Recruitment in the sea: bacterial genes required for inducing larval settlement in a polychaete worm

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Metamorphically competent larvae of the marine tubeworm *Hydroides elegans* can be induced to metamorphose by biofilms of the bacterium *Pseudoalteromonas luteoviolacea* strain H11. Mutational analysis was used to identify four genes that are necessary for met morphomic induction and encode functions that may be related to cell adhesion and bacterial secretion systems. No major differences in biofilm characteristics, such as biofilm cell density, thickness, biomass and EPS biomass, were seen between biofilms composed of *P. luteoviolacea* (H11) and mutants lacking one of the four genes. The analysis indicates that factors other than those relating to physical characteristics of biofilms are critical to the inductive capacity of *P. luteoviolacea* (H11), and that essential inductive molecular components are missing in the non-inductive deletion-mutant strains.

Introduction

Communities of benthic marine animals are established and maintained by recruitment of larvae of their member species, and larvae of most marine invertebrates recognize appropriate sites for settlement and metamorphosis by chemical cues from conspecific individuals or other associated species1. Bacteria in marine biofilms play an important role in the recruitment of many marine invertebrate species by producing cues to settlement for invertebrate larvae2–9. When invertebrate larvae select a surface on which to settle, they can differentiate between characteristics of a biofilm such as age10,11, bacterial density12,13, biochemical signals14–20, and the overall community composition7.

Hadfield and Paul1 reviewed a large literature on the topic of “settlement in response to biofilms,” citing data on the role of biofilms in settlement of larvae from 10 phyla, but finding virtually no identification of inducing substances. Subsequently, interest in the role of biofilms in recruitment of benthic marine invertebrates has been intense; a recent review of literature on the topic of marine biofilms yielded more than 1,000 references in the last 10 years. Despite this interest, there have been very few molecular components of biofilms identified as inducers of larval settlement. Studies implicating soluble substances from microbial components (e.g., amino acids, acyl homoserine lactones) of biofilms have mostly been discounted8. Two studies have identified probable bacterial products as settlement inducers for diverse invertebrates: histamine (either from an alga or bacteria on it) induces settlement and metamorphosis in larvae of the sea urchin *H. elegans* as strongly as natural, multispecies films5,25, and tetrabromopyrrole secreted by strains of *Pseudoalteromonas* isolated from the surfaces of coraline algae induces metamorphosis, but not settlement, in planula larvae of the coral *Acropora millepora*22. Related literature on possible bacterial sources of settlement inducers was recently reviewed by Hadfield8. To our knowledge, there have been no previous studies on the molecular genetic basis of bacterial induction of larval settlement.

*Hydroides elegans* is a common fouling polychaete in tropical and subtropical seas23. In the laboratory, the planktotrophic larvae of *H. elegans* become competent to settle and metamorphose in approximately 5 days24. Although the degree of settlement induced by some monospecific strains is rarely as great as with natural, multispecies films8–25, one Gram-negative bacterial strain, *Pseudoalteromonas luteoviolacea* (H11), induces metamorphosis of larvae of *H. elegans* as strongly as naturally occurring, multispecies biofilms22. Huang and Hadfield demonstrated that the inductive capacity of bacterial species is restricted to the biofilm phase and, while characteristic of only a fraction of biofilm bacterial species, is not phylogenetically constrained22. Further investigation is required to elucidate the molecular and cellular differences underlying the larval settlement-inducing capacity of bacteria that occur in biofilms.
Although the relationship between bacteria and induction of settlement in *H. elegans* has been the subject of many investigations\(^8\), the particular molecular cues and molecular mechanisms by which *P. luteoviolacea* (HI1) induces metamorphosis of *H. elegans* remain unknown. This study uses a classical genetic approach to identify genes from *P. luteoviolacea* (HI1) whose products are necessary to induce settlement and metamorphosis in those larvae. Establishing genetic markers that can be used to evaluate the inductive capacity of a wide spectrum of bacteria is a crucial step for understanding the role of biofilms in larval settlement and metamorphosis for many species that in turn may aid antifouling strategies.

**Results**

Screening for mutants of *P. luteoviolacea* (HI1) incapable of inducing larval settlement. Approximately 500 kanamycin-resistant transposon-Tn10 mutants of *P. luteoviolacea* (HI1) were screened for their capacity to induce settlement and metamorphosis of competent larvae of *H. elegans*. Two mutants that produced non-inductive biofilms, designated Plm9 and Plm45, were identified for further investigation (Fig. 1). When larvae were exposed to biofilms made by either of these two transposon mutants, their behavior was no different from that of larvae in autoclaved FSW and clean dishes (i.e., negative controls). The larvae continued to swim actively during the entire 24 hr period. Larvae that were exposed to a biofilm of wild-type *P. luteoviolacea* (HI1) immediately slowed swimming and started to crawl along the bottom of the dish. After 24 hours, 85–100% of the larvae settled and metamorphosed when they were exposed to a biofilm of wild-type *P. luteoviolacea* (HI1) or a natural biofilm.

However, fewer than 20% of larvae settled and metamorphosed when presented with biofilms made of either of the transposon mutants, which was significantly less than the positive control (p≤0.0001) (Fig. 1).

