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Genetic tools to explore function in marine symbiotic bacteria

A dissertation submitted in partial satisfaction of the requirements for
the degree Doctor of Philosophy

in

Biology

by

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The dissertation of Amanda T. Alker is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

Chair

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2022

DEDICATION

To my family for your unending support. Especially to my mother, Tese, whose sacrifices for others equate to many lifetimes worth of love. Thank you for being my proudest [and loudest] supporter. You've stoked my fire since childhood in the best ways that you could. You were honest about everything else. To my brother Jesse, for your perspectives that are both totally opposite and exactly the same as mine, thank you for taking this journey with me.

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EPIGRAPH

"Life did not take over the world by combat, but by networking."

— Lynn Margulis, Microcosmos: Four Billion Years of Microbial Evolution

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ABSTRACT OF THE DISSERTATION

Development of genetic tools for the investigation of bacteria-stimulated metamorphosis and other symbioses in marine invertebrates

by

Amanda T. Alker

Doctor of Philosophy in Biology

University of California San Diego, 2022

San Diego State University, 2022

Professor Nicholas Shikuma, Chair

My PhD research explores which bacterial products can trigger marine invertebrate metamorphosis, an irreversible transformation from free-swimming larvae to settled juvenile on the seafloor. We reviewed the existing literature for bacteria-stimulated metamorphosis in marine invertebrates (Chapter 1) to provide context for the research sections (Chapters 2-4). Taken together, this dissertation showcases the utility of using bacterial genetics to explore the molecular mechanisms underlying bacteria-induced metamorphosis and other symbiotic interactions.

In Chapter 2, we work with a tractable marine bacteria, *Pseudoalteromonas luteoviolacea* and show that it encodes the gene cluster for two different products capable of stimulating metamorphosis in different animals. We generated deletion mutations for one or both of the products and then tested their effects on two marine invertebrate models, tubeworms and *Hydractinia.* We find that the different metamorphosis-inducing products are highly specific, and do not influence metamorphosis universally across different animals.

In Chapter 3, we further explore one of the metamorphosis-inducing products from bacteria, a chemical compound called Tetrabromopyrrole (TBP). While *Pseudoalteromonas luteoviolacea* produces some TBP, other strains such as *Pseudoalteromonas* sp. PS5 produce more TBP and influence robust coral metamorphosis. To explore the genetic link between TBP production and coral metamorphosis, we established PS5 as a genetically tractable strain. We generated an in-frame deletion of a single gene, *bmp2*, and showed that the mutant strain could no longer 1) produce TBP and 2) induce metamorphosis. These results suggest that biofilms of PS5 produce enough TBP to influence coral metamorphosis *in vivo* and can be used to inform decisions for coral probiotics.

In Chapter 4, we build on modular plasmid toolkit platform to enable higher throughput genetic manipulation in diverse marine bacteria. We develop methods for genetically engineering marine bacteria using preexisting tools and parts, while adding new parts (i.e. promoters) to explore function in marine bacteria. We successfully transformed 10 strains across 2 proteobacterial classes, 4 orders and 7 genera. We developed a new method to visualize invertebrate microbiomes after the induction of metamorphosis, revealing that tubeworms ingest inductive bacteria after they undergo metamorphosis. These outcomes enable the exploration of fundamental questions surrounding marine host-microbe interactions.

CHAPTER 1: The influence of bacteria on animal metamorphosis

1.1. 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 (Knoll, 2003). Within this context, animals evolved 400 million years ago in an environment already dominated by abundant and diverse bacteria (Pradeu, 2011; Rosenberg and Zilber-Rosenberg, 2016). 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 on animal development have gained widespread appreciation, paving the way for our realization that microbes fundamentally influence animal health, development, and evolution (Moran, 2006; Gilbert *et al.*, 2012; McFall-Ngai *et al.*, 2013). For example, bacteria direct multicellular behavior in choanoflagellates—the closest living relatives to animals—(Alegado and King, 2014; Woznica *et al.*, 2016), budding in hydra (Rahat and Dimentman, 1982), light organ development in the Hawaiian bobtail squid (Koropatnick *et al.*, 2004; Nyholm and McFall-Ngai, 2004), digestive tract development in zebrafish (Bates *et al.*, 2006; Hill *et al.*, 2016) and immune system development and maturation in mammals (Mazmanian *et al.*, 2005; Bouskra *et al.*, 2008). 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 (Mcfall-ngai *et al.*, 2013). 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?

1.2. 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, environmental surface-attached bacteria serve as an indicator and provide a stimulus for the swimming larvae of many animals, promoting larval settlement on the seafloor 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 (Zobell and Allen, 1935).

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 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 (Bishop *et al.*, 2006). 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 (Bishop *et al.*, 2006). 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 (Siegel *et al.*, 2008). 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.

1.3. Biofilms 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 or mineral surfaces (H. C. Flemming, 2016). Rather than being conglomerations of cells and slime, biofilms are organized communities with functional microcolonies and channels that perform complex metabolic processes (Dang *et al.*, 2000). 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 (Hung *et al.*, 2007), and form a cohesive, three-dimensional polymer network that interconnects and transiently immobilizes biofilm cells (H. C. Flemming, 2016). EPSs are a prominent component of biofilms that has been implicated in stimulating metamorphosis (Hadfield and Paul, 2001), 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 (H. Flemming, 2016). 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 (Lau *et al.*, 2005), tidal level (Qian *et al.*, 2003; Dobretsov and P. Y. Qian, 2006), dissolved oxygen (Nocker *et al.*, 2007), hypoxia (Shin *et al.*, 2013; Cheung *et al.*, 2014; Lagos *et al.*, 2016), and habitat (Chiu *et al.*, 2007; Hung *et al.*, 2007; Lema *et al.*, 2019). Natural biofilms formed under different environmental conditions vary in their attractiveness to settling larvae (Lau *et al.*, 2005; Dobretsov and P.-Y. Qian, 2006; Chiu *et al.*, 2007; Hung *et al.*, 2007; Campbell *et al.*, 2011; Lema *et al.*, 2019). However, most factors influencing biofilm community composition, including salinity and temperature (Lau *et al.*, 2005), or succession over time (Shikuma and Hadfield, 2005; Chung *et al.*, 2010; Lema *et al.*, 2019) 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 (Nielsen *et al.*, 2015), the actual settlement cues associated with biofilm communities often remain unknown or poorly understood (Hung *et al.*, 2009; Franco *et al.*, 2019).

1.4. Known bacterial factors that induce metamorphosis

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 (Woollacottl and Hadfield, 1996; Whalan *et al.*, 2008; Wahab *et al.*, 2011; Whalan and Webster, 2014), mollusks (Fitt *et al.*, 1990; Bao *et al.*, 2007; Tamburri *et al.*, 2008; Gribben *et al.*, 2009; Kaniewska *et al.*, 2012; Wang *et al.*, 2012; Yang *et al.*, 2013; Rodriguez-Perez *et al.*, 2019) crabs (Anderson and Epifanio, 2009), barnacles (Faimali *et al.*, 2004; Khandeparker *et al.*, 2006), bryozoans (Bertrand and Woollacott, 2003; Dobretsov and P. Y. Qian, 2006), annelids (Shikuma *et al.*, 2014), urochordates (Szewzyk *et al.*, 1991), echinoderms (Huggett *et al.*, 2006; Dworjanyn and Pirozzi, 2008), and ascidians (Wieczorek and Todd, 1997; Roberts *et al.*, 2007; Karaiskou *et al.*, 2015; Chase *et al.*, 2016). 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* (Kirchman *et al.*, 1981) and larval attachment of the tunicate *Ciona intestinalis* (Szewzyk *et al.*, 1991). Histamine

isolated from algae, or the biofilm coating the algae, stimulates the metamorphosis of the sea urchin *Holopneustes purpurascens* (Swanson *et al.*, 2004, 2006).

In the study 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*.

1.5. 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. (Hadfield *et al.*, 1994) 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 (Huang and Hadfield, 2003; Shikuma and Hadfield, 2005; Lema *et al.*, 2019) and single species of bacteria (Unabia and Hadfield, 1999; Shikuma *et al.*, 2014; Freckelton *et al.*, 2017).

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 (Nedved and Hadfield, 2009). 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 embryos 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. (Hadfield *et al.*, 2014) 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 (Shikuma *et al.*, 2016) 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 (Harder *et al.*, 2002; Lau *et al.*, 2002; Huang and Hadfield, 2003; Lau *et al.*, 2004). 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.

1.6. A surprisingly different way that bacteria stimulate metamorphosis

Since the 1930s discovery that bacteria stimulate animal metamorphosis (Zobell and Allen, 1935), 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 (Hadfield, 2011). However, the stimulation of *Hydroides* metamorphosis by bacteria was shown to require physical contact with a biofilm surface (Hadfield *et al.*, 2014). 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 (Shikuma *et al.*, 2014) **(Figure 4a, b)**. We called these structures metamorphosis-associated contractile structures (MACs) because they form syringe-like protein complexes that induce tubeworm metamorphosis. To make this discovery, a pioneering study by Huang et al. (Huang *et al.*, 2012) used forward genetics to identify a set of 4 genes in the genome of *P. luteoviolacea* that are required to stimulate tubeworm metamorphosis. 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 (Shikuma *et al.*, 2014).

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 4c**)]. 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 4a, b**).

Since the discovery of MACs, related CISs have been discovered that also form multi-CIS complexes (Böck *et al.*, 2017). In addition to stimulating metamorphosis, closely related structures were found to mediate interactions between microbes and amoebae, insects, and potentially humans (Yang *et al.*, 2006; Böck *et al.*, 2017; Vlisidou *et al.*, 2019; Rojas *et al.*, 2020). While a number of pathogenic bacteria use type VI secretion systems to inject protein toxins into target cells to cause disease (Logan *et al.*, 2018), 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 (Ericson *et al.*, 2019). 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 (Rocchi *et al.*, 2019). 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 (Ding *et al.*, 2019), suggesting that MACs may benefit surface-attached bacteria by facilitating their interaction with animal larvae while deterring potential biofilm-eating predators like protozoans (Matz *et al.*, 2008).

1.7. 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 (Unabia and Hadfield, 1999; Lau *et al.*, 2002), 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 (Lau *et al.*, 2015). 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 (S. C. K. Lau *et al.*, 2003).

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) (Freckelton *et al.*, 2017). Freckelton and colleagues 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 are able to induce *Hydroides* metamorphosis (Freckelton *et al.*, 2019). 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 (Deatheragea and Cooksona, 2012;

Biller *et al.*, 2014). 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. (Hung *et al.*, 2009) 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.

1.8. 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 (Bernan, 2001). Morse and colleagues (Morse *et al.*, 1988) 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 (Morse and Morse, 1991; Morse *et al.*, 1994). Around the same time, a hypothesis arose that CCA-associated bacteria could contribute to the inductive properties of CCA (Johnson *et al.*, 1991). 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 (Sneed *et al.*, 2015). 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 (Tebben *et al.*, 2015; Gómez-Lemos *et al.*, 2018).

Approaches using natural heterogeneous (Morse *et al.*, 1988; Webster *et al.*, 2004) or isolated single-species biofilms (Negri *et al.*, 2001; Tran and Hadfield, 2011; Sharp *et al.*, 2015) 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 (Webster *et al.*, 2004). 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 (Negri *et al.*, 2001; Tran and Hadfield, 2011; Sharp *et al.*, 2015). 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 (Tran and Hadfield, 2011).

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 (Negri *et al.*, 2001) 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 (Tebben *et al.*, 2011) and PS5 (Sneed *et al.*, 2014) 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 (Tebben *et al.*, 2011; Sneed *et al.*, 2014). 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 (Agarwal *et al.*, 2014). El Gamal and colleagues (Gamal *et al.*, 2016) demonstrated that only genes *bmp1–4* are necessary to produce TBP in vitro, and further, *Pseudoalteromonas* strains A3, J010, PS5, and A757 encode 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 (Tebben *et al.*, 2015). Despite the debate, one study utilized TBP extract in comparison with CCA to attempt to differentiate the molecular processes of attachment and metamorphosis (Siboni *et al.*, 2014); 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 (Zheng *et al.*, 2018). 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 (Tran and Hadfield, 2011; Sharp *et al.*, 2015). Of note, the biofilms of *Thalassamonas agarivorans*, a gammaproteobacterium, evoked a strong metamorphic effect in the brooding coral *Pocillopora damicornis* (Tran and Hadfield, 2011). 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 (Sharp *et al.*, 2015). 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 (Sharp *et al.*, 2015).

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 (Hayward *et al.*, 2011; Meyer *et al.*, 2011; Siboni *et al.*,

2012; Strader *et al.*, 2018). 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 bacterium-animal interaction.

Hydractinia

The colonial marine hydroid *Hydractinia* is a versatile, informative cnidarian model. *Hydractinia* is a member Cnidaria—multicellular animals possessing true tissues that lie at the base of the Metazoa (Frank *et al.*, 2001). 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 (Frank *et al.*, 2001), 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 (Muller, 1969). 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 (Muller, 1973). 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* (Müller and Leitz, 2002; Guo *et al.*, 2021). However, in contrast to Müller's observations, some studies suggest that most of the bacteria tested have inductive metamorphosis capabilities, including *Escherichia coli* (Kroiher and Berking, 1999).

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 (Guo *et al.*, 2021) 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 et al. (Leitz, 1993; Leitz and Wagner, 1993a), who biochemically identified of a lipophilic fraction obtained from the marine bacterium *Alteromonas espejiana*, Guo et al. (Guo *et al.*, 2017) 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 (Muller, 1969, 1973). 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*.

1.9. 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 (Strathmann, 1993; Pechenik, 1999; Hadfield *et al.*, 2001; Nielsen, 2013; Holstein and Laudet, 2014). 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 (Jackson *et al.*, 2002). 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 (Hadfield, 2011; Antunes *et al.*, 2019). 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 sea floor, i.e. to become a bottom dwelling organism. 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 (Holmström and Kjelleberg, 1999; Egan *et al.*, 2008; Nasrolahi *et al.*, 2012). Surface-attached bacteria tend to be larger, with a higher proportion of cells with higher metabolic activity than free-living bacteria (Dang and Lovell, 2016). 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* (Holmström and Kjelleberg, 1999; Bowman, 2007; Offret *et al.*, 2016). 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 (Joel L Sachs *et al.*, 2011). Specifically, bacteria unavoidably produce publicly usable resources (e.g., toxins and antibiotics) (Sachs *et al.*, 2004; Joel L. Sachs *et al.*, 2011) 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 (Sachs, 2013). Therefore, it can be difficult to resolve by-product cooperation into clear mechanisms.

1.10. 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 (Steinberg *et al.*, 2002; Antunes *et al.*, 2019), 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 (Steinberg *et al.*, 2002). For example, a soluble proteinaceous factor and amino acids were found to stimulate oyster metamorphosis (Rittschof, 1993; Zimmer-Faust and Tamburri, 1994). Water-soluble primary metabolites may function as stimulatory factors, because they are also used as components of internal signal transduction systems (Rittschof, 1990). 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 (Ericson *et al.*, 2019). 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 (Muller, 1973; Yool *et al.*, 1986; Pearce and Scheibling, 1994; Müller and Leitz, 2002). In comparing the metamorphosis of *Hydractinia* induced by chemical versus bacterial factors, Seipp et al. (S Seipp *et al.*, 2007) 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*(Leitz and Klingmann, 1990; Yamamoto *et al.*, 1995; Biggers and Laufer, 1996; Henning *et al.*,

1996; Amador-Cano *et al.*, 2006) PKC was first implicated in the metamorphosis of *H. echinata* by Leitz et al., who were able to stimulate PKC and the metamorphosis signaling cascade using diacylglycerol, and inhibit metamorphosis using kinase inhibitors acting on PKC (Leitz and Klingmann, 1990). PKC is a lipid-sensing kinase, and Leitz et al. (Leitz and Muller, 1991; Leitz *et al.*, 1994) 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 (Liu *et al.*, 2015).

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*) (Chambon *et al.*, 2007; Wang and Qian, 2010; Shikuma *et al.*, 2016; Ueda *et al.*, 2016). 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 (Jensen and Morse, 1990; Liang *et al.*, 2018). 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 (Davidson and Swalla, 2002; Roberts *et al.*, 2007). 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 (Holmström *et al.*, 1996; Dobretsov and Qian, 2004), bryozoans (Dahms *et al.*, 2004; Dobretsov and P. Y. Qian, 2006; Rao *et al.*, 2007), barnacles (Maki *et al.*, 1988; Holmstrom, 1992; Holmström *et al.*, 1996; S. C. Lau *et al.*, 2003), and ascidians (Holmstrom, 1992) 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 (Dobretsov and Qian, 2004). 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 (Qian *et al.*, 2007; Dobretsov *et al.*, 2013)). Biochemical characterizations revealed that antifouling factors include small molecules (Bhattarai *et al.*, 2006; Li *et al.*, 2007; Dash *et al.*, 2009; Xu *et al.*, 2009, 2010) and a protease (Dobretsov *et al.*, 2007) that have been successfully embedded in paint and resins while retaining their inhibitory capabilities over some time (Yee *et al.*, 2007).

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 (Heyward *et al.*, 2002; Peixoto *et al.*, 2017). 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 (Prado *et al.*, 2010). 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 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 CIS that are produced by *Bacteroidales* bacteria commonly found in the human gut (Rojas *et al.*, 2020). 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 (Rocchi *et al.*, 2019).

1.11. 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.

1.12. ACKNOWLEDGEMENTS

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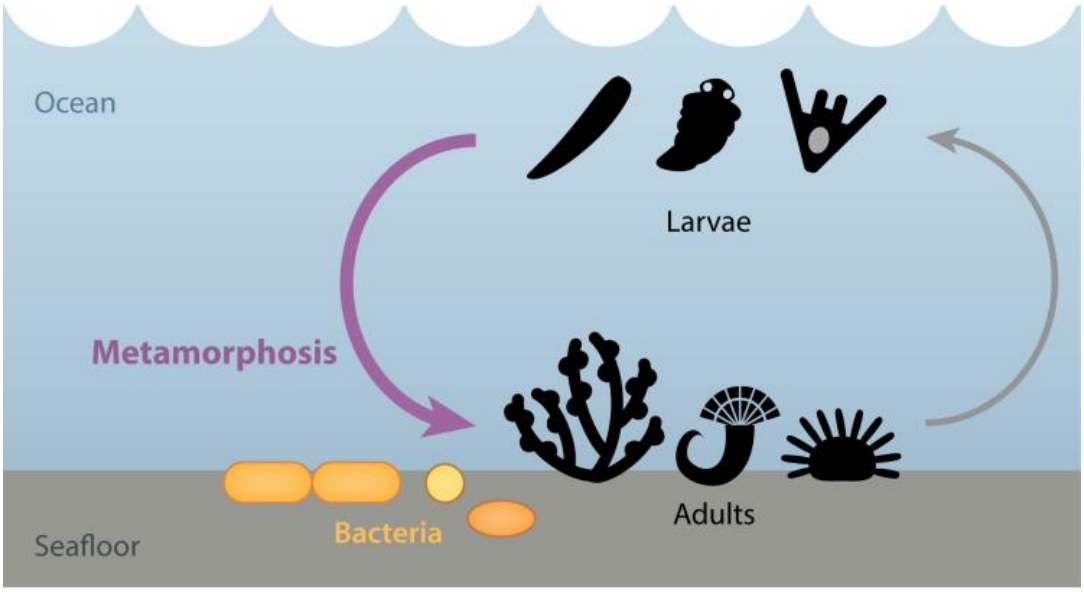

Cavalcanti GS, et al. 2020.
Annu. Rev. Microbiol. 74:137–58

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.

