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Differential gene expression in bacterial symbionts from loliginid squids demonstrates variation between mutualistic and environmental niches

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Summary

Luminescent bacteria (γ-*Proteobacteria*: *Vibrionaceae*) are found in complex bilobed light organs of both sepiolid and loliginid squids (Mollusca: Cephalopoda). Despite the existence of multiple strain colonization between *Vibrio* bacteria and loliginid squids, specificity at the genus level still exists and may influence interactions between symbiotic and free-living stages of the symbiont. The environmentally transmitted behaviour of *Vibrio* symbionts bestows a certain degree of recognition that exists prior and subsequent to the colonization process. Therefore, we identified bacterial genes required for successful colonization of loliginid light organs by examining transcripts solely expressed in either the light organ or free-living stages. Selective capture of transcribed sequences (SCOTS) was used to differentiate genes expressed by the same bacterium when thriving in two different environments (i.e. loliginid light organs and seawater). Genes specific for squid light organs included vulnibactin synthetase, outer membrane protein W and dihydroxy dehydratase, which have been associated with the maintenance of bacterial host associations in other systems. In contrast, genes that were solely expressed in the free-living condition consisted of transcripts recognized as important factors for bacterial survival in the environment. These transcripts included genes for methyl accepting chemotaxis proteins, arginine decarboxylase and chitinase. These results provide valuable information regarding mechanisms determining specificity, establishment, and maintenance of bacteria–squid associations.

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Introduction

Bacterial–host interaction during mutualistic or pathogenic symbioses can be a dynamic association where microorganisms use survival and reproduction strategies to fight the normal defence mechanisms of the host (Mekalanos, 1985; McFall-Ngai and Ruby, 1998). Because expression of virulence determinants in bacteria is regulated by both environmental and host factors (Heithoff *et al*., 1997; Jones and Nishiguchi, 2006; Soto *et al*., 2009), many novel genes that are not expressed during *in vitro* growth are known to be regulated by *in vivo* factors within the host (Heithoff *et al*., 1997) at the onset of symbiosis. For instance, Graham and Clark-Curtiss (1999) demonstrated differential gene expression of *Mycobacterium tuberculosis* upon interaction with cultured primary human microphages and during their free-living state. Likewise, studies on *Salmonella typhimurium* showed variation in expression profiles following colonization of mouse tissue when compared with growth outside of the host (Slauch *et al*., 1994). Both studies elucidated the importance of genes coding for membrane, stress and regulatory functions in the establishment and maintenance of these associations. Interactions between bacteria in the family *Vibrionaceae* and squid light organs is not an exception to this rule, with *Vibrio fischeri* undergoing differential gene expression upon colonization of the light organs of bobtail squids in the genus *Euprymna* (Mollusca: Cephalopoda) (Jones and Nishiguchi, 2006).

Associations between bobtail squids (Cephalopoda: Sepiolidae) and *Vibrionaceae* bacteria (γ-proteobacteria: *Vibrionaceae*) have been previously studied to understand the evolution and regulation of specificity of environmentally transmitted symbioses (Nishiguchi *et al*., 1998; Nishiguchi, 2002; Jones *et al*., 2006). Ruby and Asato (1993) demonstrated that luminous bacteria in squid light organs benefit from this association and exhibit higher growth rates than their free-living counterpart. Likewise, there is a benefit for the squid host, which uses the light produced by its bacterial partner for counterillumination (Jones and Nishiguchi, 2004).

Similarly, squid species in the family Loliginidae (Mollusca: Cephalopoda) are known to possess bacteriogenic light organs (Alexeyev, 1992; Anderson, 2000). The

economic importance of loliginid squid fisheries (Chotiyaputta *et al.*, 2002; Nootmorn and Chotiyaputta, 2002) has furthered scientific interest in the characterization of bacterial populations colonizing these specialized tissue complexes (Guerrero-Ferreira and Nishiguchi, 2007). Previous studies have provided evidence of an association between the marine pathogen *Vibrio harveyi* with light organs of loliginid squids. These findings have raised questions regarding the potential of this symbiosis as a temporary reservoir for pathogenic *Vibrio* species such as *V. harveyi* (Guerrero-Ferreira and Nishiguchi, 2007; Dunlap *et al.*, 2008). Considering the dual life history of *V. harveyi*, it is important to understand the genetic factors involved in its transition from pathogenic to mutualistic lifestyles to obtain valuable clues on how these unique associations have arisen.

