Transcriptome Analyses of Immune System Behaviors in Primary Polyp of Coral Acropora digitifera Exposed to the Bacterial Pathogen Vibrio coralliilyticus under Thermal Loading

Marine Biotechnology

Title

Transcriptome analyses of immune system behaviors in primary polyp of coral *Acropora digitifera* exposed to the bacterial pathogen *Vibrio coralliilyticus* under thermal loading

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Abstract

Elevated sea surface temperature associated with global warming is a serious threat to coral reefs. Elevated temperatures directly or indirectly alter the distribution of coral-pathogen interactions and thereby exacerbate infectious coral diseases. The pathogenic bacterium *Vibrio coralliilyticus* is well-known as a causative agent of infectious coral disease. Rising sea surface temperature promotes the infection of corals by this bacterium, which causes several coral pathologies, such as bacterial bleaching, tissue lysis, and white syndrome. However, the effects of thermal stress on coral immune responses to the pathogen are poorly understood. To delineate the effects of thermal stress on coral immunity, we performed transcriptome analysis of aposymbiotic primary polyps of the reef-building coral *Acropora digitifera* exposed to *V. coralliilyticus* under thermal stress conditions. *V. coralliilyticus* infection of coral that was under thermal stress had negative effects on various molecular processes, including suppression of gene expression related to the innate immune response. In response to the pathogen, the coral mounted various responses including changes in protein metabolism, exosome release delivering signal molecules, extracellular matrix remodeling, and mitochondrial metabolism changes. Based on these results, we provide new insights into innate immunity of *A. digitifera* against pathogen infection under thermal stress conditions.

Keywords: coral, *Vibrio coralliilyticus*, thermal stress, innate immunity, transcriptome analysis, immuno-suppression

Introduction

Coral reefs only occupy 0.1% of the area of the sea but harbor approximately 30% of all marine species on the planet. Therefore, they are extremely important ecosystems for the conservation of biodiversity (Moberg and Folke 1999; Roberts et al. 2002). However, many species of corals are on the verge of extinction because of increasing anthropogenic disturbances, including global warming (Sokolow 2009). Rising sea surface temperature associated with global climate change is a serious threat to coral reefs and is linked to an increasing prevalence of

infectious coral diseases (Rosenberg et al. 2007). Risk of coral disease is clearly enhanced under global warming conditions. Frequent and destructive outbreaks of coral diseases in the summer season are consistent with this notion (Bruno et al. 2007; Heron et al. 2010; Maynard et al. 2015; Sato et al. 2009). Infectious diseases of corals caused by a variety of pathogens have emerged at an accelerating rate during the last few decades, contributing to population declines and a dynamic change in community structure (Bourne et al. 2009; Sutherland et al. 2004; Harvell et al. 2002; Harvell et al. 2007; Rosenberg et al. 2007).

Elevated seawater temperature causes shifts in the coral microbiome toward potentially more pathogenic taxa (e.g. genus *Vibrio*) (Tout et al. 2015). Although *Vibrio* species also exist in the microbiome of healthy coral as minority members, elevated seawater temperatures sometimes cause an increase in prevalence of these bacteria (Arboleda and Reichardt 2009; Koenig et al. 2011; Kvennefors et al. 2010; Tout et al. 2015). Among *Vibrio* species, the gram-negative bacterium *Vibrio coralliilyticus* is best known as a causative agent of bacterial bleaching, tissue lysis, and white syndrome (Ben-Haim et al. 2003; Sussman et al. 2008; Ushijima et al. 2014). Under elevated seawater temperature, various virulence factors of *V. coralliilyticus* involved in motility, antimicrobial resistance, host degradation, and transcriptional regulation are up-regulated, enhancing bacterial phenotypes such as motility speed and acute chemotactic sensing (Garren et al. 2016; Kimes et al. 2012).

Since invertebrates, including coral, lack the adaptive immune systems of vertebrates (Cooper 2010), innate immune response to pathogens is a major factor affecting disease susceptibility. The innate immune response involves three steps: (1) recognition of bacterial infection, (2) signaling to activate appropriate defense mechanisms, and (3) an effector response (Palmer and Traylor-Knowles 2012). Whole-genome sequencing has revealed that the repertories of innate immunity in *Acropora digitifera* are more sophisticated than those of the sea anemone and Hydra (Shinzato et al. 2011). For example, the number of pattern recognition receptors encoded in the *A. digitifera* genome, such as Toll-like receptor (TLR) and nucleotide oligomerization domain (NOD)-like receptor (NLR), is much higher than that of *Nematostella* or *Hydra* (Shinzato et al. 2011; Hamada et al. 2012). Understanding the behavior of these innate

77 immune systems under elevated temperatures can provide insights into management of coral
78 diseases that are destroying many reefs. However, the effects of thermal stress on coral immune
79 response to these pathogens are poorly understood.
80 Reef-building corals maintain a symbiotic relationship with photosynthetic
81 dinoflagellates of the family Symbiodiniaceae (Yellowlees et al. 2008; LaJeunesse et al. 2018).
82 Most coral species release aposymbiotic eggs (approximately 85%), which are fertilized in the
83 water column, thereby acquiring Symbiodinium from the environment via horizontal
84 transmission (Baird et al. 2009b). Thermal stress can affect both coral host and zooxanthellae,
85 and investigating the immune response specifically in coral hosts alone is difficult. The early life
86 stages of coral larvae are the only phases that lack symbiotic algae, and therefore, coral larvae as
87 well as primary polyps can be an appropriate model to investigate the effect of thermal stress
88 solely on the coral immunity while excluding the symbiotic algae physiology (Berkelmans and
89 Van Oppen 2006; Howells et al. 2012; Inoue et al. 2012; Yorifuji et al. 2017; Motone et al. 2018).
90 In order to study molecular mechanisms of coral disease, endpoint transcriptional
91 analyses of coral (e.g. diseased versus healthy) have been conducted (Fuess et al. 2018; Libro et
92 al. 2013; Wright et al. 2015), However, there remains a gap in the understanding of the exact
93 process of coral response to bacterial infection under thermal stress. The aim of this study was to
94 reveal the effects of thermal stress solely on coral immunity against *V. coralliilyticus* infection.
95 We performed whole-genome transcriptomic analysis of aposymbiotic primary polyps of *A.*
96 *digitifera* exposed to *V. coralliilyticus* under conditions of thermal stress at early time points.
97 After gradual heat acclimatization, RNA sequencing (RNA-seq) of juvenile polyps before and
98 after experimental infection with *V. coralliilyticus* was performed on the Illumina Hiseq4000
99 platform. Based on our results, we provide new insights into innate immunity of *A. digitifera*
100 against *V. coralliilyticus* under thermal conditions.
101
102 **Materials and Methods**
103 **Coral juveniles**
104 During mass spawning in Okinawa, Japan in June 2017, the gametes of *A. digitifera*

