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Microbiomes and Obligate Symbiosis of Deep-Sea Animals

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Abstract

Microbial communities associated with deep-sea animals are critical to the establishment of novel biological communities in unusual environments. Over the past few decades, rapid exploration of the deep sea has enabled the discovery of novel microbial communities, some of which form symbiotic relationships with animal hosts. Symbiosis in the deep sea changes host physiology, behavior, ecology, and evolution over time and space. Symbiont diversity within a host is often aligned with diverse metabolic pathways that broaden the environmental niche for the animal host. In this review, we focus on microbiomes and obligate symbionts found in different deep-sea habitats and how they facilitate survival of the organisms that live in these environments. In addition, we discuss factors that govern microbiome diversity, host specificity, and biogeography in the deep sea. Finally, we highlight the current limitations of microbiome research and draw a road map for future directions to advance our knowledge of microbiomes in the deep sea.

1. INTRODUCTION

Marine microorganisms are ecosystem engineers with high physiological and genetic plasticity to adjust to environmental perturbations. Microbes have a unique capacity to survive in extreme habitats because of their large population size, fast growth rate, genome flexibility, and mutation rate. With the aid of complex microbiome communities and/or symbiotic microbial partner(s), novel metazoan biological communities have been established in some of the most extreme habitats on Earth. In the deep sea, animals adapted to cope with the absence of light, low temperatures, high pressure, and limited food supply. The restructuring of the holobiont composition, through either association with a distinct microbial community (1) or establishment of a symbiotic relationship with a specialized microbial partner (2), is a well-documented adaptive mechanism to survive in the deep sea. Many of the discovered symbiotic relationships in deep-sea organisms have broadened our appreciation of how microbes facilitate host survival (3).

The discovery of chemosynthetic symbioses, for example, expanded our understanding of how deep-sea animals use various chemical energy sources to colonize reduced chemical habitats (e.g., cold seeps, hydrothermal vents, whale falls, and shipwrecks; see the sidebar titled Deep-Sea Reducing Habitats and **Figure 1**) (2, 4). Further, in the obligate symbiosis between luminous bacterial symbionts and deep-sea organisms, the bacterial symbiont produces bioluminescent light that the host uses to attract prey and produce mating signals in the dark environment (5). Another example is the unique symbiosis between heterotrophic bacteria and the gutless bone-eating polychaeta (genus *Osedax*). The bacteria yield nutrition for both the symbionts and the *Osedax* host by producing enzymes that hydrolyze collagen and cholesterol (6–8). This host–microbiome association promotes holobiont-level adaption that widens the breadth of habitable niches in the deep sea (9, 10).

Advances in genetic tools, such as next-generation sequencing, have spurred research into how symbioses are established and led to the discovery of novel microbial symbionts and metabolic pathways (11, 12). Using metagenomic analysis, researchers recently discovered the first symbiotic relationship between chemosynthetic bacteria (SUP05) and a cnidarian host in corals and sea anemones living near cold seeps and hydrothermal vents (13, 14). These discoveries highlight the potential role of bacterial–animal symbiosis and their coevolution as a single entity to facilitate survival in deep-sea environments. Therefore, we aim to emphasize the current knowledge about obligate symbionts and microbiome communities associated with deep-sea animals in different habitats and how the microbial community varies over time and space. We discuss different bacterial–animal associations and how these relationships influence the functional profile of the animal host, enabling them to survive in the deep sea. In addition, we discuss lessons learned and challenges associated with deep-sea research and the sampling of microbiomes in the deep sea.

2. MICROBIOME ASSOCIATED WITH DEEP-SEA ANIMALS

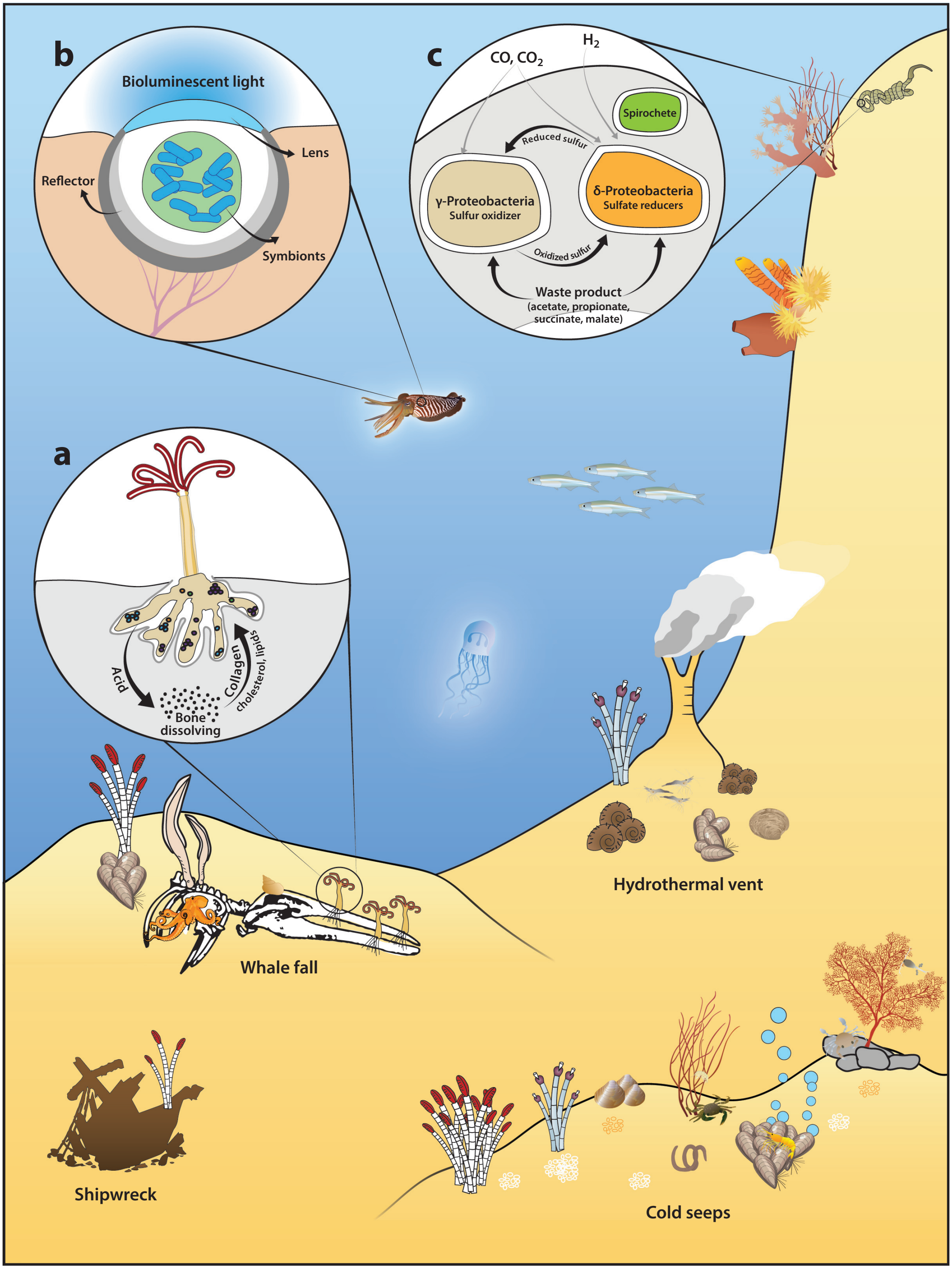
The animal microbiome refers to the consortia of microbes (e.g., bacteria, archaea, fungi, and viruses) associated with an animal host either internally (e.g., in cells, gut, or intestine) or externally on the body surface (e.g., skin or mucus). The microbiome is known to affect host fitness and homeostasis and provide protection against pathogens (15). Most deep-sea animals are associated with distinct microbial communities compared to their shallow-water counterparts and are known to have putative functions to improve host phenotypic plasticity to survive under challenging environments (16). The deep-sea bone-eating snail, *Rubyspira osteovora*, which lives in whale fall habitats, has a unique gut microbiome that is absent in the surrounding environment or other deep-sea snails, suggesting a critical digestive role (17). At hydrothermal vents, *Austinograea* sp. has a distinct microbial community compared to shallow-water crab species (*Eriocheir sinensis* and *Portunus trituberculatus*), some of which may be involved in oxidizing reduced sulfur compounds

