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Viruses in Marine Invertebrate Holobionts: Complex Interactions Between Phages and Bacterial Symbionts

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Abstract

Marine invertebrates are ecologically and economically important and have formed holobionts by evolving symbiotic relationships with cellular and acellular microorganisms that reside in and on their tissues. In recent decades, significant focus on symbiotic cellular microorganisms has led to the discovery of various functions and a considerable expansion of our knowledge of holobiont functions. Despite this progress, our understanding of symbiotic acellular microorganisms remains insufficient, impeding our ability to achieve a comprehensive understanding of marine holobionts. In this review, we highlight the abundant viruses, with a particular emphasis on bacteriophages; provide an overview of their diversity, especially in extensively studied sponges and corals; and examine their potential life cycles.

In addition, we discuss potential phage–holobiont interactions of various invertebrates, including participating in initial bacterial colonization, maintaining symbiotic relationships, and causing or exacerbating the diseases of marine invertebrates. Despite the importance of this subject, knowledge of how viruses contribute to marine invertebrate organisms remains limited. Advancements in technology and greater attention to viruses will enhance our understanding of marine invertebrate holobionts.

1. MARINE INVERTEBRATE HOLOBIONTS

Invertebrates are the dominant animal species in the oceans, and more than 181,000 species of marine invertebrates have been reported, representing approximately 88% of all extant marine animal species (WoRMS Ed. Board 2022). These animals fulfill diverse and important roles in ecology, the economy, and medicine and affect water quality, shellfish aquaculture, and natural products (Chen 2021). Marine invertebrates have evolved intimate relationships with diverse groups of microorganisms, including bacteria, archaea, fungi, protists, and viruses, and have formed symbiotic systems, also known as holobionts (Bang et al. 2018, Roossinck & Bazan 2017, van Oppen & Blackall 2019). Marine invertebrate holobionts consist of a remarkable number of animals, encompassing sponges, corals, mussels, clams, limpets, squids, ciliates, tubeworms, flatworms, nematodes, snails, shrimps, and crabs (Dittami et al. 2021, Dubilier et al. 2008). They inhabit marine environments ranging from coastal ecosystems (e.g., coral reefs, mangroves, seagrass meadows, and intertidal zones) to deep-sea habitats (e.g., hydrothermal vents, cold seeps, and wood/whale falls) (Dittami et al. 2021, Dubilier et al. 2008). The complex invertebrate holobionts of sponges and corals feature highly diverse and abundant microbial symbionts and are well-known model organisms. In contrast, some holobionts are of low complexity; these holobionts are associated exclusively with particular microorganisms that are low in diversity, for example, the association between a single species of chemosynthetic endosymbionts and deep-vent giant tubeworm holobionts (Cavanaugh et al. 1981).

Symbiosis is taxonomically widespread in every marine invertebrate phylum, and thus symbiotic microorganisms are of physiological, ecological, and evolutionary importance to invertebrate hosts (Saffo 1992). Bacterial microorganisms are common symbionts and have been well documented, significantly contributing to our knowledge of the establishment and maintenance of marine holobionts. Bacterial symbioses incorporate two opposite relationships: mutualistic and antagonistic (Roossinck & Bazan 2017). In marine invertebrate holobionts, mutualism has been widely investigated. For example, bacterial symbionts provide bioluminescent camouflage to help squid avoid predators (Nyholm & McFall-Ngai 2004) and offer their genes to invertebrate genomes by horizontal transfer to allow invertebrates to evolve new traits (Jackson et al. 2011, Starcevic et al. 2008). Symbionts in the invertebrates of the phylum Mollusca or Bryozoa can produce antibacterial compounds (Sayavedra et al. 2015) that may protect their animal hosts from invaders (Sogin et al. 2020) or competitors (Florez et al. 2015). The hosting of multiple symbiont species with metabolic diversity allows holobionts to select available energy sources in an environment (Sogin et al. 2020). By contrast, antagonistic symbionts are pathogenic and impair the health of invertebrates. For instance, *Vibrio shiloi* is responsible for the bleaching of the coral *Oculina patagonica* (Kushmaro et al. 2001), and the *Alphaproteobacteria* strain NW4327 can cause severe external tissue necrosis in the sponge *Rhopaloeides odorabile* (Webster et al. 2002).

2. VIRUSES IN MARINE INVERTEBRATE HOLOBIONTS

2.1. High Abundance and Morphology of Virus-Like Particles

Viruses, as prominent components in holobionts, have been attracting considerable interest, but few studies have shed light on the high viral abundance in animal mucus heavily colonized by microorganisms. The concentration of virus-like particles in coral mucus ranges between 10^7 and 10^8 particles mL^{-1}. In the mucus of several scleractinian species from Whale Island, located in the oligotrophic Van Phong Bay, Vietnam (12°39.1′N, 109°23.9′E), viral abundance reaches more than 10^7 particles mL^{-1}, which is higher than the viral concentrations in the surrounding seawater and exceeds that of prokaryotes in the mucus by at least one order of magnitude (Nguyen-Kim et al. 2014). Similar observations were made in two other scleractinian coral species, *Acropora formosa* and *Fungia repanda*, from Mot Island, Vietnam (Nguyen-Kim et al. 2015). In the epibiotic microbial community in the setae of deep-sea galatheids, the viral concentration can reach up to 4.5×10^9 particles g^{-1} (Yoshida-Takashima et al. 2012). In the morphological characterization of viruses in marine sponges, transmission electron microscopy observations have shown that virus-like particles from 15 coral reef sponge species collected from the Great Barrier Reef and the Red Sea have 50 morphotypes (Pascelli et al. 2018). These morphotypes include filamentous (100–1,300 nm in length and 12–60 nm in width), beaded (2–8 aligned beads, where each bead is 30–42 nm in diameter), geminate (81–95 nm in length and 37–48 nm in width), and polyhedral (60–205 nm in diameter) shapes (Pascelli et al. 2018). In *Acropora tenuis* from the Great Barrier Reef and *Porites compressa* from Kaneohe Bay, Hawaii, virus-like particles inside the cells and algal symbiotes incorporate filamentous (51–100 or 1,000–2,500 nm in length and 20–30 nm in width) and icosahedral (<50, 50–180, or >200 nm in diameter) morphologies (Lawrence et al. 2014, Weynberg et al. 2017b).