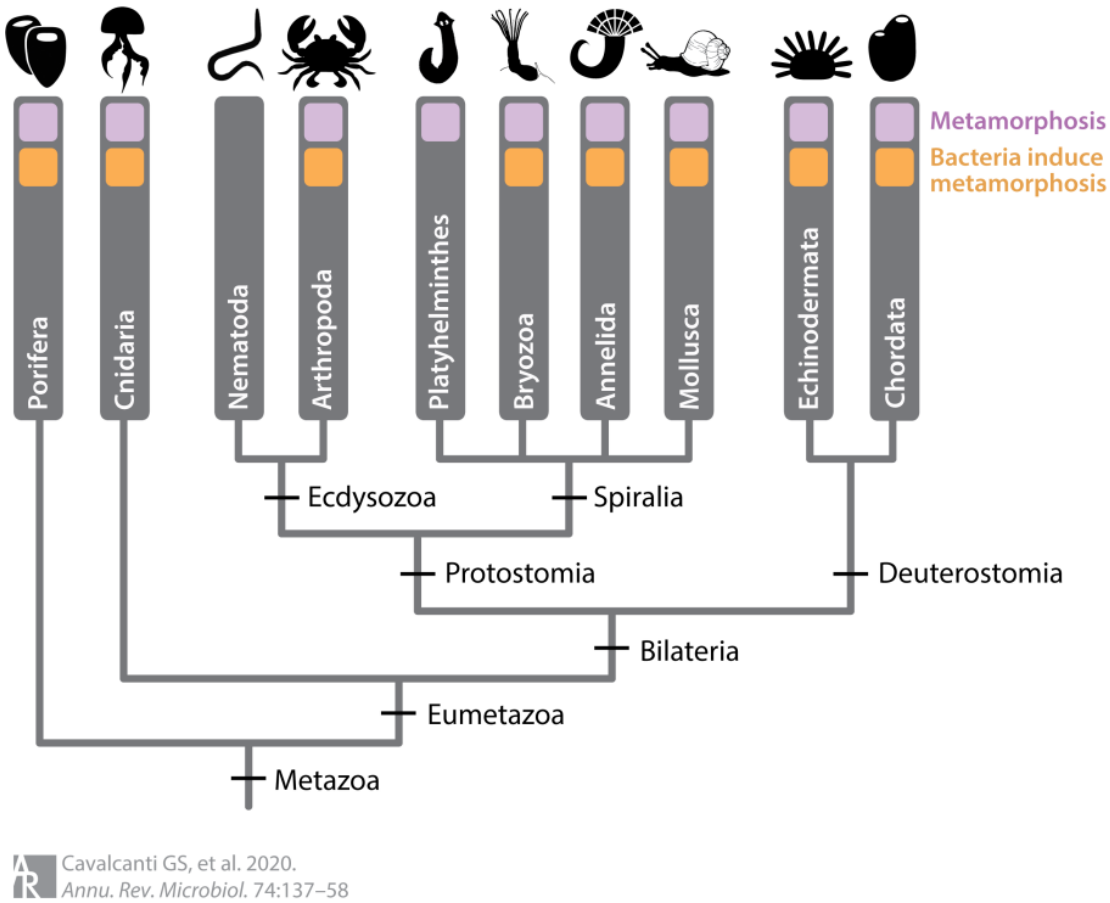

Figure 2. Bacteria-stimulated metamorphosis is widespread among diverse animal taxa.
Shown is a representation of the animal tree of life. Taxa that undergo metamorphosis are indicated in blue. Taxa that undergo metamorphosis in response to bacteria are indicated in yellow. Adapted from Reference (Shikuma *et al.*, 2016).

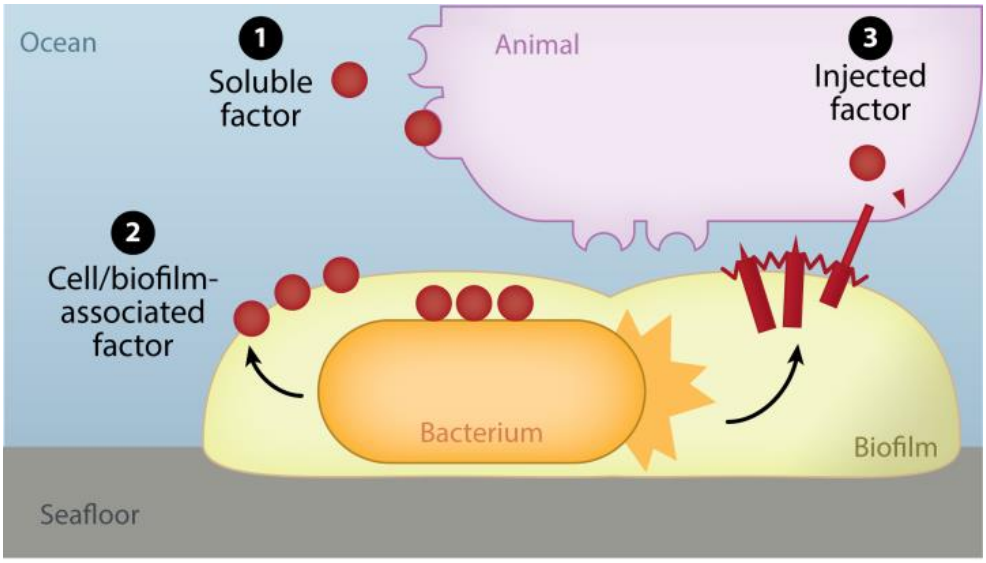

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Figure 3. Model of types of bacterial factors that stimulate animal metamorphosis.
Stimulatory factors from bacteria can be (*i*) soluble, (*ii*) bound to the bacterial cell or biofilm surface, or (*iii*) injected into host cells.

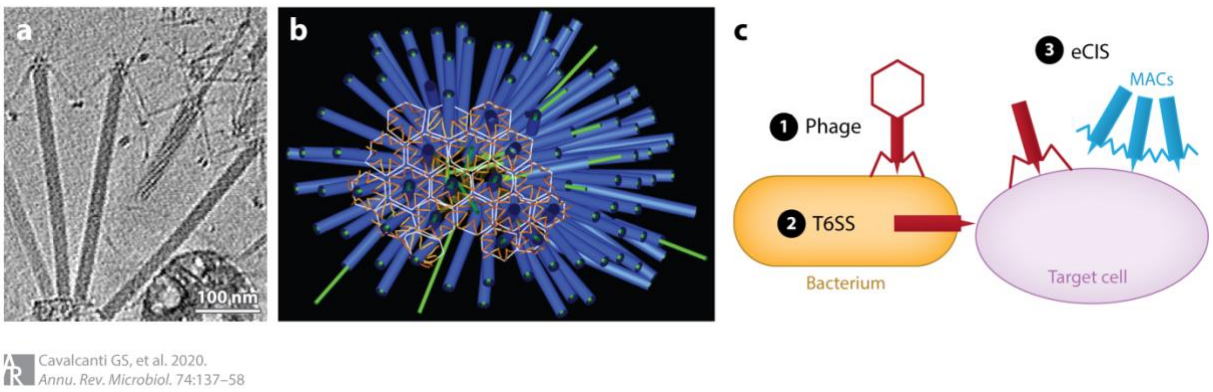

Figure 4. MACs are an example of a CIS that often injects protein effectors into target cells.
Panels (**A**) and (**B**) show a side view of MACs in extended and contracted states and a segmented model of the array, respectively. (**C**) CISs are related to the contractile tails of bacteriophage (viruses of bacteria, *i*). T6SSs (*ii*) act from within a bacterial cell, while eCISs (*iii*) 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 (Shikuma *et al.*, 2014). Reprinted with permission from AAAS.

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CHAPTER 2: Genetic examination of the marine bacterium *Pseudoalteromonas luteoviolacea* and effects of its metamorphosis-inducing factors

2.1. ABSTRACT

Pseudoalteromonas luteoviolacea is a globally distributed marine bacterium that stimulates the metamorphosis of marine animal larvae, an important bacteria-animal interaction that can promote the recruitment of animals to benthic ecosystems. Recently, different *P. luteoviolacea* isolates have been shown to produce two stimulatory factors that can induce tubeworm and coral metamorphosis; Metamorphosis-Associated Contractile Structures (MACs) and tetrabromopyrrole (TBP), respectively. However, it remains unclear what proportion of *P. luteoviolacea* isolates possess the genes encoding MACs, and what phenotypic effect MACs and TBP have on other larval species. Here, we show that 9 of 19 sequenced *P. luteoviolacea* genomes genetically encode both MACs and TBP. While *P. luteoviolacea* biofilms producing MACs stimulate the metamorphosis of the tubeworm *Hydroides elegans*, TBP biosynthesis genes had no effect under the conditions tested. Although MACs are lethal to larvae of the cnidarian *Hydractinia symbiologicarpus, P. luteoviolacea* mutants unable to produce MACs are capable of stimulating metamorphosis. Our findings reveal a hidden complexity of interactions between a single bacterial species, the factors it produces and two species of larvae belonging to different phyla.

2.2. INTRODUCTION

The free-swimming larvae of many marine invertebrates must settle and undergo metamorphosis to continue their life cycle as adults on the sea floor. Certain bacteria coating submerged surfaces may serve as important environmental cues that indicate a suitable habitat for larvae to settle and undergo metamorphosis-(Hadfield, 2011; Cavalcanti *et al.*, 2020). The larvae of diverse marine invertebrates animals undergo metamorphosis in response to stimulatory

bacteria, including cnidarians, annelids, crustaceans, urchins and tunicates (Hadfield and Paul, 2001). This phenomenon of bacteria-induced metamorphosis is critical for the biofouling of ship hulls (Schultz *et al.*, 2011), aquaculture of marine invertebrates like oysters (Yu *et al.*, 2010), and the restoration of ecosystems such as coral reefs (Negri *et al.*, 2001; Webster *et al.*, 2004; Sneed *et al.*, 2014). However, we have only just begun to understand broader mechanisms through which this beneficial microbe-animal interaction occurs.

Many larvae of marine invertebrates undergo metamorphosis in response to specific components of bacteria or products from bacteria, we call here 'factors', that are diverse in their chemical composition, physical properties, biological nature and ecological roles (Cavalcanti *et al.*, 2020). While several bacterial factors responsible for inducing metamorphosis have been identified in diverse bacteria including a small molecule (Tebben *et al.*, 2011; Sneed *et al.*, 2014), a protein (Huang *et al.*, 2012; Shikuma *et al.*, 2014; Ericson *et al.*, 2019), polysaccharides, lipopolysaccharides (Freckelton *et al.*, 2019; Guo *et al.*, 2019), and (lyso)phospholipids (Leitz and Wagner, 1993b; Guo *et al.*, 2019), only two have been both characterized and the genes that encode their biosynthesis described; the small molecule 2,3,4,5-tetrabromopyrrole (TBP) (Tebben *et al.*, 2011; Agarwal *et al.*, 2014; Gamal *et al.*, 2016) and the Mif1 protein that is carried by Metamorphosis Associated Contractile structures (MACs) (Shikuma *et al.*, 2014; Ericson *et al.*, 2019).

TBP stimulates the metamorphosis of several coral species (Tebben *et al.*, 2011; Sneed *et al.*, 2014; Tebben *et al.*, 2015) and was first identified as a metamorphosis-inducing compound through bioactivity-guided fractionation of bacterial extracts (Tebben *et al.*, 2011). Isolated

single species biofilms and organic extracts of *Pseudoalteromonas sp.* A3 (Negri *et al.*, 2001), J010 (Tebben *et al.*, 2011), and PS5 (Sneed *et al.*, 2014) can induce the metamorphosis of coral larvae. However, when the coral larvae were exposed to either the individual strains or TBP extract, metamorphosis often occurred without attachment (Negri *et al.*, 2001; Tebben *et al.*, 2011; Sneed *et al.*, 2014; Tebben *et al.*, 2015). Although TBP was proposed as a compound of ecological importance for coral reseeding and aquaculture (Tebben *et al.*, 2011; Sneed *et al.*, 2014), its ecological significance has been questioned since its discovery (Negri *et al.*, 2001; Tebben *et al.*, 2015) due to an intermediate phenotype where the larvae would metamorphose but remain unattached to the substrate. Additionally, the low abundance of pseudoalteromonads on encrusting algae may indicate its inability to provide a substantial signal for coral induction *in situ* (Tebben *et al.*, 2015). Interestingly, TBP and other cyclic halogenated moieties were lethal to several species of phytoplankton (Whalen *et al.*, 2018), demonstrating that TBP can elicit either negative or positive responses depending on the organism.

In contrast to the bioactivity-guided fractionation method used to identify TBP, MACs were discovered using bacterial genetics and functional mutants (Huang *et al.*, 2012; Shikuma *et al.*, 2014). MACs are a syringe-like complex that is evolutionarily related to type 6 secretion systems and tailed bacteriophage. MACs are composed of conserved structural components including a rigid inner tube surrounded by a baseplate complex and contractile sheath. Contraction of the sheath propels the inner tube through cell membranes often delivering protein effectors to target cells (Basler *et al.*, 2012; Shikuma *et al.*, 2014). We recently identified an effector of MACs called Metamorphosis-Inducing Factor 1 (Mif1) that is sufficient for inducing the metamorphosis of a tubeworm called *Hydroides elegans* (hereafter *Hydroides*) (Ericson *et*

al., 2019). Instead of a small cyclic molecule like TBP, Mif1 is a 943 amino acid protein loaded within a macromolecular contractile injection system. While the discovery that MACs induce *Hydroides* metamorphosis brings us a step closer to determining one way that bacteria stimulate metamorphosis, it remains unclear what proportion of *P. luteoviolacea* isolates have this capability, and if other marine larvae respond to MACs by undergoing metamorphosis.

Although many marine bacteria from diverse phyla have been shown to induce the metamorphosis of marine invertebrates (Unabia and Hadfield, 1999; Tran and Hadfield, 2011; Freckelton *et al.*, 2017; Guo *et al.*, 2017) only a handful have been studied to identify and characterize their metamorphosis-inducing properties. One of these bacteria is *P. luteoviolacea*, which is a prodigious producer of bioactive compounds (Gauthier and Flatau, 1976; Laatsch and Pudleiner, 1989) and was shown to induce the metamorphosis of corals, sea urchins, and tubeworms. *P.luteoviolacea* strain 2ta16 was isolated from the surface of corals (Rypien *et al.*, 2010) and produces halogenated compounds, such as pentabromopseudilin and tetrabromopyrrole (Agarwal *et al.*, 2014). Strains H2 and A316 were shown to induce coral (Tran and Hadfield, 2011) and sea urchin metamorphosis (Huggett *et al.*, 2006), respectively by a yet uncharacterized bacterial factor. *P. luteoviolacea* str. HI1 is genetically tractable, and is the subject of several studies showing that it is capable of inducing the metamorphosis of *Hydroides* by producing MACs (Huang and Hadfield, 2003; Huang *et al.*, 2012; Shikuma *et al.*, 2014, 2016; Ericson *et al.*, 2019), demonstrating the highly inductive nature of this bacterial species across diverse animal types. Nineteen genomes of *P. luteoviolacea* strains have been isolated and sequenced from oceans around the world (Rypien *et al.*, 2010; Tran and Hadfield, 2011; Asahina and Hadfield, 2015; Maansson *et al.*, 2016; Thøgersen *et al.*, 2016) and display a significant

diversity in gene content (Maansson *et al.*, 2016; Busch *et al.*, 2019). The chemical activity, stimulatory nature, genetic tractability and genomic diversity of *P. luteoviolacea* make this bacterium a particularly well-suited model for studying distinct metamorphosis-inducing factors in the laboratory.

The larvae of diverse marine invertebrates have been studied in the laboratory to investigate metamorphosis in response to bacteria (Hadfield, 2011; Cavalcanti *et al.*, 2020). Two prominent animals used to study this process are the spirailian tubeworm, *Hydroides*, and the cnidarian hydroid, *Hydractinia symbiolongicarpus* (hereafter *Hydractinia*). *Hydroides* has been used as a model organism to study bacteria-stimulated metamorphosis because it is easily propagated in the laboratory (Hadfield *et al.*, 1994; Nedved and Hadfield, 2009) and its larvae settle and undergo metamorphosis in response to biofilms composed of a natural consortia (Huang and Hadfield, 2003) or single strains of bacteria (Unabia and Hadfield, 1999). The colonial hydroid, *Hydractinia* has been used as an important model to study development, immunology, reproduction (Frank *et al.*, 2001), and metamorphosis in response to *Pseudoalteromonas* species (Leitz and Wagner, 1993b; Stefanie Seipp *et al.*, 2007; Guo *et al.*, 2017, 2019). While the larvae of ecologically threatened animals, like stony corals that build coral reefs, are often difficult to obtain, *Hydractinia* serves as an accessible model cnidarian to investigate bacteria-stimulated metamorphosis.

In this work, we aimed to determine what proportion of *P. luteoviolacea* isolates possess the genes encoding MACs, and what phenotypic effect MACs and TBP have on other larval species. We use comparative genomics to illustrate the distribution of the MACs biosynthesis

gene clusters across diverse isolates of *P. luteoviolacea* and find that roughly half of the sequenced *P. luteoviolacea* strains encode the genes responsible for the production of both MACs and TBP. We construct *P. luteoviolacea* mutants lacking the ability to produce TBP or MACs, and directly compare the phenotypic responses of two model animals, *Hydroides* and *Hydractinia*. We show that *P. luteoviolacea* HI1 produces the two previously characterized factors, MACs and TBP, that have very different phenotypic effects on larvae from different phyla, including eliciting no apparent response, death or metamorphosis. Taken together, these results highlight the utility in studying *P. luteoviolacea* as a model bacterium to further characterize the effect of bacterial factors on diverse animals and their phenotypic responses, including metamorphosis.

2.3. RESULTS

Many P. luteoviolacea strains possess both TBP and MACs genes

P. luteoviolacea is a globally-distributed Gammaproteobacterium that exhibits a broad genetic diversity. A previous work has demonstrated that some *P. luteoviolacea* strains possess the biosynthesis genes and the ability to produce brominated natural products (Busch *et al.*, 2019). However, a similar survey has not yet been performed for genes encoding MACs. To explore this, we identified several experimentally confirmed genes important for MACs production in strain HI1, including the baseplate (*macB*), tube (*macT*), and sheath (*macS*) structural genes (Shikuma *et al.*, 2014) and the metamorphosis-inducing effector (*mif1*) gene (Ericson *et al.*, 2019) (**Figure 5a**). The MACs genes were blasted against all nineteen complete and draft genomes of *P. luteoviolacea* available from Genbank (**Table 1**) including a *Pseudoalteromonas sp.* outgroup ATCC 29581 (Cress *et al.*, 2013). We then identified the

proportion of strains that encode MACs (*macB, macS, macT* and *mif1*) and/or TBP (*bmp1-4*) (El Gamal *et al.*, 2016) biosynthesis genes by blastn (Camacho *et al.*, 2009). We also reconstructed a *P. luteoviolacea* phylogeny using 71 bacterial ribosomal genes (Eren *et al.*, 2015; Delmont and Eren, 2018; Lee, 2019). As observed previously (Vynne *et al.*, 2012; Busch *et al.*, 2019), *P. luteoviolacea* strains fall within one of two major clades (**Figure 5b**). All *P. luteoviolacea* strains queried possessed MACs gene homologs with significant homology to characterized MACs genes from strain HI1 (represented by purple arrows in **Figure 5b** and listed in **Table 1**). Furthermore, the genomic architecture of the *bmp* genes and production of pentabromopseudilin was confirmed in roughly half of the strains (Busch *et al.*, 2019). The blue pentagons (**Figure 5b**) show that brominated natural product biosynthesis is not restricted to the phylogenetic distribution. Our results show that all *P. luteoviolacea* strains examined have the genetic capacity to produce MACs and nearly half of the strains have the genetic capacity to produce both TBP and MACs.

P. luteoviolacea strain HI1 produces both TBP and MACs

We have shown previously that *P. luteoviolacea* strain HI1 produces MACs (Shikuma *et al.*, 2014), and it was recently shown that this same strain also possesses the *bmp* gene cluster (Busch *et al.*, 2019) (**Figure 6a**). Although we have previously shown that a Δ*macB* mutant is unable to produce functional MACs (Shikuma *et al.*, 2014), the ability of *P. luteoviolacea* strain HI1 to produce TBP was unknown. We therefore tested whether strain HI1 is capable of producing TBP, and whether the brominase Bmp2 (Agarwal *et al.*, 2014; Gamal *et al.*, 2016) is required for production. *P. luteoviolacea* mutant strains were constructed using double-homologous recombination (Shikuma *et al.*, 2014; Ericson *et al.*, 2019; Rocchi *et al.*, 2019). The

mutant strains contain in-frame deletions of the *bmp2* gene (**Figure 6a;** blue), shown previously to be required for TBP production (Agarwal *et al.*, 2014; Gamal *et al.*, 2016), the *macB* gene (**Figure 5b;** purple), encoding an essential structural component of the MACs baseplate and a Δ*macB*Δ*bmp2* mutant that is unable to produce both MACs and TBP (**Table 2**). Using QToF LC-MS/MS (Agilent 6530 Accurate Mass; California, USA) we determined that *P. luteoviolacea* strain HI1 produces TBP (**Figure 6b, 9a**), and a mutation in the *bmp2* gene abrogated TBP biosynthesis (**Figure 6b**). We complemented the *bmp2* gene on a constitutively expressed plasmid, which enabled a small but detectable amount of TBP despite the absence of the gene at its native locus (**Figure 9b)**. This finding suggests that there are no other active brominases responsible for the production of TBP in *P. luteoviolacea* under the conditions tested.

