Differentially expressed genes reveal adaptations between free-living and symbiotic niches of *Vibrio fischeri* in a fully established mutualism

B.W. Jones and M.K. Nishiguchi

Abstract: A major force driving in the innovation of mutualistic symbioses is the number of adaptations that both organisms must acquire to provide overall increased fitness for a successful partnership. Many of these symbioses are relatively dependent on the ability of the symbiont to locate a host (specificity), as well as provide some novel capability upon colonization. The mutualism between sepiolid squids and members of the *Vibrionaceae* is a unique system in which development of the symbiotic partnership has been studied in detail, but much remains unknown about the genetics of symbiont colonization and persistence within the host. Using a method that captures exclusively expressed transcripts in either free-living or host-associated strains of *Vibrio fischeri*, we identified and verified expression of genes differentially expressed in both states from two symbiotic strains of *V. fischeri*. These genes provide a glimpse into the microhabitat *V. fischeri* encounters in both free-living seawater and symbiotic host light organ-associated habitats, providing insight into the elements necessary for local adaptation and the evolution of host specificity in this unique mutualism.

Key words: *Vibrionaceae*, gene expression, Sepiolidae, *Euprymna*, SCOTS.

Résumé : La principale force motrice régissant les nouvelles symbioses est le nombre d'événements adaptatifs que les deux organismes doivent acquérir en vue d'une aptitude accrue à établir un partenariat fructueux. Plusieurs de ces symbioses sont relativement dépendantes de la capacité des symbiotes à s'établir dans un hôte (spécificité), et à lui fournir de nouvelles caractéristiques lors de la colonisation. Le mutualisme entre les pieuvres de l'ordre des Sépiolidées et les membres de la famille des Vibrionacées constitue un système unique dans lequel le développement du partenariat symbiotique a été étudié en détail, mais il reste encore beaucoup de zones inconnues quant à la génétique de la colonisation du symbiote et sa persistance dans l'hôte. Grâce à une méthode qui permet de détecter des transcrits exclusivement exprimés, soit dans des souches de *Vibrio fischeri* libres, soit associées à l'hôte, nous avons identifié et vérifié l'expression de gènes exprimés de façon différentielle dans les deux formes de souches symbiotiques de *V. fischeri*. Ces gènes donnent un aperçu du micro-habitat que *V. fischeri* rencontre en eau libre ou associé aux organes de l'hôte symbiotique, donnant une idée des éléments nécessaires à l'adaptation locale et à l'évolution de la spécificité pour l'hôte dans ce mutualisme unique.

Mots clés : Vibrionacées, expression génique, Sépiolidées, *Euprymna*, SCOTS.

[Traduit par la Rédaction]

Introduction

While much recent work on mutualistic associations has focused on symbiont evolution through identification of colonization factors or identification of coevolving host–symbiont associations and their specificity to closely related hosts (parallel cladogenesis) (Hafner et al. 1994; Parker et al. 2004), few studies have examined how both ecology (abiotic) and host (biotic) factors dictate symbiont fitness (Thompson 1999; Thompson and Cunningham 2002; Barneah et al. 2004). In particular, the means of transmission (vertical, horizontal, or environmental) can determine whether bacteria are selected solely for a host species or whether there are certain trade-offs for viability in both the environment and host (Sicard et al. 2003; Stewart et al. 2005). These trade-offs between two different sets of phenotypes are crucial for determining how quickly bacterial species can adapt to novel hosts and their surroundings. Determining which adaptations are important for driving specialization between host and environmental niches can help predict such forces affecting microbial diversity through speciation (Rainey and Travisano 1998; DeLong and Karl 2005).

Symbiosis between bobtail squids (Cephalopoda: Sepiolidae) and luminous bacteria (γ-Proteobacteria: *Vibrionaceae*) has been previously studied to examine the evolution and specificity of environmentally transmitted symbioses (Nishiguchi 2002). Upon colonization of the squid host light organ, symbiotic bacteria (*Vibrio fischeri*) benefit from high growth rates compared with those free-living in seawater, while the host uses bioluminescence produced by *V. fischeri* for counter-illumination (Ruby and Asato 1993; Jones and Nishiguchi 2004). The initiation of this association has been well studied, and colonization occurs in a highly specific manner, resulting

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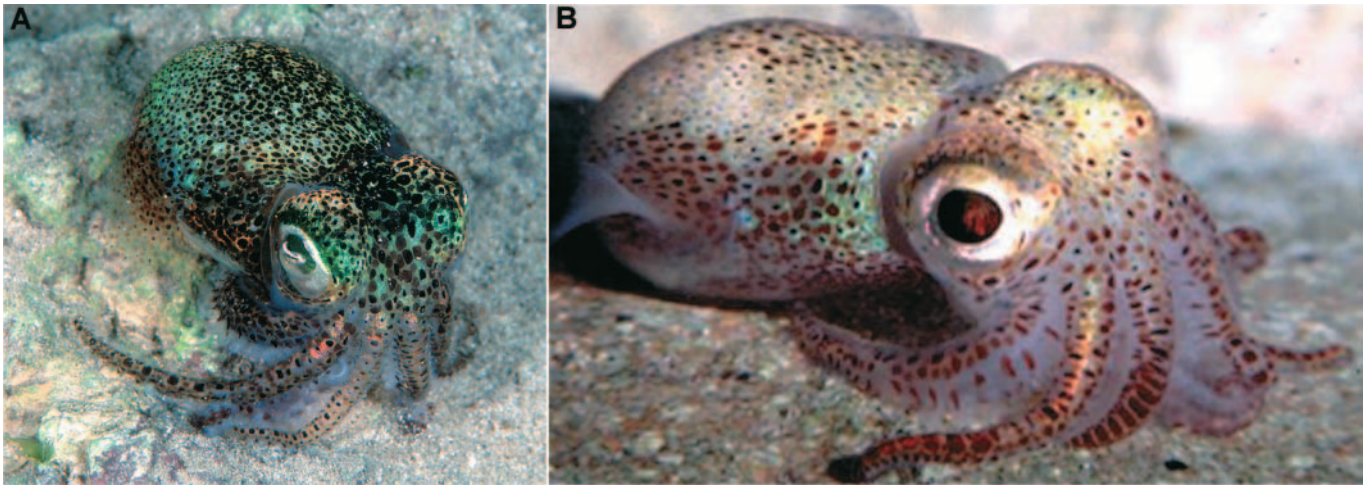
Fig. 1. Two allopatric species of bobtail squid from the Indo-west Pacific used in this study. (A) *Euprymna tasmanica* (reprinted from *Cephalopods*, *A World Guide* with the kind permission of ConchBooks, D-Hackenheim, Germany, and Mark Norman). (B) *Euprymna scolopes* (photograph provided courtesy of Ron Holcolm).



