

Establishment of Host–Algal Endosymbioses: Genetic Response to Symbiont Versus Prey in a Sponge Host

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Abstract

The freshwater sponge *Ephydatia muelleri* and its *Chlorella*-like algal partner is an emerging model for studying animal: algal endosymbiosis. The sponge host is a tractable laboratory organism, and the symbiotic algae are easily cultured. We took advantage of these traits to interrogate questions about mechanisms that govern the establishment of durable intracellular partnerships between hosts and symbionts in facultative symbioses. We modified a classical experimental approach to discern the phagocytotic mechanisms that might be co-opted to permit persistent infections, and identified genes differentially expressed in sponges early in the establishment of endosymbiosis. We exposed algal-free *E. muelleri* to live native algal symbionts and potential food items (bacteria and native heat-killed algae), and performed RNA-Seq to compare patterns of gene expression among treatments. We found a relatively small but interesting suite of genes that are differentially expressed in the host exposed to live algal symbionts, and a larger number of genes triggered by host exposure to heat-killed algae. The upregulated genes in sponges exposed to live algal symbionts were mostly involved in endocytosis, ion transport, metabolic processes, vesicle-mediated transport, and oxidation–reduction. One of the host genes, an ATP-Binding Cassette transporter that is downregulated in response to live algal symbionts, was further evaluated for its possible role in the establishment of the symbiosis. We discuss the gene expression profiles associated with host responses to living algal cells in the context of conditions necessary for long-term residency within host cells by phototrophic symbionts as well as the genetic responses to sponge phagocytosis and immune-driven pathways.

Key words: sponges, algae, symbiosis, genome expression, transcriptome.

Introduction

The host cell represents a potentially dangerous habitat, even for intracellular symbionts that provide benefits to the host (i.e., mutualists) because hosts can digest or mount immunological defenses against foreign entities (Schwarz 2008; Schmid-Hempel 2009; Davy et al. 2012; Hill and Hill 2012; Hill 2014). Despite the challenges of living inside a host cell,

intracellular habitats have been repeatedly invaded by a diverse assortment of microbes with varying degrees of specificity and duration of occupancy (e.g., Nowack and Melkonian 2010; Romano et al. 2013). However, the mechanisms that permit stable intracellular occupancy are often poorly understood. On the one hand, phagocytosis and organellar or cytoplasmic residency expose the symbiont to mortal threats; on

Significance

Intracellular habitats have repeatedly been invaded by a diversity of microbes that sometimes provide benefits to the host. However, the molecular pathways that might be targets of evolution in the maintenance of these mutualistic associations are less understood. We combined a classic experimental approach that involved exposing hosts to live symbionts, heat-killed symbionts, and other prey items with molecular and cellular methods to discern the genetic pathways that allow for long-term residency within host cells versus digestion of prey items. Our work elucidates patterns of differential gene expression to identify pathways that are common to and different from the early stages of intracellular digestion of prey and successful intracellular establishment of symbionts.

the other, each stage of phagocytotic processing presents opportunities for the symbiont to circumvent host cellular processes. Although several pathways of host-cell occupancy have been proposed (e.g., Schwarz 2008; Hill and Hill 2012), the reality is that we have limited understanding of the precise mechanisms that permit intracellular residency and allow symbionts to avoid digestion or destruction.

Understanding the mechanisms that permit stable residency is especially important for mutualistic intracellular phototroph: heterotroph symbioses given the essential role these partnerships play in many ecosystems. The most well-studied animal: algal symbionts involve members of the Symbiodiniaceae in marine environments and *Chlorella*-like green algae in freshwater systems (Venn et al. 2008). The Symbiodiniaceae include dinoflagellates that form symbioses with an astonishing diversity of mostly invertebrate hosts in tropical and sub-tropical marine habitats (LaJeunesse et al. 2018). Photosynthetically derived nutrients translocated from the dinoflagellate partner to the host can represent a large part of the host's energy budget (e.g., Muscatine 1990; Whitehead and Douglas 2003; Weisz et al. 2010; Davy et al. 2012; Hill 2014; Schlichter 1982), which contributes to the impressive coral growth in shallow oligotrophic tropical waters (Venn et al. 2008). The dinoflagellates appear to receive nutrients from the host, though the intricacies of that exchange are still poorly resolved (Davy et al. 2012). In corals, a great deal of interest has focused on the initiation of the host-symbiont partnership and the process of horizontal transmission (Ali et al. 2019). The emphasis is most often at a coarse level: Hosts "acquire" symbionts without much focus on the intricate cellular processes that permit invasion of host cells and cross-generational stability. However, several studies have provided a foundation for understanding the host genetic response and cellular mechanisms governing these mutualisms (e.g., reviewed in Weis 2019).

Chlorella-based symbioses are ecologically important features of freshwater ecosystems, and our understanding of aspects of intracellular residency has been improved through detailed study of these symbioses. For instance, the cellular and genetic pathways involved in *Chlorella* infection of *Paramecium bursaria* have begun to be elucidated (e.g., Kodama et al. 2014). *Chlorella* occupy perialgal vacuoles

derived from digestive vacuoles in *P. bursaria*, and growth of the host is impacted by algal presence (Karakashian 1963). Although the *Chlorella* symbionts appear to avoid digestion through patterns of spatial distribution within *Paramecium*, and can persist despite lysosomal fusion with the perialgal membrane, important molecular aspects of the infection process remain unclear (Kodama and Fujishima 2005, 2007, 2012; Kodama et al. 2007). Recent work in the *P. bursaria*: *Chlorella* association points to RNA-interference mechanisms to stabilize the partnership by imposing a cost for the host to break the symbiosis down (Jenkins et al. 2021). Another *Chlorella*-based symbiotic partnership involves freshwater cnidarians who receive photosynthetically fixed carbon from the algae (Muscatine and Hand 1958). *Hydra* spp. typically maintain *Chlorella* in their gastrodermal cells in organelles surrounded by a perialgal membrane of host origin. During intracellular residency by the symbiont, *Hydra* hosts engage distinct molecular and cellular pathways, including the upregulation of phosphate transporters and glutamine synthetases (e.g., Ishikawa et al. 2016; Hamada et al. 2018).

In addition to the important symbiotic systems mentioned above, members of the phylum Porifera harbor a diverse assortment of photosynthetic partners (Taylor et al. 2007), including intracellular relationships with members of the Symbiodiniaceae (Hill 1996; Hill et al. 2011; Strehlow et al. 2016; Ramsby et al. 2017) and *Chlorella*-like algae (Hall et al. 2021). We recently introduced the freshwater sponge, *Ephydatia muelleri* and its *Chlorella*-like algal symbiont as a tractable model system to study endosymbiosis (Hall et al. 2021). In this facultative association, both interacting species are easily grown alone and in symbiosis, making it possible to study the mechanisms that lead to successful partnerships in a relationship that is mutually beneficial but not fully interdependent. The exact mechanical process through which symbionts enter and disperse throughout these sponges is still relatively unknown, but they can be taken up by, and divide in, a variety of cells including choanocytes (Wilkinson 1980). Symbionts are found in intracellular compartments as early as 4 h post algal infection and persist in regions surrounding choanocyte chambers (Hall et al. 2021).

The goal of this work was to discern pathways that differentiate successful intracellular residency of a potential

mutualistic symbiont from digestion of prey/destruction of foreign entities. To answer this question, we employed a classic experimental approach combined with advanced molecular tools. We hatched sponges from gemmules, and then fed the sponges three different potential food sources: living bacteria (a typical prey item), living sponge-derived algae (a potential symbiont), and sponge-derived algal cells that had been heat-killed (a potential food item that might have intact symbiosis-related antigens). We sequenced the transcriptomes of aposymbiotic (“control”) sponges and the three feeding treatments, and then studied the differential expression of the genes between and among the four treatments. Experiments examining the function of one transcript (*abcc4*) that is downregulated in algal-infected sponges relative to aposymbiotic sponges are presented. This work represents an effort to gain important perspectives on these ecologically essential symbiotic interactions at a fine scale genetic resolution.

Results and Discussion

Our goal was to examine patterns of gene expression by sponges 4 h after exposure to native algal symbionts or potential food items (bacteria and heat-killed native algae). We infected aposymbiotic (APO) *E. muelleri* with heat-killed microalgae (HK-CHLO), bacterial food items (bacterial artificial chromosome [BAC]), and live microalgae (CHLO) to differentiate the host genetic response to establishment of native symbionts from cellular processes activated when potential food items are processed or when the host is presented with nonviable algae that may have epitopes of living algal cells (fig. 1).

