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Differential Gene Expression in Bugula Neritina during Symbiotic Association with "Candidatus Endobugula Sertula"

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DIFFERENTIAL GENE EXPRESSION IN *BUGULA NERITINA* DURING SYMBIOTIC ASSOCIATION WITH "*CANDIDATUS* ENDOBUGULA SERTULA"

by

MERIL MATHEW

Under the Direction of Dr. Nicole B. Lopanik

ABSTRACT

The colonial marine bryozoan, *Bugula neritina*, harbors an uncultured endosymbiont, "*Candidatus* Endobugula sertula" throughout its life stages. The bacterial symbiont has been proposed to be a source of complex polyketide metabolites, the bryostatins, that chemically defend *B. neritina* larvae from predation. Within a bryozoan colony, significantly higher amounts of bryostatins are found in ovicell-bearing zooids where the developing larvae are brooded, as compared to ovicell-free zooids. It is hypothesized that signaling between *B. neritina* and "*Ca.* Endobugula sertula" may be involved in the regulation of bryostatin production in different zooids, as well as in maintenance of the symbiosis. In this study, suppression subtractive hybridization (SSH) was used to identify differentially expressed host genes during this association. The identified genes suggest that the host plays a role in the distribution and localization of bacterial symbionts in different host zooids, possibly to regulate levels of bryostatin production in the zooids.

INDEX WORDS: Symbiosis, *Bugula neritina*, Bryostatins, Differential gene expression

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1 INTRODUCTION

The marine bryozoan, *Bugula neritina*, is a common member of fouling communities on boat docks, boat hulls, and submerged rocks (Gordon and Mawatari, 1992; Carlton and Hodder, 1995; Stevens *et al*., 1996). *B. neritina* colonies have been reported in tropical and temperate regions on the east and west coasts of the United States (Davidson and Haygood, 1999; McGovern and Hellberg, 2003), throughout the Australian coastline, Hong Kong, Hawaii, Curacao, and England (Mackie *et al*., 2006). Adult colonies are sessile and protected by a chitinous cuticle (Ryland, 1970). Larvae are brooded on the parent colony and released during the day (Lindquist and Hay, 1996); the non-feeding larvae are ciliated and remain in the water column from 2 to 12 hours before settling on a substrate (Keough, 1989). During this stage, larvae are vulnerable to predation because they are large, conspicuous, nutrient-rich, soft bodied, and swim slowly (Lindquist and Hay, 1996; Wendt, 2000). An uncultured symbiotic γ-proteobacterium, "*Ca.* Endobugula sertula," is associated with *B. neritina* throughout its life stages (Woollacott and Zimmer, 1975; Woollacott, 1981; Haygood and Davidson, 1997). In the larvae, the symbiotic bacterium is located within a surface invagination called the pallial sinus situated on the top of the larvae (Woollacott, 1981; Haygood and Davidson, 1997). The symbiont was not detected in seawater surrounding *B. neritina* colonies (Haygood *et al.*, 1999) and, therefore, is thought to be vertically transmitted through the generations of the bryozoan. "*Ca.* Endobugula sertula" has also been reported to be the source of metabolites called bryostatins (Davidson *et al.*, 2001; Lopanik *et al.*, 2004). These metabolites make the larvae unpalatable to particle-feeding invertebrates and fishes, thus preventing predation of larvae (Lindquist and Hay,

1996; Lindquist, 1996; Lopanik *et al.*, 2004). By producing deterrent compounds, the microbial symbiont significantly contributes to survival of the host.

Research on bryostatins increased after bryostatin 1 was reported to show activity against a variety of cancer cells (Pettit *et al.*, 1970) by modulating the activity of the protein kinase C (PKC) signal transduction pathway (DeVries *et al.*, 1988; Wender *et al.*, 1988). Bryostatin 1 alone or in combination with other chemotherapeutic agents has been found to be effective in phase I and II clinical trials for the treatment of various type of cancers, although some studies have revealed issues of toxicity that remain to be addressed (Mutter and Wills, 2000; El-Rayes *et al.*, 2006; Hayun *et al.*, 2007; Ku *et al.*, 2008; Barr *et al.*, 2009; Lam *et al.*, 2010). However, bryostatin 1 recently was demonstrated to increase memory in animal models, and therefore, is considered to be a candidate for the treatment of Alzheimer's disease (Sun and Alkon, 2005, 2006; Kuzirian *et al.*, 2006). It has also been found to be a promising therapeutic agent for the repair of neural damage caused by cerebral ischemia and hypoxia in a mouse model (Sun *et al.*, 2008, 2009). Due to its great potential for the treatment of various types of cancer, Alzheimer's disease, and neurological disorders, research on bryostatin production by "*Ca.* Endobugula sertula" in the bryozoan host has gained interest.

To date, 20 bryostatins have been characterized from different populations of *B. neritina* (Pettit, 1996; Davidson and Haygood, 1999; Lopanik *et al.*, 2004). Mitochondrial cytochrome c oxidase subunit I (*COI*) sequence data have shown three sibling species of *B. neritina*: deep-water (found on the West coast of United States), shallow-water (found on both West and East coasts) and Northern Atlantic (found on northern East coast) (Davidson and Haygood, 1999; McGovern and Hellberg, 2003). Furthermore, it was also reported that different sibling species

of *B. neritina* harbor different strains of "*Ca.* Endobugula sertula", and possess different bryostatins (Davidson and Haygood, 1999). However, neither any endosymbiont or bryostatin production was found in the Northern Atlantic sibling species (McGovern and Hellberg, 2003; Lopanik *et al.*, 2004). It has been hypothesized that the symbiotic association between the bryozoan and the bacteria is dependent on geographical differences (McGovern and Hellberg, 2003). Since the host sibling species in varying habitats harbor different strains of the symbiont, the association appears to have co-evolved in their respective environments. Further, the production of different bryostatins in these regions indicates a potential correlation between the type of bryostatins synthesized and the predators found in the surrounding habitat. The symbiotic association appears to be a tritrophic interaction between bacterial symbiont, bryozoan, and the predators in the habitat.

Studies of bryostatin distribution within *B. neritina* colonies have shown higher levels in larvae (~10 times), as well as in ovicell-bearing zooids (~3 times) where the larvae are brooded and then released (Lopanik *et al.*, 2004; Lopanik *et al.*, 2006). During the life cycle of *B. neritina*, bryostatin levels were also found to be higher after the settlement of released larvae onto a surface and metamorphosis into juveniles, which are also vulnerable to predation due to lack of structural material such as chitin (Lopanik *et al.*, 2006; Sharp *et al.*, 2007). Recently, the putative bryostatin biosynthetic gene cluster was sequenced from shallow-water (North Carolina) and deep-water (California) populations of *B. neritina*- "*Ca.* Endobugula sertula" (Sudek *et al.*, 2007). It consists of five large modular polyketide synthase (PKS) coding genes, *bryA*-*D* and *X* and a discrete four-gene cassette (*bryP*-*S*) that encodes for two acyltransferase domains on a single ORF, a β-ketoacyl synthase, a β-hydroxy-β-methyl-glutaryl CoA synthase, and a

methyltransferase. Since "*Ca.* Endobugula sertula" is, to date, uncultured, demonstration of the functionality of the proposed gene cluster by traditional gene knockout and complementation methods has not been performed. However, β-ketoacyl synthase (KS) gene fragments in larvae and symbiotic adult individuals are expressed, suggesting that a portion of the gene cluster is transcribed (Davidson *et al.*, 2001). Similarly, transcription of the entire *bryA* open reading frame was demonstrated by reverse transcription PCR on adult *B. neritina* total RNA (Hildebrand *et al.*, 2004). *In vitro* studies have also revealed the function of *bryP* for *trans*-acylation of PKS modules during elongation of the bryostatin chain (Lopanik *et al.*, 2008), suggesting that portions of the putative *bry* cluster perform biochemically as expected.