Research approaches to identify genes selectively expressed by bacteria during their symbiotic states within host animals or cells have revealed a great deal about how virulence factors are regulated (Slauch *et al.*, 1994; Graham and Clark-Curtiss, 1999; Daigle *et al.*, 2001; Somboonwiwat *et al.*, 2006), as well as identified factors required for successful colonization and persistence (Camilli and Mekalanos, 1995; Faruque *et al.*, 2004; Jones and Nishiguchi, 2006). SCOTS (Selective Capture Of Transcribed Sequences) has been successfully used to compare gene expression of the same bacterium existing in two different environments (Graham and Clark-Curtiss, 1999; Daigle *et al.*, 2001; Hou *et al.*, 2002; Jones and Nishiguchi, 2006). This technique has been recognized as a useful tool to understand selective pressures associated with persistence of bacteria in the environment as well as host colonization. Therefore, we examined differences in gene expression of environmental (seawater) and symbiotic (light organ associated) bacterial isolates from the loliginid squid *Uroteuthis chinensis* using SCOTS to determine bacterium responsiveness to either host or environment.

Results and discussion

SCOTS has been proven to be a successful method for identification of genes expressed either during symbiosis (associated with a host) or in its free-living state (Graham and Clark-Curtiss, 1999; Graham *et al.*, 2002; Dozois *et al.*, 2003; Faucher *et al.*, 2006). Successful use of SCOTS in mutualistic relationships such as the *Euprymna–V. fischeri* association has contributed to the knowledge of factors responsible for colonization and persistence of the symbiont within the light organ of the squid as well as prior to infection in the surrounding environment (Jones and Nishiguchi, 2006). We examined the utility of this method by comparing light organ-expressed genes with those solely expressed in seawater. A total of 47 genes were found, with 27 transcripts identified from light organ isolated bacteria and 20 transcripts from those isolates grown in seawater (Tables 1 and 2). Contamination by ribosomal RNA (rRNA) after capture hybridiza-

Table 1. Genes expressed by vibrio isolates in the light organs of loliginid squids.

Class	Clone name	Homology	Gene/protein coded
Cellular processes	Xba086,135	VVA1310	Vulnibactin synthetase, amide synthase subunit
Cellular processes	Xba171	VIBHAR_06625	Sensory histidine kinase CreC
Cellular processes	Xba103,014	VIBHAR_04770	Cell wall-associated hydrolase
Cellular processes	Xba064	VF-2110	Putative transporter YaaJ
Cellular processes	Xba065	VIBHAR_06639	*omp*W gene for outer membrane protein W
Cellular processes	Xba175	*Vibrio harveyi* HY01	Cell wall-associated hydrolase
Metabolism	Xba010	VIBHAR_00348	Gamma glutamyltransferase
Metabolism	Xba112	pVHA1-VHW-1	Quaternary ammonium compound resistance protein
Metabolism	Xba126	VIBHAR_00512	Partial *ilv*D gene
Metabolism	Xba106	VIBHAR_03235	Uridylate kinase (UMP kinase) PyrH
Metabolism	Xba008	VIBHAR_02101	Non-ribosomal peptide synthetase modules
Information storage and processing	Xba111	VIBHAR_00057	30S ribosomal protein S12
Information storage and processing	Xba006	VIBHAR_06651	*lys*R family transcriptional regulator
Information storage and processing	Xba005	VIBHAR_06734	Ribosomal protein S6 modification protein
Information storage and processing	Xba169	*Vibrio harveyi* HY01-A1Q_5079	Pseudouridine synthase, Rsu
Information storage and processing	Xba134	VIBHAR_0565	Integrase IntI
Poorly characterized	Xba172	VV1_1061	Orf122-like protein
Poorly characterized	Xba168	VIBHAR_00255	RNA-binding protein
Hypothetical	Xba108	VIBHAR_00327	Hypothetical protein
Hypothetical	Xba170	VV1_0932	Hypothetical protein
Hypothetical	Xba174	V12B01_06372	Hypothetical protein
Hypothetical	Xba176	A55_B0062	Hypothetical protein
Hypothetical	Xba177	A55_B0062	Hypothetical protein
Hypothetical	Xba178	VIBHAR_01012	Hypothetical protein
Hypothetical	Xba179	VC274080_B0002	Conserved hypothetical protein
Unknown	Xba173	VV20845	CMCP6 locus tag, product unknown

Table 2. Genes expressed by vibrio isolates in seawater.