The work presented here builds on previous work developing the *E. muelleri*: *Chlorella*-like symbiosis as a model lab-based experimental system (Hall et al. 2021). The *Chlorella*-like green algal symbionts can be isolated and successfully

cultured from wild *E. muelleri* populations. The pure algal cultures can be used to infect “aposymbiotic” *E. muelleri* grown from gemmules (Hall et al. 2021). Confocal and electron microscopy has verified that algae and bacteria can be found inside host cells between 1 and 4 h after infection, and that live algae persist and divide within the host while bacteria and other potential prey are digested (fig. 2; Hall et al. 2021). The RNA-Seq experiment comparing aposymbiotic and algae-infected sponges showed that a variety of conserved pathways were activated in response to the symbionts 1 day after infection (Hall et al. 2021).

RNA Sequencing and Assembly

We obtained a total of 331.4 million raw reads, and half were retained during the stringent filtering process (over 143 million reads, see [supplementary table S1, Supplementary Material](#) online). For two samples (one in the aposymbiotic—APO—and one in the living algal fed—CHLO—treatments), the sequencing was not optimal and they were both discarded for the subsequent gene expression analysis. Between 50% and 60% of the reads for each sample were mapped unequivocally to the reference transcriptome constructed with the filtered reads ([supplementary table S1, Supplementary Material](#) online). The assembly contained 218,986 transcripts and 117,619 genes, with an N50 of 1,527 bp, and a GC content of 45.99% ([supplementary table S1, Supplementary Material](#) online). The completeness of the transcriptome was high, with over 97% of eukaryotic and over 90% of metazoan conserved genes recovered as complete using BUSCO ([supplementary table S1, Supplementary Material](#) online).

Thirty two percent of the transcripts in the reference transcriptome obtained a Basic Local Alignment Search Tool (BLAST) hit against *refseq* ([supplementary table S1, Supplementary Material](#) online), and as expected, the

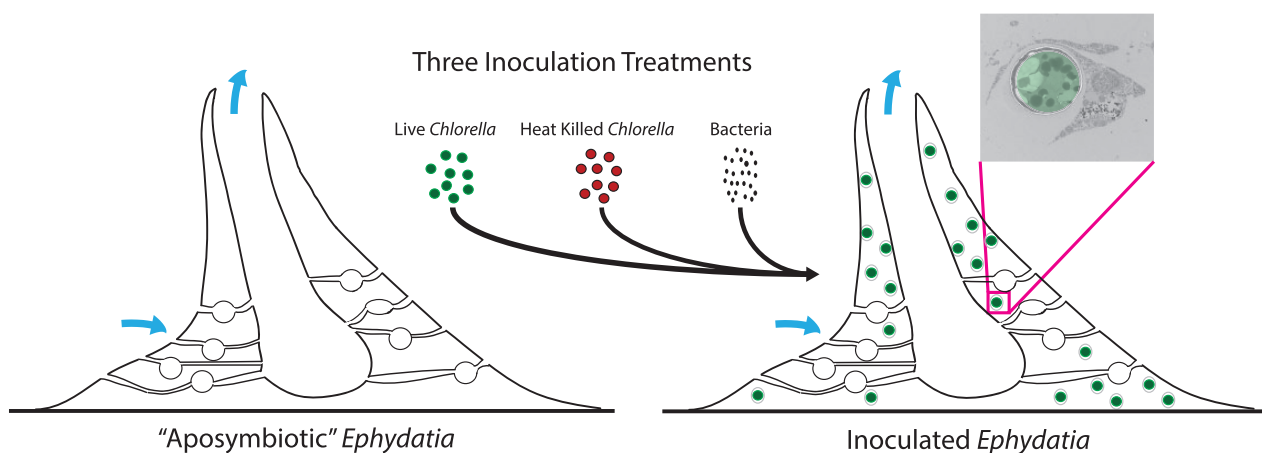


FIG. 1.—Schematic representation of infection and feeding experiments. Biological triplicate treatments of algal-free “aposymbiotic” sponges inoculated with live algae, heat-killed algae, or bacteria. After Hall et al. (2021).

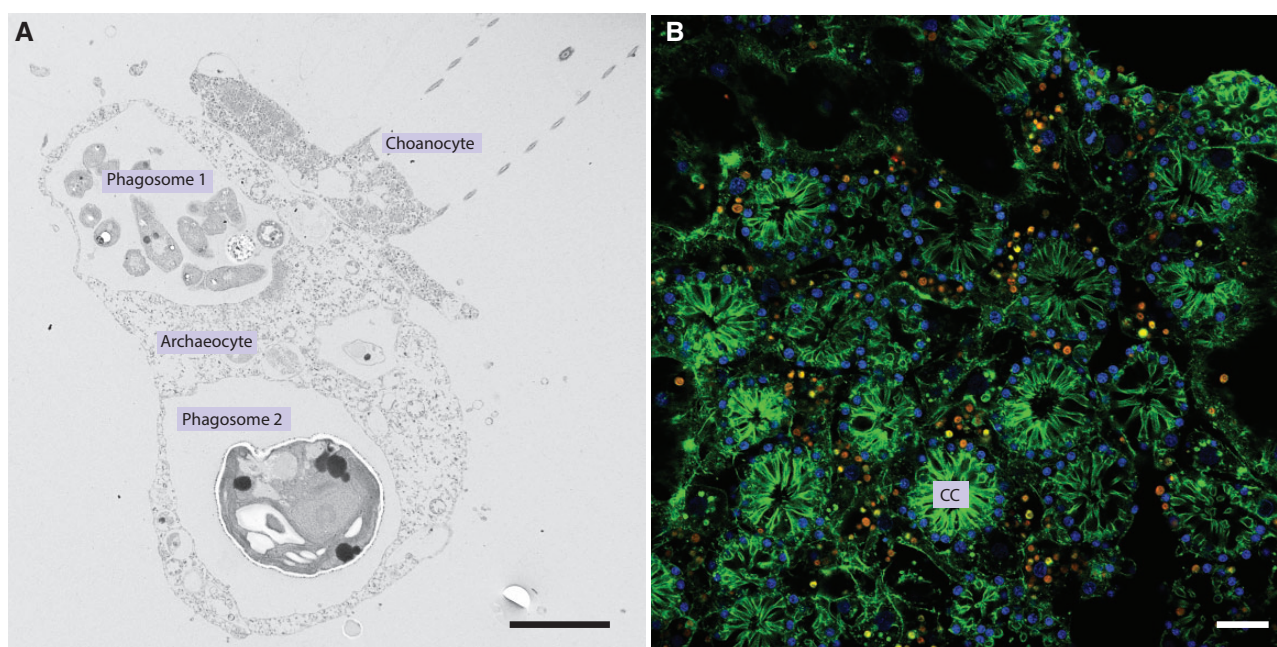


Fig. 2.—Electron and confocal microscopy of *E. muelleri* cells 4 h postinfection. (A) Transmission electron microscopy image of sponge choanocyte cell abutting an archeocyte cell containing two phagosomes. Phagosome 1 contains multiple bacteria and Phagosome 2 contains one algal cell. Scale bar 2 μm . (B) Confocal image of sponge choanocyte region showing intracellular algal symbionts (red) in and around choanocytes (CC, choanocyte chamber). Image shows DNA in blue, F-actin in green, and autofluorescence of algal cells in red. Scale bar 30 μm .

reference transcriptome contained a mix of bacterial, algal, and sponge genes (supplementary fig. S1, Supplementary Material online). The majority of genes were of sponge origin (88.9%), though we also observed transcripts representing bacterial genes from the *Escherichia coli* used in feeding treatments, the bacterial symbionts carried by the sponge gemmules, and algal genes from the native algal symbiont. The bacterial and algal genes represented 5.4% of the reference transcriptome. A very small portion of the genes was assigned to other organisms, like viruses or protists (5.7%). Almost all sequences with a BLAST hit (87%) could be further annotated with Blast2gopro (Götz et al. 2008) (supplementary table S1, Supplementary Material online).

Our analysis revealed differential gene expression (DGE) between algal-free “control” sponges (aposymbiotic or APO) and each experimental treatment (i.e., bacteria-fed [BAC], heat-killed algae fed [HK-CHLO], live native algae infection [CHLO]) at 4 h postinoculation (supplementary fig. S2, Supplementary Material online). DGE analysis in edgeR (McCarthy et al. 2012) is designed to accommodate comparisons that involve sample sizes of two or more as in our study. The majority of differentially regulated genes were found between the APO or BAC treatments and either of the algal feeding treatments (supplementary figs. S3–S8, Supplementary Material online). In fact, the APO and BAC treatments had very similar expression patterns (supplementary fig. S9, Supplementary Material online) and therefore very few genes were significantly differentially expressed

between these two treatments (i.e., only 24 genes showed expression differences between aposymbiotic and bacteria fed sponges, supplementary table S2, Supplementary Material online). This result is somewhat unsurprising given that sponge gemmules carry associated bacteria that can be digested as food so the two treatment types (APO and BAC) were very similar in terms of effects on the host sponge. Thus, given the stable expression profiles between bacteria-fed sponges and aposymbiotic sponges (supplementary figs. S2 and S7, Supplementary Material online), we treated these conditions similarly when comparing gene expression with sponges fed live or heat-killed algae.