in a number of physiological and morphological changes in both the symbiont and host (McFall-Ngai and Ruby 1991, 1998; Nyholm et al. 2000; McFall-Ngai 2002).

In vivo competition studies with the Hawaiian bobtail squid *Euprymna scolopes* have demonstrated that strains of *V. fischeri* are highly adapted to their native hosts (Nishiguchi et al. 1998; Nishiguchi 2002). In these experiments, *V. fischeri* strains isolated directly from a number of *Euprymna* hosts (including *Euprymna tasmanica* from Australia and *Euprymna morsei* from Japan) were used to inoculate newly hatched axenic *E. scolopes* individuals. While each strain was able to colonize *E. scolopes*, the native *E. scolopes* strain was always found to dominate the light organ contents after 48 h of infection (Nishiguchi et al. 1998). For example, after 48 h of infection, the ratio of the Hawaiian strain *V. fischeri* ES114 to the Australian strain ET101 was 95:5 (Nishiguchi et al. 1998), suggesting the native ES114 is able to out compete non-native strains despite their high degree of relatedness (96% sequence identity between ES114 and ET101 *gapA* sequences; Nishiguchi et al. 1998). Subsequent work has not yet revealed the underlying factors that confer this fitness advantage to local strains of *V. fischeri*.

The mode of symbiont transmission provides clues to how local adaptation has developed in this system. Environmental transmission occurs through a host-controlled daily ritual of venting excess vibrios into local waters, creating an inoculum from which naïve (axenic) juvenile squids can obtain their symbionts (McFall-Ngai and Ruby 1991; Jones et al. 2007). This cycling of local symbionts has likely set the stage for the evolution of host specificity observed in Indo-west Pacific *Euprymna* – *V. fischeri* populations (Nishiguchi et al. 1998). Since *Vibrio* fitness is higher within the light organ (doubling time of the population is approximately 4 h (Ruby and Asato 1993)) compared with seawater, selection should favor strains that are highly adapted to the light organ environment, as seen in competitive studies (Nishiguchi et al. 1998; Nishiguchi 2002). Consequently, native light organ strains should out-compete other less fit strains and potentially evade daily host expulsion. Expelled strains must also face selection for persistence in the environment until they encounter and colonize another light organ in a new host squid. These opposing selective pressures are thus likely to be common forces underlying the evolution of environmental transmission.

To better understand these selective pressures involved in environmental persistence and infection by bacteria, we have identified genes expressed exclusively either in seawater or during host infection by *V. fischeri*, using selective capture of transcribed sequences (SCOTS; Graham and Clark-Curtiss 1999), a method used to compare gene expression of the same bacterium in two distinct environments. SCOTS has the advantage over other methods, such as microarray analysis, of identifying strain-specific genes that may not be present in the genomes of sequenced conspecifics (Daigle et al. 2002). This method has successfully been implemented to identify genes expressed by *Listeria monocytogenes* growing at low temperature (Liu et al. 2002), *Mycobacterium avium*, *Mycobacterium tuberculosis*, and *Salmonella typhi* genes expressed in human macrophages (Graham and Clark-Curtiss 1999; Daigle et al. 2001; Hou et al. 2002), and *Helicobacter pylori* genes expressed in human gastric mucosa (Graham et al. 2002). Until now, SCOTS has been used to compare in vivo expression with expression of broth-grown bacteria, mostly because of the limitations of the different systems being studied. Here, we present results of experiments examining environmental (grown in seawater) and host-associated (grown in the light organ) gene expression by two strains of *V. fischeri*, each isolated directly from field-captured adult hosts *E. scolopes* (of Hawaii) and *E. tasmanica* (of Australia; Fig. 1).

Materials and methods

Bacterial growth conditions and RNA extraction

Gene expression from the identical *V. fischeri* strain grown in both the light organ of its host and in seawater was examined with SCOTS. To obtain host-associated *V. fischeri* RNA, strains were obtained directly from the light organ of *E. scolopes* (from Hawai'i-Kai Bay, O'ahu, Hawaii) or *E. tasmanica* (from Jervis Bay, New South Wales, Australia). The light

Table 1. Primers used in selective capture of transcribed sequences (SCOTS) and verification procedures.

