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

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Summary

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.

Introduction

The free-swimming larvae of many marine invertebrates must settle and undergo metamorphosis to continue their life cycle as adults on the seafloor. 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 invertebrate animals undergo metamorphosis in

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Author Contributions

A.T.A., N.D., B.S.M., and N.J.S., participated in the study design. A.T.A. and N.J.S. created the mutant strains and drafted the manuscript. A.T.A. performed the *Hydroides* and *Hydractinia* metamorphosis assays, and the phylogeny. N.D. developed the methodology and helped with the *Hydractinia* metamorphosis assays. A.T.A. and T.N.P. prepared and processed LC–MS/MS samples. T.N.P. analysed the LC–MS/MS data. B.S.M. supervised and funded the biochemical analyses. N.J.S. supervised the project and funded the genetics and metamorphosis assays. All authors edited the manuscript.

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). Several bacterial factors responsible for inducing metamorphosis have been identified in diverse bacteria and include 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, 1993; Guo *et al.*, 2019). However, only two factors 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 metamorphosis-inducing factor 1 (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, 2015; Sneed *et al.*, 2014) 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, 2015; Sneed *et al.*, 2014). Although TBP was proposed as a compound of importance for coral re-seeding 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 in *P. luteoviolacea* using bacterial genetics and functional mutants (Huang *et al.*, 2012; Shikuma *et al.*, 2014). MACs are a syringe-like complex thatisevolutionarily 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 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 *Pseudoalteromonas 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 TBP (Agarwal *et al.*, 2014). Strains H2 and A316 induce coral (Tran and Hadfield, 2011) and sea urchin metamorphosis (Huggett *et al.*, 2006) respectively by a yet uncharacterized bacterial factor. *P. luteoviolacea* strain 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). These examples demonstrate 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, 2008) 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, 1993; 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.

Results

Many P. luteoviolacea *strains encode both TBP and MACs genes*

Pseudoalteromonas 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) (Fig. 1A). The MACs genes were blasted against all 19 complete and draft genomes of *P. luteoviolacea* available from GenBank (Table S1) 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*) (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 (Fig. 1B). All *P. luteoviolacea* strains queried possessed MACs gene homologues with significant homology to essential MACs genes from strain HI1 (represented by purple arrows in Fig. 1B and listed in Table S1). 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 (Fig. 1B) 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) (Fig. 2A). 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. *Pseudoalteromonas 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 (Fig. 2A; blue), shown previously to be required for TBP production (Agarwal *et al.*, 2014; Gamal *et al.*, 2016), the *macB* gene (Fig. 1B; 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 S2). Using QToF LC–MS/MS (Agilent 6530 Accurate Mass; CA, USA) we determined that *P. luteoviolacea* strain HI1 produces TBP (Fig. 2B and Fig. S1a), and a mutation in the *bmp2* gene abrogated TBP biosynthesis (Fig. 2B). 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 (Fig. S1b). This finding suggests that there are no other active brominases responsible for the production of TBP in *P. luteoviolacea* under the conditions tested.

Pseudoalteromonas 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, 2015; Sneed *et al.*, 2014). 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. The development of *Hydractinia* larvae after exposure to TBP or bacteria was quantified after 72 h and scored positively for metamorphosis if they developed stolons and tentacles (Fig. 3A). Upon exposure to a range of TBP concentrations, *Hydractinia* did not undergo metamorphosis, and at the highest tested concentrations (1000 and 750 nM) TBP was lethal (Fig. 3B; Table S3). 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 (Fig. 3C; Table S3). Similarly, the biofilms of Δ*bmp2* resulted in the mortality of larvae. Interestingly, both mutants that lacked functional MACs (Δ*macB* and Δ*macb*Δ*bmp2*) stimulated the metamorphosis of *Hydractinia* larvae (Fig. 3C; Table S3). 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.

