ANNUAL REVIEWS

Annual Review of Microbiology

The Influence of Bacteria on Animal Metamorphosis

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Annu. Rev. Microbiol. 2020. 74:137–58

The *Annual Review of Microbiology* is online at micro.annualreviews.org

https://doi.org/10.1146/annurev-micro-011320-012753

Keywords

metamorphosis, biofilm, phage, coral, *Hydractinia*, *Hydroides*

Abstract

The swimming larvae of many marine animals identify a location on the seafloor to settle and undergo metamorphosis based on the presence of specific surface-bound bacteria. While bacteria-stimulated metamorphosis underpins processes such as the fouling of ship hulls, animal development in aquaculture, and the recruitment of new animals to coral reef ecosystems, little is known about the mechanisms governing this microbe-animal interaction. Here we review what is known and what we hope to learn about how bacteria and the factors they produce stimulate animal metamorphosis. With a few emerging model systems, including the tubeworm *Hydroides elegans*, corals, and the hydrozoan *Hydractinia*, we have begun to identify bacterial cues that stimulate animal metamorphosis and test hypotheses addressing their mechanisms of action. By understanding the mechanisms by which bacteria promote animal metamorphosis, we begin to illustrate how, and explore why, the developmental decision of metamorphosis relies on cues from environmental bacteria.

Contents

INTRODUCTION

Microbes have been evolving on Earth for more than three billion years, setting the biological and ecological foundations for the evolution of eukaryotic life (78). Within this context, animals evolved 400 million years ago in an environment already dominated by abundant and diverse bacteria (121, 133). Interactions with this microbial world shaped animal biology, whether in intimate symbioses or as organisms that share and modify a common habitat. Recently, the beneficial roles of microbes in animal development have gained widespread appreciation, paving the way for our realization that microbes fundamentally influence animal health, development, and evolution (46, 101, 103). For example, bacteria direct multicellular behavior in choanoflagellates—the closest living relatives to animals—(2, 168), budding in hydra (125), light organ development in the Hawaiian bobtail squid (79, 116), digestive tract development in zebrafish (7, 60), and immune system development and maturation in mammals (14, 100). These instances of bacteria-stimulated development stand in opposition to the conventional notion that each animal's development is directed solely by its own genome (101). Growing attention has focused on how the host microbiome drives diverse aspects of eukaryotic development. Yet, bacteria in the microbiome are not the only bacteria influencing eukaryotic development. Although often disregarded, environmental bacteria also provide cues that regulate essential developmental processes in diverse eukaryotes. However, these widespread interactions raise the provocative and, until recently, largely unaddressed question: How do environmental bacteria shape normal animal development?

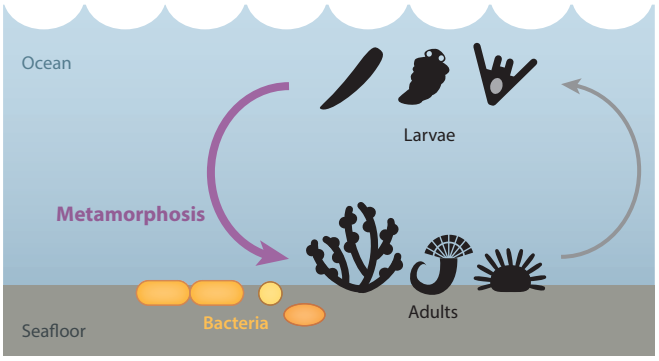

Figure 1

Model of the stimulation of animal metamorphosis by bacteria. The swimming larvae of diverse marine animals (e.g., corals, tubeworms, and urchins) are stimulated to undergo settlement and metamorphosis by the presence of bacteria bound to the seafloor.

THE INFLUENCE OF BACTERIA ON ANIMAL METAMORPHOSIS AND EVOLUTION

A widespread yet poorly understood example of bacteria shaping animal development is the stimulation of animal metamorphosis by bacteria. During these interactions in marine environments, surface-attached bacteria on the seafloor serve as an indicator and provide a stimulus for the swimming larvae of many animals, promoting larval settlement and triggering metamorphosis into the juvenile form (**Figure 1**). Once induced to undergo metamorphosis by bacteria, the larval animal undergoes a dramatic developmental transition, losing larval features and taking on adult characteristics. Bacteria that promote metamorphosis are thought to serve as a critical indicator of a preferable habitat for adult animals. While this process is fundamental to the life history of diverse animals, and likely shaped their ecology and evolution, there has still been much to learn since this phenomenon was first reported in the 1930s (178).

The diversity of animals that undergo metamorphosis is enormous. Yet apart from a few animal groups, metamorphosis is poorly characterized. Most of our knowledge of animal metamorphosis is derived from only a few model organisms, notably the fruit fly (*Drosophila melanogaster*) and African clawed frog (e.g., *Xenopus laevis*, *Xenopus tropicalis*), which are not currently believed to undergo metamorphosis in response to bacteria. Studying the metamorphosis of marine invertebrates offers valuable insight into the basis of environmental bacteria signaling in animal development in a setting where the very persistence of benthic marine ecosystems depends on it.

The complexity of settlement and metamorphosis of marine larvae invites the use of proper definitions. Here, settlement is defined as a behavioral process by which larvae that possess the ability to undergo metamorphosis (competency) reversibly bind to the substratum, while the term metamorphosis describes the transition from the attached larval stage to a sessile juvenile stage—a morphogenetic process (12). Competency permits marine invertebrate larvae to live a planktonic life and allows some flexibility in the timing for settlement and metamorphosis in response to a suitable location based on environmental cues. The developmental change of metamorphosis is often accompanied by a corresponding change from a free-swimming to a surface-associated state (12). Importantly, metamorphosis is an irreversible process. Therefore, making the decision of where and when to transition from a planktonic to a sessile state is critical for survival and reproduction as a surface-bound adult (144). Here, we explore what is known and what we hope to learn about bacteria that stimulate metamorphosis, the signaling molecules present within marine

biofilms, the chemical diversity of known bacterial cues, and challenges in identifying the animal sensory machinery that triggers this developmental transition.

