RESEARCH LETTER

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

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Abstract

While much has been known about the mutualistic associations between the sepiolid squid *Euprymna tasmanica* and the luminescent bacterium, *Vibrio fischeri*, less is known about the connectivity between the microscopic and molecular basis of initial attachment and persistence in the light organ. Here, we examine the possible effects of two symbiotic genes on specificity and biofilm formation of *V. fischeri* in squid light organs. Uridine diphosphate glucose-6-dehydrogenase (UDPDH) and mannose-sensitive hemagglutinin (*mshA*) mutants were generated in *V. fischeri* to determine whether each gene has an effect on host colonization, specificity, and biofilm formation. Both squid light organ colonization assays and transmission electron microscopy confirmed differences in host colonization between wild-type and mutant strains, and also demonstrated the importance of both UDPDH and *mshA* gene expression for successful light organ colonization. This furthers our understanding of the genetic factors playing important roles in this environmentally transmitted symbiosis.

Introduction

Mutualistic associations that occur in the marine environment have provided numerous examples of beneficial capabilities that aid in the success of both host and symbiont (Boucher *et al.*, 1982). One such relationship that has been studied over the past 20 years is the mutualistic association between sepiolid squids (*Cephalopoda*: *Sepiolidae*) and their *Vibrio* bacteria (*Gammaproteobacteria*: *Vibrionaceae*) (Nishiguchi & Jones, 2004). During the onset of the symbiosis, free-living *Vibrio* bacteria are environmentally acquired by aposymbiotic juvenile squids through initial invasion and colonization steps, and are eventually housed in a specialized bilobed light organ (Montgomery & McFall-Ngai, 1993). Once *Vibrio* bacteria successfully colonize the naïve squid light organ, those bacteria are able to bioluminescence via quorum sensing, and eventually produce diffuse, downward focused light, which is used by the squid in a behavior known as counterillumination (Jones & Nishiguchi, 2004). In return, the host provides a haven for the bacteria to reproduce at a faster rate, and eventually aid in the daily release of bacteria into the environment through a venting behavior that occurs with the onset of dawn (Lee & Ruby, 1994).

During the initial infection and colonization process *Vibrio* bacteria have developed sophisticated mechanisms to invade the host light organ and eventually persist in this complex (Nyholm & McFall-Ngai, 2004; Nyholm & Nishiguchi, 2008). During such infections they are involved in inducing a variety of extracellular factors such as adhesions, synthesis of polysaccharides, and toxin regulators (Montgomery & Kirchman, 1993). Specifically, adhesion and biofilm formation are two mechanisms that are considered to be crucial for initial colonization (O'Toole *et al.*, 2000; Darnell *et al.*, 2008; Geszvain & Visick, 2008; Hussa *et al.*,

2008). Genes that encode for proteins used in adhesion and biofilm production are oftentimes unique, and can be easily distinguished by their differential gene expression during symbiosis (prior or during colonization; Jones & Nishiguchi, 2006). Two such examples of genes that are differentially expressed by *Vibrio fischeri* are uridine diphosphate glucose-6-dehydrogenase (UDPDH), and mannose-sensitive hemagglutinin (*mshA*). UDPDH is considered to be a main factor for biofilm formation (Nesper *et al.*, 2001), while *mshA* is believed to be essential for initial adhesion and colonization (Bomchil *et al.*, 2003). *mshA* has also been demonstrated as a necessary component in *V. cholerae* for initial attachment and biofilm formation to abiotic substrates using the type IV pili (Watnick & Kolter, 1999). Earlier studies using a technique termed selective capture of transcribed sequences (SCOTS) have shown the expression of UDPDH solely in the light organ, whereas *mshA* is exclusively expressed under natural environmental conditions (Jones & Nishiguchi, 2006). Therefore, the goals of this study were to determine whether both UDPDH and *mshA* were necessary for successful colonization by *V. fischeri* in the sepiolid squid *Euprymna tasmanica*. Mutations were initiated to disrupt gene function for both genes and to determine whether colonization efficiency was decreased when squids were infected with either UDPDH or *mshA* mutants. We also examined whether these mutations had a decreased ability to attach to the brush border epithelia of *E. tasmanica* light organs. Finally, complementation of both mutant strains was used to regain loss of function, both *in vitro* and in colonization of naïve juvenile squids.