Name	Sequence (5′→3′)	Use
Xba random*	TGCTCTAGACGTCCTGATGGTT9(N)	cDNA library construction
Xba*	TGCTCTAGACGTCCTGATGGTT	Amplification of cDNA
Sal random*	ATATGTCGACTGAATTCCGTAGG9(N)	cDNA library construction
Sal*	ATATGTCGACTGAATTCCGTAGG	Amplification of cDNA
16S forward	AGAGTTTGATCMTGGCTCAG	Ribosomal operon cloning
23S reverse	ATGGTTAAGCCTCACGGGCA	Ribosomal operon cloning
flrA sense	AGGCGGCAATCAAAGTATTCGTGC	RT–PCR verification
flrA antisense	CAGCTAATGCCGCTTGTTCTTGCT	RT–PCR verification
Arginine decarboxylase sense	GTTAAACCACGTCTTGGCTTGCGT	RT–PCR verification
Arginine decarboxylase sense	ACCACCACCCACATCGAGGTATTT	RT–PCR verification
mshA sense	TAAACCTGCAATCTGATGCCGTGC	RT–PCR verification
mshA antisense	GATAGACCAACTACCGCTTCAGCA	RT–PCR verification

*Graham and Clark-Curtiss 1999.

organs were dissected immediately after capture of the animals during the evening. One-half of the light organ was plated on Luria–Bertani high-salt plates (Ruby 1993) to isolate the strain present in the host, and the other half of the light organ was placed in RNAlater® (Ambion, Austin, Tex.) until RNA extraction. The strains from *E. tasmanica* and *E. scolopes* hosts were designated ETJB1A and ESP915, respectively. Prior to RNA extraction, the organs were homogenized in RNAlater® (Ambion) and then centrifuged at 12 000*g* for 2 min to pellet eukaryotic tissue. Bacteria in the supernatant was then pelleted at 16 000*g* for 10 min.

To obtain RNA from seawater-grown *V. fischeri*, both ETJB1A and ESP915 were grown for 48 h to OD_{600} of ~0.5 (approximately 2.5×10^8 cells/mL) in sterile, filtered (0.2 μm pore size) seawater supplemented with 0.1% (*m*/*v*) chitin at 28 °C. Prior to RNA extractions, the sample was filtered through an 8 μm filter to remove excess chitin and was pelleted at 16 000*g* for 10 min.

RNA extractions were performed using a protocol modified from Mangan et al. (1997). Bacterial pellets were washed in 0.5% Tween-80 prior to resuspension in 200 μL of diethylpyrocarbonate-treated water. The cleaned cells were then homogenized in a bead beater at 4200 r/min (1 r = 2πrad) for 50 s in a suspension of 1 g zirconium–silica beads (0.1 mm diameter), 600 μL of an acid-equilibrated phenol – chloroform – isoamyl alcohol mixture (25:24:1 (by volume) pH 4.7), and 500 μL of STET buffer (8% (*m*/*v*) sucrose, 5% (*v*/*v*) Triton X-100, 50 mmol/L ethylene diamine tetraacedic acid (EDTA), and 50 mmol/L Tris (pH 6.80)). After being equilibrated at 4 °C for 5 min, each sample was centrifuged for 5 min at 16 000*g* to remove cellular debris and then chloroform extracted. RNA was then precipitated in 1× volume of alcohol solution (0.3 mL 3 mol/L sodium acetate and 49.7 mL 2-propanol) at –20 °C for 1 h. RNA was pelleted at 13 000*g* for 15 min at 4 °C and resuspended in diethylpyrocarbonate-treated water and 0.1% SDS (sodium dodecyl sulfate).

Prior to cDNA library construction for seawater and light-organ-derived RNA, contaminating DNA was removed using DNA-*free*™ (Ambion), as per manufacturer's directions. After this, removal of rRNA with MICROB*Express*™ (Ambion), following the manufacturer's protocol, was necessary to eliminate contaminating rRNA-derived cDNAs during the SCOTS procedure.

cDNA library construction

All primers used in this study are listed in Table 1. Random cDNA libraries were constructed using identical primers and the methodology of Graham and Clark-Curtiss (1999, 2000). Importantly, the seawater-derived and the light-organ-derived *V. fischeri* cDNA libraries each contained linkers enabling specific polymerase chain reaction (PCR) amplification of each library (the Xba primer was used for light organ libraries, and the Sal primer was used for seawater libraries). First-strand cDNA was synthesized with Superscript™ II Reverse Transcriptase (Invitrogen, Carlsbad, Calif.), according to manufacturer's directions, using 1 μg of each primer with a known 5′ end and random 9-mer at the 3′ end (Table 1). Second-strand cDNA was synthesized using the Klenow fragment of DNA polymerase (New England Biolabs, Beverly, Mass.), as per instructions. Double-stranded cDNA was then passed over a PCR purification column (Qiagen, Valencia, Calif.) to remove remaining salts, enzyme, and unincorporated nucleotides.

