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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 HI1. Mutational analysis was used to identify four genes that are necessary for metamorphic 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* (HI1) 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* (HI1), 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 species[1]. Bacteria in marine biofilms play an important role in the recruitment of many marine invertebrate species by producing cues to settlement for invertebrate larvae[2–9]. When invertebrate larvae select a surface on which to settle, they can differentiate between characteristics of a biofilm such as age[10,11], bacterial density[12,13], biochemical signals[14–20], and the overall community composition[7].

Hadfield and Paul[1] 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 discounted[8]. 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 *Holopneustes* purpurascens[21]; and tetrabromopyrrole secreted by strains of *Pseudoalteromonas* isolated from the surfaces of coralline 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 Hadfield[8]. 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 seas[23]. In the laboratory, the planktotrophic larvae of *H. elegans* become competent to settle and metamorphose in approximately 5 days[24]. Competent larvae are induced to settle and rapidly metamorphose by the presence of a well-developed biofilm[5]. Although the degree of settlement induced by some monospecific strains is rarely as great as with natural, multispecies films[5,25], one Gram-negative bacterial strain, *Pseudoalteromonas luteoviolacea* (HI1), induces metamorphosis of larvae of *H. elegans* as strongly as natural, multispecies biofilms[12]. 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 constrained[12]. 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 PlDM1 through PlDM7. 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 PlDM1 through PlDM7 (Fig. 4a and 4b).

Of the 7 deletion mutants, the first four, PlDM1, PlDM2, PlDM3, and PlDM4, lost the capacity to induce settlement and metamorphosis for larvae of *H. elegans* (Fig. 4c, $p < 0.0001$); percent settlement in response to PlDM5, PlDM6, and PlDM7 was not significantly
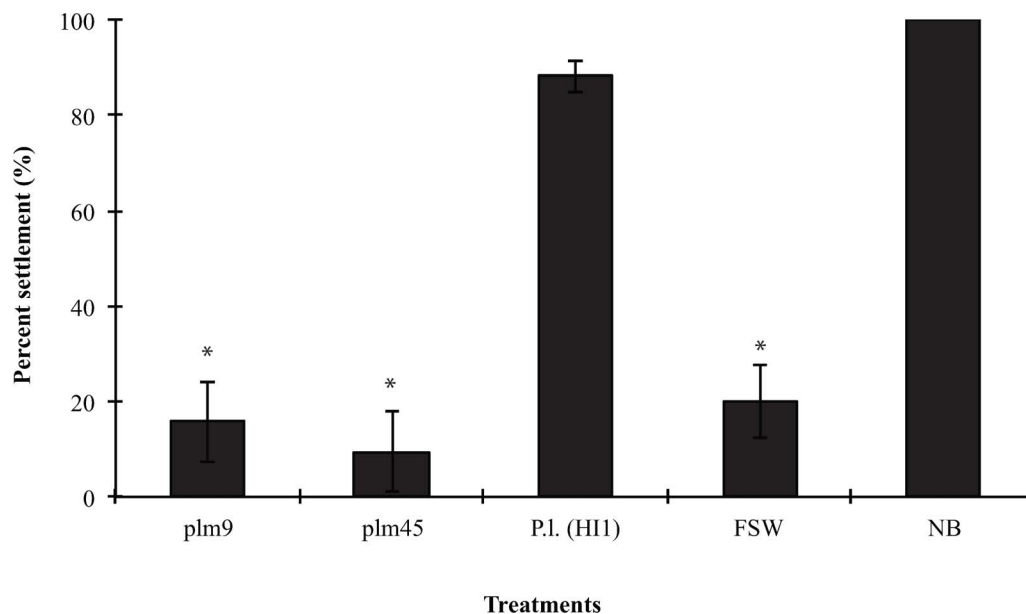