DEEP-SEA REDUCING HABITATS

- **Hydrothermal vents:** cracks in the seafloor that release hot, anoxic, and chemical-rich water to the deep ocean. Hot and chemically enriched fluid from the vent is mixed with surrounding cold seawater and establishes a range of habitats (i.e., vent chimneys, rising plumes, diffuse flow, and surrounding subfloor) that are characterized by temperature and chemical gradients at a narrow spatial scale (132, 156, 157). Chemosynthetic bacteria are the primary producers in vent ecosystems that oxidize sulfur, methane, iron, and hydrogen to produce organic carbon. Therefore, vent effluents provide chemical energy sources and provide the basis for this novel ecosystem, including animals, bacterial symbionts, and free-living pro/eukaryotes.
- **Cold seeps:** a natural phenomenon where methane-, hydrogen sulfide–, and/or hydrocarbon-rich fluid leaks from the sea floor and spreads over hundreds of meters. The interactions between an enriched methane seep and seawater establish a unique topography and a habitat that supports a broad range of faunal communities with different capabilities to survive in the presence of different concentrations of hydrocarbon seepage. Chemosynthetic microbes make cold seeps a food oasis relative to the surrounding environment. The magnitude of seepage rate and the composition of seep chemicals change over time and space, shaping the biological diversity and composition of the local ecosystem (130).
- **Whale/wood fall:** sunken whale carcass/wood in the deep sea. Whale falls are a nutrient hotspot on the deep seafloor supporting a broad range of fauna, some of which are found only on whale falls or wood. The anaerobic decomposition of lipid-rich whale bone and tissues produces a sulfidic habitat supporting chemosynthetic communities for a prolonged period; however, nonsymbiotic animals comprise a remarkable diversity at whale fall sites as well. Unlike the habitats mentioned above, whale/wood falls are present only until the organic matter provided by the fall has been consumed by other organisms.
- **Dysaerobic habitats:** areas in the seafloor that are characterized by relatively high organic input and low-oxygen concentration (0.1–1 mL/L^2). The decomposing of organic matter by microbial communities depletes dissolved oxygen in the seafloor and produces hydrogen sulfide and methane that become major sources of energy for microbial communities. Dysaerobic habitats usually occur in the subsurface of the seafloor, and because of low concentration or absence of oxygen, few benthic organisms showed resistance and could survive in this habitat, such as polychaetes, nematodes, and crustaceans.
- **Shipwrecks:** The decomposition of organic matter in shipwrecks may produce sufficient reduced inorganic chemicals to fuel the chemosymbiotic community. The rotten beams in a shipwreck (1,100 m deep) off the coast of Spain produced sufficient sulfide for vestimentiferans to grow (158). Decomposed papers in the mail room in a sunken shipwreck at 2,800 m deep in the Mediterranean Sea also supported growth of *Lamellibrachia* tubeworms (159, 160). In addition, the surface of a shipwreck can provide habitat for sessile organisms, such as corals and sponges, to settle and grow.

and sulfur metabolism, which likely facilitate survival in low temperatures and hypoxic conditions (18). This suggests that the microbiome associated with animals can govern, or at least significantly contribute to, animal survival in the deep sea.

Early studies focused on identifying the composition of the gut microbiome and how they facilitate adaption to deep-sea environments using culture-dependent techniques. Ohwada et al. (19) showed that gut microbiome diversity of deep-sea fish decreased with depth; however, experiments showed that the microbiomes of fish that live in deeper depths are better adapted to high hydrostatic pressure, suggesting that the gut microbiome contributes to survival under high pressure. Recent work on the gut microbiome, including 32 species of deep-sea fish from across



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Different forms of symbiosis that enable hosts to survive in the deep sea. Many organisms have adapted to thrive in deep-sea habitats, such as cold seeps, hydrothermal vents, and whale falls, with the aid of bacterial symbionts. (*a*) The ability of the gutless bone-eating polychaeta (genus *Osedax*) to survive in whale fall habitats is attributed to a symbiotic relationship with heterotrophic bacteria in its roots. *Osedax* produces acid to dissolve bone structure and releases lipid, cholesterol, and collagen that is absorbed back by the host and metabolized by symbiotic bacteria, which feeds both host and symbionts. (*b*) Other animals can survive in the dark environment of the deep sea by producing bioluminescent light through symbiosis with bacterial symbionts. Bacterial symbionts are housed in the photophore structure that includes a lens and reflector to magnify the bioluminescent light produced by symbiotic bacteria. (*c*) Symbiosis with chemosynthetic microbial partners is known in many deep-sea habitats; however, this discovery extended our knowledge to potential adaptive mechanisms in shallow-water animals. *Olavius algarvensis*, which lives in coastal waters in the Mediterranean, provides an example of the functional diversity of chemosynthetic symbionts. *O. algarvensis* harbors five bacterial symbionts (two *Gammaproteobacteria*, two *Deltaproteobacteria*, and one spirochete) that engage in beneficial nutrient exchange to provide the host with organic carbon from multiple energy sources. Gamma and delta symbionts exchange reduced and oxidized sulfur compounds that enable gamma symbionts to fix inorganic carbon autotrophically. Interestingly, sulfur-oxidizing *Gammaproteobacteria* use reduced sulfur compounds produced by *Deltaproteobacteria* as an internal energy source to fix CO_2 autotrophically through the Calvin–Benson cycle, allowing *O. algarvensis* to migrate between upper oxidized and lower reduced sediment layers. Also, these symbionts can use carbon monoxide and hydrogen as energy sources as well as assimilate host waste products.

the Atlantic Ocean, using metagenomic analysis, identified novel microbiome communities that have high capacity for several cellular processes, such as protein folding and DNA replication, that may facilitate survival in high pressure (20). Lian et al. (21) also investigated the hagfish gut microbiome (>900 m), and the genomic features of the dominant microbes revealed that they may contribute to host adaptation to the deep-sea environment.

In addition, the gut microbiome contributes to food digestion and to the release of metabolites that can be used by the host. For example, genomic features of two *Mycoplasma* living in the gut of a deep-sea giant isopod (*Bathynomus* sp.) revealed a greater number of genes responsible for nutrient uptake, suggesting that the symbiont may contribute to host adaption in nutrient-poor environments (22). Also, deep-sea amphipods at hydrothermal vents, *Ventiella sulfuris*, have been found feeding on Pompeii worms, *Alvinella pompejana*, with aid from a gut microbiome dominated by *Epsilonproteobacteria*, which likely has symbiotic features and implications on host nutrition (23). This pattern was also observed in herbivorous fish in freshwater, which are also dominated by cellulose-degrading bacteria (*Clostridium*, *Citrobacter*, and *Leptotrichia*), whereas carnivorous fish have *Cetobacterium* and protease-producing bacteria (*Halomonas*), suggesting potential roles in digestion (24). Herbivorous surgeon fish in shallow coral ecosystems also digest complex polysaccharides of brown macroalgae with the aid of symbiotic *Epulopiscium* gut bacteria that produce specialized enzymes (25). Similarly, Galand et al. (26) experimentally starved two deep-sea coral species, *Lophelia pertusa* and *Madrepora oculata*, and fed them on carnivorous, herbivorous, or mixed diets, finding that the microbiome was diet specific. Thus, diet is considered the main factor shaping the gut microbiome and can significantly change its composition.