Materials and methods

Strains, plasmids, and growth conditions

All strains used in this study are listed in Table 1. Wild-type *V. fischeri* strain ETJB1H was isolated from the light organs of *E. tasmanica* from Jervis Bay, Australia, as described previously (Jones *et al.*, 2006). All *V. fischeri* and *E. coli* strains used in this study were grown in seawater tryptone (SWT) and Luria–Bertani (LB) broth at 28 and 37 °C, and supplemented with appropriate antibiotics for selection of mutant strains.

Table 1. Strains and plasmids used in this study

Strains or plasmids	Relevant genotype or description
V. fischeri strains	
ETJB1H	Wild type
UDPDH$^-$	UDP-glucose-6-dehydrogenase mutant
mshA$^-$	Mannose sensitive hemagglutinin mutant
UDPDH$^+$	UDP-glucose-6-dehydrogenase complement
mshA$^+$	Mannose sensitive hemagglutinin complement
Plasmids	
pEVS122	R6K, Erm^R
pVSV105	pES213 replicon, Cm^R

Erm^R, erythromycin resistance; Cm^R, chloramphenicol resistance.

PCR

All loci in this study were amplified using 50-μL volume PCR reactions as described previously (Jones *et al.*, 2006; Table 2).

Mutant construction

Mutants were constructed using plasmid insertion where, an internal (partial) fragment of each targeted gene was PCR amplified and cloned directly into pEVS 122 (Dunn *et al.*, 2005; Dunn & Stabb, 2008). This plasmid was introduced into *V. fischeri* by triparental mating (Stabb & Ruby, 2002). The *V. fischeri* strains that had undergone homologous recombination between the genome and the internal gene fragment were selected on SWT-erythromycin plates and were verified via Southern blots.

Biofilm assays

All bacterial strains (wild type, mutant, and complement) were grown and biofilm assays were performed as described previously (Nair, 2006). Biofilm assays for every strain were completed in triplicate and statistically analyzed by Student's *t*-test.

Motility assays

All bacterial strains were grown overnight at 28 °C and 250 r.p.m. The following day, 20 μL of bacterial culture was inoculated and strains were regrown in fresh 32 p.p.t. SWT media to an $OD_{600\,nm}$ of 0.1. Ten microliters of each culture was spotted onto an SWT plate containing 0.5% agar. Motility was determined by measuring the diameter of the spot after 24 h. Each motility assay for every strain was completed in triplicate and statistically analyzed by Student's *t*-test.

Colonization assay

To determine the colonization efficiency between the mutant and the wild-type bacterial strains, colonization assays of different *V. fischeri* strains were completed as described previously (Nishiguchi, 2002). Briefly, both wild-type and mutant strains were grown in 5 mL SWT overnight in a shaking incubator (250 r.p.m.) at 28 °C. Strains were transferred the next morning and regrown in 5 mL of fresh SWT media to an $OD_{600\,nm}$ of 0.3–0.5. Cultures were diluted to approximately 1×10^3 CFU mL^{-1} and used to inoculate 5 mL of sterile seawater with axenic juvenile squids. After inoculation, seawater in all vials was changed every 12 h and bioluminescence was measured using a luminometer (Turner

Table 2. UDPDH$^-$, *mshA*$^-$, UDPDH$^+$, and *mshA*$^+$ primer sequences used for constructing mutant strains

Primers	Primer sequence 5′–3′	PCR product size (bp)
mshA-forward	AGC AGA TCT TTT ATG GTA AAG CCG CGA TT	~180
mshA-reverse	AGC AGA TCT GCT GCA GTT GGG TTA TCT GA	~180
UDP-forward	AGC AGA TCT AA AAT CGC CTA TTT CAG ATG T	~160
UDP-reverse	AGC AGA TCT GCT TCA ACA GAG CCC GTA TT	~160
mshA-Comp-F	CCC GGG GAT CAG TGA GAA TGG CCG TA	~450
mshA-Comp-R	CCC GGG CGA TTG TTG ATA CGC CAG AA	~450
UDP-Comp-F	CCC GGG ATT CAG GTC GCA GGT TTC AG	~700
UDP-Comp-R	CCC GGG TTC AGA TTG CTC ACC CAC AA	~700

F, forward primer sequence; R, reverse primer sequence; bp, base pairs.

Designs, Sunnyvale, CA) over a period of 48 h. During incubation, infected animals were kept in 12-h/12-h light/dark cycle. Infected juvenile squids were sacrificed at 48 h and plated onto SWT agar. The number of CFUs on each plate represented the colonization efficiency of each strain in a squid light organ. Results were analyzed using Student's *t*-test.

Electron microscopy

Juvenile squids infected for 48 h with either strain were fixed and processed for transmission electron microscopy (TEM) as described previously (Nair, 2006).