2.2. High Viral Diversity with Phage Dominance

Metagenomic or metatranscriptomic studies have shown that the DNA and RNA viral assemblages in sponges or corals are distinct from those of seawater viral communities (Jahn et al. 2019, Laffy et al. 2018), and some viral taxa are likely endemic to invertebrate holobionts. According to the definition of sponge-specific bacteria (Taylor et al. 2007), the phage families of *Tectiviridae* and *Inoviridae* that are not found in the surrounding seawater are likely to be specific to sponges and corals (Laffy et al. 2018, Nguyen et al. 2021, Pascelli et al. 2020, Weynberg et al. 2017a). In addition, viral communities in the holobionts of corals and sponges display a predominance of bacterial viruses (bacteriophages or phages) (Cardenas et al. 2020, Pascelli et al. 2020). The high relative abundance and diversity of phages in holobionts indicate the high probability of infection of bacterial hosts by the phages. However, understanding of these acellular symbionts (phages) is limited even though knowledge about them has grown rapidly. In the following sections, we synthesize our current state of knowledge on phage ecological characteristics, including diversity, abundance, and distribution, in a wide range of marine invertebrate holobionts, encompassing mainly corals and sponges. We then discuss phage reproduction strategies and factors driving lytic production and lysogenic replication. We discuss phage roles (**Figure 1**) in the establishment of invertebrate–bacterium symbiosis and the fitness and health of holobionts, emphasizing (*a*) how phages influence the initiation of bacterial symbiont colonization, (*b*) how phages contribute to bacterial symbiont population maintenance, and (*c*) how phages impact the health of marine invertebrates. Finally, we provide perspectives on advancements in methods and other possible functional roles and the opportunities and challenges.

The relative abundance and distribution of predominant phages have been investigated using metagenomics in coral and sponge holobionts, and the results suggested that phages of the

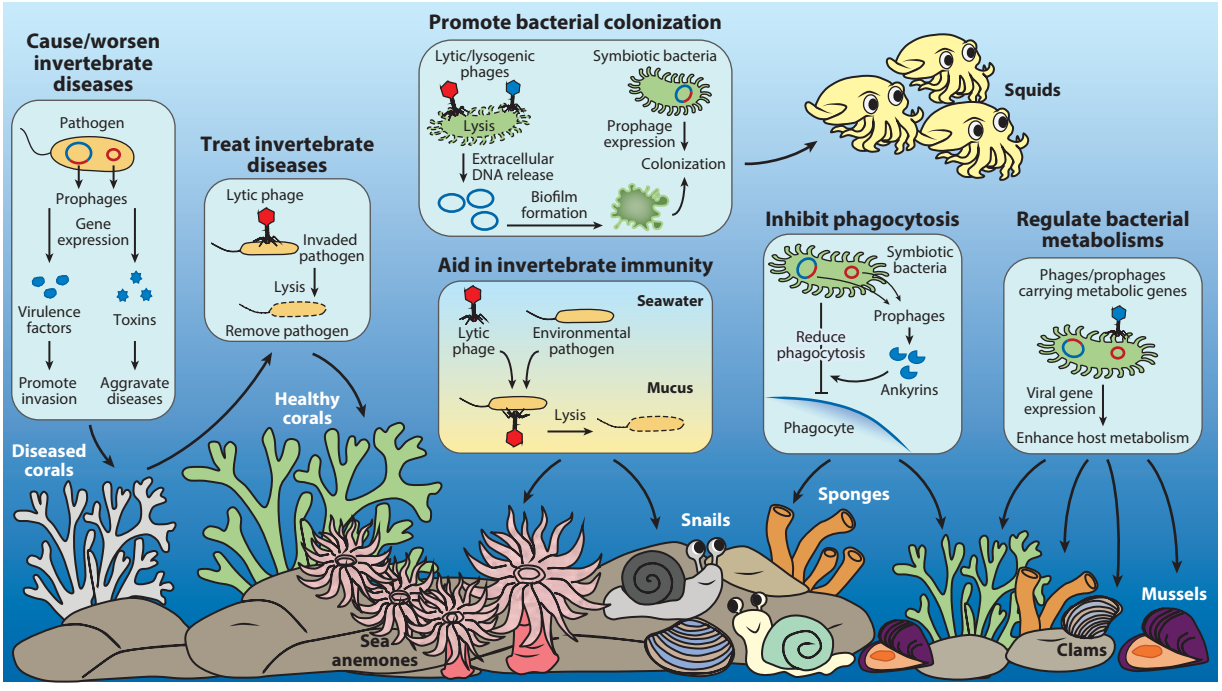

Figure 1

Examples of viral contributions to bacteria and invertebrates in marine holobionts. Based on multiomics evidence and in vitro assays, it appears that phages play a critical role in the establishment and maintenance of symbiotic systems in marine environments. During the initiation of symbiosis, phages or prophages may mediate the release of biofilm-promoting factors, such as extracellular DNA, to help bacterial populations develop biofilms. Prophages may also express their own genes, such as *smpB*, along with these released factors, contributing to the start of colonization on squids. In maintaining invertebrate holobionts, phages and prophages can act as gene reservoirs and play roles in the metabolic regulation of host pathways. Some phages even carry eukaryotic genes, such as those encoding ankyrins, which can help bacteria evade phagocytosis, as seen in sponges and corals. Viral lysis can contribute to the killing of bacterial pathogens in mucosal layers and may provide phage-borne immunity to marine invertebrate holobionts, such as sea anemones. In cases where pathogens penetrate immune defenses and cause animal diseases, phage therapy can be used to treat animal diseases by lysing pathogenic bacteria. However, temperate phages may integrate disease-related genes into pathogenic chromosomes, which can cause and worsen diseases in marine invertebrates, such as corals.

order *Caudovirales* were dominant groups (Laffy et al. 2018, Mahmoud & Jose 2017, Nguyen et al. 2021, Pascelli et al. 2020, Weynberg et al. 2017a, Wood-Charlson et al. 2015). Although *Caudovirales* (*Myoviridae*, *Siphoviridae*, and *Podoviridae*) was formally removed from the taxonomy of the International Committee on Taxonomy of Viruses in 2022 (Walker et al. 2022), we keep this classification in the present review since almost all viral diversity investigations of marine invertebrate holobionts have used it and it provides meaningful biological and ecological information. We also urge reanalysis of previously generated viral sequences in marine invertebrate holobionts.