P. luteoviolacea stimulates Hydractinia metamorphosis in the absence of MACs

TBP has been implicated as an inducer of coral metamorphosis by testing the effect of fractionated and purified TBP on coral larvae (Tebben *et al.*, 2011; Sneed *et al.*, 2014; Tebben *et al.*, 2015). To determine whether larvae of *Hydractinia* respond to purified TBP in a similar manner, we investigated whether synthesized, exogenously added TBP (Zheng *et al.*, 2018) at similar concentrations to those tested for corals (Sneed *et al.*, 2014; Tebben *et al.*, 2015) stimulates *Hydractinia* metamorphosis. Development of *Hydractinia* larvae after exposure to TBP or bacteria was quantified after 72 hours and scored positively for metamorphosis if they developed stolons and tentacles (**Figure 7a**)**.** Upon exposure to a range of TBP concentrations, *Hydractinia* did not undergo metamorphosis, and at the highest tested concentrations (1,000nM & 750nM) TBP was lethal (**Figure 7b, Table 3**). These results indicate that *Hydractinia* larvae do not undergo metamorphosis in response to TBP under the conditions tested.

We next queried whether *Hydractinia* responds to intact *P. luteoviolacea* cells within biofilms, capable of producing both TBP and MACs, TBP alone, MACs alone, or neither. To this end, we exposed *Hydractinia* larvae to biofilms of *P. luteoviolacea* wild type, Δ*macB* , Δ*bmp2*, or Δ*macB*Δ*bmp2* mutant strains. Exposure to wild type biofilms of *P. luteoviolacea* was lethal after they appeared to initiate the settlement process (**Figure 7c, Table 3**). Similarly, the biofilms of Δ*bmp2* resulted in mortality of larvae. Interestingly, both mutants that lacked functional MACs (Δ*macB* and Δ*macb*Δ*bmp2*) stimulated the metamorphosis of *Hydractinia* larvae (**Figure 7c, Table 3)**. Our results show that MACs are lethal to *Hydractinia* larvae, deletion of the *bmp2* gene in *P. luteoviolacea* has no effect under the conditions tested and *P. luteoviolacea* stimulates metamorphosis in the absence of MACs.

P. luteoviolacea biofilms stimulate Hydroides metamorphosis via MACs, not TBP

We next questioned how the larvae of a different animal, *Hydroides*, responds to *P. luteoviolacea* and its metamorphosis factors. To this end, we exposed *Hydroides* larvae to purified TBP and subsequently the same panel of mutant *P. luteoviolacea* strains with and without MACs and TBP. *Hydroides* larvae were scored after 24 hours of exposure to TBP or bacterial biofilms and assessed as metamorphosed if they developed branchial radioles and a primary proteinaceous tube (**Figure 8a**). At a 500nM concentration, TBP alone resulted in up to 30% of *Hydroides* metamorphosis compared to the acetonitrile solvent control (**Figure 8b, Table 3**; $p = 0.0054$**)**. Notably, all *Hydroides* larvae that metamorphosed were attached to the well. Higher TBP concentrations tested (1,000nM, 750nM and 500nM), resulted in death of a fraction of *Hydroides* larvae.

We next tested whether biofilms of *P. luteoviolacea* wild type and each *macB* or *bmp2* mutant elicited a phenotypic response in *Hydroides* larvae. While biofilms of wild type *P. luteoviolacea* stimulated the metamorphosis of *Hydroides*, the Δ*macB* mutant abrogated metamorphosis (**Figure 8c, Table 3**), consistent with previous findings (Shikuma *et al.*, 2014). In contrast, *Hydroides* larvae were stimulated to metamorphose by the Δ*bmp2* mutant, producing a similar response to wild type. Like the Δ*macB* mutant, the Δ*macB*Δ*bmp2* mutant did not induce metamorphosis. Our results show that *Hydroides* larvae undergo metamorphosis in response to MACs and are unaffected by mutation of the *bmp2* gene under the conditions tested.

2.4. DISCUSSION

P. luteoviolacea produces both TBP and MACs, which are factors that have been shown to stimulate the metamorphosis of corals and tubeworms respectively. However, the effect of MACs and TBP on different animal types and the distribution of MACs genes in *P. luteoviolacea* strains has not yet been determined.

Purified TBP stimulates Hydroides metamorphosis but has no effect on Hydractinia larvae

We found that purified TBP induces a moderate level of *Hydroides* metamorphosis at intermediate (500nM) concentrations and resulted in death at higher (750nM and 1000nM) concentrations. Coral larvae were found to undergo metamorphosis, many without attaching to the substrate (Negri *et al.*, 2001; Tebben *et al.*, 2011; Sneed *et al.*, 2014; Tebben *et al.*, 2015), when exposed to similar concentration ranges used in previous studies (Sneed *et al.*, 2014; Tebben *et al.*, 2015). Interestingly, we found that all *Hydroides* larvae metamorphosed with

attachment. It is currently unknown whether the cellular processes that control attachment and metamorphosis are different between corals and *Hydroides.* Although *Hydractinia* belongs to the same phylum as stony corals which undergo metamorphosis in response to TBP, *Hydractinia* larvae did not undergo metamorphosis in response to TBP and at the highest concentrations tested, TBP was lethal.

It is currently unclear whether TBP is an ecologically relevant stimulant of metamorphosis or how TBP stimulates metamorphosis in marine larvae. Studies of TBP exposure to phytoplankton reveals that TBP induces the release of intracellular calcium stores (Whalen *et al.*, 2018). TBP exposure to mammal microsomes triggers Ca^{2+} efflux by activating the Ryanodine receptor, RyR1, and inhibiting microsomal sarcoplasmic/ endoplasmic reticulum Ca^{2+} ATPase, SERCA1a (Zheng *et al.*, 2018). Calcium signaling and membrane potential depolarization have been linked to the induction of *Hydroides* (Carpizo-Ituarte and Hadfield, 1998; Holm *et al.*, 1998; Chen *et al.*, 2012). Furthermore, exposure of a calcium ionophore to the larvae of the sea urchin, *Strongylocentrotus purpuratus,* resulted in a similar percentage of metamorphosis as compared to TBP stimulated metamorphosis in *Hydroides* (Amador-Cano *et al.*, 2006). Taken together, these studies provide a potential link between TBP and calcium signaling mediating invertebrate larvae metamorphosis.

Mutation of bmp2 in P. luteoviolacea HI1 has no effect on Hydroides or Hydractinia larvae

We found that mutation of the *bmp2* brominase in *P. luteoviolacea* had no effect on *Hydroides* or *Hydractinia* larvae under the conditions tested in this work. Although we observed *Hydroides* metamorphosis in response to purified TBP, mutation of *bmp2* had no effect on

Hydroides metamorphosis in response to *P. luteoviolacea* biofilms under the conditions tested. These results suggest that MACs from *P. luteoviolacea* biofilms are the primary stimulant of *Hydroides* metamorphosis while TBP from *P. luteoviolacea* biofilms has no effect. Our results show that phenotypic responses can be very different when comparing exposure to purified factors versus live bacteria where genetic interrogation is possible.

While our investigations show that *P. luteoviolacea* can produce TBP, we found that TBP production by *P. luteoviolacea* biofilms does not impact *Hydroides* metamorphosis. One possible explanation is that TBP is not produced at the same concentration as the coral metamorphosis-inducing *Pseudoalteromonads* under the conditions tested (Tebben *et al.*, 2011; Sneed *et al.*, 2014). We found that *P. luteoviolacea* grown in different media [seawater tryptone (SWT) and Marine Broth(MB)] produced significantly different concentrations of TBP in our study (**Figure 9a**). Differences in concentrations of available bromine may account for the measured differences. SWT contains a 0.056 g/L concentration of potassium bromide while MB contains at higher concentration of 0.080 g/L. Furthermore, many strains of *P. luteoviolacea* contain the *bmp* gene cluster (*bmp1-10*) and produce a suite of polybrominated natural compounds including the antibacterial compound, pentabromopseudilin (Laatsch and Pudleiner, 1989; Busch *et al.*, 2019), and its associated monomeric molecules, such as TBP (Agarwal *et al.*, 2014). Importantly, strains of *Pseudoalteromonas* capable of inducing coral larvae from TBP extract possess a truncated version of the *bmp* gene cluster (*bmp1-4,9,10*) (Gamal *et al.*, 2016) that produce TBP almost exclusively. Future experiments with *P. luteoviolacea* and a truncated *bmp* gene cluster (*bmp1-4, 9 & 10* only) could elucidate the function of TBP-producing pseudoalteromonads and their potential effect on the larvae of different animals.

MACs are a double-edged sword, depending on the animal

Here, we show that *P. luteoviolacea* MACs stimulate *Hydroides* metamorphosis while they are lethal to *Hydractinia* larvae. MACs carry two characterized effector proteins; one effector called Mif1 that stimulates *Hydroides* metamorphosis (Ericson *et al.*, 2019), and another effector called Pne1 that is toxic to insect and mouse cell lines *ex vivo* (Rocchi *et al.*, 2019). Interestingly, MACs were also observed to be lethal in *Hydroides* at high biofilm densities and crude extract concentrations (Shikuma *et al.*, 2014). While the ecological role of MACs has not yet been determined, the range of phenotypes (i.e. metamorphosis and death) in response MACs demonstrates that they can elicit positive or negative phenotypes depending on the concentration tested and animal type. Future work into the function of Mif1, Pne1 and other putative MACs effectors could help to explain how each effector elicits a specific phenotypic response in different animals or cells.

Our finding that *P. luteoviolacea* mutants lacking *macB* induce *Hydractinia* metamorphosis suggests that *P. luteoviolacea* produces one or more additional uncharacterized factor(s) that stimulate *Hydractinia* metamorphosis. This finding may not be surprising as other *Pseudoalteromonas* and *Alteromonas* species have been previously isolated from *Hydractinia echinata* and induce their metamorphosis (Leitz and Wagner, 1993b; Klassen, Rischer, *et al.*, 2015; Klassen, Wolf, *et al.*, 2015; Guo *et al.*, 2017). Furthermore, a recent study found that purified (lyso)phospholipids and polysaccharides are strong inducers of *Hydractinia* metamorphosis (Guo *et al.*, 2019). Other recent studies have implicated Outer Membrane Vesicles (OMVs) and lipopolysaccharides as bacterial stimulants of metamorphosis (Freckelton

et al., 2017, 2019). *Hydractinia* is found in temperate oceans, while *P. luteoviolacea* was isolated from a tropical environment. Although *P. luteoviolacea* HI1 and *Hydractinia* were not isolated from the same environment, both partners of this model interaction are genetically tractable (Huang *et al.*, 2012; Sanders *et al.*, 2018) and could serve as a strong platform for determining mechanisms underlying bacteria-induced metamorphosis in cnidarians.

Genes encoding MACs are part of the core P. luteoviolacea pangenome

In this work, we found that many *P. luteoviolacea* strains possess genes required for the biosynthesis of two known metamorphosis-inducing factors (TBP and MACs). We show that MACs genes are found in all sequenced *P. luteoviolacea* strains, suggesting that MACs biosynthesis genes may be a conserved feature of the *P. luteoviolacea* pangenome. Extracellular contractile injection systems may be a common mechanism of host-microbe interactions, as genes encoding structures related to MACs have been found in diverse bacteria and archaea (Böck *et al.*, 2017; Chen *et al.*, 2019), including Bacteroidales bacteria from the human gut (Rojas *et al.*, 2020). Although the 19 strains of *P. luteoviolacea* we analyzed possess genes encoding MACs, it remains to be tested whether they are capable of producing a functional contractile injection system that deploys effectors into target cells. A previous study tested the type strain DSM 6061/ATCC 33492/ NCIMB 1893 (**Figure 5b**) and found the strain was unable to induce metamorphosis in *Hydroides* (Huang *et al.*, 2012). We find that the DSM 6061 strain possesses the known genetic components necessary to produce MACs (**Figure 5a**; bolded genes: *macB, macS, macT,* and *mif1*) with high nucleotide identity (**Table 1**). Interestingly, strain H2 (**Figure 5b**; clade 1, bold), which is within the same lineage as DSM 6061, induces the metamorphosis of the coral *Pocillopora damicornis* through a yet undetermined mechanism

(Tran and Hadfield, 2011). These results raise the possibility that *P. luteoviolacea* could elicit a positive or negative interaction with marine larvae depending on the expression of its various factor arsenal. Future studies into the regulation of MACs and TBP expression under laboratory growth conditions could explain differences between the metamorphosis inducing capabilities among *P. luteoviolacea* strains.

Future directions and challenges

The role that bioactive products play in the ecology of *Pseudoalteromonas* species still requires significant investigation. In the environment, pseudoalteromonads have been found associated with diverse marine plants and animals (Holmström and Kjelleberg, 1999; Bowman, 2007) and possess diverse antagonistic properties; for example *P. luteoviolacea* was shown to inhibit the growth of other marine bacteria, algae (Holmström *et al.*, 2002; Rypien *et al.*, 2010), fungi (Holmström *et al.*, 2002; Atencio *et al.*, 2018) and here we show that *P. luteoviolacea* is lethal to *Hydractinia* larvae. At the same time, *P. luteoviolacea* was shown to stimulate the metamorphosis of corals (Tran and Hadfield, 2011), urchins (Huggett *et al.*, 2006) and tubeworms (Shikuma *et al.*, 2014) in independent laboratory studies. The factors of *P. luteoviolacea* that facilitate or inhibit metamorphosis beyond the organisms investigated within this study remain unknown and are interesting targets for future work. For example, it has been shown that Histamine derived from algae or its associated microbes induces metamorphosis in sea urchins (Swanson *et al.*, 2004), but Huggett et al. show that *P. luteoviolacea* can induce metamorphosis as well (Huggett *et al.*, 2006). It is currently not clear whether *P. luteoviolacea* produces histamine which induces metamorphosis, or a potential other bacterial factor may be capable of inducing metamorphosis in sea urchins.

The capability of *P. luteoviolacea* to facilitate or inhibit settlement and metamorphosis leads us to question which underlying molecular processes enable bacterial factors to influence some animals to metamorphose, but not others. These results suggest that selective inductive capabilities elicited by bacteria could have an influence on animal recruitment in the environment. Future investigations into the molecular targets and regulation of these bioactive factors in natural biofilm assemblages may help shed light on their ecological role. However, before these implications can be fully interpreted, we must consider ecologically relevant concentrations of the bacteria and their factors, their distribution in varying environments, and the potential role of the bacteria and their associated factors in natural assemblages among other organisms.

2.5. CONCLUSIONS

P. luteoviolacea's ability to produce diverse bioactive compounds and distinct metamorphosis-inducing factors makes this bacterium an interesting model to study bacteria-induced metamorphosis in animals from different Phyla. Our results emphasize that there is a complex set of interactions between bacteria, the factors they produce and animal responses, even when studied under controlled laboratory conditions. Using approaches like those used in this work to compare and identify the effects of different bacterial factors on metamorphosis may aid in unraveling this complexity, and could provide a deeper understanding of the molecular underpinnings of bacteria-induced metamorphosis in divergent animals.

2.6. METHODS

Construction of Δbmp2 and ΔmacBΔbmp2 mutants

Using a double-homologous recombination technique as described previously (Shikuma *et al.*, 2014; Ericson *et al.*, 2019; Rocchi *et al.*, 2019), we created *P. luteoviolacea* HI1 in-frame deletion strains of the *bmp2* (brominase) gene, shown previously to be required for TBP production (Agarwal *et al.*, 2014; Gamal *et al.*, 2016), the *macB* gene, encoding an essential structural component of the MACs baseplate and a Δ*macb*Δ*bmp2* mutant that is unable to produce both MACs and TBP. We complemented the *bmp2* mutant by constitutively expressing *bmp2* from a plasmid *in trans*. A list of strains, plasmids, and primers constructed and used in this study can be found in **Table 2 & 4**.

Detection of TBP production by P. luteoviolacea HI1

P. luteoviolacea was grown in two growth medias, Seawater Tryptone (SWT; 35.9g/L Instant Ocean, 2.5 g/L Bacto Tryptone, 1.5g/L Bacto Yeast, 1.5 mL/L glycerol) and Marine broth (BD 2216) to address the differences in growth conditions previously used to describe MACs (Huang *et al.*, 2012; Shikuma *et al.*, 2014) and TBP (Agarwal *et al.*, 2014; Sneed *et al.*, 2014). Single colonies of wild type and Δ*bmp2* were inoculated in triplicate, grown in 5mL SWT and MB media, and incubated shaking (200rpm) at 28ºC for 16 hours. The cultures were extracted twice with an equal volume of ethyl acetate (EtOAc) and concentrated under a stream of nitrogen. The samples were resuspended in 100 µL methanol, filtered in a 0.2 µm column and 10 µL of the sample was injected into a Luna C18 reversed-phase analytical HPLC column (5 µm, 250 mm × 4.6 mm, Phenomenex; CA, USA). TBP was measured on an Accurate Mass QtoF LC-MS/MS (Agilent 6530 Accurate Mass; California, USA), run at 0.5mL/min in negative mode. Eluent was detected using electrospray ionization-mass spectrometry (ESI-MS) monitoring *m/z*

150–2,200 in negative mode with a speed of 32,500 *m/z* /s.A solvent system of acetonitrile and water both containing 0.1% formic acid (v/v) was used. Samples were eluted over a 30 minute method with a gradient from 10 to 70% acetonitrile over 15 minutes, 70 to 80% over the next 10 minutes, and then immediately to 100% for 5 minutes before returning back to 10% acetonitrile. Quantification of TBP was based on the relative intensities of 2,3,4,5-tetrabromopyrrole to synthetic standards. 2,3,4,5-tetrabromopyrrole was synthesized following the procedure as previously described (Chekan *et al.*, 2019).

Culture of Hydroides and Hydractinia

Hydroides elegans adults were collected from Quivira Basin, San Diego, California. The larvae were spawned and fed living *Isochrysis* cultures daily (Carolina Cat# 153180, North Carolina, USA) as previously described (Nedved and Hadfield, 2009; Shikuma *et al.*, 2014). Larvae were maintained in beakers containing filtered artificial seawater (ASW) (Instant Ocean Cat # SS15-10, Virginia, USA) at 35 PSU water until competent (between 6 and 8 days).

Colonies of *Hydractinia symbiolongicarpus* were maintained attached to microscope slides in ASW at 29 PSU at 20°C, a light:dark cycle of 14:10 with constant aeration, fed 4 days a week with 3 to 5 days old brine shrimp (artemia) (Carolina, Cat# 142242), and twice a week with frozen blended oyster. ASW was changed 5 days a week. Embryos were collected 2 h after the onset of light and maintained in the above mentioned conditions inside of 100 mm plastic petri dishes with ASW 29 PSU supplemented with ampicillin 100 μg/ml and kanamycin 5 μg/ml for 7 days. Prior the metamorphosis assay, competent larvae were transferred to autoclaved ASW 29 PSU to reduce the load of bacteria and antibiotics.