All bacterial strains examined using scanning electron microscope (SEM) were grown overnight at 250 r.p.m. and 28 °C in 5 mL SWT. The following day, 20 μL of each bacterial culture was used to inoculate a new test tube with 3 mL sterile SWT media and regrown at 250 r.p.m. and 28 °C, until each culture reached an OD_{600nm} of 0.1. One milliliter of each culture was transferred into 50-mL falcon tubes with a sterile cover slip half immersed in the media and incubated 24 h without shaking at 28 °C. Cover slips were then washed with 32 p.p.t. seawater to remove excess particles other than the biofilm formed by each bacterial culture. Excess seawater was blotted dry, and all cover slips were incubated in 1 mL of a 0.2% aqueous solution of crystal violet at room temperature for 1 h. Cover slips from each strain were viewed with the Hitachi S3400 SEM (Hitachi, Schaumburg, IL).

Results and discussion

Growth curves and bioluminescence

Vibrio fischeri ETJB1H and the mutant strains generated in this study are listed in Table 1.

Growth analyses were completed for both UDPDH$^-$ and *mshA*$^-$ mutants and compared with *V. fischeri* ETJB1H. ETJB1H exhibited a doubling time of 25.7 min compared with UDPDH$^-$ and *mshA*$^-$, which had doubling times of 76.2 and 56.4 min. Simultaneously, luminescence of both mutant strains and the wild-type ETJB1H was measured. Comparisons between all three strains demonstrated that mutants had lower levels of bioluminescence/OD values at 13.7 relative light units (RLU) for *mshA*$^-$ and 181 RLU for UDPDH$^-$ compared with values of > 7205 RLU for ETJB1H.

In addition, growth analyses were also completed for complemented UDPDH$^+$ and *mshA*$^+$ strains and compared with their respective mutant strains. UDPDH$^+$ exhibited a doubling time of 29.6 min, which was similar to the rate of the wild-type parental strain. *mshA*$^+$ had a doubling time of approximately 33.3 min. Luminescence of UDPDH$^+$ and *mshA*$^+$ strains was also measured simultaneously. Interestingly, luminescence/OD of complemented strains exhibited a lower amount of bioluminescence/OD compared with the parental strain, with values of 18 RLU for UDPDH$^+$ and 3.4 RLU for *mshA*$^+$.

In vitro biofilm assays

Both *V. fischeri* UDPDH$^-$ and *mshA*$^-$ strains exhibited a deficiency in biofilm formation with values of 0.1 at $OD_{595\,nm}$ when eluted with the alcohol : acetone mixture (Fig. 1a). These values were compared with the parental wild type, which had values of 0.35 at $OD_{595\,nm}$ (Fig. 1a). Test analysis between *V. fischeri* ETJB1H UDPDH$^-$ and *mshA*$^-$ compared with parental wild-type showed that average mean values were significantly different. UDPDH$^+$ and *mshA*$^+$ complemented strains also had an increase in biofilm formation with mean values of 0.3 at $OD_{595\,nm}$. *mshA*$^+$ (Fig. 1b) had a greater value than parental wild-type *V. fischeri* ETJB1H. Statistical analysis of biofilm production gave significant differences between UDPDH$^-$ and UDPDH$^+$ strains, with *P*-values of 0.0001 at $\alpha = 0.05$. Additionally, the *mshA*$^-$ and the *mshA*$^+$ strains exhibited significant differences, with *P*-values of 0.002 ($\alpha = 0.05$).

Biofilm morphology

Biofilm produced from *V. fischeri* ETJB1H parental and mutant strains were visualized using SEM. Significant morphological differences were observed between parental *V. fischeri* ETJB1H (Fig. 1c) and mutants UDPDH$^-$ (Fig. 1d) and *mshA*$^-$ (Fig. 1e). *Vibrio fischeri* ETJB1H exhibited