Identification of genes disrupted by the transposon. DNA sequencing of PCR products representing the regions flanking the Tn10 inserts in mutants Plm9 and Plm45 yielded a 21,517 bp nucleotide region of DNA comprised of 11 open reading frames (Fig. 2). The transposon in mutant Plm9 was inserted in open-reading frame 1 (ORF1) and in ORF3 for mutant Plm45. These two ORFs appear to be part of a seven-gene operon. The seven genes are in the same orientation, and intergenic regions range in length from –3 to + 100 nucleotides. Moreover, bands of appropriate sizes were obtained in reverse-transcription PCR for sets of primers flanking the individual intergenic regions of adjacent genes in the putative operon (Fig. 3) (No bands were present in all negative controls). Promoter-prediction analysis revealed a potential transcription start site around 22 bp upstream of the ORF1 start codon (sequence of –10 element ‘AGGTATGCT’ and sequence of –35 element ‘TTGACC’). Two potential transcription-factor (cAMP receptor protein and CynR, a LysR family member) binding sites were also predicted at 58 bp and 12 bp upstream of the start codon. ORF8 is oriented in the opposite direction of ORFs 1 to 7, indicating that ORF8 is part of a different transcript. This analysis suggests that ORFs 1 to 7 are in the same operon (Fig. 2). The nucleotide-sequence data for these 7 ORFs of *P. luteoviolacea* (HI1) were deposited in the GenBank database under accession numbers JQ217134, JQ217135, JQ217136, JQ217137, JQ217138, JQ217139, JQ217140.

Four ORFs required for inductive capacity. Deletion mutants were generated to confirm that the genes disrupted by transposon insertion were responsible for the lack of induction of metamorphosis of *H. elegans* by each mutant strain and to identify additional genes in the operon that may be necessary for the inductive capacity of the bacterium. Seven unmarked, in-frame deletion mutants corresponding to each of the seven ORFs were generated by allelic replacement. Depending on which open reading frame was deleted, the deletion mutants were designated PIDM1 through PIDM7. For each of the deletion-mutant strains, most of the targeted ORF was removed and the remaining 5’ and 3’ ends (51 bp) were fused in frame to avoid polar effects of the mutations. Deletions were confirmed by comparing the sizes of PCR products corresponding to each of the ORFs from wild type of *P. luteoviolacea* (HI1) and the deletion mutants PIDM1 through PIDM7 (Fig. 4a and 4b).

Of the 7 deletion mutants, the first four, PIDM1, PIDM2, PIDM3, and PIDM4, lost the capacity to induce settlement and metamorphosis for larvae of *H. elegans* (Fig. 4c, p<0.0001); percent settlement in response to PIDM5, PIDM6, and PIDM7 was not significantly
different from that of wild-type *P. luteoviolacea* (HI1) (Fig. 4c, \( P = 0.0673 - 0.1038 \)). Deletion analysis indicated that ORFs 1, 2, 3 and 4 were required for the inductive capacity of *P. luteoviolacea* (HI1).

**Comparison of the growth rate of wild-type *P. luteoviolacea* (HI1) and the deletion mutants.** Figure 5 depicts the growth curves of wild-type *P. luteoviolacea* (HI1) and its non-inductive deletion mutants (PDM1- PDM4) in liquid \( \frac{1}{2} \) SWT medium. Deletion mutants PDM1- PDM4 exhibited growth patterns similar to that of wild type *P. luteoviolacea* (HI1), which indicates the lack of inductive capacity in deletion mutants (PDM1-PDM4) was not attributable to simple differences in growth rates.

**Correlation between inductive capacity and induction genes.** The sequences of the 16S rRNA genes from the Hawaiian strain of *Pseudoalteromonas luteoviolacea* (HI1) and the strain of this bacterium held by the American Type Culture Collection (*P.
P. luteoviolacea ATCC334926) are 99.9% identical, suggesting that the two strains are very similar. However, larval settlement and metamorphosis in response to these two strains are significantly different (p<0.0004). Larval settlement on a biofilm of P. luteoviolacea ATCC334926 was less than 10% and not significantly different from that in a sterile dish filled with autoclaved, filtered seawater (negative control) (p=1.00) (Fig. 6). Conversely, settlement on both a biofilm of P. luteoviolacea (HI1) and a natural biofilm (N.B.) was above 80%.

**Figure 4** | Deletion mutants of P. luteoviolacea (HI1) and analysis of their inductive capacity. a, amplicons with primers complementary to flanking regions of each ORF from deletion mutants PlDM1 to PlDM7 (M: DNA Marker); b, amplicons with the same primer sets from wild type P. luteoviolacea (P.l. (HI1)); c, settlement (%) of H. elegans on biofilms made from deletion mutants PlDM1, PlDM2, PlDM3, PlDM4, PlDM5, PlDM6, PlDM7 and wild type P. luteoviolacea (HI1). Clean Petri dishes filled with FSW were negative controls. Bars represent mean percentages of larvae that settled in 24 h +/- SD (n = 5). * denotes significant difference compared with P. luteoviolacea (HI1) (Kruskal-Wallis test, p<0.01).