BIOFILMS AND THEIR ROLES AS SETTLEMENT CUES FOR MARINE INVERTEBRATE LARVAE

Biofilms are consortia of intimately interacting microbial cells enclosed in an extracellular matrix; biofilms cover all underwater biological, mineral, or artificial surfaces (41). Rather than being conglomerations of cells and slime, biofilms are organized communities with functional microcolonies and channels that perform complex metabolic processes (23). The microbes within biofilms produce a matrix of extracellular polymeric substances (EPSs), composed of polysaccharides, proteins, nucleic acids, and lipids, which provide mechanical stability, mediate adhesion to surfaces, and form a cohesive, three-dimensional polymer network that interconnects and transiently immobilizes biofilm cells (39). EPSs are prominent components of biofilms that have been implicated in stimulating metamorphosis (52), although this has not been shown explicitly.

Natural biofilms are composed of many microbial species including bacteria, diatoms, fungi, and protozoa. Multispecies biofilms can form stable consortia, develop physiochemical gradients, and facilitate horizontal gene transfer and intense cell-cell communication; thus, these consortia represent highly competitive environments (40). To understand the stimulation of metamorphosis by marine biofilms, a number of studies have characterized the microbial diversity within inductive biofilms. It has been shown that the bacterial community structure of natural biofilms varies in its response to environmental factors such as salinity, temperature (85), tidal level (31, 124), dissolved oxygen (115), hypoxia (19, 81, 142), and habitat (20, 70, 93). Natural biofilms formed under different environmental conditions vary in their attractiveness to settling larvae (16, 20, 31, 70, 85, 93). However, most factors influencing biofilm community composition, including salinity and temperature (85), or succession over time (21, 93, 140), did not influence settlement, whereas biofilm cell density was correlated with settlement. Importantly, denser mature biofilms support a matrix of complex molecules and morphogenic signaling compounds that are thought to contribute to larval settlement in marine invertebrates. While some studies have provided evidence that bacterial community structure might be important for settlement of marine larvae (114), the actual settlement cues associated with biofilm communities often remain unknown or poorly understood (42, 69).

FOR MOST ANIMALS, THE SPECIFIC BACTERIAL FACTORS THAT INDUCE METAMORPHOSIS ARE UNKNOWN

Animals that undergo metamorphosis represent all major branches of the animal tree of life (**Figure 2**). Of these animal types, almost all clades possess representative species that undergo metamorphosis in response to bacteria (**Figure 2**). Bacteria stimulate larval settlement and metamorphosis in diverse marine invertebrates, including sponges (160, 164, 165, 167), mollusks (6, 38, 48, 74, 131, 153, 161, 173), crabs (4), barnacles (37, 76), bryozoans (8, 31), annelids (141), urochordates (152), echinoderms (33, 68), and ascidians (18, 75, 129, 166). While the cues mediating most of these interactions are unknown, the chemical compositions of a few metamorphosis cues from laboratory-developed bacterial biofilms have been partially characterized; for example, carbohydrates induce larval attachment and metamorphosis of the polychaete *Janua (Dexiospira) brasiliensis* (77) and larval attachment of the tunicate *Ciona intestinalis* (152). Histamine isolated from algae, or the biofilm coating the algae, stimulates the metamorphosis of the sea urchin *Holopneustes purpurascens* (150, 151).

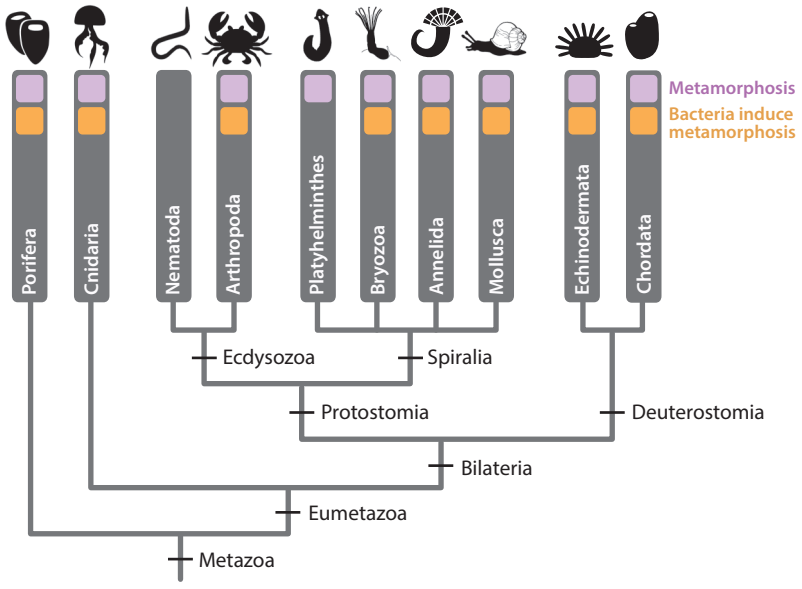

Figure 2

Bacteria-stimulated metamorphosis is widespread among diverse animal taxa. Shown is a representation of the animal tree of life. Taxa that undergo metamorphosis are indicated in purple. Taxa that undergo metamorphosis in response to bacteria are indicated in orange. Adapted from Reference 139.

In the study of bacterial factors that stimulate metamorphosis, and the animal receptors and response mechanisms, the use of simplified model systems is beginning to reveal how environmental bacteria promote animal metamorphosis. Here we review the mechanisms by which environmental bacteria influence the metamorphosis of three marine animals: (*a*) the polychaete tubeworm *Hydroides elegans* and the cnidarians, (*b*) corals, and (*c*) *Hydractinia*.