were collected. Embryos were developed to obtain planula larvae, and these larvae were kept at 25°C in a bucket containing 0.22 μm filtered seawater (FSW) with with 0.1% penicillin–streptomycin–amphotericin B suspension (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The seawater was replaced every day. Juvenile polyps were prepared by induction of settlement of planula larvae using the coral metamorphosis inducer Hym-248 (Iwao et al. 2002). Permits for coral collection were provided by the Okinawa Prefectural Government for research use (Permit 29-14).

V. coralliilyticus transformation and growth conditions

The coral pathogen *V. coralliilyticus* strain P1 (LMG 23696) was purchased from the Belgian Coordinated Collections of Microorganisms and used in bacterial challenge experiments on *A. digitifera*. A tri-parental conjugation protocol was used to transform *V. coralliilyticus* strain P1 using a plasmid carrying a gene encoding for a DsRed2 fluorescent protein [DsRed.T3(DNT)] (Dunn et al. 2006). Following transformation, colonies formed on marine agar 2216 (Difco Laboratories, Spark, MD, USA) supplemented with 20 mg/L chloramphenicol (Wako Pure Chemical Industries) were screened for DsRed fluorescence. DsRed-tagged *V. coralliilyticus* was pre-cultured in marine broth 2216 supplemented with 20 mg/L chloramphenicol at 30°C for 12 h. Cultured cells were harvested by centrifugation at 6,000 × g for 10 min at 30°C and resuspended in FSW containing 1% marine broth 2216 at 30°C for 12 h and washed with FSW.

Experimental infection of _V. coralliilyticus_ and microscopic imaging

Approximately 60–70 juvenile polyps were kept for 12 h at 25°C in 8 mL tubes (SuperClear Centrifuge Tubes; Labcon, Petaluma, CA). The tubes were kept in an inclined position to allow polyp settlement on the tube wall. The temperature was gradually increased from 25°C to 28°C, and polyps were kept at 28°C for 24 h in a cool incubator without illumination (CN-25C, Mitsubishi Electric Co., Tokyo, Japan). The juvenile polyps were exposed to thermal stress at 30°C for 48 h. The maximum rate of temperature increase was 1°C per 2 hours. Juvenile polyps were kept in FSW without antibiotics, and the seawater was replaced every day. After

exposure to thermal stress at 30°C for 48 h, the coral polyps were exposed to DsRed-tagged *V. coralliilyticus* (10^7 cells·mL^{-1}) in tubes for 5, 30, 60, or 180 min (three tubes per analysis at different time points). The bacterial cell concentration was determined with a bacteria counter (SLGC, Saitama, Japan). For all treatments and the uninfected control, juvenile polyps were immediately frozen in liquid nitrogen and stored at −80°C.

In order to confirm bacterial infection into *A. digitifera*, the infection process of DsRed-tagged *V. coralliilyticus* was observed using a Zeiss Axio Imager Z1 equipped with an AxioCamHR3 camera and EC Plan-Neofluar 10×/0.30 M27 objective lens (Carl Zeiss, Oberkochen, Germany). For microscopic imaging, juvenile polyps were settled on a 35 mm glass-base dish (IWAKI, Osaka, Japan). DsRed-based signal was detected using an HE DsRed Filter Set 43.

RNA sequencing and bioinformatics analyses

After bacterial challenge with *V. coralliilyticus,* total RNA was extracted from juvenile polyps using a specialized method combining TRIzol (Invitrogen, Carlsbad, CA, USA) and RNeasy plant kit (Qiagen GmbH, Hilden, Germany) (Rosic and Hoegh-Guldberg 2010). The homogenization step for juvenile polyps was carried out in TRIzol reagent using a Polytron (Kinematica GmbH, Kriens-Luzern, Sweden). An Illumina TruSeq RNA sample preparation kit was used for sequencing library preparation, and each library was sequenced from 150-bp paired-end libraries using the Illumina HiSeq 4000. PCR duplicates, Illumina sequence adaptors and low-quality reads were trimmed with ConDeTri v2.3 (Smeds and Künstner 2011) and Cutadapt v1.16 (Martin 2011), and retained reads were mapped to *A. digitifera* gene models using KALLISTO v0.44.0 (Bray et al. 2016) with 100 bootstrap replicates. Gene expression levels in samples at 5, 30, 60, and 180 minutes post infection (mpi) were compared to uninfected control samples. In order to eliminate the changes in gene expression caused only by heat stress, the primary coral polyps exposed to thermal stress at 30°C for 48 h (just before infection) were used as the uninfected control samples. For statistical tests, SLEUTH v0.30.0 (Pimentel et al. 2017) in RStudio version 3.5.3 was used to identify differentially expressed genes (DEGs). Expressed

genes with q-value ≤ 0.05 and absolute log2 fold change (beta value) > 1 were accepted as DEGs. Gene annotation for *A. digitifera* was performed using BLASTX analysis with an e-value cut off of 1e-5 against Swiss-Prot database (April 9, 2018). Gene ontology (GO) analysis and the identification of enriched biological themes were performed by searching the DEG list and using the DAVID web service to assign GO categories (Huang et al. 2009) (http://david.abcc.ncifcrf.gov). The UNIPROT accession identifiers of the top protein hits were used as identifiers. Similarly, functional annotation data were obtained for whole transcriptome data set using BLASTX ($e \leq 10^{-5}$) against Swiss-PROT. These annotations served as the background for enrichment analysis. We selected Biological Process (GO-BP) and Cellular Compartment (GO-CC) for our analysis. GO categories with P-values ≤ 0.05, fold Enrichment > 1.5, and number of genes ≥ 5 were considered as enriched GO terms.

