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INVERTEBRATE MICROBIOLOGY

Bacterial Nucleobases Synergistically Induce Larval Settlement and Metamorphosis in the Invasive Mussel *Mytilopsis sallei*

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ABSTRACT Marine bacterial biofilms have long been recognized as potential inducers of larval settlement and metamorphosis in marine invertebrates, but few chemical cues from bacteria have been identified. Here, we show that larval settlement and metamorphosis of an invasive fouling mussel, *Mytilopsis sallei*, could be induced by biofilms of bacteria isolated from its adult shells and other substrates from the natural environment. One of the strains isolated, *Vibrio owensii* MS-9, showed strong inducing activity which was attributed to the release of a mixture of nucleobases including uracil, thymine, xanthine, hypoxanthine, and guanine into seawater. In particular, the synergistic effect of hypoxanthine and guanine was sufficient for the inducing activity of *V. owensii* MS-9. The presence of two or three other nucleobases could enhance, to some extent, the activity of the mixture of hypoxanthine and guanine. Furthermore, we determined that bacteria producing higher concentrations of nucleobases were more likely to induce larval settlement and metamorphosis of *M. sallei* than were bacteria producing lower concentrations of nucleobases. The present study demonstrates that bacterial nucleobases play an important role in larval settlement and metamorphosis of marine invertebrates. This provides new insights into our understanding of the role of environmental bacteria in the colonization and aggregation of invasive fouling organisms and of the metabolites used as chemical mediators in cross-kingdom communication within aquatic systems.

IMPORTANCE Invasive species are an increasingly serious problem globally. In aquatic ecosystems, invasive dreissenid mussels are well-known ecological and economic pests because they appear to effortlessly invade new environments and foul submerged structures with high-density aggregations. To efficiently control exotic mussel recruitment and colonization, the need to investigate the mechanisms of substrate selection for larval settlement and metamorphosis is apparent. Our work is one of very few to experimentally demonstrate that compounds produced by environmental bacteria play an important role in larval settlement and metamorphosis in marine invertebrates. Additionally, this study demonstrates that bacterial nucleobases can be used as chemical mediators in cross-kingdom communication within aquatic systems, which will enhance our understanding of how microbes induce larval settlement and metamorphosis of dreissenid mussels, and it furthermore may allow the development of new methods for application in antifouling.

KEYWORDS invasive fouling mussels, *Mytilopsis sallei*, bacterial biofilm, larval settlement, nucleobases

Most benthic marine invertebrates produce swimming larvae that remain planktonic for minutes to months. They then settle on substrata and metamorphose into benthic juveniles. The planktonic-benthic transition is critical for the development and growth of individuals (1). Furthermore, it is a central process which is fundamental to our understanding of population dynamics, since the postlarvae after settlement lose

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or show reduced motility and subsequently form a sedentary population. Recruitment of new larval animals derived from settlement is essential to sustain benthic animal populations (2–4). Larval settlement and metamorphosis can be triggered by chemical cues associated with substrata (1, 2, 5), which are usually covered with biofilms (6, 7). An increasing body of literature indicates that bacterial biofilms on substrata within the natural habitats of marine invertebrates can induce larval settlement and metamorphosis (5, 8, 9).

Despite this, surprisingly few settlement-inducing cues from marine bacteria have been fully structurally identified, and this limits our understanding of the inducing mechanisms of bacterial biofilms. A chemical signal, tetrabromopyrrole (TBP), produced by a strain of *Pseudoalteromonas* isolated from crustose coralline algae, the natural substrate of the coral *Acropora millepora*, was reported to induce larval metamorphosis of *A. millepora* (10). Later, TBP was found to induce the settlement of multiple Caribbean corals, indicating that this compound may have more widespread importance in the colonization mechanisms of corals (11). Recently, tightly organized clusters of tailocins, termed metamorphosis-associated contractile structures (MACs), produced from a strongly inductive bacterium, *Pseudoalteromonas luteoviolacea*, were identified as a metamorphosis cue for *Hydroides elegans* larvae (12). In this work, *P. luteoviolacea* was isolated from a biofilm that accumulated on a surface submerged in a sea area where there were adults of the polychaete *H. elegans* (13). Despite these few examples, more attention should be paid to the role of bacterium-derived molecules in mediating larval settlement and metamorphosis of marine invertebrates given the variety and ubiquity of bacteria in the marine environment. This is important for understanding the relationship between prokaryotic and eukaryotic organisms in marine benthic ecosystems.

Dreissenid mussels are well-known invasive species and economic pests because they effortlessly invade new environments and foul submerged structures with highly dense and difficult-to-remove aggregations (14, 15). For example, the devastating zebra mussel *Dreissena polymorpha* and the quagga mussel *Dreissena rostriformis bugensis* have invaded industrial raw water pipelines and cause billions of dollars worth of damage due to fouling (16). In dreissenid mussels, the final pediveliger larval stage is critical, as this larval stage is responsible for searching and settling onto suitable sites (17). Following settlement, the pediveliger larva metamorphoses into a benthic juvenile. In the field, dreissenid mussels usually form clumps formed by individual mussels attaching to the shells of other adult mussels. Larval recruitment of *D. polymorpha* and *D. rostriformis bugensis* onto acrylic plastic plates covered with three types of substrate (live mussels, mussel shells, and mussel-sized stones) was investigated (18). It was found that the plates with live mussels and mussel shells, whether with or without biofilms, had more settlement than the plates with just stones, and that in all treatments, biofilm removal reduced mussel recruitment. This field study indicates that natural biofilms may play important roles in mediating larval settlement and metamorphosis of dreissenid mussels. However, the precise bacterial species which elicit settlement and the chemical identity of any microorganism-associated cues are not known. Investigations into larval settlement and metamorphosis of dreissenid mussels in response to monospecific bacterial biofilms would not only provide insights into which environmental bacteria could mediate the recruitment and colonization of these nuisance species and how they achieve it, but it may also allow the development of new methods for the control of these invasive species.