However, the microbiome communities also vary substantially between animal hosts and habitats and over time and space. For example, the microbiome of the deep-sea coral *Eguchipsammia fistula* was host specific, but its composition was restructured after one year of rearing in aquaria, suggesting that microbiome flexibility may improve host traits to survive under different environmental conditions (27, 28). Galand et al. (29) confirmed this pattern and found that *L. pertusa* bacteria changed within a day of aquarium rearing, whereas *M. oculata* bacterial communities did not change for more than six months, also suggesting host-specific response to environmental fluctuations. The microbiome is assumed to play a critical role in nutrient cycling and the metabolism of deep-sea corals owing to the lack of obligate symbiosis with photosynthetic algae, but the mechanism is poorly understood. Coral microbiomes may be involved in (*a*) regulation of the microbial community and defense against pathogens through antibiotic secretions and (*b*) food assimilation and metabolism through nitrogen/carbon fixation, amino acid production, and element cycling. Meta-analysis of seven deep-sea corals suggested that both stony and soft corals share core bacteria with potential for nitrogen and sulfur cycling, detoxification, and hydrocarbon degradation (30). Further, it was assumed that cnidarians do not get benefits from living near cold seeps as they do not feed on chemosynthetic-derived food or harbor a sulfur oxidizer symbiont to fix inorganic carbons. Vohsen et al. (13) documented the first endosymbiosis between chemosynthetic bacteria belonging to the SUP05 group and a deep-sea coral species (*Paramuricea* sp. type B3) in the Gulf of Mexico. Genomic analysis of this SUP05 phylotype revealed similarities to those symbionts associated with cold seeps and hydrothermal vent mussels, as well as reported active transcriptional genes for carbon fixation and sulfur oxidation processes, suggesting the endosymbiont's potential to supplement the corals' diet (13). Similar symbiosis was also discovered between the sea anemone *Ostiactis pearseae* and SUP05 bacteria at 3,700 m in active hydrothermal vents (14). The chemosynthetic SUB05 symbiont was housed in the tentacle epidermis of *O. pearseae* and was not (or was rarely) recovered from nearby anemones and surrounding water, suggesting a specific association. Whether similar chemosynthetic symbiosis can be found in other cnidarian species and habitats remains unknown and unexplored.

Host–microbiome specificity is evident in many deep- and shallow-water organisms (31, 32). For example, the gut microbiome communities of two amphipods (*Hirondellea gigas* and *Halice* sp.) that inhabit the Mariana Trench were host specific, and the dominant symbiont (genus "*Ca. Hepatoplasma*") was genetically divergent, despite both species inhabiting the same extreme environment (33). The microbiome of three deep-sea coral species (*Dendrophyllia* sp., *E. fistula*, and *Rhizotrochus typus*) also showed host specificity, including anaerobic bacteria that likely have metabolic functions under low-oxygen conditions (28). Interestingly, microbiomes are not only host specific but also organ specific within an animal. For example, the gills of the hydrothermal vent crab, *Austinograea* sp., have distinct microbial communities similar to those in the surrounding seawater, unlike their gut microbiome, which is dominated by anaerobic bacteria and shaped primarily by host diet (18, 34). Also, the deep-sea carnivorous sponge, *Chondrocladia grandis*, has different bacterial community composition and diversity in three distinct anatomical regions (i.e., the sphere, axis, and root), though the dominant *Tenacibaculum* was present in all anatomical regions (35). Distinct microbiome communities were also reported in both compartments of the cold-water coral *Paragorgia arborea* (mucus versus tissue), and many biomarker taxa were identified for each community (36).

Indeed, the association between the host and the microbiome community is not a stochastic process, and there are parallels between the overall microbial community and host phylogeny. This process, called phylosymbiosis, is evident in many aquatic organisms, such as sponges, ascidians, corals (37, 38), crustacea (39), and fish (40), and also terrestrial organisms (41, 42). Phylosymbiosis highlights the eco-evolutionary signals between a host and its microbial communities; however, the mechanisms that underpin this process remain to be explored (43). Interestingly, rare members of the microbiome were found consistently within the host regardless of environmental setting, forming a core microbiome that is likely essential for host survival (44). Hernandez-Agreda et al. (45) suggested that the microbiome cannot be treated as a single unit and should be categorized into (*a*) the core microbiome, (*b*) the microbiome that fills a specific environmental niche, and (*c*) the dynamic microbiome that responds to changing biotic and abiotic conditions. This highlights the need to understand microbiome dynamics (and function) more deeply to unlock host–microbe interactions under different environmental conditions. However, analysis of deep-sea microbiomes is often constrained by inaccessibility of samples and lack of sufficient replicates for further in-depth analysis.

3. OBLIGATE SYMBIOSIS IN DEEP-SEA ANIMALS

Symbiosis is a unique partnership between two cohabiting organisms, with consequences on host physiology and ecology. The coexistence between an animal and dominant microbial symbiont(s) may take several forms based on how the symbiont influences the host: (*a*) Both the host and symbiont benefit (i.e., mutualistic), (*b*) the host is harmed whereas the symbiont benefits (i.e., parasitic), or (*c*) the symbiont is benefited but the host is not harmed (i.e., commensal). In deep-sea animals, most currently understood symbiotic relationships are mutualistic, where both the symbionts and the host rely on each other to broaden their environmental niche to survive in challenging habitats. In many cases, this mutualistic relationship is necessary for both the host and symbiont, forming an obligate symbiosis in which at least one of the partners cannot complete its life cycle without the other.

Symbiosis between chemosynthetic bacteria and an animal host (known as chemosymbiosis) is widely known in many deep-sea animals that inhabit hydrothermal vents, cold seeps, whale falls, and shipwrecks (see the sidebar titled Deep-Sea Reducing Habitats and **Figure 1**). Chemosynthetic symbionts use a wide range of chemicals, such as methane, sulfide, hydrogen, iron, or carbon monoxide, to fix inorganic carbon and translocate it to their host, which consequently transfers that fixed carbon to higher trophic levels. Overall, this process revolutionized our understanding of the energy sources available to fuel animal life in the deep sea. Foundational and endemic species found in chemically reducing habitats (such as the tubeworm *Riftia* sp. and clam *Calyptogena* sp.) rely on their obligate chemosynthetic symbionts to use sulfur, carbon dioxide, iron, and hydrogen as energy sources to produce organic carbon for nutrition. Thus, chemosynthetic bacteria are considered the primary producers in several deep-sea habitats, unlike in the photic zone, in which faunal communities rely primarily on photosynthetic-derived food. Later, chemosynthetic symbionts were also observed in many shallow-water organisms, including nematodes, oligochaetes, and bivalves. *Gammaproteobacteria* is the most dominant bacterial chemosymbiont associated with diverse fauna, including shrimp (*Rimicaris exoculata*) (46), the Pompeii worm (*A. pompejana*) (47), and other *Alviniconcha* species (48). *Gammaproteobacteria* phylogeny exhibits four major clades that use sulfur or methane as energy sources (49; reviewed in 2, 50). *Alphaproteobacteria* and *Epsilonproteobacteria* are also found as endosymbionts in vent fauna (51). Interestingly, some animals allow multiple endosymbionts using different energy sources to co-occur in their tissue (see below) (52).

Bioluminescent bacteria also form obligate symbiosis with many deep-sea organisms. The symbiotic bacteria produce light to attract prey and induce mating signals, among other functions (**Figure 1**). In these relationships, symbiotic bacteria are located in specialized light organs, such as modified dorsal fins (e.g., anglerfish), esophageal pouches (e.g., pony fish), or subocular organs (e.g., flashlight fish), or in photophore structures scattered across the body (e.g., squid). The mechanism of bacterial bioluminescence is based on oxidation of the light-emitting molecule (luciferin derived from oxidized flavin mononucleotide) by a catalyzed enzyme (luciferase encoded in *lux* operon) in the presence of molecular oxygen and long-chain fatty aldehyde. This reaction produces reduced flavin mononucleotide and emits blue-green light (~490 nm) (53). This process is energetically costly; thus, to preserve energy, bacteria use quorum sensing to regulate luciferase expression and restrict light production until cell density reaches a threshold (54, 55). The animal host controls its bacterial symbionts by altering environmental conditions and nutrition supplies within light organs (56, 57). Most bioluminescent symbiotic bacteria also belong to *Gammaproteobacteria* from *Vibrio* spp., *Photobacterium* spp., *Photorhabdus* spp., and *Aliivibrio* spp. Some luminous bacteria are free-living and facultative symbionts, such as *Aliivibrio* and *Photobacterium* (58), whereas others are obligatory symbionts in mutualistic relationships, such as *Candidatus Photodesmus* in the flashlight fish of the family Anomalopidae (59). The diversity of bioluminescent bacteria was underestimated previously; however, new surveys using the *Tara* Oceans database identified 401 previously unknown luciferase-related genes, suggesting a high diversity of bioluminescent bacteria at a global scale (60).