Class	Clone name	Homology	Gene/protein coded
Cellular processes	Sal105,141	VIBHAR_01497	Arsenate reductase (*ars*C)
Cellular processes	Sal003	VIBHAR_05872	ATP-dependent chaperone *clp*B
Cellular processes	Sal198	VIBHAR_05104	Putative arsenate reductase
Cellular processes	Sal077	VIBHAR_04747	Methyl-accepting chemotaxis protein (MTA/SAH nucleosidase)
Cellular processes	Sal205	VIBHAR_05559	Heat shock protein
Cellular processes	Sal209	VIBHAR_02921	Tyrosine-phosphatase
Cellular processes	Sal005	VIBHAR_04747	Methyl accepting chemotaxis protein
Metabolism	Sal203	VIBHAR_05421	phenylalanine monooxygenase
Metabolism	Sal127	VIBHAR_06354	D-serine deaminase
Metabolism	Sal142	VIBHAR_00053	Nitrite reductase (*nir*B)
Metabolism	Sal143,180	VIBHAR_06737	Arginine decarboxylase (*spe*A)
Metabolism	Sal197,204	VIBHAR_03711	D-aspartate kinase
Metabolism	Sal103,163	VIBHAR_00698	Aminoglycoside acetyltranferase
Metabolism	Sal104	VIBHAR_05656	Chitinase
Metabolism	Sal107	VIBHAR_03431	Cellobiose phosphorylase
Metabolism	Sal 202	VIBHAR_01806	Adenosylmethionine-8amino-7-oxononanoate transaminase
Metabolism	Sal217	VIBHAR_00151	Manganese superoxide dismutase
Poorly characterized	Sal207	VIBHAR_03535	GTP-binding protein
Poorly characterized	Sal019	VIBHAR_07101	RNA binding protein
Hypothetical	Sal 090	VIBHAR_00469	Hypothetical protein
Hypothetical	Sal102	VIBHAR_00194	Hypothetical protein

tions, which is one of the concerns during SCOTS, was ruled out by completing southern blot hybridizations during primary verification. Tables 1 and 2 also show distribution of transcripts by gene types indicating that seawater-expressed genes are most associated with cellular processes (seven transcripts) and metabolism (10 transcripts). Light organ transcripts detected by SCOTS showed a more uniform distribution among different gene categories including genes for cellular processes (six transcripts), metabolism (five transcripts), and information storage and processing (five transcripts) (Fig. 1). Lack of detection of seawater transcripts under the functional category of information storage and processing (translation, ribosomal structure and biogenesis, transcription, DNA replication, recombination and repair) after SCOTS may indicate that these genes are almost equally expressed under both conditions, therefore being blocked during enrichment of each SCOTS library. However, specific genes (shown in Table 1) are selectively expressed during the bacteria symbiotic lifestyle, which highlights their importance for symbiosis.

Gene expression of vibrio isolates in the light organ of loliginid squids

Graf and Ruby (1998) argued that the crypt epithelium within the light organ provides amino acids and potentially other nutrients to the symbiotic bacteria housed therein. This might indicate that colonization of the nutrient-rich, light organ environment provides a selective advantage to the symbiont over free-living ecological variants. However, the same experiments demonstrated that auxotrophic mutants of squid symbionts were able to grow and establish themselves within the light organ environment at lower densities than wild-type isolates. Similarly, this study demonstrated that some amino acids required for bacterial growth are found in low levels within the light organ environment. Because of this, genes required for

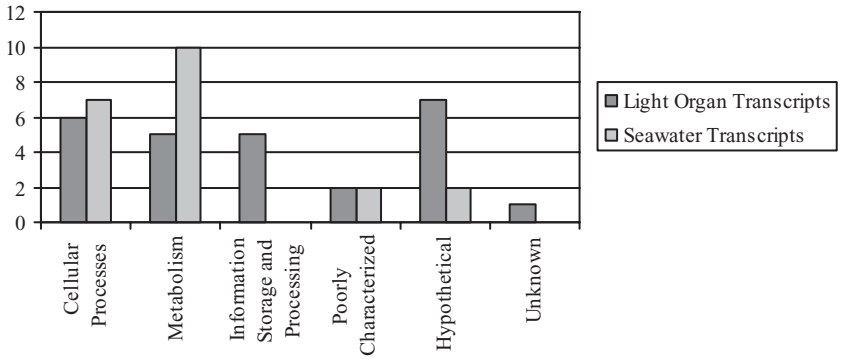

Fig. 1. Distribution of gene categories of transcripts expressed by free-living and light organ-associated bacteria captured through SCOTS.