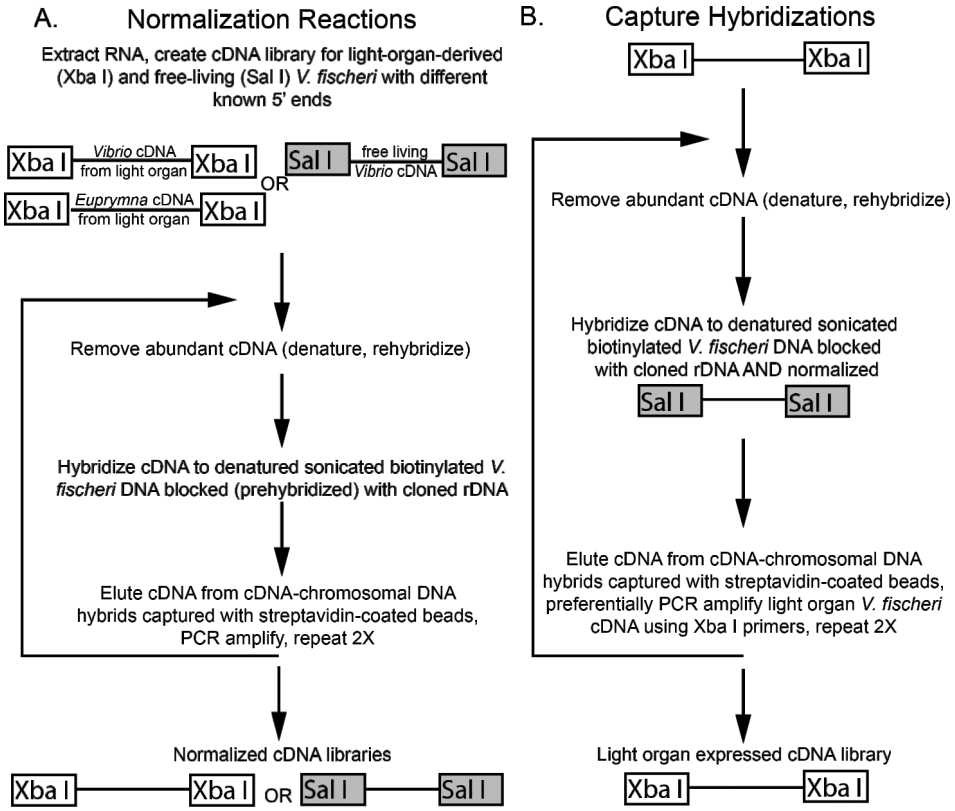
Isolation of rRNA operon

To block binding of rRNA-derived cDNA during SCOTS, the *V. fischeri* ESP915 ribosomal operon was PCR amplified in a 50 μL reaction containing 2.5 mmol/L magnesium chloride, 1× buffer, 0.2 mmol/L each dNTP, 2 U *Taq* polymerase (Continental Lab Products, San Diego, Calif.), and 0.2 μmol/L each primer (16S forward and 23S reverse; Table 1) with an annealing temperature of 48 °C and 5 min extension for 35 cycles. The ~5 kb product was excised from a 1% agarose gel and cloned using the PCR-XL TOPO cloning kit (Invitrogen), following manufacturer's directions.

SCOTS

SCOTS was performed in an identical manner for both ESP915 and ETJB1A, hybridizing the cDNA of each strain against its own DNA. In addition, subtractions were completed against both the seawater and light-organ-derived cDNA libraries to identify genes expressed in each environment by both strains. Three rounds of normalization hybridizations were used to normalize the cDNA libraries and to eliminate host cDNA contamination (Fig. 2A). Before initial normalization hybridizations, 10 μL of each cDNA library was amplified via PCR in 10 separate reactions for each library,

Fig. 2. The selective capture of transcribed sequences (SCOTS) procedure (modified from Graham and Clark-Curtiss 1999). To identify genes expressed by *Vibrio fischeri* exclusively in the light organ, cDNA libraries for light-organ-derived and seawater-derived *V. fischeri* were created. Following a series of three normalization reactions (A), seawater-derived *V. fischeri* cDNAs were used to block during capture hybridizations (B), followed by preferential amplification of the light-organ-derived *V. fischeri* cDNA library. The equivalent procedure was used to identify *V. fischeri* genes expressed exclusively in seawater.



using *Taq* polymerase with an annealing temperature of 52 °C. These PCR reactions were then pooled, and 2.5 initial PCR reactions (125 μL PCR product) were used in the first round of capture hybridizations (Fig. 2B; Graham and Clark-Curtiss 1999).

Thirty micrograms of sonicated, biotinylated, genomic *V. fischeri* ESP915 or ETJB1A DNA and 100 μg of sonicated, cloned, *V. fischeri* ESP915 rRNA operon-containing plasmids (isolated above) were suspended in 40 μL of 10 mmol/L 3-[4-(2-hydroxyethyl)-1-peperazinyl] propanesulfonic acid (EPPS; Sigma, St. Louis, Mo.) and 1 mmol/L ethylene diamine tetraacetic acid (EDTA) and were heated to 98 °C for 2 min. Similarly, 2.5 50 μL PCR reactions (125 μL total) for each library were precipitated and resuspended in 40 μL of the same buffer. Each mixture was then incubated for 30 min at 60 °C. During this time, all rDNA sites were blocked by hybridization of the cloned rRNA operon in the tubes containing genomic DNA, and common cDNAs were allowed to self-hybridize in each tube containing cDNA. After this, 10 μL of 1 mol/L sodium chloride was added to each tube. All mixtures were incubated for an additional 30 min at 50 °C, after which the genomic DNA–rRNA hybrids were added to each cDNA library for overnight hybridization at 50 °C. After hybridizations, 100 μL of water was added to each sample and placed in ice.

Biotinylated genomic DNA–cDNA hybrids were recovered with streptavidin-coated beads and a magnetic separator (Dynal, Oslo, Norway). Briefly, washed beads were resuspended in 100 μL of 10 mmol/L Tris (pH 7.5), 1 mmol/L EDTA, and 2 mol/L NaCl, and then the hybridized sample was added. cDNA was then eluted from genomic DNA with 100 μL each of 0.5 mol/L NaOH and 0.1 mol/L NaCl. Eluted cDNAs were then amplified in 10 parallel PCR reactions (using the same conditions as above). After PCR, the cDNA was once again passed over a PCR purification column (Qiagen). Rounds two and three of normalization hybridizations used the cDNA from the previous round, but all volumes were one-tenth of those from the first round.