1.1 Purpose of the Study

Molecular signaling between partners has been shown to be necessary for initiation, establishment and maintenance of various mutualistic interactions. Differential gene expression using suppression subtractive hybridization (SSH) has been utilized in several symbiotic systems to identify signals and genes that are specifically expressed during the association. SSH is a powerful molecular technique to enrich differentially expressed mRNAs (Diatchenko *et al.*, 1996). In this technique, the mRNA containing the differentially expressed transcripts is the tester, while the one without the transcript (reference) is the driver. Tester cDNA molecules are divided into two portions and each portion is ligated with different adaptor oligonucleotide molecules. Following a series of hybridizations between adaptor-ligated-tester cDNA molecules and driver cDNA molecules, the hybridized cDNA molecules are amplified using primers complementary to the adaptor sequences. Only differentially expressed cDNA molecules with different adaptors on each strand will be exponentially amplified. The enriched cDNAs can be

cloned into a suitable vector to create a cDNA library of differentially expressed genes. Steindler and coworkers (2007) screened differentially expressed genes in the symbiotic association between the marine sponge, *Petrosia ficiformi* and cyanobacteria compared to the non-symbiotic form using SSH. The study identified a novel gene, *PfSym2*, which was proposed to be either involved in recognition of the symbiont or facilitate adhesion of the symbiont to the sponge cells. Similarly, Yuyama and coworkers (2005), reported higher expression of *AtSym-02* in symbiotic populations of the coral, *Acropora tenuis*, thought to be involved in recognition of the dinoflagellate symbionts, *Symbiodinium* spp. In another example, SSH revealed host proteins that are specifically involved in metabolite exchanges occurring in the brachial plume tissue and trophosome between the symbiotic partners, the hydrothermal vent tube worm, *Riftia pachyptila* and chemolithotrophic sulfide-oxidizing bacteria (Sanchez *et al.*, 2007).

The biosynthesis of bryostatin is reported to be extremely low in *B. neritina* colonies without "*Ca.* Endobugula sertula" (Davidson *et al.*, 2001; Lopanik *et al.*, 2004). Aposymbiotic *B. neritina* were obtained by treating *B. neritina* larvae with gentamicin for 4 - 10 days to cure the host of symbionts. Previous studies have also identified significantly higher bryostatin production in the ovicell-bearing zooids where the larvae are brooded before being released (Lopanik *et al.*, 2006). Sharp and coworkers (2007) studied localization of the symbionts and bryostatins during the bryozoan life cycle using fluorescence *in situ* hybridization (FISH) and a PKC-based detection method, respectively. Strong bryostatin signals, as well as higher symbiont density, were detected inside the ovicell-bearing zooids in and around the funicular cords, which transport nutrients to the developing larvae. This difference in bryostatin concentrations in different life stages of *B. neritina* is intriguing when considering that the microbial symbiont

"*Ca*. Endobugula sertula", found in all life stages, is most likely responsible for producing the bryostatins. In this study, it is hypothesized that host signals result in the upregulation of the production and distribution of bryostatins in ovicell-bearing zooids. It is also hypothesized that *B. neritina* genes may also be differentially expressed in the symbiotic state of the host for maintenance of the symbiosis by recognizing the symbiont as non-pathogenic, suppressing its immune response against the symbiont, and allowing distribution and localization of symbionts in its tissues. The goal of this study is to identify host genes involved in these processes by examining differentially expressed *B. neritina* mRNAs in the symbiotic and aposymbiotic ovicell-bearing (with larvae) and ovicell-free zooids.

2 MATERIALS AND METHODS

2.1 Collection of *B. neritina* larvae and creation of aposymbiotic larvae

B. neritina colonies attached to floating docks in Beaufort and Morehead City, NC were collected by hand during November 2009 and housed in wet lab facilities at the UNC-CH Institute of Marine Sciences in Morehead City. The colonies were maintained in flowing seawater tables in the dark overnight (~16 hours), and the next morning (~9 AM), were transferred to large glass jars filled with seawater. The colonies were exposed to sunlight to stimulate release of larvae. The released larvae were collected with a wide tip glass pipette and divided into two groups. Each group of larvae was allowed to settle and metamorphose onto six-well polystyrene plates (N = 6 replicate plates per group). One group of newly metamorphosed juveniles was treated daily for 10 hours with the antibiotic gentamicin (75µg/mL; MP Biomedicals, LLC., Solon, OH) in sterile filtered seawater for four consecutive days to cure the

symbiont bacteria. Following the 10 hour antibiotic treatment on each day, the developing juveniles were placed in an indoor artificial environment supplied with unfiltered sea water from Bogue Sound, Morehead City, NC. The second group of larvae and juveniles were handled in a similar manner as the antibiotic-treated group, except they were not treated with the antibiotic and served as a control. After four days the antibiotic-treated (aposymbiotic) and control (symbiotic) group of juveniles were permanently placed in the indoor artificial environment and allowed to mature.

2.2 Nucleic acid extraction from *B. neritina* tissues

The mature symbiotic and aposymbiotic *B. neritina* colonies were collected in April 2010. Colonies from the different replicate plates for each group (antibiotic-treated and control) were processed separately. The colonies were randomly picked from the wells of the plate and the ovicell-bearing and ovicell-free zooids were dissected (Figure 1). The ovicell-bearing and ovicell-free zooids were homogenized separately and stored in RNA lysis buffer containing β-mercaptoethanol (Pure Yield RNA Midiprep System, Promega, Madison, WI). Some of the dissected ovicell- bearing and ovicell-free zooids from each plate were pooled together for DNA extraction (ZR Fungal/ Bacterial DNA MiniPrep, Zymo Research, Orange, CA) to determine the levels of bacterial symbiont, "*Ca.* Endobugula sertula," in the antibiotic-treated and control *B. neritina* colonies.

2.3 Symbiont quantification in antibiotic-treated colonies

The DNA extracted from *B. neritina* colonies from each replicate plate of antibiotic-treated and control groups were subjected to PCR and quantitative real-time PCR (Q-PCR) using

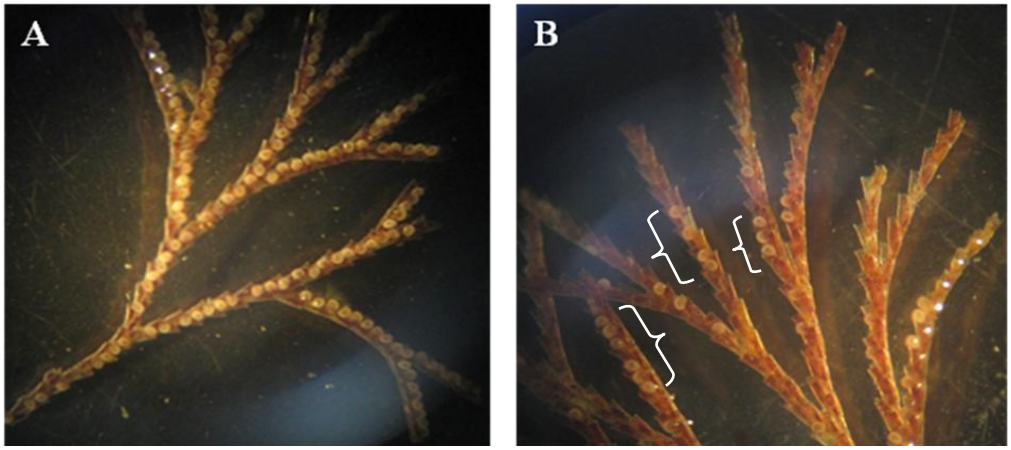

Figure 1: Adult *B. neritina* zooids. A: ovicell-bearing zooids. B: mostly ovicell-free zooids with a few ovicell-bearing zooids (white brackets).