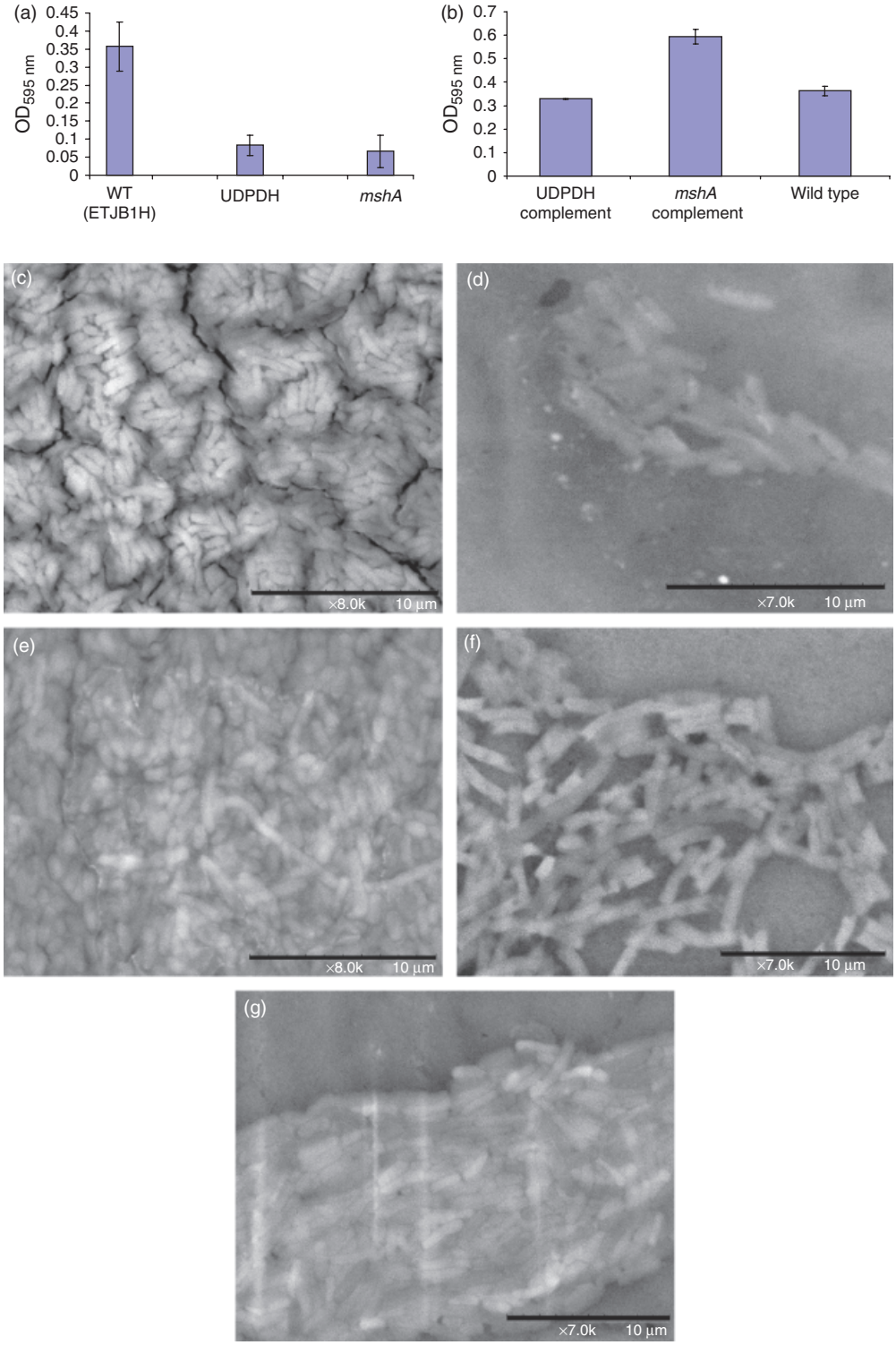

Fig. 1. (a) Biofilm produced by *Vibrio fischeri* ETJB1H wild-type and *V. fischeri* ETJB1H UDPDH$^-$ had a *P*-value of 0.0034 at $\alpha = 0.05$ and *mshA*$^-$ mutant strains had a *P*-value of 0.005 ($\alpha = 0.05$) Bars represent ± 1 SE. (b) Biofilm produced by *V. fischeri* ETJB1H parental wild type and both *V. fischeri* ETJB1H UDPDH$^+$ and *mshA*$^+$ complemented strains (bars represent ± 1 SE). (c) Scanning electron micrograph (SEM) of biofilm formed by the wild-type strain *V. fischeri* ETJB1H. Scale bar = 10 μm. (d) SEM of biofilm formed by *V. fischeri* ETJB1H UDPDH$^-$ strain. Scale bar = 10 μm. (e) SEM of biofilm formed by *V. fischeri* ETJB1H *mshA*$^-$ strain. Scale bar = 10 μm. (f) SEM of biofilm formed by *V. fischeri* ETJB1H UDPDH$^+$ complement strain. Scale bar = 10 μm. (g) SEM of biofilm formation by *V. fischeri* ETJB1H *mshA*$^+$ complement strain. Scale bar = 10 μm.

compact bacterial cells that were attached to one another in a three-dimensional structure. This was in contrast to both mutant strains, which were thinner and less dense than the wild type. Additionally, bacterial biofilms from the complemented UDPDH$^+$ (Fig. 1f) and *mshA*$^+$ strains (Fig. 1g) regained the ability to form well-organized biofilms, and appeared to form complexes similar to their wild-type parent (Fig. 1c).