Data availability

Raw RNA sequencing data reported are available in the DDBJ Sequenced Read Archive under the accession number DRA010139.

Results and discussions

Microscopic observation of DsRed-labeled *V. coralliilyticus* infection

For confirmation of bacterial infection of primary coral polyps, the infection process of *V. coralliilyticus* was observed using fluorescence microscopy. To overcome the background autofluorescence of primary coral polyps, we transformed the coral pathogen *V. coralliilyticus* strain P1 with a plasmid encoding the DsRed fluorescent protein (Dunn et al. 2006). Although the autofluorescence of the coral host was high, DsRed-tagged *V. coralliilyticus* cells could be distinguished from background (Fig. 1 and Supplemental movie 1). When coral primary polyps were exposed to *V. coralliilyticus,* intensely fluorescent DsRed-tagged pathogenic cells accumulated around the mouth of the polyp (stomodaeum), and a few cells adhered to the tentacles (Supplemental movie 1).

In a previous study of host-pathogen visualization, the scleractinian coral *Pocillopora*

damicornis was used for microscopic observation (Shapiro et al. 2016). They induced "polyp-bail-out" of *P. damicornis* by applying stress and produced coral micropropagates. However, this method inevitably requires applying environmental stress (e.g. salinity and pH) to coral fragments (Kvitt et al. 2015; Shapiro et al. 2016). Conversely, we showed that it is possible to conduct a pathogen-infection experiment without additional stress using aposymbiotic primary coral polyps.

Transcriptional response of coral to *V. coralliilyticus* infection

To better understand the effects of thermal stress on the coral immune response to the pathogen, host gene expression changes were determined following infection with *V. coralliilyticus* (Fig. 1 and Supplemental movie 1). At early time-points (5, 30, 60, and 180 mpi), whole transcriptome expression profiles were compared between uninfected controls and *V. coralliilyticus*-infected juvenile coral polyps. An average of 12.1 million paired-end reads per sample were retained after quality and adaptor trimming, and on average, 81.5% of reads were successfully mapped to the *A. digitifera* genome (Supplementary Table S1).

Volcano plots of each time-point were used to select DEGs (Fig. 2 and Supplementary Fig. 1). Distinct transcriptomic responses were identified in 30 min and 60 min post-infected groups (1960 and 2061) (Fig. 2), whereas a few DEGs were detected in 5 min and 180 min post infected groups (69 and 1) (Supplementary Fig. 1). At 30 mpi, a total of 1960 (8.8%) genes were differentially expressed, of which 455 (2.0%) and 1505 genes were up-regulated or down-regulated, respectively. At 60 mpi, a total of 2061 (9.2%) genes were differentially expressed, of which 1060 (4.7%) and 1001 genes were up-regulated or down-regulated, respectively. Among the DEGs in *V. coralliilyticus*-infected polyps at 30 and 60 min, 1425 and 1331 had reliable Swiss-Prot annotation, respectively (Supplementary Table S2, S3, S4, and S5). Furthermore, in order to extract the biological function of the annotated DEGs, a relative ranking of various GO category associations was performed with respect to the gene list using DAVID (Huang et al.2009) (http://david.abcc.ncifcrf.gov).

Among the genes up-regulated at 30 mpi, 4 Biological Process (GO-BP), and 7 Cellular Component (GO-CC) categories showed significant enrichment (Table 1). Up-regulated

genes at 60 mpi showed significant GO enrichment with respect to 5 GO-BP, and 14 GO-CC terms (Table 1). For down-regulated genes at 30 mpi, 73 GO-BP and 48 GO-CC categories showed significant enrichment (Table S6). Down-regulated genes at 60 mpi showed significant GO enrichment with respect to 36 GO-BP, and 16 GO-CC terms (Table S7).

Promotion of protein synthesis during the early stage of *V. coralliilyticus* infection

The majority of the commonly enriched GO terms were associated with protein translation including the categories "translation" (GO:0006412), "cytoplasmic translation" (GO:0002181), "cytosolic large ribosomal subunit" (GO:0022625), "ribosome" (GO:0005840), and "cytosolic small ribosomal subunit" (GO:0022627) (Table 1). Furthermore, the GO-BP term "proteolysis involved in cellular protein catabolic process" (GO:0051603) was enriched at 60 mpi, indicating an up-regulation of the host protein response, including protein synthesis and metabolism.

These responses have been documented in pathogen exposure of other invertebrates, including Caribbean sea fan, abalone, clams, and urchins (Burge et al. 2013; Travers et al. 2010; Gestal et al. 2007; Nair et al. 2005). Conversely, Mohamed et al. (2016) reported that protein synthesis was down-regulated during the initial coral-*Symbiodinium* interaction. Furthermore, the long-term exposure of *Acropora millepora* larvae to thermal stress also resulted in down-regulation of ribosomal proteins (Meyer et al. 2011). In the case of the Caribbean coral *Orbicella faveolata*, down-regulation of protein synthesis has also been observed during thermal stress (DeSalvo et al. 2008). Thus, the up-regulation of host protein response, including the synthesis and breakdown of proteins, may be a specific response to counter the early stages of pathogen infection.

Regulation of exosomal and extracellular matrix transcripts

Of the up-regulated GO categories at 30 and 60 mpi, the GO-CC terms "extracellular exosome" (GO:0070062) and "extracellular matrix" (GO:0031012) were commonly enriched (Table 1, Supplementary Table S8, S9, S10, and S11). Exosomes are small membrane vesicles of

endosomal origin with a diameter of 40−100 nm that are secreted by many cell types into the extracellular environment (Pan and Johnstone 1983; Stoorvogel et al. 2002). Exosomes mediate intercellular communication through direct binding and transport of biochemical cues (e.g. microRNA, proteins and lipids) to target-cells (Théry et al. 2002; Valadi et al. 2007). Extracellular matrix (ECM) can provide structural support for tissues, basement membranes, and individual cells as substrates for migration (Hynes 2009). Among major ECM components such as collagen, proteoglycans, and adhesive glycoproteins (Helman et al. 2008), collagen transcripts (e.g. collagen type I alpha 2 chain, type IV alpha 2 chain, and type XII alpha 1 chain) are mostly included in "extracellular matrix" (GO:0031012) category and were enriched at 60 mpi (Supplementary Table S11). There are direct interactions between exosomes and the ECM through matrix metalloproteinases (MMPs) and integrins (Clayton et al. 2004; Hakulinen et al. 2008; Vrijsen et al 2010). In fact, we found that MMP2 (Q90611) and MMP10 (P09238) are included in the "extracellular matrix" (GO:0031012) category and were enriched at 30 and 60 mpi, respectively (Table S10 and S11). MMPs are zinc-dependent endo-peptidases, which degrade ECM and play important roles in tissue remodeling during physiological and pathological processes, including cell migration, matrix remodeling, and tumor invasion (Hakulinen et al. 2008). These results justify the investigation of whether interactions between exosomes and the ECM may play a key role in the immune response of coral against *V. coralliilyticus* infection.

