Short Communication

MARINE BIOTECHNOLOGY

Differential Gene Expression in a Marine Sponge in Relation to Its Symbiotic State

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Abstract

The molecular mechanisms involved in the establishment and maintenance of sponge photosymbiosis, and in particular the association with cyanobacteria, are unknown. In the present study we analyzed gene expression in a common Mediterranean sponge (*Petrosia ficiformis*) in relation to its symbiotic (with cyanobacteria) or aposymbiotic status. A screening approach was applied to identify genes expressed differentially in symbiotic specimens growing in the light and aposymbiotic specimens growing in a dark cave at a short distance from the illuminated specimens. Out of the various differentially expressed sequences, we isolated two novel genes (here named *PfSym1* and *PfSym2)* that were up-regulated when cyanobacterial symbionts were harbored inside the sponge cells. The sequence of one of these genes (*PfSym2*) was found to contain a conserved domain: the scavenger receptor cysteine rich (SRCR) domain. This is the first report on the expression of sponge genes in relation to symbiosis and, according to the presence of an SRCR domain, we suggest possible functions for one of the genes found in the sponge-cyanobacteria symbiosis.

Keywords: cyanobacteria — differential gene expression — sponge — suppression subtractive hybridization — symbiosis

Introduction

The initiation, establishment, and maintenance of a mutualistic symbiosis are most likely based on a sophisticated molecular signaling between the partners. A small number of studies have investigated the molecular cross-talk between some marine invertebrates and their prokaryotic symbionts. The best-studied model among invertebrates is the marine squid–*Vibrio* extracellular symbiosis (Visick and McFall-Ngai 2000; Davidson et al. 2004; Kimbell and McFall-Ngai 2004). Other studies characterized genes that were expressed in sea anemones in relation to their symbiotic state (harboring or not harboring eukaryotic microalgae called zooxanthellae; Reynolds et al. 2000; Schwarz and Weis 2003; Rodriguez-Lanetty et al. 2006). Recently, Grant and co-workers demonstrated the presence of two sponge host-factors involved in the carbon metabolism of the symbiotic red alga in the *Haliclona cymiformis–Ceratodictyon spongiosum* association (Grant et al. 2006), but the chemical identity of these compounds remains unknown. To date, nothing is known about the genetic regulation of the symbiosis between sponges and prokaryotic organisms, probably both because of the high complexity of microbial consortia in sponges and because of the difficulties encountered in growing both marine sponges and their bacterial symbionts under laboratory conditions.

Sponge–bacteria interactions have been suggested to be the oldest host–bacteria interactions, dating back more than 500 million years (Wilkinson 1983). Several studies have revealed that permanent associations exist between certain host sponges and specific microorganisms (Preston et al. 1996; Althoff et al. 1998; Hentschel et al. 2002, 2006; Steindler et al. 2005). Further, sponges may also succumb to microbial and fungal infections, resulting in sponge death (Lauckner 1980; Vacelet et al. 1994; Perović-Ottstadt et al. 2004; Olson et al. 2006; Wiens et al. 2007). Therefore a balance is needed in which the sponge establishes healthy and stable associations with microorganisms while maintaining its integrity against pathogenic microorganisms. The cellular location of the symbionts with regard to their host is likely to influence the type of reciprocal signaling between the partners, and thus the intimacy of their

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relationship. Sponge cyanobacteria may reside either extra- or intracellularly and, in the latter case, either inside symbiosomes (specialized compartments) or free in the cytosol (Rützler 1990; Wilkinson 1992).

The marine demosponge *Petrosia ficiformis* (Poiret 1789) is a Mediterranean sponge species that lives in symbiosis with cyanobacteria. Its cyanobacteria were first described as *Aphanocapsa feldmanni* (Feldmann 1933) and later as *Synechococcus feldmanni* (Usher et al. 2004). The facultative nature of the association of *P. ficiformis* with the cyanobacteria makes it an ideal species for the study of symbiosis-specific gene expression in sponges. Specimens of symbiotic and aposymbiotic *P. ficiformis* have two slightly different morphotypes, the first being more massive and the second more slender (Vacelet and Donadey 1977; Sarà et al. 1998). Also, based on a biochemical analysis of parameters linked to cell reducing power (such as glucose-6-phosphate dehydrogenase), it was proposed that in *P. ficiformis* the cyanobacteria effectively participate in controlling the redox potential of the host cells (Arillo et al. 1993). In this study of the marine sponge *P. ficiformis* we used a differential screening approach to recognize sponge genes that are being expressed only during symbiosis in order to unravel molecular communication between the sponge and its endosymbiotic cyanobacteria.