In 40 coral and sponge species, eight phage families (*Myoviridae*, *Siphoviridae*, *Podoviridae*, *Microviridae*, *Tectiviridae*, *Inoviridae*, *Corticoviridae*, and *Plasmaviridae*) are widely distributed (**Table 1**). *Myoviridae*, *Siphoviridae*, and *Podoviridae* phages are always present across the 40 investigated species. *Myoviridae* phages are dominant in eight sponge species (*Carteriospongia foliascens*, *Cymbastela marshae*, *Ianthella basta*, *Stylissa carteri*, *R. odorabile*, *Stylissa* sp. 445, *Amphimedon ochracea*, and *Hyrtios erectus*) (Laffy et al. 2018, Nguyen et al. 2021, Pascelli et al. 2020, Weynberg et al. 2017a) and only one coral species (*Pocillopora acuta*) (Laffy et al. 2018, Weynberg et al. 2017a),

Table 1 Relative abundances of diverse phages in marine corals and sponges

Species	*Myoviridae*	*Podoviridae*	*Siphoviridae*	*Microviridae*	*Inoviridae*	*Tectiviridae*	*Corticoviridae*	*Plasmaviridae*	Reference(s)
Corals									
Acropora downingi	✓	✓	✓	✓					Mahmoud & Jose 2017
Acropora hyacinthus	✓	✓	✓						Wood-Charlson et al. 2015
Acropora millepora (postbleach)	✓	✓	✓	✓		✓			Wood-Charlson et al. 2015
Acropora millepora (prebleach)	✓	✓	✓	✓					Wood-Charlson et al. 2015
Acropora palmata	✓	✓	✓						Wood-Charlson et al. 2015
Acropora tenuis	•	•	•	•					Weynberg et al. 2017a, Wood-Charlson et al. 2015
Diploria strigosa (bleached)	✓	✓	✓						Wood-Charlson et al. 2015
Diploria strigosa (healthy)	✓	✓	✓		✓				Wood-Charlson et al. 2015
Fungia fungites	•	●	•	•					Weynberg et al. 2017a
Galaxea fascicularis	•	•	•	•					Weynberg et al. 2017a
Goniastrea aspera	●	•	•	•					Weynberg et al. 2017a
Montastraea cavernosa	✓	✓	✓	✓	✓	✓			Wood-Charlson et al. 2015
Montastraea faveolata	✓	✓	✓	✓					Wood-Charlson et al. 2015
Pocillopora acuta	●	●	●	●					Laffy et al. 2018, Weynberg et al. 2017a
Pocillopora damicornis	✓	✓	✓	✓	✓	✓		✓	Wood-Charlson et al. 2015
Pocillopora verrucosa	•	•	•	•					Weynberg et al. 2017a
Porites astreoides	✓	✓	✓	✓	✓				Wood-Charlson et al. 2015
Porites compressa	✓	✓	✓	✓	✓		✓		Wood-Charlson et al. 2015
Porites harrisoni	✓	✓	✓	✓					Mahmoud & Jose 2017
Porites lutea	●	●	●	●		•			Laffy et al. 2018
Sponges									
Amphimedon ochracea	●	●	●	•	•	•			Pascelli et al. 2020
Amphimedon queenslandica	●	●	●	●	•	•			Pascelli et al. 2020
Callyspongia sp.	●	●	●	•					Pascelli et al. 2020

(*Continued*)

Table 1 (*Continued*)

Species	*Myoviridae*	*Podoviridae*	*Siphoviridae*	*Microviridae*	*Inoviridae*	*Tectiviridae*	*Corticoviridae*	*Plasmaviridae*	Reference(s)
Carteriospongia foliascens (Great Barrier Reef)	●	●	●	•	•				Pascelli et al. 2020
Carteriospongia foliascens (Red Sea)	●	●	●	•	•	•			Pascelli et al. 2020
Cinachyrella schulzei	●	•	●	•	•	•			Pascelli et al. 2020
Crella cyathophora	●	●	●	•		•			Pascelli et al. 2020
Cymbastela concentrica	✓	✓	✓						Nguyen et al. 2021
Cymbastela coralliophila	✓	✓	✓						Nguyen et al. 2021
Cymbastela marshae	●	●	●	•	•	•			Pascelli et al. 2020
Echinochalina isaaci	●	●	●	•	•				Pascelli et al. 2020
Hyrtios erectus	●	●	●	•	•	•			Pascelli et al. 2020
Ianthella basta	●	●	●	•		•			Pascelli et al. 2020
Lamellodysidea herbacea	●	●	●	•	•	•			Pascelli et al. 2020
Mycale sp.	●	●	●	•	•				Pascelli et al. 2020
Niphates rowi	●	●	●	•					Pascelli et al. 2020
Pipestela candelabra	●	●	●	•	•	•			Pascelli et al. 2020
Rhopaloeides odorabile	•	●	•	●		•			Nguyen et al. 2021
Scopalina sp.	✓	✓	✓						Nguyen et al. 2021
Stylissa carteri	●	●	●	•					Pascelli et al. 2020
Stylissa sp. 445	✓								Nguyen et al. 2021
Tedania anhelans	✓	✓	✓		✓				Nguyen et al. 2021
Xestospongia testudinaria	●	●	●	●	•	•			Pascelli et al. 2020

Each row represents a different species or treatment of corals or sponges, and each column represents a different phage family. Circles and check marks indicate relative abundances: large circle, >10%; medium circle, >1% and ≤10%; small circle, ≤1%; check mark, present but not quantified. It is important to note that all phages in the samples were identified before 2022, when the polyphyletic families of *Myoviridae*, *Siphoviridae*, and *Podoviridae*, as well as the random amplification of viral genomes, were widely used, and the estimated abundances may be biased due to these limitations.