THE TUBEWORM *HYDROIDES ELEGANS* AS A MODEL ANIMAL

The marine tubeworm *Hydroides elegans* (hereafter *Hydroides*) is a powerful model organism to investigate how bacteria stimulate animal metamorphosis. In the 1990s, Hadfield et al. (55) first documented that the larvae of *Hydroides* respond to bacterial biofilms by undergoing metamorphosis. In the laboratory, *Hydroides* larvae undergo metamorphosis in response to biofilms composed of multispecies communities of microorganisms (66, 93, 140) and single species of bacteria (45, 141, 158).

Hydroides was first developed as a model organism for biofouling because it forms thick crusts of calcified tubes on submerged boat hulls, causing corrosion and higher fuel consumption when ships are underway (111). The properties that make this tubeworm a pest also make it an effective model organism for studying how bacteria stimulate metamorphosis. Specifically, *Hydroides* is easily propagated in the lab, each female can yield thousands of eggs per spawning, and the larvae have a short development period (six days) before acquiring the ability to sense bacteria and undergo metamorphosis (i.e., become competent). To demonstrate that *Hydroides* is adapted to respond to surface-bound bacteria, Hadfield et al. (54) showed that *Hydroides* changes its swimming and settlement behavior when in direct contact with biofilms.

A valuable feature of model organisms is that they have genes and molecular pathways that are conserved among diverse animals. To further develop *Hydroides* as a model organism, we sequenced

its genome (139) and found that the gene content of this tubeworm more closely resembles that of anemones, sea squirts, and humans than it does other model invertebrates such as the fruit fly (*Drosophila melanogaster*) or nematode (*Caenorhabditis elegans*). Therefore, insights into how *Hydroides* senses and responds to bacteria may be applicable to diverse animal lineages.

Diverse bacteria have been shown to induce *Hydroides* metamorphosis, including those belonging to gram-negative (*Gammaproteobacteria* and *Alphaproteobacteria* classes, *Cytophaga-Flexibacter-Bacteroides* group) and gram-positive (*Firmicutes* phylum) groups (56, 66, 83, 87). However, so far bacterial taxonomy has not been correlated with the induction of metamorphosis. In fact, different isolates belonging to the same genus can differ tremendously in their ability to induce metamorphosis, varying from no induction to moderate induction to very strong induction. For example, the marine bacterium *Pseudoalteromonas luteoviolacea* is a potent inducer of metamorphosis, while diverse other *Pseudoalteromonas* species show little stimulatory effect on *Hydroides* metamorphosis. *Hydroides* is well suited for the reductionist approach of studying the effect of one bacterium on one animal to identify specific bacterial factors that stimulate metamorphosis. Identifying these factors and the different mechanisms by which they stimulate metamorphosis will provide significant insight into the diversity and mechanisms of how bacteria influence animal development.

A SURPRISINGLY DIFFERENT WAY THAT BACTERIA STIMULATE METAMORPHOSIS

Since the 1930s discovery that bacteria stimulate animal metamorphosis (178), the prevailing model has been that animals respond to factors that are bound to the surface of bacterial cells or released nearby (**Figure 3**). For many marine animal larvae, dissolved factors have been shown to stimulate metamorphosis (52). However, the stimulation of *Hydroides* metamorphosis by bacteria was shown to require physical contact with a biofilm surface (54). These findings hinted that the bacterial factors that induce metamorphosis are diverse in their biological and physical properties.

Recently, we discovered a surprisingly different way that bacteria stimulate animal metamorphosis—the first known bacterial injection system that stimulates the metamorphosis of an animal (141) (**Figure 4*a*,*b***). We called these structures metamorphosis-associated contractile structures (MACs) because they form syringe-like protein complexes that induce tubeworm metamorphosis. A pioneering study by Huang et al. (67) used forward genetics to identify a set of 4 genes in the genome of *P. luteoviolacea* that are required to stimulate tubeworm metamorphosis.

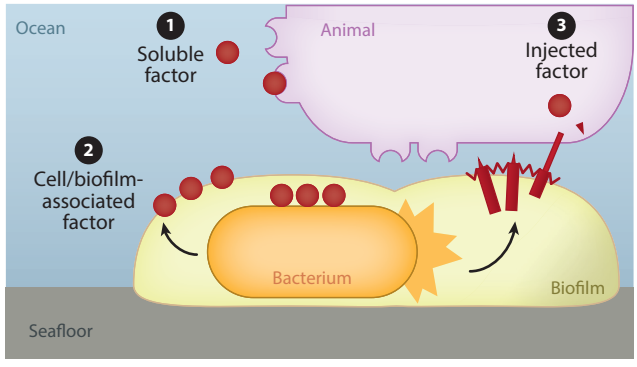

Figure 3

Model of types of bacterial factors that stimulate animal metamorphosis. Stimulatory factors from bacteria can be (❶) soluble, (❷) bound to the bacterial cell or biofilm surface, or (❸) injected into host cells.