In this study, we therefore examined the larval settlement and metamorphosis responses of the Caribbean false mussel *Mytilopsis sallei* to marine bacteria and the active compound(s) which these bacteria secrete. This mussel is an invasive dreissenid species that was introduced into the Pacific-Indian Oceans via the Panama Canal (19). The recruitment and aggregation of *M. sallei* have led to serious fouling problems on aquaculture facilities and have caused changes in local aquatic community structure (20, 21). Here, we hypothesized that some bacteria from the natural habitat of *M. sallei* may have inducing effects on its larval settlement and metamorphosis, and that

TABLE 1 16S rRNA gene affiliations of 24 bacterial strains with strong biofilm-forming capacity

Code	Phylum	Class	Closest relative	GenBank accession no.	Identity (%)	Source[a]
MS-1	*Proteobacteria*	*Gammaproteobacteria*	*Pseudoalteromonas* sp.	HE983366.1	100.0	Shell/substrate
MS-2	*Proteobacteria*	*Gammaproteobacteria*	*Pseudoalteromonas* sp.	FJ425222.1	100.0	Substrate
MS-3	*Proteobacteria*	*Gammaproteobacteria*	*Pseudoalteromonas profundi*	NR_152699.1	100.0	Shell/substrate
MS-4	*Proteobacteria*	*Gammaproteobacteria*	*Vibrio parahaemolyticus*	CP023248.1	99.9	Substrate
MS-5	*Proteobacteria*	*Gammaproteobacteria*	*Vibrio vulnificus*	CP019320.1	100.0	Shell
MS-6	*Proteobacteria*	*Gammaproteobacteria*	*Vibrio sinaloensis*	KU525100.1	100.0	Shell
MS-7	*Proteobacteria*	*Gammaproteobacteria*	*Vibrio natriegens*	KR347293.1	99.7	Shell/substrate
MS-8	*Proteobacteria*	*Gammaproteobacteria*	*Vibrio nereis*	KR347307.1	100.0	Shell
MS-9	*Proteobacteria*	*Gammaproteobacteria*	*Vibrio owensii*	CP025796.1	100.0	Shell/substrate
MS-10	*Proteobacteria*	*Gammaproteobacteria*	*Vibrio fluvialis*	LC420091.1	100.0	Shell
MS-11	*Proteobacteria*	*Gammaproteobacteria*	*Vibrio alginolyticus*	MH879822.1	99.9	Shell
MS-12	*Proteobacteria*	*Gammaproteobacteria*	*Hafnia paralvei*	CP014031.2	99.9	Substrate
MS-13	*Proteobacteria*	*Gammaproteobacteria*	*Shewanella amazonensis*	KX950815.1	100.0	Shell/substrate
MS-14	*Proteobacteria*	*Gammaproteobacteria*	*Psychrobacter celer*	FJ613610.1	100.0	Shell/substrate
MS-15	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonas sp.*	AB571943.1	99.0	Substrate
MS-16	*Proteobacteria*	*Alphaproteobacteria*	*Paracoccus homiensis*	KU845384.1	100.0	Shell/substrate
MS-17	*Proteobacteria*	*Alphaproteobacteria*	*Yangia pacifica*	KJ009554.1	100.0	Shell/substrate
MS-18	*Proteobacteria*	*Alphaproteobacteria*	*Ruegeria atlantica*	EU624444.1	99.9	Shell/substrate
MS-19	*Proteobacteria*	*Alphaproteobacteria*	*Leisingera daeponensis*	NR_044026.1	99.9	Shell
MS-20	*Firmicutes*	*Bacilli*	*Bacillus altitudinis*	MG651513.1	100.0	Shell/substrate
MS-21	*Firmicutes*	*Bacilli*	*B. altitudinis*	MK120884.1	100.0	Shell
MS-22	*Firmicutes*	*Bacilli*	*Exiguobacterium profundum*	KM215140.1	100.0	Shell/substrate
MS-23	*Bacteroidetes*	*Flavobacteriia*	*Tenacibaculum* sp.	KF801479.1	99.6	Shell
MS-24	*Bacteroidetes*	*Flavobacteriia*	*Arenibacter palladensis*	KC534324.1	100.0	Shell/substrate

[a]Shell, shells of *M. sallei* adults collected from Maluan Bay, Xiamen, China; substrate, polypropylene ropes submerged in seawater in Maluan Bay and settled with *M. sallei* adults.

compounds released by the inducing bacteria may play a role in the inducing activity. To test these hypotheses, we isolated bacteria from the shells and natural substrates of *M. sallei* and examined larval settlement and metamorphosis in response to monospecific bacterial biofilms. Furthermore, the inducing chemical cues were isolated from a strongly inductive bacterium, *Vibrio owensii* MS-9, by bioassay-guided fractionation. The release of the inducing cues into seawater by bacteria, the synergistic effect of the inducing cues on larval settlement and metamorphosis, and the possibility that the inducing cues also play a role in the inducing activity of other bacteria were investigated in this study.

RESULTS

Identification of bacterial strains with strong biofilm-forming capacity. Overall, 54 bacterial strains were isolated from the surfaces of *M. sallei* shells and their settlement substrates from the marine environment, of which 24 strains produced biofilms with an optical density at 600 nm (OD_{600}) of >1.0, showing strong biofilm-forming capacity (see Table S1 in the supplemental material). As shown in Table 1, 16S rRNA analysis of these 24 strains indicated that they belong to 14 different genera. Noticeably, among the 19 isolates affiliated with the *Proteobacteria*, most of them (16) were from *Gammaproteobacteria*.