The gutless bone-eating polychaeta, genus *Osedax*, has a unique symbiosis with heterotrophic bacteria that does not rely on chemosynthesis (6). *Osedax* has an intracellular bacterial symbiont from order *Oceanospirillales* and *Gammaproteobacteria* located in the trophosome in the vascular bone-boring root system (**Figure 1**). *Osedax* produces acid to dissolve bone and release collagen and cholesterol that are metabolized by symbionts, yielding nutrition to both the symbionts and the *Osedax* host (7; reviewed in 8). *Osedax* colonization is observed mainly on whale falls worldwide, but other sister taxa are associated with cow, bird, and teleost fish bones in the ocean, indicating a widespread role in the ocean ecosystem. This unique symbiotic relationship enables *Osedax* to use the nutrition from bones and thus survive in an unusual habitat. Further, a bone-eating snail, *R. osteovora*, has a unique gut microbiome relative to the surrounding environment and to counterparts in other deep-sea habitats (17).

Parasitic symbionts have also been reported in deep-sea animals; however, these relationships have received little attention and are not well characterized. Zielinski et al. (61) reported an intranuclear gammaproteobacterial parasite, "Ca. *Endonucleobacter bathymodioli*," in the chemosynthetic mussel *Bathymodiolus puteoserpentis* collected from cold seeps and hydrothermal vents. This parasitic symbiont infects, proliferates, and then swallows the nucleus of host tissue cells, causing damage to host organs prior to release. Notably, gill tissue, where sulfur and methane oxidizer symbionts reside, is not affected, suggesting a defensive role against the parasitic symbionts. Further, a novel intracellular *Oceanospirillales* phylotype associated with the hydrothermal vent snail *Alviniconcha* spp. is located in membrane-bound vacuoles of the gills. This phylotype is associated with *Gammaproteobacteria* rather than *Epsilonproteobacteria*, suggesting that it could be parasitic or symbiotic with unknown functions (62). Another instance of parasitism is the intracellular bacteria *Rickettsia* (*Alphaproteobacteria*), which is known to infect the digestive and gill epithelia of several deep-sea animals, such as limpets and mussels, at cold seeps and hydrothermal vents (63, 64). Parasitic fungi have also been reported in association with deep-sea benthic nematodes at methane seeps. The fungi are of a novel and host-specific phylogenetic microsporidium fungal clade, *Nematocenator marisprofundi*, suggesting a unique evolutionary pattern for this parasitism (65).

Further, cold-water corals also harbor many parasitic and/or pathogenic bacteria that have yet to be explored in much detail (reviewed in 32). For example, *Mycoplasma* is an abundant intracellular bacteria associated with deep-sea octocorals that is likely mutualistic or commensal. However, *Mycoplasma* in the cold-water scleractinian, *L. pertusa*, is located extracellularly next to the spirocysts, suggesting that *Mycoplasma* opportunistically benefit from hemolymph leaking from prey captured by the animal, without affecting host health (66). It remains unknown whether other *Mycoplasma* associated with octocorals have a similar role, as they belong to a different phylogenetic cluster (67). Furthermore, *Vibrio* spp. can be mutualistic bacteria or pathogens with implications for animal health. Close phylotypes to *Vibrio shiloi* (the common coral pathogen) were found consistently in healthy corals, suggesting that it may be an opportunist rather than a specialized pathogen (68, 69). Interestingly, other low-abundant *Vibrio* spp. are common and a core member in nearly all investigated Mediterranean gorgonians and the red coral (68). Some of those phylotypes are close relative to the putative symbiont, *Vibrio gigantis*, that also aids its host in food digestion (70, 71). The genome of a *V. gigantis*–related bacterium (99.8% shared identity) isolated from the gorgonian *Eunicella verrucosa* suggested that it is likely a generalist and opportunistic commensal symbiont (72). This highlights that further effort is needed to identify the functions of potentially parasitic symbionts that may influence the fitness and functional profile of many deep-sea organisms. Further, whether deep-sea corals are engaged in other symbiotic relationships with microbes, similar to photosynthetic algae found in shallow-water corals, remains unclear, and this field of study is still in its infancy. *Apicomplexa* is a phototrophic symbiont associated with many shallow-water corals. Vohsen et al. (73) expanded on *Apicomplexa* distribution and reported 23 *Apicomplexa* (corallicolid) plastotypes associated with 14 coral species found in the deep sea, down to 2,200 m in depth. Despite the presence of photosynthetic genes in *Apicomplexa* found in the deep sea, it is unlikely that these genes have a photosynthetic role in the absence of light, which suggests they play a different role(s) in dark environments that are yet to be explored.

Interestingly, symbionts may reside within animal tissues (i.e., endosymbiont), such as the esophageal glands of gastropods, the trophosome of tubeworms, or the bacteriocytes in the gills of bivalves, or exist on the internal or external surfaces of an animal (i.e., episymbiosis or epibiont), such as the feet of gastropods, shrimps, crabs, nematodes, and mollusks (50, 74). In all cases, symbionts take advantage of relatively stable microhabitats with the animal host that maintain a sufficient supply of nutrients to sustain the symbionts' functionality, regardless of external environmental settings (75). Epibionts attached to gastropod surfaces in hydrothermal vents (e.g., *Chrysomallon squamiferum*) deposit mineralized scales that likely act as protective armors, whereas other sulfur-oxidizing epibionts produce natural products to protect their hosts from parasites (76, 77). Presumably, epibionts often represent a more primitive evolutionary stage than endosymbiosis, but they still exhibit functional roles for their animal hosts (50). This is unlike endosymbionts, which show integration with the host genome to enable the host to survive in extreme environments, emphasizing the coevolution between host–symbiont partners (10). Further, obligate symbionts cannot survive in a free-living state and thus must reside in host tissue to establish the symbiotic relationship. In contrast, facultative symbionts, such as bioluminescent bacteria associated with deep-sea anglerfish, can be either free-living or symbiotic (78). Obligate symbionts have reduced genomes, relative to free-living ones, and encode essential functional genes to retain energy-efficient metabolism for the holobiont (79, 80). Hendry et al. (81) identified two novel obligate luminous bacterial symbionts (*Candidatus Enterovibrio luxaltus* and *Enterovibrio escacola*) associated with two anglerfish species with extreme genome reduction and loss of metabolic abilities. In conclusion, association with microbial symbiont(s) is a key strategy for animals to survive in the deep sea habitats, and more effort is needed to characterize and understand the functionality of these various symbiotic relationships.