Motility assay

Wild-type ETJB1H exhibited the highest degree of motility compared with both mutant strains (Fig. 2). Both UDPDH$^-$ and the wild-type strains exhibited significant differences (Student's *t*-test). Motility of complemented strains was also measured and exhibited greater motility compared with their respective mutant strains.

Colonization assay

Axenic juvenile *E. tasmanica* squids were infected with both mutants as well as wild-type *V. fischeri* ETJB1H. Seven animals were used in this study for each strain. Single strain infections were measured by homogenizing juvenile squid after 48 h of infection and calculating the number of CFUs per milliliter (Nishiguchi, 2002). *Vibrio fischeri* ETJB1H parental wild type had a mean colonization efficiency of 1×10^5 CFU mL^{-1} compared with UDPDH$^-$ and *mshA*$^-$, which had values of 1×10^3 CFU mL^{-1} and 1×10^4 CFU mL^{-1}, respectively (Fig. 3).

TEM

Examination of the crypt regions of juvenile *E. tasmanica* that were infected with either parental wild-type *V. fischeri* ETJB1H, *V. fischeri* ETJB1H UDPDH$^-$ or *V. fischeri* ETJB1H *mshA*$^-$ was completed using TEM. Parental wild-type ETJB1H (Fig. 4a) had complete colonization of the crypt region, whereas *V. fischeri* ETJB1H UDPDH$^-$ (Fig. 4b) exhibited little or no change in microvillar density to the brush border epithelia. *Vibrio fischeri* ETJB1H *mshA*$^-$ mutant (Fig. 4c) showed deficiency in colonization of the crypt region, where few bacteria were found along the brush border epithelial lining.

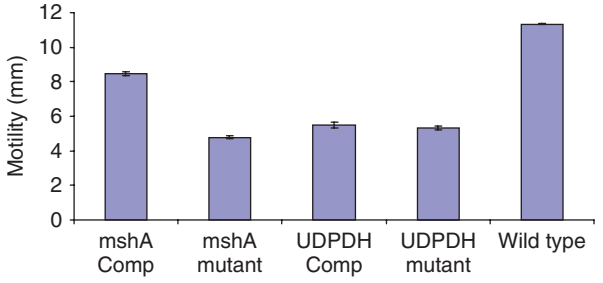

Fig. 2. Mean average motility between *Vibrio fischeri* ETJB1H *mshA*$^-$ and *mshA*$^+$ strains, *V. fischeri* ETJB1H UDPDH$^-$, and UDPDH$^+$ strains and the wild-type *V. fischeri* ETJB1H strains. Each sample was run in triplicate. Both UDPDH$^-$ and *mshA*$^-$ strains exhibited *P*-values of 0.001 and 0.0007 accordingly at $\alpha = 0.05$ compared with the wild-type strains. The UDPDH$^-$ and UDPDH$^+$ did not show significant difference, whereas the *mshA*$^-$ and *mshA*$^+$ had significant *P*-values of 0.001 at $\alpha = 0.05$. Bars represent ± 1 SE.

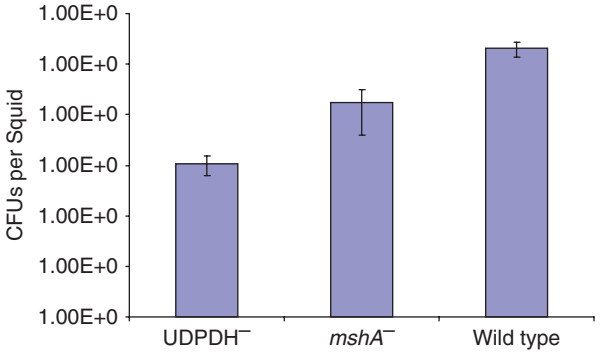

Fig. 3. Colonization assay 48 h postinfection of juvenile *Euprymna tasmanica* by the parental wild-type *V. fischeri* ETJB1H and both *V. fischeri* ETJB1H mutant strains (UDPDH$^-$ and *mshA*$^-$). Both UDPDH$^-$ and *mshA*$^-$ compared with the wild-type strains exhibited significant differences with *P*-values of 1.4E-10 and 7.8E-5 at $\alpha = 0.05$. Bars within each graph represent the SE within ± 1 of the mean.