The cDNA capture procedure was performed in a nearly identical manner to the normalization hybridizations, with the exception that 25 μL of the third round normalized cDNA was added to the sonicated, cloned rRNA plasmid and sonicated, biotinylated, genomic DNA mixture. When identifying cDNAs exclusively from the light organ, normalized cDNA from the seawater library was used, blocking each site expressed in seawater. Similar to the normalization hybridizations, this sample, along with the third round normalized cDNA (the light-organ-derived cDNA library when identifying light-organ-expressed genes), was denatured and allowed to partially hybridize before the addition of 1 μL of 1 mol/L NaCl. These two mixtures were then combined and allowed to hybridize overnight. The hybrids were collected with streptavidin-coated beads, and cDNA was eluted and cleaned as described above. After PCR amplification with

the primer appropriate for the differentially expressed sequences of interest, the products were cloned with the TOPO Cloning kit (Invitrogen), as per manufacturer's directions.

Primary verification using Southern hybridizations and sequencing

To eliminate false-positive cDNA sequences from recovered seawater or light-organ-derived clones, Southern hybridizations were employed. Clone libraries were denatured by heating each sample to 98 °C for 10 min in denaturing solution (0.4 mol/L NaOH, 10 mmol/L EDTA, final concentration), blotted onto a positively charged nylon membrane using a 96 well blotting apparatus (Bio-Rad Laboratories, Hercules, Calif.), and cross-linked using a UV Stratalinker (Stratagene, La Jolla, Calif.). The clones were then hybridized against a digoxigenin-labeled probe created from the third round captured sequences used to block during the enrichment reactions. In doing so, only the clones expressed in the final enrichment reactions that were expressed in both capture hybridizations would bind during this procedure, allowing elimination of false-positive sequences that escaped the subtraction process.

To perform Southern hybridizations, the membranes were hybridized overnight at 42 °C with 25 ng of probe in 3.5 mL DIG EasyHyb hybridization solution that was added to the membrane after prehybridization in 10 mL hybridization solution for 30 min at 42 °C. The membranes were then washed twice for 5 min each in low stringency buffer (2× SSC, 0.1% SDS), followed by two 15 min washes at 65 °C in high stringency buffer (0.5× SSC, 0.1% SDS). Hybridizations were detected using the chemiluminescent substrate CSPD-Star (Roche Diagnostics Corporation, Indianapolis, Ind.) and a ChemiDoc XRS System (Bio-Rad Laboratories).

Hybridization signals indicated a false-positive result, since not all sequences were exclusive to one library. No hybridization signal indicated a gene expressed exclusively in the light organ or seawater. Distinct clones were sequenced with an ABI 3100 sequencer (Applied Biosystems, Foster City, Calif.) and previous protocols (Nishiguchi and Nair 2003). The identity of the sequences was determined by using the National Center for Biotechnology Information BLAST function to search GenBank for sequences similar to those discovered using SCOTS. In addition, the newly published *V. fischeri* genome (Ruby et al. 2005) was searched to find identical sequences. Clones were placed in one of five categories, including metabolic genes, stress-related genes, regulatory genes, membrane-associated genes, and genes of unknown function. Among the genes of unknown function, genes were categorized as hypothetical proteins and those having no known homology.

Secondary verification using reverse transcriptase PCR

Based on the function of discovered clones, three clones were picked for secondary verification. These included the seawater-expressed mannose-sensitive hemagglutin (*mshA*), the light-organ-expressed arginine decarboxylase, and the flagella regulator *flrA*. Based on the *V. fischeri* genomic sequence of each of these genes, primers were developed (Table 1) to amplify 428 bp (*flrA*), 294 bp (arginine decarboxylase), and 145 bp (*mshA*) fragments by reverse transcriptase (RT)–PCR using the SuperScript™ III One-Step RT–PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen).

Each 25 μL of reaction mixture contained 12.5 μL of 2× buffer (supplied by manufacturer), sense and anti-sense primer final concentrations of 4 μmol/L, 1 μL of SuperScript™ III Reverse Transcriptase and Platinum® *Taq* DNA polymerase mixture, and either 100 or 1 ng of seawater or light-organ-derived RNA. The reaction consisted of 30 min of incubation at 55 °C for first strand synthesis, denaturation at 94 °C for 2 min, 40 cycles each of 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 30 s, followed by a final extension of 68 °C for 7 min.

Results and discussion

SCOTS has been used to identify exclusively expressed cDNA of bacteria cultivated in a natural environment and in broth (Graham and Clark-Curtiss 1999, 2000; Daigle et al. 2001, 2002; Graham et al. 2002). These experiments have a minor limitation in that bacteria growing in broth undoubtedly express many genes identical to those of bacteria in the natural environment, resulting in a cDNA library that does not completely describe the ecological state being studied. Here, we have applied SCOTS to examine gene expression in two natural environments: *V. fischeri* grown in the light organs of field-caught sepiolid squid and the same strain isolated from these hosts grown in chitin-supplemented seawater. This being so, the expressed genes identified in this study provide a unique chance to examine the microhabitats to which *V. fischeri* must adapt (Tables 2 and 3).