The individual components of the ECM are substrates that pathogens directly bind to or degrade, facilitating adhesion and penetration into the host (Tomlin and Piccinini 2018). Genomic sequencing of *V. coralliilyticus* revealed that this bacterium has 17 putative metalloproteases, including collagenase, metallopeptidase, vibriolysin, and bacterial leucyl aminopeptidase (Santos et al. 2011). Among them, the collagenase metallopeptidase (U32) can degrade type I collagens and facilitate the invasion of the host (Santos et al. 2011). Furthermore, based on the microscopic observation, we confirmed that *V. coralliilyticus* adhered to the surface of primary coral polyps, especially around the mouth (Fig. 1 and Supplemental movie 1). This might indicate that *V. coralliilyticus* can adhere to the coral surface via binding of adhesion proteins and components of the ECM early in the infection process and degrade substrates to

penetrate the host. Subsequently, coral significantly enhanced biosynthesis of ECM component transcripts in response to *V. coralliilyticus* infection.

Exosomes not only act as key mediators of cell-to-cell communication, but also directly transport antimicrobial peptides (Hu et al 2013). Damicornin is well-known as an antimicrobial peptide in *P. damicornis* (Vidal-Dupiol et al 2011). Damicornin is active against some Gram-positive bacteria (*Staphylococcus aureus, Micrococcus luteus, Corynebacterium stationis*); however, it has a limited activity against Gram-negative bacteria (*Vibrio coralliilyticus, Vibrio aesturianus, Vibrio splendidus, Vibrio shiloi*) (Vidal-Dupiol et al 2011). Although the existence of antimicrobial activity of several species belonging to the genus *Acropora* (including *A. digitifera*) has been suggested, the active compounds have not been identified (Sato et al 2013). Antimicrobial peptides could potentially be used as therapeutic agents, tools for monitoring the health condition of cultured animals, or as selection markers for improving resistance to microbial infections (Bachère 2003). Therefore, further investigations of antimicrobial transcripts or peptides expressed by members of the genus *Acropora* are strongly justified.

V. coralliilyticus infection negatively regulated the innate immune response

GO enrichment analysis revealed negative effects on molecular processes in coral primary polyps under thermal stress following *V. coralliilyticus* infection, including metabolism, cell cycle, and apoptosis (Supplementary Table S6 and S7). Among them, we found that "innate immune response" (GO:0045087) genes were enriched as down-regulated GO categories at 60 mpi (Supplementary Table S7). Major genes in this category that were down-regulated included Toll-like receptor 1 (TLR1) (Q15399), Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) (Q8BHB0), NOD2 (Q8K3Z0), NLR family CARD domain-containing protein 4 (NLRC4) (F1MHT9, F6R2G2, Q3UP24), and NLRC5 (C6FG12, C3VPR6) (Figure 3a and Supplementary Table S12). As shown in Fig. 3a, expression levels of these genes began to decrease at 30 mpi and reached a minimum at 60 mpi. Subsequently, a gradual up-regulation was observed at 180 mpi. In the bacterial challenge experiment, not only microbe-sensing protein TLRs but also MYD88 (A2TF48), which activates signaling pathways downstream of TLRs, was down-regulated at 30 and 60 mpi (Fig. 3a).

TLRs are transmembrane proteins that are responsible for recognition of extracellular microbial pathogenic components and mediate the activation of appropriate response genes (Medzhitov et al 1997; O'Neill 2013). For example, a TLR in the sea anemone *Nematostella vectensis* (Nv-TLR) is capable of directly recognizing *V. coralliilyticus*. A functional study in human cells revealed that Nv-TLR can activate canonical NF-κB signaling (Brennan et al. 2017). The activation of NF-κB signaling occurs through the interaction between the intracellular Toll/IL-1 receptor (TIR) domain of Nv-TLR and TLR adapter proteins (e.g. MYD88 and MAL). Our results indicated that *V. coralliilyticus* infection under thermal stress conditions may have negatively regulated the TLR-to-NF-κB pathway of *A. digitifera*.

In addition to genes related to the TLR-to-NF-κB pathway, many NODs and NLRCs were negatively regulated at 30 and 60 mpi (Fig. 3a). NODs and NLRCs detect the cytosolic presence of microbial components such as peptidoglycan fragments, meso-DAP, and muramyl dipeptide, and drive the activation of mitogen-activated protein kinase and the transcription factor NF-kB (Kanneganti et al. 2007). Vidal-Dupiol et al. (2014) has reported similar results in adult *P. damicornis*, showing that genes involved in innate immunity were significantly down-regulated by bacterial infection under thermal stress. These observations are consistent with the hypothesis that high temperature favors infection because it has negative impacts on coral immune systems (Bruno et al. 2007; Lesser et al. 2007; Bourne et al. 2009; Mydlarz et al. 2010; Muller et al. 2012). Coral mass spawning in Okinawa typically occurs in the early summer (Hayashibara et al. 1993), and our results indicate that global warming has negative effects on not only the immune response of adult corals but also their early life stages, thereby increasing dispersal limitations of coral larvae. Moreover, higher temperatures not only affect the host's immune systems, but also improve some virulence functions of pathogenic microbes such as motility and chemotaxis (Garren et al. 2016). Therefore, these host-microbe interactions caused by higher temperature may increase microbial disease during the summer season (Bruno et al. 2007; Heron et al. 2010; Maynard et al. 2015; Sato et al. 2009).