suggesting that they may preferentially inhabit sponge hosts. Members of *Siphoviridae* are dominant in *A. tenuis* and *Goniastrea aspera* (Weynberg et al. 2017a). Podoviruses are dominant phages in *Fungia fungites*, *Galaxea fascicularis*, *Pocillopora damicornis*, *Pocillopora verrucosa*, *Acropora downingi*, and *Porites harrisoni* (Mahmoud & Jose 2017, Weynberg et al. 2017a). *Caudovirales* seem to be dominant in marine sponges and corals, consistent with the observation that most characterized phages belong to this order (Dion et al. 2020). *Microviridae* is another common phage family. Polyhedral single-stranded DNA (ssDNA) phages are harbored by more than 30 species of corals and sponges from coral reefs in the northern Arabian Gulf, Papua New Guinea, the Red Sea, and the Great Barrier Reef (Laffy et al. 2018, Mahmoud & Jose 2017, Pascelli et al. 2020, Weynberg et al. 2017a, Wood-Charlson et al. 2015). The polyhedral double-stranded DNA *Tectiviridae* and filamentous *Inoviridae* with ssDNA genomes have been detected in many species, such as *Diploria strigosa* and *Acropora millepora* (Wood-Charlson et al. 2015). By contrast, polyhedral *Corticoviridae* and pleomorphic *Plasmaviridae* are less frequently detected; only three coral species contain their genomic signals (Wood-Charlson et al. 2015). *Corticoviridae* and *Plasmaviridae* in marine invertebrates are less known, possibly because of their low distributions.

It is worth pointing out that there are uncertainties in the above comparison due to variability and bias of technique applied in previous studies. For example, random amplification after DNA extraction may introduce bias in viral community composition, leading to an overrepresentation of certain viruses, such as circular ssDNA viruses. To address this issue, new approaches should be developed to reflect the true abundance of certain viruses in communities.

Even though numerous sponge and coral species have been investigated, the viral compositions of thousands of sponge and coral species at various sites in the oceans remain unexplored, particularly in species in habitats with high endemism. Marine invertebrate symbiotic viral reservoirs can be enlarged by studying the viromes in endemic species (e.g., *Suberites luna* and *Chalinula qatari*) (Giraldes et al. 2020). The exploration of a wide range of species would contribute substantially to expanding the reservoir of viral genetic diversity due to the species-specific signatures of viromes in their animal hosts (discussed in detail in Section 2.3).

2.3. Intra- and Interspecies Patterns

Resembling symbiotic bacteria, the viromes in sponges and corals exhibit species-specific signatures and individual-unique fingerprints (Jahn et al. 2019, Laffy et al. 2018, Pascelli et al. 2020). In *Petrosia ficiformis*, *Chondrosia reniformis*, *Agelas oroides*, and *Aplysina aerophoba* on the Montgrí coast and at Portlligat (Catalunya, Spain), a large proportion of cross-assembled viral sequences (~35%) are unique to an individual, and approximately 28% of the sequences are prevalent in a specific sponge species (Jahn et al. 2019). However, many of the specific sequences cannot be annotated because of the limitations of viral genome databases, and thus deeply understanding the intra- and interspecies patterns and the composition of unique viruses at the species level remains challenging. Taxonomic investigation has shown that sponges and corals are colonized mainly by tailed phages and that the major community variation is subject to the composition of less abundant *Microviridae*, *Tectiviridae*, *Inoviridae*, *Corticoviridae*, and *Plasmaviridae*. For example, *Inoviridae* phages are present only in *Amphimedon queenslandica* and are absent in other sponge and coral species at the Great Barrier Reef (Laffy et al. 2018). Additionally, *Tectiviridae* phages are present in *Pipestela candelabra* at the Great Barrier Reef and *Xestospongia testudinaria* in the Red Sea but are absent in other sponge species (Pascelli et al. 2020).

Recently, a few studies have shown that viruses inhabit various organs. For example, the esophageal glands of the deep-sea snail *Gigantopelta aegis* harbor four populations of tailed phages (Zhou et al. 2021b), and the intestines and hepatopancreases of the mussels *Mytilus edulis* and

Modiolus modiolus and the oyster *Ostrea edulis* contain enteric viruses (Myrmel et al. 2004). Tissue-specific signatures can be found by sampling viruses from different tissues and organs and performing sequencing for further analysis. In a specific tissue, phages are likely to be present in extracellular and intracellular environments, as evidenced by the filamentous virus-like particles in the extracellular mesohyls and tissue cells of sponges, such as *X. testudinaria* (Pascelli et al. 2018). However, no specific signatures are present in sponge tissues (Jahn et al. 2019), possibly due to the lack of true tissues and organs. The tissue-specific nature of other marine invertebrates with specialized tissues has not been identified.

3. PHAGE LIFE CYCLES

Viruses reproduce through three pathways: lytic, lysogenic, and chronic infections. In the lytic pathway, virulent or temperate viruses produce progeny and destroy host cells. The lysogenic cycle enables temperate viruses to integrate their genomes into host chromosomes as proviruses or exist as extrachromosomal plasmid-like elements to replicate themselves during host reproduction (Correa et al. 2021, Howard-Varona et al. 2017). In chronic infection, temperate viruses are productive but also leave host survival. Viruses can undergo various life cycles in marine invertebrate holobionts. Nguyen-Kim et al. (2015) showed that the lytic production rate in the mucus of corals is 10 times that in the ambient seawater. Moreover, prophage genomic signals have been identified in marine invertebrate holobionts, indicating that phages also reproduce in a lysogenic cycle (Jahn et al. 2021, Zhou et al. 2022). Finally, culture-independent microscopy observation of marine sponge cells has shown potential chronic infections. In *C. foliascens*, *Inoviridae*-like particles seem to have injected their DNA into the host cells of cyanobacteria and been extruded without inducing cell lysis (Pascelli et al. 2018), which indicates a chronic infection. However, the prevalent infection strategies of viruses in symbiotic environments remain unclear, and findings discussed below also make them controversial.

In sponge holobionts, lysogeny is considered typical and predominant due to the detection of temperate phage markers in viral metagenomes and the relatively high abundance of predicted temperate phage genome sequences (Jahn et al. 2021). However, the microbiomes of sponges in deep hydrothermal vents have revealed an absence of complete prophage genomes, a low abundance of incomplete prophage sequences, and a small number of associations between incomplete prophages and free-living phages (Zhou et al. 2021a). Intriguingly, the lytic production and the fraction of lysogenic cells have a positive correlation in coral mucosal layers, and both are affected by environmental conditions (e.g., temperature and salinity) (Nguyen-Kim et al. 2015). The fraction of lysogenic cells can be raised by the lytic-to-lysogenic switching of temperate viruses, and the lysogenic switching is considered driven by diverse factors (Correa et al. 2021). For example, the initiation of lysogenic switching in viral infections of the eukaryotic symbiont *Symbiodinium* in stony corals is related to temperature, nutrients, and salinity (Lawrence et al. 2014, 2017; Weynberg et al. 2017b). Another factor, density, has been proposed in the piggyback-the-winner model that links lysogeny with high microbial density and predicts a lytic-to-lysogenic switch when microbial density increases (Knowles et al. 2016). However, the lack of detected intact prophages and lysogeny indicators in the genomes of dominant bacterial groups in sponge microbiomes challenges this model (Zhou et al. 2021a).