Only around 30% of the genes that were uniquely upregulated in CHLO or HK-CHLO treated sponges were annotated with proteins of known function (fig. 3). The remaining genes were either uncharacterized proteins or putative sponge-specific proteins of unknown function (supplementary table S2, Supplementary Material online). It should be noted that 74% of the genes in the recently sequenced chromosomal-level genome of *E. muelleri* are known to have similarity to other organisms, and half of all *E. muelleri* genes can be assigned to full functional annotations using BLAST2GO (Kenny et al. 2020), and *E. muelleri* gene identification numbers for annotated proteins of known function discussed below are provided (supplementary table S2, Supplementary Material online). The symbiosis-specific genes here are thus enriched regarding the smaller portion of the freshwater sponge genome that is uncharacterized at the protein level.

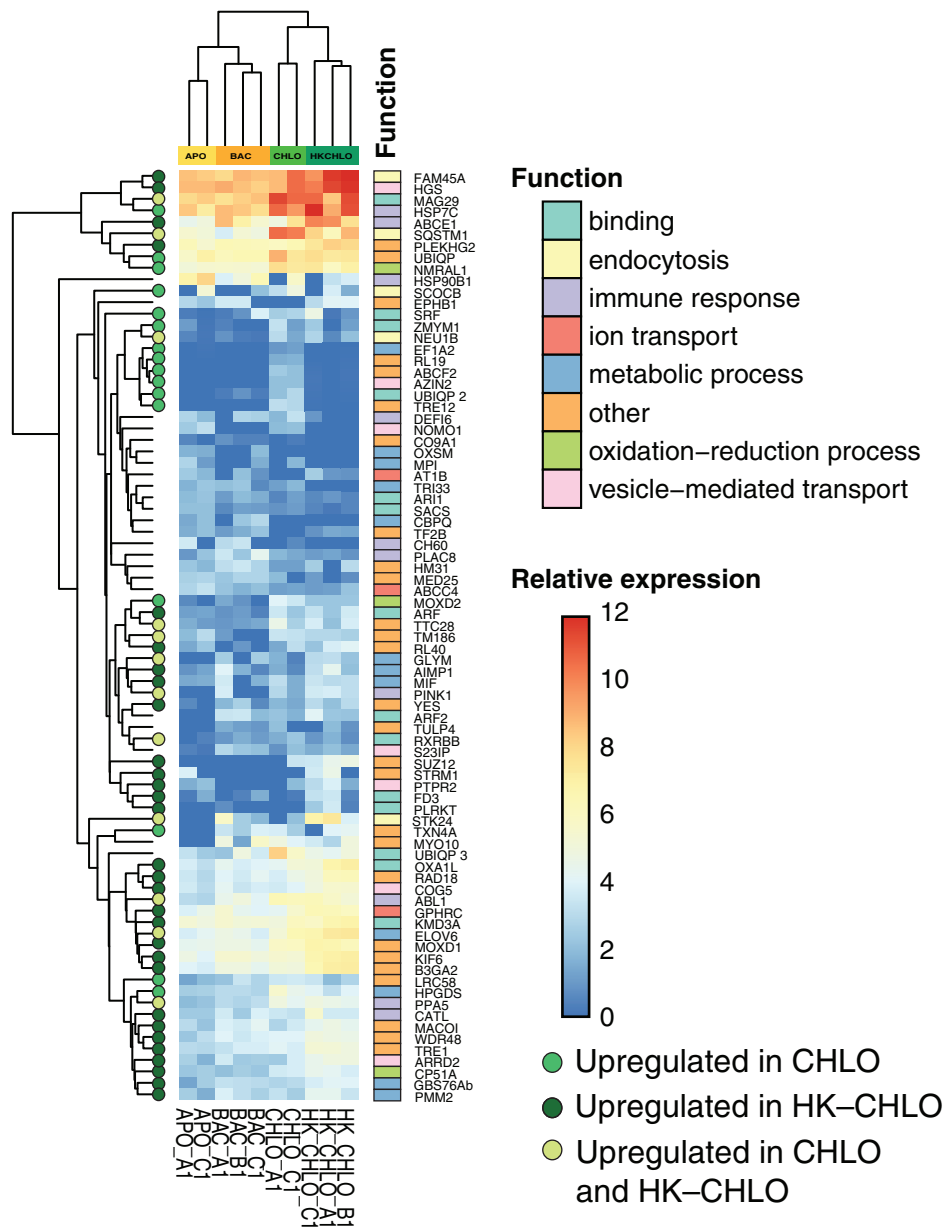


FIG. 3.—Heat map of subset of differentially expressed genes with BLAST annotations. Heatmap shows significantly (≥ 2 -fold-change and a P -value of ≤ 0.001) differentially expressed genes across four treatments (relative expression color coding: blue, low expression; red, high expression). Dark green dots show genes uniquely upregulated in HK-CHLO treatment, medium green dots show genes uniquely upregulated in CHLO treatment, and light green dots show genes upregulated in both treatments. Category functions of genes are represented with different colors. Note that HK-CHLO and BAC had three replicates whereas APO and CHLO had two replicates.

Thus, our data can only describe a subset of the genes that likely play roles in the regulation of stable symbioses until further work on this large body of uncharacterized protein-encoding genes moves forward. Most of the genes upregulated in CHLO and HK-CHLO treated sponges were involved in vesicle-mediated transport, endocytosis, protein binding, immune system, and oxidation–reduction, whereas most genes upregulated in APO or BAC treated sponges were involved in metabolic processes (fig. 3).

Putative Symbiosis-Related Genes Involved in Stable Residency

The association of freshwater sponges with *Chlorella*-like algae is facultative because the host and symbiont have the capacity to (and in fact must) live some portion of their life cycles separate from one another. This is the case in many phototroph: heterotroph partnerships that involve vertical transmission (e.g., Baird et al. 2009). When they do associate,

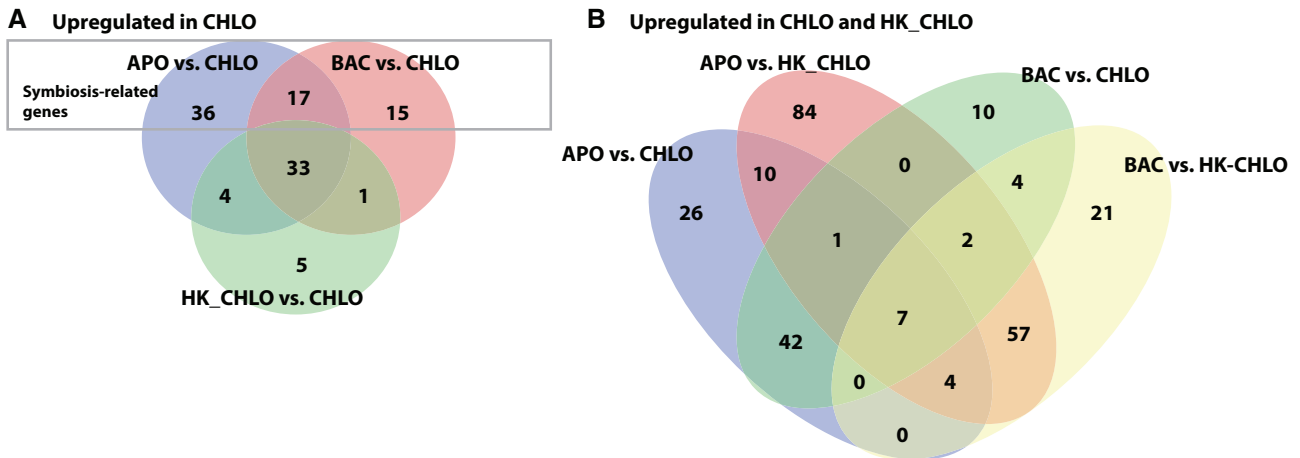


FIG. 4.—Venn Diagram comparing differentially expressed genes. (A) Genes upregulated in CHLO sponges when compared with APO sponges, BAC sponges, and HK-CHLO sponges. (B) Genes upregulated in either CHLO, HK-CHLO sponges or both.