amino acid synthesis are expected to be expressed in the light organ. In our study, we confirmed that genes required for the production of specific amino acids are being expressed during symbiosis, with *ilv*D (dihydroxyacid dehydratase) being solely expressed in the light organ. *ilv*D (Tarleton and Ely, 1991) catalyses the fourth step in the metabolic pathway leading to production of isoleucine (from pyruvate) and valine (from alpha-oxobutyrate). The product of the reaction catalysed by dihydroxyacid dehydratase is also the starting point in the alternative pathway producing leucine (Gottschalk, 1986). Production of leucine and valine is necessary within the light organ matrix where levels of these two amino acids are low, reaching only 0.07 and 0.09 mM respectively in *Euprymna scolopes* (Graf and Ruby, 1998). Conversely, concentrations of the same amino acids measured within symbiotic cells (including free and peptide forms) are among the highest, with 1.58 mM of leucine and 1.26 mM of valine, indicating that these molecules are being synthesized within the squid light organ. This suggests a major role for this amino acid in the proliferation of the association. Production of *ilv*D mutants of *Vibrio* bacteria and further colonization studies on squids would give insights on the role of *ilv*D on the persistence of the association.

An underlying role of *ilv*D is evident in *Vibrio cholerae*, the causative agent of cholera. In a human *in vivo* expression technology study (IVET), Lombardo and colleagues (2007) identified *ilv*D as one of the *V. cholerae* genes selectively expressed during human infection. In a different study, a large-scale signature-tagged mutagenesis screen (Merrell *et al*., 2002) also demonstrated the requirement of *ilv*D for human colonization by *V. cholerae*. This study showed that mutations affecting the entire *ilv* operon were associated with decreased virulence in the mouse model. Contribution of *ilv*D to the capability of *Vibrio* bacteria to colonize loliginid squid is yet to be characterized. However, since *V. cholerae* requires this gene for colonization of epithelial tissues, it may also indicate a connection between *ilv*D expression and colonization of eukaryotic tissue (such as squids) by *Vibrio* bacteria in general.

Our study also demonstrates selective expression of the gene for the amide synthetase subunit of vulnibactin synthetase, the enzyme responsible for production of vulnibactin, a siderophore first isolated from low-iron cultures of *Vibrio vulnificus* (Noriyuki *et al*., 1994). Acquisition of iron from the host is a requirement for colonization by host tissue-associated microbes. It is a common feature in pathogenic systems such as *Vibrio anguillarum* (Actis *et al*., 1986; Wolf and Crosa, 1986), *V. cholerae* and *V. harveyi* (Henderson and Payne, 1994; Owens *et al*., 1996). In mutualistic associations, studies with *V. fischeri* mutants with depleted siderophore production revealed that the ability to respond to limited iron levels may be connected to symbiosis efficiency (Graf and Ruby, 2000). Owens and colleagues (1996) also established a correlation between siderophore production and the type of bacterial isolates, and determined that free-living strains of *V. harveyi* produce higher amounts of siderophore compared with isolates associated with invertebrates. This difference in siderophore production was in contrast with our findings that light organ-associated vibrios selectively expressed the vulnibactin synthetase gene, necessary for bacterial siderophore production within the light organ. However, because Owens and colleagues (1996) focused on measurements of *V. harveyi* siderophore activity on agar plates prepared, their results do not reflect the expression of genes related with siderophore-producing pathways *in vivo*. Few studies have focused on iron sequestration and regulation of sidephore production of symbiotic bacteria during mutualistic associations with animal tissues (Bagg and Neilands, 1987; Aznar and Alcaide, 1992; Naidu and Yadav, 1997; Moeck and Coulton, 1998). Since bacteria harboured in the light organ are found in high concentrations (~10^8–10^{10} in an adult *Euprymna*), the necessity of having siderophores to capture iron resources may be fuelled by the competition among this community (i.e. limited resources in the light organ). Further examination of siderophore activity and regulation of gene expression associated with iron assimilation is necessary to fully comprehend the role of iron sequestration in colonization of loliginid light organs by *Vibrionaceae* bacteria.