Biofilm metamorphosis assay

Single species biofilms of *P. luteoviolacea* were produced and tested for their ability to induce metamorphosis as previously described (Huang and Hadfield, 2003; Shikuma *et al.*, 2014). Briefly, bacterial strains were struck onto SWT and MB agar plates and incubated overnight at 28ºC. Single colonies were inoculated into 5mL of SWT and MB broth and incubated overnight for 14-16 hours with agitation (200rpm). Cells were removed from the culture, pelleted at 4000g, and washed twice and resuspended with ASW. Cell density was adjusted to OD_{600} of 1 (approximately 10^7 -10^8 cells/ml) for *Hydractinia* and were further diluted to OD 0.5 for *Hydroides* (1:1 with ASW) to evoke optimal metamorphic responses. Aliquots of 100µL were added to 96-well plates to form biofilms over a two-hour incubation period at room temperature. The excess culture and unattached cells were removed from each well. Each well contained 20-40 competent larvae (6-8 days old) and filtered ASW with a final volume of 100µL for *Hydroides* and 200µL for *Hydractinia*. The percentage of metamorphosis was scored after 24 hours (*Hydroides*) and 72 hours (*Hydractinia*). While the complete metamorphosis of *Hydractinia* can occur 24 hours post induction (Stefanie Seipp *et al.*, 2007), the death phenotype produced similar phenotypes during early differentiation. This influenced qualitative scoring daily and quantitative scoring after 72 hours. Four technical replicates of each treatment and three biological replicates were performed on separate occasions. Both media conditions produced similar metamorphosis outcomes for *Hydroides* and *Hydractinia*. Graphs displayed for metamorphosis assays represent the media that produced the most prominent results. We display biofilm metamorphosis assays with *Hydroides* grown in Marine Broth media and *Hydractinia* grown in SWT media.

Exogenous TBP metamorphosis assays

Pure 2,3,4,5-tetrabromopyrrole standard was synthesized as previously described (Zheng *et al.*, 2018). Eleven milligrams of TBP was resuspended in acetonitrile (0.0059M) and was diluted into ASW (35 PSU for *Hydroides* and 29 PSU for *Hydractinia*), which contained a final acetonitrile concentration of 2% (v/v). The concentrations tested were: 1000nM, 750nM, 500nM, 250nM, 100nM, 10nM, 1nM and 0.1nM. We saw no phenotype for the lowest concentrations tested and therefore did not include them in the final analysis. The diluted TBP was aliquoted (100µL) into 96-well plates. Approximately 20-40 competent larvae (*Hydroides* and *Hydractinia*) were added to each well. Four technical replicates were performed for each treatment with *Hydractinia* and eight technical replicates were performed with *Hydroides.* Three and five biological replicates were repeated on different occasions for *Hydractinia* and *Hydroides,* respectively.

Comparative genomics

P. luteoviolacea draft and complete genomes were downloaded from NCBI (**Table 1**)**.** The representations of the MACs and TBP gene clusters in *P. luteoviolacea* were created using Easyfig (Sullivan *et al.*, 2011) (v2.2.2_OS). Fasta files containing the gene clusters for MACs (Shikuma *et al.*, 2014) and TBP (Gamal *et al.*, 2016) were used to perform a tblastx on the *P. luteoviolacea* HI1 strain. MACs genes, *macB, macS, macT*$_1$, and *mif1* were selected to perform a blastn (Camacho *et al.*, 2009) against all of the other sequenced strains for *P. luteoviolacea.* The sequenced genomes were formatted into a blast database through the galaxy server (Cock *et al.*, 2015).

All *P.luteoviolacea* genomes were then analyzed through the Anvi'o pangenomics (v6) pipeline (Eren *et al.*, 2015; Delmont and Eren, 2018) using their publicly available tutorials. The pangenome was generated using the parameter values (Van Dongen and Abreu-Goodger, 2012; Benedict *et al.*, 2014): –minbit 0.5; mcl-inflation –10; –use-ncbi-blast. Once the pangenome was generated, the sequences for bacterial hmm hits were concatenated. 71 ribosomal bacterial genes(Lee, 2019) (modified as noted here: https://github.com/merenlab/anvio/tree/master/anvio/data/hmm/Bacteria_71) were used to create the phylogeny. The concatenated sequences were aligned using MAFFT and the alignment algorithm G-ins-I (Katoh *et al.*, 2019). A maximum likelihood phylogeny was constructed using the LG+G+I+F substitution model (Guindon *et al.*, 2010) with the Smart Model Selection (Lefort *et al.*, 2017) feature for PhyML from the ATGC bioinformatics webserver. Bootstrap values (100 resamples) were calculated to ensure tree robustness. The tree was manipulated and viewed in iTOL (Letunic and Bork, 2016).

2.7. ACKNOWLEDGEMENTS

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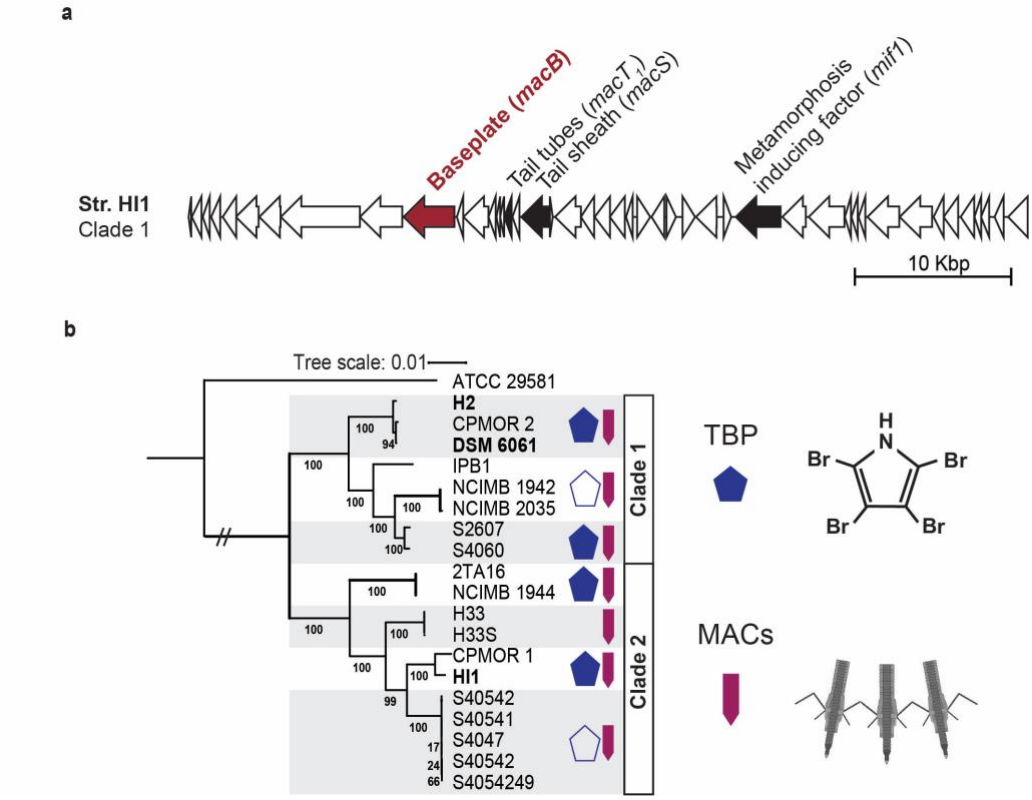

Figure 5. Diverse *Pseudoalteromonas luteoviolacea* strains encode the biosynthesis genes for TBP and MACs. **(A)** MACs gene cluster from strain HI1. The bolded arrows denote the genes interrogated by blast and are previously shown to be necessary for MACs function. The red filled arrow represents the *macB* gene, which is knocked out to create the nonfunctional MACs (Shikuma *et al.*, 2014) in the biofilm metamorphosis assays. **(B)** Maximum likelihood phylogeny of 19 sequenced *P. luteoviolacea* genomes including *Pseudoalteromonas* sp. ATCC 29581 as an outgroup. The bootstraps values represent 100 resamples. The banded boxes indicate highly conserved subgroups for which symbol representations apply throughout the group. The blue pentagon denotes strains that produce TBP. Hollow pentagons represent strains that encode some genes in the *bmp* gene cluster, but are nonfunctional. Purple arrows indicate the presence of MACs genes (*macB, macS, macT, and mif1*).

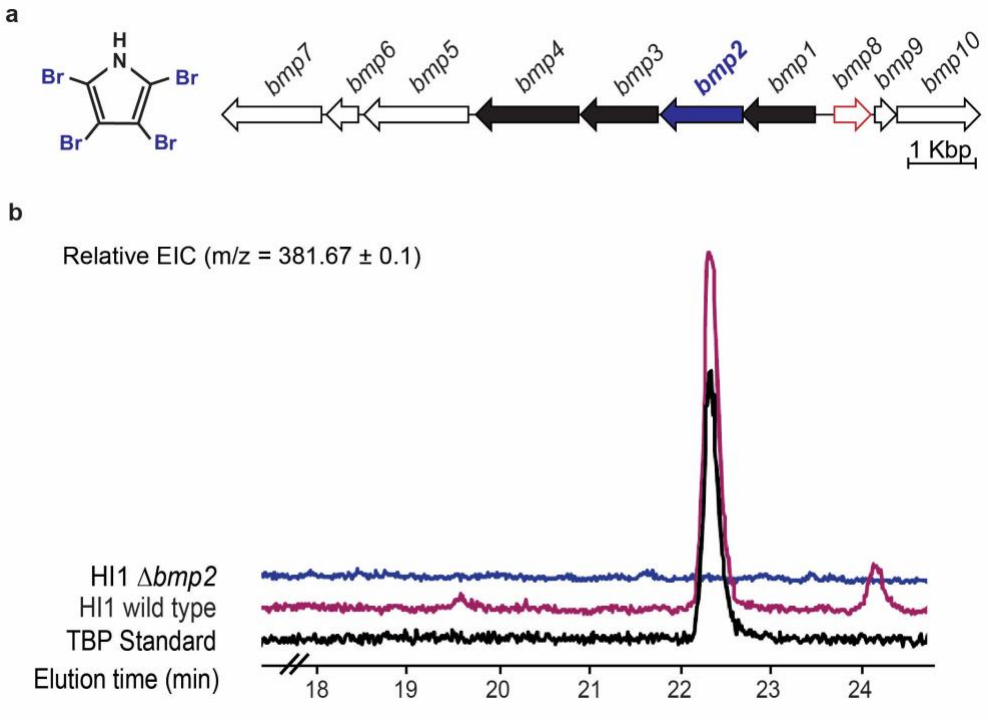

Figure 6. ***Pseudoalteromonas luteoviolacea*** **HI1 wildtype produces TBP, while the Δ*bmp2* strain does not.** **(A)** genomic arrangement of the *bmp* gene cluster in strain HI1. Bolded genes *bmp1-4* have previously been validated to code for TBP biosynthesis. The blue bolded gene *bmp2* was deleted to create a nonfunctional TBP mutant in *P. luteoviolacea.* The gene outlined in red *bmp8* is a pseudogene. **(B)** Representative Ion Chromatogram (EIC = 381.67) overlaid comparison of an organic extraction of wildtype and Δ*bmp2* strains to the TBP standard. Cultures were grown in Marine Broth and SWT media overnight in triplicate to quantify TBP production in *P. luteoviolacea* (see Figure 9a).

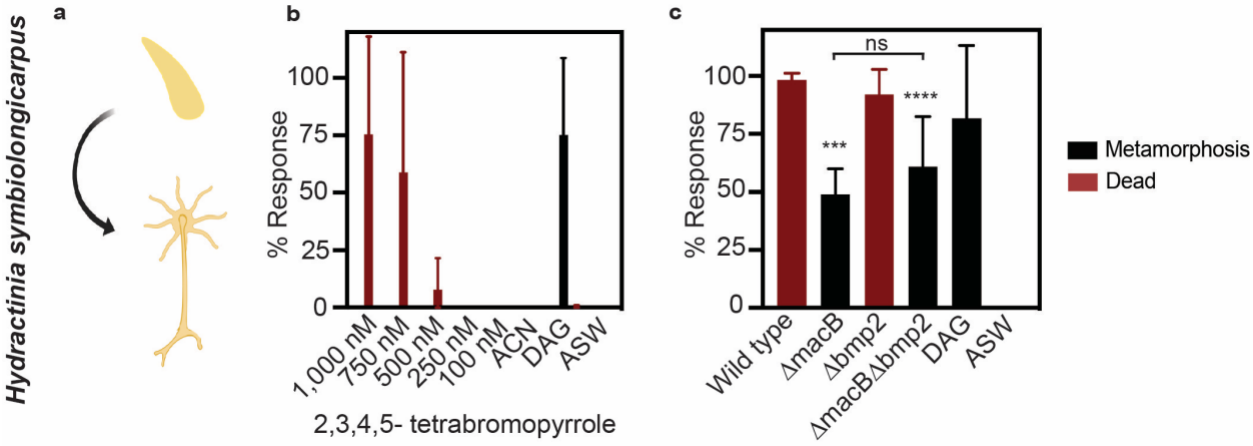

Figure 7. *P. luteoviolacea* stimulates *Hydractinia* metamorphosis in the absence of MACs.
(A) Schematic of larval and metamorphosis phenotypes scored for *Hydractinia* larvae. All metamorphosis assays are performed in 96-well plates with either **(B)** the addition of synthesized TBP or **(C)** monoculture biofilms of *P. luteoviolacea* wild type or mutant strains. Phenotypic response of *Hydractinia* larvae to treatments containing **(B)** increasing concentrations of purified TBP and **(C)** *P. luteoviolacea* wild type or mutant biofilms. The bars represent the average of 3 biological replicates (n=3). Values for the biological replicates were determined by averaging 4 technical replicates per treatment. The biological replicates were performed on different batches of larvae on different days. Error bars denote standard deviation. Asterisks above the bars denote statistical significance compared to the **(B)** ACN and **(C)** ASW controls. ACN is a 2% (v/v) acetonitrile solvent control. ASW is filtered artificial seawater and is used as the negative control in all assays. DAG (1,2-Diocanoyl-sn-glycerol) is a chemical stimulant of metamorphosis and is used as a control for competency of *Hydractinia* larvae at a concentration of 100µM. **(C)** Statistical analyses were performed with a one-way ANOVA corrected for multiple comparisons by false discovery rate (FDR) using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (GraphPad® Prism), where *** p=0.0029, and **** is p=0.0006. No statistical difference was found between treatments Δ*macB* and Δ*macB*Δ*bmp2*.

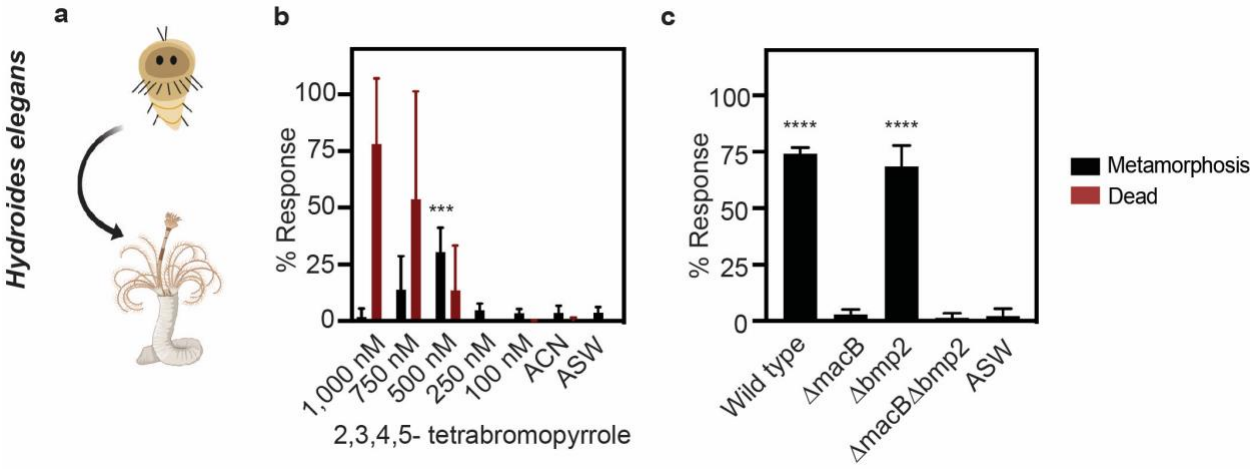

Figure 8. *P. luteoviolacea* MACs stimulate the metamorphosis of *Hydroides* larvae.
(A) Schematic of larval and metamorphosis phenotypes scored for *Hydroides*. Metamorphosis assays are performed as described previously (Shikuma *et al.*, 2014)**.** Phenotypic response of *Hydroides* larvae to treatments containing **(B)** increasing concentrations of purified TBP and **(C)** mutant biofilms. The bars represent the average of **(B)** 5 biological replicates (n=5) and **(C)** 3 biological replicates (n=3). A statistical power analysis aided with the determination for the appropriate number of biological replicates. Values for the biological replicates were found by averaging 4 technical replicates per treatment. The biological replicates were performed on different batches of larvae on different days. Error bars denote standard deviation. Asterisks above the bars denote statistical significance compared to the **(B)** ACN and **(C)** ASW controls. Statistical analyses were performed with a **(B)** Kruskal-Wallis ANOVA and **(C)** one-way ANOVA both corrected for multiple comparisons by FDR using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (GraphPad® Prism), where *** p=0.0054, and **** is p<0.0001.

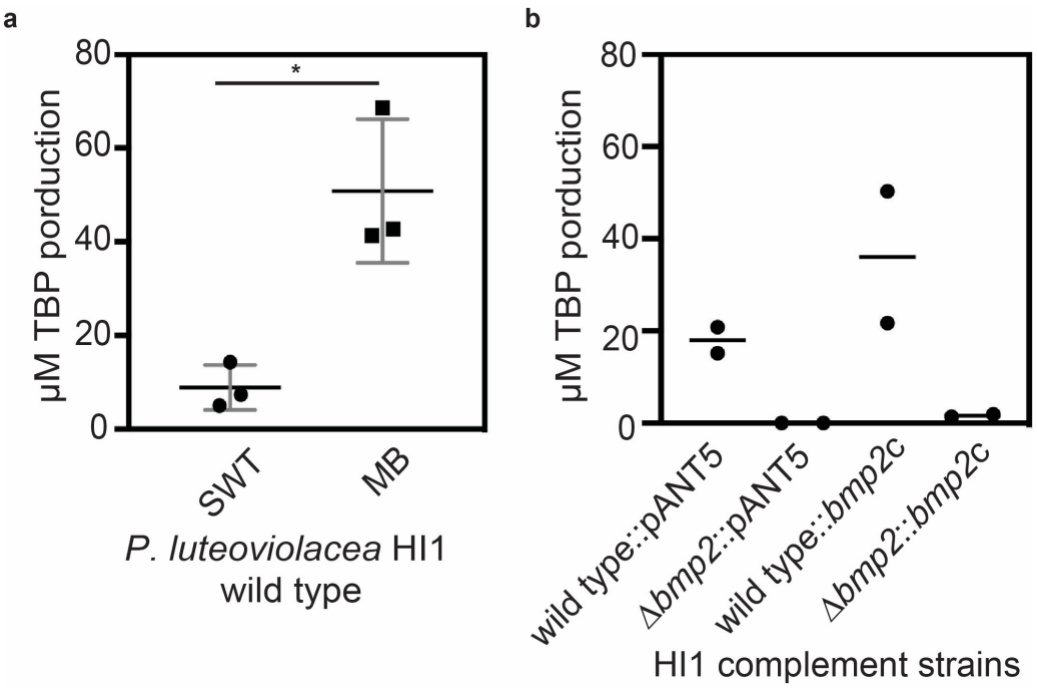

Figure 9. ***Pseudoalteromonas luteoviolacea*** **HI1 complement restores some TBP production, while the Δ*bmp2* strain does not.**

(**A**) Wild type cultures were grown in triplicate in SWT and MB media overnight. The cultures were extracted with two volumes of ethyl acetate and concentrated under a stream of nitrogen gas. Extracts were resuspended in methanol, filtered and injected into a C18 reversed-phase analytical HPLC column. TBP was measured on an Accurate Mass QToF LC-MS/MS, run at 0.5mL/min in negative mode. Quantification of TBP was determined by integrating the 381.67 mass (m+4) peak from the isotope distribution of the mass spectrum to enhance the signal to noise ratio. Gaussian statistics were calculated using a unpaired t-test with Welch's correction, where * $p \leqq 0.0325$. Error bars represent the standard deviation. (**B**) The *bmp2* complemented cultures were grown overnight in MB media in duplicate and the same extraction and analysis procedures from (**A**) were used to determine presence of TBP in the complemented strains. Horizontal lines represent the mean in both (**A**) and (**B**).