**Figure 5** | Growth of wild type of P. luteoviolacea (HI1) and 4 deletion mutants for four open reading frames, PlDM1, PlDM2, PlDM4 and PlDM4. Y-axis represents optical density of bacterial broth at 600 nm.
significantly greater than the negative control (*p* = 0.0045 and *p* = 0.0004) (Fig. 6). Probes to each of the four induction genes from strain HI1 were developed and used in Southern blot analysis to determine if there are corresponding genes in *P. luteoviolacea ATCC334926* with significant sequence similarity. As seen in figure 7, no bands appeared in digests of the *P. luteoviolacea ATCC334926* with probes to ORF1, ORF2 and ORF4, although one light and much smaller fragment for ORF3 was present. This evidence indicates that this area of the genome of *P. luteoviolacea ATCC334926* is very different from that of strain HI1, perhaps even missing, which may account for the difference in metamorphic induction capability of the two strains.

**Prediction of putative gene functions.** Putative protein functions were determined by BLAST analysis of the translated nucleotide sequences of ORFs 1 – 7 with NCBI's protein data bank; a cut-off *e*-value of 0.001 was used. Proteins corresponding to both ORFs 1 and 2 contain the conserved domain TIGR02243, which is a large, conserved hypothetical phage-tail-like protein that is similar to components of Type VI secretion systems. ORF2 encodes a multi-domain protein. In addition to the TIGR02243 domain, the protein encoded by ORF 2 belongs to superfamily CL09931, NADB_Rossmann superfamily, which is found in numerous dehydrogenases of metabolic pathways such as glycolysis, and many other redox enzymes.

ORF3 encodes a putative protein that may function in cell adhesion or aggregation; it has similarity to YadA domain-containing proteins or adhesion-like proteins. YadA domain has been shown to be a major adhesin and is necessary for virulence of some strains of *Yersinia*. However, ORF3 does not appear to contain the YadA domain itself. The translated ORF4 sequence lacked recognizable domains and is similar to only hypothetical proteins of unknown function.

**Comparison of biofilm characteristics between wild type *P. luteoviolacea* (HI1) and the deletion mutants.** The putative functions of the ORFs shown to be required for induction of morphogenesis suggested that they might be involved in biofilm production. Accordingly, cell density, biofilm thickness, biofilm cell biomass and EPS biomass of biofilms composed of wild type *P. luteoviolacea* (HI1) were compared with those of the 7 deletion mutants (Table 1). Cell densities in biofilms from mutant strains PlDM3 and PlDM5 (> 18 × 10^3 cells mm^-2) were significantly greater than in the wild type (*p* = 0.0003 and *p* = 0.0014), while...
those from the other deletion mutant strains were not shown to be significantly different from that of the wild type \((p=0.1279 – 1.00)\). Mean biofilm thicknesses of the strains were similar, and none of the deletion-mutant strains was significantly different from the wild type \(P. luteoviolacea\) \((HI1)\) \((p=0.2703)\). Mutant strain PDM3 was the only strain among seven deletion mutants whose total biomass and EPS biomass were significantly greater than those of wild type of \(P. luteoviolacea\) \((HI1)\) \((p=0.0082 \text{ and } p=0.0097)\) (Table 1). These results indicate that ORF3 appears to negatively regulate with thickness and density of biofilm.

**Discussion**

Ubiquitous components of shallow water marine biofilms, *Pseudoalteromonas* species have been found to provide cues for larval settlement and metamorphosis for a variety of marine invertebrate species.\(^6\,9\,21\,22\) However, the inductive capacity of the bacteria varies both between species of a single genus\(^12\) and between strains of single species, such as those of *P. luteoviolacea* evaluated here (Fig. 6). Variation in inductive capacity between these strains suggests that small genetic changes can result in disparate inductive capacities.

In the present study, two mutant strains, Plm9 (ORF1 disrupted) and Plm45 (ORF3 disrupted), created by random transposon insertion were found to have lost the capacity to induce larval settlement in the tubeworm. Although it was possible that these genes are essential for induction, it was also possible that the insertion of the transposon disrupted the functions of genes downstream from those with the inserted transposon. To examine this possibility, deletion mutants were generated that were non-polar and thus would be less likely to affect transcription and translation of downstream genes.

Deletion mutants lacking one of the first four open-reading frames in a single putative operon lost the capacity to induce settlement of *H. elegans*, and deletion mutants of the remaining three downstream open-reading frames remained inductive (Fig. 4c). The growth curves of deletion-mutant strains for the first 4 open-reading frames and for wild type *P. luteoviolacea* \((HI1)\) reveal that all these strains reach stationary phase after 4 hrs in broth culture (Fig. 5), strongly suggesting that the loss of inductive activity in the deletion mutants is not due simply to differences in either growth stage or density of the bacteria in cultures (our biofilms are made from overnight bacterial broth cultures). This evidence suggests that the products of these genes are required in a more fundamental manner for the settlement process of *H. elegans*. Significantly, ORF1, ORF2 and ORF4 identified in *P. luteoviolacea* \((HI1)\) are absent in the non-inductive strain *P. luteoviolacea* ATCC3349\(^26\). These results provide strong evidence that the region of the genome identified in the study contains important genes whose products are necessary for inducing the settlement and metamorphosis of *H. elegans* and perhaps larvae of other invertebrate species. It will be of great interest to learn if products of the same genes are involved in metamorphic induction of an Australian sea urchin whose larvae are known to respond to *P. luteoviolacea*\(^6\) and the coral *Pocillopora damicornis*\(^45\). We have very preliminary data (not shown) suggesting that the mutants of *P. luteoviolacea* (Plm9 and Plm45) created in the current study fail to induce larval settlement of *P. damicornis*.