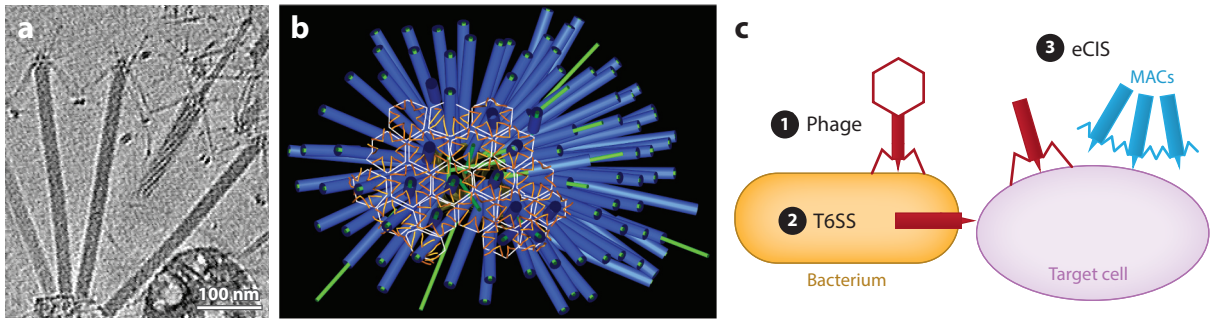

Figure 4

MACs are an example of a CIS that often injects protein effectors into target cells. Panels *a* and *b* show a side view of MACs in extended and contracted states and a segmented model of the array, respectively. (*c*) CISs are related to the contractile tails of bacteriophage (viruses of bacteria, ❶). T6SSs (❷) act from within a bacterial cell, while eCISs (❸) are released by bacterial cell lysis and autonomously bind to target cells. MACs are one example of an eCIS. Abbreviations: CIS, contractile injection system; eCIS, extracellular CIS; MAC, metamorphosis-associated contractile structure; T6SS, type VI secretion system. Panels *a* and *b* adapted from Reference 141 with permission.

They did this by using a transposon to randomly mutagenize the bacterial genome and then screen for mutants deficient in inducing metamorphosis. We subsequently found that the 4 genes identified in this screen belong to a cluster of over 40 genes that encode the syringe-like MACs (141).

Instead of soluble or surface-bound factors produced by bacteria, MACs are complex syringe-like structures that inject protein effectors into target cells. MACs are one example of contractile injection systems (CISs), which are related to the contractile tails of some bacteriophage [the viruses of bacteria (**Figure 4*c***)]. Like other CISs, MACs are composed of a rigid inner tube surrounded by a contractile sheath, a tail spike, and a baseplate complex. Contraction of the sheath propels the inner tube and tail spike into target cells and delivers effector proteins that elicit a host response. While other CISs typically form individual syringe-like structures, MACs are the first example of a CIS forming arrays of about 100 CIS structures arranged in a star conformation (**Figure 4*a*,*b***).

Since the discovery of MACs, related CISs have been discovered that also form multi-CIS complexes (13). In addition to stimulating metamorphosis, closely related structures were found to mediate interactions between microbes and amoebae, insects, and potentially humans (13, 132, 159, 172). While a number of pathogenic bacteria use type VI secretion systems to inject protein toxins into target cells to cause disease (97), MACs are the first CIS to promote a beneficial microbe-animal interaction. Such a mechanism of bacteria stimulating metamorphosis is unprecedented and provides a paradigm shift in our thinking about how microbes stimulate animal development.

While we identified MACs as the structures stimulating tubeworm metamorphosis, it remained unclear how MACs influenced *Hydroides*' metamorphic transition. Recently, we used cryo–electron tomography (cryo-ET) to directly observe a protein effector loaded within the inner tube lumen of the MAC's syringe-like needle (36). We identified the protein effector and named it metamorphosis-inducing factor 1 (Mif1) because it is sufficient for stimulating tubeworm metamorphosis when delivered to tubeworm larvae by electroporation. Although Mif1 is the first identified bacterial protein that stimulates metamorphosis, we do not yet know its mechanism of action, and its protein sequence possesses no identifiable domains that could yield clues to its function. However, Mif1 still provides an intriguing entry point into understanding how a bacterial factor, particularly a proteinaceous factor, stimulates metamorphosis.

It is unclear how bacteria benefit from producing MACs. One clue is a second protein effector that MACs deliver to target cells in vitro (130). Paradoxically, this second effector, which we termed *Pseudoalteromonas* nuclease effector 1 (Pne1), is toxic to insect and murine cells in vitro but had no observable effect on *Hydroides* larvae. Reciprocally, we did not observe an effect of Mif1 on the cell lines in vitro. We currently hypothesize that the two MAC effectors target different organisms to promote the *P. luteoviolacea* lifestyle as a free-living yet host-associated marine bacterium. A recent study exploring the distribution and diversity of MACs' structural gene homologs in the marine environment found them to be more abundant in biofilms than in the water column (28), suggesting that MACs may benefit surface-attached bacteria by facilitating their interaction with animal larvae while deterring potential biofilm-eating predators like protozoans (99).

DIFFERENT BACTERIAL FACTORS STIMULATE METAMORPHOSIS IN THE SAME ANIMAL

A surprising finding derived from studying *Hydroides* is that chemically different factors from bacteria may be able to stimulate the same developmental process of metamorphosis. Diverse bacterial strains that are able to induce *Hydroides* settlement have been isolated (83, 158), which shows that the inductive chemical(s) can be produced by many different bacterial families and classes. For instance, *Loktanella hongkongensis*, a marine alphaproteobacterium that induces *Hydroides* metamorphosis, does not possess genes that produce MACs (84). Instead, it has been suggested that *L. hongkongensis* produces low-molecular-weight compounds associated with the exopolymeric matrix of the bacterial cells that are able to induce *Hydroides* metamorphosis (82).

Hydroides metamorphosis is also triggered by taxonomically distant strains of *Cellulophaga lytica* (*Flavobacteriia* class), and the gram-positive bacteria *Bacillus aquimaris* and *Staphylococcus warneri* (*Bacilli* class) (45). Freckelton and colleagues (44) revealed that the gene assemblies for MACs are lacking in these bacteria, but they observed the presence of inductive extracellular vesicles from *C. lytica*, *B. aquimaris*, and *S. warneri*. Employing a biochemical structure-function approach, they recently showed that lipopolysaccharide extracted from *C. lytica* cultures is able to induce *Hydroides* metamorphosis (44). Interestingly, extracellular vesicles from both gram-positive and gram-negative species have been found to provide a mechanism for cell-to-cell interaction, including the transfer of DNA, protein, and small signaling molecules (11, 27). Thus, membrane vesicles are potentially a widespread mechanism of interaction between biofilm bacteria and invertebrate larvae.