Figure 1 | Settlement (%) of *H. elegans* on biofilms made by transposon mutants Plm9, Plm45, and wild type (P.l.) of *P. luteoviolacea* (HI1). Dishes coated by natural biofilm (NB) served as positive controls; untreated Petri dishes filled with autoclaved filtered seawater (FSW) were negative controls. Bars represent mean percentages of larvae that settled in 24 h +/− SD (n = 5). * denotes significant differences compared with the positive control (Kruskal-Wallis test, $p < 0.0001$).
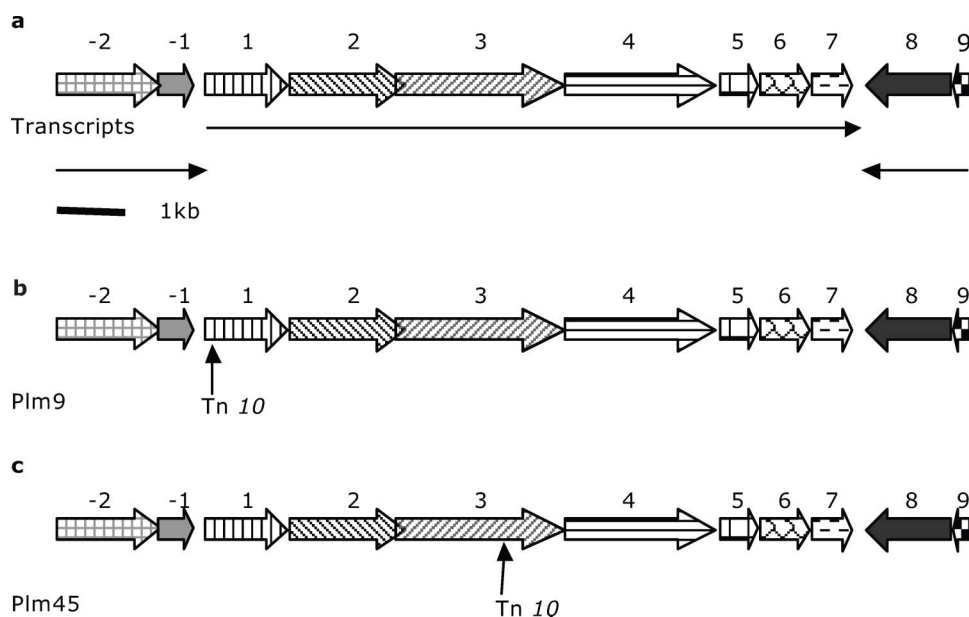
Figure 2 | Predicted operon in *P. luteoviolacea* (HI1) showing sites of transposon insertions: a, schematic diagram of putative transcription units with numbers 1–7 indicating putative open reading frames (ORFs) within the operon, −1 and −2 ORFs upstream of the operon; and No.8 and No.9 ORFs downstream from the operon; b, transposon insertion site in transposon mutant Plm9; c, transposon insertion site in transposon mutant Plm45. The scale bar represents approximately 1Kb. Horizontal arrows represent the direction of transcription and the approximate lengths of transcript units.

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 (PlDM1- PlDM4) in liquid ½ SWT medium. Deletion

mutants PlDM1- PlDM4 exhibited growth patterns similar to that of wild type *P. luteoviolacea* (HI1), which indicates the lack of inductive capacity in deletion mutants (PlDM1-PlDM4) 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.*
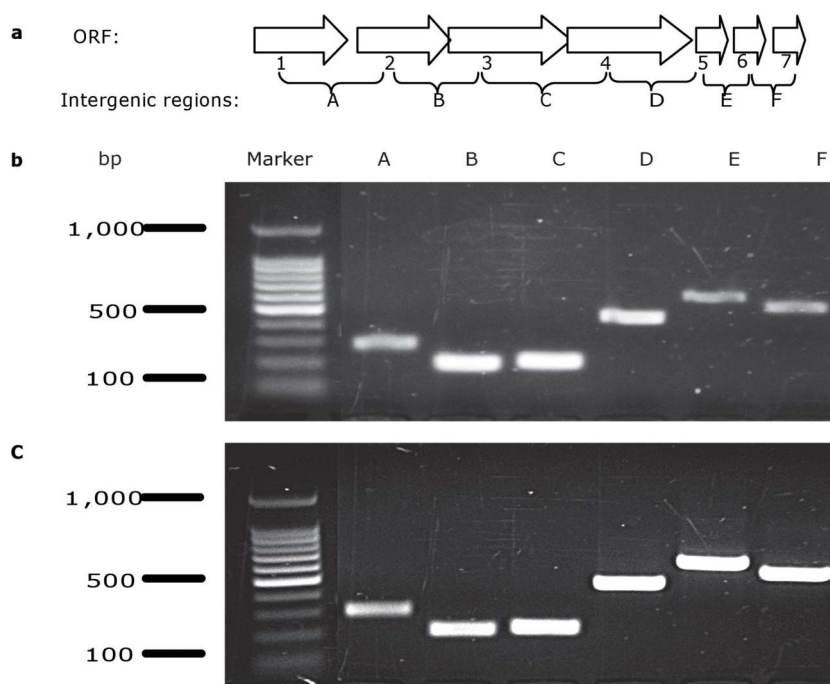