symbiont-specific 16S rDNA primers (EBn16S_254F: 5'-TAC TCG TTA ACT GTG ACG TTA CTC-3' and EBn16S_643R: 5'-ACG CCA CTA AAT CCT CAA GGA AC-3') and *B. neritina* cytochrome c oxidase I (*COI*) primers (BnCOIF: 5'-TTG ATA CTG GGG GCT CCT GAT ATG-3' and BnCOIR: 5'-AAG CCC GAT GAT AAG GGA GGG TA-3'). Each Q-PCR reaction was performed in triplicate and each experiment was repeated three times. A hot start version of modified *Tbr* DNA polymerase alongwith SYBR Green I fluorescent dye, and ROX passive reference dye (DyNAmo HS SYBR Green qPCR kit, Finnzymes, Espoo, Finland) was used for the Q-PCR reactions. The threshold cycle (C_T) value was determined for each reaction for each of the six replicate plates of each group. The mean C_T value of each reaction was used to calculate the ratio of mean C_T value for symbiont 16S rDNA gene to the mean C_T value for host *COI* gene for each replicate. The aposymbiotic *B. neritina* colonies possessing a higher ratio of symbiont 16S rDNA to host *COI* genes (indicating a lower amount of 16S rDNA) were used for RNA extraction. Because of low total RNA yields of some plates, the RNA from each replicate of antibiotic-treated and control ovicell-bearing and ovicell-free zooids RNA were pooled. The poly(A) mRNA was purified from each of the combined RNA samples using MicroPoly(A)

Purist (Ambion, Austin, TX). The oligo(dT) cellulose was hybridized to poly(A) sequences found on the bryozoan mRNA and separated from ribosomal RNA and other RNAs by spin column chromatography. The purified poly(A) mRNA was eluted in the RNA storage solution provided in the kit.

2.4 Enrichment of differentially expressed genes

The purified mRNA from ovicell-bearing and ovicell-free zooids of antibiotic-treated and control *B. neritina* colonies was used for cDNA synthesis using SMARTer PCR cDNA synthesis kit (Clontech Laboratories, Inc., Mountain View, CA) according to the manufacturers protocol. cDNA was generated from mRNA using poly-dT primers and SMARTScribe Reverse Transcriptase. The cDNA template was amplified using Advantage 2 PCR kit (Clontech Laboratories, Inc., Mountain View, CA) and purified using CHROMA SPIN-1000+DEPC-H_2O columns (Clontech Laboratories, Inc., Mountain View, CA). The purified cDNA was digested with the restriction enzyme *RsaI* to generate shorter, blunt-ended cDNA fragments for adaptor ligation. Differentially expressed genes in different zooids were enriched using PCR-Select cDNA Subtraction kit (Clontech Laboratories, Inc., Mountain View, CA).

The cDNA subtraction was performed in forward and reverse directions for each type of zooid. The cDNA sample used as tester in forward subtraction was used as driver in the reverse subtraction, while the driver cDNA sample in forward subtraction was used as tester in reverse subtraction. For each type of zooid, the forward subtraction was performed to enrich genes that are differentially expressed in the symbiotic zooid, while the reverse subtraction was done to identify differentially expressed gene in the aposymbiotic zooid. Following five cDNA subtraction reactions were performed:

(1) Ovicell-bearing forward subtraction (OB-FS)

- Control ovicell-bearing cDNA (Tester) and antibiotic-treated ovicell-bearing cDNA (Driver)

(2) Ovicell-bearing reverse subtraction (OB-RS)

- Antibiotic-treated ovicell-bearing cDNA (Tester) and Control ovicell-bearing cDNA (Driver)

(3) Ovicell-free forward subtraction (OF-FS)

- Control ovicell-free cDNA (Tester) and antibiotic-treated ovicell-free cDNA (Driver)

(4) Ovicell-free reverse subtraction (OF-RS)

- Antibiotic-treated ovicell-free cDNA (Tester) and Control ovicell-free cDNA (Driver)

(5) Control subtraction

- Mixture of human placental control cDNA and *HaeIII*-digested λX174 DNA (Tester), and human placental control cDNA (Driver)

The tester sample in each of the subtraction reaction was split into two portions: One portion was ligated to Adaptor 1, while the second was ligated to Adaptor 2 oligonucleotide molecule. In a separate reaction tube, unsubtracted tester control was prepared by mixing equal portions of adaptor 1-ligated-tester cDNA with adaptor 2-ligated-tester cDNA. Following the ligation reactions, the first hybridization reaction was performed. Adaptor 1-ligated-tester cDNA and adaptor 2-ligated-tester cDNA were hybridized with excess of driver cDNA in separate tubes. The first hybridization was followed by a second hybridization in which both the samples

from the first hybridization were hybridized together in presence of fresh denatured driver cDNA. A primary PCR reaction using primers complementary to the adaptor sequences was performed on the subtracted cDNA and unsubtracted tester cDNA template to selectively amplify differentially expressed cDNAs. A secondary PCR using an aliquot of primary PCR product was performed with nested primers complementary to the adaptor sequences to further enrich the amount of subtracted cDNA in the sample.

2.5 Preparation of subtracted cDNA library

The amplified subtracted cDNAs from forward and reverse subtraction reactions (OB-FS, OB-RS, OF-FS, and OF-RS) were separately ligated into pGEM-T vector (Promega, Madison, WI) and transformed into electrocompetent 10-β *E. coli* (New England BioLabs, Ipswich, MA). The transformed cells containing subtracted cDNA inserts were screened on LB agar plates supplemented with carbenicillin (Cellgro, Mediatech, Inc., Manassas, VA), IPTG (Promega, Madison, WI) and X-gal (Promega, Madison, WI). In total, 1995 clones (475 clones each from OF-FS, OF-RS, and OB-RS and 570 clones from OB-FS reactions) were randomly selected and subjected to colony PCR using M13 forward and reverse primers to confirm the presence subtracted cDNA inserts. The selected clones were grown in 96-well plates containing LB broth and carbenicillin. Glycerol stocks of the selected clones were prepared and stored at -80°C.

2.6 Screening of differentially expressed genes

The subtracted cDNA library created for ovicell-bearing and ovicell-free forward and reverse subtractions were screened for differentially expressed genes using the PCR-Select Differential Screening kit (Clontech Laboratories, Inc., Mountain View, CA). The subtracted cDNA amplified by colony PCR was denatured and arrayed as dot blots on a nylon membrane

(GE Healthcare, Buckinghamshire, UK). The hybridization probes were biotinylated using the NEBlot Phototope kit (New England BioLabs, Ipswich, MA). The cDNA blots were hybridized with an excess of forward-subtracted cDNA, reverse-subtracted cDNA, unsubtracted tester (primary PCR product of unsubtracted tester control from the forward subtraction), and unsubtracted driver (primary PCR product of unsubtracted tester control from the reverse subtraction) probes in separate hybridization reactions. The biotinylated probes hybridized to the target gene sequences were detected by chemiluminescence using the Phototope-Star Detection kit (New England BioLabs, Ipswich, MA). The target genes (putatively differentially expressed genes) which displayed approximately twice the level of hybridization with the probes were sequenced (3100 Genetic Analyzer, Applied Biosystems). The sequences were compared to those in the GenBank database (National Center for Biotechnology Information) using nucleotide and protein BLAST programs to identify possible functions of the genes. The gene sequences were also investigated for known conserved domains using the conserved domain search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The sequence similarity was considered significant for e-values less than 1.0×10^{-5}. PCR primers for the gene of interest were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3) for the confirmation of differential expression by reverse transcription PCR analysis using initial total RNA from ovicell-bearing and ovicell-free zooids as the template.

2.7 Confirmation of differential expressed genes

Differences in transcript levels of the identified genes were confirmed by reverse transcription PCR analysis. Single stranded cDNA was synthesized from equal amounts of total RNA from the different types of zooids using reverse transcriptase (SuperScript III, Invitrogen,

Carlsbad, CA) and random hexamer primers. The cDNA generated from each type of zooids was quantified and three dilutions of cDNA were prepared (1X, 0.1X, and 0.01X). Equal quantities of cDNA from each dilution were used as template for PCR analysis using primer sets synthesized (Integrated DNA Technologies, Coralville, IA) for each of the putative differentially expressed genes. Genomic DNA from symbiotic *B. neritina* was used as template for positive control PCR reactions. The PCR products obtained from the cDNA of different types of zooids for each of the putative differentially expressed gene fragment were electrophoresed on 1% agarose gel. The expression level of the target gene in different zooids was analyzed by visual comparison and agarose gel densitometric analysis (FluorChem 8800 Imaging System, Alpha Innotech Corporation) of PCR product obtained for each dilution of the cDNA template.