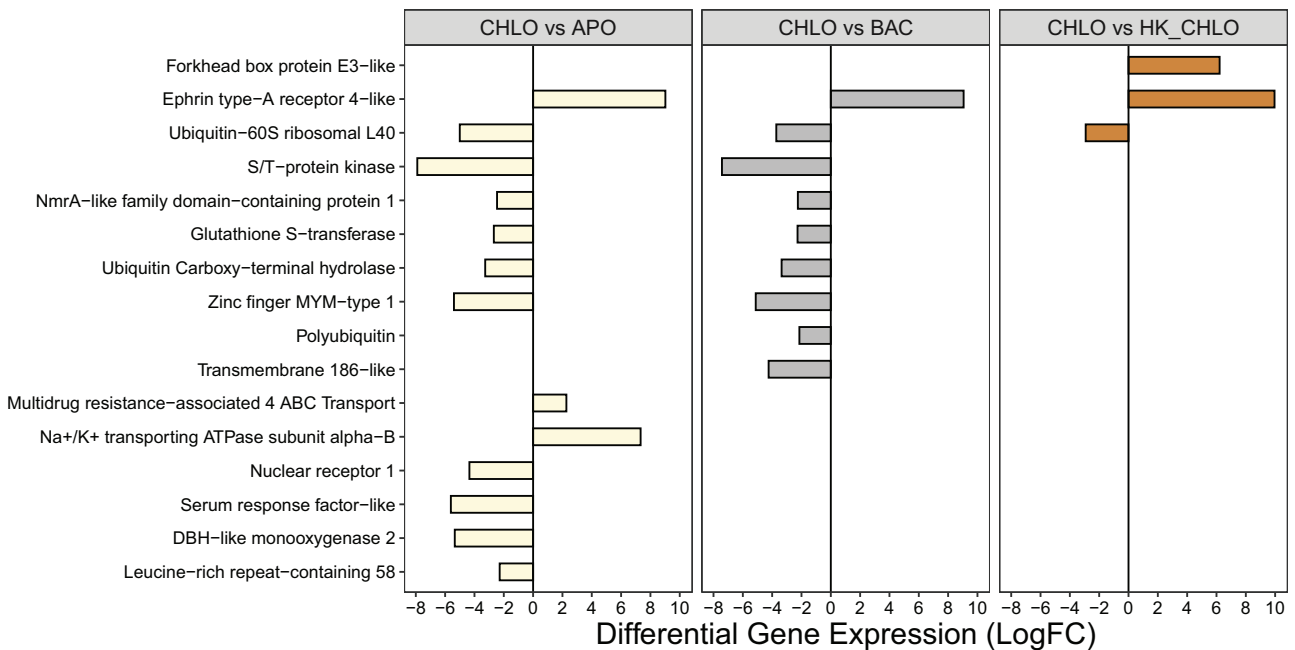


FIG. 5.—Symbiosis-specific differentially expressed genes. Log-fold changes in expression ($P < 0.001$) when comparing CHLO with each of the other three treatments of algal-free APO, BAC, and HK-CHLO. Genes with increased expression in live algal-infected sponges compared with given treatments have negative LogFC values and are shown to the left of the solid line in the graph. Only genes with clear BLAST annotations are shown.

it is unclear how the phagotrophic sponge differentiates potential mutualists from potential prey. We studied the gene expression profiles among the “digesting” treatments (BAC or HK-CHLO) compared with the live algal treatment (CHLO). We examined any differentially regulated genes exclusively found to CHLO compared with APO or BAC but not found in HK-CHLO. We found 68 genes that were uniquely regulated in CHLO (fig. 4A). We considered these genes to be putative “symbiosis related genes” involved in stable residency (figs. 3 and 4A).

Among this set of differentially regulated genes ($P < 0.001$), some had been previously found to be differentially regulated at 24 h postinfection in symbiotic compared with aposymbiotic *E. muelleri* (Hall et al. 2021). Genes significantly upregulated at both 4 and 24 h postinfection include a *DBH-like monooxygenase (moxd2)*, *thioredoxin 4 (txn4a)*, *leucine-rich repeat containing 58 (lrc58)*, a *glutathione S-transferase (Hematopoietic prostaglandin D synthase, hpgds)*, and an *NmrA-like family domain-containing protein 1 (nmr11)* (figs. 3 and 5, supplementary table S2, Supplementary

Material online). MOXD2, TXN4A, and NMRL1 may be involved in cellular oxidative–reductive systems. For example, in humans, the NmrA-like family domain-containing protein 1 (NMRL1) functions as a redox sensor that is responsive to NADPH/NADP⁺ levels and helps moderate the production of nitric oxide and reduce apoptosis (Zhao et al. 2008). Indeed, when the photosynthesis of the algae starts, reactive oxygen species (ROS) are released, with the potential of damaging host cell elements (Hamada et al. 2018). Therefore, an appropriate oxidative stress response is usually deployed by the host, as it occurs in the symbiotic partnership between *Chlorella* and *Hydra* (Hamada et al. 2018). But NMRL1 is also known to negatively regulate the activity of NF- κ B (Gan et al. 2009), a protein whose negative regulation has been implicated in the host response to symbionts in corals (Weis 2019). The finding that multiple DBH-like monooxygenases were also regulated at 24 h postinfection (Hall et al. 2021) points to a possible role for these enzymes in the onset of algal symbiosis or to a more generalized role of ROS signaling in phagocytosis events. Indeed, the finding that multiple glutathione S-transferase genes are upregulated at 24 h postinfection (Hall et al. 2021), along with the upregulated *glutathione S-transferase* found here, suggests that this gene family may be important for cellular detoxification or for promoting cell growth and viability during symbiosis.

On the other hand, several genes that were uniquely upregulated at significant levels in the 4 h postinfection treatment were present but not differentially expressed in the 24 h algal symbiosis reference transcriptome (Hall et al. 2021). One of these genes is a *MADS box-containing serum response factor* (*Serum response factor-like*, figs. 3 and 5, and [supplementary table S2, Supplementary Material](#) online). These genes have been implicated as immediate early genes (IEGs) that can be activated in response to intrinsic and extrinsic cellular signals and be transcribed rapidly after stimulation (Bahrami and Drabløs 2016). Also included in the upregulated genes is a *Nuclear receptor (nr1) or Retinoic acid receptor RXR-beta-B (rxrbb)* gene (figs. 3 and 5, and [supplementary table S2, Supplementary Material](#) online). These genes are known to function as transcriptional regulators involved in homeostasis, metabolism, and development (Hwang et al. 2014) and can directly translate the message of signaling molecules into a transcriptional response (Miglioli et al. 2021). The upregulation of a probable *ubiquitin carboxy-terminal hydrolase (FAM188b)* in algal-fed sponges as compared with aposymbiotic and bacterial fed sponges is notable (figs. 3 and 5, and [supplementary table S2, Supplementary Material](#) online). This enzyme likely has both ligase and hydrolase activity to recycle and remove ubiquitin from degraded proteins as well as linking molecules of ubiquitin for tagging proteins to be discarded. The possibility that ubiquitin carboxy-terminal hydrolase can suppress activation of NF- κ B and increase cellular ROS is particularly interesting, indicating a possible role in oxidative stress and immune reaction (reviewed in

Matuszczak et al. 2020). This protein is also known to play roles in regulation of apoptosis and extrinsic apoptotic signaling and immune defense response. Coupled to this finding, we see upregulation of two ubiquitin genes (likely *polyubiquitin* and *ubiquitin-60S ribosomal L40 [ubiqp]*, figs. 3 and 5, and [supplementary table S2, Supplementary Material](#) online), both of which are implicated in the peroxisome proliferator-activated receptor (PPAR) signaling pathway which has roles in metabolism, cell proliferation/differentiation, as well as immune cell function (Le Menn and Neels 2018). This is of particular interest because we find upregulation of an *nr1* gene. The upregulated *nr1* gene is part of a superfamily that includes PPARs, suggesting that these genes may be part of the same pathway activated by infection with algal symbionts.

We found relatively few genes that are downregulated in response to symbiosis at 4 h postinfection (fig. 5, [Supplemental table S2, Supplementary Material](#) online). Interestingly, two of the downregulated genes are active membrane transporters. One, a *Na⁺/K⁺ transporting ATPase subunit alpha-1* gene, codes for a P-type cation transport ATPase integral membrane protein that can serve to regulate electrochemical gradients by pumping cations across the membrane using primary active transport (Zhiqin and Langhans 2015). The other is a primary active transporter of the *multidrug resistance-associated 4 ABC transporter group (mcr4 or abcc4-like)* which is explored in greater detail as described below. We wonder if the downregulation of these transporters may be involved in a mechanism that initiates phagosome arrest, leading to sustained residency in the host cell. Another gene that is decreased in expression in live algal-infected sponges is an *ephrin type-A receptor 4-like* gene (*epb1a*). These proteins are members of a large family of receptor tyrosine kinases that mediate cellular processes, including components of immune function as well as phagocytosis, by interacting with membrane-bound ephrin ligands (Darling and Lamb 2019). Expression of *epb1a* was decreased by 9–10 fold in the CHLO treated sponges when compared with every other treatment (fig. 5). The role of these receptors in immune cell activation, as well as mediating immune cell trafficking and implication as receptors for some viral and other pathogens (e.g., malaria parasite) (Darling and Lamb 2019), makes them interesting candidates to explore in regard to establishing this mutualism. Furthermore, downregulation of *epb1a* in CHLO is contrasted to the upregulation of a distinct, other ephrin-type receptor-like gene in HK-CHLO (discussed below), indicating possible roles for these types of receptors in distinguishing symbionts from food.

