Vol. 84: 31–42, 2020
https://doi.org/10.3354/ame01925

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online January 30

# Bacteria known to induce settlement of larvae of *Hydroides elegans* are rare in natural inductive biofilm

**Nidhi Vijayan[1,2], Michael G. Hadfield[1,*]**

[1]Kewalo Marine Laboratory, Pacific Biosciences Research Center, University of Hawai'i Manoa, Honolulu, HI 96813, USA

[2]*Present address:* Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06268, USA

ABSTRACT: Like those of many benthic marine invertebrates, larvae of the polychaete *Hydroides elegans* settle in response to complex, multi-species biofilms in the field and to certain monospecific bacterial biofilms in the laboratory. This study characterized the bacterial diversity of complex natural biofilms from Pearl Harbor, Hawai'i, USA, using high-throughput 16S rRNA-amplicon sequencing and further characterized those bacterial species that could be isolated using standard culture methods. Community analysis of the biofilms revealed dominance of *Bacteroidetes*, *Verrucomicrobiae*, *Alpha-* and *Gammaproteobacteria* and, at lower taxonomic levels, *Rubritalea* spp., *Hyphomonas* spp. and members of *Sphingomonadaceae* and *Rhodobacteraceae*. These natural biofilms induced settlement in nearly 100% of larvae of *H. elegans* exposed to them. We identified 1636 different amplicon sequence variants in the field biofilm from the normalized data set. The culture-based methods with one media type produced only 40 isolates, of which 36 were unique based on 97% sequence similarity cut-off. The cultured isolates were assigned to 11 genera. Single-species biofilms of only 6 of the 40 isolates, all in the genus *Pseudoalteromonas*, induced settlement by larvae of *H. elegans.* However, *Pseudoalteromonas* was present at very low relative abundance (<0.01%) in the biofilm. Crude suspensions of 0.22 µm filtered supernatant from cultures of these 6 *Pseudoalteromonas* strains were not settlement-inductive, while those from *P. luteoviolacea* strain HI1 were inductive as previously reported. These findings reinforce the conclusion that larvae of *H. elegans* respond to different cues from different bacteria.

KEY WORDS: Biofilm · 16S rRNA microbiome · Metamorphosis · *Hydroides elegans* · *Pseudoalteromonas* spp.



## 1. INTRODUCTION

The life cycles of many benthic marine invertebrates are dependent on external chemical and/or physical environmental cues to induce settlement and metamorphosis of their larvae. Bacterial biofilms have been found to be an important source of these cues (Hadfield 2011, Harder et al. 2018). A majority of marine invertebrate larvae including corals (Webster et al. 2004, 2011), marine sponges (Whalan & Webster 2014), bryozoans (Dobretsov & Qian 2006), molluscs (Gribben et al. 2009, Alfaro et al. 2011), tunicates (Roberts et al. 2007) and crustaceans (Anderson & Epifanio 2009) settle specifically on surfaces coated with bacterial biofilms. However, it is unclear if larvae respond to different bacterial cues or one type of cue produced by all inductive bacterial biofilms.

Studying the interaction of larvae and biofilms is not a trivial task, because biofilms are complex structures. Biofilms found on all submerged marine surfaces are made up of microbial communities embed-

*Corresponding author: hadfield@hawaii.edu

ded in a bacteria-produced matrix of slimy extracellular polymeric substances (EPS) (Flemming et al. 2016). Marine biofilms form when planktonic bacteria attach to a suitable substratum, reproduce and secrete EPS. Maturation of a biofilm is often accompanied by bacterial production of quorum-sensing acyl-homoserine lactones (AHL) and increase of EPS. The EPS, which may contain nucleic acids, lipopolysaccharides, proteins and humic acids, serves the resident bacteria for defense, retention in a favorable habitat or community, and it may be a default mode of growth (Jefferson 2004). Once a biofilm is established, it is followed by detachment and dispersal of bacteria, which may be a result of starvation or other cues not yet well understood (Monds & O'Toole 2009). Single or multiple bacterial species in a natural wild biofilm may cue larvae to settle and metamorphose.

The serpulid polychaete *Hydroides elegans* has been extensively studied because of its dependence on marine biofilms for cues to settle and metamorphose and its ease of culture in the laboratory (Nedved & Hadfield 2009). The worm is widely distributed globally in shallow tropical and subtropical waters where it is a member of the biofouling community. When 5 d old competent nectochaete larvae of *H.* elegans swim very near sufficiently dense natural bacterial biofilm of at least 10 000 cells $mm^{-2}$ (Shikuma & Hadfield 2005), they contact the biofilm, adhere to the substratum and quickly secrete a thin organic primary tube along the length of their bodies (Hadfield et al. 1994, 2014). The larvae metamorphose within the tube, and from its anterior opening secrete the calcified tube of the juvenile and adult worm (Carpizo-Ituarte & Hadfield 1998). In controlled laboratory experiments, larvae of *H. elegans* have been found to settle and metamorphose on monospecific films of a few bacterial species, including *Pseudoalteromonas luteoviolacea* HI1 strain, *Cellulophaga* (*Cytophaga*) *lytica*, *Bacillus aquimaris* and *Staphylococcus warneri*, all isolated from marine biofilms in Hawai'i, USA (Unabia & Hadfield 1999, Huang & Hadfield 2003, Freckelton et al. 2017). Additional bacterial strains belonging to the genera *Alteromonas*, *Vibrio*, *Brevibacterium* and *Micrococcus* were found to induce metamorphosis of *H. elegans* in Hong Kong (Lau et al. 2002). However, the overall bacterial diversity of the biofilms from which inductive bacteria were isolated in these studies is unknown.