3 RESULTS

3.1 Symbiont quantification in antibiotic-treated colonies

Total DNA extracted from *B. neritina* colonies from each replicate plate of antibiotic-treated and control group was subjected to PCR (Figure 2) and Q-PCR using symbiont-specific 16S rDNA and *B. neritina COI* gene primers. The amount of symbiont DNA normalized to host DNA was calculated using C_T values of 16S rDNA and *COI* in each replicate as a proxy. The aposymbiotic *B. neritina* colonies demonstrating higher ratio of symbiont 16S rDNA to host *COI* C_T values (indicating less symbiont DNA per unit of host DNA) were used for RNA extraction. The comparison of mean C_T values obtained for symbiont-specific 16S rDNA primed reactions among different antibiotic-treated *B. neritina* colonies indicate greater than 64-fold less symbiont DNA in colonies from plates 1, 5, and 6 (C_T value difference of more than 6) (Table 1) and

therefore RNA was extracted from colonies in these plates for SSH analysis. The ratio of symbiont 16S rDNA to host *COI* C_T values in the symbiotic *B. neritina* colonies in all the control plates was equal (~1.06). RNA was extracted from control *B. neritina* colonies on plates 3, 4, and 5. The differences in the ratios for 3 different Q-PCR experiments could be due to the use of different machines for the first experiment and experiments 2 and 3. However, the pattern of ratios in all the experiments was similar. The ratios obtained for all the plates containing symbiont colonies were similar, while the ratios for colonies in plate T1, T5, and T6 were higher than those obtained for colonies in plate T2, T3, and T4. Dissociation curves generated for each Q-PCR reaction were used to determine if there was formation of non-specific products, which could result in a false C_T value. The melting temperatures of *COI* and 16S rDNA Q-PCR product were 77.5°C and 84°C respectively. One of the replicates of T5_16S reaction in experiment# 2 had lower C_T value due to formation of a non-specific product at 73°C, while the C_T values for other replicates were undetermined (Table 1).

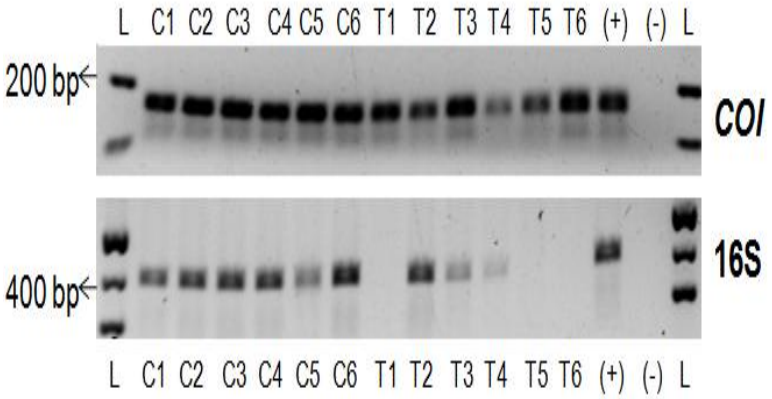

Figure 2: Confirmation of symbionts in *B. neritina* colonies. PCR using *COI* and 16S rDNA primers on *B. neritina* colonies from the control (C) and antibiotic-treated (T) plate replicates. Symbiont-specific 16S rDNA was not amplified in aposymbiotic colonies from plates T1, T5, and T6.

Table 1: Symbiont quantification in *B. neritina* colonies. Quantitative real time PCR on symbiotic and aposymbiotic *B. neritina* DNA samples using symbiont-specific 16S rDNA primers and *B. neritina* cytochrome c oxidase (*COI*) primers. C= Control (symbiotic), T= Antibiotic-treated (aposymbiotic), and C_T= Threshold cycle.

Sample	Experiment # 1 $\frac{C_T(16S)}{C_T(COI)}$	Experiment # 2 $\frac{C_T(16S)}{C_T(COI)}$	Experiment # 3 $\frac{C_T(16S)}{C_T(COI)}$	Mean $\frac{C_T(16S)}{C_T(COI)}$	Standard Deviation
C1_16S	0.98	1.16	1.08	1.07	0.09
C1_*COI*					
C2_16S	0.97	1.07	1.07	1.04	0.06
C2_*COI*					
C3_16S	0.94	1.12	1.07	1.05	0.09
C3_*COI*					
C4_16S	1.00	1.13	1.07	1.06	0.06
C4_*COI*					
C5_16S	0.98	1.13	1.08	1.06	0.08
C5_*COI*					
C6_16S	0.94	1.13	1.08	1.05	0.10
C6_*COI*					
T1_16S	1.67	1.84	1.64	1.72	0.11
T1_*COI*					
T2_16S	1.00	1.16	1.13	1.10	0.09
T2_*COI*					
T3_16S	1.14	1.33	1.27	1.25	0.10
T3_*COI*					
T4_16S	1.18	1.37	1.28	1.28	0.10
T4_*COI*					
T5_16S	1.73	NA*	1.50	1.62	0.16
T5_*COI*					
T6_16S	1.79	1.87	1.77	1.81	0.05
T6_*COI*					

*: C_T (16S) value for one replicate was 21.03 due to non-specific product formation, while C_T values for other two replicates were undetermined.

3.2 Enrichment of differentially expressed genes

Differentially expressed genes in ovicell-bearing and ovicell-free zooids were enriched using the PCR-Select cDNA Subtraction kit (Clontech Laboratories, Inc., Mountain View, CA). Following the primary and secondary PCR reactions, the control subtracted cDNA in the SSH experiment was expected to be the *HaeIII*-digested λX174 DNA, which was added just to the

control tester sample (Figure 3). In contrast, the control unsubtracted tester cDNA should have both the *HaeIII*-digested λX174 DNA and human placental control cDNA. Similarly, in the experimental SSH experiments (OB-FS, OB-RS, OF-FS, and OF-RS), the subtracted cDNA should contain the cDNAs which are only present in the tester sample but not in the driver. The subtracted cDNAs showed fewer distinct bands or just a smear (potential differentially expressed cDNAs) than the corresponding unsubtracted tester cDNAs. Although the results from the controls suggested that the reaction was not optimal, the presence of fewer cDNA bands or just a smear in the subtracted samples indicates successful subtraction of cDNAs. Since the subtraction of cDNA by SSH was suboptimal, the presence of differentially expressed genes in the subtracted cDNAs was confirmed by further screening procedures.

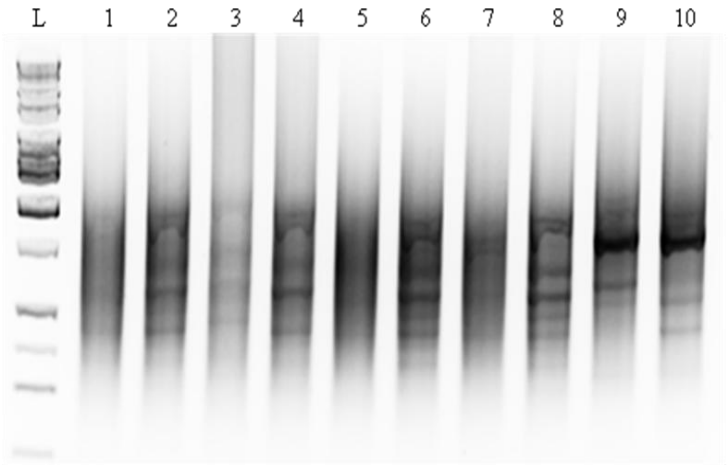

Figure 3: Suppression subtractive hybridization. Lane L= HiLo DNA ladder, Lane 1= Ovicell-bearing (OB)-Forward subtracted cDNA, Lane 2= OB-Forward unsubtracted tester cDNA, Lane 3= OB-Reverse subtracted cDNA, Lane 4= OB-Reverse unsubtracted tester cDNA, Lane 5= Ovicell-free (OF)-Forward subtracted cDNA, Lane 6= OF-Forward unsubtracted tester cDNA, Lane 7= OF-Reverse subtracted cDNA, Lane 8= OF-Reverse unsubtracted tester cDNA, Lane 9= Control subtracted cDNA, and Lane 10= Control unsubtracted tester cDNA. The subtracted reactions in the experimental subtractions show fewer distinct cDNA bands or just a smear than the corresponding unsubtracted reactions. However, the subtracted reaction in the control subtraction do demonstrate distinct *HaeIII*-digested λX174 DNA band pattern. This indicates that the subtraction of cDNA for all the subtraction reactions was suboptimal.