Genes Expressed in Heat-Killed Algal Infections

Phagocytosis is an essential cellular process that typically involves the internalization of particles > 0.5 μ m. Phagocytotically driven processes shape many dynamic cellular operations, including development and the destruction of

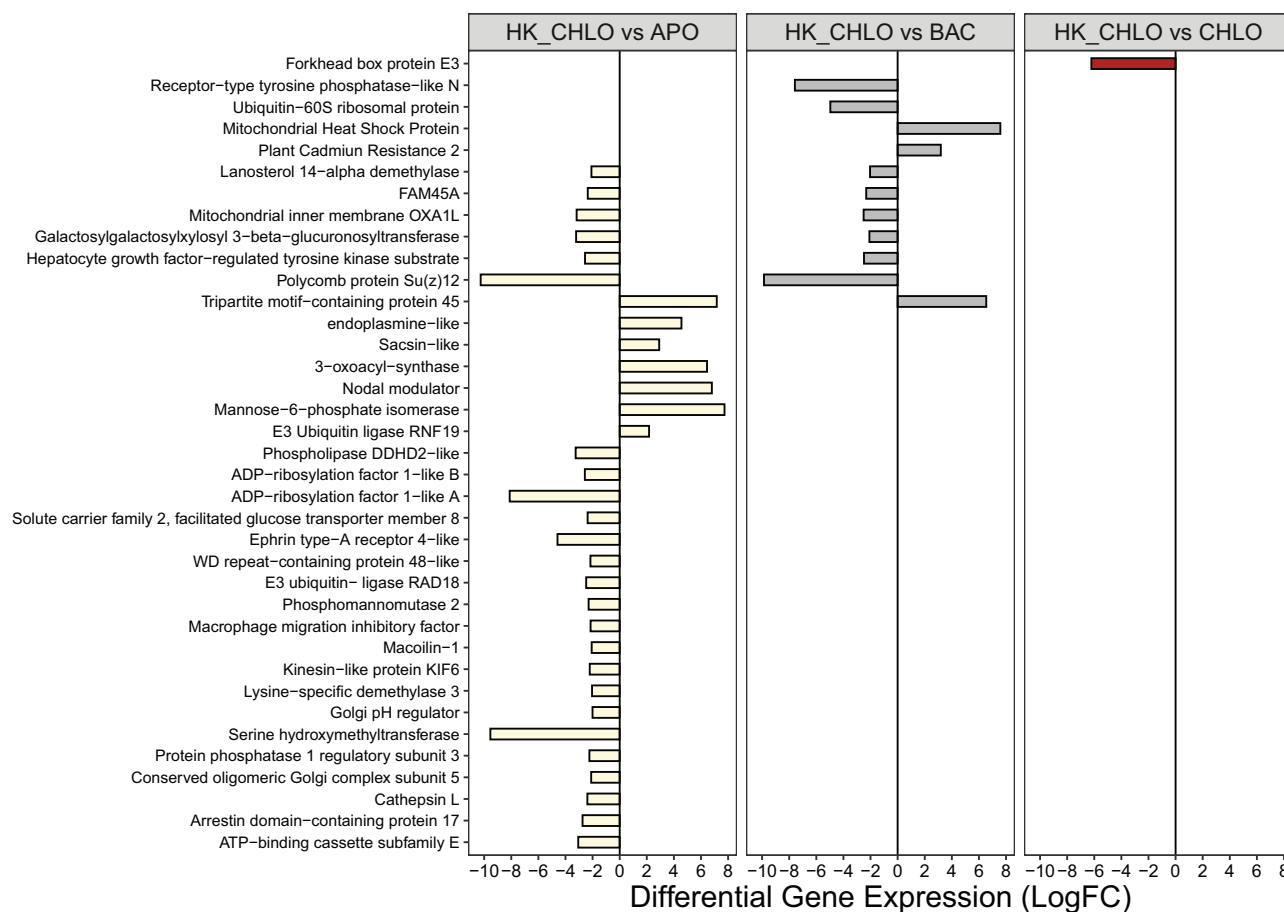


Fig. 6.—Heat-killed specific differentially expressed genes. Log-fold changes in expression ($P < 0.001$) when comparing sponges-fed heat-killed algae to each of the other three treatments of algal-free APO, BAC, and CHLO sponges. Annotated genes with increased expression in heat-killed algae-fed sponges compared with given treatments have negative LogFC values and are shown to the left of the solid line in the graph.

invading pathogens (Kerr et al. 1972; Ren and Savill 1998; Vaux and Korsmeyer 1999). In the case of the latter, phagocytosis plays a role in linking innate and acquired immunity (Savina and Amigorena 2007). For example, Boulais et al. (2010) compared the proteomes of 39 taxa (from protists to mammals) and identified an ancient core of phagosomal proteins primarily involved in phagotrophy and innate immunity. For some heterotrophic organisms, like bacterivorous sponges, phagocytosis is the primary strategy available to capture food. However, phagocytosis is also the major route of entry for symbionts during vertical transmission (e.g., Koutsouveli et al. 2020), and phototrophic symbionts may co-opt cellular machinery using strategies that are similar to those used by parasites to invade eukaryotic cells (Schwarz 2008). Given the fact that *E. muelleri* sponges infected with live algae (CHLO) turn green after several days of incubation and have growing populations of intracellular algae in their cells, whereas *E. muelleri* sponges infected with heat-killed algae (HK-CHLO) do not turn green after incubation, we assume that the heat-killed algae are readily digested by phagocytosis.

Interestingly, we found a larger number of genes ($n = 84$) to be differentially regulated in the APO versus HK-CHLO comparison than in the APO versus CHLO comparison (fig. 4). The genes uniquely regulated in response to HK-CHLO (fig. 6) were considered to be candidates for phagocytosis and immunity-related pathways that may have subtle but important roles in the creation of stable symbiotic states. Among these, differentially regulated genes with receptor-related activities were identified.

One upregulated gene was an *arrestin domain-containing protein* (supplementary table S2, Supplementary Material online). Arrestins are known to selectively bind to G protein coupled receptors (GPCRs) and other classes of cell surface receptors to control information flow by binding to phosphorylated receptors and blocking further signaling (Chen et al. 2018). The complexes formed between β -arrestin and GPCRs occupied by ligands become targeted for endocytosis and, ultimately, induce internalization of the receptors (Ferguson et al. 1996). We also found a *hepatocyte growth factor-regulated tyrosine kinase substrate* (*hrs*) to be increased in

expression in HK-CHLO. These proteins are known to regulate trafficking, degradation, and recycling of GPCRs as part of protein complexes localized on endosomal membranes (Roux et al. 2017). In vertebrate cell lines, HRs associate with phagosomes prior to endosome/lysosome fusion, and depletion of HRs impairs the maturation of phagosomes. We find parallels in the infection of cells by mycobacteria—particularly avirulent mycobacteria—which leads to impaired recruitment of HRs to phagosomes and contributes to the arrest of phagosomal maturation (Vieira et al. 2004). The increase in *hrs* expression we observed may point to a role for this protein in phagosomal maturation leading to digestion of the heat-killed prey. This gene had decreased but nonsignificant expression in CHLO treated sponges, and may deserve greater attention to determine if it has a role in arresting the phagosome, which might lead to persistent intracellular residency.

Additionally, a *receptor-type tyrosine phosphatase-like N* gene and an *ephrin type receptor* gene (*tyrosine-protein kinase Yes, yes*) were expressed at higher levels in HK-CHLO treatments (figs. 3 and 6). As discussed earlier, another transcript encoding an *ephrin type-A receptor 4-like* gene was downregulated in sponges infected with live algae. These proteins are members of a large family of receptor tyrosine kinases that play a role in immune function as well as phagocytosis, by interacting with membrane bound ephrin ligands (Darling and Lamb 2019). Activation of these receptors, especially the *ephrin receptor-like* gene, may indicate a role for immune or phagocytic pathways in response to the heat-killed algae, and these genes might be activated during digestion of prey. Hamada et al. (2018) found that an *ephrin type-A receptor* was increased in expression in symbiotic *Hydra* compared with aposymbiotic *Hydra*, but not in a light dependent manner. The significance of the divergent results remains unclear, but the *ephrin receptor-like* gene deserves greater attention in this symbiosis.