Materials and Methods

Sponge specimens were collected by SCUBA diving inside (n=4) and outside (n=4) a submarine cave located along the rocky cliffs of Paraggi (Portofino Promontory, Ligurian Sea, Mediterranean), and were immediately transferred into ice-cold absolute ethanol. The color of the sponge surface (purple-red or white) depends on the presence or absence of these autotrophic symbionts, whose abundance is controlled by irradiance (Sarà and Vacelet 1973; Regoli et al. 2000). Sponges occurring in sun-exposed locations harbor the cyanobacteria immediately below the sponge ectosome in a 1- to 2-mm thick layer known as the symbiocortex, whereas sponges found in dark caves or rock crevices are aposymbiotic, i.e., lack symbiotic cyanobacteria. RNA extracted from both types of specimens was used for creating a suppression subtractive hybridization (SSH, PCR-Select cDNA Subtraction kit, Clontech, Mountain View, CA). The latter is a powerful method to identify unknown genes that are uniquely expressed in one experimental sample, but not in another. To enrich eukaryotic sponge mRNA, while excluding most bacterial mRNA, we isolated poly(A) mRNA; prokaryotic organisms lack a relatively stable poly(A) tail. The final polymerase chain reaction (PCR) products of both symbiotic and aposymbiotic libraries, produced with the SSH procedure, were separated on a 6% TBE (TBE 10×: 0.9 M Tris base, 0.9 M Boric acid, 20 mM EDTA) acrylamide gel. These PCR products were cloned into the pGEMT vector (Promega, Madison, WI). Following amplification of 480 isolated clones by M13 forward and reverse primers, dot blot analysis was performed on these PCR products to test their differential expression with regard to the symbiotic state of the sponge. All samples were dotted in replicates, for hybridization with ^{32}P-labeled cDNA from symbiotic and aposymbiotic *P. ficiformis* specimens. Clones found to be evidently up-regulated in the symbiotic library were further examined by Northern analysis using RNA that was isolated from specimens of *P. ficiformis* that were either symbiotic (n=3) or aposymbiotic (n=3, the RNAs of each type were pooled) and hybridized with ^{32}P-labeled DNA representing the different clones. Two clones (termed *PfSym1* and *PfSym2*) that had a major differential expression between the two symbiotic states were characterized further. To compare the expression of these two genes in two genetically identical specimens that either harbored or did not harbor the symbiotic cyanobacteria, a symbiotic sponge was cut into two parts: one was transferred to the inside of a dark cave and the other kept in an illuminated environment. The specimen kept in the light remained with symbionts, while that in the cave showed a gradual loss of cyanobacterial symbionts. After 5 months (when the specimen in the cave appeared completely white), RNA was isolated from the sponge bodies of these two genetically identical specimens, and the expression of *PfSym1* and *PfSym2* was compared via Northern analysis.

The clones obtained from the subtractive hybridization include only a part of the transcribed genes; therefore the RACE (rapid amplification of cDNA ends) technique was applied to try and isolate the 5′ and 3′ ends of the genes. For gene *PfSym2*, the introns were determined by performing a PCR reaction on genomic DNA template using primers that matched the cDNA sequence available.

To investigate the possible function of *PfSym1* and *PfSym2*, we searched the GenBank database using the BLAST algorithm (Altschul et al. 1990) both with the DNA and with the predicted amino acid sequences. The amino acid alignment was performed using Clustal X, version 1.83 (Thompson et al. 1994).