In this study we examined how mutations in either UDPDH and *mshA* genes in *V. fischeri* affect infection and colonization in juvenile *E. tasmanica* light organs. As an environmentally transmitted bacterium, symbiotic *V. fischeri* must be capable of adapting to the selective environment of the squid light organ upon initial infection. Following successful colonization, squids will vent 90–95% of the bacteria from their crypt spaces every morning at dawn (McFall-Ngai, 2000), which may allow the streamlining of better adapted *V. fischeri* strains to be maintained in the colonized light organ. Earlier studies have provided evidence that bacterial biofilms are present in squid light organs (Nair, 2006). Therefore, the question of whether biofilm formation is important for colonization and persistence within the squid light organ may be of interest for determining specificity.

UDPDH

Biofilm formation has been shown to be one factor responsible for persistence of the bacterium *Pseudomonas aeruginosa* within infected lungs of human patients (Lam *et al.*, 1980). In addition, studies using *E. coli* and *P. aeruginosa* indicate the importance of colanic acid biosynthesis for capsular formation, which increases the ability of those bacteria to adhere to substrates. Colonic acid production is also believed to enhance the ability of bacteria to form

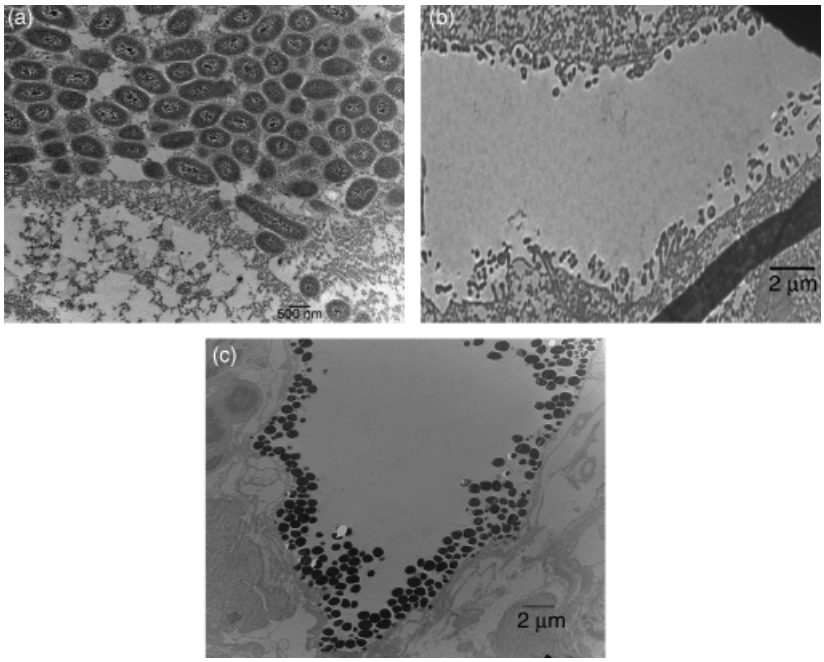

Fig. 4. Transmission electron micrographs of the squid light organ infected by (a) *Vibrio fischeri* ETJB1H wild-type strain (scale bar = 500 nm), (b) *V. fischeri* ETJB1H UDPDH$^-$ mutant (scale bar = 2 μm), and (c) *V. fischeri* ETJB1H *mshA*$^-$ mutant strains (scale bar = 2 μm).

biofilm production upon initial infection of epithelial cells in the lungs of cystic fibrosis patients (Davies *et al.*, 1993). Biosynthesis of this acid first begins with the pathways of four different nucleotide sugars, UDP-galactose, UDP-glucose, UDP-D-glucuronate, and guanine-diphosphate-L-fucose, where UDPDH catalyzes UDP-glucose to UDP-D-glucuronic acid (Stevenson *et al.*, 1996). Interestingly, this gene was only expressed in the symbiotic state in both *E. tasmanica* and *Euprymna scolopes* light organs (Jones & Nishiguchi, 2006). Because biofilm production is also linked to increased infectivity in a number of pathogenic bacteria (Austin & Zhang, 2006; Allegrucci *et al.*, 2006), it may also be involved in colonization by mutualistic bacteria such as *V. fischeri*. Additional studies have demonstrated that biofilms in *V. fischeri* are highly regulated and important for symbiosis to occur in the *E. scolopes* light organs (Yip *et al.*, 2005, 2006; Darnell *et al.*, 2008; Geszvain & Visick, 2008; Hussa *et al.*, 2008). Thus, biofilms are an integral part of the infection, colonization, and persistence processes of mutualistic vibrios in this dynamic symbiosis.