Mitochondrial oxidative metabolism

The appropriate maintenance of redox homeostasis is crucial for biological cellular processes and cell survival (Gostner et al. 2013). Changes in redox balance in the tissue are often related to diseases that are characterized by chronic immune activation such as infections, allergies, autoimmune disorders, and malignancies in humans (Dalle-Donne et al. 2006; Murr et al. 2002). We found that the "oxidation-reduction process" (GO:0055114), which is strongly related to redox homeostasis, was enriched as an up-regulated Go category (Table 1). Moreover, we discovered that the major genes in this category were commonly included in "mitochondrion" (GO:0005739), "mitochondrial respiratory chain complex I" (GO:0005747), and "mitochondrial inner membrane" (GO:0005743) at 60 mpi (Table1, Supplementary Table S13, S14, S15, and S16). These results indicated that the major genes in the up-regulated "oxidation-reduction process" (GO:0055114) strongly related to mitochondrial functions. In contrast to the innate immune response (Fig. 3a), expression levels of these genes began to increase at 30 mpi and peaked at 60 mpi. Subsequently, a gradual decrease was confirmed at 180 mpi (Fig. 3b).

Mitochondria are dynamic double-membrane-bound organelles that are engaged in a wide variety of cellular processes, including ATP generation, calcium homeostasis regulation, programmed cell death, and the biosynthesis of amino acids, lipids, and nucleotides (West et al. 2011). Numerous studies have recently highlighted the importance of mitochondria and mitochondrial functions as central in the regulation of the host innate immune response (West et al. 2011; Arnoult et al. 2011; Weinberg et al. 2015). Mitochondrial oxidative metabolism is a major cellular source of reactive oxygen species (ROS) generation. Approximately 1–2% of oxygen consumed during physiological respiration is converted into superoxide. However, when electrons prematurely leak from the electron transport chain under specific conditions such as pathologic and stress conditions and are aberrantly transferred to molecular oxygen, they can further augment mitochondrial ROS (mROS) generation (Koopman et al. 2010; Orrenius et al. 2007). Among the five multi-subunit protein complexes comprising the mitochondrial respiratory chain, complex I (NADH: ubiquinone oxidoreductase) and complex III (cytochrome b-c1 complex) are the major sites of superoxide generation within mitochondria (Koopman et al. 2010; Orrenius et al. 2007). The up-regulated "oxidation-reduction process" (GO:0055114) category

genes at 60 mpi included core subunit S3 (P23709), core subunit S7 (P42026), subunit A4 (Q62425), subunit A6 (Q4R5X8), subunit A9 (Q5BK63), and subunit A13 (Q95KV7) of the mitochondria respiratory chain complex I (Fig 3b and Supplementary Table S13). In addition, the up-regulated DEG list at 60 mpi contained the cytochrome b-c1 complex subunit 7 (P00129) (Table S4). These results indicated that mROS generation may be promoted during *V. coralliilyticus* infection.

mROS not only directly contribute to bacterial killing (Hall et al. 2013), but also facilitate antibacterial innate immune signaling, such as that through NF-κB and MAPK signaling pathways, which augment pro-inflammatory cytokine production (Nishio et al. 2005; Bai et al. 2005; Emre et al. 2005; West et al. 2011). Additionally, mROS promote the release of mitochondrial DNA (mtDNA) into the cytosol, which binds to TLRs triggering an innate response in human cell lines (Williamson et al. 2019). In concordance with this observation, we found that the expression level of several TLRs and NLRs returned to normal at 180 mpi. This was subsequent to the enrichment of GO terms associated with mitochondrial functions ("mitochondrion" [GO:0005739], "mitochondrial respiratory chain complex I" [GO:0005747], and "mitochondrial inner membrane" [GO:0005743]) at 60 mpi (Table 1, Fig. 3a, and Fig. 3b). Therefore, mitochondria of coral may also play crucial role in the regulation of the host innate immune response. For example, the nematode *Caenorhabditis elegans* is often used to model innate immunity, leading to evidence that mitochondria participate in the *C. elegans* immune response against pathogen infection (Kwon et al. 2018). As the position of cnidarians in the animal phylogenetic tree is more basal than that of nematodes, the innate immunity of corals is an expanding field in basic research. Investigation of these systems is critical for understanding the evolution of defense systems against pathogen infection. Although several studies have shown that marine invertebrates up-regulate mitochondrial functions in response to pathogen infection (Gestal et al., 2007; van Rensburg and Coyne, 2009; James et al., 2010; Burge et al. 2013), this study provides the first evidence that mitochondria and mitochondrial functions may play important roles in the regulation of host innate immune response in the scleractinian coral.

Conclusions

Herein we report transcriptome analysis of aposymbiotic primary polyps of *A. digitifera* exposed to *V. coralliilyticus* at early time points during thermal stress. Based on our microscopic observation and gene expression analysis, we present a schematic summary of early interactions between aposymbiotic primary coral polyps and pathogens in Fig. 4. We propose a model where the coral pathogen *V. coralliilyticus* accumulates around the mouth (stomodaeum) of the coral and degrades components of coral surface to facilitate invasion into tissue. Subsequently, invading bacteria suppress gene expression related to the innate immune response such as that of TLRs, MyD88, and NLRs under thermal stress. In order to eliminate the infected pathogens, the coral undergoes complex changes, including altered mitochondrial metabolism, altered protein metabolism, exosome release for delivering signal molecules, and ECM remodeling. Further investigations and analysis of changes of gene expression in bacterial challenge experiments will contribute to the elucidation of molecular mechanisms in coral innate immunity and the development of diagnostic tools to manage coral disease outbreaks.

Declarations

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of Interest

The authors declare that they have no conflict of interest.

Funding

This study was supported by JSPS Research Fellowship for Postdoctoral Researchers (TT, 17J05024), Grant-in-Aid for Young Scientists (KAKENHI) (TT, 18K14479), and a research grant from the Yamaguchi Educational and Scholarship Foundation (TT). This study was also partially

supported by the Collaborative Research of Tropical Biosphere Research Center, University of the Ryukyus.

Acknowledgements

For technical assistance with experiments and sequencing, we would like to thank Shinichi Yamasaki (DNA Sequencing Section, Okinawa Institute of Science and Technology Graduate University). We thank Prof. E. Stabb (Franklin College, University of Georgia) for generously providing conjugation protocols and materials.