We were also intrigued by a variety of genes that play roles in endocytosis and lysosomal processes and were upregulated in the HK-CHLO treatment (figs. 3 and 6) including *macrophage migration inhibitory factor* (*mif*), a gene with many roles in mediating immune response (Calandra and Roger 2003), a *conserved oligomeric Golgi complex subunit 5* gene (*cog5*), involved in intra-Golgi vesicle mediated transport, as well as a *Golgi pH regulator* gene (*gphrc*). Golgi pH regulators are anion channels that are required for acidification and other Golgi functions (Maeda et al. 2008). Two *ADP-ribosylation factor 1-like* genes (*arf* and *arf2*), which are members of the Ras superfamily and play roles in post-Golgi membrane trafficking (Shin et al. 2005), and a *fam45a* gene, which is involved in regulation of early to late endosomal transport and is a guanine nucleotide exchange factor for Rab small GTPases (Zhang et al. 2019), were upregulated. The upregulation of these genes support the hypothesis that digestive processes, such as the engulfment of the heat-killed algae

via endocytosis and digestion via lysosomal fusion, are uniquely upregulated in the heat-killed algae treatment and suppressed in the live-algae treatment.

Finally, among the sponge genes upregulated in response to HK-CHLO (figs. 3 and 6), *ATP-binding cassette subfamily E* (*abce1*) is known to act as an inhibitor to block the activity of ribonuclease L. Ribonuclease L inhibits protein synthesis involving interferons, which are released during a viral infection to alert nearby cells to increase immune activity. As such, expression of *abce1* can promote activity leading to an increase in antiviral defense mechanisms. Although there is much work to be done to examine the roles of differentially regulated sponge genes in response to HK-CHLO, it is clear that many of these genes are candidates for phagocytosis pathways and are related to the digestion of food particles.

Genes Regulated in Live and Heat-Killed Algal Treatments Are Enriched for Immune Function Roles

We hypothesized that comparing sponges-fed heat-killed algae with those fed live algae might allow us to identify genes with altered expression in both treatments that result as a response to algal-specific epitopes (figs. 3, 4, and 7). These genes may be candidates for understanding species-specific recognition pathways or common pathways in initiation of phagocytosis.

The protein Cathepsin L, known for roles in innate immunity as an endolysosomal protease, is upregulated in response to both CHLO and HK-CHLO (fig. 3, [supplementary table S2, Supplementary Material](#) online) and was also found upregulated in response to live algal symbionts 24 h after infection (Hall et al. 2021). This is interesting because *cathepsin L* has also been found upregulated in response to *Vibrio* spp. in mollusks and freshwater prawn in response to bacterial and viral infections. It plays a role in cell death in the superficial light-organ tissue in *Euprymna scolopes* colonized by *Vibrio fischeri* at the onset of symbiosis (reviewed in Peyer et al. 2018). Cathepsin L plays numerous roles in immune function, including phagocytosis and nonoxidative killing of bacteria (Müller et al. 2014), and at least one obligate intracellular pathogen is known to manipulate Cathepsin L activity as a strategy to alter neutrophil function so that the pathogen maintains infection and can proliferate inside the host cell (e.g., Thomas et al. 2008).

The other genes that were regulated in both HK-CHLO and CHLO treatments are enriched for roles in immune function (figs. 3 and 7). *Tyrosine protein kinase Lyn* (*abl1*), upregulated both in HK-CHLO and CHLO treatments (fig. 3), is often involved in regulation of signaling pathways resulting in modulation of immune cell activation (Xu et al. 2005). *Tartrate-resistant acid phosphatase 5* (*ppa5*) encodes a lysosomal metalloenzyme expressed known to be expressed in macrophages to promote inflammation and immune cell activity and is involved in gene ontologies such as negative regulation

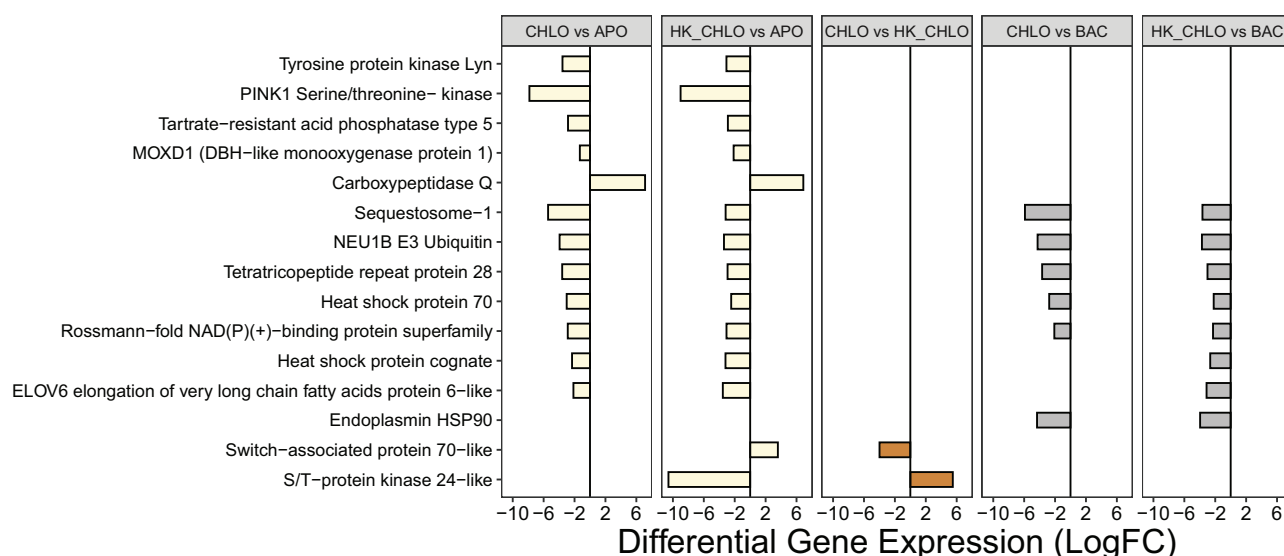


Fig. 7.—Algal-specific differentially expressed genes. Log-fold changes in expression ($P < 0.001$) when comparing sponges-fed heat-killed algae and live algae to each of the other three treatments of algal-free APO, BAC, and CHLO sponges. Annotated genes with increased expression in heat-killed algae or live algal-fed sponges compared with given treatments have negative LogFC values and are shown to the left of the solid line in the graph.

of tumor necrosis factor production and negative regulation of nitric oxide biosynthetic process (The UniProt Consortium 2021). Both of these genes continue to be upregulated at 24h postinfection in this symbiosis (Hall et al. 2021). Furthermore, the gene coding for a *serine/threonine kinase* (*pink1*) is upregulated in heat-killed and algal-fed sponges (figs. 3 and 7). This gene plays roles in protecting cells from stress-induced mitochondrial dysfunction by setting the stage for mitophagy as part of the innate immune response (Song et al. 2020). Recently, PINK1 was shown to modulate antiviral immune response through the regulation of *TNF receptor-associated factor 3* (Zhou et al. 2019), another gene we find to be upregulated in algal-infected sponges (Hall et al. 2021). We also find *sequestosome-1* (*sqstm1*), an autophagy receptor to be upregulated in heat-killed and algal-infected sponges (figs. 3 and 7). This gene may be involved in regulating the activation of NF-kappaB as well as cell differentiation (The UniProt Consortium 2021) and is involved in isolation of cargos degraded by autophagy, induction of antioxidant responses, and regulation of inflammation, apoptosis and endosomal trafficking. Interestingly, sequestosome-1 also plays important roles during PINK1-mediated mitophagy and is linked with some E3 ubiquitin ligases for its roles in ubiquitylation, autophagy, and recruitment of endocytotic vesicles (reviewed in Sánchez-Martín and Komatsu 2018). Of note, we do find an *E3 Ubiquitin Protein Ligase 1B-like* gene (*neu1b*) among the upregulated transcripts in our heat-killed and live algal-fed sponge treatments (figs. 3 and 7). This may indicate conserved connections between these immune pathway regulators that are yet to be elucidated.

The upregulation of expression of several heat shock proteins in live and heat killed algal-fed sponges is not surprising

(figs. 3 and 7), as heat shock proteins play crucial roles as components of cellular response to stress, modulation of phagocytosis and immune function (e.g., reviewed in Lee and Repasky 2012; Zininga et al. 2018). Here, we find a *heat shock protein 70* (*hsp70*), known to stimulate phagocytosis of internalized antigens in macrophages (Wang et al. 2006), a *heat shock protein cognate* (*Heat shock cognate 71 kDa, hsp7c*), which can mediate LPS-induced inflammatory responses through modulation of NF-kappaB in macrophages (Sulistyowati et al. 2018), and *endoplasmic hsp90* (*hsp90b1*), which is an essential immune chaperone acting to fold proteins in secretory pathways and protect the integrity of associated antigens (Calderwood et al. 2016) all to be differentially expressed in response to algae. The role of heat shock proteins has been explored in coral symbioses, mostly in relation to thermal stress and bleaching (e.g., Leggat et al. 2011; Ross 2014; Ishii et al. 2019), but it is known from this literature that both algae and coral employ heat shock proteins during stress response (Weis 2008). The role of these proteins in modulating sponge immune responses or phagocytosis remains to be explored, but recent work by Schmittmann et al. (2021) showed that when the marine sponge *Halichondria panicea* is exposed to LPS, a complex set of innate immune genes are regulated that include signaling molecules and those involved in post-translational regulation of immune components like ubiquitylation and phosphorylation. Comparisons between marine and freshwater sponge symbioses will shed further light on the evolutionary conserved mechanisms of immune signaling.