Figure 3 | Intergenic regions of adjacent genes in the putative operon were checked by Rt-PCR. a, diagrammatic representation of the operon with open reading frames 1 – 7 and their intersections indicated with A – F; b, amplicons with primers connecting intersections of the neighboring ORFs on cDNA; and c, amplicons with primers connecting intersections of the neighboring ORFs on chromosomal DNA positive control. No bands appeared in negative controls which employed cDNA amplified without reverse transcriptase in reactions.
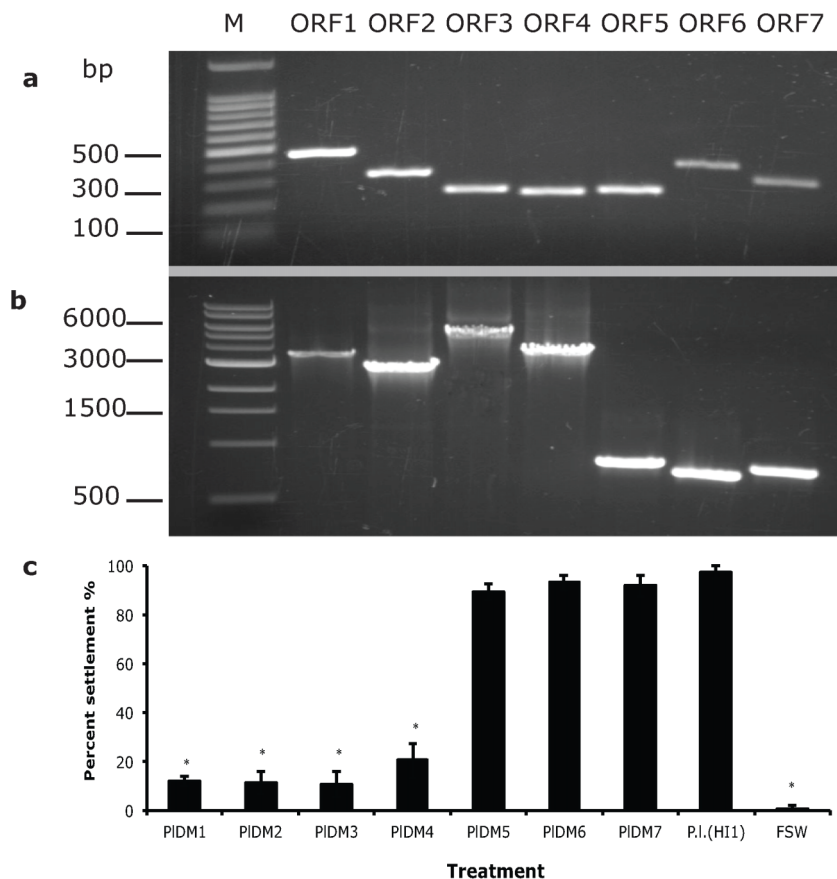
Figure 4 | Deletion mutants of _P. luteoviolacea_ (HI1) and analysis of their inductive capacity. a, amplicons with primers complementary to flanking regions of each ORF from deletion mutants PlDM1 to PlDM7 (M: DNA Marker); b, amplicons with the same primer sets from wild type _P. luteoviolacea_ (P.l. (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).

luteoviolacea ATCC3349[26]) 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_

ATCC3349[26] 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%,
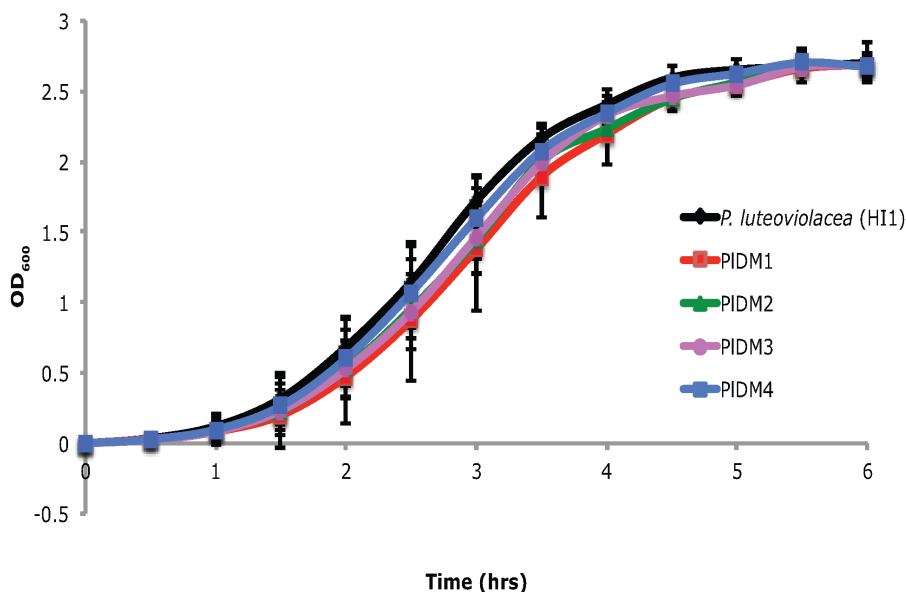