Comparable to most siderophores, vibriobactin is a small cyclic peptide with the ability to bind environmental iron. This siderophore–iron complex is recognized by bacterial surface receptors such as *omp*W and transported into the cytoplasm (Moeck and Coulton, 1998). Expression of the *omp*W gene for outer membrane protein W was also detected in our SCOTS analysis. The importance of iron sequestration in symbiosis, specifically with regards to growth and replication of cytochrome systems of bacterial symbionts residing in eukaryotic hosts, has been previously observed (Bagg and Neilands, 1987; Henderson and Payne, 1994; Dozois *et al*., 2003). It is also known that production of siderophore is frequently accompanied by the synthesis of outer membrane proteins acting as receptors for the iron–siderophore complex (Bagg and Neilands, 1987; Dai *et al*., 1992).

In addition, outer membrane proteins have been shown to enhance adhesion and colonization when tested in animal models. However, their exact role in virulence is not completely understood (Faruque *et al*., 2004). In mutualistic associations, Jones and Nishiguchi (2006) reported *V. fischeri* expression of an integral membrane protein during colonization of *E. scolopes* light organs. Moreover, Aeckersberg and colleagues (2001) presented

evidence connecting the presence of a specific bacteria outer membrane protein (i.e. OmpU) for the establishment of successful mutualistic associations with sepiolid squids. Results from our SCOTS screening indicate that expression of genes for membrane proteins, such as *omp*W in symbiotic bacteria, may be necessary for successful colonization of the loliginid light organ, because of their direct relevance to *in vivo* recognition of iron–siderophore complexes.

Equally important for maintenance of colonization efficiency is the expression of the *pyr*H gene. This gene codes for the protein uridine monophosphate kinase (UMP kinase) that participates in pyrimidine metabolism catalysing the conversion of UMP into UDP (Voet and Voet, 2004). Kim and colleagues (2003) identified *pyr*H as one of the genes in *V. vulnificus* that is expressed preferentially *in vivo*. *Vibrio vulnificus* mutants unable to produce UMP kinase exhibited retarded growth on media and a reduction in the ability to infect HeLa cells. Therefore, *pyr*H expression has relevance in host colonization by *Vibrio* bacteria by directly reducing bacterial growth and decreasing infectivity.

Gene expression of vibrio isolates in seawater

Analysis of gene expression profiles of seawater-grown bacteria yielded a set of genes dominated by those responsible for bacterial metabolism. Among transcripts identified by SCOTS, a chitinase gene was hypothesized to be present, since *Vibrio* species use chitin as a carbon source (Yu *et al*., 1991; Svitil *et al*., 1997). Vibrios play a critical role in transforming chitin, a highly insoluble polysaccharide, into a form usable to other organisms (Bassler *et al*., 1991). Moreover, it has been reported that some species in the family *Vibrionaceae* are able to monitor surrounding environmental conditions by recognizing and migrating towards low concentrations of chitin oligosaccharides (Yu *et al*., 1991). Similarly, in most cooperative interactions studied in nature, hosts provide nutrient-rich environments for bacterial growth, whereas the bacteria contribute by providing the host with products of specific bacterial processes. It might be expected that free-living vibrios would use chitin metabolic machinery to monitor host tissue until successfully detecting chitin contained in the squid light organ (McFall-Ngai, 1998). This type of mechanism would potentially be part of the interactions that account for the first encounter between bacteria and host in the squid–*Vibrio* symbiosis. Upon first contact, other processes such as mucus and nitric oxide production occur (Nyholm *et al*., 2000; Davidson *et al*., 2004) that allow for the successful and permanent establishment of the symbionts within the crypts of the light organ (McFall-Ngai and Ruby, 1998). Our results not only support the importance of host-produced chitin on the initiation of squid-vibrio symbiosis but also imply a more active role of the symbiont in the establishment of this association.

In addition, our screening exhibited arginine decarboxylase (ADC) expression by *V. harveyi* loliginid light organ isolates grown in seawater. ADC degrades arginine to produce agmatine, and increases pH by removing acidic carboxyl groups and releasing CO_2 from their substrates (Stim and Bennett, 1993). A potential role of ADC in the environment is the formation of biofilm. ADC, similar to other amino acid decarboxylases, is involved in the production of polyamines (Stim and Bennett, 1993) that modulate bacterial biofilms within *Vibrionaceae* species (Kierek-Pearson and Karatan, 2005; Patel *et al*., 2006). Studies have shown that factors that modulate biofilm formation (such as ADC and uridine diphosphate dehydrogenase, UDPDH) by *Vibrionaceae* bacteria are important in light organ symbiosis. For example, analysis of *V. fischeri* UDPDH mutants demonstrated that their biofilm-forming ability is reduced *in vitro*. In addition, these mutants were not detected in any part of the crypt region during colonization assays (Ariyakumar, 2007; Ariyakumar and Nishiguchi, 2009).