Larval settlement and metamorphosis in response to monospecific biofilm. As shown in Fig. 1, the monospecific biofilms of six strains, *Pseudoalteromonas profundi* MS-3, *Vibrio parahaemolyticus* MS-4, *V. owensii* MS-9, *Psychrobacter celer* MS-14, *Paracoccus homiensis* MS-16, and *Arenibacter palladensis* MS-24, significantly induced the larval settlement of *M. sallei*. Among these strains, *V. owensii* MS-9 showed the highest settlement-inducing activity throughout the bioassays. Besides the inducing effect on settlement, strains *V. parahaemolyticus* MS-4, *V. owensii* MS-9, *P. celer* MS-14, and *P. homiensis* MS-16 also induced a significantly higher metamorphosis response (over 50%) than did the control (21.1%) at 48 h, among which strain *V. owensii* MS-9 again showed the highest inducing activity (67.8%). Of the 24 bacterial strains tested here, there was one strain, *Vibrio fluvialis* MS-10, which showed significant lethal toxicity to *M. sallei* and inhibited settlement and metamorphosis. As strain *V. owensii* MS-9 exhibited

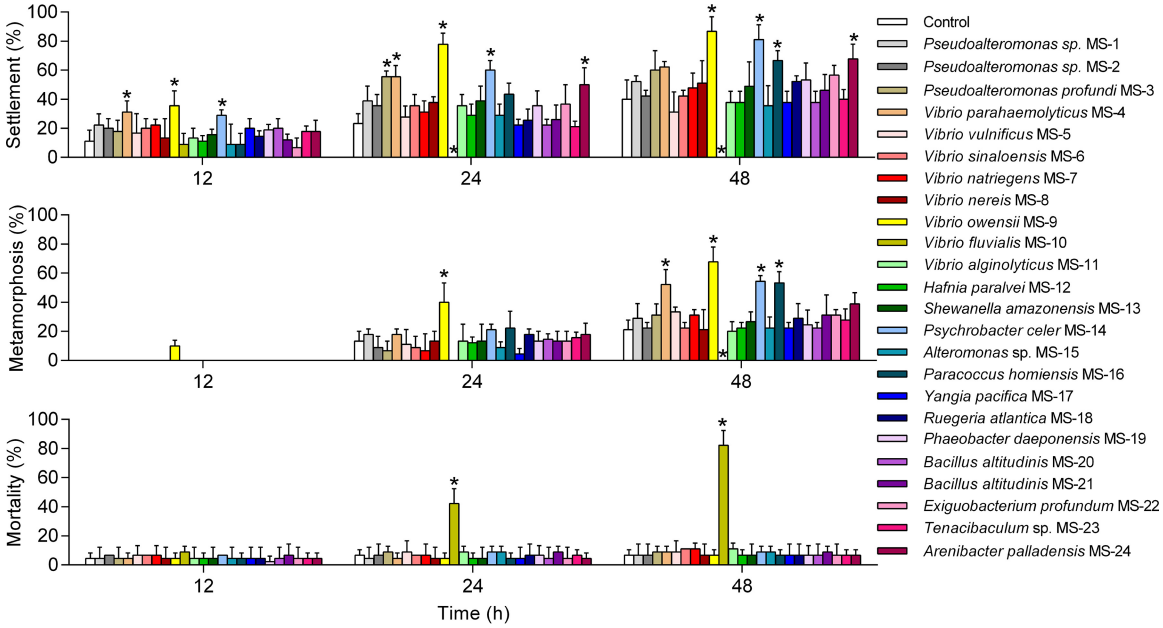

FIG 1 Effects of monospecific bacterial biofilms on larval settlement, metamorphosis, and mortality of *M. sallei*. Larvae exposed to FSW were used as a control. Data are the mean values of three replicates, with standard deviations indicated by vertical bars. *, significant difference between a treatment and the control ($P < 0.05$, one-way ANOVA).

the highest inducing activity for larval settlement and metamorphosis, this strain was used for the further bioassay-guided isolation of the inducing cue(s).

Isolation and characterization of the inducing cue(s). The bioassay-guided isolation of inducing cues from *V. owensii* MS-9 and characterization of the inducing cues are shown in Fig. 2. The crude extract of *V. owensii* MS-9 significantly induced settlement and metamorphosis of *M. sallei* larvae at concentrations of 20 to 100 μg ml^{-1}, confirming the existence of inducing cue(s) in this strain (Fig. 2). The active extract was further subjected to bioassay-guided fractionation. Among the eight fractions obtained by fractionation of the extract using reverse-phase octadecyl-silica (ODS) column chromatography, only the polar fraction F2 induced a significant amount of settlement and metamorphosis compared with the control. This fraction was subsequently subjected to Sephadex LH-20 column chromatography to yield three subfractions, and it was found that only subfraction F2-2 significantly induced larval settlement and metamorphosis at 5 to 10 μg ml^{-1}.

Five nucleobases, namely, Ura, Thy, Xan, Hyp, and Gua, were detected in F2-2 by liquid chromatography mass spectrometry (LC-MS). A bioassay using these five compounds separately showed that Hyp significantly induced larval settlement of *M. sallei* at 5 to 50 μM and larval metamorphosis at 1 to 50 μM. Thy and Gua also exhibited inducing activity on larval settlement but at a relatively high concentration (50 μM), and these two compounds did not show an inducing effect on larval metamorphosis. For Ura and Xan, the percentages of larval settlement and metamorphosis did not significantly differ from those of the control under the concentrations tested here.

Determination of the synergistic effect of nucleobases in biofilm-conditioned seawater of *V. owensii* MS-9. As shown in Fig. 3A, compared with the negative control (filtered seawater [FSW]), the biofilm-conditioned seawater (BCS) of strain *V. owensii* MS-9 significantly induced larval settlement and metamorphosis of *M. sallei*, suggesting that the biofilm of *V. owensii* MS-9 has the potential to release inducing cues into seawater. The inducing activity of BCS on larval settlement and metamorphosis was similar to that of the positive control (the biofilm of *V. owensii* MS-9), which indicated that BCS also contained chemicals responsible for the inducing activity of the *V. owensii* MS-9 biofilm.