In addition to proteinaceous MACs, small-molecule compounds have been demonstrated to stimulate *Hydroides* metamorphosis. Hung et al. (69) described two lipid moieties isolated from a mixed bacterial biofilm that also induce metamorphosis. These two compounds were a long-chain fatty acid (12-octadecenoic acid) and a hydrocarbon (6,9-heptadecadiene) that induced *Hydroides* larval settlement to a similar extent as natural biofilms. These two compounds are quite distinct from proteinaceous MACs, and it is currently unclear whether each bacterial factor stimulates metamorphosis through the same pathway. Thus, inducers that have been discovered indicate that there are a variety of modes that bacteria can use to stimulate their animal hosts, demonstrating that diverse mechanisms of interaction can promote the same developmental process.

BACTERIA-INDUCED METAMORPHOSIS OF CNIDARIANS; CORALS AND *HYDRACTINIA*

Corals

Many cnidarians have free-swimming planula larvae that settle and develop into sessile polyps. Larval settlement and metamorphosis of reef-building corals are of particular interest due to the

decline of coral reef ecosystems. Understanding how bacteria stimulate coral metamorphosis could have implications for reef restoration through the recruitment of larvae and survival of newly metamorphosed juveniles. Both bacteria and crustose coralline algae (CCA), which are encrusting red algae, have been described as natural inducers of coral metamorphosis (51). Morse and colleagues (104) were the first to demonstrate that CCA induce agariciid coral metamorphosis. Further studies went on to characterize the morphogen as an insoluble CCA-associated cell wall fraction that appears to be a large polysaccharide (105, 106). Around the same time, a hypothesis arose that CCA-associated bacteria could contribute to the inductive properties of CCA (73). This hypothesis relied on the premise that CCA host distinct microbial assemblages, which was supported by a recent study that characterized CCA-associated bacteria using molecular techniques (145). While multiple studies have attempted to determine whether it is the algae or bacteria that stimulate metamorphosis, a consensus in the field has not been reached (47, 154).

Approaches using natural heterogeneous (104, 163) or isolated single-species biofilms (112, 138, 156) demonstrated that bacteria alone are sufficient to induce metamorphosis in corals. Age, location, and depth of the biofilm are considered important factors for natural biofilm-induced coral metamorphosis (163). Systematic isolation and culturing of bacteria from inductive substrata (i.e., CCA, coral host, and biofilmed slides), and subsequent laboratory assays utilizing single-species biofilms, have led to the identification of several bacteria that can induce metamorphosis in broadcasting and brooding coral larvae (112, 138, 156). Interestingly, the ability of diverse bacteria to stimulate coral metamorphosis suggests that taxonomy and the source of isolation are not indicative of a bacterium's capacity for stimulating coral metamorphosis (156).

To date, there is one well-characterized chemical compound, 2,3,4,5-tetrabromopyrrole (TBP), from bacteria that is capable of stimulating coral metamorphosis. Negri and colleagues (112) first identified a single bacterium, *Pseudoalteromonas* sp. A3, that when grown in a monospecific biofilm elicits a strong but mixed coral larval response. Some larvae would undergo partial metamorphosis (metamorphosis but unattached), while others fully attached and metamorphosed. Characterization of inductive and phylogenetically related *Pseudoalteromonas* sp. A3, J010 (155), and PS5 (146) strains identified TBP as an inducer of metamorphosis in globally distributed species of coral larvae. Exposure of the larvae to the extracted chemical cue recapitulated similar levels of attached and unattached metamorphosis in multiple species of coral larvae when compared to the monospecific biofilm metamorphosis assays (146, 155). Genetic and biochemical analyses identified the *bmp* biosynthetic gene cluster (*bmp1–10*) as being responsible for the production of a suite of brominated natural products, including TBP (1). El Gamal and colleagues (35) demonstrated that only genes *bmp1–4* are necessary to produce TBP in vitro, and further, *Pseudoalteromonas* strains A3, J010, PS5, and A757 have a version of the *bmp* cluster that produces TBP almost exclusively.

While it was shown that extracted TBP is sufficient to induce metamorphosis, the significance of TBP as an ecologically relevant metamorphosis-inducing factor remains debated because TBP stimulates some coral larvae to undergo metamorphosis without settlement and attachment. Furthermore, Tebben and colleagues argue that the predicted abundance of pseudoalteromonads on the surface of CCA would not be sufficient to induce metamorphosis in the environment (154). Despite the debate, one study utilized TBP extract in comparison with CCA to attempt to differentiate the molecular processes of attachment and metamorphosis (143); however, the underlying molecular mechanism by which TBP can induce metamorphosis in corals has not been characterized. A potential lead from a study utilizing mammalian microsomes demonstrated that ryanodine receptors bind TBP, which triggers Ca^{2+} efflux (176). Understanding the breadth of molecular triggers capable of initiating metamorphosis in corals may enable us to more effectively harness them for potential restoration uses.