Previous studies in *V. cholerae* also indicate the importance of UDP glucose dehydrogenase for the synthesis of lipopolysaccharide in addition to colanic acid biosynthesis. UDP glucose dehydrogenase mutant strains also exhibit a deficiency in capsular formation in pathogenic strains of *V. cholerae* (Nesper *et al.*, 2001). Similar results were observed during colonization assays with *V. fischeri* ETJB1H UDPDH$^-$ mutant strains, which exhibited a 10^{-2} decrease in colonization compared with wild-type parental strains (Fig. 3). This may provide initial evidence that UDPDH has an additional role in the biosynthetic pathway producing lipopolysaccharide via glucuronic acid synthesis in *V. fischeri*. Previous studies have shown the role between lipopolysaccharide and peptidoglycan that together trigger morphogenesis in juvenile squid light organs (Foster *et al.*, 2000; Koropatnick *et al.*, 2004). Peptidoglycan is also known to be essential for mucin synthesis (Nyholm *et al.*, 2000, 2002; Nyholm & McFall-Ngai, 2003). Induction of mucus secretion in *E. scolopes* demonstrates the presence of gram-negative bacteria such as *V. fischeri*. Together the morphogenesis and mucus production enhances the capability of specific *V. fischeri* to recognize and infect their squid hosts. This is one of the mechanisms that is responsible for selecting symbiotically competent bacteria from all others in the surrounding seawater.

TEM observations in this study also support the hypothesis that UDPDH is responsible for colanic acid biosynthesis thereby affecting capsular and biofilm formation in symbiotic *V. fischeri*. UDPDH$^-$ mutant *V. fischeri* were not found in any part of the crypt region of the light organs upon examination (Fig. 4b). In addition, the ability to produce biofilm *in vitro* was measured to verify whether biofilm production was reduced in the UDPDH mutant strains. *In vitro* biofilm assays exhibited a threefold reduction in biofilm production by *V. fischeri* ETJB1H UDPDH$^-$ mutants compared with parental wild-type *V. fischeri* ETJB1H (Fig. 1). Similar deformations in biofilm production were observed in UDP glucose mutants in *V. cholerae* where mutant strains are not capable of inducing biofilm production (Nesper *et al.*, 2001).

Additionally, motility assays were completed to verify whether the UDPDH gene is linked to motility, because

motility is associated with a decrease in colonization by *V. fischeri* (Millikan & Ruby, 2002). *Vibrio fischeri* UDPDH$^-$ mutants did exhibit significant differences in motility compared with the wild-type parental strain. This result indicated that mutating the UDPDH locus did effect the motility of the bacterium, providing evidence that colonization deficiencies at this locus were linked not only to the decreases in biofilm production but also motility.

mshA

Studies examining *V. cholerae* have provided information regarding the role of *mshA* during initial infection before biofilm formation (Watnick *et al.*, 1999). This gene is responsible for the formation of type IV bundle-forming pili, which are crucial for the initial attachment to abiotic substrates. In addition, type IV bundle pili are vital for twitching motility in *P. aeruginosa*, which is considered to be an essential trait for the spread of infection following initial attachment and biofilm production (O'Toole & Kolter, 1998; Skerker & Berg, 2001). Earlier studies using SCOTS in symbiotic *V. fischeri* detected two genes (*pilM* and *mshA*) that were responsible for pili formation and attachment, and are solely expressed in seawater cultures (Jones & Nishiguchi, 2006). Expression of both *pilM* and *mshA* before colonization (in the free-living stage) suggests that both have important roles in initiating symbiosis with *E. tasmanica*. For example, another *pil* locus (*pilA*) has decreased colonization when mutant (*pilA*$^-$) *V. fischeri* were infected in *E. scolopes* juveniles (Stabb & Ruby, 2003), as well as establishing specificity during the early stages of colonization in enteropathogenic *E. coli* (Hicks *et al.*, 1998). It has also been demonstrated that a high degree of variability exists at this operon when comparing symbiotic and free-living strains of *V. fischeri* (Browne-Silva & Nishiguchi, 2008). This is yet another example of how subtle differences at a particular symbiotic locus can determine specificity in this association, and may be responsible for the differences observed in infection (commencement of the interaction) and colonization (growth and persistence of the association) between a number of symbiotically competent *V. fischeri* isolated (Nishiguchi *et al.*, 1998; Nishiguchi, 2002).