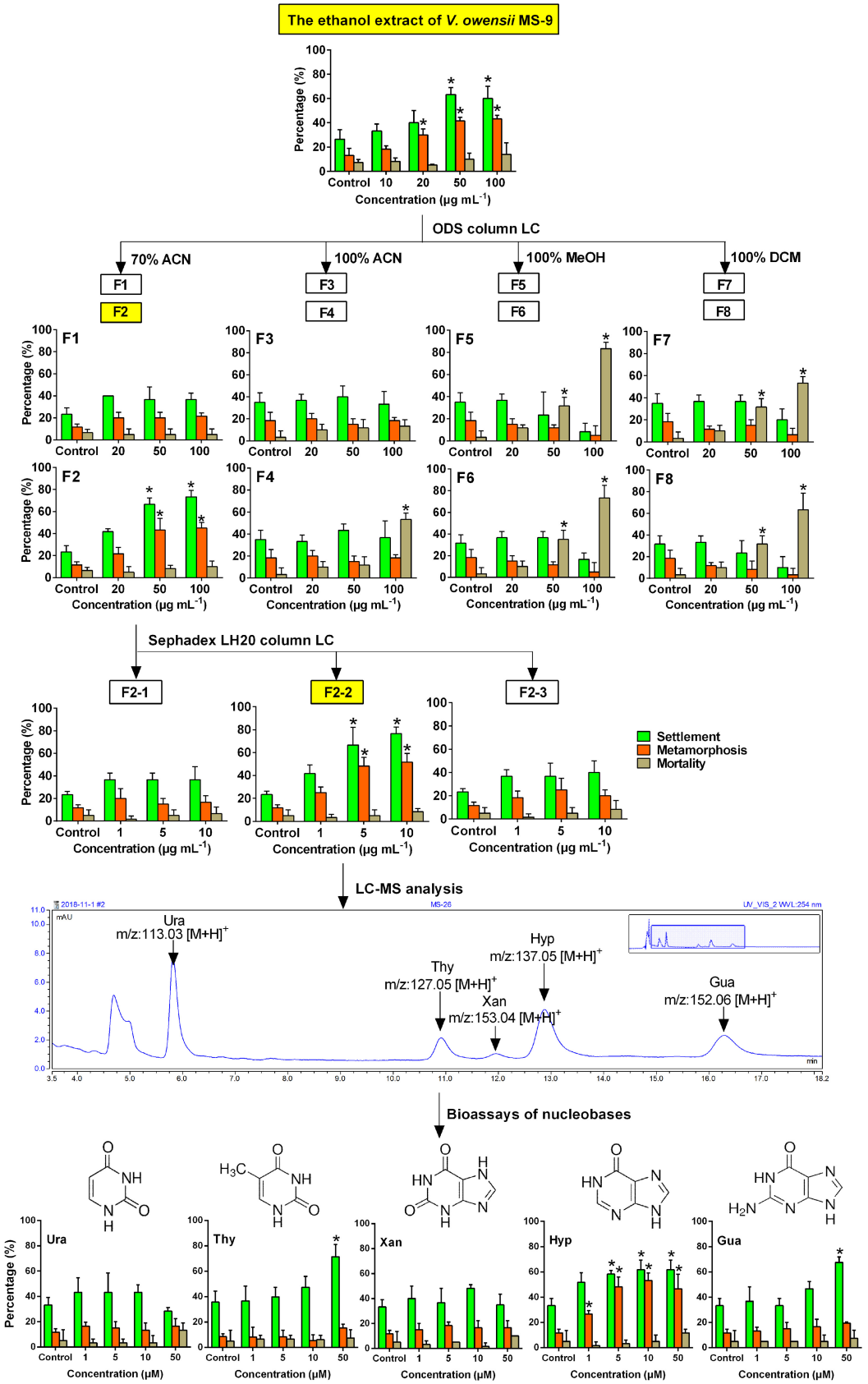

FIG 2 Bioassay-guided isolation and characterization of the inducing cues from *V. owensii* MS-9. Depending on the solubility of the tested substances in seawater, a 0.5% (vol/vol) solution of DMSO in FSW was used as control for the bioassays with

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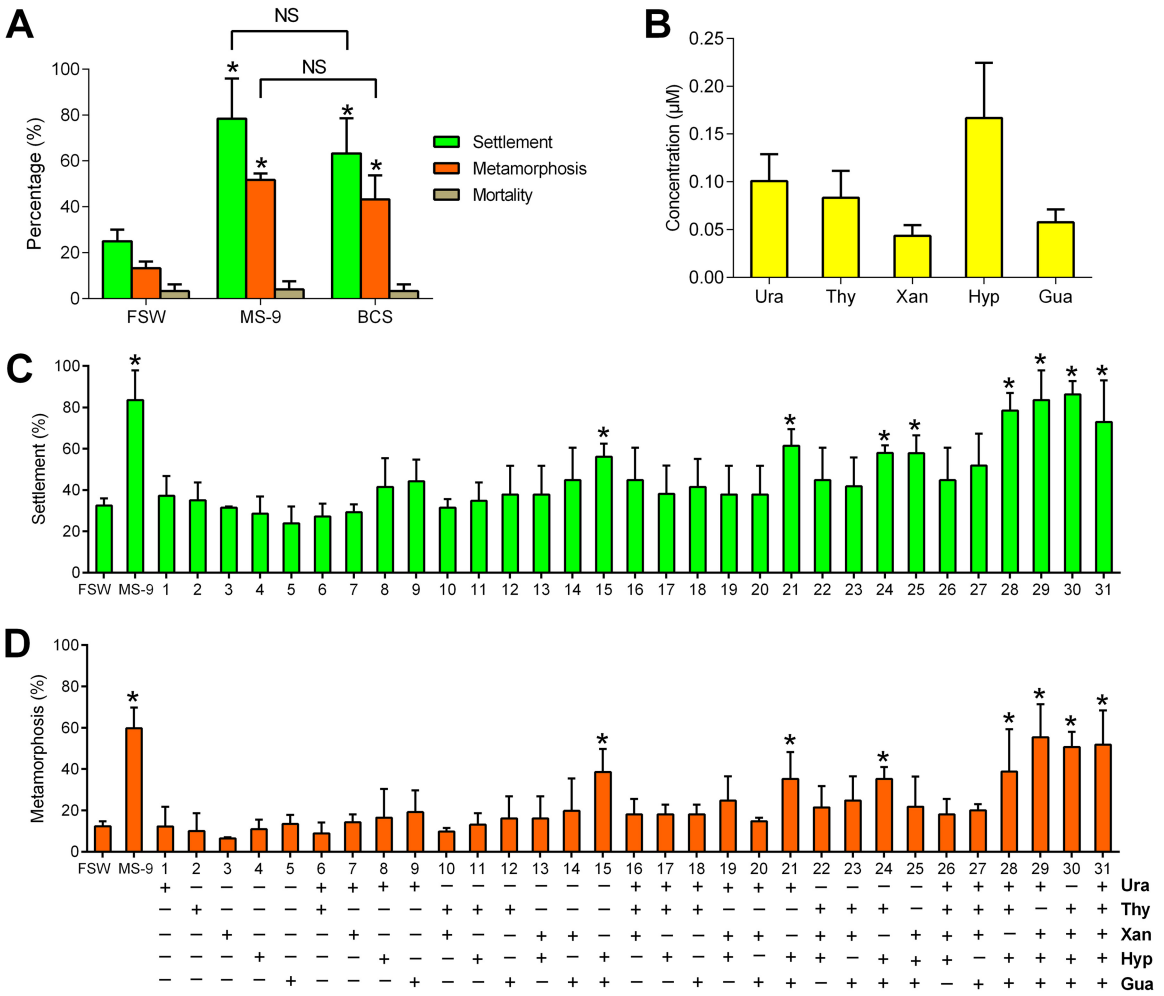