Figure 5 | Growth of wild type of _P. luteoviolacea_ (HI1) and 4 deletion mutants for four open reading frames, PlDM1, PlDM2, PlDM4 and PlDM4. Y-axis represents optical density of bacterial broth at 600 nm.
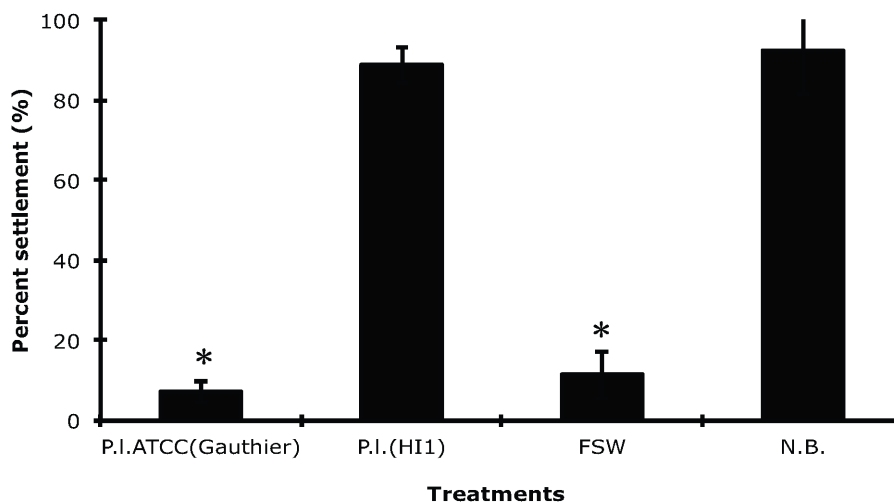
Figure 6 | **Comparison of settlement (%) of larvae of *H. elegans* on biofilms made by *P. luteoviolacea* ATCC3349[26] and *P. luteoviolacea* (HI1).** A, dishes coated by a natural biofilm (N.B.) were positive controls; untreated Petri dishes filled with autoclaved filtered seawater (FSW) were negative controls. Bars represent mean percent of larvae that settled in 24 h +/− SD (n = 5). * denotes significant difference compared with positive control (Kruskal-Wallis test, p<0.01).

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* ATCC3349[26] with significant sequence similarity. As seen in figure 7, no bands appeared in digests of the *P. luteoviolacea* ATCC3349[26] 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* ATCC3349[26] 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[27,28]. 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[29].

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*[30]. 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 × 10³ cells mm⁻²) were significantly greater than in the wild type (p=0.0003 and p=0.0014), while
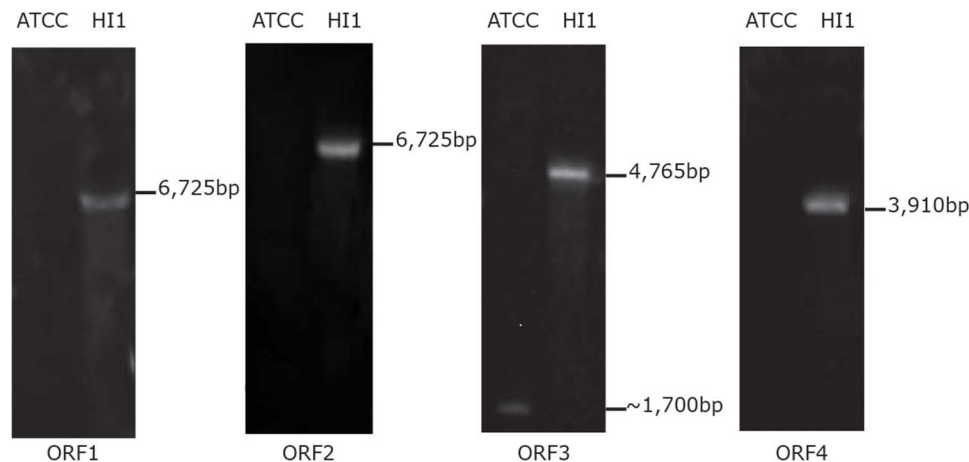

Figure 7 | **Southern blot analysis to determine the presence of inductive genes in *P. luteoviolacea* ATCC3349[26].** Probes designed from ORFs 1–4 were hybridized with genomic DNA of *P. luteoviolacea* (HI1) and *P. luteoviolacea* ATCC3349[26] that had been digested with restriction enzymes and run in parallel lanes on a gel.