4. SYMBIONT DIVERSITY AND HOST–SYMBIONT SPECIFICITY

The environmental niche for biological communities can be broadened through partnership with microbes; however, the genetic identity of microbial members plays a crucial role in determining this process. Early attempts to explore the diversity of symbionts using culture-based methods greatly underestimated the diversity of microbiome and symbionts associated with deep-sea animals. The gutless mussels, genus *Bathymodiolus*, are among the first residents to inhabit cold-seep habitats, forming a dense population, and rely primarily on chemosymbiotic symbionts to gain their nutritional demands. Initially, *Bathymodiolus childressi* was found to be associated with a methanotrophic endosymbiont that uses methane (4; see the sidebar titled Methane-Consuming Microbes). Later, five species of *Bathymodiolus* were found to have dual symbiosis with both methanotroph and sulfur-oxidizing symbionts in their tissue (e.g., *Bathymodiolus brooksi* and *Bathymodiolus heckerae*) (52, 82). Sulfur-oxidizing bacteria are the most dominant endosymbionts in clams, snails, mussels, and vestimentiferan tubeworms in chemically reduced habitats (83–85). Notably, sulfur-oxidizing symbionts (SUP05 clade, *Gammaproteobacteria*) from the cold-seep mussel *Bathymodiolus* are genetically similar to those associated with fauna in hydrothermal vents (86). Later, *B. heckerae* was found to have four co-occurring symbionts in its gills: two distinct thiotrophs

METHANE-CONSUMING MICROBES

Methanotrophs are a unique group of microbes that have the potential to use methane as a sole energy source. Aerobic and anaerobic methanotrophs oxidize methane to obtain the required energy and carbon to synthesize organic carbon. These microbes are important players in climate regulation, as they consume more than 80% of produced methane before it reaches the atmosphere. Thus, they are found extensively in reducing habitats in the deep sea, such as cold seeps. However, methanotrophs are not technically chemosynthetic microbes, as they do not fix CO_2 and/or dissolved inorganic carbon. In contrast, methane-oxidizing microbes that use methane exclusively as an energy source to fix CO_2 and inorganic carbon from surrounding environment to produce organic carbon are technically true chemosynthetic microbes.

(i.e., sulfur oxidizers), methanotroph, and methylotroph phylotypes, suggesting that microbial symbionts provide a high degree of metabolic plasticity to survive in different habitats (87).

This pattern is evident in several deep-sea animals in different habitats. The hydrothermal vent snail, *Ifremeria nautilei*, has thiotrophic and methanotrophic symbionts, whereas gutless oligochaetes have six endosymbionts belonging to *Gammaproteobacteria*, *Deltaproteobacteria*, and *Alphaproteobacteria*, as well as the *Spirochaeta* (88, 89). Interestingly, the gutless coastal oligochaete, *Olavius algarvensis*, also relies on its five co-occurring symbionts for nutrition and waste assimilation (90, 91) (**Figure 1**). Indeed, advances in sequencing technology have improved the taxonomic resolution of many symbiotic phylotypes. For example, Ansorge et al. (11) identified 16 strains of sulfur-oxidizing symbionts coexisting together in a single *Bathymodiolus* mussel living at a hydrothermal vent. This suggests that more efforts are needed to unlock the diversity of symbionts associated with deep-sea animals.

Host–symbiont specificity is a well-documented pattern in many organisms. However, the factors that govern specificity versus flexibility remain unclear. Whether specificity is attributed to (*a*) acquisition mode of symbionts, (*b*) environmental selection for symbiont traits that better fit with the surrounding environment, or (*c*) the recognition mechanism between host and symbiont regardless of environmental setting is not understood clearly. Symbiont acquisition mode plays an essential role in shaping host–symbiont specificity: (*a*) vertical transmission, whereby the symbiont is inherited from parents and passed to offspring directly during the reproduction process; (*b*) horizontal transmission, whereby the symbiont is acquired from the surrounding environment after development and independent of parents; and (*c*) a mixed transmission mode, in which hosts can acquire their symbionts both vertically and horizontally (**Figure 2**).

In vertical transmission, offspring cannot acquire their symbiont(s) from the surrounding environment. Thus, animal hosts that use the vertical transmission mode exhibit symbiont specificity and congruent evolution with the obligate symbiont(s) (50). As such, this mode limits genetic exchange (i.e., gene flow and horizontal gene transfer) with the free-living symbiont pool, potentially leading to biogeographic isolation. In contrast, symbionts acquired through horizontal transmission are obtained freely from the surrounding environment, which may enhance symbiont diversity within the host. *Bathymodiolus* mussels and oligochaetes, for example, are known for their horizontal transmission mode and harbor up to six co-occurring endosymbionts (50). Mixed transmission was observed in a few animals, such as *Vesicomya* spp. (92, 93) and *Solemya velum* (94), that were previously known to undergo restricted vertical transmission. The flexibility of symbiont acquisition allows for the introduction of new genetic diversity to the host genome that subsequently influences host traits and symbiont diversity/evolution (95).

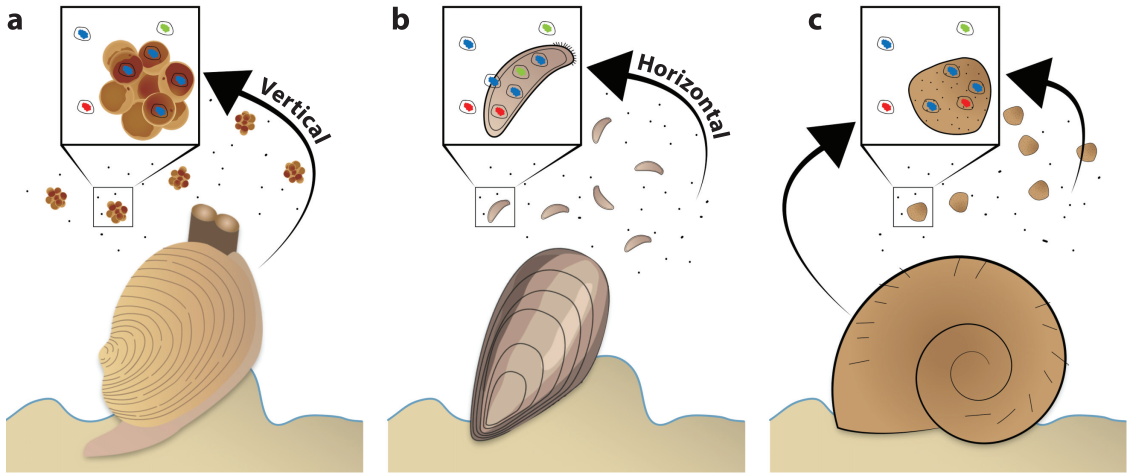


Figure 2

Different modes of symbiont transmission in deep-sea animals. (*a*) In vertical transmission mode, parents pass symbionts to their offspring (particularly eggs), and thus adults have identical symbiont(s) to their parents. (*b*) Horizontal transmission mode is the opposite pattern, in which parents produce symbiont-free larvae that acquire symbionts from the environment independent from parents. (*c*) Mixed transmission mode has been reported in some hosts known to obtain their symbionts vertically, but they show flexibility and uptake symbiont(s) horizontally from the environment to introduce new genetic diversity to the inherited symbionts.

Selection of bacterial symbionts may be a factor determining host specificity. The mechanisms and factors that govern symbiont selection are well documented in model organisms (e.g., squid–*Vibrio* symbiosis; see 96); however, host–symbiont specificity remains an active area of research for many non-model taxa. Gastropods from the genus *Alviniconcha* that colonized different vent areas with varying geochemical characteristics are associated with different symbiont phylotypes, suggesting environmental selection (9, 97). In contrast, many chemosymbiotic bivalves (family *Lucinidae*) are associated with a single bacterial symbiont regardless of region (98). Beinart et al. (99) revealed that distinct symbiont phylotypes in *Alviniconcha* and *Ifremeria* exhibit similar chemoautotrophic gene content, suggesting that symbiont genotypes alone likely do not explain the environmental niche and thus selection. Notably, hosts that use horizontal transmission also have a high level of symbiont specificity, such as vestimentiferan tubeworms that are associated with similar *Gammaproteobacteria* symbionts despite various geographic locations highlighting host specificity rather environmental selection (100, 101). In contrast, horizontally acquired symbionts in *B. brooksi* mussels constitute genetically isolated subpopulations within the same site, suggesting self-infection rather than uptake from the environment (102). This reveals that selection is a regulated process and not a random pattern; however, selection alone may not be the exclusive factor governing host–symbiont specificity.