FIG 3 Nucleobases synergistically induce larval settlement and metamorphosis of *M. sallei*. (A) Effect of BCS from *V. owensii* MS-9 biofilm on larval settlement and metamorphosis. (B) Concentrations of nucleobases in BCS of the *V. owensii* MS-9 biofilm. (C and D) Percentages of larval settlement (C) and metamorphosis (D) of *M. sallei* in response to different combinations of nucleobases (indicated by plus and minus signs). The tested concentrations were 0.1 μM for Ura, 0.1 μM for Thy, 0.05 μM for Xan, 0.2 μM for Hyp, and 0.05 μM for Gua, near their "natural" concentrations in BCS. Data are mean values of three replicates, with standard deviations indicated by vertical bars. FSW, filtered seawater (negative control); MS-9, the *V. owensii* MS-9 biofilm (positive control); NS, no significant difference between the treatments; *, significant difference between a treatment and the FSW control ($P < 0.05$, one-way ANOVA).

To further examine whether the water-soluble nucleobases in BCS were responsible for the inducing activity of the *V. owensii* MS-9 biofilm, we first determined the concentrations of the five nucleobases mentioned above in BCS by high-performance liquid chromatography (HPLC). The mean concentrations of Ura, Xan, Thy, Hyp, and Gua in the BCS were 0.101, 0.044, 0.086, 0.177, and 0.048 μM, respectively (Fig. 3B). Surprisingly, the concentrations of the three aforementioned inducing active compounds Thy, Hyp, and Gua in BCS were found to be much lower than the lowest effective concentration of each compound when tested individually (50 μM for Thy, 1.0 μM for Hyp, and 50 μM for Gua; Fig. 2), indicating there may be a synergistic effect of mixtures of nucleobases in the BCS. To examine this possibility, we investigated larval responses to all possible combinations of the five nucleobases at concentrations similar to their

FIG 2 Legend (Continued)
the ethanol extract and the fractions F3 to F8, and FSW was used as control for the bioassays with the fractions F1, F2, F2-1, F2-2, and F2-3 and nucleobases. The yellow frames indicate the extract and the fractions showing inducing activity. Data are mean values of three replicates, with standard deviations indicated by vertical bars. *, significant difference between a treatment and the control ($P < 0.05$, one-way ANOVA). ACN, acetonitrile; MeOH, methanol; DCM, dichloromethane.

TABLE 2 Nucleobase content in BCS of bacterial strains

Nucleobase	Nucleobase concn (μM) by strain type (mean ± SD)[a]						
	Inducing strains				Noninducing strains		
	Vibrio owensii MS-9	*Pseudoalteromonas profundi* MS-3	*Psychrobacter celer* MS-14	*Arenibacter palladensis* MS-24	*Pseudoalteromonas* sp. MS-1	*Pseudoalteromonas* sp. MS-2	*Vibrio fluvialis* MS-10
Uracil	0.101 ± 0.018 A	0.042 ± 0.018 A	0.080 ± 0.039 A	0.466 ± 0.144 B	0.013 ± 0.003 A	0.011 ± 0.008 A	0.010 ± 0.007 A
Thymine	0.086 ± 0.027 A	0.052 ± 0.009 A	0.061 ± 0.022 A	0.069 ± 0.006 A	ND B	ND B	ND B
Xanthine	0.044 ± 0.011 A	ND B	ND B	ND B	ND B	ND B	ND B
Hypoxanthine	0.177 ± 0.058 A	0.072 ± 0.008 B	0.050 ± 0.012 BC	0.042 ± 0.004 BC	0.014 ± 0.003 BC	0.013 ± 0.003 BC	0.010 ± 0.008 C
Guanine	0.048 ± 0.013 AB	0.022 ± 0.013 C	0.061 ± 0.022 B	0.028 ± 0.011 AC	0.003 ± 0.003 C	0.003 ± 0.003 C	ND C
Total	0.456 ± 0.117 AB	0.188 ± 0.031 CD	0.252 ± 0.095 AC	0.605 ± 0.144 B	0.030 ± 0.117 D	0.028 ± 0.002 D	0.020 ± 0.017 D

[a]$n = 3$. Different letters represent significant differences ($P < 0.05$, one-way ANOVA) among strains. ND, compound not detected.