It has been found that metamorphosis of *H. elegans* larvae occurs in response to different characteristics of different bacterial species (Freckelton et al. 2017). The bacterium *P. luteoviolacea* HI1 produces complex arrays of tailocins, referred to as 'metamorphosis-associated contractile structures' (MACs) by Shikuma et al. (2014). Tailocins are structures derived from T4-type phage tail assemblies that have bactericidal properties (Gill & Young 2011). However, these structures are not found in bacterial species in the genera *Cellulophaga*, *Bacillus* or *Staphylococcus*, where inductive activity appears to be associated with external membrane structures called outer membrane vesicles (OMVs) in the case of Gram-negative bacteria or extracellular vesicles (EVs) in the case of Gram-positive bacteria (Freckelton et al. 2017). OMVs, small spherical structures budded from the outer membrane of Gram-negative bacteria, have various functional attributes such as secretion of metabolites, delivery of toxins, defense mechanisms and nutrient acquisition (Kulp & Kuehn 2010, Manning & Kuehn 2011, Biller et al. 2014). However, both of these cell-free preparations (tailocins and OMVs) are laboratory preparations, whereas with either wild biofilms or single bacterial species biofilms, larvae must physically contact the biofilm to be induced to settle (Hadfield et al. 2014). That is, soluble products are not involved.

The diversity of bacteria in natural biofilms can range from approximately 30 to several thousand species, depending on the environment (Leary et al. 2014, Schmeisser et al. 2017). Natural marine biofilms formed on submerged surfaces may be highly diverse, including many thousands of different operational taxonomic units (OTUs) (Lema et al. 2019, Zhang et al. 2019). While as many as a few tens of bacterial species from these biofilm communities can be cultured on standard media, it is not known how many of them naturally induce settlement of larvae of *H. elegans* (Unabia & Hadfield 1999, Lau et al. 2002, Lema et al. 2019), and we have no understanding of their proportional representation in complex marine biofilms. To increase understanding of the bacterial–larval interactions that underlie most larval recruitment to the benthos, we employed field-collected marine biofilms (3 replicates) to determine (1) the total bacterial diversity of a natural biofilm using high-throughput 16S rRNA amplicon sequencing; (2) the identity of culturable bacteria from the natural biofilm using half-strength seawater tryptone (½ SWT) media; (3) the identity of the culturable bacterial strains that induce metamorphosis in larvae of *H. elegans*; and (4) the presence of inductive elements such as tailocin complexes in cell-free preparations of bacteria that induce metamorphosis like that of *P. luteoviolacea* HI1.

## 2. MATERIALS AND METHODS

### 2.1. Biofilm collection

Cleaned glass slides (25 × 75 × 1 mm) were deployed in a slide holder (described by Lema et al. 2019) for 12 d in April 2017 at Ford Island, Pearl Harbor, Hawai'i, USA, at a depth of approximately 1 m below sea surface at low tide, after which the slides were placed in a container of ambient seawater and brought to the Kewalo Marine Laboratory for immediate processing. It was previously established that a dense biofilm of a minimum of 20 000 cells $mm^{-2}$ forms in 7 d at this site (Lema et al. 2019). Each slide (in 3 replicates) was rubbed on one side with a sterile polyester-tipped swab, and the swabs were placed in individual microfuge tubes and stored at −80°C until extraction of total DNA and sequencing. The reverse side of each slide was rubbed with another sterile swab, which was suspended in a microfuge tube containing 1 ml of double-filtered (at 0.22 µm) autoclaved seawater (DFASW) for immediate bacterial culture.

### 2.2. 16S rRNA gene high-throughput amplicon sequencing and analysis

Genomic DNA was extracted from the biofilm swabs stored at −80°C using a MasterPure Complete DNA and RNA Purification kit (Epicenter Technologies). The biofilm swabs were incubated in the cell lysis solution from the kit at 65°C for 45 min and vortexed for 30 s every 10 min to remove cells from the swab. The swabs were removed, and the DNA was extracted from the suspended cells according to the manufacturer's instructions. The extracted DNA was stored in Tris-EDTA buffer at −20°C. Extracted DNA was quantified using a dsDNA HS Assay kit (Thermofisher) and a Qubit 2000 UV fluorometer (Invitrogen). Aliquots of the community DNA (20 ng $ml^{-1}$) were sent to the Argonne National Laboratory, IL, USA, for sequencing and library preparation. The hypervariable V3-V4 region of the microbial 16S rRNA gene (primers 515F 5'-GTG YCA GCM GCC GCG GTA A-3' and the 806R 5'-GGA CTA CNV GGG TWT CTA AT-3') (Walters et al. 2015) was amplified and sequenced on a Miseq 500× DNA sequencer (Illumina).

QIIME 2 was used in the initial processing to de-noise sequences (Kuczynski et al. 2012, Bolyen et al. 2019). The sequences were further denoised using DADA2 and trimmed at 250 bp (Callahan et al. 2016). Chimeric sequences were filtered out using UCHIME (Edgar et al. 2011). The reference database SILVA-128 was amended to include the sequences from the 40 isolates prepared using Sanger sequencing to determine presence of the isolates in the high-throughput data. The amplicon sequence variants (ASVs) were aligned against the amended SILVA-128 reference database at a 99% or higher identity threshold. Chloroplast and mitochondrial sequences were removed from the table.

The resulting ASV-abundance-matrix table was rarefied to the minimum number of reads in the sample set (21 826) at 100 permutations with R v.3.5.1 using the 'phyloseq' package (McMurdie & Holmes 2013) to normalize the samples. ASVs at <0.1% relative abundances were assigned as 'Rare' to identify the less abundant microbial communities at the phylum level. The ASVs that made up a relative abundance ≥0.5% in at least one sample replicate were identified to the genus level, while the remaining ASVs were assigned as 'Rare'. The community composition plots were visualized using the 'ggplot2' (Wickham 2016) package in RStudio.

The sequences were also processed using QIIME 1.9 as described previously (Vijayan et al. 2019), and an OTU abundance matrix was constructed using 'uclust' based open-reference OTU picking. Briefly, the sequences that met the following parameters were processed for further analysis: (1) ≥200 and ≤300 nucleotides in length, (2) ≤1 ambiguous bases, (3) homopolymer runs with ≤6 nucleotides and (4) a phred-quality score ≥19. The sequences were aligned at a 97% or higher identity threshold of the SILVA-128 database (included with sequences from the 40 isolates). Chloroplast and mitochondrial sequences were removed from the data set. The resulting OTU table was used to search for the presence of cultured isolates.