Also important for bacterial colonization of eukaryotic hosts is the expression of genetic factors responsible for DNA methylation (Mahan *et al*., 2000). This study provides evidence for expression in seawater of the 5′-methylthioadenosine/*S*-adenosylhomocysteine (MTA/SAH) nucleosidase gene. Methylation reactions depend on the appropriate methyl donor to be available when necessary. While *S*-adenosylmethionine (SAM) is the main donor of methyl groups in the cell, the end-product of the reaction is *S*-adenosylhomocysteine (SAH) that negatively regulates SAM-dependent methyltransferases. Therefore, SAH needs to be readily metabolized by MTA/SAH nucleosidase to adenine and *S*-ribosylhomocysteine. The activity of MTA/SAH nucleosidase is indispensable for the maintenance of methylation reactions in bacteria (Stepkowski *et al*., 2005). It has been demonstrated that severe defects in colonization of eukaryotic tissue occur when methylation processes are affected in methylation-defective *S. typhimurium* mutants (Heithoff *et al*., 1999). Such increases in methylation events and efficient regulation of methylation reactions may be accomplished by preferential expression of genes coding for MTA/SAH nucleosidases. Also, with methylation reactions being linked to successful eukaryotic colonization by bacteria, selective expression of methylation genes may be important for improving the likelihood of successful colonization of squid tissue by *Vibrio* bacteria.

Also important in metabolic processes in bacteria is the expression of chaperone molecules such as ClpB. The gene coding for this chaperone molecule was exclusively expressed during seawater growth of symbiotic bacteria.

ClpB is important for the refolding of luciferase (Zavilgelsky *et al.*, 2004), the molecule responsible for light emission and important for quorum sensing in bacteria (Bassler, 1999). Zavilgelsky and colleagues (2004) demonstrated that presence of ClpB accelerated the refolding of luciferase and increased its efficiency approximately 10-fold. Considering that luciferases of *Vibrionaceae* bacteria are sensitive to thermoinactivation (Zavilgelsky *et al.*, 2004), activity of ClpB would be considerably important in locations where water temperatures can change between winter and summer months (Jones *et al.*, 2006; Soto *et al.*, 2009), or where temperatures can exceed 28°C (Soto *et al.*, 2009). Since bioluminescence is an important aspect of light organ symbioses, this phenomenon is extremely important for the fitness of light-producing bacteria.

Physiological adaptation is essential to maintain homeostasis. Metabolic processes responsible for such adaptations are regulated by the activity of sensory and regulatory proteins that control gene expression and enzymatic activity. Some of these mechanisms are conserved across bacterial species, while others are the result of microbial adaptation to specific environmental niches. Our research presents evidence of gene expression profiles that may explain the ability of a bacterium to transition between a free-living to mutualistic lifestyle, given its mechanisms of transmission (environmental). This study also provides support for differential gene expression relative to the ecology of *V. harveyi* bacteria and their ability to occupy multiple niches when grown in seawater and in squid hosts. Examining the processes that select for the evolution of symbiosis in the ocean can be better understood by defining important genetic factors that contribute to successful initiation and persistence of symbiotic associations. Future studies will include detailed investigations of these genes and the metabolic pathways related to synthesis or degradation of their products, and will address questions concerning host specificity and the array of mechanisms necessary for beneficial associations.

Experimental procedures

Bacterial growth conditions and extraction of total RNA

Specimens of *U. chinensis* were obtained from squid trawls off the coast of Cairns, Australia during the 2008 summer season. Light organs were dissected from the mantle cavity and a portion of it homogenized in sterile seawater. The rest of the light organs were kept in RNA Later (Applied Biosystems/Ambion, Austin, TX) for further extraction of mRNA. Serial homogenate dilutions were incubated on seawater tryptone agar (70% seawater v/v, 0.5% tryptone w/v, 0.3% yeast extract w/v, 0.3% glycerol v/v and 1.5% technical grade agar) at room temperature for 16 h. Individual colonies of luminous bacteria were isolated and used to inoculate 5 ml of SWT media and shaker incubated (250 r.p.m.) overnight. An aliquot (900 μl) of bacterial suspension was used for glycerol stocks. To identify transcripts selectively expressed by the *U. chinensis* isolates in seawater, bacteria were grown in sterile seawater (non-artificial) in a 28°C shaker incubator (250 r.p.m.) for 48 h. At this time an optical density (OD_{600}) of about 0.5 was reached. Seawater was obtained from Santa Catalina Island, CA. Bacteria were concentrated by centrifugation and RNA extracted following the protocol described below.