While pseudoalteromonads have gained considerable attention for their role in coral metamorphosis, there are other isolates of bacteria capable of inducing metamorphosis whose genomes do not appear to encode characterized inducers of metamorphosis, e.g., TBP (138, 156). Of note, the biofilms of *Thalassamonas agarivorans*, a gammaproteobacterium, evoked a strong metamorphic effect in the brooding coral *Pocillopora damicornis* (156). Cell cultures and filtrates of an *Alphaproteobacteria* strain, *Roseivivax* sp. 46E8, induced metamorphosis of the brooding coral *Porites astreoides*, albeit at a lower rate than that of CCA or natural biofilms (138). These findings suggest that bacteria produce other factors besides TBP that can induce coral metamorphosis or may synthesize TBP using a mechanism that has yet to be determined. Further, there could be synergistic effects of multiple bacterial factors resulting in the metamorphosis of coral larvae in the environment (138).

The current state of research in bacteria-stimulated coral metamorphosis could benefit from a bilateral approach that aims to understand both the bacterial factors responsible for inducing metamorphosis and the cellular responses that mediate metamorphosis in the coral larvae. Recent advancements in high-throughput sequencing of coral genes have identified gene products with potential for surface/biofilm recognition (57, 102, 143, 148). On the bacterial side, a comprehensive approach for testing bacteria and identifying their factors that are described to induce metamorphosis in other organisms may reveal universal underlying mechanisms for bacteria-stimulated metamorphosis. Despite the importance of corals as animals of ecological concern, the limitation of coral spawning events and lack of molecular tools make closely related model organisms (e.g., *Hydractinia*) of key importance for the elucidation of this bacteria-animal interaction.

Hydractinia

The colonial marine hydroid *Hydractinia* is a versatile, informative cnidarian model. *Hydractinia* is a member of Cnidaria—multicellular animals possessing true tissues that lie at the base of the Metazoa (43). Members of the *Hydractinia* genus (*H. echinata* and *H. symbiolongicarpus*) have served as important models to understand the origins of cell and tissue differentiation, histocompatibility, and development (43), but they have also provided important, early insights into the phenomenon of bacteria-stimulated animal metamorphosis.

The first account of bacteria inducing metamorphosis of *Hydractinia* was published in 1969 by Müller (107). During these pioneering studies, Müller provided evidence that only some bacteria produce cues that trigger *Hydractinia* metamorphosis through direct interaction and only under specific growth conditions (108). Enrichments of bacterial communities from shells inhabited by hermit crabs, the natural substrate colonized by some *Hydractinia* species, were more effective at inducing metamorphosis when harvested closer to stationary phase. From tests with isolated bacterial strains, Müller determined that the inductive capabilities depended on the type of bacterium, growth media, growth phase, density, and duration of exposure. In later studies, bacteria belonging to the genera *Alteromonas* and *Pseudoalteromonas* were found to induce the metamorphosis of larvae of *Hydractinia* (50, 109). However, in contrast to Müller's observations, some studies suggest that most of the bacteria tested have inductive metamorphosis capabilities, including *Escherichia coli* (80).

In a recent study, the microbiome of *H. echinata* was characterized for the first time. Using 16S rRNA deep sequencing as well as a culture-dependent approach, Guo and colleagues (50) investigated the microbial secondary metabolite repertoire and the settlement and metamorphosis-inducing activity of *H. echinata*–associated strains. Six isolated strains were able to induce rapid settlement and metamorphosis (within 24 h); two *Pseudoalteromonas* strains exhibited the strongest

induction capabilities. Another ten strains could induce slower settlement in 60–80% of larvae within 48 h. Additionally, they reported four *Pseudoalteromonas* strains that caused lysis of larvae.

Consistent with a previous study by Leitz & Wagner (88, 92), who biochemically identified a lipophilic fraction obtained from the marine bacterium *Alteromonas espejiana*, Guo et al. (49) recently found that bacterial (lyso)phospholipids and polysaccharides from *Pseudoalteromonas* sp. P1–9 and *Alcaligenes faecalis* stimulate *Hydractinia* metamorphosis. Interestingly, exposure of *Hydractinia* to both phospholipids and polysaccharides induced higher rates of metamorphosis than either type of compound on its own, which the authors hypothesize could provide important environmental context for *Hydractinia* larvae to select an optimal habitat.

Anecdotal observations suggest that *Hydractinia* larvae will not metamorphose in the absence of bacteria (107, 108). However, the degree to which *Hydractinia* larvae rely on bacteria to complete their life cycle has not been explicitly addressed experimentally. Results of such a study could help determine whether bacteria play an essential role in the metamorphosis of *Hydractinia*.

COSTS AND BENEFITS OF STIMULATING ANIMAL METAMORPHOSIS

The interactions between bacteria and animals during bacteria-stimulated metamorphosis are not intimate, long-term symbioses. Rather, these interactions occur transiently as an animal larva searches for a location to settle and metamorphose. It is interesting to contemplate what evolutionary pressures led marine invertebrate larvae to evolve a reliance on bacterial cues for metamorphosis. While these interactions may be circumstantial, there may be significant selective pressures that promote this interaction for one or both partners.

It is currently debated whether a biphasic (larva and adult) life history was an ancestral characteristic of the first animals or it arose multiple times among major animal clades (53, 65, 113, 119, 149). Similarly, it is unknown whether the ability to undergo metamorphosis in response to bacteria was an ancestral characteristic of the first animals or whether it is a convergent trait among diverse metazoans with a biphasic life cycle. Nonetheless, the widespread nature of this phenomenon suggests that a strong selective pressure exists to evolve and maintain this microbe-animal interaction.

As bottom-dwelling and often immobile adults, marine invertebrates may benefit from using bacteria as a metamorphosis cue. Because metamorphosis is an irreversible process, the decisions of where and when to undergo metamorphosis are critical for survival of the juvenile and adult (71). Certain bacteria may serve as proxies for specific environmental conditions and a suitable habitat, thus avoiding a switch to the benthic lifestyle in an unfavorable environment (5, 52). This response may be especially important in aquatic environments where biotic and abiotic conditions are constantly changing. Nonetheless, it is important to note that all underwater surfaces are coated with dense microbial biofilms, and thus, animal larvae must interact with biofilms to settle and metamorphose on the seafloor, i.e., to become bottom-dwelling organisms. It is, therefore, reasonable to expect that larvae actively select attachment sites with certain biofilm characteristics.