Notably, the presence of symbionts throughout all life stages is a hot topic, particularly in bioluminous symbionts. Bioluminescence was not reported throughout all life stages of the animal hosts, suggesting horizontal transmission for the acquisition of bacterial symbionts from the environment. Larval-stage anglerfish, for example, do not develop light organs that are able to house symbiotic bacteria until after metamorphosis and juvenile development (78, 103). Freed et al. (104) confirmed that juveniles do not harbor symbiotic bacteria, suggesting that vertical transmission is unlikely to be the transmission mode in anglerfish. These relationships can be difficult to study due to the limitations involved with collecting and investigating various life stages of mobile deep-sea species. Thus, environmental DNA approaches and metabarcoding may advance our knowledge and be useful tools for studying these groups in the future.

The recognition mechanism between host and bacterial symbiont is another critical factor influencing specificity. Studies investigating this mechanism(s) use model organisms in experimental settings rather than non-model or wild animals. Chemotaxis, in which chemoreceptors in symbionts sense host-produced chemical signals and subsequently direct the movement toward (or against) the chemical cue, is a pivotal process for host–symbiont recognition. The composition of chemical cues is diverse and varies between animal hosts but generally includes DMSP (dimethylsulfoniopropionate), amino acids, acrylate, *N*-acetylglucosamine, glucose, galactose, citrate, fumarate, and glycolate (105, 106). The specific and exclusive symbiosis between the Hawaiian bobtail squid, *Euprymna scolopes*, and its bioluminescent symbiont, *Vibrio fischeri*, is governed by the chemical dialog between the host and the bacterial symbiont (96). Therefore, initiation of chemical signaling at the molecular/genomic level and how these signals are integrated to work in harmony remain active areas of research. Further, symbiont mobility is also critical to establish symbiosis, as mobile bacteria have a higher chance of interacting with a host and are likely to establish symbiosis if both partners are compatible. However, host mobility and size may dilute chemical signal concentration and minimize symbiont mobility. Large and mobile hosts usually obtain their symbionts through water pumping, feeding, and/or swimming. Therefore, chemotaxis is more effective after the symbiont is ingested, and consequently, chemotaxis aides the symbiont in recognizing the target organs within the animal host, such as the photophores in squid (107). Overall, host–symbiont specificity is a complex process and is unlikely to be governed by a single factor. Multi-omic and genomic approaches will be key tools to further explore factors that determine the host–symbiont recognition process.

5. FUNCTIONAL DIVERSITY OF SYMBIOSIS

The metabolic capacity of animals is limited to a narrow range of environmental settings, and nutrient-limited habitats challenge the survival and growth of many deep-sea animals. However, association with a unique microbiome community or symbiotic partners is a strategy to cope with nutrient deficiency (91). The microbiome is a complex community composed of hundreds, if not thousands, of phylotypes, and owing to technical limitations, it is rarely feasible to identify the functional role of each member. Therefore, most studies rely on the correlation between host phenotypic traits (e.g., resistance) and the shift of microbiome composition and diversity. Notably, some research infers the putative functions of the community based on taxonomy using different tools (e.g., PICRUSt, Tax4Fun, METAGENassist); however, this remains a controversial and active area of discussion. In contrast, dominant symbionts associated with many animal hosts are well studied, such as zooxanthellae in shallow corals, bioluminous *V. fischeri* in squid, and gammaproteobacterial SUP05 in vent/seep animals. Single, or a few, symbionts can be easily targeted and tested to unlock its physiological and genomic traits and identify its functions within the host. This restricts our knowledge of the microbiome functions to the dominant (or few targeted) symbiont(s), while the function of the entire microbiome community remains poorly understood.

Deep-sea corals, for example, have a distinct metabolic signature relative to shallow-water corals (108); however, how this links to their microbiome composition remains unclear. For shallow-water corals, Imbs et al. (109) reported many bacterial fatty acid biomarkers in the metabolome, and Sogin et al. (110) reported a correlation between metabolome profile and microbial community composition. Despite an emphasis on understanding the functional role of the microbiomes associated with deep-sea animals, the mechanisms remain unclear. Therefore, functional microbiome research focuses on the few members of the microbiome that likely play important roles in host fitness, such as *Endozoicomonas*, which is associated with many shallow-water and deep-sea corals and can make up to 95% of total bacterial abundance (69, 111). Given their reoccurrence and abundance across coral species, there has been an effort to investigate their potential in holobiont functions. Bayer et al. (112) used fluorescence in situ hybridization (FISH) and found *Endozoicomonas* aggregates within the endoderm of coral tissues with different aggregate densities. Analysis of different phylotypes of *Endozoicomonas* showed an enrichment of genes associated with carbon sugar transport and utilization and protein secretion (113, 114). This indicates that *Endozoicomonas* plays a central role in metabolism that is essential for holobiont functioning; however, how these functions are linked to coral fitness is not fully understood. Also, Vohsen et al. (13) targeted the dominate gammaproteobacterial SUP05 associated with a deep-sea coral, *Paramuricea* sp., using metagenome and metatranscriptome approaches and reported the genomic potential to oxidize reduced sulfur and fix carbon. They showed that these pathways were transcriptionally active, suggesting that microbiome diversity may infer metabolic diversity. However, more effort is required to establish a comprehensive database of microbiome functional profiles to improve the current knowledge of host–microbiome interactions.

Variation in environmental conditions drives the establishment of symbiosis with single (or few) symbionts that are phylogenetically and physiologically distinct. These symbionts may have the capability to use various energy sources and metabolic flexibility to aid in host survival in different environmental niches (115). Chemosynthetic symbionts, for example, fix inorganic carbon using energy from oxidation of different reduced chemicals (e.g., thiosulfate, hydrogen sulfate, CO_2) via various metabolic pathways: (*a*) reductive acetyl-coenzyme A, (*b*) reductive citric acid cycle (reverse Krebs cycle), (*c*) the 3-hydroxypropionate cycle, and (*d*) reductive tricarboxylic acid (rTCA) pathways. Advances in metabolomic, metaproteomic, and metagenomic techniques have revolutionized our understanding of symbiont metabolic diversity and have identified new energy sources and novel pathways for organic carbon fixation, particularly those that harbor multiple symbionts (**Figure 1**).

Sulfur-oxidizing symbionts in *Riftia pachyptila* are autotrophic, but genomic analysis showed genes that potentially allow symbionts to live heterotrophically. Reveillaud et al. (116) showed that symbionts can switch between autotrophy, heterotrophy, and likely mixotrophy, with diverse metabolic potential allowing the host and symbionts to live in different environmental conditions. Proteomic analysis of tubeworm, *Escarpia laminate*, symbionts revealed that they use the rTCA pathway to fix carbon, which requires less energy than the Calvin–Benson cycle, explaining the presence of symbionts in low-sulfur environments (117). Further, the gastropod *Alviniconcha hessleri*, which inhabits two different hydrothermal vents, harbors two distinct symbionts using different pathways for carbon fixation: *Gammaproteobacteria* that mediate the Calvin–Benson cycle and *Epsilonproteobacteria* that use the rTCA cycle. Further, deep-sea *Bathymodiolus* mussels have dual symbiosis; sulfur and methane oxidizer symbionts each use different energy sources and pathways to fix organic carbon. Ikuta et al. (118) reported subpopulations of a sulfur-oxidizing symbiont (SUP05) in *Bathymodiolus* with a heterogeneous genome, with genes related to hydrogen oxidation and nitrate reduction processes, suggesting metabolic flexibility. Ansorge et al. (11) found that individual *Bathymodiolus* mussels living at deep-sea hydrothermal vents in the Atlantic can harbor up to 16 strains of sulfur-oxidizing symbionts coexisting together. These symbionts have extensive variations in key functional genes and metabolic pathways, suggesting high metabolic diversity and low-cost symbiosis with the animal host. Interestingly, symbionts may function differently as they grow and age within a host. Small symbionts in *R. pachyptila* are actively divided and may establish cellular symbiont–host interactions, whereas large symbionts apparently no longer divide but still replicate DNA and prioritize carbon fixation and biomass production (119). This adds another

FACTORS AFFECTING MICROBIAL BIOGEOGRAPHY

- **Selection:** the retention of certain taxa (or traits) that are physiologically and genetically fit to survive in certain environmental conditions better than other taxa.
- **Dispersal:** species movement over space to establish a new population(s) with potential metabolic and reproductive capacity in a new location.
- **Ecological drift:** stochastic differences in birth, death, and migration rates that cause changes in population demography. The success of species drift depends on (*a*) environmental filtering (i.e., selection against certain species), (*b*) competition potential with existing species (i.e., prior residents), (*c*) population size (i.e., abundant taxa have higher probability for dispersal), (*d*) habitat suitability (i.e., availability of essential needs), and (*e*) physiological traits (i.e., the acclimation capacity to survive under new conditions).
- **Genetic mutation (i.e., speciation):** genetic change that occurs under certain conditions and rarely happens twice and therefore causes genetic dissimilarity between regions.

layer of complexity to our understanding of the functional diversity of symbiosis and highlights the urgent need for models and tools that combine multiple factors governing metabolic diversity of symbionts.