Finally, we find a *switch-associated protein 70-like* gene (*Differentially expressed in FDCP 6 homolog, defi6*) to be upregulated in both treatments (figs. 3 and 7). This phosphatidylinositol 3,4,5-trisphosphate-dependent guanine nucleotide

exchange factor is known to be recruited to phagosomes, to transduce signals from tyrosine kinase receptors to Rac, and to mediate signaling of membrane ruffling by organization of actin cytoskeleton as an essential process for phagocytosis (Baranov et al. 2016). The differential regulation of the genes described above in live and heat-killed algal treatments here show that regardless of whether sponge cells will form stable symbioses with live algae or digest heat-killed algae, a variety of putative immune and phagocytosis response pathways are activated before the phagosome arrests or digests algae.

ABC Transporter Suppression during Onset of Symbiosis

ABC (ATP-Binding Cassette) transporters encompass the largest family of transmembrane proteins and originated in the first unicellular organisms (Rees et al. 2009). Using energy released by ATP hydrolysis, these transport proteins are responsible for the movement of ions and other molecules into the cell (influx) and out of the cell (efflux), and are respectively classified as importers and exporters (Vasilou et al. 2009). The ABCC subfamily consists of transporters that have a diverse functional spectrum, including ion transport, acting as cell surface receptors, and secretion of toxins (Dean et al. 2001). One distinct feature that many ABCC transporters possess that distinguish them from other ABC transporters is they are responsible for the transportation of glutathione conjugates or cotransport glutathione combined with a substrate (Stolarczyk et al. 2011).

Although there is limited evidence describing the role of ABC transporters in animal symbiotic relationships, many plant–bacteria interactions are facilitated by the host’s ABC transporters (Zhang et al. 2010) and, in animals, there is evidence of downregulated ABC transporter expression as a result of numerous types of infections (Hinoshita et al. 2001). In rats, an increase in a cytokine immune response results in a decrease of expression of ABC transporters and one ABC transporter (ABCC2) is downregulated by NF- κ B (Nakamura et al. 1999). We previously showed that a *TNF receptor-associated factor* in the NF- κ B signaling pathway was found to be significantly increased in expression at 24 h postinfection with live native algae (Hall et al. 2021). This *TNF receptor associated factor* is also known to be directly involved in cytokine production, which could potentially downregulate certain ABC transporters in *E. muelleri* as is suggested to occur in other model systems.

The *E. muelleri abcc4* gene (genome id Em0024g219) is 10,820 base pairs long and consists of 30 exons and 29 introns. There are multiple domains that identify it as a predicted ABCC transporter, including the multidrug resistance-associated protein domain with its highly conserved C-terminal region, the PLN3130 and ATP-binding cassette domain found on the nucleotide-binding (NBD) domains, and the Md1B domain encoding for the ATPase and permease component of the transporter. Included is the transmembrane (TMD) region of the protein that is composed of six transmembrane helices, thus indicating that a TMD is present.

The AAA domain also codes for an ATPase. Both of the ATPases encoded by separate domains are located in the NBD subunit, which is responsible for the hydrolysis of ATP to ADP. The N-terminal helix, which is poorly conserved across species, is not present in this sequence.

Although our sample size for each treatment is small ($n = 3$), *E. muelleri abcc4* expression was downregulated in CHLO treatment (fig. 5) 4 h after infection, but was further evaluated using qRT-PCR at four postinfection time points and compared with expression levels of aposymbiotic sponges cultured for the same time period under identical experimental conditions (fig. 8). It appears that after the initial downregulation of *abcc4* expression following symbiont introduction at 4 h postinfection, the levels of expression continued to decrease at 8 h postinfection and reached a minimum at 24 h postinfection. When measured at 48 h postinfection, the levels of *abcc4* expression began to increase again back to aposymbiotic levels. This trend was consistent when the data were normalized with *GAPDH* and *Ef1a* housekeeping genes (fig. 8).

To determine if the downregulation of *abcc4* in *E. muelleri* was a generalized response to live algal symbionts, or specific to native algal infection, we infected sponges hatched from gemmules collected from Bryant Park, VA, with native algae and with *Chlorella*-like algae isolated from *E. muelleri* sponges collected from Lake O’Connor, Canada (provided by Dr Sally Leys). Relative levels of gene expression for *E. muelleri abcc4* were evaluated for the heterologous cross infections. For Bryant Park sponges infected with algae isolated from Lake O’Connor *E. muelleri*, *abcc4* expression levels increased, but not significantly, when compared with aposymbiotic Bryant Park sponges (fig. 8). The change in *abcc4* expression in the infected sponges was not statistically significant with either housekeeping gene used for normalization. This data suggest that the geographic variant did not elicit the same response (downregulation of *abcc4*) as the native algae and that these sponges may form symbiotic relationships with closely related, but distinct algal populations.

Conclusions

Further work is needed to better understand the nature of these facultative phototroph: heterotroph partnerships in terms of how stable residencies within host cells can be achieved, but the possibilities provided by *E. muelleri* and its algal symbionts as a model for studying symbiotic relationships is promising. We have identified a suite of genes that are regulated early on the establishment of a stable symbiosis between *E. muelleri* and its native green algal symbionts. We have also begun to differentiate these genes from those involved in generalized phagocytosis events related to feeding and/or immunity. These include cellular oxidative–reductive systems, response to cellular signals, membrane and vesicle-mediated transport, metabolism, cell proliferation/differentiation, as well as immune cell function.

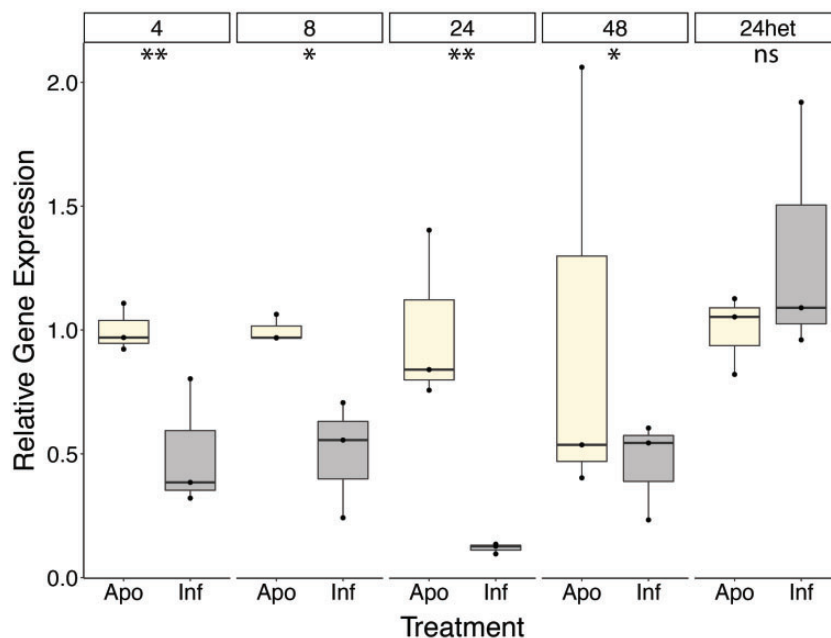


FIG. 8.—Differential *ABCC4* expression. Relative gene expression for *ABCC4* is shown for five different experiments for sponges infected with live algal symbionts relative to aposymbiotic age-matched sponges. Three independent replicates were compared between each treatment. Sponges infected with native symbionts at 4, 8, 24, and 48 h postinfection or with heterologous symbionts at 24 h postinfection are given. Error bars represent standard deviation. Two-tailed, two-sample independent *t*-tests were performed on deltaCt values (** $P < 0.001$; * $P < 0.05$; ns, not significant).

Materials and Methods

Sample Collection

Sponge gemmules were collected and processed as described in Hall et al. (2021). Sponges for this study were either collected in Richmond, VA, in Bryan Park (37°35'53.0" N, 77°28'06.3" W) under Virginia Department of Game and Inland Fisheries Permit #047944 or were obtained from Dr Sally Leys (Alberta) from a site at O'Connor Lake, Saskatchewan, Canada (61.3129° N, 111.8608° W). Gemmules were isolated, washed, and stored as described in Leys et al. (2019).

Algae Isolation

Native sponge algae were isolated as described in Hill et al. (2020). The algae were isolated either from adult tissue (Hall et al. 2021), in samples from Bryan Park, or from hatched gemmules, in samples from Lake O'Connor. Algal cultures from the isolates were grown at ± 22 °C under fluorescent light for 16 h per day in either Basal Medium (Sigma-Aldrich, Milwaukee, WI) or in Modified Bolds 3N Medium (UTEX, Austin, TX).