The physical and chemical attributes of bacterial biofilms are known to affect the settlement and metamorphosis of marine invertebrates.\(^7\,9\,11\) Thus, the biofilm phenotypes of wild type *P. luteoviolacea* \((HI1)\) and the deletion mutants were compared to

### Table 1 | Biofilm characteristics of wild type *P. luteoviolacea* \((HI1)\) and its deletion mutants

| Bacterial strains | Biofilm cell density \(\times 10^3\) cells mm \(^{-2}\) | Biofilm thickness (µm) | Biofilm cell biomass (µm\(^2\)·µm \(^{-2}\)) | EPS biomass (µm\(^3\)·µm \(^{-2}\)) |
|-------------------|-----------------------------|------------------|----------------|------------------|
| *P. luteoviolacea* \((HI1)\) | 1.61±0.81 | 0.40±0.36 | 0.17±0.11 | 0.09±0.07 |
| PDM1 | 6.33±4.13 | 0.39±0.17 | 0.18±0.07 | 0.14±0.06 |
| PDM2 | 3.62±3.95 | 0.10±0.10 | 0.09±0.05 | 0.05±0.04 |
| PDM3 | 27.55±18.30* | 0.62±0.26 | 0.50±0.18* | 0.33±0.14* |
| PDM4 | 5.83±6.58 | 0.56±0.28 | 0.25±0.09 | 0.21±0.07 |
| PDM5 | 18.34±7.79* | 0.45±0.31 | 0.28±0.15 | 0.19±0.11 |
| PDM6 | 8.11±6.12 | 0.71±0.47 | 0.31±0.13 | 0.25±0.11 |
| PDM7 | 7.05±4.62 | 0.44±0.30 | 0.20±0.11 | 0.17±0.11 |

Comparison were made between characteristics of wild type *P. luteoviolacea* \((HI1)\) and its deletion mutants: PDM1.7. Means of biofilm cell density were compared with a Kruskal-Wallis chi-square test, followed by Bonferroni correction for multiple comparisons. Biofilm thickness, biofilm cell biomass and EPS biomass were compared with a One-Way ANOVA, followed by Bonferroni correction for multiple comparisons also. Data listed in the table represent mean value ± SD (n=3–5).

### Table 2 | Bacterial strains and plasmids used in this study

| Bacterial strain | Description | Source or reference |
|------------------|-------------|---------------------|
| **E. coli**      | Δ[ara-leu] araD ΔlacX74 galE galK phoA20 thiI rpsE rpoB argE(Am) recAl λpir | 48 |
| CC118 [λpir]    | thiI thr leu tonA lacY supC recA::RP4-2-Tc::Mu, Km, λpir | 48 |
| SM10 [λpir]     | Wild-type isolate | 12 |
| **P. luteoviolacea** | Isolated from seawater, France, ATCC number: 33492 | 26 |
| HI1              | Spontaneous streptomycin-resistant, mutant of Hawaii strain | This study |
| ATCC3349         | Plasmids | 48 |
| pLOF/Km | Amp\(^r\) \((Tn10 based delivery plasmid, Km\(^r\))\) | 48 |
| pCVD442         | Ap\(^r\), Sucrose\(^r\), 1·2 kb Sac\(^b\) gene inserted into pG704 at cloning site PstI | 41 |
| pCVD443         | Ap\(^r\), Km\(^r\), Sucrose\(^r\), pCVD442 with Km\(^r\) gene inserted at Smal site | This study |
| pDM1            | Ap\(^r\), Km\(^r\), Sucrose\(^r\), pCVD443 with 1.6 kb of DNA flanking ORF1 | This study |
| pDM2            | Ap\(^r\), Km\(^r\), Sucrose\(^r\), pCVD443 with 1.6 kb of DNA flanking ORF2 | This study |
| pDM3            | Ap\(^r\), Km\(^r\), Sucrose\(^r\), pCVD443 with 1.6 kb of DNA flanking ORF3 | This study |
| pDM4            | Ap\(^r\), Km\(^r\), Sucrose\(^r\), pCVD443 with 1.6 kb of DNA flanking ORF4 | This study |
| pDM5            | Ap\(^r\), Km\(^r\), Sucrose\(^r\), pCVD443 with 1.6 kb of DNA flanking ORF5 | This study |
| pDM6            | Ap\(^r\), Km\(^r\), Sucrose\(^r\), pCVD443 with 1.6 kb of DNA flanking ORF6 | This study |
| pDM7            | Ap\(^r\), Km\(^r\), Sucrose\(^r\), pCVD443 with 1.6 kb of DNA flanking ORF7 | This study |
examine the possibility that the products of the corresponding genes are involved in the physical structure of the biofilms. However, biofilm characteristics, including cell density, average biofilm thickness, biofilm biomass, and EPS biomass (evaluated from specific carbohydrates) of the 7 deletion mutants were not significantly lower than those of wild type *P. luteoviolacea* (HI1), indicating that it is not simply the gross physical characteristics of the biofilms that make *P. luteoviolacea* (HI1) indusive for settlement and morphogenesis of larval *H. elegans*. Therefore, it is most likely a missing or altered specific molecular component in the biofilm that results in loss of inductive action in the deletion mutants of *P. luteoviolacea* (HI1).