Results

SSH was applied on mRNA isolated from the sponge *P. ficiformis* to discover the genes that are differen-

tially expressed between its symbiotic and aposymbiotic states with endosymbiotic cyanobacteria. Dot-blot analysis was performed to screen a large number of clones for differential expression, and an example of such a blot is shown in Figure 1. Overall, we observed genes that were up- or down-regulated in the symbiotic versus the aposymbiotic specimens. We subsequently concentrated on seven genes that appeared up-regulated in the symbiotic sponge, and tested their expression by Northern analysis. The Northern analysis results did not correspond in all cases to the dot-blot results (in some the differential expression was not as evident). Therefore, two clones (here termed *PfSym1* and gene *PfSym2*; *Pf* standing for *Petrosia ficiformis*, and *Sym* for symbiosis related), which appeared to be clearly up-regulated in both analyses, were chosen as candidates for further examination (Figure 2, left part for each clone). For these two clones, the Northern analysis was repeated with RNA extracted from additional symbiotic and aposymbiotic individuals, and the results were thus confirmed (data not shown). From Northern blot analysis, the estimated sizes of the corresponding cDNAs of genes *PfSym1* and *PfSym2* were found to be approximately 1.5 and 2 kb, respectively.

To analyze the involvement of these two genes in symbiosis, their expression was compared also in two genetically identical specimens of *P. ficiformis*, one harboring cyanobacteria and the other aposymbiotic. These specimens were obtained by cutting a single symbiotic individual into two parts and transferring one part to the inside of a dark cave (see

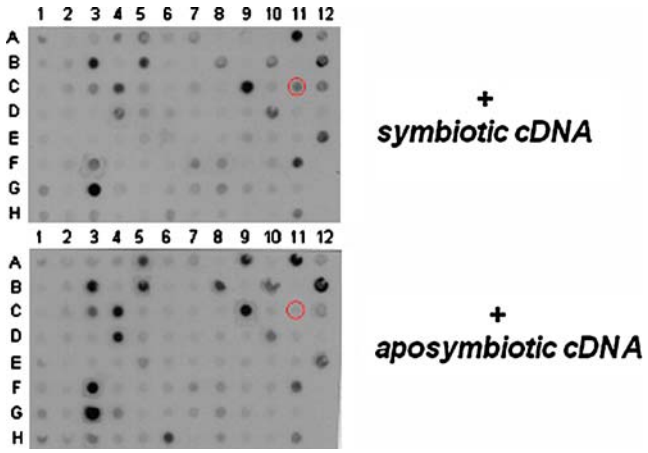

Figure 1. Dot-blot analysis: an example. Each dot represents a clone derived from the SSH library and all clones were dotted in replicates, top and bottom blots. The top one was hybridized to ^{32}P-labeled single-stranded cDNA from symbiotic *P. ficiformis* specimens, while the bottom one was hybridized to ^{32}P-labeled single-stranded cDNA from aposymbiotic *P. ficiformis* specimens. The circles (at position C11) show a clone that is up-regulated in the symbiotic states of *P. ficiformis*.

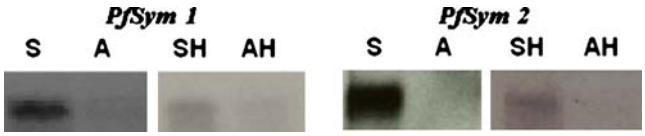

Figure 2. Expression of selected genes in symbiotic and aposymbiotic specimens. Poly(A) (4 μg) pooled from three individuals of symbiotic (S) and aposymbiotic (A) *P. ficiformis*, and from two halves of a single individual, a symbiotic half (SH) and an aposymbiotic (AH) were separated on 1% formaldehyde agarose gel (Sambrook et al. 1989). mRNA was transferred onto a Hybond nylon membrane (Amersham Biosciences) and hybridized with ^{32}P-labeled clones *PfSym1* or *PfSym2*. Equal loading of RNA in each lane was confirmed via methylene blue staining (data not shown).

earlier). During 5 months post-transfer, we could observe the gradual loss of the purple pigmentation of the individual found in the cave, indicating the loss of cyanobacteria probably due to the darkness (Figure 3). The RNA extracted from sponge tissue deriving from these two genetically identical specimens was used for Northern analysis, and the results indicated both genes *PfSym1* and *PfSym2* to be up-regulated in the symbiotic state of the individual (Figure 2, right part for each clone), confirming the results obtained both in the Dot Blot analysis and in the previously mentioned comparison (with Northern analysis) of genetically different individuals, naturally symbiotic and aposymbiotic.