### 2.3. Isolation and identification of culturable bacteria

Swabs from the field biofilms suspended in 1 ml DFASW were vortexed, and after removing the swabs, the DFASW containing collected bacteria was serially diluted at a ratio of 1:10 for 5 dilutions. Each dilution was plated (50 µl) on a ½ SWT agar plate (Boettcher & Ruby 1990). This was repeated with swabs from each of the 3 biofilmed slides. The plates were observed for 3 d, and different bacteria were selected for isolation on the basis of colony color and

morphology. The isolates were purified by subculturing on ½ SWT agar, and 1:1 glycerol stocks were prepared with ½ SWT liquid cultures and stored at −80°C. Two sterile swabs were also plated at no dilution as a control and no bacterial growth was observed.

Genomic DNA was extracted from overnight broth cultures of bacterial cells with a MasterPure™ Complete DNA Purification Kit (Epicentre®). Approximately 1400 bp of the 16S rRNA gene was amplified using forward primer 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) (Weisburg et al. 1991). PCR conditions were the same for all bacterial strains isolated: an initial denaturation step at 94°C for 3 min, followed by 35 cycles at an annealing temperature of 50°C for 90 s, extension of 72°C for 120 s, ending the cycle at 72°C for 10 min. Amplified products were aliquoted into new tubes and cleaned with 1:1 exonuclease-I: shrimp alkaline phosphatase (ExoSAP-IT Affymetrix™) before being sent for Sanger sequencing at the Advanced Studies in Genomics Proteomics and Bioinformatics sequencing facility at University of Hawai'i at Mānoa (Honolulu, HI).

### 2.4. Accession numbers

The raw amplicon sequence data sets from the Pearl Harbor biofilms were uploaded to the Sequence Read Archive under Bioproject Accession No. PRJNA509913. The sequences of 40 cultured bacterial isolates were submitted to NCBI with Accession Nos. MG819683-92 and MG819694–MG819724.

### 2.5. Sequence alignment and phylogenetic analysis

Reads from Sanger sequencing of pure culture bacterial isolates were manually inspected and trimmed prior to assembling the forward and reverse sequences. The resulting consensus sequences were aligned in Geneious v.10.1.9 using built-in ClustalW parameters (gap opening: 10; gap extension: 0.2). A maximum-likelihood (PHYML) tree with Jukes Cantor model was constructed on 1120 bp of the aligned sequences with a bootstrap of 1000 replicates (Guindon et al. 2009). Similarly, 1460 bp were used to construct the phylogenetic tree for the inductive strains, with *Escherichia coli* (Accession No. NR_024570.1) used as the outgroup taxon.

### 2.6. Larval production and settlement assays

Pieces of Vexar mesh (approximately 30 $cm^2$) were immersed in Pearl Harbor at Ford Island (21° 21′ 27.2″ N, 157° 57′ 34.8″ W) for more than 1 mo while they accumulated adult specimens of *Hydroides elegans.* The screens and tubes were brought to the Kewalo Marine Laboratory, Honolulu, HI, where they were maintained in aquaria receiving a continuous flow of unfiltered seawater. Male and female worms were spawned *in vitro*, the gametes were collected and larvae were cultured according to existing protocols (Nedved & Hadfield 2009). Larvae were cultured in 2 l beakers of 0.22 µm filtered seawater (FSW) (10 000 larvae $l^{-1}$), with daily water changes and feeding of *Isochrysis galbana* (Tahitian strain) at a concentration of approximately 60 000 cells $ml^{-1}$. Five days after fertilization, larvae become competent and readily undergo metamorphosis upon exposure to inductive biofilms.

To test the 40 isolated bacterial strains for inductivity, the strains were prepared as liquid cultures in 100 ml of ½ SWT broth grown overnight at 27°C with shaking (170 rpm). The overnight cultures were centrifuged at 4000 rpm (5976 × *g*) for 20 min, after which the supernatant was removed and the cell pellet re-suspended in DFASW. Bacterial concentrations were adjusted with the addition of DFASW to achieve the desired inoculation densities of approximately $10^8$ cells $ml^{-1}$. The bacterial suspension (500 µl) was pipetted into sterile 4 ml Petri dishes and left for 1 h to allow bacteria to develop a suitable monospecific biofilm as described by Huang & Hadfield (2003). The dishes were rinsed 3 times with sterile seawater, filled with 3 ml DFASW, and >10 larvae (6 d old) were added. A total of 3 replicate Petri dishes were prepared for each bacterial isolate. Larvae were exposed to the biofilms for 24 h, after which the number of larvae that had undergone metamorphosis was determined. Each induction experiment also included a positive control (3 wk old wild biofilm on glass slide) and a negative control (new glass slide in DFASW) (Carpizo-Ituarte & Hadfield 1998).

### 2.7. Preparation of crude extracts from inductive bacteria

Preliminary bioassays revealed that 6 of the 40 isolated strains induced larvae of *H.* elegans to settle. To determine if these 6 bacterial strains pro-

duce the same tailocin complexes as those isolated from *Pseudoalteromonas luteoviolacea* HI1 (Shikuma et al. 2014) and found to be inductive, they were subjected to the same published isolation method for bioassays. That is, cells centrifuged from overnight cultures were re-suspended in 1/10$^{th}$ volume of DFASW. This seawater–cell suspension was then centrifuged at 4000 rpm (5976 × *g*) for 25 min to remove bacterial cells, and the supernatant was carefully transferred to a fresh tube. Ampicillin (100 µg ml$^{-1}$) was added to the supernatant samples to kill any remaining viable cells, and the samples were incubated at 4°C for 2 h. The supernatants (10 µl) were then plated on ½ SWT agar plates and examined after 24 h to confirm the absence of live bacterial cells. If present, the number of cells was counted to determine colony-forming units (CFUs) per ml. These preparations were then assayed for their capacity to induce settlement of competent 6 d old larvae at 1:10 and 1:100 dilutions with DFASW. The positive controls for these assays were cell-free extracts containing tailocin complex arrays prepared from cultures of *P. luteoviolacea* HI1 and 3 wk old natural biofilms on glass slides; the negative control was new/clean glass slides in DFASW.