**Table 4 | Sequences of oligonucleotides used in overlapping extension PCR to generate plasmids pDM 1-7**

Table 3 | Sequences of oligonucleotides used in genome walking

| ORF1 | Pldm1-1L | 5'-TTTGTCGACACAGCAATCTGCAGGCGAAAGC-3' |
| ORF1 | Pldm1-1R | 3'-CAATCCCTCTGCGACCCCTTACGGTGTCAGAT-5' |
| ORF1 | Pldm1-2L | 5'-CGGTAAAGGGGCGCGAGGAGGATIGGAAATGGA-3' |
| ORF1 | Pldm1-2R | 5'-TTTCGATGCAGGGGTTAATTAGCGCAATA-5' |
| ORF2 | Pldm2-1L | 5'-TTTGTCGACCTGCACTATATGCAGGCGTCAT-3' |
| ORF2 | Pldm2-1R | 3'-CGATAAAAACTCCAGGATTTACGGGAGAT-5' |
| ORF2 | Pldm2-2L | 5'-AACCCCTGGGAAATTTATCGTCGACCGCAACG-3' |
| ORF2 | Pldm2-2R | 3'-TTTGTCGACACCTACACGGCGCCCTCA-5' |
| ORF3 | Pldm3-1L | 5'-TTTGTCGACACCGATCTACTGGGAAATG-3' |
| ORF3 | Pldm3-1R | 3'-GGGCATGCCAATTGCTGAATGGCATGACTTCT-5' |
| ORF3 | Pldm3-2L | 5'-ATTTGCCAATCTGGCAGGCCCTTGTTATTTAG-3' |
| ORF3 | Pldm3-2R | 3'-TTTGTCGACAAAACTCTGGGCTGCTAC-5' |
| ORF4 | Pldm4-1L | 5'-GTCGACCATCGGCCAGTGGTTCTCTAGAGC-3' |
| ORF4 | Pldm4-1R | 3'-CTAACCAAGGTTTGCGAGGGCTTTACTATT-5' |
| ORF4 | Pldm4-2L | 5'-CAGCTGCAGGCCTTGTAAGCAGCAGGATCTC-3' |
| ORF4 | Pldm4-2R | GTCAACCCAAAGCAGTTGATTGCACTT-5' |
| ORF5 | Pldm5-1L | 5'-GTCGACGCTGGGCGTGAGCTATAGTTCT-3' |
| ORF5 | Pldm5-1R | 3'-GAAAATCGGGAAATATGTTTTGGGGCTGGTG-5' |
| ORF5 | Pldm5-2L | 5'-AACCACATTCTCGGAGTTCATGCTTCTAG-3' |
| ORF5 | Pldm5-2R | GTGACACTTGTGACGTGATTTTGAG-5' |
| ORF6 | Pldm6-1L | 5'-GCTACGACGCTGGGCGTGAGCTATAGTTCT-3' |
| ORF6 | Pldm6-1R | 3'-TTTCGATGCAGGGGTTAATTAGCGCAATA-5' |
| ORF6 | Pldm6-2L | 5'-TCATATGCTGCTATTGAAATATGGCGGAAT-3' |
| ORF6 | Pldm6-2R | 3'-GTCGACGACTGGTCTTCTTTTGGCAGACTT-5' |
| ORF7 | Pldm7-1L | 5'-TTTGTCGACTGGTCTTCTTTTGGCAGACTT-3' |
| ORF7 | Pldm7-1R | 3'-TTGACACTTGTGACGTGATTTTGAG-5' |
| ORF7 | Pldm7-2L | 5'-AGAAGGGAACCTGCCCAGATGACAGATTT-3' |
| ORF7 | Pldm7-2R | 3'-TTGACACTTGTGACGTGATTTTGAG-5' |
secretion system or biofilm formation, either of which may be important in inducing larval settlement and metamorphosis in *H. elegans*. The exact mechanism of induction remains an open and interesting question, but may be difficult to determine. Finally, investigation of the importance of the identified gene cluster in inducing settlement of larvae from many different phyla known to be induced by biofilms will yield interesting phylogenetic implications.

### Methods

**Spawning, larval culture and larval settlement bioassay.** Specimens of *Hydroides elegans* were spawned and larvae were cultured as previously described23,25. Wild type and mutant strains of *PseudolOTORONUS lutoviolacea* were subjected to a larval settlement bioassay as described by Huang and Hadfield46.

**Bacterial strains, plasmids, media and growth conditions.** Bacterial strains and plasmids used in this study are described in Table 2. The Hawaii strain, *P. lutoviolacea* (HII), was previously isolated from a seawater table at Kewalo Marine Laboratory (KML), Honolulu, HII. A spontaneously occurring streptomycin-resistant mutant of *P. lutoviolacea* (*P. lutoviolacea* (HII, Str³)) was experimentally selected from the Hawaii strain for mutagenesis experiments; a larval settlement assay showed that it maintained the capacity to induce settlement and metamorphosis in larvae of *H. elegans*. Another strain, *P. lutoviolacea* ATCC3349⁷, obtained from the American Type Culture Collection, is 99.99% identical with *P. lutoviolacea* (HII) in the 16S rDNA gene sequence.