3.3 Screening of differentially expressed genes

The subtracted cDNA library was screened for differentially expressed genes using PCR-Select Differential Screening kit (Clontech Laboratories, Inc., Mountain View, CA). The screening procedure resulted in identification of sixty putatively subtracted genes, which displayed more than twice the level of hybridization intensity (Figure 4). Bioinformatic analysis of these subtracted cDNA sequences revealed significant similarity to known proteins and conserved domains of interest in 11 subtracted cDNAs. The identified proteins had key functions and roles such as initiation and maintenance of symbiosis, bacterial pathogenesis and localization within the host, and signal transduction. Primer sets for these eleven genes of interest were designed (Table 2) to confirm differential expression of the subtracted genes by reverse transcription PCR analysis.

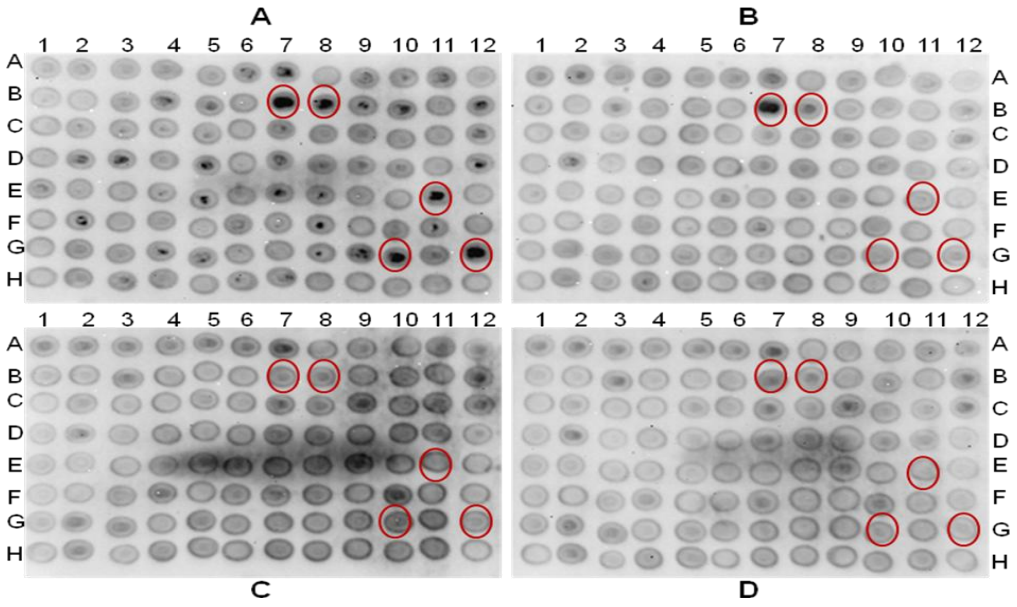

Figure 4: Screening of differentially expressed genes. Hybridization of ovicell-bearing reverse subtracted clones with (A) reverse subtracted cDNA, (B) unsubtracted driver cDNA, (C) forward subtracted cDNA, and (D) unsubtracted tester cDNA probes. Clones within the red circle indicate subtracted clones because they only hybridized to reverse subtracted cDNA and unsubtracted driver cDNA probes.

Table 2: Primers used for reverse transcription PCR analysis.

Primer	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GH9	CTATGGCCTCCTCAGCTACG	CTGGTCTTCCCCACCAACTA
ACT1	ATCAGGGTGTCATGGTTGGT	AGGGTTAAGGGGAGCTTCTG
ACT2	ATCCTTACCGAGAGGGGCTA	GAAGAGTGCTTCTGGGCATC
RGDI	AATGGAGCTTCGTTCTGCAT	AAATACTTCGCATGGCAACC
DYN	GTGTCGTCCGTGACATTGAT	TTCCACTGTGGTAGCTGCTG
PTP	GGCTAGCTCGAGGACTTAATGA	TGATATGGCAAGAACATCCAAG
PH	TCTTGCCCCAAATCTTCAAC	CCTGCATTATGCGAAAGTCA
GH20	TGTGCAAAATTCTGGTTTCG	TCTTCATCCATCCCAAAAGC
TDO	GTACATGGGGAGAGTGTGTCAA	AATCAACAGCATCAGTGGTTTG
VDAC	ATGGCAAAGTCCTTGGAGTG	GCTGTGAGTTTCAGCCCTTC
PRT	GGCATTTGCAATTTGCTTTC	GCGGTAACGATCAACAGGAT

3.4 Confirmation of differential expressed genes

Reverse transcription PCR was conducted to verify differences in expression of the nine putative subtracted genes in different *B. neritina* zooids. Single stranded cDNA was synthesized from an equal amount of initial RNA sample from different zooids (32 ng). Three dilutions of synthesized cDNA were prepared (200 pg/µL, 20 pg/µL, and 2 pg/µL) and 2 µL of each dilution was used as template for PCR amplification using primers designed for each of the screened putative differentially expressed gene fragments. Differential expression in ovicell-bearing and ovicell-free zooids was confirmed for 6 of 11 of the genes identified by SSH (Table 3 and Table 4).was used as template for PCR amplification using primers designed for each of the screened putative differentially expressed gene fragments. Differential expression in ovicell-bearing and ovicell-free zooids was confirmed for 6 of 11 of the genes identified by SSH (Table 3 and Table 4). Two out of 6 differentially expressed transcripts encoded actin protein. Alignment of cDNA sequences for both the actin gene fragments using Lasergene 6 SeqMan program showed no

Table 3: Conserved domains present in the identified differentially expressed gene transcripts.

Clone	Sequence length (bp)	Closely related domain	E-value	Possible functions in the host
OBRS#4_H5	765	Glycosyl hydrolase family 9	1×10^{-54}	Cellulase prevents attachment and flocculation of symbionts within the funicular cords
OBFS#2_E12	645	Glycosyl hydrolase family 20	2.23×10^{-9}	β-hexosaminidase and β-1,6-N-acetylglucosaminidase degrade biofilm matrix
OBRS#1_B7	602	Actin (1)	1.24×10^{-59}	Distribution and localization of symbionts to different zooids through funicular cords
OFRS#4_C7	425	Actin (2)	1.73×10^{-48}	Distribution and localization of symbionts to different zooids through funicular cords
OBFS#1_B8	552	Dynein	2.07×10^{-13}	Distribution of symbionts using host microtubule network
OBFS#2_H3	572	Rho GDP dissociation inhibitor	2.5×10^{-25}	Regulates host actin reorganization for distribution of symbionts

Table 4: Proteins encoded by the differentially expressed genes as identified by BLASTx alignment.