The clones obtained by the SSH included only a part of the transcribed genes; gene *PfSym1* included 393 nucleotides and gene *PfSym2*, 362 nucleotides (Figures 4 and 5; GenBank accession nos. EF507683 and EF507684, respectively). Various methodological approaches using cDNA or genomic DNA (e.g., RACE and Vectorette Gene-Walking, Riley et al. 1990) were applied to isolate additional parts of the isolated genes. Using cDNA, *PfSym1* was extended to 508 nucleotides. Genomic DNA was used to extend *PfSym2* to 938 nucleotides. The intron/exon borders were identified in *PfSym2* (Figure 5). All introns presented the consensus GT-AG splicing sites, and their AT% was 76%, higher than the 59% found in the exons. We are presently designing new approaches to recover the complete sequence of *PfSym1* and *PfSym2*.

PfSym1 did not show any significant homology with any known sequence, neither at the nucleotide level nor at the translated databases. *PfSym2* showed similarity to proteins containing the scavenging receptor cystein rich (SRCR) domain. The *PfSym2* deduced amino acid sequence shares 36% identity in a 68 amino acids overlap with the speract protein of *Strongylocentrotus purpuratus* (accession no. AAD08654); 40% identity in a 55 amino acids

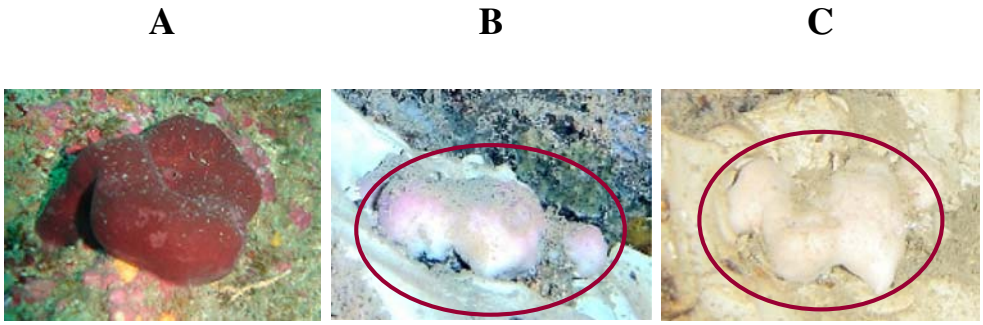

Figure 3. *P. ficiformis*. (**A**) A symbiotic specimen. The red-purple color derives from the cyanobacterial symbionts present near the sponge surface. (**B**) A specimen (circled) photographed 1 month after its transfer into a dark cave. (**C**) A specimen (circled) photographed 5 months after its transfer into a dark cave. The latter has lost its cyanobacterial symbionts, and therefore appears white.

overlap with a SRCR protein from *Danio rerio* (accession no. CAI11836) and 51% identity in a 31-amino-acid overlap with a SRCR protein from the marine sponge *Geodia cydonium* (accession no. CAA75175). Alignment of part of the deduced amino acid sequence for gene *PfSym2*, including the beginning of the SRCR domain, with the above mentioned protein sequences is shown in Figure 6.

Discussion

The molecular regulation that underlies the relationship between the partners of the sponge-cyanobacteria symbiosis has not been previously described. The present study thus describes the first two sponge genes whose regulation is suggested to be linked to the presence/absence of cyanobacterial endosymbionts. Although factors other than the presence of symbionts (i.e., light or water currents) could potentially account for the differential gene expression between the symbiotic sponges outside the cave and aposymbiotic sponges inside it, cyanobacteria present inside the sponge cells will most likely be a major contributor to the differential gene expression found in the experiment. Gene *PfSym1* is a novel gene, and the sequence obtained to date does not include known domains [as revealed by the Conserved Domain Architecture Retrieval Tool (CDART), available on NCBI, http://www.ncbi.nlm.nih.gov/]. There are generally very few sponge cDNA sequences in GenBank, so the overall lack of homology to sequences in the database is not unexpected. Gene

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1    TCAGAATTCGGCACGAGGTGAATACGTTTTTCATTAAACAAGTGTACATGATACAATAAAGGCCTATTA

                                                                        M
70   AAAAATAAACTAAAAAAAGGTAACAAAAAAGGAATTTAGTACATAGTCACTACATGTGCTTGTTTAATG

     K  V  F  C  S  L  D  I  L  T  S  S  F  L  R  I  N  N  L  E  T  N  F
140  AAGGTCTTTTGCAGTCTTGATATACTGACGAGCTCATTTTTGAGGATCAACAATTTGGAAACTAACTTC