"natural" concentrations observed in BCS (Table S2). The results showed that different combinations of nucleobases produced quite different larval responses (Fig. 3C and D). The nucleobases did not elicit significant inducing activity when assayed individually (treatments 1 to 5). In contrast, treatments 15, 21, 24, 25, 28, 29, 30, and 31, which consisted of at least two nucleobases, gave significant inducing activity for larval settlement or metamorphosis, suggesting that there is a synergistic effect of mixtures of nucleobases. Interestingly, the other treatments consisting of at least two nucleobases did not show any effect on larval settlement or metamorphosis, which indicated that the precise combination of the different nucleobases is important for their physiological effects on *M. sallei*. It was found here that all the inducing combinations contained Hyp and Gua (Fig. 3C and D), suggesting that the coexistence of Hyp and Gua was sufficient for the inducing activity. Among the inducing combinations, treatment 15 (containing Hyp plus Gua) and treatments 21, 24, and 25 (containing three nucleobases of Hyp plus Gua plus one other nucleobase) all had lower levels of settlement and metamorphosis (although not significantly lower) than did the *V. owensii* MS-9 biofilm (the positive control), while treatments 28 to 31 (containing four or five nucleobases of Hyp plus Gua plus two or three other nucleobases) almost fully recovered the bioactivity of the positive control, which suggested that the presence of two or three other nucleobases could enhance to some extent the activity of the Hyp-Gua mixture, and that the mixture of the five nucleobases in BCS was responsible for the inducing activity of the *V. owensii* MS-9 biofilm.

Investigation of larval responses to synthetic nucleobase mixtures. Table 2 lists the concentrations of Ura, Thy, Xan, Hyp, and Gua in the BCS of three inducing strains (*P. profundi* MS-3, *P. celer* MS-14, and *A. palladensis* MS-24) and three noninducing strains (*Pseudoalteromonas* sp. strain MS-1, *Pseudoalteromonas* sp. strain MS-2, and *V. fluvialis* MS-10). These can be compared to those in the BCS of the inducing strain *V. owensii* MS-9. As shown in Table 2, there are diverse mixtures and concentrations of nucleobases in the BCS of the different bacterial strains. It is noteworthy that the total concentrations of the five tested nucleobases in the four inducing strains (0.188 to 0.605 μM) were generally much higher than those in the three noninducing strains (0.020 to 0.030 μM). Larval responses to the synthetic nucleobase mixtures which have compositions that are similar to the natural nucleobase mixtures present in the BCS (referred to as SNB) of the inducing and noninducing strains are shown in Fig. 4. The SNBs of the inducing strains *V. owensii* MS-9, *P. profundi* MS-3, and *A. palladensis* MS-24 exhibited inducing activity on larval settlement and metamorphosis of *M. sallei*, while the SNBs of the noninducing strains *Pseudoalteromonas* sp. MS-1, *Pseudoalteromonas* sp. MS-2, and *V. fluvialis* MS-10 exhibited no significant activity on larval settlement and metamorphosis. These results suggest that the higher concentrations of nucleobases produced by the inducing strains might be responsible for their inducing activity. The production of nucleobases by marine bacteria may be quite common, and so it is likely that larval settlement and metamorphosis of *M. sallei* have evolved to use these bacterial signals as indicators of suitable places to settle and grow. Interestingly, an

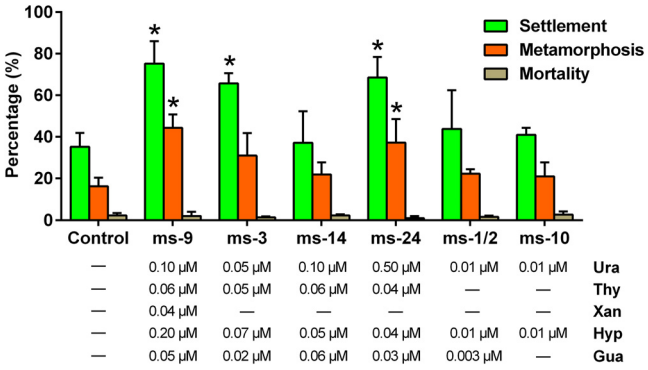

FIG 4 Larval responses of *M. sallei* to the synthetic nucleobase mixtures which were prepared with concentrations of nucleobases similar to the natural nucleobase mixtures present in the BCS from the different bacterial strains. The concentrations of each nucleobase in the synthetic nucleobase mixtures for each treatment are listed below the graph. Since the nucleobase contents in the BCS from strains *Pseudoalteromonas* sp. MS-1 and *Pseudoalteromonas* sp. MS-2 were very similar (Table 2), one treatment (ms-1/2) was set for these two strains to copy the nucleobase mixture composition in their naturally produced BCS. Data are the mean values of three replicates, with standard deviations indicated by vertical bars. Control, filtered seawater; *, significant difference between a treatment and the control ($P < 0.05$, one-way ANOVA).

exception was found in the treatment of the inducing strain *P. celer* MS-14, in which the concentrations of nucleobases in BCS were relatively high, but its SNB showed no significant activity on larval settlement and metamorphosis, indicating that there might be inducing cue(s) other than these nucleobases in *P. celer* MS-14.

DISCUSSION

There is a growing interest in the role of metabolites as chemical mediators in cross-kingdom interactions within aquatic systems (22). Molecular cues from environmental bacteria can influence the growth, development, and morphogenesis of the eukaryotes (23). The importance of bacterial biofilms in inducing larval settlement and metamorphosis of marine invertebrates has been described, but few molecular cues produced by bacteria have been identified. The present study demonstrates that nucleobases from bacterial biofilms play an important role in larval settlement and metamorphosis in marine invertebrates.