### 2.8. Whole genome analysis

Although crude culture preparations from the 6 strains isolated in this study did not have the settlement-inducing capacity of the same preparation of *P. luteoviolacea* HI1, we also endeavored to examine published genomes for the bacterial species to determine if genes known to be responsible for their assembly (Shikuma et al. 2014) were present. The qualities of these genome assemblies were analyzed using the web-based program QUAST (Gurevich et al. 2013) (Table S1 in the Supplement at www.int-res.com/articles/suppl/a084p031_supp.pdf). The average nucleotide identity (ANI) of each isolate against the genome of the inductive bacterium *P. luteoviolacea* HI1 (Asahina & Hadfield 2015) was determined using the python module for ANI analysis (PyANI) aligned with a minimum length of 700 bp and a minimum identity of 60% (Pritchard et al. 2016). Basic local alignment search tool (BLAST+ 2.5.0) was used to search the genomes for homologues of 6 open reading frames previously identified to encode phage tail elements making up the tailocin assembly in *P. luteoviolacea* HI1 (Shikuma et al. 2014) (Table S2). Additionally, the translated genomes were searched for 12 hypothetical proteins involved in the initiation of larval metamorphosis (Shikuma et al. 2016).

## 3. RESULTS

### 3.1. Diversity of 16S rRNA gene high-throughput amplicon sequences in field-collected biofilms

The total number of amplicon reads from biofilms from 3 replicate slides were (1) 30 032, (2) 21 826 and (3) 63 710. The rarefied data set with 21 826 reads per sample contained 1636 different ASVs and 4627 OTUs. The relative abundances of the dominant phyla/classes were similar with both methods (DADA2 and open-reference OTU picking): *Bacteroidetes* (22–26%), *Alphaproteobacteria* (25–28%), *Gammaproteobacteria* (17–28%), *Verrucomicrobiae* (9–10%), *Deltaproteobacteria* (2–5%), *Planctomycetacia* (2.5–5%) and *Oxyphotobacteria* (1%) (data from ASVs are represented in Fig. 1).

The ASVs that made up more than 0.5% of the whole community were classified at the generic level as *Hyphomonas*, *Tistila*, clade BD1-7, *Kangiella*, *Aureispira*, *Lewinella*, *Saprospira*, *Lutolibacter*, *Rubritalea*, *Syenochococcus* and uncultured *Rhodobacteraceae*, *Rhizobiales*, *Enterobacteriaceae*, *Flavobacteriaceae* and *Saprospiraceae* (Fig. 1B). These ASVs made up approximately 28% of the entire data set from 3 biofilms.

### 3.2. Identification of bacterial isolates

From the original biofilm swabs that were spread on culture plates, we distinguished 40 distinct colonies based on morphology. These 40 bacterial isolates were identified via PCR amplification of the 16S rRNA gene, and 36 of the isolates were found to be less than 97% similar to each other. Phylogenetic analyses with multiple models confirmed the identity of the strains (Fig. 2). Of the 40 isolates, 18 strains belonged to the genus *Vibrio* and 8 strains to the genus *Pseudoalteromonas*. Other bacterial genera included *Photobacterium* (n = 3), *Tenacibaculum* (n = 3), *Bacillus* (n = 1), *Pseudomonas* (n = 1), *Psychrobacter* (n = 2), *Ruegeria* (n = 1), *Aquimarina* (n = 1), *Mesoflavibacter* (n = 1) and *Thalassomonas* (n = 1). However, we cannot conclusively confirm the number of species isolated in this study because 16S rRNA gene alone cannot distinguish between species (Rossi-Tamisier et al. 2015, Chung et al. 2018).

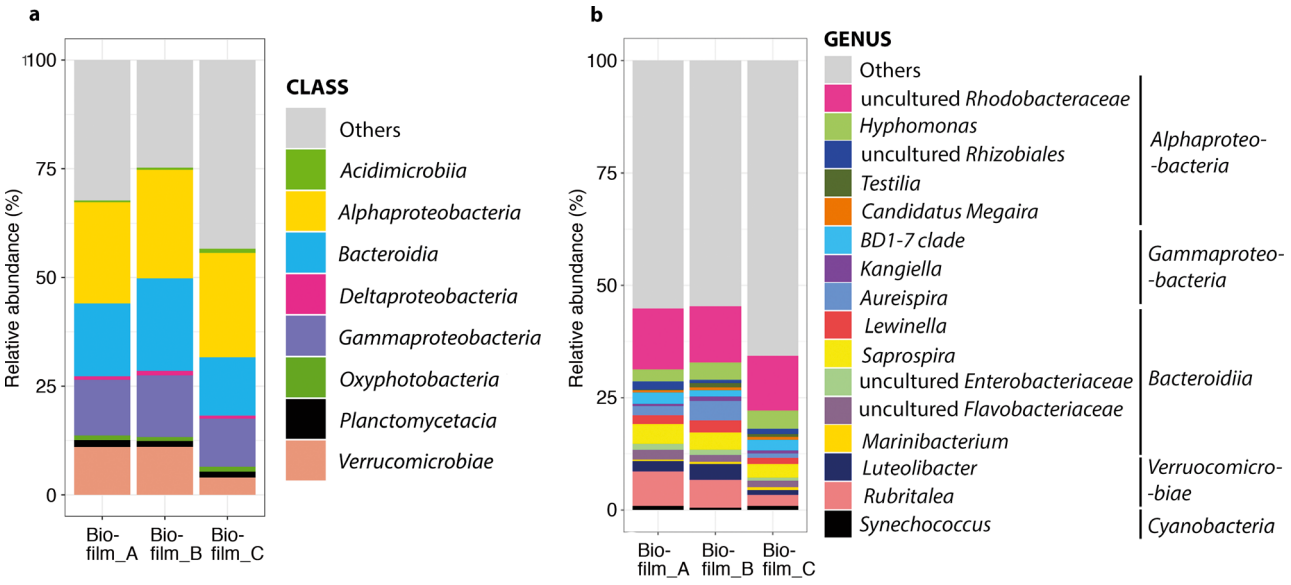


Fig. 1. Relative abundance of microbial communities derived from microbial 16S rRNA amplicon sequence variants (ASVs) of three 12 d old biofilms (A, B and C) formed on glass slides. Chimeric sequences as well as sequences identified as chloroplast and mitochondria were removed from analysis. (a) ASVs that were >0.1% of the entire data set were classified at the class level and the remaining ASVs were assigned as 'Rare'. (b) ASVs that were >0.5% relative abundance were classified at the genus level and remaining ASVs were assigned as 'Rare'