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745
746 **Figure legends**
747 **Fig. 1** Visualization of *V. coralliilyticus* infection using microscopic imaging
748 (a) DIC image of a coral juvenile polyp (b) Fluorescence imaging of a coral juvenile polyp and
749 DsRed-tagged *V. coralliilyticus.* White arrows show *V. coralliilyticus* attached to mouth of the
750 coral juvenile polyp. Scale bars denote 100 μm.
751

Fig. 2 Volcano plots showing coral genes that are differentially expressed at (a) 30 mpi (b) and 60 mpi after *V. coralliilyticus* infection compared to uninfected control

The red plots show a q-value ≤ 0.05 and a log2 fold change in transcript of > 1 (up-regulated genes). The blue plots showed a q-value ≤ 0.05 and a log2 fold change in transcript of < -1 (down-regulated genes).

Fig. 3 Heatmap of genes related to the innate immune response (a) and the oxidation-reduction process (b)

The color key indicates the log2 fold change in expression levels compared with uninfected control sample. TLR, Toll-like receptor; NOD, nucleotide oligomerization domain; NLRC, NOD-like receptor (NLR) family CARD domain-containing protein; LGR, leucine-rich repeat-containing G-protein coupled receptor; POLR3C, DNA-directed RNA polymerase III subunit RPC3; MYD88, myeloid differentiation primary response protein MyD88; UCHL1, ubiquitin carboxyl-terminal hydrolase; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein; TBK1, serine/threonine-protein kinase; DDX60, probable ATP-dependent RNA helicase DDX60; MX1, interferon-induced GTP-binding protein MX1; ALKBH1, AlkB homolog 1, histone H2A dioxygenase; AIFM1; apoptosis inducing factor, mitochondria associated 1; CYB5R3, cytochrome b5 reductase 3; CYB5A, cytochrome b5 type A; DEGS1, delta 4-desaturase, sphingolipid 1 DES1; HSD17B10, hydroxysteroid 17-beta dehydrogenase 10; MDH2, malate dehydrogenase 2; PCBD2, pterin-4 alpha-carbinolamine dehydratase 2, SDHD, succinate dehydrogenase complex, subunit D, integral membrane protein; FDX1, ferredoxin 1, NDUFA4, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4; NDUFS3, NADH: ubiquinone oxidoreductase core subunit S3; NDUFS7, NADH: ubiquinone oxidoreductase core subunit S7; NDUFA13, NADH: ubiquinone oxidoreductase subunit A13; NDUFA9, NADH: ubiquinone oxidoreductase subunit A9; NDUFS6, NADH: ubiquinone oxidoreductase subunit S6; KDSR, 3-ketodihydrosphingosine reductase; IFI30, lysosomal thiol reductase; DEGS2, putative sphingolipid delta(4)-desaturase/C4-monooxygenase; BLVRA, biliverdin reductase A; DHRSX, dehydrogenase/reductase X-linked; GRIF, grixazone synthase; GMPR2, guanosine

780 monophosphate reductase 2; MOXD1, monooxygenase, DBH-like 1; PIPOX, pipecolic acid and
781 sarcosine oxidase; RDH8, Retinol dehydrogenase 8; SCD5, stearoyl-CoA desaturase 5.
782
783 **Fig. 4** Schematic summary representing a working model of the early interactions between
784 aposymbiotic primary coral polyp and pathogen
785 Up-regulated coral functions in response to pathogen are in blue text, while down-regulated
786 functions and *V. coralliilyticus* functions are in red text. The coral pathogen *V. coralliilyticus*
787 accumulates around the mouth (stomodaeum) of the coral, degrading components of the coral
788 surface and ECM to facilitate invasion into tissue. Subsequently, invading bacteria suppress coral
789 gene expression related to the innate immune response, including TLRs, MyD88, and NLRs,
790 under thermal stress. To eliminate the infected pathogens, the coral undergoes complex changes,
791 including altered mitochondrial metabolism, altered protein metabolism, exosome release for
792 delivering signal molecules, and ECM remodeling.
793
794 **Supplemental movie** Visualization of *V. coralliilyticus* infection using microscopic imaging.
795
796 **Supplementary figure legend**
797 **Supplementary Fig. S1** Volcano plots showing coral genes that are differentially expressed at (a)
798 5 mpi (b) and 180 mpi after *V. coralliilyticus* infection compared to uninfected control.
799 The red plots show a q-value ≤ 0.05 and a log2 fold change in transcript of > 1 (up-regulated
800 genes). The blue plots showed a q-value ≤ 0.05 and a log2 fold change in transcript of < -1 (down-
801 regulated genes).
802
803 **Supplementary tables**
804 **Table S1** Summary of quality trimming and mapping rates
805 **Table S2** Up-regulated DEGs at 30 mpi
806 **Table S3** Down-regulated DEGs at 30 mpi
807 **Table S4** Up-regulated DEGs at 60 mpi

Table S5 Down-regulated DEGs at 60 mpi

Table S6 Down-regulated GO categories enriched at 30 min post infection (with corrected *P*-value ≤ 0.05; fold Enrichment > 1.5; and number of genes ≥ 5)

Table S7 Down-regulated GO categories enriched at 60 min post infection (with corrected *P*-value ≤ 0.05; fold Enrichment > 1.5; and number of genes ≥ 5)

Table S8 Genes listed in "extracellular exosome" (GO:0070062) enriched at 30 mpi

Table S9 Genes listed in "extracellular exosome" (GO:0070062) enriched at 60 mpi

Table S10 Genes listed in "extracellular matrix" (GO:0031012) enriched at 30 mpi

Table S11 Genes listed in "extracellular matrix" (GO:0031012) enriched at 60 mpi

Table S12 Genes listed in "innate immune response" (GO:0045087) enriched at 60 mpi

Table S13 Genes listed in "oxidation-reduction process" (GO:0055114) enriched at 60 mpi

Table S14 Genes listed in "mitochondrion" (GO:0005739) enriched at 60 mpi

Table S15 Genes listed in "mitochondrial respiratory chain complex I" (GO:0005747) enriched at 60 mpi

Table S16 Genes listed in "mitochondrial inner membrane" (GO:0005743) enriched at 60 mpi