Studies have also shown that juvenile *E. scolopes* contain mannose along the crypt region of the light organs, enhancing colonization by *V. fischeri* (Visick & McFall-Ngai, 2000). Symbiotic *V. fischeri* were also able to agglutinate to guinea pig red blood cells and that exogenous mannose blocked colonization, indicating the presence of mannose-recognizing adhesions (McFall-Ngai *et al.*, 1998). In addition, further studies have provided evidence for mannose residues on the contact surface of host epithelial lining, implicating a receptor–ligand interaction between symbionts and the brush border epithelia of squid light organs (Visick & McFall-Ngai, 2000). Interactions such as those between symbiotic bacteria and mannose or other sugar-containing residues are commonly found in environmentally transmitted associations (Nishiguchi *et al.*, 2008; Nyholm & Nishiguchi, 2008), providing yet another mechanism for recognizing specific partners. This is particularly important in associations where a few bacteria are responsible for initiating the colonization of a new host amidst a number of abiotic and biotic forces that may delay or deter colonization from ever occurring (Soto *et al.*, 2009a, b).

In this study, *mshA* mutants were generated to examine whether this gene was involved in colonization processes. Results indicate that *V. fischeri* ETJB1H *mshA*$^-$ mutants had deficiencies in their colonization ability compared with the parental wild type, with a reduction of mutants observed inside the crypt region after infection (Fig. 4c). *Vibrio fischeri* ETJB1H *mshA*$^-$ mutants also exhibited severe deficiencies in biofilm production when compared with the wild-type parent. These results were similar to *V. cholerae* *mshA*$^-$ mutants where the *mshA*$^-$ mutants lack biofilm production and are severely deficient in motility (Watnick *et al.*, 1999). Motility is believed to be essential for the colonization of the squid light organ, because nonmotile *V. fischeri* are incapable of successful colonization (Millikan & Ruby, 2002). The lack of motility in *V. fischeri* *mshA*$^-$ mutants is additional support that movement to the light organ pores as well as through the duct region is required to colonize the crypts of the light organ after initial infection (Millikan & Ruby, 2002, 2003).

Complementation of mutant strains

Both *V. fischeri* UDPDH$^+$ and *mshA*$^+$ were able to regain the loss of function significantly for biofilm production, growth rate, and motility. The only phenotype that was not regained by complementation was bioluminescence with either UDPDH$^+$ or *mshA*$^+$. This suggests that both UDPDH and *mshA* genes may be linked to luminescence-related behavior (quorum sensing), and disruption of these genes interferes not only with bioluminescence production, but the ability for bacteria to quorum sense. Quorum sensing is activated through a cascade of genes in the *lux* operon, so that disruption of UDPDH may have possibly interfered with other potential downstream genes, regulated through bioluminescence production (Callahan & Dunlap, 2000). Although *lux* genes were not found to be downstream from either UDPDH or *mshA* in *V. fischeri* ES114 (Ruby *et al.*, 2005), it may differ in the *V. fischeri* ETJB1H strain that was used in this study. Further studies examining whether UDPDH is transcriptionally or translationally regulated via the *lux* operon would be required to verify the presence of either *cis*- or *trans*-acting regulatory elements linked to quorum sensing.

Conclusions

This study has examined whether UDPDH and *mshA* genes were involved in the early events of colonization of squid light organs. Results from our experiments indicate that both genes are involved in the establishment of the mutualism between *V. fischeri* and *E. tamanica*, where early attachment, colonization, and biofilm production are important for a successful environmentally transmitted symbiosis to occur. Experimental comparisons of environmentally expressed genes to those that are expressed solely in symbiosis can help us better understand whether trade-offs evolve to benefit either situation, such as the expression of *mshA* in the free-living state and UDPDH in the squid light organ, and how both genes are beneficial during different steps in establishing mutualistic relationships. Additionally, our understanding of how different pathways are co-opted from either a benign or pathogenic association can provide clues as to the evolution of virulence, and whether those genes involved in the symbiosis have been selected for among a wide variety of genotypes (Nishiguchi *et al*., 2008). The selective pressures that bacteria are exposed to (abiotic and biotic) have an incredible amount of influence that determines the evolutionary trajectory of a specific genotype; balancing those forces is one feat that symbiotic bacteria have overcome to be successful in nearly all ecological niches, including those that invade eukaryotic host tissues (Soto *et al*., 2009a, b). Continued research in the genetic basis behind *V. fischeri*'s adaptations to biotic and abiotic factors will continue to illuminate the underpinnings of symbiosis in the years to come.

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