Primary verification using Southern hybridizations provided evidence that there was no rRNA contamination after three rounds of capture hybridizations. Furthermore, using this procedure, a number of clones were eliminated from the libraries (Fig. 3). After elimination of the clones that escaped the subtraction procedure, a total of 53 clones were sequenced from seawater-expressed libraries. Nineteen of these belonged to *V. fischeri* derived from *E. scolopes*, whereas the other 34 were from *V. fischeri* derived from *E. tasmanica*. Among the 54 light-organ-derived clones, 33 were from *V. fischeri* of *E. tasmanica*, and 21 were from *V. fischeri* of *E. scolopes* (Tables 2 and 3). Interestingly, host-associated *V. fischeri* gene expression was very consistent between hosts. Southern hybridizations using the enriched cDNA from *E. scolopes* light-organ-derived *V. fischeri* as a probe against the analogous *V. fischeri* library from the light organ of *E. tasmanica* indicated that each clone was expressed in both hosts (data not shown). The remarkable similarity of *V. fischeri* expression between hosts indicates that local adaptation is not due to exclusive expression of single or a few genes but is likely due to changes in levels of expression of different genetic pathways coupled with small mutational changes; a view of adaptation that is prevalent in the literature (Johnson and Porter 2001; Johannesen and Hansen 2002; West-Eberhard 2005). Many of the genes and pathways identified here represent a starting point for future studies that will identify the specifics of local adaptation by *V. fischeri* at the genetic level.

Vibrio fischeri that colonize host tissues are at an advantage over their environmental counterparts for a number of reasons. Bacteriovory is limited, the light organ is nutrient

Table 2. Genes expressed by *Vibrio fischeri* in the light organs of *Euprymna scolopes* and *Euprymna tasmanica*.

Class	Clone name*	ES114 open reading frame	Genes coding for protein
Metabolism	etx17	VFA0839	Arginine decarboxylase
	etx20	VF1740	3-Oxoacyl-[acyl-carrier protein] reductase
	etx35	VF1932	Acyl-CoA dehydrogenase
	etx38	VF0772	Pyruvate dehydrogenase E1 component
	etx42	VF2440	Oligopeptide transport ATP-binding protein (OppD)
	etx46	VF1262	Glutaredoxin
	etx52	VF1590	Formate acetyltransferase
	etx57	VF1363	Formate hydrogenlyase (FdhF)
	etx60	VFA0004	Peptidase T
	etx65	VF2251	GatB/Yqey domain protein
	etx67	VF0060	3-Polyprenyl-4-hydroxybenzoate decarboxylase
	etx68	VF1302	Cytochrome-*c* oxidase, diheme subunit
	etx19	VF0439	*S*-Adenosylmethionine synthetase
	eslo4	VF0198	UDP glucose 6-dehydrogenase
	eslo5	VF0480	Dihydropteroate synthase (FolP)
	eslo15	VF2402	Uroporphyrinogen decarboxylase (HemE)
	eslo18	VF2340	Allosteric 6-phosphofructokinase
	eslo20	VF1590	Formate acetyltransferase
	eslo34	VF2258	Pyruvate kinase
	eslo7	VFA1078	Acetyltransferase and (or) hydrolase family protein
Stress	etx24	VF2539	Sporulation-control protein
	etx66	VF1263	Mercuric reductase
Membrane	etx29	VFA0971	Vitamin B12 transport ATP-binding protein (BtuD)
	eslo78	VFA1047	Mg^{2+} transporter MgtE
	etx47	VFA0189	Cytochrome-*c*-type protein TorC
	etx23	VF1065	Integral membrane protein with TRKA (tyrosine kinase *a*) domains
	eslo3	VF1856	Transcriptional activator FlrA
	eslo31	VF1193	Mechanosensitive ion channel
	eslo61	VFA0736	ABC transporter permease protein
	etx16	VF2108	Mechanosensitive ion channel
Regulatory	etx22	VF0551	tRNA (Guanine-N(1)-)-methyltransferase
	etx26	VF2477	33 kDa chaperonin
	etx49	VF0241	Large subunit ribosomal protein L22P
	etx27	VF1784	tRNA methyltransferase
	etx63	VF1759	Small subunit ribosomal protein S1P
	etx64	VF0074	DNA polymerase I
	etx31	VF0231	Small subunit ribosomal protein S7P
	eslo17	VF1689	Hnr protein
	eslo23	VF1748	Ribonuclease E
	eslo28, etx37, etx40	All VF2412	DNA-directed RNA polymerase beta chain
	eslo33	VF1891	Cell division protein ZipA
	eslo38	VF1204	DNA gyrase subunit A
Unknown	etx28, etx43, eslo30	VF0882, VF1653, VF0882	Hypothetical proteins
	etx51, etx62	Both VFA1018	Hypothetical cytosolic proteins
	eslo75	VF2401	Hypothetical protein YhgL
	eslo8, eslo9, etx45	Not applicable	No known homology
	eslo66	pES213 OR5	*V. fischeri* plasmid open reading frame

*Clones eslo and etx were derived from *Euprymna scolopes* and *Euprymna tasmanica*, respectively.

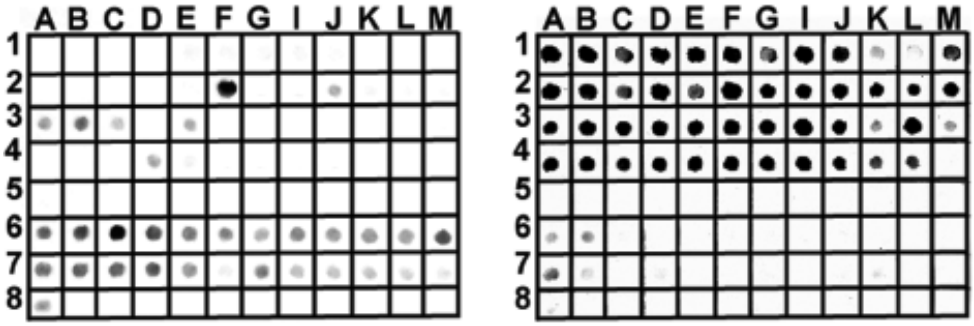
rich (Graf and Ruby 1998), and growth rates are much higher (Ruby and Asato 1993). For these reasons, environmental expression of genes necessary for adhesion during the initiation of colonization is critical. The seawater-expressed pilin synthesis gene, *pilM*, and a mannose-sensitive hemagglutin, *mshA*, are two genes that are likely important to the initial steps of colonization. Significantly, *V. fischeri* with *pilA* deletions have a decreased competitive ability against parent wild-type strains when both strains are introduced to uninnoculated *E. scolopes* juveniles (Stabb and Ruby 2003). In addition, comparative genetic studies of *pilA* (which is in the same operon as *pilM*) suggest this gene may be involved in local adaptation to host species (Brown-Silva and Nishiguchi in review), creating the competitive advantage