Additionally, the genome size of bacterial hosts is associated with lysogeny in nature (Touchon et al. 2016). Obligate bacterial symbionts usually undergo genome reduction and evolve streamlined genomes (McCutcheon & Moran 2011). For instance, the genome sizes of the dominant symbiotic bacteria (SUP05) range from 1.07 Mb to 1.18 Mb in vent demosponges (Zhou et al. 2019, 2021a) and are much smaller than the threshold for high prophage frequency (6 Mb)

(Touchon et al. 2016). Genome reduction can result in the loss of sequences essential for phage integration; indeed, attachment sites have not been detected in symbiont genomes (Zhou et al. 2021a), and this would cause symbiotic bacteria to be nonlysogenic.

Notably, while microbiome or virome studies have generated much of our knowledge of viral infection strategies in symbiotic environments, it is important to recognize that genomic information can only provide limited insights into viral infection strategies. The evaluation of lysogenic versus lytic infections needs ecological data, such as the measurement of viral lytic production and the proportion of lysogens.

4. CONTRIBUTIONS TO HOLOBIONTS

Diverse viruses with dynamic infection strategies account for the intertwined nature of virus–host interactions in marine invertebrate holobionts. Recently identified interactions have suggested that viral reproduction and genetic traits can affect bacterial hosts in immunity, physiology, ecology, and evolution. The diverse roles of phages include participation in the establishment, maintenance, and breakdown of symbiosis and are discussed in detail below.

4.1. Roles of Phages in the Establishment of Bacterial Symbiosis

Similar to other symbiotic systems, the surfaces of epithelial cells of marine invertebrate animals, such as sea squids (Nyholm & McFall-Ngai 2004) and corals (Ainsworth et al. 2010), are colonized by microbial communities. Bacterial colonization in corals is subject to multiple factors, including quorum-sensing signals, chemical compounds, motility, biofilm formation, and glycosidases (Alagely et al. 2011, Krediet et al. 2013b, Li et al. 2017). Recently, life cycle shifting in prophages in symbiotic bacteria has been recognized as another factor controlling the bacterial colonization of corals (Wang et al. 2022). Under thermal stress (32°C), the induction of GfP2 prophages of a commensally symbiotic *Vibrio* sp. is triggered by LodAB encoded by the pathogen *Vibrio coralliilyticus* in *G. fascicularis* and then induces phages to enter a lytic cycle and lyse host cells, leading to the death of the *Vibrio* sp., which then fails to colonize the coral (Wang et al. 2022). However, the induction of prophages may promote bacterial colonization because lysogenic-to-lytic switching contributes to the production of extracellular DNA and promotes biofilm formation (Gödeke et al. 2011). In vitro biofilm assays of the gut-associated *Shewanella fidelis* of the marine tunicate *Ciona intestinalis* showed that extracellular DNA released by the lysis of phages can cover 50% of the area, which is larger than that in untreated control culture, and a robust biofilm forms in 24 h (Leigh et al. 2017). Biofilm formation is related to the initiation of bacterial colonization in corals and sea squids (Alagely et al. 2011, Krediet et al. 2013a, Nyholm et al. 2000). In the squid *Euprymna scolopes* and its symbiotic bacterium *Vibrio fischeri*, an essential step in the initiation of *V. fischeri* transmission into a light organ is biofilm development on a ciliated epithelium surface (Brooks et al. 2014, Visick 2009). Accordingly, inducible prophages and virulent phages are other factors that influence bacterial colonization because of the nature of the lytic infection strategy (**Figure 2**).

In addition to the life cycle switching of prophages, genetic elements in the prophage genomes of symbionts may influence the establishment of symbiosis. Though the impact of prophage genes on symbiosis initiation in marine invertebrates has not been explored, studies of the bacterial colonization of humans and chicks have provided a basis for our assumptions. Functional genes encoded by prophages play a role in adhesion or biofilm formation, including *ssa*, *speC*, and *spd1* in prophage ΦHKU.vir of *Streptococcus pyogenes* (Brouwer et al. 2020), *pblA* and *pblB* in prophages of *Streptococcus mitis* (Bensing et al. 2001), the prophage gene for STM2699 protein in *Salmonella* (Shah et al. 2014), the gene for B cell–stimulating protein B in prophages of *Neisseria meningitidis*