Clone	Sequence length (bp)	Closely related protein	E-value	Query coverage (%)	Maximum identity (%)
OBRS#4_H5	765	β-1, 4-endoglucanase	7×10^{-61}	84	52
OBFS#2_E12	645	β-hexosaminidase	8×10^{-12}	66	33
OBRS#1_B7	602	Cytoskeletal β-actin	1×10^{-87}	88	91
OFRS#4_C7	425	Cytoplasmic actin	8×10^{-73}	99	97
OBFS#1_B8	552	Dynein, light chain roadblock-type 2	2×10^{-33}	51	75
OBFS#2_H3	572	Rho GDP dissociation inhibitor	2×10^{-23}	43	56

similarity and were completely different from each other. It is possible that both the fragments are two non-overlapping distant portions of a single gene or different genes encoding actin. The PCR products obtained for each of the six differentially expressed genes was electrophoresed on 1% agarose gel and visualized (Figure 5). The percent density of PCR products in agarose gel was measured by agarose gel densitometric analysis (Figure 6).

The positive control PCR reactions with symbiotic *B. neritina* genomic DNA as template, amplified bigger gene products in glycosyl hydrolase family 9 (GHF9), glycosyl hydrolase family 20 (GHF20), and dynein primed reactions as compared to the products amplified for experimental cDNA samples (Lane 13, Figure 5). This could be due to presence of introns in the DNA copy of these genes, which is later spliced from transcribed mRNA. The gene encoding GHF9 proteins showed more product formation in both the symbiotic zooids and aposymbiotic ovicell-free cDNA samples (Figure 5A and 6A). Similar trend was observed for GHF20 transcript in the reactions with template cDNA concentrations of 400 pg and 4 pg (Figure 5B and 6B). The gene expression for one of the two actin transcripts, actin (1), varied in different zooids at different concentrations of cDNA template (Figure 5C and 6C). However, the expression of actin (2) appeared to be similar in all types of zooids (Figure 5D), but was highest in symbiotic ovicell-free zooids at template concentrations of 400 pg and 40 pg (Figure 6D). Differences in dynein encoding transcript were also noticed in reactions with 40 pg cDNA template (Figure 5E). Dynein expression was highest in symbiotic ovicell-bearing zooids (Figure 6E). Rho GDP dissociation inhibitor (GDI) expression was found to be varying in different types of zooids, but it was not consistent for every cDNA template dilution (Figure 5F and 6F). An extremely faint band of Rho GDI gene fragment was also detected for 4 pg symbiotic OB zooid cDNA template.

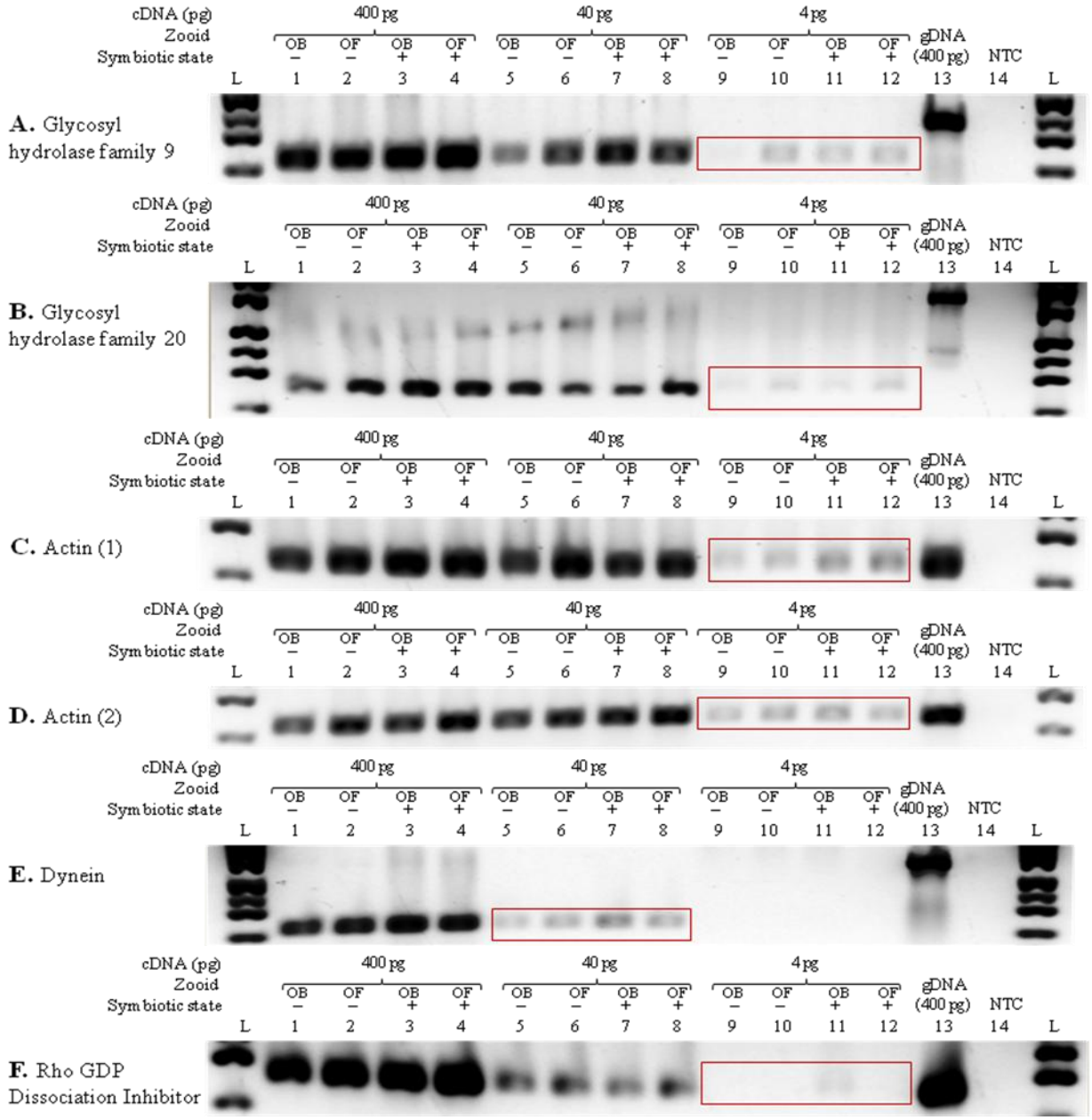

Figure 5: Reverse transcription PCR analysis. Lane L= HiLo DNA ladder, Lane 1= aposymbiotic (aposymb.) ovicell-bearing (OB) cDNA (400 pg), Lane 2= aposymb. ovicell-free (OF) cDNA (400 pg), Lane 3= symbiotic (symb.) OB cDNA (400 pg), Lane 4= symb. OF cDNA (400 pg), Lane 5= aposymb. OB cDNA (40 pg), Lane 6= aposymb. OF cDNA (40 pg), Lane 7= symb. OB cDNA (40 pg), Lane 8= symb. OF cDNA (40 pg), Lane 9= aposymb. OB cDNA (4 pg), Lane 10= aposymb. OF cDNA (4 pg), Lane 11= symb. OB cDNA (4 pg), Lane 12= symb. OF cDNA (4 pg), Lane 13= symb. *B. neritina* genomic DNA (400 pg), and Lane 14= negative PCR control. The red boxes indicate the reactions in which the difference in the gene transcript was observed.