Pseudoalteromonas luteoviolacea biofilms stimulate Hydroides metamorphosis via MACs, not TBP

We next questioned how the larvae of a different animal, *Hydroides*, respond 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 h of exposure to TBP or bacterial biofilms and assessed as metamorphosed if they developed branchial radioles and a primary proteinaceous tube (Fig. 4A). At a 500 nM concentration, TBP alone resulted in up to 30% of *Hydroides* metamorphosis compared to the acetonitrile solvent control (Fig. 4B; Table S3; $p = 0.0054$). Notably, all *Hydroides* larvae that metamorphosed were attached to the well. Higher TBP concentrations tested (1000, 750 and 500 nM), resulted in the 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 (Fig. 4C; Table S3), 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 the 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.

Discussion

Pseudoalteromonas 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 have 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 (500 nM) concentrations and resulted in death at higher (750 and 1000 nM) concentrations. Coral larvae were found to undergo metamorphosis, many without attaching to the substrate (Negri *et al.*, 2001; Tebben *et al.*, 2011, 2015; Sneed *et al.*, 2014), when exposed to similar TBP 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 that 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 signalling and membrane potential depolarization have been linked to the induction of *Hydroides* metamorphosis (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 signalling mediating invertebrate larvae metamorphosis.

Mutation of bmp2 *in* P. luteoviolacea *HI1 has no apparent 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 *Pseudoalteromonas* 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 (Fig. S1a). 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* and *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, 1993; Klassen *et al.*, 2015a; Klassen *et al.*, 2015b; 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 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 analysed 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 (Fig. 1B) 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 (Fig. 1A; bolded genes: *macB, macS, macT*, and *mif1*) with high nucleotide identity (Table S1). Interestingly, strain H2 (Fig. 1B; 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.

Conclusion

Pseudoalteromonas 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 unravelling this complexity and could provide a deeper understanding of the molecular underpinnings of bacteria-induced metamorphosis in divergent animals.

Experimental procedures

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* (Lee *et al.*, 1998). A list of strains, plasmids, and primers constructed and used in this study can be found in Tables S2 and S4.

Detection of TBP production by P. luteoviolacea *HI1*

Pseudoalteromonas luteoviolacea was grown in two growth medias, SWT (35.9 g/L Instant Ocean, 2.5 g/L Bacto Tryptone, 1.5 g/L Bacto Yeast and 1.5 ml/L glycerol) and MB (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 5 ml SWT and MB media, and incubated shaking (200 rpm) at 28°C for 16 h. The cultures were extracted twice with an equal volume of ethyl acetate (EtOAc) and concentrated under a stream of nitrogen. The samples were re-suspended 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), run at 0.5 ml/min in negative mode. Eluent was detected using electrospray ionization-mass spectrometry monitoring *m/z* 150–2200 in negative mode with a speed of 32 500 *m/z* per second. A solvent system of acetonitrile and water both containing 0.1% formic acid (v/v) was used. Samples were eluted over a 30 min method with a gradient from 10% to 70% acetonitrile over 15 min, 70%–80% over the next 10 min, and then immediately to 100% for 5 min before returning back to 10% acetonitrile. Quantification of TBP was based on the relative intensities of 2,3,4,5-TBP to synthetic standards. 2,3,4,5-TBP 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, CA, USA. The larvae were spawned and fed living *Isochrysis* cultures daily (Carolina Cat# 153180, NC, USA) as previously described (Nedved and Hadfield, 2008; Shikuma *et al.*, 2014). Larvae were maintained in beakers containing filtered artificial seawater (ASW) (Instant Ocean Cat # SS15–10, VA, USA) at 35 PSU water until competent (between 6 and 8 days).