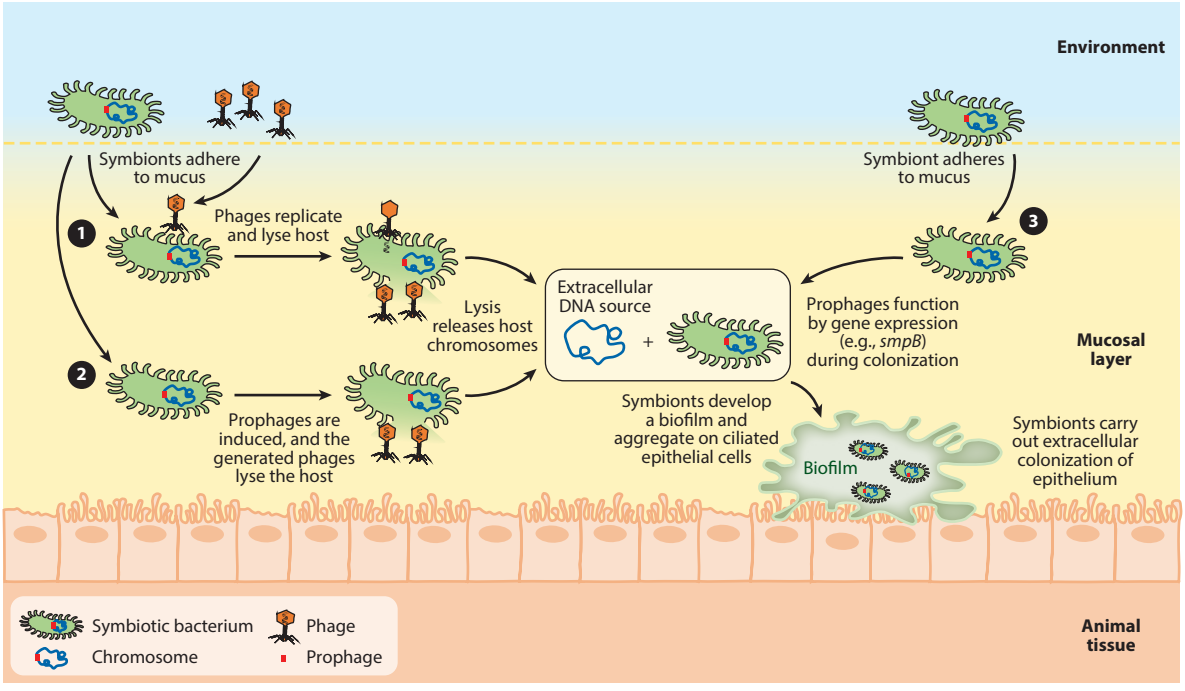

Figure 2

Phage-involved episymbiosis initiation in the mucosal layers of marine invertebrate animals. Microbial symbionts are acquired by animals through vertical and/or horizontal transmission. Vertical transmission is the transfer from parents to the next generation, and horizontal transmission involves selection from the environment in each generation. Most symbionts living on the surfaces of epithelial cells are thought to be horizontally transmitted from the surrounding environments to the animal hosts, where specific symbionts are retained by screening. Phages from seawater can infect symbiotic bacteria and trigger cellular DNA release (Leigh et al. 2017) (route ①). Prophages in symbiotic bacteria may be induced and lead to host lysis, resulting in cellular DNA release (Gödeke et al. 2011) (route ②). Prophages of symbiotic bacteria may encode factors, such as SmpB, that are likely to contribute to animal colonization (route ③). The released extracellular DNA and prophage-born proteins may enable symbionts to develop a biofilm and successfully colonize invertebrates.

(Müller et al. 2013), and a gene encoding endo-alpha-sialidase in the prophage of *Escherichia coli* DE205B (Liu et al. 2020). Therefore, similar functional genes in prophages may induce bacterial colonization in marine invertebrates. In a study of sea squids and their extracellular symbiont *V. fischeri*, 380 colonization determinants were identified in symbiont genomes (Brooks et al. 2014). To investigate a potential relationship between the determinants and prophages, we searched for prophage sequences in the *V. fischeri* genomes using PHASTER, a phage database search tool (Arndt et al. 2016). Intriguingly, we found that two determinants, VF_2002 and VF_2642, were located within a putative prophage region. Thus, the symbionts of marine invertebrates likely contain prophages carrying genes relevant to bacterial colonization. Additionally, the gene VF_2002, encoding the SsrA-binding protein (SmpB) for binding to the transfer-messenger RNA, is unique to symbiont chromosomes according to whole-genome annotation, indicating that prophages play an indispensable role in the *V. fischeri* colonization. According to these observations, we hypothesized that the expression of prophage genes regulates cellular activity and contributes to colonization (**Figure 2**). This enables the aggregation of specific symbionts on epithelial surfaces, where the symbionts remain as extracellular symbionts.

4.2. Roles of Phages in the Maintenance of Bacterial Symbiosis

After forming close relationships with invertebrate hosts, bacterial symbionts must avoid being pursued, engulfed, and killed by the eukaryotic immune system to establish permanent associations, in which phages may play a role. In addition, the auxiliary metabolic genes in phage genomes are considered to contribute to the general metabolic genetic diversity of marine invertebrate holobionts.

4.2.1. Phages protect bacterial symbionts from eukaryotic immune systems. Phagocytosis is an animal immune response to interlopers, such as invading bacteria; however, bacterial symbionts in invertebrates can evade this response with ankyrin-repeat proteins encoded by phages (Nguyen et al. 2014). A study of sponge viromes demonstrated that phages enriched in the pinacoderm and mesohyl encode repeat-domain-containing ankyrins, including Ank2 (PF12796), Ank3 (PF13606), and Ank5 (PF13857) (Jahn et al. 2019). These domains in ankyrin-encoding phages (ankyphages) are relatively abundant in sponge-associated viromes but are absent in surrounding seawater viromes, indicating that ankyrin genes are unique to phages in sponges. Macrophage infection assays have shown that the expression of phage ankyrin genes downregulates proinflammatory signaling, reduces phagocytosis rates, and increases bacterial survival (Jahn et al. 2019). This finding suggests that phage-born ankyrins can modulate animal immune responses to bacteria and provide an alternative way for microbial symbionts to evade immunity and survive in holobionts (**Figure 1**). The presence of ankyphages may promote the maintenance of a symbiotic relationship between marine invertebrates and their harbored bacteria. Moreover, the phage-encoded ankyrin-domain-containing protein is widely distributed in viromes in host-associated contexts, ranging from shallow-water corals (Cardenas et al. 2020) to deep-sea mussels and tubeworms (Zhou et al. 2022). Virus-encoded antiphagocytosis genes have been reported in viromes generated from seawater collected from coral reef boundary layers (Silveira et al. 2020). This finding highlights the significant role phages may play in symbiosis in marine habitats, and future research should examine the ubiquity of this mechanism across marine invertebrate phyla.

4.2.2. Phages grant hosts access to metabolic genetic diversity. Metagenomic and metatranscriptomic analyses of the microbiomes of sponges and corals have found a variety of metabolic genes in viral genomes, including genes related to photosynthesis, chemosynthesis, and nutrition biosynthesis. In photosynthetic sunlit oceans, symbiont phages encode the homologs of photosystem II (such as *psbA* and *psbD*) (Nguyen et al. 2021, Pascelli et al. 2020). The presence of these auxiliary metabolic genes suggests that phages increase light reaction activity and energy output and produce additional energy sources to feed symbiotic autotrophs, thereby contributing to symbiotic systems. In addition, phages participate in many other pathways, including thiamine metabolism and cobalamin biosynthesis, which may produce the vitamin B_1 and B_{12} necessary for animals to benefit holobiont fitness (Nguyen et al. 2021, Pascelli et al. 2020).

