantly reduced 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 and 3e, e1, f, f1). 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

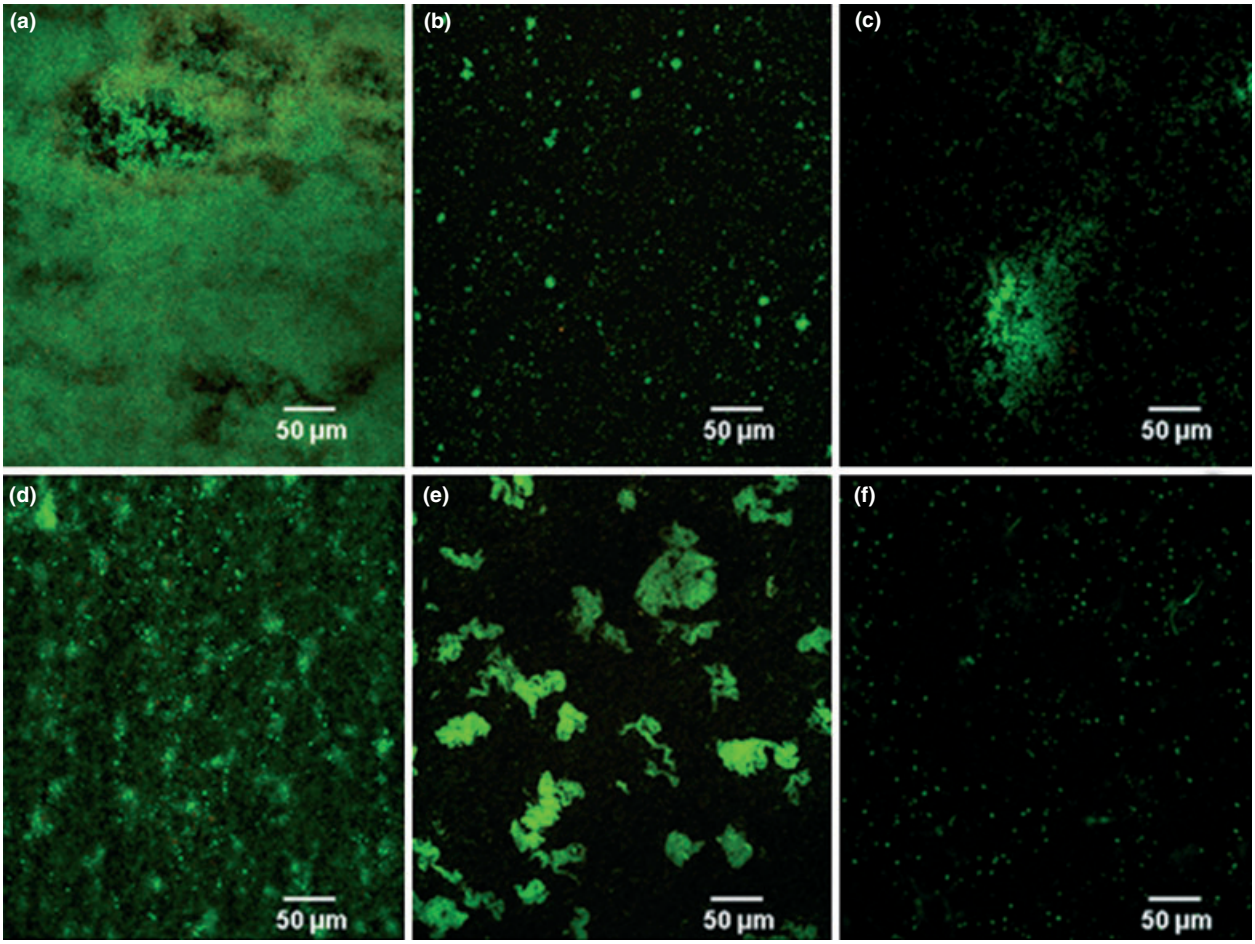

Fig. 6. Confocal scanning laser microscopyof different *Vibrio fischeri* strains. (a) Wild-type ETJB1H (thickness 19.35 ± 3.30 μm), (b) *pilU*$^{-}$ (thickness 3.45 ± 0.18 μm), (c) *pilT*$^{-}$ (thickness 3.66 ± 0.12 μm), (d) *motY*$^{-}$ (thickness 4.84 ± 0.59 μm), (e) *flgF*$^{-}$ (thickness 2.14 ± 0.67 μm), (f) *ibpA*$^{-}$ (thickness 0.93 ± 0.27 μm). Strain *mifB*$^{-}$ is not shown as it looks the same as *ibpA*$^{-}$ (thickness 0.88 ± 0.42 μm). Scale bar = 50 μm at 10× magnification. Mean thickness was calculated from five different image stacks (or 'z' stack).

of IbpA in adhesion and colonization of the squid light organ.

MifB

The second regulatory gene analysed in this study was *mifB*, which is responsible for synthesis of c-di-GMP (Wolfe & Visick, 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 and 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 exopolysaccharide production in this mutant. A recent study determined that c-di-GMP in *Vibrio 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* catalyses 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 to 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 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.

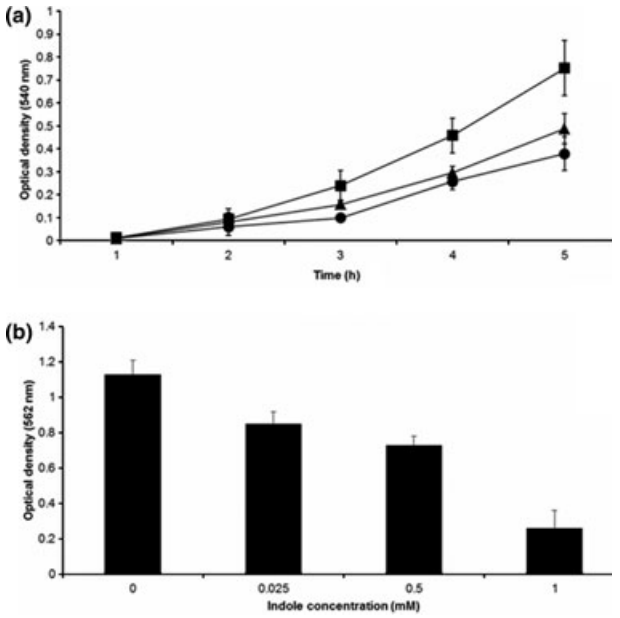

Fig. 7. Indole assays. (a) Indole production test; squares represent the mean for *ibpA*$^-$, triangles represent the mean for the wild-type, and circles represent the mean for the complement (*ibpA*$^+$) strain. Indole production was calculated in triplicate (three different clones from the same strain). Production of indole from the *ibpA*$^-$ strain was significantly different from the other two strains. (b) Effect of indole on formation of biofilms formed from the ETJB1H wild-type strain. Different concentrations of indole were added and biofilm mass was reduced significantly when concentration increased twofold or higher.

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. Other 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.*, 2006), *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 focus 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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