### 3.3. Comparison of amplicon sequence data with cultured isolates

The bacterial genera that we were able to culture were of very low abundance or not detected in the amplicon communities using both DADA2 and an open-reference OTU picking method: *Pseudoalteromonas* spp. (<0.01%), *Vibrio* spp. (0.5%), *Bacillus* spp. (<0.1%), *Photobacterium* spp. (<0.5%), *Tenacibaculum* spp. (<0.2%), *Pseudomonas* spp. (<0.5%), *Ruegeria* spp. (<0.1%), *Aquimarina* spp. (0.1%) and *Thalassomonas* sp. (<0.1%). Furthermore, analysis of high-throughput amplicon data against the reference database amended with the 40 sequences of the cultured bacteria yielded no matches except for *Thalassomonas* sp. (B24). Additionally, the bacterial genera previously identified as inductive by others were absent: *Cellulophaga* (*Cytophaga*) spp., *Staphylococcus* spp., *Micrococcus* spp., *Brevibacterium* spp. and *Alteromonas* spp. (Lau et al. 2002, Huang & Hadfield 2003, Freckelton et al. 2017).

### 3.4. Inductive capacities of monospecific biofilms

Biofilms of each of the 40 bacterial isolates were tested for their capacity to induce settlement and metamorphosis by larvae of *Hydroides elegans* (Fig. 3). In total, 6 of the 40 cultured bacteria induced settlement and metamorphosis. All of the 6 inductive strains with an inductive capacity greater than 50% belonged to the genus *Pseudoalteromonas*. The remaining 34 strains induced metamorphosis in less than 20% of larvae tested. The inductive *Pseudoalteromonas* strains were further analyzed for their closest BLAST identifications (Fig. 4, Table 1).

### 3.5. Metamorphosis-inducing capacities of crude extracts from cultures of inductive isolates

Inductive strains extracted the same way as that of *Pseudoalteromonas luteoviolacea* HI1 (Shikuma et al. 2014) did not induce significant metamorphosis of the larvae, whereas the preparation from *P. luteoviolacea* HI1 induced more than 70% larval metamorphosis, as expected (Fig. 5). The extracts tested at a higher dilution (1:10) also did not induce metamorphosis, and the extract of *P. rubra* killed the larvae. Colony proliferation from intended cell-free sample extracts spread on ½ SWT agar was observed for *P. spongiae* A23, *P. piscicida* C06 and *P. phenolica* C21, demonstrating that living bacterial cells remained in the samples, but not at high enough densities to induce metamorphosis (CFUs: A23 = $5 \times 10^3$ cells ml$^{-1}$, C06 = $10^3$ cells ml$^{-1}$, C21 = $10^3$ cells ml$^{-1}$).

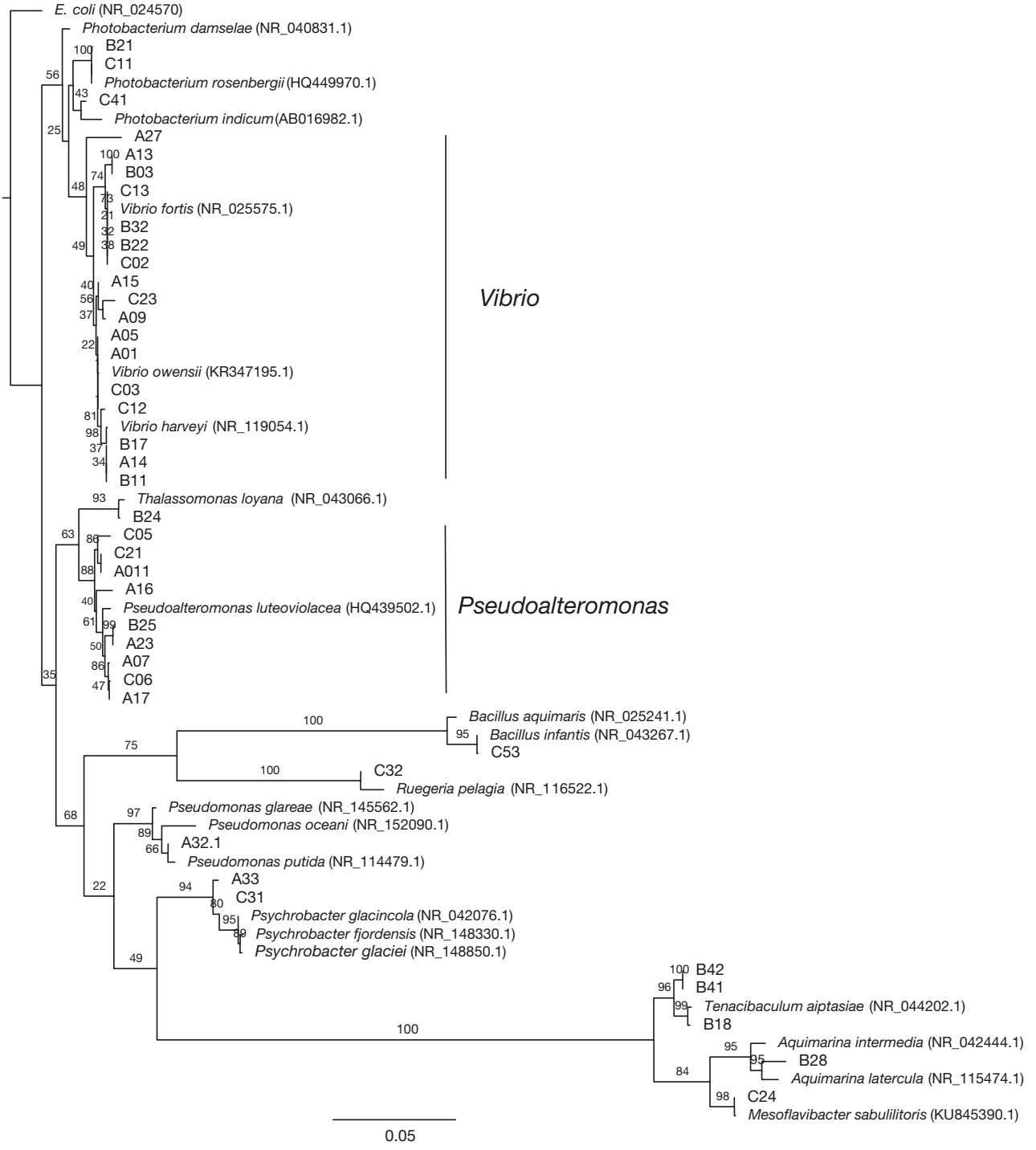


Fig. 2. Maximum likelihood phylogenetic tree (Jukes Cantor model) from the ClustalW alignment of 1120 bp of 16S rRNA gene sequences of 40 bacterial isolates. Numbers on nodes: bootstrap values based on analysis of 1000 re-sampled data sets. Accession numbers are in parentheses to the right of the organism name. Scale bar: 0.05 substitutions per nucleotide position. Only support values >65% are shown