All strains of *P. lutoviolacea* were maintained on agar or in liquid seawater media containing 0.25% tryptone (W/V), 0.15% yeast extract (W/V) and 0.15% glycerol (V/V) (1/2 SWT), at room temperature (~25°C). Luria-Bertani (LB) medium was routinely used to grow strains of *E. coli* on agar plates or in liquid culture at 37°C.

### DNA sequence analysis

Homology searches and sequence comparisons were performed in GenBank, National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) using the BLAST-search algorithm. Open reading frames (ORFs) were determined by the ORF Finder program through the NCBI website. Promoter prediction was performed using the BPROM program, which predicts bacterial promoters and is available through the Softberry website (http://linux1.softberry.com/berry.phtml?topic=index&group=program &subgroup= promoter).

### DNA manipulation and sequencing

The primers and DNA oligonucleotides used in this study are listed in Table 3. Genomic DNA of each candidate mutant was extracted with a MoBio UltraClean™ Microbial DNA kit following the manufacturer’s instructions. For transposon mutants, the region of DNA flanking the transposon insertion site was isolated by successive rounds of panhandle PCR27. Two primers, Tn10T and Tn10C, that were complementary to the 5′ and 3′ ends of the mini-Tn10 transposon28, were used individually in PCR reactions with the Adapter 1 primer in the first round of PCR. Subsequent rounds of panhandle PCR for genome walking were conducted using genome-specific primers, which were designed in the transposon flanking regions elucidated with earlier rounds of PCR, and Adapter primer 1 as primer pairs to obtain the upstream and downstream regions. The steps above were repeated until the sequence of the entire region was obtained. PCR products were excised and purified agarose ethidium bromide gels using a MinElute Gel Extraction Kit (Qiagen) according to the manufacturer’s protocol.

DNA sequence analysis. Homology searches and sequence comparisons were performed in GenBank, National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) using the BLAST-search algorithm. Open reading frames (ORFs) were determined by the ORF Finder program through the NCBI website. Promoter prediction was performed using the BPROM program, which predicts bacterial promoters and is available through the Softberry website (http://linux1.softberry.com/berry.phtml?topic=index&group=program &subgroup= promoter).

### Southern blot

Genomic DNA of wild type *P. lutoviolacea* (HII) was performed in TRI REAGENT (Invitrogen) following the manufacturer’s instructions. One microgram of genomic DNA of both strains of *P. lutoviolacea* was digested with restriction enzyme Hind III or Kpn I at 37°C overnight. Hybridization probes were designed from ORFs 1–4 (Probe primers are listed in Table 4), amplified by PCR, and biotinylated with NERLOT® Photoprobe kit (NEB®) according to the manufacturer’s instructions. Southern blot was performed with standard technique32,42. Blots were detected with a LightShift® Chemiluminescent RNA EMSA Kit (Thermo Scientific®) following the manufacturer’s instructions.

### Reverse transcription PCR

Total RNA isolation from overnight cultures of *P. lutoviolacea* (HII) and *P. lutoviolacea* ATCC3349⁷ was extracted with a MoBio UltraClean™ Microbial DNA kit following the manufacturer’s instructions. One microgram of genomic DNA of both strains of *P. lutoviolacea* was digested with restriction enzyme Hind III or Kpn I at 37°C overnight. Hybridization probes were designed from ORFs 1–4 (Probe primers are listed in Table 4), amplified by PCR, and biotinylated with NERLOT® Photoprobe kit (NEB®) according to the manufacturer’s instructions. Southern blot was performed with standard technique32,42. Blots were detected with a LightShift® Chemiluminescent RNA EMSA Kit (Thermo Scientific®) following the manufacturer’s instructions.
Each time point, the OD values were read 3 times, and the mean value was recorded. The optical density at 600 nm was recorded every half hour for 6 hours. At phenylindole, dihydrochloride (DAPI, Sigma SCIENTIFIC) was replaced reverse transcriptase with H2O and served as negative controls.

Growth rate determination. One colony of wild type P. luteoviolacea (HI1) and each of the 4 deletion mutant strains (PIDM 1–4) were inoculated into 2 ml of ½ SWT broth medium and incubated overnight. 100 μl of overnight culture of each strain was then inoculated into 10 ml of ½ SWT broth. Three replicates were prepared for each strain. The optical density at 600 nm was recorded every half hour for 6 hours. At each time point, the OD values were read 3 times, and the mean value was recorded.

Biofilm preparation and biofilm staining or labeling. Biofilms of P. luteoviolacea (HI1) and the 7 deletion mutants were formed on coverslips as described by Huang and Hadfield12. Biofilms on the coverslips were fixed in 3% formaldehyde in FSW for at least 10 minutes. Bacterial cells were stained by 100 ng/ml of 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Sigma®). EPS in the biofilms were labeled by 10 μg/ml of lectins conjugated with fluorescein isothiocyanate (FITC) as describe by Strathmann et al.14. Lectins used were concanavalin A (Con A, Sigma®) (10 μg/ml) and wheat germ agglutinin (WGA, Sigma®).