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

Bacterial strains	Biofilm cell density (X10^3 cells·mm^{-2})	Biofilm thickness (μm)	Biofilm cell biomass (μm^3·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
PlDM1	6.33±4.13	0.39±0.17	0.18±0.07	0.14±0.06
PlDM2	3.62±3.95	0.10±0.10	0.09±0.05	0.05±0.04
PlDM3	27.55±18.30*	0.62±0.26	0.50±0.18*	0.33±0.14*
PlDM4	5.83±6.58	0.56±0.28	0.25±0.09	0.21±0.07
PlDM5	18.34±7.79*	0.45±0.31	0.28±0.15	0.19±0.11
PlDM6	8.11±6.12	0.71±0.47	0.31±0.13	0.25±0.11
PlDM7	7.05±4.62	0.44±0.30	0.20±0.11	0.17±0.11

Comparisons were made between characteristics of wild type *P. luteoviolacea* (HI1) and its deletion mutants: PlDM1-7. 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).
*denotes significant difference compared with *P. luteoviolacea* (HI1) (p<0.01).

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 PlDM3 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 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[6,8,21,31–33]. 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*[33]. 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[7,9–11,34–36]. Thus, the biofilm phenotypes of wild type *P. luteoviolacea* (HI1) and the deletion mutants were compared to

Table 2 | Bacterial strains and plasmids used in this study

Bacterial strain	Description	Source or reference
E. coli		
CC118 (λpir)	Δ(ara-leu) araD ΔlacX74 galE galK phoA20 thi-l rpsE rpoB argE(Am) recAl λpir	[48]
SM10 (λpir)	thi-l thr leu tonA lacY supE recA::RP4-2-Tc^r::Mu, Km^r, λpir	[48]
P. luteoviolacea		
HI1	Wild-type isolate	[12]
ATCC3349	Isolated from seawater, France, ATCC number: 33492	[26]
HI1, Str^r	Spontaneous streptomycin-resistant, mutant of Hawaii strain	This study
Plasmids		
pLOF/Km	Amp^r (Tn*10*-based delivery plasmid, Km^r)	[48]
pCVD442	Ap^r; Sucrose^s, 1.2-kb SacB gene inserted into pGP704 at cloning site PstI	[41]
pCVD443	Ap^r, Km^r, Sucrose^s, pCVD442 with Km^r gene inserted at SmaI site	This study
pDM 1	Ap^r, Km^r, Sucrose^s, pCVD443 with 1.6 kb of DNA flanking ORF1	This study
pDM2	Ap^r, Km^r, Sucrose^s, pCVD443 with 1.6 kb of DNA flanking ORF2	This study
pDM3	Ap^r, Km^r, Sucrose^s, pCVD443 with 1.6 kb of DNA flanking ORF3	This study
pDM4	Ap^r, Km^r, Sucrose^s, pCVD443 with 1.6 kb of DNA flanking ORF4	This study
pDM5	Ap^r, Km^r, Sucrose^s, pCVD443 with 1.6 kb of DNA flanking ORF5	This study
pDM6	Ap^r, Km^r, Sucrose^s, pCVD443 with 1.6 kb of DNA flanking ORF6	This study
pDM7	Ap^r, Km^r, Sucrose^s, pCVD443 with 1.6 kb of DNA flanking ORF7	This study

Table 3 | Sequences of oligonucleotides used in genome walking

Adaptor 1	5′-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCA GGT-3′
Adaptor 2	3′-H2N-CCCGTCCA-P-5′
Adaptor 1 primer	5′-GATCCTAATACGACTCACTATAGGGC-3′
Adaptor 1 nested primer	5′-A ATA GGG CTC GAG CGG C- 3′
Tn10C	5′-GCTGACTTGACGGGACGGCG-3′
Tn10D	5′-CCTCGAGCAAGACGTTTCCCG-3′

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) inductive 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).