For light organ-expressed transcripts, three (partial) *U. chinensis* light organs were homogenized in RNA Later (Applied Biosystems/Ambion, Austin, TX) and centrifuged at 12 000 *g* for 2 min to pellet eukaryotic tissue, separating bacterial cells from the rest of the squid light organ. RNA extraction was completed using a protocol modified from Mangan and colleagues (1997). This protocol was incorporated in the RiboPure™ kit (Applied Biosystems/Ambion, Austin, TX), and manufacturer instructions were followed. Following RNA isolation, removal of rRNA from total RNA samples was performed with MICROB*Express* (Applied Biosystems/Ambion, Austin, TX). This additional step removes considerable amounts of rRNA from bacterial total RNA samples, reducing the chance of rRNA-derived complementary DNAs (cDNAs) being generated during SCOTS.

cDNA library construction

Complementary DNA libraries were constructed using the method proposed by Graham and Clark-Curtiss (1999; 2000). The first strand of cDNA was synthesized from total RNA using a Superscript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA), following manufacturer's instructions. Oligonucleotides with a random 9-mer at the 3′ end were used for this first step of cDNA library construction (Table 3). Second-strand synthesis was performed with the Klenow fragment of DNA polymerase (New England Biolabs, Beverly, MA) following manufacturer's instructions. Double-stranded cDNA was purified from remaining salts, enzymes and unincorporated nucleotides using the PCR purification kit (Qiagen, Valencia, CA).

Isolation of rRNA operon

In order to block cDNA derived from rRNA during SCOTS, the full ribosomal operon was PCR-amplified from total DNA using primers 16S Forward (Edwards *et al.*, 1989) and 23S Reverse (Jones and Nishiguchi, 2006) (Table 3). PCR reactions yielded a product of approximately 5 kb in length, which was excised from a 1% agarose gel in 1× TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8.0) using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified ribosomal operon was subsequently inserted into the pCR® 2.1-TOPO® cloning vector (3.9 kb) from the TOPO® TA Cloning kit (Invitrogen Corporation, Carlsbad, CA).

Selective Capture Of Transcribed Sequences (SCOTS)

Normalization of cDNA libraries was conducted by three rounds of capture hybridization following a protocol from

Table 3. Primers for SCOTS protocol and verification.

Name	Sequence (5′–3′)	Application
Xba random	TGCTCTAGACGTCCTGATGGTT9(N)	cDNA library
Xba	TGCTCTAGACGTCCTGATGGTT	cDNA amplification
Sal random	ATATGTCGACTGAATTCCGTAGG9(N)	cDNA library
Sal	ATATGTCGACTGAATTCCGTAGG	cDNA amplification
16S forward	AGAGTTTGATCMTGGCTCAG	rRNA cloning
23S reverse	ATGGTTAAGCCTCACGGGCA	rRNA cloning
*spe*A sense	GTTAAACCACGTCTTGGCTTGCGT	Secondary verification
*spe*A antisense	ACCACCACCCACATCGAGGTATTT	Secondary verification
Chitinase sense	TCAGTCGGTGGATGGACGCT	Secondary verification
Chitinase antisense	GACGCTGATAATGGCGACAT	Secondary verification
*ilv*D sense	TCCGTCGAATACATGGTCAA	Secondary verification
*ilv*D antisense	TTTCAAACGCTGCTTTGTTG	Secondary verification
*omp*W sense	CCACCTACCTTTATGGTCC	Secondary verification
*omp*W antisense	GGTTTGTCGAATTAGCTTCACC	Secondary verification
*vib*H sense	TTGATGGCTACAGCTTGCAC	Secondary verification
*vib*H antisense	ATTGATCCACAGCGGTAAGG	Secondary verification