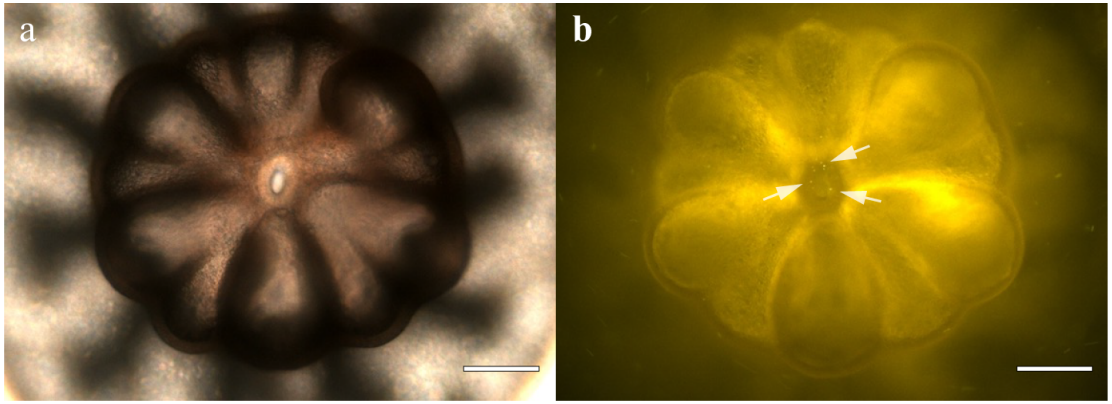


Figure 1 Takagi et al.

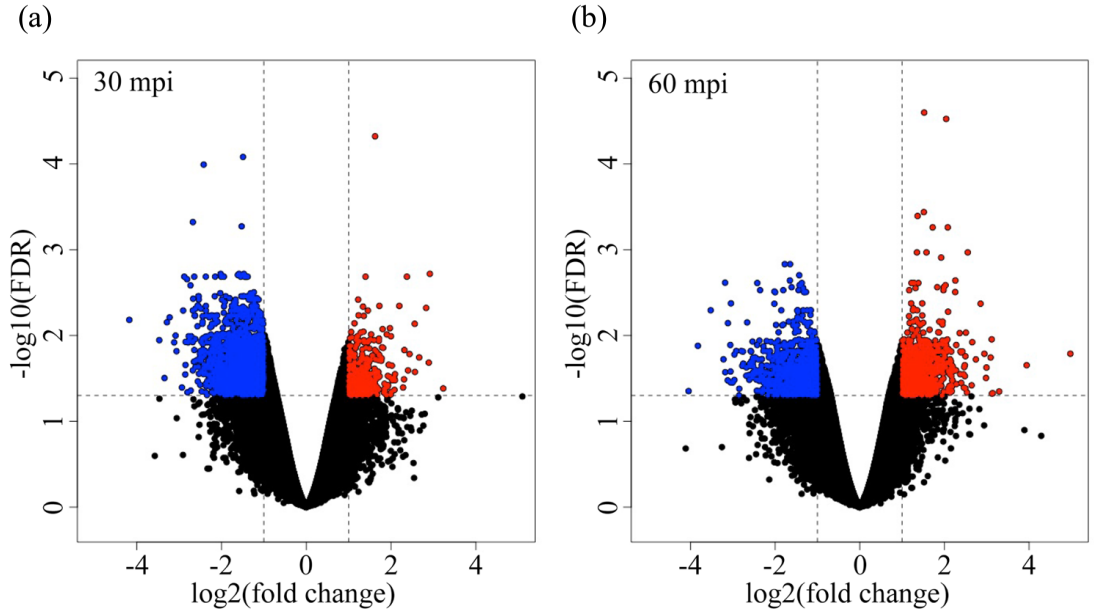


Figure 2 Takagi et al.