Table 1. List of *Pseudoalteromonas luteoviolacea* strains used for phylogeny and MACs genes used for blastn

		macB		***macS***		***macT*$_1$**		***mif1***	
Strain	**WGS Acc.**	% ID	E-value	% ID	E-value	% ID	E-value	% ID	E-value
S4054249	CP015411	83.904	0	86.327	0	92.208	0	65.765	1.45E-71
S4054	CP015415	83.904	0	86.327	0	92.208	0	65.765	1.45E-71
S40542	CP015413	83.904	0	86.327	0	92.208	0	65.765	1.45E-71
H2	VIGJ01	67.025	0	75.934	0	76.392	2.73E-90	73.211	0
DSM 6061	AUYB01	66.912	0	75.764	0	76.392	2.73E-90	73.465	0
HI1	JWIC01	100	0	100	0	100	0	100	0
IPB1	MAUJ01	66.943	0	75.297	0	76.392	2.73E-90	72.347	0
S2607	AUXV01	67.375	0	75.424	0	75.056	3.79E-82	72.269	0
S4060-1	AUXX01	67.312	0	75.48	0	74.833	1.61E-80	72.596	0
NCIMB 1944	AUXS01	72.818	0	77.501	0	87.013	8.89E-160	76.19	0
CPMOR-1	AUYC01	96.576	0	89.347	0	96.753	0	98.835	0
2ta16	AUSV01	72.818	0	77.501	0	87.013	8.89E-160	76.19	0
H33	AUXZ01	77.033	0	93.986	0	94.156	0	80.734	0
H33-S	AUXY01	77.033	0	93.986	0	94.156	0	80.734	0
CPMOR-2	AUYA01	66.933	0	75.764	0	76.392	2.73E-90	73.465	0
S4047-1	AUXU01	83.904	0	86.327	0	92.208	0	65.765	1.45E-71
S4054	AUXW01	83.904	0	86.327	0	92.208	0	65.765	1.45E-71
NCIMB 1942	AUXT01	70.259	0	74.732	0	75.501	7.33E-85	72.488	0
NCIMB 2035	JPWZ01	70.179	0	74.732	0	75.501	7.33E-85	72.488	0

Table 2. List of strains and plasmids used in Chapter 2

Strain No.	Strain	Genotype	Source
NJS 002	HI1 Str^R	*P. luteoviolacea* HI1, Str^R	(Huang *et al.*, 2012)
NJS 213	Δ*macB*	*P. luteoviolacea* HI1 Δ*macB*, Str^R	(Shikuma *et al.*, 2014)
NJS 448	Δ*bmp2*	*P. luteoviolacea HI1* Δ*Bmp2,* Str^R	This study
NJS 450	Δ*macB*Δ*bmp2*	*P. luteoviolacea HI1* Δ*macB*Δ*Bmp2,* Str^R	This study
NJS 482	HI1::EV	*P. luteoviolacea HI1::pANT5_EV,* Str^R	This study
NJS 483	Δ*bmp2*::EV	*P. luteoviolacea* Δ*bmp2::pANT5_EV,* Str^R	This study
NJS 484	HI1::*bmp2*	*P. luteoviolacea HI1::bmp2,* Str^R	This study
NJS 485	Δ*bmp2*::*bmp2*	*P. luteoviolacea* Δ*bmp2::bmp2,* Str^R	This study
Plasmid No.	**Plasmid**	**Genotype**	**Source**
pNJS 007	pCVD443	Amp^R, Km^R, sacB, pGP704 derivative	(Huang *et al.*, 2012)
pNJS 032	pANT5	*bla*$^+$*mob*$^+$*lacI*^{q}::Km^R *ptac; ori*RSF1010 derivative	(Lee and Falkow, 1998)
pNJS 539	pCVD443_ΔHI1_*bmp2*	pCVD443::Δ*bmp2* Amp^R, Km^R	This study
pNJS 592	pANT5Δ*bmp2::bmp2*	pANT5::Δ*bmp2* Amp^R Km^R	This study

Table 3. Summary table of metamorphosis assay results for Chapter 2

		Hydractinia			*Hydroides*		
	average (stdev)	% metamorphosis	% death	n	% metamorphosis	% death	n
TBP	1000nM	0	75.34 (42.71)	3	1.76 (3.76)	78.08 (29.01)	5
	750nM	0	58.83 (52.29)	3	13.89 (14.75)	53.75 (47.57)	5
	500nM	0	7.88 (13.65)	3	30.48 (10.78)	13.42 (19.92)	5
	250nM	0	0	3	4.86 (2.85)	0	5
	100nM	0	0	3	3.48 (1.85)	0.09 (0.19)	5
	ACN	0	0	3	3.52 (3.19)	0.47 (1.05)	5
	ASW	0	0	3	3.64 (2.66)	0	5
	DAG	75.16 (29.70)	0.40 (0.69)	3	--	--	
Bacteria	Wild Type	0	98.34 (2.88)	3	74.08 (8.37)	0	3
	Δ*macB*	49.1 (10.90)	0	3	2.85 (6.04)	0	3
	Δ*bmp2*	0	92.19 (10.77)	3	68.59 (17.79)	0	3
	Δ*macB*Δ*bmp2*	60.87 (21.63)	0	3	1.50 (4.34)	0	3
	ASW	0	0	3	2.29 (3.93)	0	3
	DAG 100µM	81.89 (31.36)	0	3	--	--	

Table 4. List of Primers used in Chapter 2

Primer	Sequence
p443_bmp2_dA	TAAAAAGGATCGATCCTCTAGAACAAAGGCTTCACTGATGGC
p443_bmp2_dB2	AAGGTGGATATGGCATGAGCGAGTACGACCGCAAGCAAAT
p443_bmp2_dC2	ATTTGCTTGCGGTCGTACTCGCTCATGCCATATCCACCTT
p443_bmp2_dD	GTGAATTCAAAGGGAGAGCTCTGCCTACAGAAAACGAGCCT
p443_BMP2_SeqF1	CCTGCTTCAGCTTCCGATAT
p443_BMP2_SeqR1	GTCATCCACATTGCGTTCTG
pANT5_bmp2_F	TTTAAGAAGGAGATATACATATGAGCGCATTTAAAAGTTA
pANT5_bmp2_R	ATGCCTGCAGGTCTGGACATCTAACCTACCATTTGCTTGC
pANT5_F	GGAAGCTGTGGTATGGCTGT
pANT5_R	CTGGCAGTTCCCTACTCTCG
pANT5_F2	GTCGTAAATCACTGCATAATTCGTG
pANT5_bmp2c_F2	AGGAAACAGAATTCGAGCTCTGAAGATTTCTTAACTAAGGTGG
pANT5_bmp2c_R2	ATCTCCTTCTTAAATCTAGACTAACCTACCATTTGCTTGC
bmp2_seq_F1	TTGTTAACATTGACGCGGCT
bmp2_seq_R1	AGAACGCAATGTGGATGACC
bmp2_seq_F2	ACGTGTTAAAGGGGATGGCC
bmp2_seq_R2	AGCCAATCACATTTACGCCA
bmp2_seq_F3	TCCACGTTTTCATATCGGTGA
bmp2_seq_R3	ACCAACACGCTCAGCATTTT

2.8. APPENDIX

While Chapter 2 (as it appears in *Environmental Microbiology*) set out to develop *P.luteoviolacea* as a model for both MACS and TBP-induced metamorphosis in diverse animals, it fell short on being able to test the strains in an ecologically relevant system (i.e. corals). It also inspired deeper questions about the natural genetic variability of MACS and how these differences may influence animals in the environment.

I started my PhD research journey convinced that *P.luteoviolacea* induces robust coral metamorphosis based on a study from Tran & Hadfield in 2011. This study led me to suggest the hypothesis that MACS, TBP or both from *P.luteoviolacea* may influence coral metamorphosis. This was the premise used for an NSF GRIP internship, which was funded in early 2020. The Coronavirus pandemic delayed field opportunities and forced us to publish the *Environmental Microbiology* paper using the data with the two metamorphosis models, *Hydroides* and *Hydractinia.* A year after publication, we were finally able to test out the strains and hypotheses on the brooding coral, *Porites astreoides* with collaborators at Smithsonian.

Considering both the previous literature and the results of the *Environmental Microbiology* paper, we reasoned that there may be one of two different outcomes: either *P.luteoviolacea* induces coral metamorphosis as suggested by Tran, or it would kill the corals, as it did to the Cnidarian model animal, *Hydractinia* (**Figure 8**). The outcome of the biofilm metamorphosis assays followed the rationale of the latter, showing that *P.luteoviolacea* HI1 kills coral larvae (**Figure 10**). While it is known that there is a "goldilocks" density of *P.luteoviolacea* that induces robust metamorphosis in *Hydroides* (Shikuma et al. 2014), it appears unlikely that the resulting death follows this same paradigm. Assays were performed using various biofilm concentrations, culturing techniques and tested both brooding and broadcasting coral species,

with little change in the coral death phenotype. Despite the methodological differences tested, it is clear that removal of the *macB* gene abrogated the death response to *P.luteoviolacea*, leaving the majority of the larvae swimming in planula form. The results of this experiment suggests that MACS may be lethal to corals and led us to investigate the differences in environmental strains of *P.luteoviolacea* and the possibility that different strains could elicit different phenotypes in corals (and perhaps even *Hydroides*).

To explore the variability of *P.luteoviolacea* strains, we queried the available genomes and generated a representative gene neighborhood (**Figure 11**) for all ANI subclades (previously defined by Busch and colleagues in 2019). Notably, all strains contained some version of the MACs gene cluster. The structural genes were highly conserved, while the effector clusters were variable across ANI subgroups. On the other hand, the previously characterized effector genes, metamorphosis inducing factor 1 (*mif1*) (Ericson et al. 2019) and endonuclease effector (*pne1*) (Rocchi et al 2019), were not equally conserved in the genetic architectures. The gene *mif1* was at least partially conserved amongst all strains, while half of strains lost *pne1*. Interestingly, the loss of *pne1* occurs in both clades of the *P.luteoviolacea* phylogeny, suggesting that these events happened independently. *Pne1* was described as an endonuclease effector capable of killing murine macrophages, and the natural loss of this gene could have implications for the effect of MACS-associated killing in corals. These results support the hypothesis that strain-level differences have the potential to culminate in different phenotypes among host-microbe interactions.

The killing phenotype in corals combined with the recurring loss of *Pne1* in various environmental strains led us to explore the role of *pne1* in coral death by *P.luteoviolacea.* When exposed to mutant *P.luteoviolacea* strains lacking the effector genes, we found that neither *mif1*

nor *pne1* contributed to the death phenotype alone. While the results in **Figure 12** seem very clear, it is important to note that previous trials with Δ*pne1* on both *Hydractina* and corals suggested that there may be a phenotype associated with *pne1* and death. If this phenotype is of interest to others in the future, I would recommend reproducing these results (either *Hydractinia* or corals) with more biological replicates.

To continue exploring the effects of MACS in the context of environmental strain variability, I isolated and sequenced or acquired several strains of *P.luteoviolacea* throughout my dissertation research period. I then performed metamorphosis assays with them on *Hydroides* to gauge if the MACS genetic architecture, had an effect on the induction of metamorphosis (**Figure 13**). Strains CCR1 and WFS2.3 (MMG019) were isolated from the surface of crustose coralline algae and from surrounding sediment in coral reef ecosystems, respectively. Strain 2TA16 (Rypien et al. 2010) was provided to us by the Moore Lab at Scripps Institution of Oceanography and was the subject of studies to characterize brominated natural product synthesis (Agarwal et. al 2014). This strain has an interesting insertion just upstream of the effector cluster (**Figure 11**) that seems to have little effect on the ability to stimulate metamorphosis in *Hydroides* (**Figure 13**). Echoing previous studies (Huang and Hadfield, 2012), strain DSM6061 does not induce robust metamorphosis (**Figure 13**) and would be a good candidate to explore MACS regulation/function in the context of *Hydroides* metamorphosis. Strain NCIMB 2035 (purchased through a strain collection) is a particularly interesting strain for variability and exploring the death phenotype because it falls within clade 1 and has experienced a natural loss of *pne1*. While we were unable to test this strain on corals, this effector architecture does not seem to impact the ability to induce metamorphosis in *Hydroides* (**Figure**

13). If pursuing the toxic effects of MACS on corals or other animals, I would recommend incorporating this strain in future studies.

Taken together, this data highlights the importance of strain and host selection for investigating phenotypes in the context of bacteria-stimulated metamorphosis and other host-microbe interactions. While some of this work is incomplete or leave hypotheses posed but untested, I hope they provide a jumping off point of new and ecologically-informed questions in the context of MACS-host interactions.

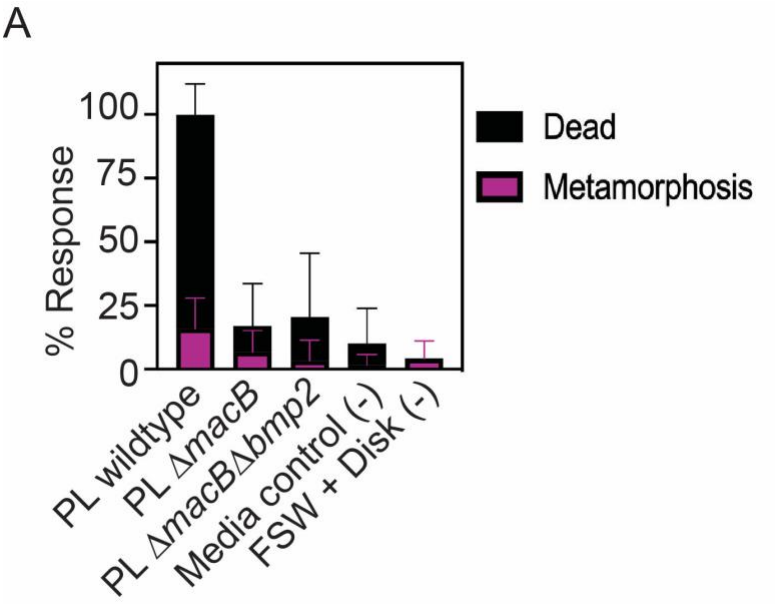

Figure 10. MACS kill coral larvae and TBP from *P. luteoviolacea* does not evoke significant coral metamorphosis.
Coral metamorphosis assays performed on settlement fragging disk with brooding coral species *Porites astreoides*. Settlement disks were incubated for 24 hours in MB media. The assay was performed with the same batch of larvae and used the same conditions as document in Chapter 3. Data shown is a single biological replicate containing 6 technical replicates with 10 larvae per well.

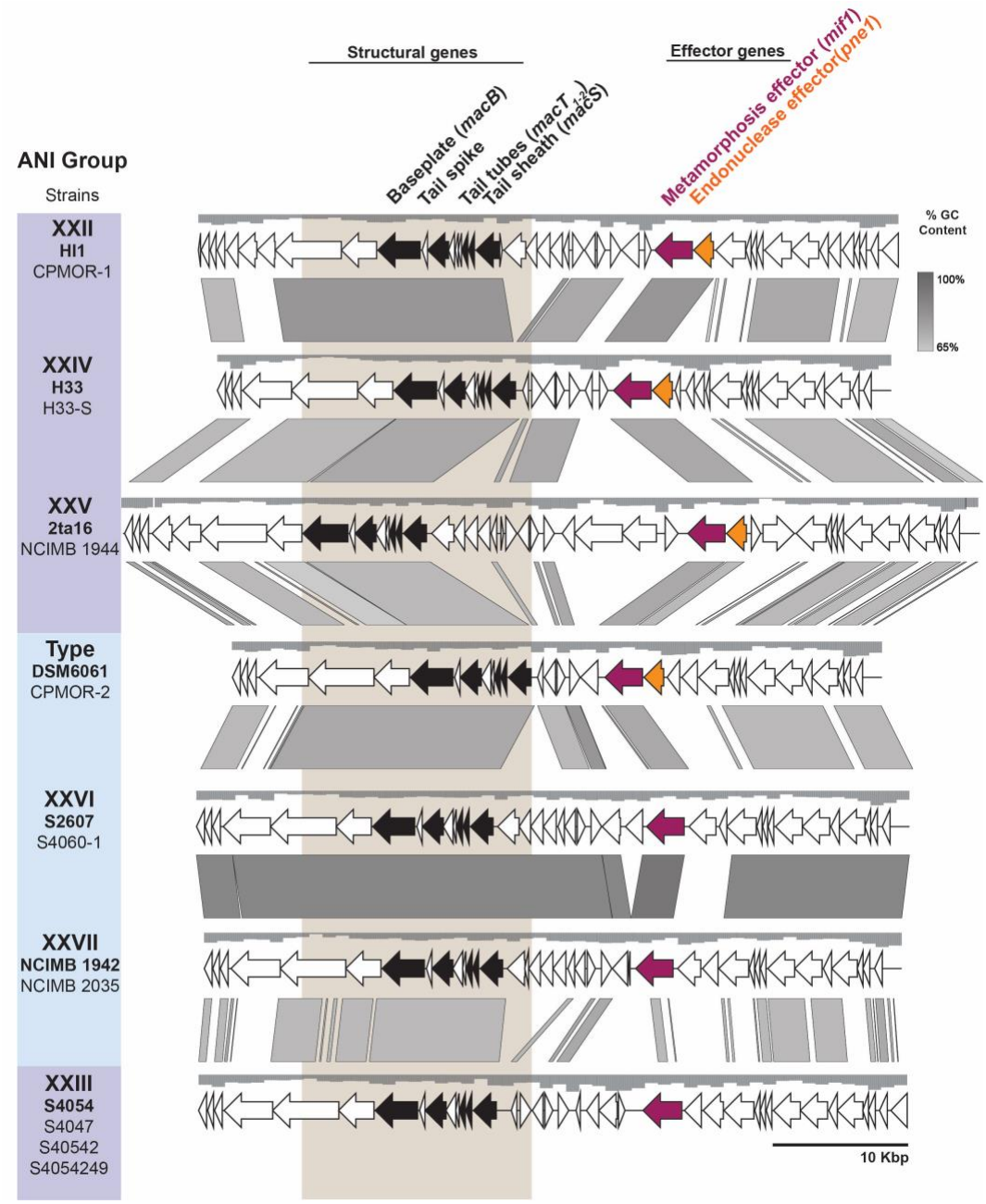

Figure 11. MACS gene synteny of sequenced strains of *P. luteoviolacea* show repeated loss of nuclease effector Pne1.

The P.luteoviolacea gene synteny was generated using EasyFig v2.2.2_OSX. The MACs gene cluster was searched by using the annotated HI1 gene cluster as the query and performing a tblastn against the sequenced strains of *P.luteoviolacea*. Subject hits on each matching contig were manually inputted into Easyfig to generate the alignment. The ANI group designations are adapted as previously described (Busch *et al.*, 2019). The black filled arrows represent experimentally important MACs structural genes within the brown background box highlighting the conserved structural genes across strains. The purple and orange arrows label previously described effector genes from the model strain Hi1.

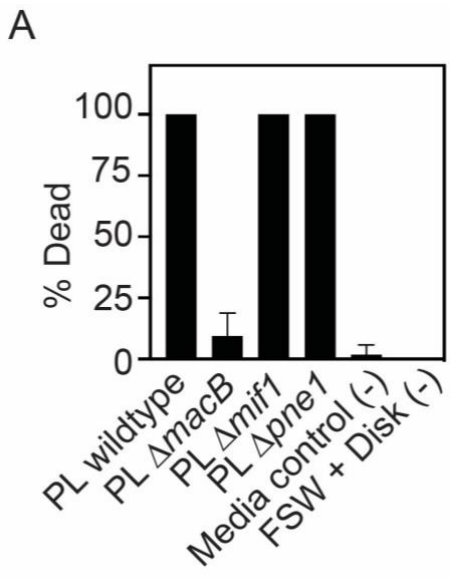

Figure 12. MACs effector knockouts do not change the outcome of *P.luteoviolacea*-induced death in corals. Coral metamorphosis assays performed on settlement fragging disk with brooding coral species *Porites astreoides*. Settlement disks were incubated for 24 hours in MB media. The assay was performed with the same batch of larvae and used the same conditions as document in Chapter 3. Data shown is a single biological replicate containing 6 technical replicates with 10 larvae per well. There may have been too high of a bacterial load for this assay, considering that other control strains did not perform well during this assay.