### 3.6. Genome analysis

Published whole genomes of 17 *Pseudoalteromonas* species were examined for the presence of the genes reported to code the proteins in tailocins in *P. luteoviolacea* HI1 (6 genomes of *P. piscicida*, 2 of *P. spongiae*, 2 of *P. phenolica* and 7 of *P. rubra*). A genome of *P. viridis* was not available. BLAST analysis of the whole genomes found no matches with >30% query cover for the 6 ORFs implicated in

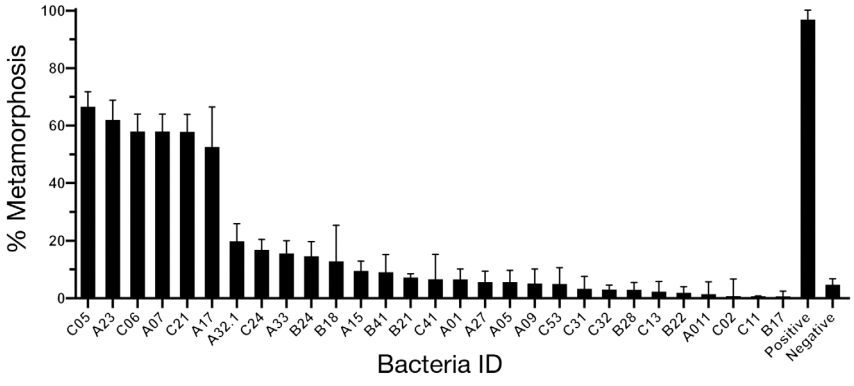


Fig. 3. Induction of *Hydroides elegans* larvae by mono-specific bacterial biofilms. The mean (±SD) percentage of larvae that metamorphosed were plotted for 40 different bacterial strains. Larvae (>10) were introduced to 3 replicates of bacterial biofilms (see Fig. 2 for identification of 'Bacteria ID') and observed for metamorphosis after 24 h. Isolates C05, A23, C06, A07, C21 and A17 belong to the genus *Pseudoalteromonas*. No metamorphosis of larvae were observed in biofilms of A16, B42, C12, B25, B03, B32, C03, A13, A14, C23 or B11. Positive controls: natural 3 wk wild biofilms; negative controls: autoclaved double filtered seawater

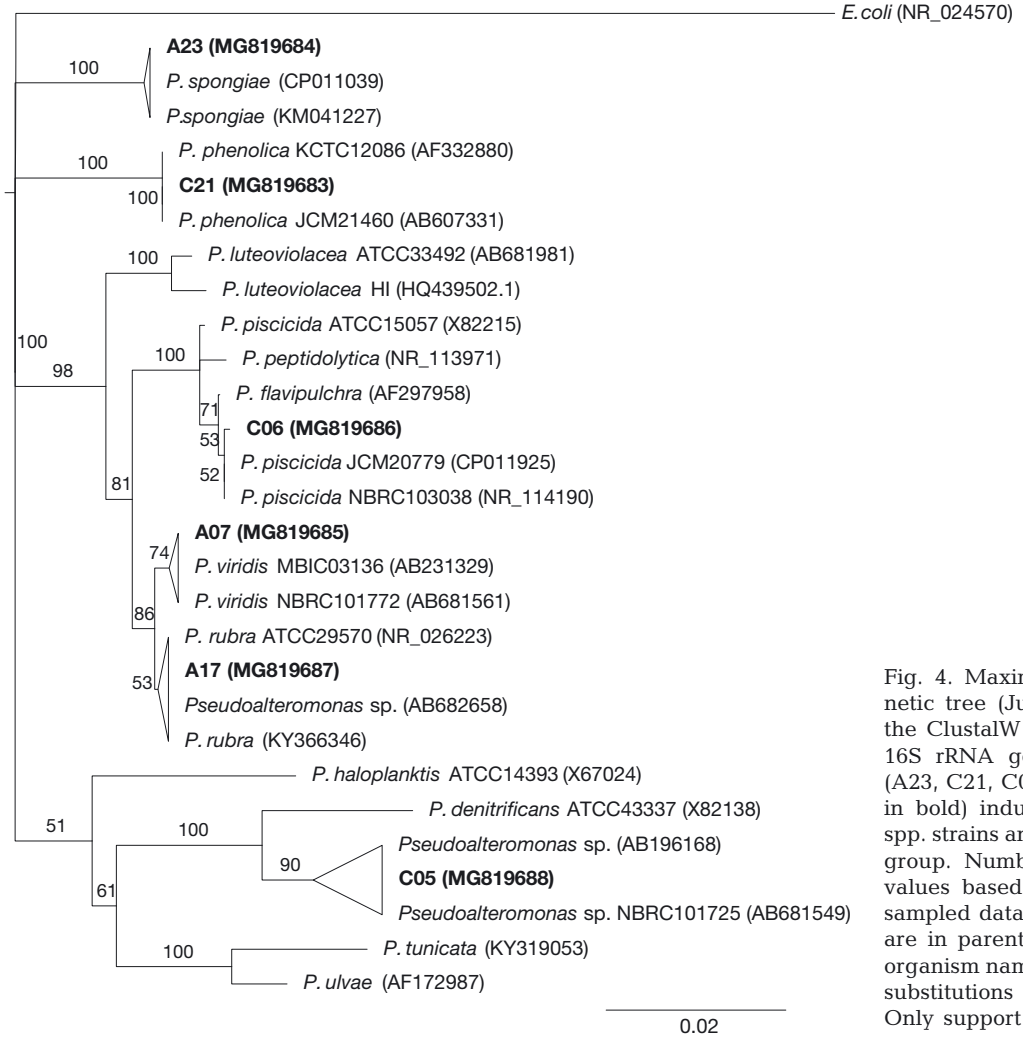


Fig. 4. Maximum likelihood phylogenetic tree (Jukes Cantor model) from the ClustalW alignment of 1461 bp of 16S rRNA gene sequences of the 6 (A23, C21, C06, A07, A17, C05, shown in bold) inductive *Pseudoalteromonas* spp. strains and an *Escherichia coli* outgroup. Numbers on nodes: bootstrap values based on analysis of 1000 resampled data sets. Accession numbers are in parentheses to the right of the organism name. Scale bar: 0.02 units of substitutions per nucleotide position. Only support values >65% are shown