Colonies of *H. 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 3–5 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^{-1} and kanamycin 5 μg ml^{-1} for 7 days. Prior to 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 28°C. Single colonies were inoculated into 5 ml of SWT and MB broth and incubated overnight for 14–16 h with agitation (200 rpm). Cells were removed from the culture, pelleted at 4000 g, and washed twice and re-suspended with ASW. Cell density was adjusted to OD_{600} of 1 (approximately 10^7–10^8 cells ml^{-1}) for *Hydractinia* and was further diluted to OD 0.5 for *Hydroides* (1:1 with ASW) to evoke optimal metamorphic responses. Aliquots of 100 μl were added to 96-well plates to form biofilms over a 2-h 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 100 μl for *Hydroides* and 200 μl for *Hydractinia*. The percentage of metamorphosis was scored after 24 h (*Hydroides*) and 72 h (*Hydractinia*). While the complete metamorphosis of *Hydractinia* can occur 24 h post-induction (Seipp *et al.*, 2007), the death phenotype produced similar phenotypes during early differentiation. This influenced qualitative scoring daily and quantitative scoring after 72 h. 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 MB media and *Hydractinia* grown in SWT media.

Exogenous TBP metamorphosis assays

The pure 2,3,4,5-TBP standard was synthesized as previously described (Zheng *et al.*, 2018). Eleven milligrams of TBP was re-suspended in acetonitrile (0.0059 M) 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: 1000, 750, 500, 250, 100, 10, 1 and 0.1 nM. We saw no phenotype for the lowest concentrations tested and therefore did not include them in the final analysis. The diluted TBP was aliquoted (100 μl) 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

Pseudoalteromonas luteoviolacea draft and complete genomes were downloaded from NCBI (Table S1). 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 analysed 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. A total of 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).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability Statement

The *P. luteoviolacea* genomes were downloaded from NCBI GenBank, and Table S1 provides a list of accessions.

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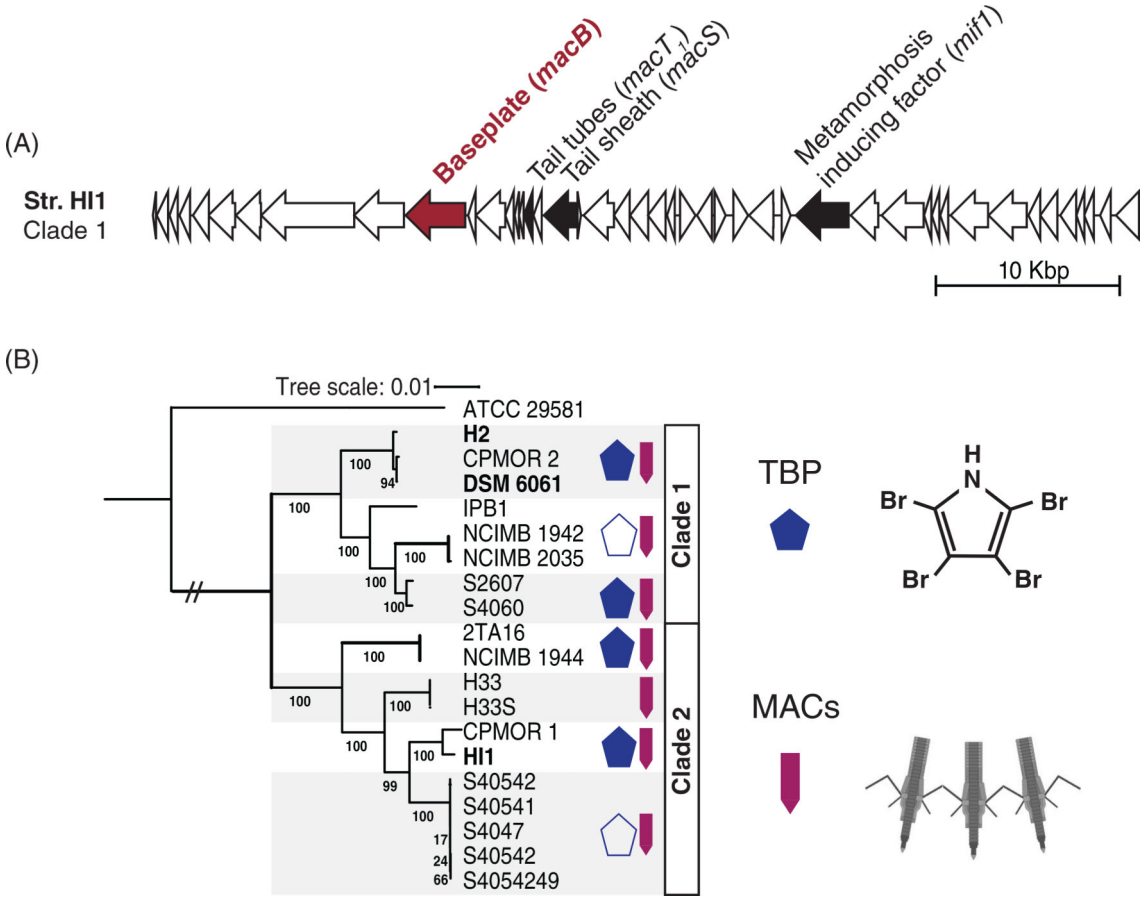