It is currently unknown whether bacteria benefit or are harmed from stimulating animal metamorphosis. Many of the bacteria that induce animal metamorphosis frequently associate with eukaryotes, for example, by accumulating on surfaces of invertebrates as epibiotic biofilms (34, 62, 110). Surface-attached bacteria tend to be larger, with a higher proportion of cells with higher metabolic activity than free-living bacteria (24). Because these bacteria produce exoenzymes that could help them utilize animal-derived molecules for nutrition, it is possible that inducing eukaryotic development allows specific bacteria to rapidly colonize a valuable niche, i.e., the settled animal.

Interestingly, antimicrobial metabolites are produced by many bacteria associated with marine invertebrates, for example, several members of *Pseudoalteromonas* (15, 62, 117). These properties—inducing metamorphosis, producing antimicrobial metabolites, association with macroorganisms—may, in fact, be interconnected. An intriguing hypothesis is that an evolutionary arms race is imposed among sessile invertebrates: As larvae, they must locate and colonize a surface in order to metamorphose; yet as adults they must keep their own surfaces clean and ward off settlement of other larvae. The association with the bioactive bacteria might therefore offer a favorable trade-off. The bacteria that promote settlement/metamorphosis might colonize a valuable niche, the adult animal, through which they can obtain nutrients via exoenzyme production. But they also produce antimicrobials that protect their animal niche from being colonized by other bacteria. Further characterization of marine invertebrate microbiomes could help illuminate this hypothesis.

Alternatively, it is possible that the stimulation of animal metamorphosis does not directly benefit the bacterium. Because surfaces in the ocean are often limiting, the bacterial partner might be influencing marine animal metamorphosis through by-product cooperation, i.e., cooperation as an incidental consequence of selfish action (135). Specifically, bacteria unavoidably produce publicly usable resources (e.g., toxins and antibiotics) (135, 136) that become available to their local community and might be interpreted by the animal larvae as a cue to an appropriate environment for settling down. By-product mutualism might not seem like a typical form of cooperation, since the cooperative phenotype carries no cost and because the trait need not evolve in the context of the interaction (134). Therefore, it can be difficult to resolve by-product cooperation into clear mechanisms.

CURRENT AND FUTURE CHALLENGES

The Biological Nature of Factors Inducing Metamorphosis

Identifying the chemical nature of bacterial factors that stimulate animal metamorphosis is a compelling endeavor. Biofilms are abundant sources of chemical cues (5, 147), and we have only scratched the surface when it comes to identifying specific metamorphosis cues, deciphering their chemical nature, and determining their ecological roles within natural biofilm communities. A few described inducers of invertebrate settlement are primary metabolites such as carbohydrates or peptides that are water-soluble (147). For example, a soluble proteinaceous factor and amino acids were found to stimulate oyster metamorphosis (128, 177). Water-soluble primary metabolites may function as stimulatory factors, because they are also used as components of internal signal transduction systems (127). Thus, the receptor machinery for responding to similar but externally derived signals is already present in the larval animal. Additionally, some bacteria are able to inject stimulatory factors, like Mif1, into larvae and stimulate metamorphosis (36). The mode of delivery and chemical properties of bacterial factors that stimulate metamorphosis are clearly diverse and likely have significant ecological implications for both microbe and animal. Our understanding of the role that bacteria and biofilms play in larval attachment and metamorphosis would be substantially enhanced if the chemical cues originating from natural biofilms were characterized.

Animal Sensing and Response Machinery

How animals directly sense bacterial factors that stimulate metamorphosis is currently unknown for any animal. However, there are chemicals known to artificially stimulate metamorphosis, and a few eukaryotic signal transduction pathways that mediate metamorphosis have been identified.

Excess concentrations of potassium or cesium ions, or perturbations of potassium channels, have been shown to induce metamorphosis in several animal species, and these ions have been used as tools to study eukaryotic pathways that mediate metamorphosis (108, 109, 118, 175). In comparing the metamorphosis of *Hydractinia* induced by chemical versus bacterial factors, Seipp et al. (137) showed that these processes occur in a similar manner. However, the larvae settled earlier when induced with *Pseudoalteromonas espejiana* compared to exposure of cesium ions. Moreover, the apoptotic process of the cells on the anterior end also occurs earlier in the presence of *P. espejiana* bacteria.

The protein kinase C (PKC) pathway has been heavily implicated in metamorphosis signaling in a variety of marine organisms including *H. echinata*, the sea urchin *Strongylocentrotus purpuratus*, the barnacle *Balanus amphitrite*, multiple Red Sea coral planulae (*Heteroxenia fuscescens*, *Xenia umbellata*, *Dendronephthya hemprichii*, *Litophyton arboretum*, *Parerythropodium fulvum fulvum*, and *Stylophora pistillata*), and the annelid *Capitella* sp. 1 (3, 10, 58, 89, 171). PKC was first implicated in the metamorphosis of *H. echinata* by Leitz & Klingmann et al. (89), who were able to stimulate PKC and the metamorphosis signaling cascade using diacylglycerol and inhibit metamorphosis using kinase inhibitors acting on PKC. PKC is a lipid-sensing kinase, and Leitz et al. (90, 91) have additionally implicated several lipids regulating metamorphosis such as lysophosphatidylcholine and arachidonic acid, a known PKC-sensitizing lipid. While it is unclear exactly how universal the PKC pathway is in regulating metamorphosis in marine invertebrates, even the distantly related insect *Aedes aegypti* metamorphic factor juvenile hormone was demonstrated to stimulate its metamorphic induction through the PKC pathway (96).