ORF1 and ORF2 both contain the conserved domain TIGR02243, encoding hypothetical phage-tail or phage-baseplate proteins, which have been found in at least in 6 bacterial genomes. Pell, *et al.*[27] and Leiman, *et al.*[28] reported a remarkable level of structural and presumed functional similarity between phage tail and baseplate proteins and components of type VI secretion systems. Type VI secretion systems mediate contact dependent transfer of effector molecules from the bacterium to the cytoplasm of other prokaryotic or eukaryotic cells[37]. Interestingly, the N-terminal deduced amino acid sequence of ORF7, which appears to be in the same operon

as ORFs 1 and 2, has homology with VgrG proteins from *Vibrio mimicus* (VM603), a group of proteins proposed to be extracellular appendages in the bacterial type VI secretion system with shared structural features of phage-tail spike proteins[38]. This suggests that the TIGR02243 domain may be associated with secretion pathways that transport specific molecules into the biofilm matrix or directly into larval cells, and that loss of inductivity in mutants with disrupted or deleted ORFs 1 and 2 might be due to interruption of transport of necessary compounds from the bacterium to the tubeworm. This question will be pursued in our future studies.

In conclusion, this study has identified the first bacterial genes required for induction of settlement and metamorphosis of a marine invertebrate animal. Four of the seven open reading frames in the identified operon were necessary for induction. In addition, sequences corresponding to three of the four ORFs necessary for induction of metamorphosis were not found in the non-inductive strain *P. luteoviolacea* ATCC3349[26]. However, other bacterial species are known to induce settlement and metamorphosis in *H. elegans*[5,12], and it remains to be learned if their genomes include this same inductive operon. The gene-function analysis of four essential genes suggests that the operon may be involved in the formation of a

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

ORF1		
	Pldm1-1L	5′-TTTGTCGACCAATCTCGTTAGGCGAAAGC-3′
	Pldm1-1R	3′-CAATCCCTTCGTCGACCCCTTTACCGTTGC CAGAGAT-5′
	Pldm1-2L	5′-CGGTAAAGGGGTCGACGAAGGGATTGGAA AATGGA-3′
	Pldm1-2R	3′-TTTGCATGCACGGGTTAAATTAGGCGATA-5′
ORF2		
	Pldm2-1L	5′-TTTGTCGACGTCGCTTATATCGGCGTGTC-3′
	Pldm2-1R	3′-CGATAAATTTCCCAGGGTTTAGCACAGCATC-5′
	Pldm2-2L	5′-AAACCCTGGGAAATTTATCGTGGCGACCAG-3′
	Pldm2-2R	3′-TTTGCATGCACCTAACATCGGCGCTTCTA-5′
ORF3		
	Pldm3-1L	5′-TTTGTCGACAGCGATCTACTGGGGAATGA-3′
	Pldm3-1R	3′-GCGCATGCCATTGGTCACATTCGACTAACACTT-5′
	Pldm3-2L	5′-ATGTGACCAATGGCATGCGCTGTATTTAG-3′
	Pldm3-2R	3′-TTTGTCGACGAAAACTCTGGCTGCGCTAC-5′
ORF4		
	Pldm4-1L	5′-GTCGACCATCGCCAGGTTTCTAGAGC-3′
	Pldm4-1R	3′-CTAACCAAGGGTGTGCAGGGGCTTCTAATT-5′
	Pldm4-2L	5′-CCCTGCACACCCTTGGTTAGACACCGATCTT-3′
	Pldm4-2R	GTCGACCCAAGACGTGTATTTGACCTTT-5′
ORF5		
	Pldm5-1L	5′-GTCGACGTCGGGTCGAACGTATTTCT-3′
	Pldm5-1R	3′-GAAAATCCGAATAATTGGTTTTGGGCGTTG-5′
	Pldm5-2L	5′-AACCAATTATTCGGATTTTCTAAGCCTGGTT-3′
	Pldm5-2R	GTCGACTTTGAGCTGCGATGTTTGAG-5′
ORF6		
	Pldm6-1L	5′-GCATGCACGCCCAAAACCAATTATCA-3′
	Pldm6-1R	3′-TTTCAATTCTCGCATATTGAAATGCCTCTTG-5′
	Pldm6-2L	5′-TCAATATGCGCTCATTGAAAATAGGCGATTA-3′
	Pldm6-2R	3′-GTCGACTGGTCACTTTTTGCATACTTTCA-5′
ORF7		
	Pldm7-1L	5′-TTTGTCGACTGCAGTTTCCACATCTACCC-3′
	Pldm7-1R	3′-TTGGCACTGTTTGCCCTTCTTGTTCAAAGG-5′
	Pldm7-2L	5′-AGAAGGGCAAACAGTGCCAAGTGAACGATTT-3′
	Pldm7-2R	3′-TTTGCATGCTAACACCATTTGGGCGATTT-5′