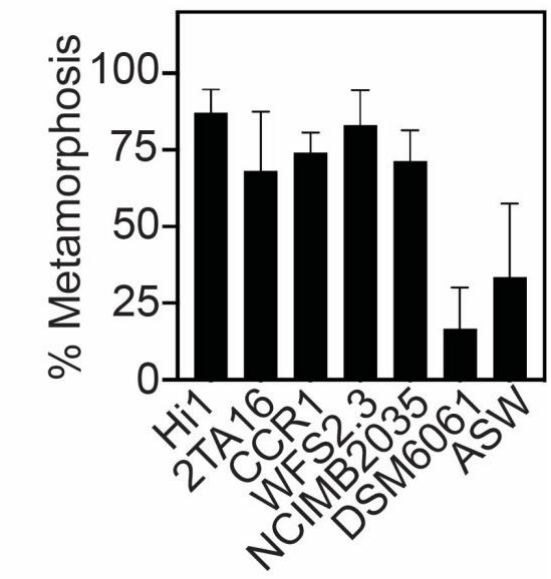

Figure 13. Diverse strains of *P.luteoviolacea* induce robust metamorphosis in *Hydroides elegans* with DSM6061 as an exception.

Different representative environmental isolates were isolated or collected as described above. These strains were used to perform a *Hydroides* biofilm metamorphosis assay as described previously in Chapter 2. The assay plotted is a single biological replicate of OD 0.2 concentration biofilms, however I was able to perform 2 biological replicates of OD 0.2 and OD 0.5 for this assay.

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CHAPTER 3: The genetic manipulation of *Pseudoalteromonas* sp. PS5 links tetrabromopyrrole biosynthesis genes to coral metamorphosis

3.1. ABSTRACT

Climate change and human impacts have resulted in a rate of coral reef decline that has outpaced mitigation efforts, which has recently culminated in a call for new interventions to save coral reefs. An important factor dictating coral fitness is the quality of microbial symbionts that reside on and within corals, which has led coral restoration biologists to employ techniques that manipulate the coral microbiome such as treatments with probiotic bacteria. A desirable quality of microbes that are currently being considered as coral probiotics are those that generate cues that stimulate larval settlement and metamorphosis. One of the best characterized molecules produced by bacteria that induces metamorphosis in a range of coral species is tetrabromopyrrole (TBP). While previous studies have shown that TBP purified from marine bacteria stimulates coral metamorphosis, the genetic tools have not been available in TBP-producing bacteria to link the TBP biosynthesis genes directly with the stimulation of coral metamorphosis. In this study, we establish the genetic techniques to manipulate the bacterium, *Pseudoalteromonas* sp. PS5, to explore TBP-induced metamorphosis in the hard coral *Porites astreoides*. We find that a deletion of the brominase gene, *bmp2,* disrupts TBP production in *Pseudoalteromonas* sp. PS5 and ablates the bacterium's ability to stimulate the metamorphosis of *P. astreoides* larvae. Our results attribute TBP production from live bacteria to the stimulation of metamorphosis in corals and bring us closer to realizing the use of genetically modified bacteria for studying and improving bacteria for use as coral probiotics.

3.2. INTRODUCTION

The health of coral reef ecosystems worldwide has been declining drastically for the past few decades, leading to a crescendo of warnings from scientists about the fate of coral reefs as

we know them. Since the 1950s, global coral reef coverage and the services provided by coral reef ecosystems have declined by half (Eddy *et al.*, 2021). A dire outlook on the future progression and rate of coral decline has led scientists to realize the limitations of passive conservation strategies, such as the regional implementation of no-take zones (Anthony *et al.*, 2017; van Oppen *et al.*, 2017). Integrated approaches combining passive strategies with active interventions that incorporate cutting edge technologies, such as assisted evolution, gene flow and synthetic biology have been proposed to help offset further coral reef decline (Anthony *et al.*, 2017; van Oppen and Blackall, 2019).

An important facet of coral reef health is the coral holobiont, defined as the community of microorganisms, including dinoflagellates, prokaryotes, viruses and fungi that live on and within the corals (Rohwer *et al.*, 2002). These associations are crucial for a number of evolutionary, developmental and ecological interactions (reviewed in (Thompson *et al.*, 2014)). While the consortia of microbes within the coral holobiont has been extensively studied thanks to advancements in culture-independent DNA sequencing technology (Rohwer *et al.*, 2002; Bourne *et al.*, 2016), many of the insights regarding microbial function remain hypothesized and still untested.

The coral probiotic hypothesis suggests that corals maintain a dynamic relationship with their holobiont depending on environmental conditions, allowing the coral to select for the most advantageous microbial community resulting in a fast-acting adaptive advantage for the corals to resist against threats like disease (Reshef *et al.*, 2006). One novel solution that combines the Coral Probiotic Hypothesis with strategies used in agricultural engineering is coral microbiome

manipulation, whereby Beneficial Microorganisms for Corals (BMCs) are isolated and screened for potential probiotic mechanisms (Peixoto *et al.*, 2017, 2021; Deutsch *et al.*, 2022). The uncultured fraction of coral-associated microbes remained a barrier to the diversity of BMCs until recently, where significant inroads towards consolidating the information about already isolated strains (Sweet *et al.*, 2021) and advancements in genomic and metabolomic screening(Deutsch *et al.*, 2022) have closed the gap significantly. Currently, probiotic bacterial strains are already being tested (Damjanovic *et al.*, 2019; Santoro *et al.*, 2021), and in some cases implemented in the field (Will Probiotics Save Corals or Harm Them? - Scientific American) to mitigate the decline of coral health. However, we lack functional data that supports the proposed beneficial mechanisms of coral-associated probiotic bacteria. One of the desirable characteristics among BMCs is their ability to produce products that promote the settlement of larvae and metamorphosis from larval to juvenile stages, which could be utilized in both environmental (Damjanovic *et al.*, 2017) and aquaculture settings (Chamberland *et al.*, 2017; Joseph Pollock *et al.*, 2017).

Some bacteria forming biofilms composed of a single species can induce coral metamorphosis (Negri *et al.*, 2001; Tran and Hadfield, 2011; Cavalcanti *et al.*, 2020). Bacterial species from the genus *Psuedoalteromonas* have been shown to stimulate coral metamorphosis and appear to be promising candidates for probiotic use (Negri *et al.*, 2001; Tebben *et al.*, 2011; Sneed *et al.*, 2014; Shikuma *et al.*, 2014). The strain *Pseudoalteromons* sp. PS5 was first isolated from the surface of *Paragoniolithon solubile,* a species of crustose coralline algae found on a fringing reef in near Looe Key, FL (Sneed *et al.*, 2014). *Pseudoalteromonas* sp. PS5 and related strains are capable of producing a compound called tetrabromopyrrole (TBP), which has been

associated with coral metamorphosis. Biofilms, fractionated extracts and synthesized TBP all induce robust metamorphosis in both Pacific (Tebben *et al.*, 2011; Busch *et al.*, 2019) and Atlantic (Sneed *et al.*, 2014) corals. Although TBP induces both attached and unattached metamorphosis phenotypes and is the subject of debate as an ecologically relevant signal (Tebben *et al.*, 2015), TBP has remained a consideration as a potential signal for coral development.

The brominated marine pyrroles/phenols (*bmp*) gene cluster was identified and characterized previously (Agarwal *et al.*, 2014) and the genes *bmp1-4* were determined to be important for TBP biosynthesis in *Pseudoalteromonas* sp. PS5 (Gamal *et al.*, 2016). The gene *bmp2* is a flavin-dependent halogenase capable of tetrabromination, via a novel decarboxylative bromination reaction demonstrated through total *in vitro* reconstitution (Gamal *et al.*, 2016). While *Pseudoalteromonas* sp. PS5 biofilms were shown to influence metamorphosis (Sneed *et al.*, 2014), it has not been shown whether the TBP biosynthesis genes are responsible for stimulating coral metamorphosis. The functional link between the *bmp* biosynthesis genes and coral metamorphosis has not yet been explicitly tested because (1) genetic manipulation techniques in a metamorphosis-inducing bacterium with the TBP biosynthesis genes has not been developed and (2) our ability to capture and rear coral larvae has become comprehensive and predictable only in the last decade (Marhaver).

3.3. RESULTS & DISCUSSION

To test whether *Pseudoalteromonas* sp. PS5 stimulates coral metamorphosis through the production of TBP, we set out to generate a genetically tractable strain lacking a key *bmp*

biosynthesis gene. We searched the sequenced *Pseudoalteromonas* sp. PS5 genome (Busch *et al.*, 2019) and identified the *bmp* gene cluster (Genbank accession KR011923) by blastn. Using conjugation to deliver a suicide plasmid we performed double homologous recombination resulting in an in-frame deletion that includes the first two and last three amino acids of the *bmp2* gene, thus generating a truncated *bmp2* knockout strain.

We next quantified the production of TBP from the *Pseudoalteromonas* sp. PS5 wild type and Δ*bmp2* strains using LCMS-MS. When grown in liquid media for 24h, *Pseudoalteromonas* sp. PS5 produces 1.47 $\mp$ .69 mM TBP in culture while a Δ*bmp2* mutant is unable to produce TBP (**Figure 14a, b**). When compared to a different *Pseudoalteromonas* species *Pseudoalteromonas luteoviolacea* that carries a homologous *bmp* gene cluster, we find that *Pseudoalteromonas* sp. PS5 produces 30x more TBP when using the same culturing, extraction and quantification conditions (Alker *et al.*, 2020). These results show that removing the *bmp2* gene from *Pseudoalteromonas* sp. PS5 stops TBP production and indicates that *bmp2* is the only brominase genes contributing to TBP biosynthesis in *Pseudoalteromonas* sp. PS5 under the conditions tested.

To determine whether bacteria lacking the ability to biosynthesize TBP are unable to promote coral metamorphosis, we then compared the ability for *Pseudoalteromonas* sp. PS5 wild type and Δ*bmp2* strains to stimulate the metamorphosis of *Porites astreoides* coral larvae. *P. astreiodes* larvae were chosen for this study because they brood larvae predictably and have been used as a model for metamorphosis in previous studies (Edmunds *et al.*; Sneed *et al.*, 2014). When exposed to *Pseudoalteromonas* sp. PS5 wild type, we observed the metamorphosis of

coral larvae, both attached to the substrate and floating (**Figure 14c**). Our observations are consistent with previous findings showing that purified TBP promotes both floating and attached larvae (Negri *et al.*, 2001; Tebben *et al.*, 2011; Sneed *et al.*, 2014; Tebben *et al.*, 2015). In contrast to the wild type, biofilms of the *Pseudoalteromonas* sp. PS5 Δ*bmp2* strain exhibited a significantly reduced ability to stimulate the metamorphosis of coral larvae. However, we did observe a small amount of metamorphosis even when coral larvae were exposed to the Δ*bmp2* strain. Our results suggest that the stimulatory effect of PS5 on coral metamorphosis is predominantly due to the production of TBP.

Our results establish the functional link between the presence of the *bmp* biosynthesis genes and the induction of coral metamorphosis by *Pseudoalteromonas* sp. PS5. While TBP may not be an ecologically relevant inducer of metamorphosis (Tebben *et al.*, 2015), strains that produce TBP may still be useful as probiotics. TBP was shown to have specificity towards the induction of metamorphosis in corals, while not eliciting robust metamorphosis in two other types of invertebrate larvae (Alker *et al.*, 2020). The mechanism of action and effect of causing both attached and unattached coral recruits may have significant implications for its usage as a probiotic, especially considering the evidence that TBP elicits phytoplankton mortality (Whalen *et al.*, 2018) and halts sea urchin development (Akkipeddi *et al.*, 2021). While many questions remain regarding how TBP induces metamorphosis in corals (Siboni *et al.*, 2012), the establishment of new genetic tools both on the bacteria side (this study) and the animal side (Cleves et al. 2018) will enable future studies aimed to determine the mechanism by which TBP induces metamorphosis.

3.4. CONCLUSIONS

The genetic manipulation of *Pseudoalteromonas* sp. PS5 provides a proof-of-concept that scientists can use genetically modified bacteria to test hypotheses about the mechanistic effects of probiotic bacteria on coral larvae or adults. Knowledge gained using such methodologies may ultimately help restoration managers make informed choices (van Oppen *et al.*, 2017) about active interventions for coral reef restoration. The ability to genetically manipulate probiotic bacteria opens the door to the production of enhanced strains with the ability to produce stimulatory products that amplify their probiotic effects for reef restoration and biotechnology applications.

3.5. METHODS

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in **Table 5**. *Pseudoalteromonas sp.* PS5 was cultured with natural seawater tryptone media NSWT for the cloning and Marine Broth 2216 (BD, Difco) for the metamorphosis assays. All PS5 cultures were incubated between 25-28C. *E.coli* were grown in LB media and cultured at 37C. All liquid cultures were inoculated with a single colony and incubated between 14-18 hours while shaking at 200rpm unless otherwise indicated. Plasmids were selected and maintained on LB Kanamycin 100µg/mL.

Cloning and generation of mutant strains

Primers used to generated strains in this study are listed in **Table 6**. The in-frame deletion was generated following a previously published protocol (Shikuma *et al.*, 2014). Briefly, Gibson primers were ordered from integrated DNA technologies (IDT) and were designed to amplify

1400 base pair homology arms up and downstream of the *bmp2* gene in *Pseudoalteromonas sp.* PS5. The homology arms were amplified using a high-fidelity DNA polymerase (Primestar, TaKaRa) and the resulting fragments were purified using a DNA Clean and Concentrator kit (Zymo Research). The suicide vector pCVD443 (Huang *et al.*, 2012) was digested with Sph1, XbaI and SacI. To assemble the digested plasmid and the PCR products, a three fragment Gibson Assembly was performed using the NEBuilder HiFi DNA Assembly Master Mix at a ratio of 2:1 for inserts:backbone vector. Resulting assemblies were diluted and electroporated into SM10 *pir* electrocompetent cells and selections were performed on LB Kanamycin 100µg/mL. Clones were PCR screened using p443_F and p443_R and positive clones containing a band around 3000 base pairs were cultured, miniprepped using the Zippy DNA Miniprep Kit (Zymo Research). Minipreps of the positive clones sent for were confirmed by Sanger sequencing (Eton Biosciences). The pCVD443_PS5Δ*bmp2* plasmid was conjugated with PS5 according to a previously published double homologous recombination protocol (Shikuma *et al.*, 2014). Selections were performed on NSWT Streptomycin/Kanamycin 200µg/mL and counter selections were performed on NSWT + 10% sucrose.

Coral collection and culturing

Reproductively mature colonies of *Porites astreoides* were collected three days before the new moon via SCUBA by the Mote Marine Laboratory (Summerland Key, FL) in June 2021. Coral colonies were placed in a flow-through seawater table within larval capture bowls. Water lines pushed overflow water containing larvae through the handle where the larvae were collected sieve buckets submerged in the table over the course of the night. Larvae were collected through the day after the new moon, and were pooled for experimentation. Larvae were

maintained in filtered natural seawater within clear polystyrene containers until use in experiments. Larvae selected for experiments were between 3 and 6 days old and were actively swimming.

Metamorphosis assay methods

Wildtype and mutant strains were struck out onto MB media and incubated overnight at 28C. The next day, single colonies were inoculated into 2mL culture and incubated with agitation at 150 rpm for 18 hours. The optical density of the cultures were measured and standardized to OD 0.5. Ceramic fragging disks (Bulk Reef Supply) were sterilized by autoclave and placed into each well of a sterile, untreated 6-well plate (Falcon). Each well of the plate contained 5mL of sterile Marine Broth and was inoculated with 100µL of OD 0.5 diluted culture. The plates were then incubated at 28C for 48 hours with slow agitation (35 rpm). The biofilmed disks were removed from the wells and rinsed under a steady stream of 0.2 filtered seawater to eliminate unattached cells. Biofilmed disks were then placed into 6 replicate deep petri dishes containing 60mL of 0.2 FSW. 10 larvae were added to each petri dish in 10mL, bringing the final volume of the petri dishes to 70mL. N=6. **Figure 14** represents one biological replicate.

HPLC methods

Quantification of TBP was performed as previously described (Alker *et al.*, 2020). Briefly, replicate cultures of PS5 were grown in 5mL overnight for 16 hours and extracted with 2x volume of ethyl acetate. Extracts were resuspended in 100µL methanol, filtered in a 0.2µm column and 10µL was injected into a Luna C18 reversed-phase analytical HPLC column (5µm, 250mm x 4.6 mm, Phenomenex; CA, USA). TBP was quantified on an Accurate Mass QtoF LC-

MS/MS (Agilent 6530 Accurate Mass), run at 0.5 ml/min in negative mode. The same solvent system of acetonitrile and water (+ 0.1% formic acid (v/v)), elution profile and quantification methods were followed as previously reported (Chekan *et al.*, 2019; Alker *et al.*, 2020)

Statistics

Data was plotted and analyzed using Prism v9 (Graphpad). Nonparametric statistics were performed on all data. The statistics for the biofilm metamorphosis assays were performed on the combined morphogenesis phenotype (attached and unattached) and a 2-tailed Mann Whitney test was performed to compare PS5 wildtype and PS5Δbmp2 strains ($p=0.0079$).

3.6. ACKNOWLEDGEMENTS

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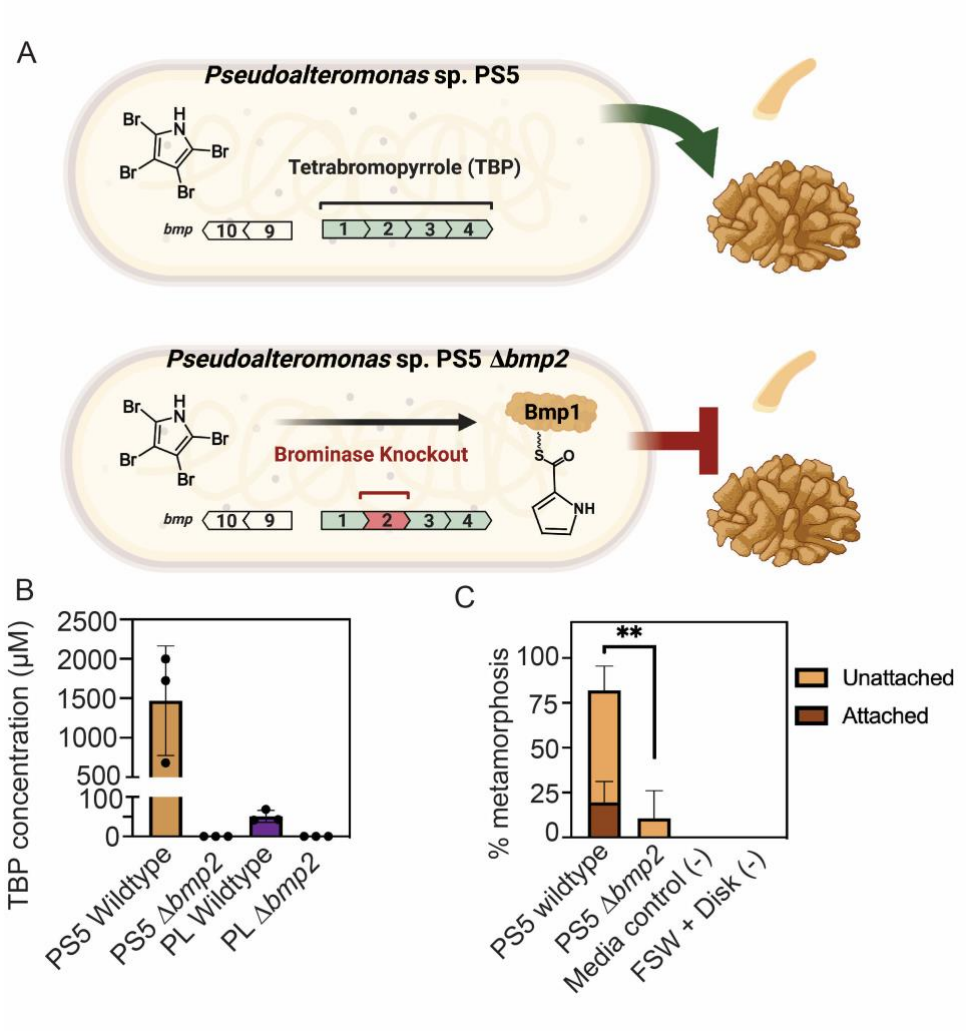

Figure 14. *Pseudoalteromonas* sp. PS5 strain lacking the brominase gene *bmp2* is unable to produce TBP and does not induce coral metamorphosis

(**A**) A model of the TBP biosynthesis gene cluster in *Pseudoalteromonas* sp. PS5 including *bmp*1-4 and *bmp*9-10 genes. The *bmp2* brominase gene is highlighted in red. (**B**) *Pseudoalteromonas* sp. PS5 produces 30-fold more TBP than *P. luteoviolacea* strain HI1 and mutation of the *bmp*2 gene ablates TBP production in both strains. Error bars represent standard deviation of the mean in plots B-C. (**C**) Metamorphosis (%) of *Porites astreoides* larvae in response to *Pseudoalteromonas* sp. PS5 wild type and Δ*bmp2* strains. Marine Broth 2216 growth media or Filtered Sea Water (FSW) alone are included as controls. Statistical significance was determined by a two-tailed Mann Whitney test where p=0.0079. N=6, 10 larvae/well.