Jones and Nishiguchi (2006), modified from Graham and Clark-Curtiss (1999). Briefly, 30 μg of sonicated, biotinylated, genomic DNA (gDNA) and 50 μg of sonicated, cloned, ribosomal operon (rRNA) were precipitated and re-suspended in 40 μl of 10 mM 3-[4-(2-Hydroxyethyl)-1-piperazinyl] propanesulfonic acid (EPPS) (Sigma-Aldrich, St. Louis, MO). Simultaneously, 125 μl of amplified cDNAs from each condition were equally precipitated and re-suspended in 40 μl of EPPS. Then, hybrids and cDNAs were denatured (2 min at 98°C), normalized (30 min at 55°C) and mixed with 10 μl of 1 M sodium chloride. After an additional incubation at 55°C for 1 h, a single gDNA–rRNA hybridization reaction was added to each cDNA library and incubated at 55°C overnight.

Following overnight hybridizations, gDNA–rRNA hybrids were recovered using Dynal streptavidin-coated magnetic beads (Dynal/Invitrogen Corporation, Carlsbad, CA) with a magnetic stand (Applied Biosystems/Ambion, Austin, TX). Biotinylated gDNA–cDNA hybrids were washed and cDNA eluted from gDNA using 100 μl of 0.5 M NaOH and 0.1 M NaCl.

Eluted cDNA was PCR-amplified with a non-random primer (Table 3) specific for each growth condition, then purified and normalized through four additional rounds of capture hybridizations. Conversely, all reactions were completed using 1/10 of the volumes used in the first round.

Enrichment of cDNA was conducted in a way similar to capture hybridizations. However, biotinylated gDNA, blocked with rRNA, was additionally blocked with 25 μl of cDNA that was amplified after the last round of capture hybridization (seawater-derived cDNA to examine genes expressed in the light organ and vice versa). Hybrids were collected using Dynal streptavidin-coated magnetic beads. cDNA was eluted, PCR-amplified and purified using the aforementioned protocols. PCR products (Xba-amplified libraries for light organ-expressed transcripts and Sal libraries for seawater-expressed transcripts) were cloned using the TOPO® TA Cloning kit (Invitrogen Corporation, Carlsbad, CA).

Southern hybridizations for primary verification

Southern hybridizations were carried out to eliminate false-positive sequences that escaped the subtraction process. Both Xba (light organ-expressed) and Sal (seawater-expressed) libraries were denatured with denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 5 min and treated with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.4) for 5 min and 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) for 5 min. Samples were then transferred onto a positively charged nylon membrane and cross-linked utilizing a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). Hybridization was then completed against DIG-labelled probes created from the fifth round captured sequences used to block the cross-linked sequences during the enrichment reactions.

Membranes were pre-hybridized in 15 ml of hybridization solution at 40°C and then hybridized at 40°C overnight. Hybridization was performed with 20 ng ml^{-1} of probe in DIG EasyHyb hybridization solution that was added to the membrane. After hybridization, two 2 min washes were completed using a low-stringency buffer (2× SSC, 0.1% SDS), followed by a 20 min washes at 65°C (with rotation) in high-stringency buffer (0.1× SSC, 0.1% SDS). A final wash with maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) was executed for 5 min at room temperature.

Detection was completed by exposing the membrane to 0.5 ml of anti-DIG antibody/alkaline phosphatase enzyme conjugate for 20 min and washing twice with 4 ml of maleic acid buffer for 10 min. Detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$) was then used for 5 min to start visualization with the enzyme substrate solution [4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4chloro-3-indolyl phosphate]. No signal indicated that the clone was exclusively expressed in either the light organ or the seawater condition.

Sequence analysis

Positive clones were sequenced using an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA), and edited using Sequencher v 4.6 (Gene Codes Corporation, Ann Arbor, MI). Sequences were then compared with the National Center for Biotechnology Information (NCBI) database using BLAST 2.2.11 (Basic Local Alignment Search Tool, NCBI, NLM, NIH, Bethesda, MD) for initial confirmation of sequence identity. In addition, the presence and location of transcripts in

the *V. harveyi* genome was determined (Tables 1 and 2) by comparisons with the *V. harveyi* genome (Bassler *et al*., 2007).

Secondary verification using reverse transcriptase PCR

Four clones were picked for secondary verification using reverse transcription with the SuperScript™ One-Step RT-PCR system (Table 3). Primers were developed for each gene based on their corresponding sequence to the *V. harveyi* genome (Bassler *et al*., 2007). PCR reactions were executed as described previously by Jones and Nishiguchi (2006).

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