Confocal laser scanning microscopy (CLSM) and image analysis. All microscopic observations and image acquisition were performed on a Zeiss LSM 710 confocal laser scanning microscope (Zeiss®@, NY, USA). Three to five replicate biofilms were examined for each strain of bacteria. Ten non-overlapping fields of view of each biofilm were chosen randomly for imaging and analysis. Each field of view had an area of 220 μm×220 μm. 16–20 image stacks of varying thicknesses were generated to determine the full thickness of the biofilm in each field of view. Bacterial densities were counted using Image J software (http://rsweb.nih.gov/ij/). Other biofilm parameters were quantified using COMSTAT software16.

Statistical analysis. The data were arcsine-transformed according to the needed and tested for normality and homogeneity of variance by using Shapiro-Wilk’s test and Cochran’s C-test. If the assumptions for a parametric test were met, then means were compared among strains using ANOVA; if not, a nonparametric Wilcoxon analysis was performed using a Kruskal-Wallis chi-square approximation to indicate the significance of differences using the untransformed data15.

Table 7 | Sequences of the primers used for cDNA synthesis and PCR

| Amplified region | Nucleotide sequence (5’→3’) |
|------------------|---------------------------|
| Primers for cDNA synthesis | AATACATTGTGGTGCTGCTCCA CCAGAGACGGCTTAAACCTC GCTGCTACAGCCAGTATAA CCAGAGGCAGTTAAGAC TGCCTTTGTCAGAGGG TGCTACACCTTCTGATACTTCA |
| Upstream of ORF2 | TAAGTTCCGACACGCATCTA CTGTGCTGGAAACACATCTA AAATTATCGGGGCACCGAG TCCAATACCTCCCCGTGCG TGGAGCAGCCGACCATTTAC |
| Upstream of ORF3 | AAGCCATTAGGCTAATATCTG CGCCATCGATGCATTTAGAC TGCCGCACCTGATACTGCTG |
| Upstream of ORF4 | CGGACTCGGATATGCAAGTCC TGACCCATCGTGAAGTTGGA |
| Upstream of ORF5 | TGATTCTTGCTGCTAAAACTTGA |
| Upstream of ORF6 | TGGACATCCTCTCAGGATACTT |

1. Unabia, C. R. C. & Hadfield, M. G. Role of bacteria in larval settlement and metamorphosis of the polychaete Hydroides elegans. Mar. Biol. 133, 55–64 (1999).
2. Huggett, M., Williamson, J. E., Nys, R., Kjelleberg, S. & Steinberg, P. D. Larval settlement of the common Australian sea urchin Heliocidaris erythrogramma in response to bacteria from the surface of coralline algae. Oecologia 149, 604–619 (2006).
3. Webster, N. S. et al. Morphogenesis of a scleractinian coral in response to microbial biofilms. Appl. Environ. Microbiol. 70, 1231–1237 (2004).
4. Hadfield, M. G. Biofilms and marine invertebrate larvae: What bacteria produce microbial films: effects of different types of films. J. Exp. Mar. Biol. Ecol. 185, 235–253 (1995).
5. Wieczorek, S. K., Clare, A. S. & Todd, C. D. Inhibition and facilitatory effects of different types of films on settlement of Balanus amphitrite amphitrite larvae. Mar. Ecol. Prog. Ser. 119, 221–228 (1995).
6. Huang, S., Hadfield, M. G. Composition and density of bacterial biofilms determine larval settlement of the polychaete Hydroides elegans. Mar. Ecol. Prog. Ser. 260, 161–172 (2003).
7. Maki, J. S., Rittschof, D., Costlow, J. D. & Mitchell, R. Inhibition of attachment of larval barnacles, Balanus amphitrite, by bacterial surface films. Mar. Biol. 97, 199–206 (1988).
8. Kirchman, D. G., Graham, S., Reish, D. & Mitchell, R. Lectins may mediate in the settlement and metamorphosis of Janua (Dexiosis) brasiliensis Grube (Polychaeta:Spionidae). Inv. Reprod. Dev. 31, 109–122 (1997).
9. Hofmann, D. K. & Brand, U. Induction of Metamorphosis in the Symbiotic Scyphozoan Cassiopea andromeda: Role of Marine Bacteria and of Biochemicals. Symbiosis 9, 99–116 (1987).
10. Zimmer-Faust, R. K. & Tamburini, M. N. Chemical identity and ecological implications of a waterborne, larval settlement cue. Limnol. Oceanogr. 39, 1075–1087 (1994).
11. Hung, O. S. et al. Characterization of cues from natural multi-species biofilms that induce larval attachment of the polychaete Hydroides elegans. Aquat. Biol. 4, 253–262 (2009).
12. Swanson, R. L. et al. Induction of Settlement of Larvae of the Sea Urchin Holopneustes purpurascens by Histamine From a Host Alga. Biological Bulletin 206, 161–172 (2004).
13. Tebben, J. et al. Induction of larval metamorphosis of the coral Acropora millepora by tetrabromopyruvole Isolated from a Pseudoalteromonas Bacterium. PloS one 6, 1–8 (2011).
36. Dobretsov, S. & Qian, P. Y. Facilitation and inhibition of larval attachment of the polychaete Hydroides elegans Haswell (Serpulidae). *Biol. Bull.* **194**, 14–24 (1998).

23. Nedved, B. T. & Hadfield, M. G. in Marine and Industrial Biofouling (eds H. C. Flemming, R. Venkataram, S. P. Murthy, & K. Cooksey) (Springer, 2008).