Table 5. List of strains and plasmids used in Chapter 3

Strain No.	Strain	Genotype	Source
NJS 595	PS5 wildtype	*Pseudoalteromonas sp.* PS5	[Sneed et al. 2014]
NJS 597	PS5 StrR	*Pseudoalteromonas sp.* PS5, *StrR*	This study
NJS 602	PS5Δ*bmp2*	*Pseudoalteromonas sp.* PS5 Δ*Bmp2, Str*^{R}	This study
NJS 700	PS5Δ*bmp2::bmp2*	*Pseudoalteromonas sp.* PS5 Δ*Bmp2::F+ p402-PS5bmp1-bmp2c*	This study
NJS 703	PS5Δ*bmp2::bmp2*	*Pseudoalteromonas sp.* PS5 Δ*Bmp2::F+ p402-CP25-bmp2c-T7*	This study
NJS 641	PS5 GFP	*Pseudoalteromonas sp.* PS5*::F+ pBTK402-CP25-GFP-T7*	This study
NJS 702	PS5Δ*bmp2*::GFP	*Pseudoalteromonas sp.* PS5Δ*bmp2::F+ pBTK402-CP25-GFP-T7*	This study
Plasmid No.	**Plasmid**	**Genotype**	**Source**
pNJS 007	pCVD443	Amp^{R}, Km^{R}, sacB, pGP704 derivative	[Huang and Hadfield, 2012]
pNJS 677	pCVD443 PS5Δ*bmp2*	pNJS 007 including 1500 bp homology arms flanking PS5*bmp2*	This study
pNJS 710	pBTK107_PS5_*bmp1*	GGA compatible PS5 *bmp1* UTR type 2 promoter	This study
pNJS 747	pYTK034_PS5_bmp2c	GGA compatible PS5 *bmp2* type 3 CDS	This study
pNJS 636	pBTK402	RSF1010 *ori*, Km^{R}, pMMB67EH derivative	[Leonard et al. 2018]
pNJS 665	pBTK402 GFP dropout	GGA assembly with RSF1010 *ori*, Km^{R}, pMMB67EH derivative with 234 GFP dropout (inverted bsaI cut sites)	[Leonard et al. 2018]
pNJS 790	p402_PS5_bmp2c	GGA assembly with p402 GFP dropout-PS5_bmp1-bmp2c-T7 parts	This study
pNJS 791	p402_PS5_bmp2c	GGA assembly with p402 GFP dropout-CP25-bmp2c-T7 parts	This study

Table 6. List of primers used in Chapter 3

Primer	Sequence
p443_PS5_dbmp2_A1	GGTTAAAAAGGATCGATCCTCTAGACGAACCACCACATTCTCCTT
p443_PS5_dbmp2_B1	TGGGTAATTCCCTTAACTTGCGTTCATTACATTGCCACCTTATTTA
p443_PS5_dbmp2_C1	TAAATAAGGTGGCAATGTAATGAACGCAAGTTAAGGGAATTACCCA
p443_PS5_dbmp2_D1	CAACGTGAATTCAAAGGGAGAGCTCACGGCATGACTTGTCTACCC
PS5_dbmp2_seq_F1	CACCATTGCTTGAACTTGGT
PS5_dbmp2_seq_F2	AGGCTTTGGTTTGGTTGATG
PS5_dbmp2_seq_F3	AGCAGAAGCAGGTTCCGATA
PS5_dbmp2_seq_F4	ATCGCCGATATGGAAGTGAG
PS5_dbmp2_seq_R1	TGTGCCTCATTCCATTCAAA
PS5_dbmp2_seq_R2	CTGCCATTGGTCACATAGGA
PS5_dbmp2_seq_R3	TGCCTTTGACTCGTAGCGTA
pYTK034_PS5bmp2_gbsn_F	GGATAACCGTAGTCGGTCTCATATGAACGGATTTACACATTATGACG
pYTK034_PS5bmp2_gbsn_R	TTTTATTGGTCTGGTCTCAGGATTTAACTTGCCATTTGTTTACGG
PS5_bmp2_R1	GCATCCATATCCTCCGCTAA
pCVD443_F	CACTAAATAATAGTGAACGGCAGGT
pCVD443_R	CAAGACGTTTCCCGTTGAAT
pBTK107_PS5bmp1_promoter_gbsn_F1	ACCGTAGTCGGTCTCAAACGCGAACCACCACATTCTCCTT
pBTK107_PS5bmp1_promoter_gbsn_R1	GTTTTTTATTGGTCTGGTCTCACATAGCAGCACCTTCGAGTAGATCG

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CHAPTER 4: Broadening the genetic manipulation of diverse marine bacteria with a modular plasmid toolkit

4.1. ABSTRACT

Marine bacteria play significant roles in symbiotic and ecosystem-level processes in the sea. Although harnessing the genetic potential of these microbes could open numerous avenues for biotechnology, aquaculture and environmental restoration, many marine bacteria remain genetically intractable. A significant bottleneck in genetically modifying marine bacteria from diverse lineages is the broad variation in natural antibiotic resistances and genetic features that different bacteria require to stably replicate or integrate foreign DNA. A tangible solution to this problem is to rapidly create and iteratively test potential genetic modification strategies, which would allow for the identification of targeted tools that fit the requirements of specific species. In this work, we describe a Marine Modification Kit (MMK) to streamline a mix-and-match workflow to genetically modify marine bacteria. Specifically, we adapt existing and add new standardized genetic parts plasmids that can be assembled by golden gate assembly that facilitate fluorescent tagging, luminescence detection and CRISPR interference (CRISPRi) capabilities in species from two alphaproteobacterial lineages. To demonstrate the MMK's utility for studying host-microbe interactions, we perform live cell imaging during and after the stimulation of the metamorphic development of a marine invertebrate host. The MMK provides a strategy for unlocking our ability to genetically engineer diverse marine microbes, opening significant avenues for fundamental research and biotechnology applications in previously intractable marine microbes.

4.2. INTRODUCTION

The sheer diversity and abundance of microbes within the ocean remains as an untapped reservoir for genetic discovery and biotechnology(Paulsen *et al.*, 2019; Bech *et al.*, 2020;

Varrella *et al.*, 2020). This potential for discovery is exemplified by the fact that as much as 40% of the ocean's core protein functions remain unknown(Sunagawa *et al.*, 2015). Harnessing marine microbes for their unexplored functions serves as the bedrock of rapidly developing microbial biotechnology ventures, including bioprospecting for natural product discovery(Lozada and Dionisi, 2015; Paulsen *et al.*, 2019), the aquaculture of critical food stock(Prado *et al.*, 2010; D'Alvise *et al.*, 2012), probiotic treatments for ecological restoration(Peixoto *et al.*, 2017) and green energy initiatives(Nozzi *et al.*, 2013; Kracke *et al.*, 2015). Considering that marine microbes play critical roles in the origins of life on Earth through their involvement in biogeochemical cycles(Madsen, 2011) and symbioses with eukaryotes(Mcfall-ngai *et al.*, 2013), we have much to learn about their biology and mechanistic potential for biotechnology.

An era of metagenomic sequencing has opened the door to explore the diversity and functional potential of microbial life in the ocean (e.g. the Global Ocean Sampling Expedition (Gross, 2007; Yooseph *et al.*, 2007), Earth Microbiome Project(Thompson *et al.*, 2017) and Tara Oceans(Sunagawa *et al.*, 2015)). However, a significant limitation with the massive and growing volume of sequencing information is a lack of comparable genetic tools to elucidate protein function and genetic mechanisms that promote the interaction between microbes and their environment. While we are able to make predictions about marine microbial gene products, physiology and behavior, many of these predictions are based on data using model organisms (e.g. *Escherichia coli*, *Saccharomyces cerevisiae*) that may be taken out of context. As a result, these predictions often remain untested due to a lack of relevant and tractable microbial systems. While whole genome sequencing has become faster and more affordable, synthetic biology in

this field remains expensive, time-consuming and largely limited to model strains of bacteria. As the fraction of ecologically relevant cultured strains increases and become more accessible (Sweet *et al.*, 2021), it is imperative that there are comparable genetic tools and open access methodologies to explore the functionally important marine microbes.

Plasmid cloning is a historically time-consuming process, recent advances in synthetic biology revolutionized our ability to engineer model microbial species. One effective genetic engineering approach that significantly reduced cloning time was the standardization of molecular cloning systems, such as the BioBrick(Shetty *et al.*, 2008), which generated plasmid parts based on the ordered pairings of Type II restriction enzymes. As the standardization movement gained traction, advancements in cloning techniques based on Golden Gate technology(Engler *et al.*, 2008) enabled scarless assembly in a single tube reaction. The Golden Gate Modular Cloning Kit (MoClo), designed for the transformation of plants, utilized a set of vector parts which contained flanking Type IIS recognition sites. Functional vector parts with overlapping 4bp overhangs are seamlessly ligated together to generate a transcriptional unit vector(Engler *et al.*, 2014). Single transcriptional units can now be linked with two or more units, generating recursive options to build genetic circuits(Pollak *et al.*, 2019). When standardized vectors are combined in a predictable manner as modular components, they enable innumerous mix-and-match assembly possibilities that allow for rapid experimentation.

Standardized genetic cloning systems were first restricted to a small number of relatively well definedl chassis for synthetic biology such as *E. coli* and plants. Recently, the Yeast Tool Kit (YTK), Bee Tool Kit (BTK) and CyanoGate were developed to address deficiencies in

synthetic biology tools for microbial, eukaryote and animal microbiome bacteria systems(Lee *et al.*, 2015; Leonard *et al.*, 2018; Vasudevan *et al.*, 2019). New standardized molecular cloning tools in non-model microbes provide a framework for experimentally testing previously untestable questions in diverse microbes. Although unlocking the ability to genetically engineer diverse marine microbes would open significant avenues for fundamental research and biotechnology applications, the vast majority of microbial taxa from the ocean remain genetically intractable.

To explore the tractability of various bacteria, we isolated or acquired a collection of strains thought to be important their critical role in the symbioses with marine plants or animals in the ocean. For example, *Vibrio* species are critical symbionts in light organ development of the Hawaiian bobtail squid(Nyholm and McFall-Ngai, 2021; Visick *et al.*, 2021) and are pathogens of corals(Ushijima *et al.*, 2016), shrimp and fish (Zhang *et al.*, 2020). *Pseudoalteromonas* species produce diverse secondary metabolites that could be harnessed for biotechnology purposes(Maansson *et al.*, 2016; Paulsen *et al.*, 2019; Alker *et al.*, 2021) and stimulate the metamorphosis of animal larvae, such as tubeworms that promote biofouling of ship hulls(Shikuma *et al.*, 2014) and corals that build reef ecosystems(Sneed *et al.*, 2014). *Endozoicomonas* species are globally distributed and are often the most abundant bacterial symbionts in certain species of corals and other marine hosts, but their functional roles in the coral holobiont largely remain restricted to genomic predictions(Neave *et al.*, 2016, 2017; Pogoreutz *et al.*, 2018). *Roseobacter* group strains, compose up to 20% of bacterial communities in some marine environments (Moran *et al.*, 2007) and have been shown to control the microbial community structure on marine diatoms and selectively target specific cell types of

phytoplankton *Emiliania huxleyi* (Bramucci *et al.*, 2018; Dittmann *et al.*, 2019; Majzoub *et al.*, 2019). Despite the critical and diverse roles marine bacteria have been shown to play in ocean symbioses and ecosystems, we currently lack a standardized genetic system for the manipulation of marine bacteria.

In this work, we describe a Marine Modification Kit (MMK) that adds to the standardized genetic parts from prominent platforms like the YTK and BTK for use in a range of marine bacteria. We demonstrate the functionality of the MMK in marine bacteria that perform important symbiotic functions with marine plants or animals. We demonstrate that a number of previously tractable and intractable species stably carry broad host range plasmids and express fluorescent proteins and nanoluciferase genes. Specifically, we manipulated 10 marine strains across 2 proteobacterial classes, 4 orders and 7 genera. With the MMK, we quantify and compare the expression from promoters driving genes that are key for host-microbe interactions. We show that CRISPR interference (CRISPRi) can be used in *Pseudoalteromonas* bacteria to knock down secondary metabolite gene expression. Finally, we use the MMK to perform live cell imaging of *Leisingera* bacteria present within the gut of the biofouling tubeworm *Hydroides elegans*, a discovery that has significant implications for the process of bacteria-stimulated metamorphosis.

4.3. RESULTS & DISCUSSION

A modular plasmid toolkit for genetic engineering of marine bacteria

The MMK system utilizes and builds upon the standardized parts and golden gate assembly principles from BTK and YTK platforms, allowing integration with parts available from both toolkits (**Figure 15**). Stage 0 plasmids include Type-1 and Type-5 connector parts for

Stage 2 assembly, a Type-2 promoter part with ribosome binding site (RBS), Type-3 protein coding sequence (CDS) part, Type-4 terminator part, Type-6 repressor part, Type-7 promoter with RBS part and a Type-8 backbone part. Standardized Stage 0 parts are combined into Stage 1 plasmids via golden gate assembly to create a functional unit. Multiple stage 1 plasmids may be combined together into Stage 2 plasmids as described in BTK and YTK systems (Lee *et al.*, 2015; Leonard *et al.*, 2018). Importantly for the purposes of engineering diverse marine bacteria, specific parts with antibiotic resistance markers and origins of replication may be combined into Stage 1 or Stage 2 plasmids that are compatible with the target marine bacterium based on natural properties such as antibiotic susceptibility. In this work, we focus on the genetic engineering of marine bacteria. However, the tools described could in principle be applied to a range of bacteria outside of the ocean as long as they are amenable to genetic transformation.

Diverse marine bacteria stably replicate Stage 1 plasmids and express fluorescent proteins

To determine whether specific proteobacterial species are amenable to genetic manipulation using a standardized molecular cloning system, we first screened for natural antibiotic susceptibility to three commonly used antibiotics (kanamycin, gentamycin, streptomycin). We observed that many marine bacteria are susceptible to at least one of the antibiotics tested (Table X) and might therefore be amenable to antibiotic selection after conjugation of modular plasmids.

Type-8 origin of replication parts from the BTK system utilize broad host range plasmids containing an RSF1010 origin of replication for the conjugative transfer and stable replication into the marine bacteria of interest. To test whether alpha and gamma proteobacterial species are

amenable to conjugation and stable replication of existing BTK and YTK plasmids, we assembled Stage 1 plasmids comprised of a Type-2 broad host range CP25 promoter, Type-3 GFP or mRuby protein coding sequence (CDS), Type-4 terminator, Type-1 and Type-5 connector parts, with the Type-8 RSF1010 backbone. These Stage 1 plasmids were tested for their ability to be conjugated into a set of marine species. We observed conjugation and multiple variants that express GFP-optim-1, mRuby or Nanoluciferase were generated in numerous Alpha- and Gammaproteobacteria species (**Figure 16a, b**). Fluorescence was variable between strains as we observed differences in the uniformity of expression among bacteria within the same genus (i.e. *Pseudoalteromonas luteoviolacea* and PS5). These findings suggest that it is important to characterize promoter efficacy on a strain-level basis.

CRISPRi knockdown of secondary metabolite expression in Pseudoalteromonas luteoviolacea

Pseudoalteromonas species are known for their ability to produce diverse secondary metabolites and their ability to stimulate animal metamorphosis. To demonstrate the compatibility of the MMK system in studying marine bacteria, we tested whether *Pseudoalteromonas luteoviolacea* is susceptible to CRISPR interference (CRISPRi) (Qi *et al.*, 2013) within the BTK standardized molecular cloning framework (Leonard *et al.*, 2018). We targeted well-characterized gene clusters important for Violacein(Balibar and Walsh, 2006) (*vioA*) and MACs(Shikuma *et al.*, 2014) (*macB*) production in *Pseudoalteromonas*. To ensure that the sgRNA part was constitutively expressed in *Pseudoalteromonas*, we added a ptac promoter. We then designed an ptac-sgRNA part plasmid targeting the non-template strand of the *vioA* and *macB* genes in the coding sequence near the transcription start site. To increase the assembly efficacy, we added an Ampicillin antibiotic resistance gene *bla* to the pBTK614 dCas9

part, thus increasing plasmid assembly efficiency via dual selection with kanamycin and ampicillin. Knockdown of *vioA* resulted in visible absence of the purple pigment associated with violacein production on the plate (**Figure 17a**) and in culture (**Figure 17b).** Overnight cultures were ethanol extracted and the production of violacein was quantified by an absorbance reading at 580nm. The CRISPRi-*vioA* construct reduced violacein production comparable to an in-frame deletion of *vioA* (**Figure 17b**). Compared to a *P.luteoviolacea* CRISPRi-GFP plasmid-containing control, violacein production was significantly reduced (p=0.0007). However, it is important to note that expression of the plasmid seems to affect the rate of growth in *P.luteoviolacea*, highlighting the importance of including comparable plasmid-containing controls.

Bacteria that promote tubeworm metamorphosis are present within the gut of juvenile animals

To showcase the utility of the MMK platform in host-microbe interactions, we tested whether a set of marine bacteria with MMK plasmids expressing fluorescent proteins would be able to stimulate the metamorphosis of the tubeworm, *Hydroides elegans*. Indeed, *Pseudoalteromonas luteoviolacea* strain HI1 has been previously demonstrated to stimulate the metamorphosis of *Hydroides*(Ericson *et al.*, 2019), and was able to stimulate metamorphosis while carrying the MMK plasmid. Additionally, two *Roseobacter* species that have not previously been shown to stimulate animal metamorphosis, *Leisingera* sp. 204H and *P. gallaeciensis* ATCC 700781, were able to stimulate the metamorphosis of *Hydroides* larvae (**Figure 18**). These results substantiate the use of the MMK system in marine bacteria during a host-microbe interaction.

To test whether marine bacteria harboring MMK plasmids with fluorescent proteins are amenable to live cell imaging during a host-microbe interaction, we created microcosms containing biofilms of *Leisingera* sp. 204H or *P. gallaeciensis* ATCC 700781 bacteria and added competent *Hydroides* larvae. After incubation of bacteria and larvae for 24 hours, biofilms of *Leisingera* sp. 204H or *P. gallaeciensis* ATCC 700781 bacteria were clearly visible when strains expressed *gfp* or *mRuby* from respective MMK plasmids and visualized by fluorescent microscopy (**Figure 18**). As suspected, the bacteria and their biofilms were difficult to visualize by light microscopy without fluorescence expressing bacteria(Vijayan *et al.*, 2019)(**Figure 18c**). Many *Hydroides* larvae had undergone metamorphosis into juvenile animals on the biofilmed surface. Intriguingly, fluorescent *Leisingera* sp. 204H or *P. gallaeciensis* ATCC 700781 bacteria could be prominently observed within the gut of *Hydroides* juveniles (**Figure 18a,b**). These results provide a proof-of-concept that the MMK system provides experimental tools for observation and experimentation of marine host-microbe interactions.