Studies have implicated other signaling systems in addition to PKC in the induction of metamorphosis. The MAPK signaling pathway, which can be activated by various upstream signals, including PKC, has also been demonstrated to be necessary for metamorphosis through the use of pharmacological inhibitors in a sponge (*Amphimedon queenslandica*), an annelid (*Hydroides*), and an ascidian (*Ciona intestinalis*) (17, 139, 157, 162). An alternative signaling pathway has been shown in the annelid *Phragmatopoma californica* and mussel *Mytilus coruscus*, where the alterations of cAMP levels have been shown to contribute to metamorphosis induction (72, 95). Additionally, in *M. coruscus*, both inhibitors and activators of cAMP induced metamorphosis, implying that there is a delicate balance required for cAMP to regulate metamorphosis.

How multiple eukaryotic signaling systems evolved to orchestrate metamorphosis in response to bacteria is unclear. An intriguing possibility is that the ability to sense bacteria and proceed with metamorphosis is linked to innate immunity. In a few instances, larval competency is correlated with the expression of genes related to innate immunity, suggesting a possible role for Toll-like receptors or other sensing machinery of the innate immune system (26, 129). How diverse animals evolved the ability to recognize bacterial factors and subsequently signal the induction of metamorphosis has been pondered by scientists for decades and is a clear grand challenge for future investigations.

Bacteria Inhibiting Metamorphosis

Many studies have shown that in addition to stimulating metamorphosis, microbial biofilms inhibit settlement and metamorphosis of a suite of fouling macroorganisms, such as tubeworms (30, 61), bryozoans (22, 31, 126), barnacles (61, 64, 86, 98), and ascidians (64), when in the presence of an inductive cue or condition. Despite the presence of inductive bacteria, antifouling properties of certain bacteria can render experimentally mixed biofilms inhibitive (30). This finding suggests that the presence of certain inductive cues is not sufficient to overcome inhibitory factors in laboratory settings. Understanding the microbial ecology of natural heterogeneous biofilms

containing both inducers and inhibitors will help us better understand the influence of microbes on larval fate outside of laboratory conditions.

Despite uncertainty of the effects of the microorganisms when outside of laboratory conditions, the need for green antifoulant solutions has motivated the identification of antifouling factors from inhibitive bacteria (reviewed in 29, 123). Biochemical characterizations revealed that antifouling factors include small molecules (9, 25, 94, 169, 170) and a protease (32) that have been successfully embedded in paint and resins while retaining their inhibitory capabilities over some time (63, 174).

Applied Potential of Studying How Bacteria Stimulate Animal Metamorphosis

Animal metamorphosis in response to bacteria has several applied implications. For example, knowledge of bacterial factors that stimulate metamorphosis can inform probiotic treatments that promote the recruitment of new animals to degraded benthic ecosystems such as coral reefs (59, 120). This knowledge could also improve the husbandry protocols for aquaculture animals for commercial use, such as oysters, that may depend on our knowledge of specific bacteria that stimulate metamorphosis in captivity (122). In addition, knowledge of the bacterial factors that stimulate metamorphosis could inform new strategies for preventing biofouling, for example, through embedding of antifouling compounds within paints for boat hull surfaces. Finally, bacteria-stimulated metamorphosis is a widespread example of a beneficial host-microbe interaction, yet it is a largely unexplored space for mining of biomedical and biotechnology applications. For example, based on our discovery of MACs, we identified a new and previously undescribed family of CISs that are produced by *Bacteroidales* bacteria commonly found in the human gut (132). Such systems inject contents into diverse animal cell types and could someday be modified as nanometer-scale devices for the delivery of specific proteins into target cells (130).

CONCLUSION

As we learn more about the astonishing ubiquity and diversity encompassing the microbial world and the vast range of bacteria-animal interactions, it has become clear that microbes are often essential for animal development. Although nearly all animals have stable associations with bacteria, investigating how these interactions shape animal development has been difficult, partially because of a dearth of tractable and phylogenetically relevant model systems. Only a few investigations of these interactions have unraveled the specific mechanisms by which environmental bacteria influence the life cycles of animals. Studying mechanisms by which environmental bacteria stimulate the metamorphosis of diverse animals may begin to provide explanations of why stable associations with bacteria, once considered anathema to human health, are indispensable for animals. Thus, there is still a great need to interrogate the molecular dialogue that mediates microbe-animal interactions in diverse contexts, such as the stimulation of animal metamorphosis by bacteria.

SUMMARY POINTS

1. Bacteria stimulate the metamorphosis of phylogenetically distant animals like corals, tubeworms, and urchins.
2. The stimulation of metamorphosis by bacteria is an example of bacteria promoting animal development.

3. Bacteria-stimulated metamorphosis is critical for coral reef formation, aquaculture, and biofouling.
4. Bacteria stimulate animal metamorphosis by producing stimulatory factors that can be biochemically very different (e.g., protein, lipid, diffusible small molecules).
5. Bacteria can stimulate metamorphosis by producing phage-tail-like structures that inject a stimulatory protein.
6. For most marine animals that undergo metamorphosis, we still do not know the identity of bacterial factors that stimulate metamorphosis, their mechanisms of action, or how the animal senses these factors.

DISCLOSURE STATEMENT

N.J.S. has a patent application pending related to Contractile Injection Systems in the United States, Application Number: 5810.133691PCT.

ACKNOWLEDGMENTS

This work was supported by the Office of Naval Research (N00014-17-1-2677, N.J.S. and N00014-16-1-2135, N.J.S.), the Alfred P. Sloan Foundation, a Sloan Research Fellowship (N.J.S.), and the National Science Foundation (1942251, N.J.S., and GRFP 2017232404, A.T.A.).

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