Fig 1.

Diverse *Pseudoalteromonas luteoviolacea* strains encode the biosynthesis genes for TBP and MACs. (A) MACs gene cluster from strain HI1. The filled arrows denote the genes interrogated by the 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) strain used in the biofilm metamorphosis assays. (B) Maximum likelihood phylogeny of 19 sequenced *P. luteoviolacea* genomes including *Pseudoalteromonas* sp. ATCC 29581 as an outgroup. The bootstrap values represent 100 re-samples. 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 do not experimentally produce halogenated compounds. Purple arrows indicate the presence of MACs genes (*macB, macS, macT*, and *mif1*).

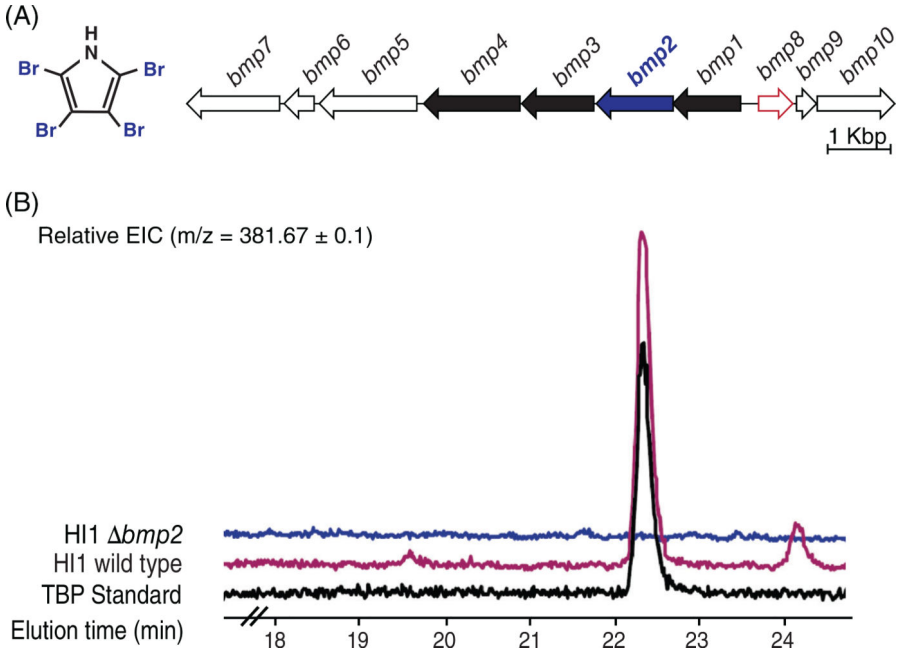

Fig 2.

Pseudoalteromonas luteoviolacea HI1 wild type 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 the comparison of an organic extraction of wild type and Δ*bmp2* strains to the TBP standard. Cultures were grown in MB and SWT media overnight in triplicate to quantify TBP production in *P. luteoviolacea* (see Fig. S1a).