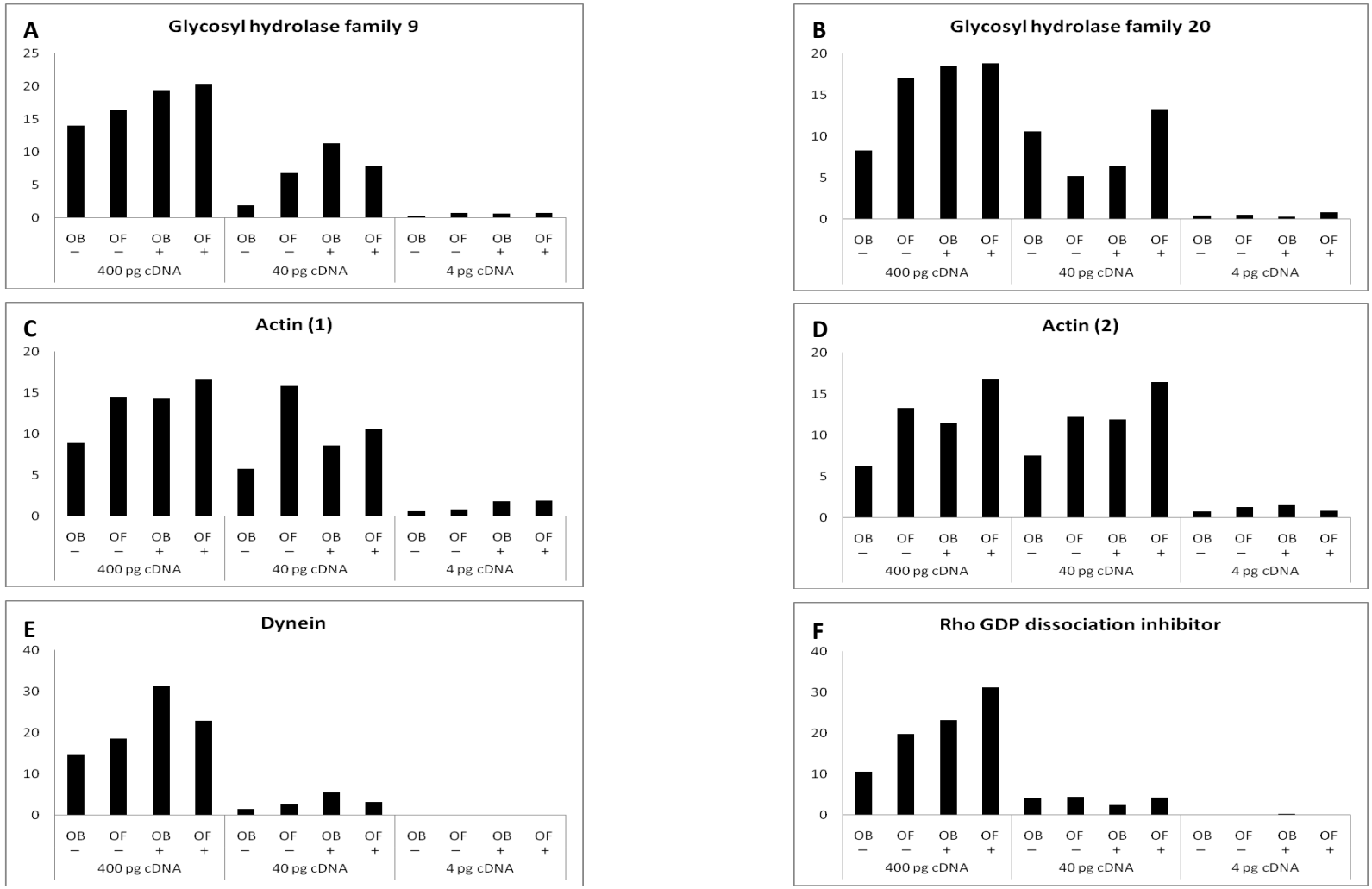

Figure 6: Agarose gel densitometric analysis of reverse transcription PCR products. PCR products obtained from symbiotic (+) and aposymbiotic (-) ovicell-bearing (OB) and ovicell-free (OF) zooid cDNAs. Solid bars represent percent density of PCR products in 1% agarose gel.

4 DISCUSSION

The defensive symbiosis between *B. neritina* and "*Ca.* Endobugula sertula" is thought to result in production of bryostatins, which provide protection to bryozoan larvae from predation. Since the bryostatins are complex polyketide compounds and similar to bacterial secondary metabolites (Pettit, 1991), they are thought to be synthesized by the endosymbiotic bacterium. This hypothesis has been supported by studies which have reported decreased bryostatin levels in antibiotic-treated *B. neritina* colonies (Davidson *et al.*, 2001; Lopanik *et al.*, 2004). Since attempts to pure culture the bacterial symbionts has not been successful, the true source of bryostatins is unknown. Furthermore, complete genetic information about both partners in this symbiotic association is lacking. Therefore, SSH, which does not require a *priori* knowledge of genes, was used to identify differentially expressed genes.

The goal of this study was to identify host genes that are differentially expressed during association with the endosymbiont bacterium. Aposymbiotic juveniles lacking the bacteria were prepared by treating larvae with gentamicin during the course of larval metamorphosis into juveniles to cure them of the symbiont. Both control and antibiotic-treated *B. neritina* colonies appeared to be healthy and were similar size. However, the aposymbiotic adult colonies appeared to have less ovicell-bearing zooids as compared to the control adult colonies. This suggests that the symbiont may influence the onset of embryogenesis in the host. Recent studies of the symbiotic association between the phytopathogenic fungi, *Rhizopus microsporus* and the endobacterium *Burkholderia rhizoxinica* have demonstrated the reliance of the host on its symbiont for sporulation (Lackner *et al.*, 2010). Quantification of symbiont DNA in DNA

extracted from antibiotic-treated colonies confirmed removal of symbionts in colonies from plates T1, T5, and T6.

The enrichment of differentially expressed genes was successful, although not optimal, in experimental SSH reactions. This was indicated by presence of fewer distinct bands or just a smear in subtracted cDNAs as compared to unsubtracted cDNAs. However, the *HaeIII*-digested λX174 DNA was not successfully subtracted in the control SSH reaction, which implies possibility of suboptimal subtraction of cDNAs in all of the SSH reactions. To ensure selection of differentially expressed clones, screening of subtracted cDNA was performed by hybridization of subtracted cDNA dot blots with specific biotinylated probes. Sixty clones were selected by screening and their gene sequence was compared to other sequences in GenBank for potential functions and conserved domains. Bioinformatic analysis revealed 17 subtracted gene sequences with significant homology to known proteins and conserved domains, and 11 transcripts were identified with important roles in interaction between host and symbiont. Reverse transcription PCR using primers for the 11 transcripts suggested differential gene expression in 6 transcripts (Table 3).

The six differentially expressed transcripts identified in this study have homology to conserved domains of proteins involved in the regulation of interactions between symbiotic organisms, as well as between hosts and pathogens. The results obtained suggest that the identified host proteins function in distribution and localization of the symbiont bacteria within the host funicular cords. The contig, OBRS#4_H5, encodes a GHF9 protein. Members of this protein family are capable of degrading cellulose (Henrisat, 1991) (also: http://www.cazy.org/GH9.html). Some gram-negative bacteria have been shown to form

cellulose fibrils, which allow flocculated growth and formation of bacterial aggregates (Deinema and Zevenhuizen, 1971). Cellulose fibrils have also been reported to play an important role in attachment of the plant pathogen, *Agrobacterium tumefaciens*, to plant tissue (Matthysse *et al.*, 1981; Matthysse, 1983). Similarly, *Rhizobium leguminosarum* utilizes cellulose fibrils and Ca^{2+}-dependent adhesin(s) to attach itself to the root hair tip of pea plants (Smit *et al.*, 1987). In addition to a GHF9 protein, a protein belonging to the GHF20 family was encoded by contig OBFS#2_E12. GHF20 includes β-hexosaminidase and β-1,6-N-acetylglucosaminidase (http://www.cazy.org/GH20.html). Recently, a soluble β-N-acetylglucosaminidase, Dispersin B, produced by *Actinobacillus actinomycetemcomitans* was shown to disrupt and detach biofilms formed by several Gram-negative and Gram-positive species of bacteria (Kaplan *et al.*, 2003; Kaplan *et al.*, 2004a; Kaplan *et al.*, 2004b; Itoh *et al.*, 2005). The levels of both GHF9 and GHF20 protein encoding genes were found to be higher in symbiotic *B. neritina* zooids. In adult *B. neritina* colonies, the symbionts are present in the funicular cords (Woollacott and Zimmer, 1975; Woollacott and Zimmer, 1977). The funicular cords serve as a vascular system for the transport of nutrients and wastes within the colony (Woollacott and Zimmer, 1975; Carle and Ruppert, 1983). Sharp and coworkers (2007) demonstrated presence of symbionts in the funicular cords by FISH using symbiont-specific probes and hypothesized the possibility of symbiont transmission from one zooid to other within the colony via funicular cords. By upregulating production of proteins capable of degrading cellulose and disintegrating biofilm matrix, the host may prevent attachment of symbionts that could form large bacterial aggregates or inhibit the formation of bacterial biofilms within the funicular cords, which could otherwise block the passage of nutrients through the cords resulting in death of host. The pathogenicity of

Xylella fastidiosa has been discovered to be due to formation of biofilms in the xylem vessels, which causes vascular occlusion leading to water stress in plants (de Souza *et al.,* 2005). The accumulation of symbionts in a biofilm in the colony could also result in triggering virulence factor production by quorum sensing, which could be detrimental to the host.