Our study found six bacterial strains, isolated from shells or substrates of *M. sallei*, that could induce larval settlement and metamorphosis of *M. sallei*, suggesting these strains may play roles in promoting the recruitment and aggregation of *M. sallei* in the field. The marine bacteria previously reported to have activity in inducing larval settlement and metamorphosis of mussels belong mainly to the *Gammaproteobacteria*. For the mussel *Mytilus galloprovincialis*, members of the genera *Pseudomonas* and *Alteromonas*, belonging to the *Gammaproteobacteria*, were found to induce larval attachment and metamorphosis (24). Yang et al. (25) reported that of the eight isolates found with inducing activity for larval settlement and metamorphosis of *Mytilus coruscus*, six isolates belonged to *Gammaproteobacteria*. Consistently, our study found that four of six inducing strains were gammaproteobacteria, suggesting that gammaproteobacteria are important inducers of larval settlement and metamorphosis for *M. sallei*. Because colonizable surfaces in the ocean are often very limited, cues from biofilm bacteria growing on these surfaces might indicate to animals that there is a surface on which to settle (23). Furthermore, the presence of biofilms on surfaces indicates that the environment of the surfaces is suitable for the survival of organisms. Gammaproteobacteria usually flourish in nutrient-rich environments (23) and may thus serve as proxies for favorable (nutrient-rich) environmental conditions for the growth and survival of mussels. Thus, it might be advantageous for mussel larvae to respond to bacterial signals.

Since the species *P. profundi*, *V. parahaemolyticus*, *V. owensii*, and *P. celer*, which

induced settlement and metamorphosis of *M. sallei* larvae in this study, are widespread in estuarine or marine environments (26–29), it is suggested here that the inducing activity of these bacteria may also help the invasion of *M. sallei* to new areas. Furthermore, since microbial communities are highly dynamic in natural marine environments (7), the present finding that larval settlement and metamorphosis of *M. sallei* can be induced by multiple environmental bacteria indicates that it may be beneficial for *M. sallei* to interpret the cues inducing important developmental decisions from different environmental bacteria, which would promote the promiscuous colonization and survival of *M. sallei* onto a broad variety of surfaces, rather than only settling on a narrow range of surfaces.

Vibrio species are ubiquitous in aquatic ecosystems and are well known to form biofilms (30). In the present study, the strain *V. owensii* MS-9 showed the strongest inducing activity for larval settlement and metamorphosis of *M. sallei*. Although there are no previous reports on the effect of *V. owensii* on larval settlement or metamorphosis in marine invertebrates, the genus *Vibrio* has been reported to play a positive or negative role in mediating larval settlement or metamorphosis in mussels (25), polychaetes (31), and bryozoans (32). In this study, different *Vibrio* species elicited different larval responses in *M. sallei*. Two *Vibrio* strains (*V. parahaemolyticus* MS-4 and *V. owensii* MS-9) significantly induced larval settlement and metamorphosis, five *Vibrio* strains (*V. vulnificus* MS-5, *V. sinaloensis* MS-6, *V. natriegens* MS-7, *V. nereis* MS-8, and *V. alginolyticus* MS-11) had no significant effects, and one *Vibrio* strain (*V. fluvialis* MS-10) showed significant inhibiting effects and lethal toxicity. Many bacterial species have been reported to have antifouling activity against larvae of fouling organisms, and a few bacterial secondary metabolites have been found to be potential antifoulants (33). The present finding of the inhibitory effect of *V. fluvialis* on larval settlement has also been noted in a widespread marine fouling organism, the bryozoan *Bugula neritina* (32). *V. fluvialis* has also been reported as an enteric pathogen in humans (34). *V. fluvialis* produces diverse toxins that may be important in pathogenesis, including lipase, protease, cytotoxin, and hemolysin (35). Thus, compounds produced by *V. fluvialis* may have potential in preventing marine biofouling.

Studies on the polychaete *H. elegans* found that its larval settlement and metamorphosis were not triggered in response to conditioned seawater of bacterial biofilms but were effectively mediated upon contact with the surface of bacterial films, suggesting that in this species, the chemical cue is not released into seawater but is surface bound (36, 37). In addition, it has been reported that the seawater conditioned by an *Alteromonas* sp. strain 1 biofilm did not induce larval metamorphosis of the mussel *M. galloprovincialis* but evoked larval settlement behavior, while the presence of an *Alteromonas* sp. biofilm could induce larval settlement and metamorphosis normally, which indicated that a potential or partial waterborne cue existed but remained inactive when the biofilm was absent (38). In contrast to these studies, the results presented here further confirm that waterborne cues from bacterial biofilms can induce mussel settlement and metamorphosis.

Although nucleobases are ubiquitous in bacteria and eukaryotes (39), no nucleobases have previously been implicated in the regulation of a cross-kingdom interactions in aquatic systems. The behavioral and morphogenetic interactions described here indicate that chemical communication using nucleobases can occur between marine invertebrates and bacteria in the marine environment. Bacterially produced nucleobases may also play an important role in the chemical signaling that influences larval settlement and metamorphosis of other invasive dreissenid mussels, which requires further investigation. Since nucleobases are most likely to occur as mixtures in nature (40), our finding that the combination effect of nucleobases is critical for signaling in an aquatic mussel has significant implications for the study of nucleobase signaling in other organisms.

Although the role of nucleobases as chemical signals in animals is substantially underestimated (40), purines as signal molecules have been reported in aquatic systems. Recently, it has been reported that nucleobase-containing compounds such as