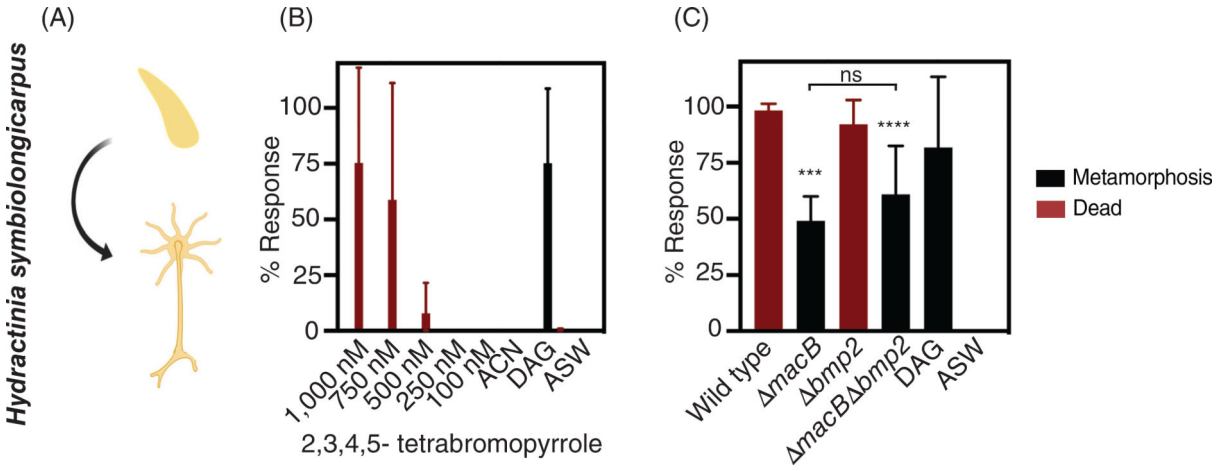

Fig 3.

Pseudoalteromonas 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 three biological replicates ($n = 3$). Values for the biological replicates were determined by averaging four 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 a 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 the competency of *Hydractinia* larvae at a concentration of 100 μM. (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 ****$p = 0.0006$. No statistical difference was found between treatments Δ*macB* and Δ*macB*Δ*bmp2*.

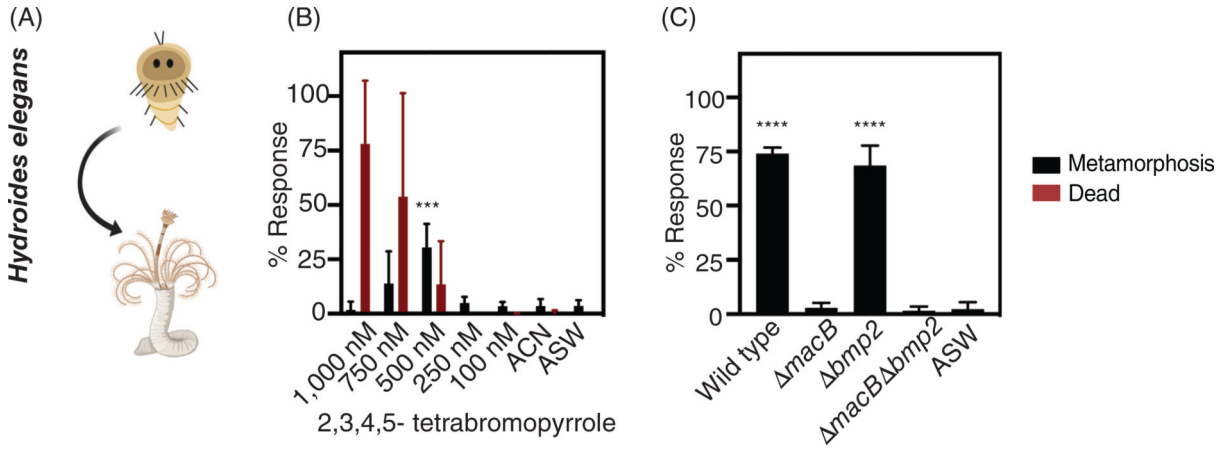

Fig 4.

Pseudoalteromonas 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) five biological replicates ($n = 5$) and (C) three 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 four 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 ****$p < 0.0001$.