In chemosynthetic hydrothermal vent environments, some phages that potentially infect symbionts associated with invertebrates (e.g., the gill endosymbionts of mussels and clams) contain genes for reverse dissimilatory sulfite reductase (*rdsr*), which converts elemental sulfur to sulfite (Anantharaman et al. 2014). This infection may enhance the efficiency of sulfur oxidation in cellular metabolism and contribute to the biogeochemical cycling of symbiotic systems. Apart from *rdsrA*, host-derived *iscS* is present in phages infecting SUP05 bacteria in hydrothermal vent sponges; these phages may participate in iron–sulfur cluster assembly and contribute to host sulfur metabolism (Zhou et al. 2019). The expression of phages or prophages carrying metabolic genes can be a driving force that enhances host metabolism (**Figure 1**). However, relying only on

genomic data hinders our understanding of virus–host interactions; experimental metabolic analyses should also be performed when exploring viral complementation to host metabolism.

The complementation between phages and their hosts' metabolic processes has been widely investigated in nonsymbiotic environments (Breitbart 2012). For example, cyanobacterial viruses (cyanophages) carry various photosynthesis-related auxiliary metabolic genes, including *psbA* (encoding the protein D1 in the photosystem II reaction), *hli* (encoding high-light-inducible proteins), *cp12* (encoding an inhibitor for the Calvin cycle), *zwf* (encoding glucose-6-phosphate dehydrogenase), and *gnd* (encoding 6-phosphogluconate dehydrogenase) (Lindell et al. 2005, Thompson et al. 2011). These genes are coexpressed and function in photosynthetic reactions, particularly reducing Calvin cycle activity, enhancing light reaction activity, and leading to a twofold increase in host NADPH/NADP ratio in infected cells (Thompson et al. 2011). A methodology for investigating non-host-associated viruses not only demonstrates viral gene functions but also provides a detailed methodology for developing laboratory-based systems for symbiotic viruses. The coupling of dry and wet laboratory experiments will enhance our understanding of host–virus interactions in metabolic processes.

4.3. Roles of Phages in the Health of Marine Invertebrate Holobionts

Phages have a significant impact on enhancing the fitness of animal hosts. Their lytic nature confers phage-born immunity or phage therapy by eliminating pathogens. Conversely, lysogenic infection results in the integration of virulence genes into the chromosomes of bacterial pathogens, potentially inducing or exacerbating diseases in marine invertebrate holobionts.

4.3.1. Assisting animal immunity. Animal mucus is considered a part of the immune system due to its role as a physical barrier. Additionally, mucus serves as a critical site for phage–host interplay. When pathogenic bacteria invade, in theory, phage predation can form a defense line and develop into an immune system. Based on an environmental survey of sea anemones and hard corals and assays of T4 phages, *E. coli*, and cultured tissue cells, Barr et al. (2013a,b) proposed a possible contribution of phages in mucus to innate immunity. The hypervariable immunoglobulin-like domains on phage capsid proteins [such as highly antigenic outer capsid (Hoc) in T4] can promote the adherence of phages to animal mucin glycans and create a suitable environment for phage activity (Barr et al. 2013a). Lytic phages predate and disrupt pathogenic bacterial populations, acting as a non-host-derived innate immune system against invading pathogenic bacteria in animal cells. Temperate phages may contribute to acquired immunity by infecting and killing a certain range of pathogens in memory (Barr et al. 2013b). Temperate phages with a broad host range can be carried by invading pathogenic lysogens; when entering mucus, they are released after spontaneous induction and integrate their genomes into the chromosomes of commensal bacteria residing in mucosal layers; and the spontaneous induction of commensal lysogens releases temperate phages and maintains a few virions that readily attack pathogens attempting to reinfect metazoans (Barr et al. 2013b). However, to date, information about phage-mediated immunity in marine invertebrates remains limited. Nevertheless, phage-mediated immunity can be tested in marine invertebrate model systems by using a method similar to that used for rainbow trout (*Oncorhynchus mykiss*) (Almeida et al. 2019).

4.3.2. Treating invertebrate diseases. Mucosal layers and other immune systems of marine invertebrates eliminate a wide range of microbial pathogens. However, some pathogens can penetrate immune defenses and cause animal diseases. Phage therapy can treat animal diseases by lysing pathogenic bacteria (**Figure 1**), as demonstrated in vibriophages in corals. In the treatment of bleached corals, the *V. coralliilyticus*–induced photoinactivation of *Symbiodiniaceae* and the

formation of coral tissue lesions can be inhibited by the vibriophage YC (Cohen et al. 2013). Phages are potential agents for treating other diseases, such as coral black band disease, which is closely associated with the filamentous cyanobacterium *Roseofilum reptotaenium* (Casamatta et al. 2012). In diseased tissues, cyanophage PRSM6 is highly abundant and likely infects *R. reptotaenium*, as demonstrated in an investigation of CRISPR-Cas spacer targets (Buerger et al. 2019). Thus, PRSM6 may act as a killer in the control of *R. reptotaenium* and treatment of black band disease. In addition, phage therapy has been applied to the control of diseases in the aquaculture of sea cucumbers, shrimps, lobsters, oysters, and black abalone; pathogens are removed by using vibriophages (Ninawe et al. 2020) or rickettsial bacteriophages (Friedman et al. 2014). Multiple antiviral defense systems, such as CRISPR-Cas and restriction–modification systems, have been identified in sponge- or snail-associated bacteria (Zhou et al. 2021a,b). The presence of defense systems indicates that phage resistance occurs in the pathogens of marine invertebrate animals. To overcome this resistance, a mixture of multiple phages can be used in phage therapy (Doss et al. 2017).