     S  T  Q  A  F  H  F  R  W  L  K  N  I  N  V  V  V  V  G  I  G  L  P
210  AGCACTCAAGCCTTCCACTTTAGGTGGCTTAAGAATATCAACGTGGTAGTTGTTGGGATTGGTCTGCCC

     R  Q  L  G  S  F  K  G  G  I  V  Y  T  I  V  T  E  F  N  F  E  S  F
280  AGGCAACTTGGTAGCTTTAAAGGTGGGATAGTCTATACCATTGTAACTGAATTCAATTTTGAATCCTTC

     T  R  E  L  N  C  V  K  T  T  H  I  S  C  A  S  S  S  N  V  G  F  I
350  ACCAGGGAACTGAACTGTGTAAAAACCACTCACATATCCTGTGCCAGTAGCTCCAATGTGGGCTTCATC

     N  Y  S  G  R  V  I  C  V  K  P  L  M  N  F  S  V  F  T  G  I  E  N
420  AATTACAGTGGTAGGGTTATCTGTGTCAAACCATTGATGAACTTCTCCGTATTCACCGGTATAGAAAAT

     T  C  S  L  I  S
490  ACTTGCAGTCTCATTAGT
```

Figure 4. Nucleotide sequence of the gene *PfSym1* from *P. ficiformis*. The clone was sequenced via the dideoxy chain termination method (Sanger 1981). The predicted protein sequence is denoted by a single-letter code. The original sequence obtained by sequencing the SSH-obtained clone is underlined.

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CGTTATAGCTATTATGGACTTGCGACCATCAGTTTGTGTGAAATGAGTGAACTGTTTCACTGCAAGGAT

TCCAGCCCTGACAAAAATTAATACTTGTTTATATATATCATTTTATGAAGCTTTAGTAGTGCTGAAGGA

AGTGCGTATAACACAACCACGCAGTGCTTACCACGAATCATAATAAATTCAGTCTCTGAGGACATACTG

                                        D  W  Q  K  Q  V  C  R  I  L  K
CAGCATATGCATCAAGTACAAAAGCTGCGACTCTGAGACTGGCAAAAGCAAGTGTGTAGAATATTGAAA

 Q  R  S  S  K  S  S  R  I  F  H  S  V  Y  E  M  K  M  W  I  A  L  A
CAAAGATCCTCCAAATCCAGCAGAATATTCCATTCAGTATACGAGATGAAGATGTGGATTGCTTTAGCT

 L  L  A  I  C  A  L  Q  A  N  A  S  P  [1] Intron
CTATTGGCAATTTGTGCTCTCCAGGCCAATGCCTCACCAAGTAAATGAAATGCTCAATTTCTACAGTAA

                                     Intron [1] S  G  K  L  D  D  L  K  N
TTTTGCATAATTCAATTCTATTATACTTAATTGTACTCATTTAGGTGGTAAACTTGACGACTTGAAGAA

  A  F  E  A  F  M  Q  T  E  E  [2] Intron
TGCTTTTGAAGCTTTCATGCAAACTGAAGAAGGTATATATACAATTATTGTTATACTTTTAATTATTAT
                                                                Intron [2
AGTGTATAAAGCCTTAATGTTTATATTGTGTAACAAAAAGATGAGAATAGATTCTCATTCACAATACAG

D  G  G  N  K  A  K  I  N  E  I  E  H  A  P  L  A  K  E  [3] Intron
ATGGTGGTAACAAAGCCAAAATTAATGAAATAGAACATGCTCCTCTTGCAAAAGAAGGTGTTTAACTAT

                                                       Intron [3]E  S  D
ATTGAATTCATTTTGCAAAATTTAACTTTTCTTTCTTTTTAATTCTCTCCCTTTTAAAAGAGTCTGATG

G  N  G  L  A  H  S  E  A  L      [4] Intron
GAAATGGACTTGCTCATTCTGAGGCACTTAGTAAGTGCGATATGCTGTATGTGTTTTGANCTATTATTA

                                     Intron [4]S  Y  R  L  V  D  G  G
TAGATATAATGTTCCTATTCATTATTTTAACTCGCACCTTTTCAGGCTATCGTCTAGTTGATGGAGGTT

S  P  S  R  G  R  V  E  V  F  F  N  G  Q
CTCCCTCACGTGGACGTGTTGAGGTATTCTTTAATGGACAGTG
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Figure 5. Nucleotide sequence of the gene *PfSym2* from *P. ficiformis*. The four intron sequences are shown with gray background and the intron numbers are given in brackets [n]. The predicted protein sequence from exons is denoted by a single-letter code.

PfSym2 was also found to be novel, and to contain a conserved domain: the scavenger receptor cysteine rich (SRCR) domain. Proteins featuring 1 to 11 repeats of the SRCR domain form a superfamily that includes a few invertebrate and several vertebrate proteins (reviewed in Resnick et al. 1994). The first molecule identified as a member of this superfamily in invertebrates, speract, was found in the sea urchin *Strongylocentrotus purpuratus* and displays four SRCR repeats (Dangott et al. 1989). All cloned members of the SRCR superfamily are either cell-surface or secreted proteins. In vertebrates they have functions related to host-defense during endocytosis (e.g., the macrophage scavenger receptor); they are expressed on T- and B-cells, or are involved in adhesion, for example binding to lectins (Resnick et al. 1994). The SRCR domain consists of a *ca*. 110 amino acid residues motif with conserved spacing of six to eight cysteines, which apparently participate in intradomain disulfide bonds. The first two forms of