Table 3. Genes exclusively expressed by *Vibrio fischeri* strains isolated from *Euprymna tasmanica* and *Euprymna scolopes* in seawater.

Class	Clone name*	ES114 open reading frame	Genes coding for protein
Metabolism	essw35	VF2554	1-Acyl-*sn*-glycerol-3-phosphate acyltransferase
	essw11	VF2537	Phosphoribosylaminoimidazole carboxylase
	essw7	VF2383	Acetyl-CoA synthetase
	essw37	VF0480	Phosphoglucomutase and (or) phosphomannomutase family protein MrsA
	essw36	VF0214	Phosphoribulokinase
	essw40	VF2461	Thioredoxin
	ets05	VF1743	Fatty acid and (or) phospholipid synthesis protein PlsX
	ets10	VF0758	PhoH-like protein
	ets13	VF0543	Glutamate–cysteine ligase
	ets18	VF2271	Maltose operon periplasmic protein precursor
	ets27	VF0531	Cytosine deaminase
	ets38	VF0772	2-Dehydro-3-deoxyphosphooctonate aldolase
	ets39	VF0063	HemY protein
	ets52	VF1972	Isocitrate lyase
	ets56	VFA0099	Glutamate synthase (NADPH) small chain
	ets53	VF2234	D-Glycero-D-manno-heptose-7-phosphate 1-kinase
	ets61	VF0406	Aspartate carbamoyltransferase
	ets66	VF1070	CDP-4-dehydro-6-deoxy-D-glucose 3-dehydratase
	ets71	VF0077	2-Polyprenyl-3-methyl-5-hydroxy-6-metoxy-1,4-benzoquinol methylase
Stress	essw16	VFA0916	Na^+ driven multidrug efflux pump
	essw29	VF2341	Cobalt–zinc–cadmium resistance protein CzcD
	essw30	VF0720	*ampG* protein
Membrane	essw23	VF0031	Chloride channel protein
	essw24	VF2518	Dipeptide-binding protein
	essw38	VF0322	Transporter
	ets75	VF1304	Copper-exporting ATPase
	ets14	VFA1022	Transporter
	ets40	VF2440	Na^+ driven multidrug efflux pump
	essw17	VF2297	Pili assembly protein PilM
	essw28	VF1137	Putative lipoprotein
	ets16	VF0436	Surface protein
	ets20	VF0366	Mannose-sensitive hemagglutin MshA
	ets36	VF0367	Type II secretory pathway pseudopilin PulG
Regulatory	essw39	VF0454	Transcriptional regulator
	essw20	VF1707	Ribonuclease D
	ets03	VFA0750	Ribosomal-protein-alanine acetyltransferase
	ets17	VF0548	Signal recognition particle, subunit FFH/SRP54
	ets22	VF2345	23S rRNA methyltransferase
	ets19	VF2545	16S rRNA m(5)C 967 methyltransferase
	ets23	VF0425	Xanthosine triphosphate pyrophosphatase
	ets67	VF0971	GTP-binding protein HflX
Unknown	essw18	VF1638	Nif3 family protein
	essw19, ets12, ets15, ets63	VF0898, VFA0908, VF1166, VF2500	Hypothetical proteins
	essw33	VF2071	Hypothetical cytosolic protein
	ets24	VFA0834	*nirV* precursor
	ets64, ets65	Not applicable	No known homology
	ets76, ets70	VFA0756, VF1495	Conserved hypothetical protein

*Clones ets and essw were derived from *Euprymna tasmanica* and *Euprymna scolopes*, respectively.

Fig. 3. Primary verification of all *Vibrio fischeri* clones derived from *Euprymna scolopes*. Lanes 1A-4L are light organ expressed cloned cDNAs (right panel), while lanes 6A–8A are seawater expressed cloned cDNAs (left panel). In the left panel, a probe created from the normalized seawater libraries shows a number of light organ clones that escaped the selection procedure (2F, 2J, 3A–3C, 3E, 4D), whereas every seawater-exclusive clone gives signal. In the right panel, a probe created from normalized light organ libraries produces a signal in every light-organ-exclusive library as well as six clones that escaped the selection procedure (6A, 6B, 7A, 7B, 7K, 8A). The analogous procedure was performed for clones derived from *Euprymna tasmanica*.