6. BIOGEOGRAPHY OF THE DEEP-SEA MICROBIOME

Microbes play a critical role in biogeochemical cycles and the linkage between different trophic levels. Deep-sea microbes vary in composition over space and time and exhibit biogeographical patterns in which some microbial taxa are ubiquitous, and others are restricted to particular regions (120). Studying factors that influence microbial biogeography is critical to understanding their ecological functions and connectivity between habitats (121). The biogeographical distribution of free-living microbes is governed by four main processes: selection, dispersal, drift, and speciation. These processes interplay simultaneously to shape the biogeography of microbes; however, their relative importance remains unclear (see the sidebar titled Factors Affecting Microbial Biogeography; reviewed in 122, 123).

The current literature suggests that selection is likely the most critical process influencing microbial biogeography. Environmental heterogeneity (e.g., depth, temperature, nutrients, and oxygen concentrations) is a major force of microbial distribution because only taxa that can survive under certain conditions can inhabit a particular space (124–127). Selection therefore alters the relative abundances of microbial communities and shapes their composition between regions/habitats (128). At local scale, cold seeps have distinct microbial communities that are less abundant (or absent) in adjacent non-seep sediments, forming island-like habitats along a spatial scale of meters (129, 130). Interestingly, the key functional taxa at methane cold seeps showed differential relative abundance among different seeps in respect to depth and temperature variations (129). Similarly, temperature and geochemistry gradients at hydrothermal vents were the main factors shaping microbial composition and their relative abundance within the same vent site over a narrow spatial range (131, 132). This highlights the niche specificity of these chemosynthetic habitats and the fact that environmental heterogeneity promotes selection regardless of the available species pool in the surrounding environment (133).

In addition, the movement capability of microbial communities is always limited (if not absent), and thus, microbial dispersal is governed mainly by passive movement (i.e., water circulation and/or a mobile host) and restricted by seafloor geomorphology (e.g., oceanic ridges and land masses). Limitation of microbial dispersal is an important factor that causes biogeographical pattern. Djurhuus et al. (134) found that microbial biogeography in the deep sea is linked to the water masses, resulting in regional patterns of microbial biogeography that correspond to the regional-scale physical oceanography. Therefore, biogeographical pattern can be minimum if high dispersal rates take place. Similarly, drifting could also shape microbiome biogeography (e.g., 135, 136); however, it must align with high dispersal rate to create biogeographic patterns rather than patchy distribution of microbes (137). Therefore, drifting alone has limited effect in shaping microbial pattern and must be combined with high dispersal rate and selection to significantly influence biogeographical pattern at large geographical scale.

Further, selection may also drive substantial local mutation/diversification over long timescales. Anaerobic methane oxidation bacteria show a degree of endemism to cold seep habitats, highlighting that selection may promote local diversification over time (129). In addition, fine-scale genomic analysis of microbial populations at two geochemically distinct hydrothermal vents (20 km apart) exhibited discrete evolutionary pressures that correlate with genes related to local conditions such as nutrient uptake, biofilm formation, and viral invasion (138). We therefore advocate that selection pressure likely promotes local diversification, and these combined processes shape biogeographical patterns of microbes over time and space. Interestingly, selection processes are not restricted to species selection but also extend to the selection of specific genes or phenotypic traits that improve an organism's capacity to survive in particular environments (139).

Free-living microbes in seawater serve as an inoculum for animals in the deep sea, and thus the presence and survival of free-living symbionts are critical to establish symbiosis. This means that the four main processes that govern free-living microbes can also influence the biogeography of animal-associated microbiomes and symbionts. Ücker et al. (140) found that the genetic variability of symbionts was better explained by geographic location rather than host identity, suggesting that geographic structuring of the free-living symbiont populations influences the composition of the microbiome in these deep-sea *Bathymodiolus* mussels (see also 141). However, this is not always the case, and other factors may play essential roles in shaping microbiome composition, including host identity. For example, three different mussel species (*Bathymodiolus earlougheri*, *Bathymodiolus billschneideri*, and *Bathymodiolus nancyschneideri*) that live side-by-side in methane seeps have distinct thiotrophic symbionts, whereas the symbionts in tubeworms (*Escarpia spicata*, *Lamellibrachia barhami*, and *Lamellibrachia donwalshi*) were identical regardless of host species or sample location (142). Further, tissue chemistry plays a critical role in structuring microbiome composition and may represent selective pressures on the microbiome (143, 144). Microbiome/symbiont biogeography can also be governed by host physiology, chemical composition of surface/gut mucus, diet, and habitat type. Ultimately, many factors govern the biogeography of animal microbiomes, and a deeper understanding of these processes, both independently and together, is needed.

7. LESSONS OF DEEP-SEA MICROBIOME RESEARCH

Novel microbial communities have been discovered through deep-sea exploration and have greatly influenced the global perspective about bacterial–animal symbioses and the different energy sources that can support life on Earth. The discovery of the gutless tubeworm, *R. pachyptila*, at deep-sea hydrothermal venting sites along the Galapagos Rift was the first described symbiosis between an invertebrate host and chemosynthetic bacteria. Histological and enzymatic analysis of *R. pachyptila* revealed that the tubeworm harbored endosymbiotic chemoautotrophic bacteria that oxidize sulfur compounds to fix organic carbon. Tissue stable isotope analyses first suggested that *R. pachyptila* obtains the bulk of its nutrition from its symbionts (145). Following this realization, many other chemosynthetic bacteria were discovered that fix organic carbon from different energy sources, such as methane, hydrogen, iron, manganese, ammonia, nitrate, and carbon monoxide. Even recently, a thermophilic genus of archaea (Ca. *Ethanoperedens*) in the sediment of hydrothermal vents was discovered to use ethane as a carbon source and shown to be highly divergent from methanotrophs and methanogenic archaea (146), suggesting that additional energy sources are yet to be discovered in the deep sea. The revelation of chemosynthetic symbiosis in *R. pachyptila* ignited an effort to characterize chemosynthetic associations in other fauna in deep-sea and shallow-water habitats. Currently, chemosynthetic symbiosis has been reported at hydrothermal vents and sulfur-rich habitats around the world, including shallow-water sediments, seagrasses, sewage outfalls, and mud flats, and has been observed in at least seven different phyla (reviewed in 2 and 50 and references therein; see **Figure 1**). The initial discovery of chemosymbiosis in the deep sea led to groundbreaking discoveries about the variability of energy sources and unique utilization mechanisms of host–microbial interactions. Although some of the most pivotal initial discoveries were made in chemosynthetic habitats, more recent research is starting to illuminate how significant the microbiome is to organismal health across the tree of life.