4.3.3. Aggravating invertebrate diseases. Their lytic nature enables phages to control pathogens and treat diseases. However, lysogenic conversion can act in an opposite manner and cause and worsen diseases, as observed in prophages that induce pathogenesis by enhancing virulence and resistance in animal-associated *E. coli* (Kwon et al. 2013, Lai et al. 2018, Wiles et al. 2013) and *Pseudomonas aeruginosa* (Sweere et al. 2019). Phages in coral reefs carry abundant virulence factors, including *ail*, which encodes attachment–invasion locus protein; *pla*, which serves as a plasminogen activator; and *bepA*, which produces adenylyltransferase, an enzyme that promotes bacterial recognition and invasion of metazoan epithelium (Silveira et al. 2020). Additionally, a proposed spatial pattern of infection predicts that bacteria tend to be lysogenized in upper mucus layers (Silveira & Rohwer 2016), which can promote pathogens to acquire virulence genes from coral reef phages for invasion. On this basis, the acquisition and expression of prophage virulence genes enable pathogens (e.g., *Vibrio* bacteria) to attach to epithelial cells and successfully invade animals (e.g., corals) (**Figure 1**). Prophage-like sequences have been consistently detected in the chromosomes of pathogenic *V. coralliilyticus* in corals (Santos et al. 2011, Weynberg et al. 2015). The presence of genes for zonula occludens toxins and accessory cholera enterotoxins in these prophage sequences indicates that prophages contain virulence factors that might cause coral diseases (Weynberg et al. 2015). This finding is consistent with the prophages of the coral black band disease–associated *R. reptotaenium* AO1 (Buerger et al. 2016) and *Geitlerinema* sp. BBD 1991 (Den Uyl et al. 2016), which contain putative virulence genes. Phage genes encoding transcription regulators and homologs are located at staphylococcal pathogenicity islands and are more highly expressed in bacterial genomes from diseased corals than those from healthy corals (Daniels et al. 2015). Additionally, phage genes for RNA polymerase sigma factors are predominantly expressed by *Planctomycetaceae* in diseased corals (Daniels et al. 2015). Notably, the presence of *Tectiviridae* and *Inoviridae* is inconsistent in healthy and bleached *D. strigosa* and *A. millepora* (**Table 1**), indicating that distributions and potential disease-associated phages vary. Overall, lysogenic phages might play a key role in pathogenicity and contribute to the diseases of corals and other marine invertebrates.

5. CONCLUDING REMARKS AND PERSPECTIVE

In recent years, many studies have focused on interactions between animals and cellular microorganisms, whereas phage–holobiont interactions have not been comprehensively explored. The presence of abundant and diverse phages in marine invertebrates raises fundamental questions

about their ecological functions in holobionts. Depending on their infection strategies and genetic traits, phages may actively participate in the establishment, maintenance, and breakdown of the symbiotic systems of marine invertebrates. Among marine invertebrates, viromes in sponges and corals are highly diverse and relatively well studied, generating a large volume of genomic information that expands our knowledge of phage diversity, phage–host interactions, and phage life strategies (Engelberts et al. 2022, Zhou et al. 2021b). Given the high diversity of marine invertebrate animals, novel ecological interactions between phages and bacteria and their influences on marine invertebrate hosts will be revealed, particularly for underexplored marine invertebrates from extreme marine habitats, such as hydrothermal vents and cold seeps (Bass et al. 2021, Holt et al. 2022).

Apart from genomic datasets, the transcriptomes of holobionts can provide information on unknown RNA phages (single- and double-stranded RNA) in marine invertebrate holobionts. However, the following issues in meta-omics study should be addressed: (*a*) No efficient and accurate binning tools are available for clustering fragmental contigs, especially plasmid-like and integrated prophage sequences (Maguire et al. 2020), from metagenomes and (*b*) in silico predictions and in vitro assays for precisely reconstructing virus–host networks are lacking. To improve the accuracy of virus–host predictions, complementary approaches have recently been developed, including single-cell viral tagging (Dzunkova et al. 2019), high-throughput chromosomal confirmation capture (known as Hi-C) (Marbouty et al. 2021), and ribosome cross-linking and sequencing (a newly proposed method based on associations between host ribosomes and viral transcripts) (Ignacio-Espinoza et al. 2020). The isolation of viruses and their hosts from marine invertebrates warrants detailed investigations at the molecular, cellular, and physiological levels. However, many of the approaches have not been applied to marine holobionts. Developments in analytic tools will increase our knowledge of the roles of viruses in marine invertebrates, particularly in the following directions:

1. Sustaining a stable symbiotic microbial community: The kill-the-winner viral infection model stabilizes a complex microbial community and ensures the coexistence of bacterial competitors (Thingstad 2000). In addition, prophages regulate colonization competition among symbiont candidates (Wang et al. 2022), indicating their potential roles in symbiont selection. Monitoring phage and bacterial abundance, diversity, and community structure over time will provide empirical evidence of the regulatory effect of phages on microbial communities in symbionts and the roles of viral killers as non-animal-derived factors in the regulation of the population size of symbiotic bacteria.
2. Diverse functions in marine invertebrates: Coral and sponge viromes contain more than 500 gene categories (Swiss-Prot keywords), which are composed of core viral genes and accessory genes that may be specific to animal species (Laffy et al. 2018, Pascelli et al. 2020). These accessory genes might be important for holobiont adaptation to specific environments, but this has not been experimentally verified. Therefore, wet laboratory experiments following metagenomic and metatranscriptomic analysis are urged to demonstrate the diverse functions of these marine invertebrate viromes.
3. Sources of nutrient acquisition for animal hosts: Marine worms, crustaceans, cnidarians, tunicates, and sponges can significantly remove viruses in water columns (Hadas et al. 2006, Welsh et al. 2020). The high removal rate of viruses by marine invertebrates indicates that viruses are potential nutrient supplies. Stable isotope analysis can provide direct evidence of the nutritional roles of marine viruses.
4. Symbiont genome evolution: Lytic and lysogenic infections, as well as their switching under certain conditions, offer opportunities for the integration of viral genomes into host

chromosomes. The integration of virus genomes can reduce the effect of genome reduction in evolution because genome recombination serves as a driver for maintaining symbiont genome size (Russell et al. 2020).

5. Phage therapy: Phage therapy is still in its infancy for aquaculture, and foreseeable challenges include a narrow host range in specificity, removal of remaining phages from invertebrate animals, an efficient way to properly deliver lytic phages to infected tissues, knowledge gaps on marine phages, and the lack of regular treatment guidelines (Kowalska et al. 2020). However, these challenges can be overcome according to the recommendations of Richards (2014) and Kowalska et al. (2020). Genome engineering and synthetic genomics are also potential strategies for designing phages that can target specific pathogens (Strathdee et al. 2023). Multiple levels of bioinformatic and biological experiments can provide novel methods for phage therapy in marine systems.

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Errata

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