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                                                  *
Pf EAFMQTEEDGGNKAKINEIEHAPLAKEES---DGNGLAHSEAL-SYRLVDGGSPSRGRVEVFFNGQ
Gc ----------GDEFFIQDCNHADIGENNCGHYEDVGLRCLPNTLDVRLMNGGNTSAGRVEVNYNGE
Sp EIVLDDVECTGDEVSLIECQHAGLGTNNCGHSEDAGVICSVN---VRLADGNSPAEGRVEVYFDGQ
Dr ----------GNESQIHLCPTSTLYENNCTHNNSVRLICADAR-NVRLVDGNSPCAGRVEVFHRGQ
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Figure 6. Alignment of part of the deduced protein sequence of gene *PfSym2* from *P. ficiformis* (Pf), with the corresponding protein sequences from *Geodia cydonium* (Gc, sponge; CAA75175), *Strongylocentrotus purpuratus* (Sp, sea urchin; AAD08654), and *Danio rerio* (Dr, zebrafish; CAI11836). Conserved (identical) residues in all sequences are shown in white on black and those in at least three sequences, including Pf, in black on gray. A star shows the beginning of the SRCR domain.

SRCR molecules characterized in sponges were reported from *Geodia cydonium* (Pancer et al. 1997). The function of the putative *G. cydonium* SRCR protein was not elucidated, but it was proposed that this molecule could be involved in recognition of bacteria, which often live in symbiosis with sponges (Pancer et al. 1997). The largest reported form of sponge protein containing SRCR repeats was proposed to be the cell surface receptor that interacts with extracellularly localized aggregation factors, which are responsible for species-specific reaggregation of sponge cells (Blumbach et al. 1998).

According to the aforementioned studies, SRCR domains in sponges appear to be linked to adhesion and cell recognition. We suggest that the presence of such a domain in a gene that is expressed in a sponge during a symbiotic state indicates that the *PfSym2* gene may have a function in the recognition of the sponge cyanobacterial symbiont, or in the activation of an immune response enabling discrimination of true symbionts from potential pathogenic microorganisms. An alternative explanation is that this gene has adhesion properties and therefore a potential role in connecting the photosymbionts to the sponge cells. Clearly, we are at the early stages of describing the genetic regulation of sponge symbiosis. The analysis of the expression of additional symbiosis related genes, in other sponge-cyanobacteria associations, will unravel their uniqueness to a specific association or their generality throughout the Porifera phylum, or even in other eukaryotic–prokaryotic associations. Further studies are required to explore additional differentially expressed genes and their regulation. The understanding of how beneficial associations between invertebrates and prokaryotic organisms are genetically regulated can also, in the future, help biologists to understand how cooperative associations differ from pathogenic ones, and how the latter could possibly be prevented.

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