Improvements of DNA sequencing technologies have revolutionized our understanding of the power of symbionts for animal survival. Although older, traditional methods identified significant relationships between animal hosts and symbionts, many of the mechanisms and host–symbiont interactions were unclear and not fully understood. For example, early analysis of cnidarians (e.g., *L. pertusa*) living near cold seeps showed presence of SUP05 phylotypes (147, 148), but no evidence was reported to support the idea that SUP05 could provide the host with nutrition. In addition, cnidarians have no specialized respiratory structures or oxygen-transport mechanisms, and therefore, it was suggested that they would not form associations with chemosynthetic symbionts (149). Despite this, metagenomic approaches revealed that cnidarians can have symbiotic relationships with chemosynthetic symbionts, and tissue stable isotope analyses suggest that the symbionts provide some nutritional input to the host (13, 14). Further, the hydrothermal vent shrimp, *R. exoculata*, can live in various proximity to hydrothermal vents, regardless of geochemical conditions. Initially, it was assumed that *R. exoculata* was associated with a single bacterial epibiont belonging to *Campylobacteria*. Recently, advanced sequencing technologies refined this assumption and indicated higher microbial diversity, including *Campylobacteria* (up to 90%) and *Gammaproteobacteria* (10–30%), whereas *Alpha*-, *Beta*-, *Delta*-, and *Zetaproteobacteria*, as well as *Bacteroidetes*, *Flavobacteria*, *Bacilli*, *Clostridia*, and some *Aquificae* were also reported at lower abundances (150, 151; reviewed in 152). The recent use of a multi-omics approach not only identifies the components of deep-sea-associated microbiomes but also provides insight into their functionality and has greatly advanced our understanding of the microbes making up these communities. The continued use and further development of these technologies will be instrumental in unlocking the many unknowns that still lie ahead in the field of symbiosis.

8. CHALLENGES OF DEEP-SEA MICROBIOME RESEARCH

The deep sea remains one of the least explored and sampled realms on Earth, largely because it is logistically challenging to access. The earliest forms of deep-sea research were conducted using trawling nets or through fishing bycatch, which gave only a very rudimentary understanding of the types of organisms and microbes inhabiting the seafloor. Advances in deep-sea exploration technology have enabled human beings to physically reach and/or remotely explore deep-sea habitats. However, the current knowledge of deep-sea ecosystems is shaped largely by snapshots of exploration that cover a relatively small portion of the deep seafloor (132). As a result, temporal and spatial changes in biodiversity, microbial ecology, and habitat composition can be very difficult to monitor or interpret. To address these gaps, there are concerted efforts to obtain continuous deep-sea ocean observations in certain areas, such as the Deep Ocean Observing Strategy (153). In addition, we advocate for stronger partnerships between academic, industrial, governmental, and nongovernmental organizations to maintain regular observations and monitoring of deep-sea ecosystems at fixed sites. This will minimize time, effort, and logistical challenges for deep-sea exploration. Also, it will provide invaluable data about temporal changes to deep-sea habitats and how these ecosystems might respond to climate change and anthropogenic activities and thus enable effective management plans to sustain these ecosystems.

Furthermore, the state of current sampling technologies is one of the major challenges facing microbiome research in the deep sea. Microbiome sampling protocols have not been standardized for different types of sampling (animal, seawater, and sediment), which can make it difficult to compare results across sites, regions, or studies. Although in situ sample preservation and/or storage in temperature- and pressure-controlled chambers would be ideal, it is impractical to assume all operations could meet these conditions. Even though all conditions cannot be controlled, scientists have created numerous workarounds for microbiome sampling in the deep sea, such as the high-pressure serial sampler, hydrothermal fluid and particle sampler, hydraulic benthic interactive sampler, and many others (see the **Supplemental Appendix**). In lieu of complex sampling chambers, combining a high number of samples and characterizing the microbial communities associated with the immediate surrounding environment (e.g., seawater, sediment, rock) can also be a way to confirm the dominant components of the microbiome of a specific organism. Notably, recent research has shown that although preservation and extraction protocols can result in slight differences in coral-associated microbiome analysis, these differences do not mask the differences observed between different coral species (154). However, to achieve cross-study compatibility, there should be a standard protocol for all microbial sampling conducted in the deep sea, which would also work to assure an accurate representation of the target habitat or organisms and maximize sampling efficiency.

Supplemental Material >

In addition, laboratory techniques for cultivation of microbes to assess their physiological traits under different conditions that mimic the deep-sea environment remain a big challenge. The classic isolation and cultivation methods (e.g., agar medium) for deep-sea microbes are time and effort consuming and largely have failed to grow members of animal-associated microbiomes. However, promising new technologies are emerging, such as the Prospector® System (GALT.inc, USA), which uses nanoscale cultivation chambers that can grow thousands of microcolonies in parallel in a benchtop automated system. The Prospector® System was used successfully on different microbiome samples (soil, roots, corals, and human), and approximately 2,000 human gut microbiomes were cultured and isolated in only 7 days. This technique may provide a future direction for deep-sea microbiome research and could revolutionize our understanding of microbial physiology and metabolism under laboratory conditions, but it has yet to be tested using deep-sea bacteria. Further, location of bacterial symbionts in the host tissue have been detected via FISH; however, this technique was unable to spatially link the genotype and metabolic phenotype from various microorganisms. The metaFISH technique was developed recently, combining FISH microscopy and high-resolution atmospheric-pressure matrix-assisted laser desorption/ionization mass spectrometry to image host–microbe symbioses and their metabolic interactions (155). Together, the advances in field and laboratory technology are important to modernize our understanding of host–microbiome interaction in the deep sea.

9. CONCLUSIONS

The exploration of deep-sea habitats has resulted in the discovery of novel microbes that play critical roles in facilitating animal survival in challenging environments. The microbiome communities associated with deep-sea animals are unique and largely host specific but vary between habitats and environmental conditions, suggesting critical functional roles. Further, deep-sea organisms establish symbiotic relationships with diverse symbionts to either obtain (or facilitate) their nutrition or produce bioluminescence, enabling animals to cope and survive in a variety of extreme deep-sea habitats. Mutualism is the most common form of symbiosis in deep-sea animals; however, other forms of symbiosis have also been reported but have received less attention. Biogeographical patterns of free-living microbes in the deep sea are likely driven primarily by environmental selection that leads to local diversification and geographical isolation. However, other host factors, such as physiology, diet, tissue chemistry, and chemical composition of surface/gut mucus, can also govern biogeography of animal microbiomes and symbionts. Microbiome research in the deep sea is limited by sampling logistics, inaccessibility, non-standardized sampling protocols, and technical laboratory challenges. This highlights the need to continue efforts to develop technological tools to address challenges associated with assessing and understanding deep-sea microbiomes.

FUTURE DIRECTIONS

1. Strong partnerships between academic, industrial, governmental, and nongovernmental organizations are strongly recommended in the future to allow for regular sampling and monitoring of deep-sea microbial biodiversity and investigation of temporal and spatial variation in the long term. This would produce invaluable data to investigate symbiosis in different seasons and life stages of animals, rather than sporadic sampling and exploration.
2. Standardization of microbiome sampling protocols is necessary for reproducible deep-sea research. We must optimize the field sampling conditions and techniques that allow cross-comparison between sites, habitats, and studies.
3. The development of sequencing technology and genomic pipelines has already improved our knowledge of animal symbiosis, diversity, and functions of deep-sea microbiomes. However, we recommend further extensive use of meta-omic approaches in the future to further modernize the current knowledge of host–bacterial interactions and how bacterial symbionts expand the environmental niche for their hosts.
4. Cultivation-based experiments in laboratory settings remain an essential technique to assess physiological traits of deep-sea microbes and microbiomes. We therefore advocate innovation/use of new laboratory tools to establish a biobank of cultured deep-sea microbes to enhance the current knowledge of microbial traits under certain environmental conditions, which may also have implications in biotechnology.
5. Host–symbiont/microbiome specificity is evident despite the symbiont transmission mode; however, the recognition mechanism between bacteria and animal partners remains a hot topic that needs continued focus and further research.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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