Table 5 | Sequences of oligonucleotides to check the presence of 7 ORFs

ORF1	5′-CGCACCGCTTATCCTTTTAC-3′
	3′-TTTGGCGTCGCTTTAAATGT-5′
ORF2	5′-GAAGGGATTGGAAAATGGA-3′
	3′-TTGGTCACATTCGACTAACACTT-5′
ORF3	5′-AAATTTATCGTGGCGACCAG-3′
	3′-GTGTGCAGGGGCTTCTAATT-5′
ORF4	5′-GGCGTAACGACCACGTTTA-3′
	3′-ATAATTGGTTTTGGGCGTTG-5′
ORF5	5′-CCTTGGTTAGACACCGATCTT-3′
	3′-CCAAGACGTGTATTTGACCTTT-5′
ORF6	5′-TCGGATTTTCTAAGCCTGGTT-3′
	3′-TTGCCCTTCTTGTTCAAAGG-5′
ORF7	5′-TGAAGGTTTTCCCAGTCCAC-3′
	3′-TGGTCACTTTTTGCATACTTTCA-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 very interesting phylogenetic implications.

Methods

Spawning, larval culture and larval settlement bioassay. Specimens of *Hydroides elegans* were spawned and larvae were cultured as previously described[23,25]. Wild type and mutant strains of *Pseudoalteromonas luteoviolacea* were subjected to a larval settlement bioassay as described by Huang and Hadfield[12].

Bacterial stains, plasmids, media and growth conditions. Bacterial strains and plasmids used in this study are described in Table 2. The Hawaii strain, *P. luteoviolacea* (HI1), was previously isolated from a seawater table at Kewalo Marine Laboratory (KML), Honolulu, HI[12]. A spontaneously occurring streptomycin-resistant mutant of *P. luteoviolacea* (*P. luteoviolacea* (HI1, Str^R)) 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. luteoviolacea* ATCC3349[26], obtained from the American Type Culture Collection, is 99.99% identical with *P. luteoviolacea* (HI1) in the 16S rDNA gene sequence.

All strains of *P. luteoviolacea* 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.

Table 6 | Sequences of oligonucleotides to generate probes for southern blot

ORF1	5′-CAACCTGGCACTCAGTTTGA-3′
	3′-CAGCTGAGGGGTAAAACCAA-5′
ORF2	5′-CGCGCATTTGCTGGCACGTTTTG-3′
	3′-TCTTCGCTAAACCCATCAGG-5′
ORF3	5′-GGTGATAGCGAAATGCAGGT-3′
	3′-GACAGCTTGCCGTTGACATA-5′
ORF4	5′-CACCAGCCAGCGTTATTTTT-3′
	3′-CAATTCACCGGCAGTAACAT-5′

When required, the final concentrations of antibiotics in growth media were as follows: kanamycin, 50 μg ml⁻¹ (for *E. coli* strains) and 200 μg ml⁻¹ (for *P. luteoviolacea* strains); ampicillin, 100 μg ml⁻¹; and streptomycin, 200 μg ml⁻¹.

Transposon mutagenesis. Overnight cultures of the donor strain, SM10 λpir containing pLOF/Km, and the recipient strain, *P. luteoviolacea* (HI1, Sm^r), were washed in LB or ½ SWT liquid media, respectively, to eliminate antibiotics. Plasmids were transferred from the *E. coli* strain into *P. luteoviolacea* by conjugation as described by Egan, et al.[39]. Colonies that arose were screened in larval settlement assays.

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 PCR[40]. Two primers, Tn10D and Tn10C, that were complementary to the 5′ and 3′ ends of the mini-Tn10 transposon[39], were used individually in .PCR reactions with the Adaptor 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 Adaptor 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 examined on 1% agarose ethidium bromide gels and purified with a MinElute Gel Extraction Kit (Qiagen®) according to the manufacturer's protocol. Purified PCR products were sent to the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) facility at University of Hawaii for sequencing.

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).