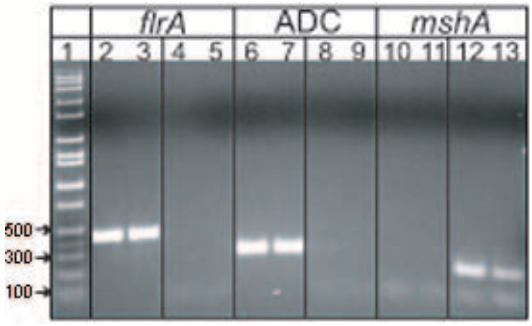


over non-natives seen in colonization experiments (Nishiguchi et al. 1998). The contribution of *mshA* to the ability of *V. fischeri* to colonize *E. scolopes* has yet to be examined, but it is known that in *V. cholerae*, *mshA* is necessary for biofilm formation (Watnick and Kolter 1999; Watnick et al. 1999; Moorthy and Watnick 2004), suggesting a role in adherence to host cells. Secondary verification using RT–PCR further supported expression of *mshA* (Fig. 4).

In addition to *pilA* and *mshA*, the functional identity of a number of other genes found in this study is supported by previous work on *E. scolopes* and *V. fischeri* ES114 (a native Hawaiian strain). For example, Arora et al. (1997, 1998) demonstrated that the expression of *flrA*, a motility master regulator, is necessary for mucin adhesion during *Pseudomonas aeruginosa* infection. Mucin adhesion is also critical to *V. fischeri* because it must adhere to a string of mucus from the host ciliated epithelia appendages before moving to the light organ pores upon initial host colonization (Nyholm et al. 2000, 2002; Nyholm and McFall-Ngai 2003). Previous infection studies of *E. scolopes* using *V. fischeri flrA* mutants have shown that expression is essential to normal colonization in the symbiosis (Millikan and Ruby 2004). Since *flrA* was also found to be expressed in adult light organs (further confirmed using RT–PCR; Fig. 4), it can be hypothesized that flagellar expression may be necessary to avoid daily expulsion by the host. Interestingly, previous studies have shown that *V. fischeri* lose their flagella upon colonization (Ruby and Asato 1993). It is not known, however, if flagellation in the light organ may differ depending on symbiont spatial orientation (associated with host epithelial cells or in the middle of the host crypt space) or time of day (growth rates are increased as the light organ is repopulated during the day). It is worth noting that hosts were collected during the evening when *Vibrio* growth rates are near their maximum rate. Further examination of flagellation within the host light organ is necessary to understand the expression of this regulator.

Colonization of host tissues also appears to be accompanied by a shift in metabolism. In contrast to genes for aerobic respiration found exclusively in seawater (Table 3), *V. fischeri* expresses a number of genes involved in anaerobic pathways, respiration, and oxidative metabolism inside the light

Fig. 4. Secondary verification of *flrA* (lanes 2–5), arginine decarboxylase (ADC, lanes 6–9), and *mshA* (lanes 10–13) transcripts using reverse transcriptase polymerase chain reaction. Lane 1: 1 kb ladder. Lane pairs 2–3, 6–7, and 10–11, 1× and 1:100× dilutions, respectively, of *Euprymna tasmanica* light-organ-derived *Vibrio fischeri* RNA. Lane pairs 4–5, 8–9, and 12–13, 1× and 1:100× dilutions, respectively, of seawater-derived *V. fischeri* RNA. *flrA* and ADC are expressed exclusively in the light organ, while *mshA* is expressed only in seawater. Controls using DNA polymerase verified the absence of DNA contamination in the RNA samples (not shown).



organs of both hosts (Table 2). This diverse metabolism by *V. fischeri* inside the host may be due to microhabitat differences within the light organ and the fact that bioluminescence reactions consume large amounts of oxygen. This could result in anaerobic microenvironments in which *V. fischeri* must persist.

If fermentative processes are common in the light organ, the process can be potentially hazardous for *Vibrio* bacteria, since acidic by-products excreted into the local environment may accumulate, lowering the pH of the light organ. It appears that *Vibrio* may tolerate this acidity through the expression of arginine decarboxylase, an enzyme involved in pH regulation as part of homeostasis (Park et al. 1996). As with *mshA*, secondary verification by RT–PCR of arginine decarboxylase confirmed exclusive expression in the light organ (Fig. 4). The potential reduction of the light organ pH may also have unidentified effects in terms of persistence of the symbiont. For example, expression of *toxR*, a virulence

regulator in *V. cholerae*, changes dramatically with pH (Skorupski and Taylor 1997). This implies that the shift to fermentation may be a signal for bacteria to express genes, exploiting a novel environment during infection.

Along with shifting metabolic activities upon host contact, *Vibrio* must also adapt to dramatic changes in the physical architecture of their environment and to the presence of dense populations of con-specifics during symbiotic colonization. uridine diphospho (UDP)-glucose-6-dehydrogenase, which is involved in colanic acid biosynthesis during capsular formation in addition to starch and sugar metabolism, has been demonstrated to be expressed when *Pseudomonas aeruginosa* or *Escherichia coli* contact surfaces, suggesting its importance for bacterial adhesion (Davies et al. 1993). Colanic acid also appears to play a role in maintaining the three-dimensional architecture of biofilms (Hanna et al. 2003). Given this, light organ expression of colanic acid may be of importance in the localization and three dimensional structure of clonal *Vibrio* communities.

This study provides evidence to support the importance of differential gene expression for the evolutionary ecology of a bacterium that inhabits two distinct environmental niches. Our results indicate that *V. fischeri* displays radically different expression patterns in seawater versus in the light organs of their adult squid hosts, and expression is remarkably consistent in two different strains from *E. tasmanica* of Australia and *E. scolopes* of Hawaii. Future studies will examine the extent to which each discovered gene and associated pathway is important for persistence in these ecological habitats, providing insight into the genetics to host colonization by environmentally transmitted bacteria. In addition, studies using these genes will hopefully identify the elements necessary for local adaptation and the evolution of host specificity as research further determines the ecological genetics of these two interacting species.

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