Whether and how bacteria and the animal are harmed or benefit from bacteria-stimulated metamorphosis remains a prominent question in the field(Aldred and Nelson, 2019; Freckelton and Nedved, 2020; Shikuma, 2021). Live bacteria within the gut of *Hydroides* juveniles have never been observed previously. The presence of bacteria within the gut of *Hydroides* juveniles opens a possibility that *Rhodobacterales* bacteria might benefit from stimulating *Hydroides* to undergo metamorphosis because they can later occupy the tubeworm's digestive tract. Previous work by Gosselin et al. have shown that *Hydroides* is able to feed on bacteria as the sole food source(Gosselin and Qian, 2000). Our present observation that *Hydroides* juveniles ingest marine bacteria substantiates these findings and suggest that *Hydroides* might undergo metamorphosis in

response to some bacteria because they might provide a source of food. Advancements in metaparental mating (Cuív *et al.,* 2015) combined with the resources developed in this study open the possibility of characterizing native *Hydroides* gut symbionts and their potential physiological and ecological importance.

4.4. CONCLUSIONS

The current modular platform for genetic engineering brings us new abilities to uncover genetic function of marine bacteria and harness marine microbes for applied purposes. In this study, we characterize features of previously described bacteria that serve as models for marine symbiosis (i.e. *Pseudoalteromonas*) and make key symbiosis bacteria tractable for the first time (i.e. *Leisingera*, *Endozoicomonas* and *Nereida*). The tools and methods described in this study serve as a basis for the genetic manipulation of marine bacteria and provide a framework to characterize new and important protein functions and underlying molecular mechanisms in developing areas such as probiotic development, aquaculture, bioprospecting, and fundamental research.

4.5. METHODS

All cloning and MMK protocols used in this study can be accessed on the Shikuma Lab SDSU protocols.io page.

Bacterial Culture

A list of strains used in this study, their isolation sources, accession numbers, and their minimum inhibitory concentration can be found in Table X. Environmental strains of marine bacteria were isolated and cultured on Marine Broth 2216 (Difco) and seawater tryptone media. The marine bacteria were incubated at 25ºC, and cultures were shaken at 200rpm. Antibiotic

selections *E.coli* SM10pir and S17-1pir were cultured in LB at 37ºC, shaking at 200rpm. *E. coli* MFDpir (Ferrieres et al. 2010) was cultured in LB supplemented with 0.3mM Diaminopimelic acid (DAP). For *E.coli,* antibiotic selections with Ampicillin, Kanamycin, Chloramphenicol were performed using a concentration of 100µg/mL.

Plasmid construction & Assembly

New plasmid parts were made using PCR-amplified fragments and Gibson Assembly. The list of new, BTK and YTK plasmid parts used in this study is available in Table XX. Fragments were then assembled using Golden Gate Assembly and either BsaI or BsmBI, depending on the construct. The kanamycin backbone assemblies were electroporated into S17 cells and shuttled to MFD cells for conjugation. The CRISPRi assemblies were electroporated into SM10 cells and shuttled to MFD cells before conjugation.

Biparental conjugation in marine bacteria

Donor strains of *E.coli* (MFD *pir* or SM10 *pir*) containing the mobilizable plasmids were grown under antibiotic selection in LB with the appropriate supplements (including 0.3mM DAP for *E.coli* MFD *pir)*. The biparental mating was performed as previously described (Leonard et al. 2018) with modifications for the marine bacteria. Several colonies of the recipient strains were inoculated and grown overnight in liquid culture. Recipient and donor cultures were spun down (4000 x g for 2 minutes) in a 1:1 OD ratio. All donor supernatant was removed leaving only the cell pellet. All but 100µL of the recipient supernatant is removed and the cell pellet is resuspended. The recipient suspension is transferred to the donor pellet, which is resuspended with the recipient cells. Two 50µL spots are plated onto Marine broth media (supplemented with

0.3mM DAP for MFD). Spots are resuspended in liquid media and plated onto Marine Broth media containing kanamycin (300µg/mL) (unless otherwise noted in Table X).

Violacein extraction

P.luteoviolacea containing the CRISPRi plasmid targeting the *VioA* gene was struck onto NSWT media containing 200µg/mL of Streptomycin and Kanamycin and incubated overnight at 25C. Single colonies were inoculated into 5mL of liquid media containing the same antibiotic concentrations. Cultures were incubated at 25C, shaking at 200rpm between 18 and 20 hours. Cultures were removed from the incubator and standardized to an OD of 1.5. The cells were then pelleted and the supernatant was removed. The cell pellet was resuspended in 200µL of 100% ethanol. The resuspended cells were pelleted and the supernatant containing the crude extract was recorded on a Biotek Synergy HT plate reader (Vermont, USA) using the Gen5 program (v2.00.18) with an endpoint reading at 580nm.

Microscopy

Microscopy was performed using a Zeiss Axio Observer.Z1 inverted microscope equipped with an Axiocam 506 mono camera and Neofluar10x/0.3 Ph1/DICI (*Hydroides* co-cultures) or Apochromat 100x/1.4 Oil DICIII (bacteria only) objectives. The Zeiss HE eGFP filter set 38 was used to capture GFPoptim expression and Zeiss HE mRFP filter set 63 was used to capture mRuby2 expression. For Nanoluciferase controls, images were captured using the same fluorescence exposure times as the GFPoptim and mRuby2 labeled strains of the same species. Bacterial culture (2 µl) were added to 1% saltwater low-melt agarose (Apex, Bioresearch products) pads on glass slides and coverslips were placed on top. *Hydroides elegans* with

bacteria were imaged in the visualization chambers (Lab-Tek Chambered Coverglasses catalog #155411PK) they were prepared in.

Phylogeny

Strains or close representative strains used in this study were compiled into a genome group on PATRIC v3.6.12 (Wattam *et al.*, 2017). A whole genome phylogenetic codon tree composed of 100 single copy genes (Davis *et al.*, 2016) was performed using the Phylogenetic Tree Service (Edgar, 2004; Cock *et al.*, 2009). A Maximum liklihood phylogeny was generated using the best protein model found by RaxMLv8.2.11 (Stamatakis, 2014) which was LG. Bootstraps were generated using the rapid bootstrapping algorithm (Stamatakis *et al.*, 2008). The tree was visualized with FigTree v1.4.4. The tree was rooted at the mid-line.

Hydroides elegans culture

Hydroides elegans adults were collected from Quivira Basin, San Diego, California. The larvae were cultured and reared as previously described (Nedved and Hadfield 2008, Shikuma et al 2014). The larvae were maintained in beakers containing filtered artificial seawater (35 PSU) and were given new beakers with fresh water daily. The larvae were fed living *Isochrysis* daily. The larvae were used for metamorphosis assays once they reached competency (between 5 and 7 days old).

Metamorphosis assays

Biofilm metamorphosis assays were performed using previously described methods (Huang and Hadfield 2003, Shikuma et al. 2014, Alker et al. 2020). Bacteria were struck onto

Marine Broth plates and incubated overnight at 25C. Up to 3 single colonies were inoculated into MB broth and incubated overnight (between 15 and 18 hours), shaking at 200rpm. Cultures were pelleted at 4000g for 2 minutes, the spent media was removed and the cell pellets were washed twice with filtered ASW. The concentration of the cells was diluted to OD600 of 1 and four 100µL aliquots of the cell concentrate were added to 96-well plates. The cells were given between 2 and 3 hours to form biofilms, then the planktonic cells were removed and the adhered cells were washed twice with filtered ASW. Between 20 and 40 larvae were added to each well in 100µL of filtered ASW. Metamorphosis was scored after 24 hours. Four biological replicates were performed on different days using separately spawned batches of larvae.

Chambered metamorphosis assays were performed using the same preparation principles as described above with few modifications. Visualization chambers (Lab-Tek, Cat# 155411) were used for setting up the metamorphosis assay, then subsequently imaged. Inductive strains containing constitutively expressed GFP/mRuby/nanoluc plasmids were struck out onto MB media containing 300µg/mL Kanamycin. Several colonies were inoculated into 5mL MB media with antibiotics. Cells were washed and allowed to form biofilms as described above. Cell concentrations ranging between OD 0.2 and OD 1 were used to elicit optimal metamorphosis depending on the bacterial species being probed for colonization. Larvae were concentrated and the resident filtered ASW was treated with 300µg/mL Kanamycin. Larvae were imaged 24 hours later.

4.6. ACKNOWLEDGEMENTS

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dissertation author is the primary investigator and author of this material.

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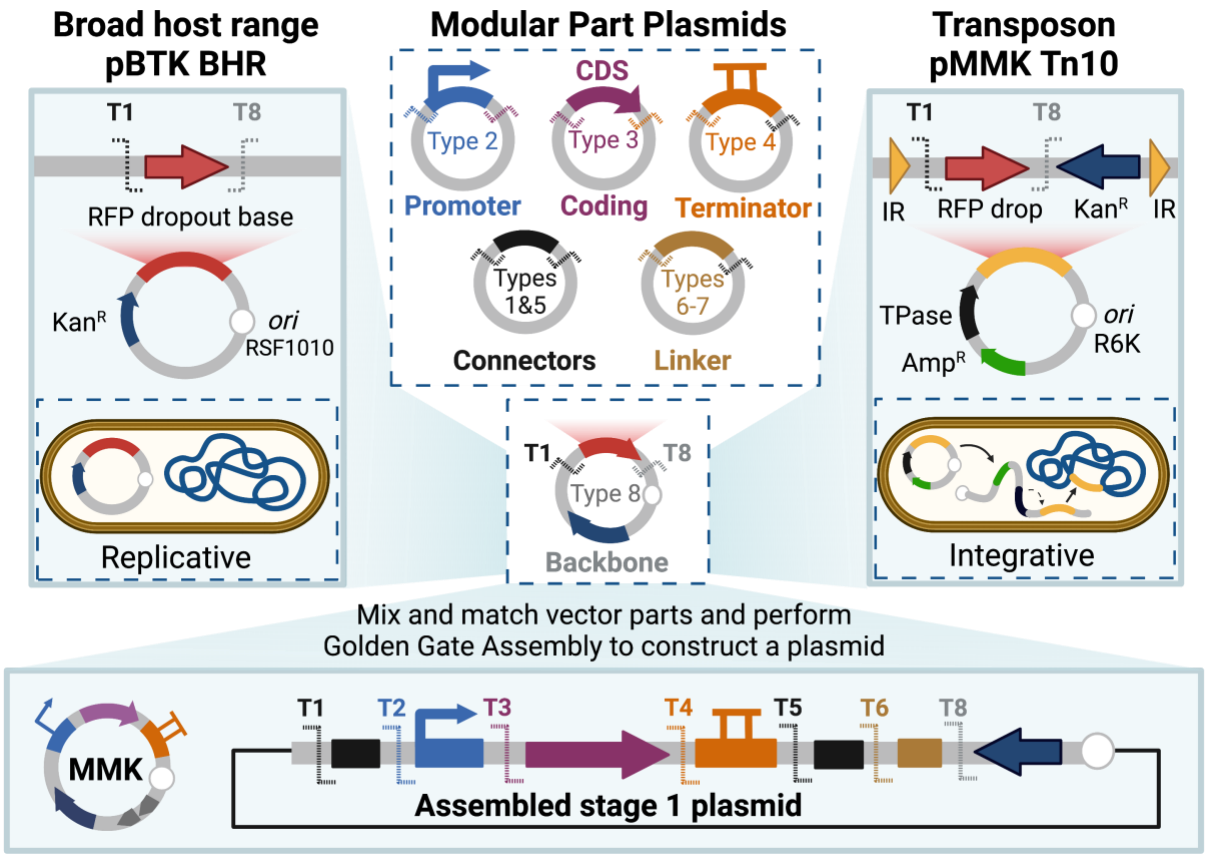

Figure 15. Schematic overview of the modular MMK system and integration into diverse bacteria for experiment testing.
The MMK compatible plasmids can be bsaI digested to generate ordered 4bp overhangs. Functional plasmid parts (backbone, promoter, coding sequence or terminators) were selected from the BTK and YTK libraries or were generated (i.e. ptac, macS, and macB promoters). Golden gate assembly was performed on the backbone and insert plasmids in a one-tube reaction.

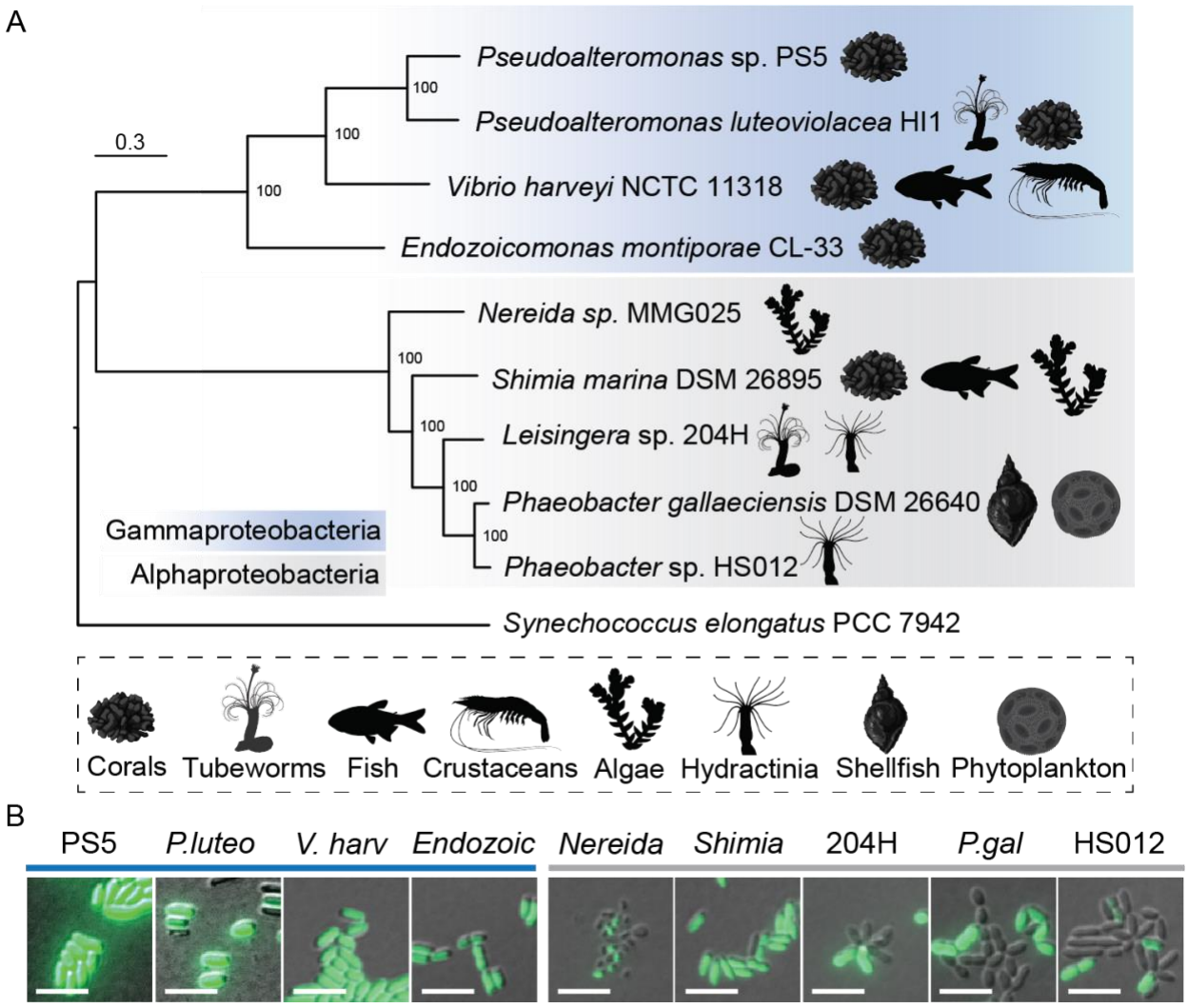

Figure 16. Diversity of strains amenable to plasmid uptake and stably replicate RSF1010 *ori* plasmids. (**A**) Maximum likelihood whole genome phylogeny of strains selected for manipulation in this study. The Gamma proteobacteria strains are in the purple box and the orange box shows the Alpha proteobacteria strains. *Synechococcus elongatus* serves as the outgroup and is not manipulated in this study. Scale bar is 0.3 and bootstraps were generated using the rapid-bootstrapping method(Stamatakis *et al.*, 2008). (**B**) Fluorescence microscopy of overnight cultures containing constitutively expressed RSF1010 *ori* fluorescence vectors (CP25-GFP-T7, CP25-mRuby-T7). Scale bar is 5µm.

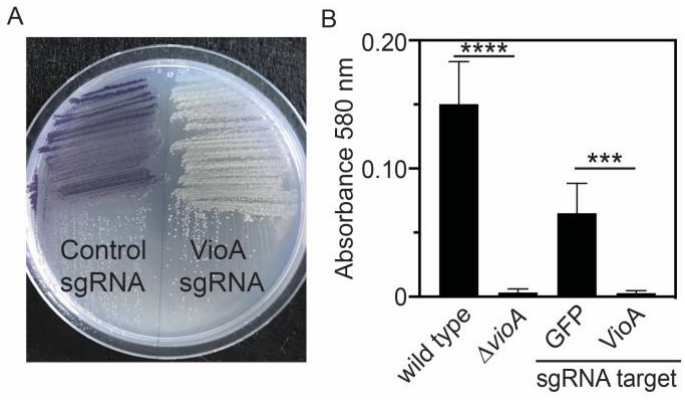

Figure 17. CRISPRi reduces gene expression in *Pseudoalteromonas*
(**A**) Agar plate of *P.luteoviolacea* comparing the control (sgRNA-GFP) to the violacein knockdown (sgRNA-VioA5). (**B**) Quantification of Violacein extracted from overnight cultures of *P.luteoviolacea* containing a gfp control sgRNA plasmid versus an sgRNA targeting VioA. Absorbance was measured at 580 nm. N=8. Error bars represent standard deviation. Statistical significance was determined by a Brown-Forsythe and Welch's ANOVA with Dunnetts multiple comparisons.

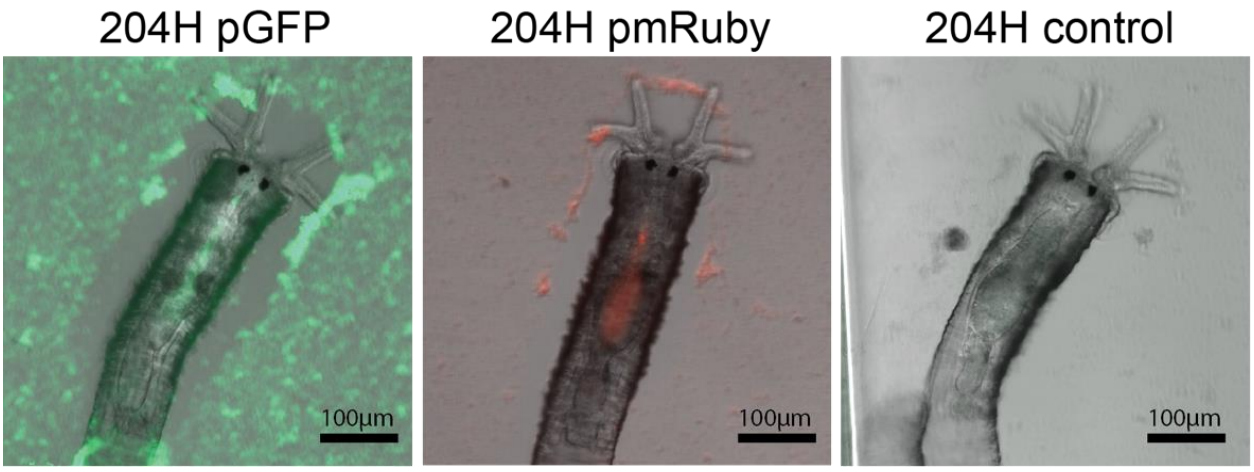

Figure 18. *Roseobacter* species stimulate *Hydroides* metamorphosis and are present within the gut of juvenile tubeworms

Fluorescence microscopy of juvenile *Hydroides elegans* imaged 24 hours after the larvae were exposed to inductive biofilms of *Leisingera* sp. 204H containing constitutively expressed (**A**) CP25-*gfp* (**B**) CP25-*mRuby* and (**C**) CP25-Nanoluciferase. Scale bar is 100µm. Nanoluciferase expressing plasmids were used as the negative control to minimize autofluorescence documented in the tubeworms.

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