Infection Experiments on Sponges Hatched from Gemmules

Gemmules of *E. muelleri* from the same individual were used from stocks that were either frozen at -80 °C or held at 4 °C. Gemmules were hatched and cultured as described in Leys et al. (2019). Gemmules in 1X Strekal's were grown to

sponges up to the stage where a functioning osculum had developed. For each type of infection, triplicate samples with ~ 20 – 30 sponges per treatment were used. Live sponge-derived algal cells ("CHLO" treatment), algal cells that had been previously heat-killed by boiling for 3 min ("HK-CHLO" treatment), or living nonpathogenic K12 *Escherichia coli* bacteria ("BAC" treatment) were introduced into the water surrounding the sponge. In addition, one treatment of sponges received no infection and were considered the "control" (APO). Infections were conducted as described in Hill et al. (2020). Briefly, infections were initiated with 130,000 cells ml^{-1} harvested during the logarithmic portion of their growth phase. We estimated cell densities and population growth characteristics using optical density measurements at 425 and 675 nm for algae and 600 nm for bacteria. Algae or bacteria were resuspended in sponge culture media and slowly pipetted around and above the tissue to inoculate sponges and placed under fluorescent grow lights at room temperature for 4 h. Infected sponges used at later time points were placed under a 12:12 light: dark exposure.

Microscopy

Sponges were grown on 35 mm glass bottom dishes (MatTek Life Sciences) and either infected with live sponge-derived algae or left untreated. Fixation and imaging were conducted as described in Hall et al. (2021). Briefly, sponges were fixed in 4% paraformaldehyde and 1/4 Holtfreter's Solution overnight at 4 °C. After washing and permeabilization, tissue was stained

with Hoescht 33342 (1:200 dilution, Thermo Fisher Scientific, Waltham, MA) and Phalloidin Alexa 488 (1:40 dilution, Thermo Fisher Scientific, Waltham, MA) and imaged using an Olympus FV1200 laser scanning microscope using FluoView software.

Electron microscopy was performed as described in Hall et al. (2021). Briefly, sponge samples infected with live algae were fixed in 2.5% glutaraldehyde, washed in 0.2 M cacodylate buffer (pH 7.4), and postfixed with 1% OsO₄ and 1% uranyl acetate. Samples were dehydrated, infiltrated in propylene oxide, and embedded in Embed 812 plastic resin. Ultrathin sections were stained with uranyl acetate and lead citrate. Micrographs were taken using a JEOL 1010 transmission electron microscope at the University of Richmond with an Advanced Microscopy Techniques XR-100 Digital CCD system.

RNA Isolation for Library Construction, and Sequencing

Sponge tissue was collected after 4 h exposure to algae, heat-killed algae, bacteria or no exposure, washed several times to remove algae or bacteria from the surrounding water and the pinacoderm, and stored at –80 °C after RNA^{later} treatment (Thermo Fisher Scientific, Waltham, MA). Total RNA was isolated from three replicates of each treatment using TRIzol Reagent (ThermoFisher Scientific) and the standard protocol according to the guidelines of the manufacturer. Then, mRNA purification was performed with Dynabeads mRNA DIRECT kit (ThermoFisher Scientific), only performing the final stage of the protocol “Elimination of rRNA contamination” with the purified total RNA as input. The quantity and overall quality of mRNA were assessed on a NanoDrop 2000 (ThermoFisher Scientific), and only those extractions with A260/280 with values around 2 were used. The cDNA synthesis for the libraries was performed with Scriptseq v2 kit (Illumina) (according to the manufacturer’s instructions), using an initial mRNA quantity of 50 ng. For the final cDNA libraries, we selected a fragment size of approximately 250–350 bp with a BluePippin (Sage Science, MA). The final concentration of the cDNA libraries was assessed with a Qubit dsDNA HS Assay kit (ThermoFisher Scientific) and the quality with an Agilent TapeStation 2200 system (Agilent Technologies). The sequencing of all libraries was done in several runs in an Illumina NextSeq 500 platform at the Natural History Museum of London sequencing facility (Molecular Core Labs) with 150 bp paired-end settings.

Transcript De Novo Assembly and Annotation

The raw reads were filtered based on quality with Trimmomatic (Bolger et al. 2014), cutting when the average quality in a 4-base window dropped below 28. Then, high quality reads were used for de novo assembly, which was done with Trinity v2.8.4 (Grabherr et al. 2011). Completeness of the assembly was calculated with Benchmarking Universal Single-Copy Orthologs (BUSCO V2/3) against metazoan and eukaryotic cassettes (Simão et al. 2015) in gVolante (Nishimura et al. 2017).

To annotate the transcriptome, we first did a blastx search (Altschul et al. 1997) of the transcriptome using the *refseq* database (Pruitt et al. 2007) (accessed in 2018) with a cut-off e-value of 1e–5. The sequences with BLAST hits were visualized in MEGAN (Huson et al. 2007) for taxon assignment and further annotated by Blast2GOPRO (Conesa et al. 2005) to retrieve the functional information from the Gene Ontology (GO) terms. Gene models of annotated sponge-specific loci were mapped to the *E. muelleri* reference genome.

Transcript Quantification and DGE Analysis

To obtain the gene expression metrics associated to each treatment, we mapped the reads to the reference assembly with Bowtie2 (Langmead and Salzberg 2012), performed transcript quantification with RSEM (Li and Dewey 2011), and then analyzed DGE analysis with edgeR (Robinson et al. 2010; McCarthy et al. 2012) using a minimum fold change of 2 and a maximum adjusted *P* value of 0.001. Three independent replicates for HK-CHLO and BAC were used and two independent replicates for CHLO and BAC were used as edgeR can handle different numbers of replicates and can be performed with two samples without use of special parameters. We analyzed the differences in gene expression in pairwise comparisons between all four treatments.

RNA Isolation, cDNA Synthesis and Differential Expression Analysis by qRT-PCR

Sponge tissue from time points post-algal infection or from “control” sponges was harvested by scraping tissue from the petri dish using a pipette and placed in RNA^{later} solution (Thermo Fisher Scientific, Waltham, MA). Per biological replicate, sponges were pooled as 12–24 samples per time point. RNA was isolated from tissue samples using the Aurum Total RNA Mini Kit (Qiagen, Hilden, Germany) using the Spin Format in biological triplicate according to the manufacturer’s instructions. Additional on-column DNase I steps were performed to limit DNA contamination. RNA quantity was determined using a Nanodrop Spectrophotometer. If sponge tissue was not immediately processed into RNA, it was placed in RNA^{later} and stored at 4 °C. RNA^{later} was removed after 24 h and tissue was stored at –80 °C until use.

cDNA was synthesized from three independent replicates using the AffinityScript qPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions. Starting RNA sample concentration ranged depending on the set of experiments, but was typically in the range 125–250 ng/μl.

The PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA) was used for the qRT-PCR reactions which were performed on an Agilent AriaMX Real-Time PCR System (Agilent Technologies, Santa Clara, CA). qRT-PCR was run at the following conditions: 5 °C for 10 min, 40 cycles at 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min, and lastly

one cycle at 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. Triplicate technical reactions were run for each sample in addition to a nontemplate control triplicate. Primer sequences for *ATP Binding Cassette Subfamily C Member 4 (abcc4)* were: forward-ATG CAT GTC TCG TCT CCT CG, reverse-AAT GCC ACA TTG GTG TCT GC (primer efficiency 2.09). Both *glyceraldehyde 3-phosphate dehydrogenase (gapdh)* and *elongation factor 1-alpha (ef1α)* were run with each gene tested as housekeeping genes, and their expression was stable across treatments. The primer sequences for *gapdh* were: forward-GGT CGA GTC ACA GGT GTT, reverse-CCA AGC AGT TGG TAG TGCA (primer efficiency 2.09). The primer sequences for *ef1α* were: forward-GCG GAG GTA TCG ACA AGC GT, reverse-AGC GCA ATC GGC CTG GGA CG (primer efficiency 1.91). Triplicate Ct values were averaged and the expression of *abcc4* was normalized to the geometric mean of the reference housekeeping genes. Gene expression was analyzed using a modification to the delta delta-Ct method described by Vandesompele et al. (2002) and Hellemans et al. (2007) to allow the use of multiple housekeeping genes and for efficiency-corrected $\Delta\Delta\text{Ct}$ values. Relative quantities on the \log_2 scale were used for statistical analysis (two-tailed, two-sample independent *t*-test) using R (version 3.6.3).

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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Data availability

The transcriptome sequences and gene annotations are accessible at EphyBase. Raw sequence files are available at NCBI SRA under BioProject PRJNA781218.

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