Gene deletions. The plasmid pCVD443 is a suicide plasmid derived from pCVD442[41], which encodes the *sacB* counter-selectable marker, by adding the 977 bp kanamycin-resistance gene cassette from plasmid pLOF/Km in the SmaI site. This plasmid was used to facilitate deletion of the majority of the DNA corresponding to each of the seven ORFs individually. To create the plasmids for deletion mutants pDM1 – pDM7, chromosomal DNA upstream and downstream of each ORF was amplified by PCR, joined by overlap extension PCR (Table 4), and cloned into pCVD443 as a SalI – SphI fragment. Each deletion fragment consisted of approximately 750 bp of DNA upstream of the targeted ORF plus 51 bp of the 5′ end joined in-frame to the last 51 bp of the ORF followed by approximately 750 bp of downstream DNA. Plasmids pDM1 - 7 were transferred into *E. coli* SM10 (λpir) to serve as donor strain and conjugated with *P. luteoviolacea* (HI1, Sm^r). Single recombinants were selected on ½ SWT agar plates supplemented with kanamycin (200 μg/ml) and streptomycin (200 μg/ml). After checking the success of the first recombination, one colony was inoculated into ½ SWT broth medium without antibiotics and incubated at room temperature (25°C) overnight. The bacterial broth was diluted 10 fold, spread onto ½ SWT agar plates with 5% sucrose (W/V), and incubated at room temperature overnight. The resulting colonies were screened for kanamycin sensitivity and sucrose resistance. PCR was applied to confirm that the ORFs were deleted in selected strains. Primers were designed to complement the flanking region of each of the ORFs of interest (Table 5). The sizes of amplified DNA fragments were compared using chromosomal DNA of wild type *P. luteoviolacea* (HI1) and chromosomal DNA of the deletion mutants as templates.

Southern blot. Genomic DNA of wild type *P. luteoviolacea* (HI1) and *P. luteoviolacea* ATCC3349[26] was extracted with a MoBio Ultraclean™ Microbial DNA kit following the manufacturer's instructions. One microgram of genomic DNA of both strains of bacteria 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 6), amplified by PCR, and biotinylated with NEBLOT® Phototope® kit (NEB®) according to the manufacturer's instructions. Southern blot was performed with standard technique[42–44]. 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. luteoviolacea* (HI1) was performed in TRI REAGENT® according to the manufacturer's instructions. Contaminating chromosomal DNA in RNA samples was digested with DNase I for 3 times. First strand complementary DNA was synthesized from DNase I-treated total RNA using gene specific primers that complement the sequence of each targeted open reading frame (Table 7). Two μg of total RNA were mixed with 40 μM gene specific primers and 2.5 mM dNTP, incubated at 65°C for 5 minutes, and placed promptly on ice for 2 minutes. 200 units of M-MuLV reverse transcriptase, 10X RT buffer, and 1 μl of RNase inhibitor were

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

Amplified region	Nucleotide sequence (5′ -3′)
Primers for cDNA synthesis	
Upstream of ORF2	AATACATTGTGGTCGCTCCA
Upstream of ORF3	CCAGAGACGCGTAAAACCTC
Upstream of ORF4	GCTGCTCAACCGCAGTATAA
Upstream of ORF5	CCAAGACGTGTATTTGACCTTT
Upstream of ORF6	TTGCCCTTCTTGTTCAAAGG
Upstream of ORF7	TGGTCACTTTTTGCATACTTTCA
Primer pairs for PCR	
Intergenic region between ORF1 and ORF2	TTAGTTCCGACACGCAATCA
	CTGTGCTCGCAAACCATCTA
Intergenic region between ORF2 and ORF3	AAATTTATCGTGGCGACCAG
	TTCAAAATCATCCCCTGTCG
Intergenic region between ORF3 and ORF4	TTAGAAGCCCCTGCACACTT
	AACGCATTTAGGTCAATATCTGG
Intergenic region between ORF4 and ORF5	GCCCATCGATGCATTTAGAC
	TCCGCAACTTGATACTGCTG
Intergenic region between ORF5 and ORF6	CCGACTGCGGATATGCAGTTTCCA
	TGAGCCACTCGATGAGTTTG
Intergenic region between ORF6 and ORF7	TGATTCTTGCTGCTAAAACTTGA
	TGAAGGTTTTCCCAGTCCAC

added to the tube and incubated at 42°C for one hour. Another set of reactions replaced reverse transcriptase with H_2O and served as negative controls.

To determine the organization of the gene cluster between ORF1 to ORF7, primer pairs were designed to complement the 3′-end of one ORF and 5′-end of the succeeding ORF (Table 7). cDNAs synthesized with each gene specific primer were used as templates. Chromosomal DNA was used as a template in the same PCR as a positive control.

Growth rate determination. One colony of wild type *P. luteoviolacea* (HI1) and each of the 4 deletion mutant strains (PlDM 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 Hadfield[12]. 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.[45]. 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://rsbweb.nih.gov/ij/). Other biofilm parameters were quantified using COMSTAT software[46].

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 data[47].

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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.

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