24. Carpizo-Inuarte, E. & Hadfield, M. G. Stimulation of metamorphosis in the polychaete *Hydroides elegans* Haswell (Serpulidae). *Biol. Bull.* **194**, 14–24 (1998).

25. Hadfield, M. G., Unabia, C. C., Smith, C. M. & Michael, T. M. in Recent Developments in Biofouling Control (eds M. F. Thompson, R. Nagabhushanam, R. Sarojini, & M. Fingerman) 65–74 (Oxford and IBH Publishing Co., 1994).

26. Gauthier, G., Gauthier, M. & Christen, R. Phylogenetic analysis of the genera *Alteromonas*, *shewanella*, and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (Emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations. *Int. J. Syst. Bacteriol.* **45**, 755–761 (1995).

27. Pell, L. G., Kanelis, V., Donaldson, L. W., Lynne Howell, P. & Davidson, A. R. The phase major tail protein structure reveals a common evolution for long-tailed phages and the type VI bacterial secretion system. *Proc. Natl. Acad. Sci. USA* **106**, 4160–4165 (2009).

28. Leiman, P. G. et al. From the Cover: Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. *Proc. Natl. Acad. Sci. USA* **106**, 4154–4159 (2009).

29. Richter, N., Breicha, K., Hummel, W. & Niefiend, K. The three-dimensional structure of AKR11B, a glycerol dehydrogenase from *Gluconobacter oxydans*, reveals a tryptophan residue as an accelerator of reaction turnover. *J. Mol. Biol.* **304**, 335–362 (2010).

30. El Tahir, Y. & Skurnik, M. YadA, the multifaceted Yersinia adhesin. *Int. J. Med. Microbiol.* **291**, 209–218 (2001).

31. Holmstrom, C. & Kjelleberg, S. Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiology Immunology* **30**, 285–293 (1999).

32. Skovhus, T. L., Holmstrem, C., Kjelleberg, S. & Dalhoff, I. Molecular investigation of the distribution, abundance and diversity of the genus *Pseudoalteromonas* in marine samples. *Fems Microbiology Ecology* **61**, 348–361 (2007).

33. Tran, C. & Hadfield, M. G. Settlement and metamorphosis of the larvae of *Pocillopora damicornis* (Anthozoa) in response to surface-biofilm bacteria. *Marine Ecology Progress Series* **333**, 85–96 (2011).

34. Maki, J. S., Rittschof, D., Schmidt, A. R., Snyder, A. C. & Mitchell, R. Factors controlling attachment of bryozoan larvae: A comparison of bacterial films and unfilmed surfaces. *Biol. Bull.* **177**, 295–302 (1989).

35. Qian, P. Y., Thiayagarajan, V., Lau, S. C. K. & Cheung, S. C. K. Relationship between bacterial community profile in biofilm and attachment of the acorn barnacle *Balanus amphitrite*. *Aquat. Microb. Ecol.* **33**, 225–237 (2003).

36. Dobretsov, S. & Qian, P. Y. Facilitation and inhibition of larval attachment of the bryozoan *Bugula neritina* in association with mono-species and multi-species biofilms. *J. Exp. Mar. Biol. Ecol.* **333**, 263–274 (2006).

37. Hayes, C. S., Aoki, S. K., and Low, D. A. Bacterial contact-dependent delivery systems. *annual Review of Genetics* **44**, 71–90 (2010).

38. Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D. & Mekalanos, J. J. Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proc. Natl. Acad. Sci. USA* **104**, 15508–15513 (2007).

39. Egan, S., James, S. & Kjelleberg, S. Identification and characterization of a putative transcriptional regulator controlling the expression of fouling inhibitors in *Pseudoalteromonas tunicata*. *Appl. Environ. Microbiol.* **68**, 372–378 (2002).

40. Siebert, P. D., Chenchik, A., Kellogg, D. E., Lukyanov’, K. A. & Lukyanov’, S. A. An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Res.* 23, 1087–1088 (1995).

41. Donnenberg, M. S. & Kaper, J. B. Construction of an eae deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infec. Immun.* **59**, 4310–4317 (1991).

42. Ausubel, F. M. et al. Current Protocols in Molecular Biology. (John Wiley & Sons, Inc., 1989).

43. Sambrook, J., Fritsch, E. F. & Maniatis, T. Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor Laboratory Press, 1989).

44. Heydorn, A. et al. Quantification of biofilm structure by the novel computer program COMSTAT. *Microbiology* **146**, 2395–2400 (2000).

45. Sokal, R. R. & Rohlf, F. J. Biometry: the principles and practice of statistics in biological research. 2nd edn, 859 (W. H. Freeman and Company, 1981).

46. Herrero, M., Lorenzo, V. d. & Timmis, K. N. Transposon Vectors Containing Non-Antibiotic Resistance Selection Markers for Cloning and Stable Chromosomal Insertion of Foreign Genes in Gram-Negative Bacteria. *Journal of Bacteriology* **172**, 6557–6567 (1990).

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**Author contributions**

YH carried out all laboratory procedures under the guidance of SC and MGH. All authors contributed to writing the manuscript.

**Additional information**

**Competing financial interests:** The authors declare no competing financial interests.

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