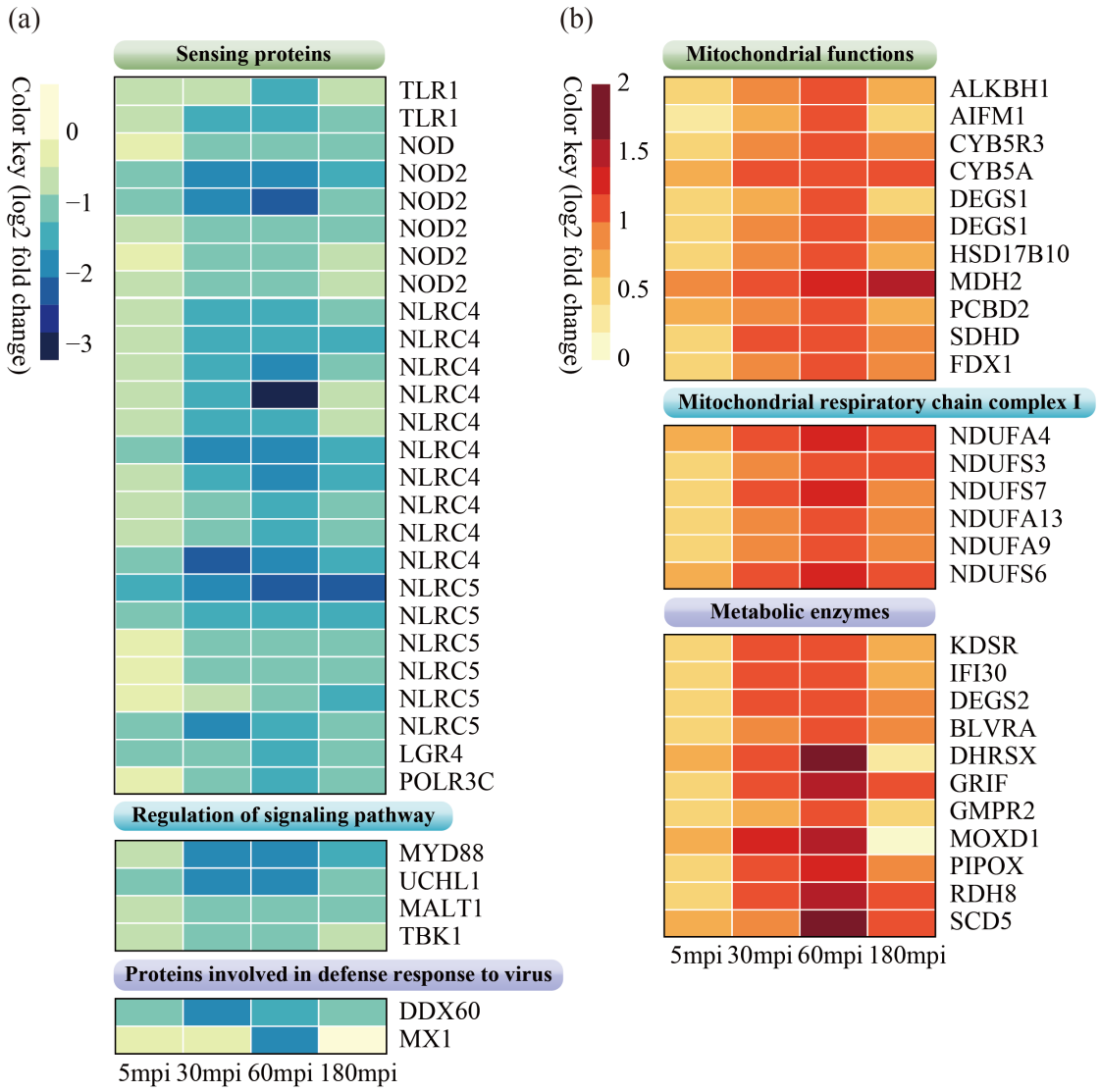


Figure 3 Takagi et al.

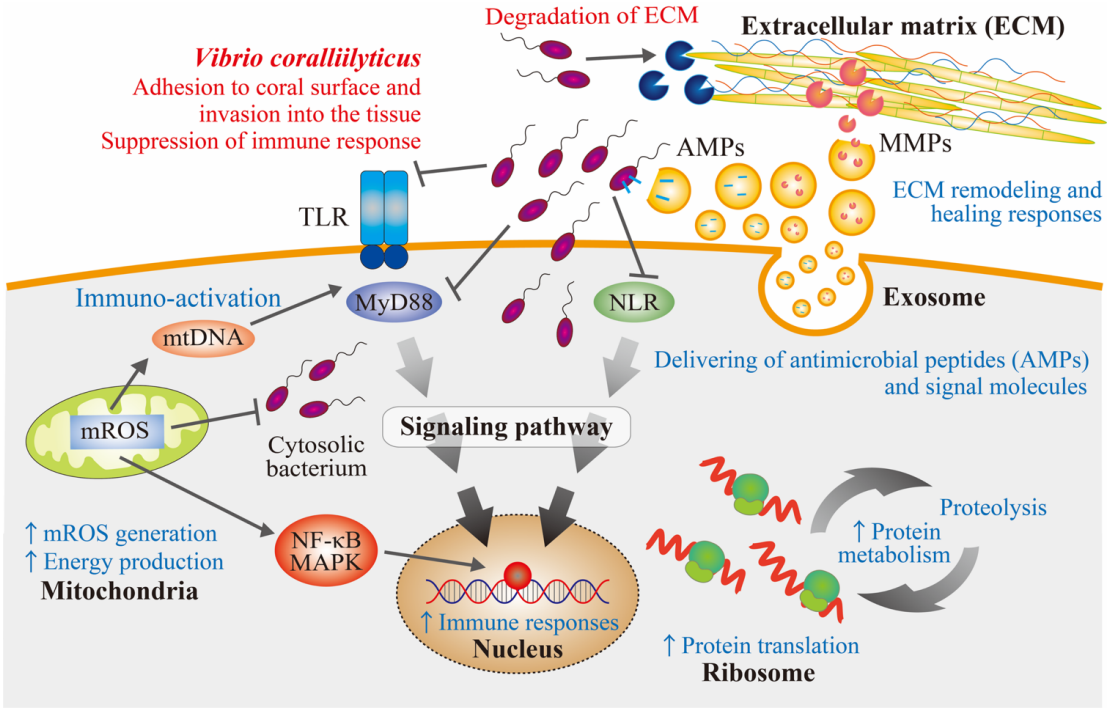
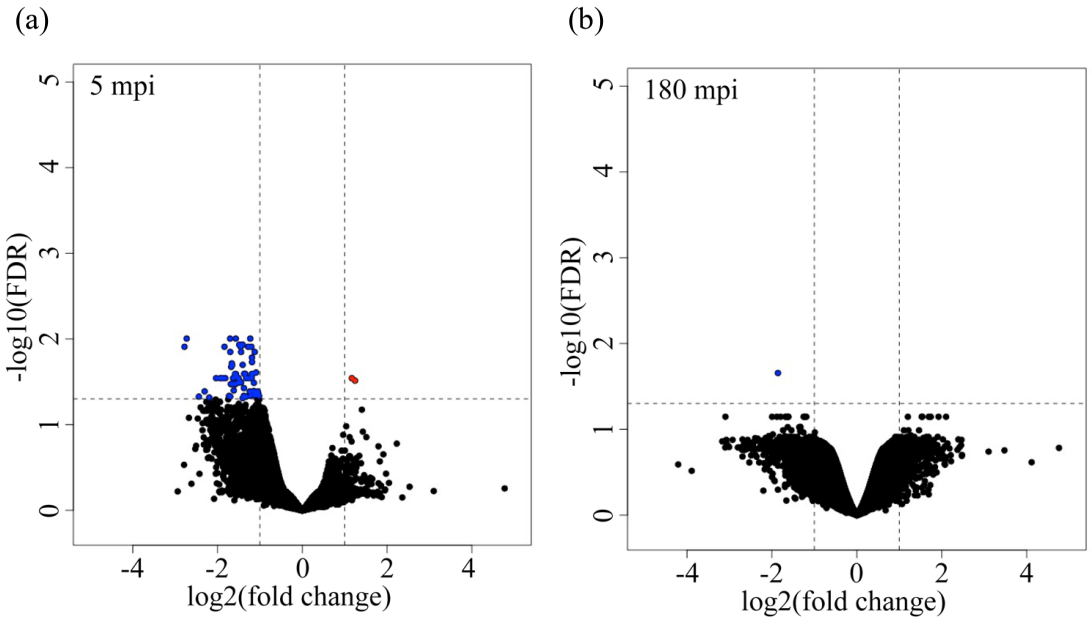


Figure 4 Takagi et al.



Supplementary Fig. S1 Takagi et al.