inducing larval metamorphosis (Shikuma et al. 2014). Additionally, BLAST analysis found only 3 of the 17 strains (*P. rubra* ATCC 29570, T7 and S2471) with >70% query cover for any of the proteins involved in the tailocin assemblage (Tables S3–S9). Of them, the translated genome of *P. rubra* (ATCC 29570) had significant matches (>75% query cover and >50% identity) for 2 of the hypothetical proteins

Table 1. Inductive *Pseudoalteromonas* strains. Pigment color: color of the bacterial colony grown on half-strength seawater-tryptone agar. Closest BLAST match: the species with the closest percent similarity from the 1460 bp 16S rRNA gene sequence

| Strain ID | Pigment color | Closest BLAST match | % similarity |
|---|---|---|---|
| C05 | Purple | *Pseudoalteromonas* sp. (AB681549.1) | 96.4 |
| C06 | Yellow | *P. piscicida* | >99.5 |
| C21 | Brown | *P. phenolica* | 100 |
| A07 | Pink | *P. viridis* | >99.5 |
| A23 | Orange | *P. spongiae* | 100 |
| A17 | Red | *P. rubra* | >99.7 |

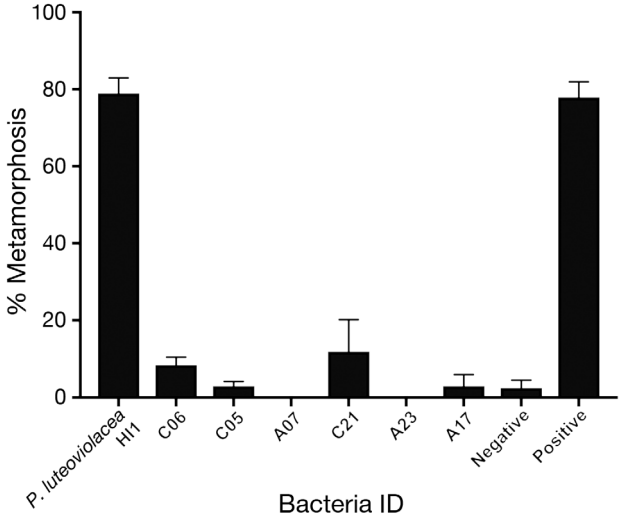


Fig. 5. Mean (±SD) percentage metamorphosis of *Hydroides elegans* larvae exposed to 4 replicates of extracts from *Pseudoalteromonas* spp. inductive strains at 1:100 dilutions in double filtered autoclaved seawater (C21 *P. phenolica*, A23 *P. spongiae*, C06 *P. piscicida*, A07 *P. viridis*, A17 *P. rubra*, C05 *Pseudoalteromonas* sp.). Positive controls: cell-free extracts of *P. luteoviolacea* HI1 and Wild natural biofilm (Positive); negative controls: autoclaved double filtered seawater

(macS and macT2) present in the tailocin assembly (Table S6).

## 4. DISCUSSION

It is widely recognized that natural biofilms induce larvae of the tubeworm *Hydroides elegans* to settle; however, only few bacterial species are known to induce metamorphosis, and the mechanisms of the interactions are unclear. This study focused on marine biofilms from Pearl Harbor, Hawai'i, USA, a habitat rich in this polychaete species, to answer a series of questions regarding total microbial diversity, diversity of culturable bacteria from the wild biofilm and the capacities of the cultured bacterial strains to induce settlement in the tubeworm larvae. We found that amplicons from the cultured bacteria that induced larvae to metamorphose, all belonging to the genus *Pseudoalteromonas*, were rare in the high-throughput sequencing data. Additionally, the mechanism(s) by which these *Pseudoalteromonas* strains induce larval metamorphosis is not similar to that of the previously described inductive species, *P. luteoviolacea* HI1.

The field biofilms we examined were composed mostly of species in the phyla *Proteobacteria* (*Alpha-* and *Gamma-*), *Bacteroidetes* and *Verrucomicrobia*. These phyla were abundant in previous analyses of biofilms from Pearl Harbor in June and July 2016 (Lema et al. 2019) and other biofilms from different geographic locations (Zhang et al. 2019). From the Pearl Harbor biofilm communities, we were able to isolate bacteria from 4 phyla and 11 genera: *Gammaproteobacteria* (genera *Vibrio*, *Pseudoalteromonas*, *Photobacterium*, *Pseudomonas*, *Psychrobacter* and *Thalassomonas*), *Alphaproteobacteria* (*Ruegeria* spp.), *Bacteroidetes* (genera *Tenacibaculum*, *Aquimarina* and *Mesoflavibacter*) and a firmicute, *Bacillus infantis*. The genus-level community composition plot shows approximately 70% of the ASVs having less than 0.5% relative abundance in the biofilm, which suggests a diverse bacterial community. Furthermore, we were unable to isolate representatives of the most dominant bacterial groups. The generic ASVs of greatest abundance in the Pearl Harbor biofilms were *Rubritalea*, *Hyphomonas* and *Aureispira* belonging to phyla *Verrucomicrobia*, *Proteobacteria* (*Alpha-*) and *Bacteroidetes*, respectively. While these phyla are ubiquitous in the marine environment (Zinger et al. 2011, Hamdan et al. 2013), only few of the abundant genera identified here have been cultured previously: *Rubritalea* spp. with M1 agar (Scheuermayer et al. 2006, Kasai et al. 2007), *Hyphomonas* spp. with Zobell marine medium (Weiner et al. 1985) and *Aureispira* spp. with half-strength tryptic soy media (Hosoya et al. 2007). To the best of our knowledge, theses bacterial strains have not been tested for production of cues that induce larval settlement.