The contigs OBRS#1_B7 and OFRS#4_C7 encode for actin proteins. These actin transcripts appeared to be greater in symbiotic zooids of *B. neritina* as compared to aposymbiotic zooids. A symbiont-induced change in host actin synthesis has been found to facilitate winnowing in the establishment of *Euprymna scolopes-Vibrio fischeri* symbiosis (Kimbell and McFall-Ngai, 2004). The passage of *V. fischeri* through the ducts of the host light organs results in upregulation of actin synthesis. Increase in actin production in the ducts results in constriction and limits entry of other microorganisms. Modification and reorganization of the host actin cytoskeleton for the benefit of bacterial pathogenesis and transmission has been reviewed by Finlay and Cossart (1997) and Barbieri *et al.* (2002). Similarly, the difference in level of actin transcripts in symbiotic *B. neritina* zooids could be symbiont-induced as in the case of squid-*Vibrio* symbiosis or completely independent of the symbiont. Rearrangement of the actin cytoskeleton in the funicular cords could regulate the distribution or movement of symbionts to different zooids within the colony. The differential expression of actin protein is further supported by differences in expression of GDI protein. Overexpression of human GDI proteins has been reported to result in damage of the actin cytoskeleton (Leffers *et al.,* 1993). Disruption of actin cytoskeleton by bacterial toxins has been found to be regulated by GDI protein (Barbieri *et al.,* 2002).

Increased expression of dynein (OBFS#1_B8) in symbiotic zooids also supports the assumption that *B. neritina* influences distribution and localization of "*Ca.* Endobugula sertula." Ferree and coworkers (2005) explored interaction between host microtubule cytoskeleton and *Wolbachia*. The bacterium was found to associate itself to a host microtubule network via dynein so that it was localized in the oocytes of *Drosophila*. Such interaction is believed to facilitate bacterial motility and maternal transmission to offspring.

The interaction between *B. neritina* and "*Ca.* Endobugula sertula" seems to have co-evolved through strict vertical transmission to sustain the relationship. The phylogenetic analyses of the host (*COI*) and symbiont (16S rRNA) sequences using neighbor-joining method suggest that this association is ancient and demonstrated parallel diversification of both the host and symbiont (McGovern and Hellberg, 2003). In such a specific and co-evolved symbiotic association, it is expected that the host's immune response evolves to allow infection of only the symbiont microorganism, while the symbiont may evolve to combine mutualistic and pathogenic properties to benefit and invade the host respectively (Moran, 2006). Therefore, changes in expression of *B. neritina* immune genes were also expected in the symbiotic and aposymbiotic state of the organism. The SSH study identified genes potentially involved in regulation of distribution and localization of the symbiont by the host, but did not reveal any host genes of known immune function those might be differentially expressed during the association with the symbiont. Similar SSH study on bacterial-challenged *Acyrthosiphon pisum* detected very few insect genes involved in immune response (Altincicek *et al.*, 2008). Furthermore, the genome of *A. pisum* has been reported to be missing immune genes, suggesting that aphids have a reduced immune repertoire (Gerardo *et al.*, 2010). One of the hypotheses proposed for the lack of

immune defense in aphids was symbiont-mediated host protection, in which the host relies on the symbiont for its defense against pathogenic microorganisms. *A. pisum* has been found to be defended by its secondary symbionts, *Regiella insecticola* and *Hamiltonella defensa*, against fungal pathogens and parasitoid wasp *Aphidius ervi* respectively (Scarborough *et al.*, 2005; Oliver *et al.*, 2005). Similarly, *B. neritina* may have evolved to maintain the symbiosis with "*Ca.* Endobugula sertula" by reducing or altering its immune responses, while the symbiont in turn may have coevolved to safeguard the host against pathogens. However, discovery of genes encoding anti-attachment and anti-biofilm forming proteins by SSH suggest that the host does possess some defensive strategies to protect itself from being harmed by the symbiont.

As discussed above, the interaction between *B. neritina* and "*Ca.* Endobugula sertula" seems to have co-evolved and co-diversified in the deep and shallow/Southern sibling species of bryozoan. However, it is interesting that no such association is found in the Northern Atlantic species of bryozoan. The neighbor-joining phylogenetic studies suggest that both the symbiotic forms of *B. neritina* are monophyletic and that the association with the symbiont may have started after the divergence of the Southern and Northern Atlantic forms (McGovern and Hellberg, 2003). A general higher amount of predation in southern habitats is also thought to act as a major selective pressure on the southern bryozoan populations to establish and maintain association with the symbiont, which benefits the host larvae with chemical defense against predation (McGovern and Hellberg, 2003). This suggests a role of predators in the evolution of this association.

The association between bryozoan and "*Ca.* Endobugula sertula" is similar to the aphid-*Buchnera* symbiosis as in both the symbionts are vertically transmitted to the offspring and the

partners have co-evolved, as well as demonstrate parallel diversification to establish and maintain the association. However, the aphid-*Buchnera* relationship appears to have co-evolved more because both the partners have evolved to be completely dependent on each other (Brinza *et al.*, 2009). The insect host has evolved to provide nutrition and accommodation to the symbionts in specialized cells (bacteriocytes) within the host (Sabeter Muñoz *et al.*, 2001), while the symbiont has evolved to synthesize essential amino acids for the host (Febvay *et al.*, 1999). Since the bacterial symbiont of *B. neritina* was not found free-living in the environment (Haygood *et al.*, 1999), nor has it been cultured in laboratory, the symbiont may have evolved to form an obligate association. However, the relationship may not be obligate for bryozoan host at least in case of Northern Atlantic forms, which lack the symbionts. In the case of horizontally transmitted symbiotic associations such as that of squid-*Vibrio*, the interaction among the partners has evolved together for a successful symbiosis. However, both the lineages do not show strong evidence of co-diversification (Moran, 2006). Since the juvenile squid acquires the symbionts from the surrounding water, the host utilizes a variety of developmental, biochemical, and immunogenic mechanisms to prevent colonization of the light organ by other environmental microorganisms except *V. fischeri*, which has evolved to utilize its pathogenic mechanisms to avoid these host strategies to successfully establish the association (Moran, 2006). These evolutionary trends among the partners in symbiotic associations suggest that such relationships potentially play a critical role in determining the evolutionary and ecological processes of symbiotic organisms.

5 CONCLUSION

The goal of this study was to identify host genes that are differentially expressed among symbiotic and aposymbiotic *B. neritina* ovicell-bearing and ovicell-free zooids which may be involved in the establishment and maintenance of the association between *B. neritina* and "*Ca.* Endobugula sertula." Using SSH, host genes were identified that may regulate the distribution and localization of the endosymbiont in the host. The results of this study suggest that *B. neritina* regulates distribution and localization of the symbiont within its funicular cords by actin cytoskeleton rearrangement and upregulates production of proteins to prevent attachment or biofilm formation by symbionts within the funicular cords. Using these mechanisms, the host is potentially capable of transporting more symbionts to ovicell-bearing zooids, where higher levels of bryostatin production are needed to impart the developing larvae with its chemical defense before it is released. This study extends our understanding about interaction between the host and the symbiont in the bryozoan-bacteria symbiosis and also serves as a model for the study of other symbiotic relationships.

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