The 40 bacterial isolates that we were able to culture from the field biofilms were low in abundance or not detected in the bacterial community analyses. Most of the cultured bacteria belonged to the genera *Vibrio* and *Pseudoalteromonas*, both *Gammaproteobacteria*, which together made up less than 1% of the total bacterial community in the biofilm. The genus *Pseudoalteromonas* was also relatively rare in other

microbial environments and when analyzed using other tools such as metagenomics (Lema et al. 2019) and fluorescent *in situ* hybridization (Eilers et al. 2000). Similarly, there was no correlation between the presence of *P. luteoviolacea*, which also induces sea urchin larval metamorphosis, and its inductive microbial community (Nielsen et al. 2015). Other bacterial species known to induce settlement in *H. elegans* belonging to the genera *Bacillus*, *Cellulophaga*, *Staphylococcus* and *Thalassotalea* (Huang & Hadfield 2003, Freckelton et al. 2017) were also either of low abundance (<0.5%) or not detected in our Pearl Harbor biofilms, although all were originally isolated from biofilms collected at the same site. However, because we cannot absolutely quantify individual bacterial species using 16S rRNA amplicon sequencing, the total number of inductive individuals of *Pseudoalteromonas* spp. in the biofilm may be sufficient to induce larval metamorphosis. Quantification tools such as qPCR or fluorescent *in situ* hybridization may provide an accurate estimation of *Pseudoalteromonas* spp. in these biofilms. Also, because our success with isolating single biofilm species followed the 'great plate count anomaly' (Staley & Konopka 1985), i.e. only small fractions of natural bacterial communities are typically cultured *in vitro*, we have yet to test the most abundant bacterial genera for their capacities to induce metamorphosis of marine invertebrate larvae. In the future, use of different media and methods may serve to isolate the more abundant strains and test their inductive capacities.

As noted above, all 6 of the cultured bacterial species that induced metamorphosis in larvae of *H. elegans* belong to the genus *Pseudoalteromonas*. Of these 6, *P. rubra*, *P. piscicida* and *P. spongiae* have been previously reported to induce metamorphosis of *H. elegans* in Australia and Hong Kong (Holmström et al. 2002, Huang et al. 2007). Notably, species of the genus *Pseudoalteromonas* also induce larval metamorphosis in other invertebrates, including cnidarians (Negri et al. 2001, Seipp et al. 2007, Tran & Hadfield 2011, Tebben et al. 2011), bryozoans (Dobretsov & Qian 2006), molluscs (Alfaro et al. 2011, Pachu et al. 2012) and echinoderms (Huggett et al. 2006). Many species in the genus *Pseudoalteromonas* produce an array of organic compounds with a broad range of biological activities, reviewed in detail by Bowman (2007), but biological functions for most of them have not been reported.

To the best of our knowledge, only for the induction of metamorphosis in larvae of *H. elegans* by *P. luteoviolacea* HI1 is there significant information on the host's reaction to bacterial products (Shikuma et al. 2014, Ericson et al. 2019). However, we did not find evidence that settlement induction by the *Pseudoalteromonas* spp. isolated here involves a mechanism similar to that of the well-characterized tailocin assemblies in *P. luteoviolacea* HI1. The bacteria lack the necessary genes. This indicates that these inductive *Pseudoalteromonas* strains possess another, unidentified characteristic that induces larvae of *H. elegans* to settle. This is not surprising in light of the discoveries of Freckelton et al. (2017) that other strongly inductive bacteria produce none of the elements of the tailocin arrays reported for *P. luteoviolacea* HI1 by Shikuma et al. (2014). Instead, membrane vesicles from multiple bacterial genera are implicated in the inductive process. Tetrabromopyrrole extracted from *Pseudoalteromonas* strains J010 and PS5 was also reported to induce coral larval metamorphosis, but the compound did not first induce settlement in all coral species (Tebben et al. 2011). Different coral species had different responses to tetrabromopyrrole; for example, tetrabromopyrrole induced only metamorphosis and not settlement in *Acropora millipora*, but it did induce settlement in *A. palmata* (Sneed et al. 2014). Metamorphosis without prior attachment to the substratum would quickly lead to loss of the propagule. Another example is histamine produced by bacteria found on red algae induces larvae of 4 Australian echinoid species (Swanson et al. 2012).

Biofilms are complex systems with a number of characteristics that might contribute to larval settlement and metamorphosis. Future efforts should be made to explore the components of the biofilms of the inductive *Pseudoalteromonas* species to identify the attributes of their biofilms that cause the larvae to metamorphose, e.g. OMVs as reported by Freckelton et al. (2017) for other inductive bacteria. The consequence of interactions of bacteria in mixed species biofilms may also contribute to inducing larval metamorphosis. Alternatively, it could be a still yet to be cultured abundant bacteria that is driving settlement. The parsimonious larvae of biofouling species, known to inhabit bays and harbors around the world, have surely not evolved to settle in response to a single bacterial species, and surely not to the rarest ones. However, assuming that larvae have not evolved multiple recognition-response systems, it remains to be discovered just how larvae are cued by many different bacteria and their different cueing factors. Or are the mechanisms really that different? Until we know in detail the structures produced by most, perhaps all, inductive bacterial species, we will not be able to deduce what larvae actually 'do' with those cues.

*Acknowledgements*. Research reported here was supported by funds from grants to M.G.H. from the Gordon and Betty Moore Foundation (no. 5009) and the US Office of Naval Research (no. N00014-15-1-2658). We are pleased to acknowledge Grant Batzel for his assistance in sequencing bacterial isolates. We also thank Dr. Marnie Freckelton for providing useful comments and suggestions during the early drafting of the manuscript and Dr. Brian Nedved for making certain we had a continuous supply of *Hydroides elegans*.

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*Editorial responsibility: Jed Fuhrman, Los Angeles, California, USA*

*Submitted: April 23, 2019; Accepted: October 30, 2019*
*Proofs received from author(s): January 21, 2020*