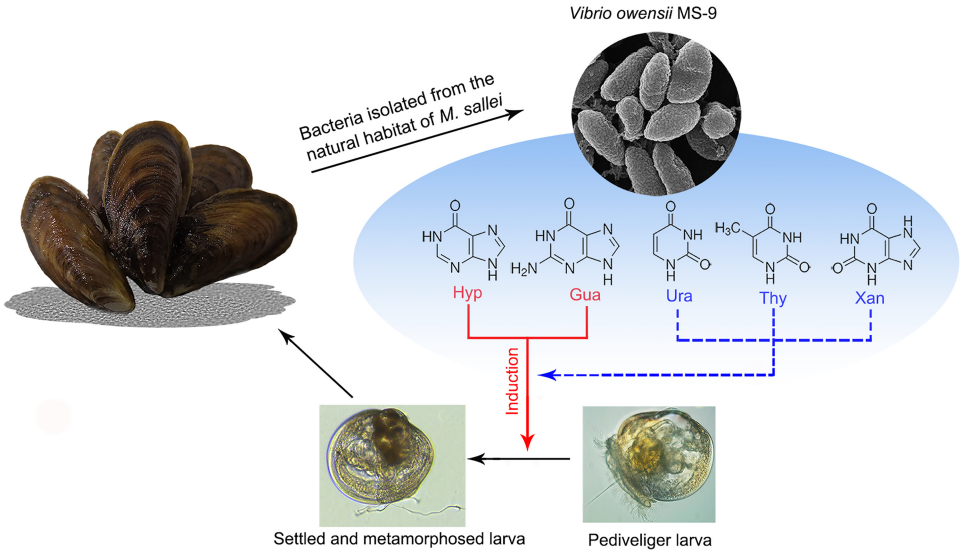

FIG 5 A schematic diagram of the synergistic induction by multiple nucleobases released from *V. owensii* MS-9 on larval settlement and metamorphosis of *M. sallei*. Strain *V. owensii* MS-9, isolated from the natural habitat of *M. sallei*, releases five nucleobases, Hyp, Gua, Ura, Thy, and Xan. The synergistic effect of Hyp and Gua could significantly induce larval settlement and metamorphosis of *M. sallei*, which is indicated by the red arrow with a solid line. The presence of the other nucleobases, Ura, Thy, and Xan, could enhance the inducing activity of the Hyp-Gua mixture, which is indicated by the blue arrow with a dotted line.

ATP, ADP, AMP, and adenosine evoke attraction or feeding (appetitive) responses in fish (40, 41). *M. sallei* larvae may have evolved sensitivity to certain kinds and certain combinations of nucleobases. This sensitivity may help *M. sallei* colonize new areas where inducing bacteria grow and which produce relevant nucleobases. It will be interesting to determine whether less-invasive species are sensitive to a more restricted range of settlement-inducing compounds.

Furthermore, our results suggest that bacteria producing higher concentrations of nucleobases are more likely to induce larval settlement and metamorphosis of *M. sallei* than are those producing lower concentrations. Here, however, it should be noted that the inducing activity of bacterial nucleobase mixtures varies considerably depending on the combination of different nucleobases, and the synergistic effect of Hyp and Gua plays a key role in the inducing activity of *V. owensii* MS-9. The synergism between bacterial cues on development has also been found in the choanoflagellate *Salpingoeca rosetta*, in which multicellular rosette development is regulated by an environmental bacterium, *Algoriphagus machipongonensis* (42). Here, there is synergism between the bacterial sulfonolipids in a mixture of rosette-inducing factors 1 and 2 (RIF-1 and RIF-2, respectively), which can initiate rosette development in *S. rosetta*. Interestingly, rosettes induced by RIFs have much lower levels than those induced by live *Algoriphagus* spp., while the further combination of bacterial lysophosphatidylethanolamines (LPEs; with no detectable activity on their own) and the RIF mixture can enhance the activity, which recovers the full bioactivity of live *Algoriphagus* spp.

Here, we also found that the presence of Ura and Xan (with no detectable inducing activity on their own) could enhance the activity of the Hyp-Gua mixture, suggesting that multiple bacterial cues can converge to activate and enhance bioactivity in a prokaryote-eukaryote interaction. Based on the results, a schematic diagram showing the induction of larval settlement and metamorphosis of *M. sallei* by several nucleobases released from *V. owensii* MS-9 is shown in Fig. 5. On the other hand, for *M. sallei*, besides nucleobases, there may be other bacterial chemicals which may serve as inducing cues, as suggested in the assay with the inducing strain *P. celer* MS-14, in which the concentrations of nucleobases in BCS were high but the nucleobase mixtures showed no inducing activity.

In conclusion, our data show that larval settlement and metamorphosis of *M. sallei* could be induced by 6 of 24 bacterial strains isolated from its natural habitat. Strain *V. owensii* MS-9 exhibited the highest inducing activity, and it was found that the synergistic blend of nucleobases released from this strain into seawater was responsible for the inducing activity of *V. owensii* MS-9. Furthermore, a comparison of the nucleobase content of biofilm-conditioned seawater and the bioactivity of their mixtures produced by inducing strains and noninducing strains suggested that bacteria producing higher concentrations of nucleobases are more likely to induce larval settlement and metamorphosis of *M. sallei*. The precise composition of the nucleobases present was also found to be important for inducing activity. This study identifies a bacterial metabolite mixture of simple nucleobases used as chemical mediators in cross-kingdom interactions within aquatic systems. This new knowledge may allow the development of methods for the control of invasive dreissenid mussels.

MATERIALS AND METHODS

Isolation of marine bacteria. Marine bacteria were isolated from the surfaces of *M. sallei* shells and its settlement substrate (submerged polypropylene ropes) collected from Maluan Bay, Xiamen, China (24°33′N, 118°01′E). These surfaces were washed with autoclaved sterile seawater to remove sediment and then sampled using sterile cotton swabs. The cotton swabs were stored in sterile tubes and kept on ice during transport. Once at the laboratory, 5 ml autoclaved sterilized seawater was added to each tube with the cotton swab, and the tubes were vortexed. Serial dilutions (100 μl) of the suspension were spread on Zobell 2216E agar plates and incubated at 28°C for 48 h. Bacterial colonies with different morphotypes were then isolated and purified. All strains were stored in 30% glycerol–70% Zobell 2216E broth at −80°C.

Evaluation of biofilm-forming capacity. The assay for evaluating the biofilm-forming capacity of the isolated bacterial strains was performed according to O'Toole et al. (43). Each bacterial strain was incubated in Zobell 2216E broth for 24 h (28°C, 180 rpm). Bacterial culture (2 μl) was added to 96-well microtiter plates containing 200 μl fresh 2216E broth. After incubating at 28°C for 24 h, the supernatant was removed from each well, after which 4% formaldehyde was added into each well to fix the biofilms. After 10 min, the supernatant was removed, and the plates were air dried. Then, 50 μl of 0.1% (wt/vol) crystal violet was added to each well. After 10 min, the excess crystal violet was removed by washing the plates thoroughly with distilled water. After air drying, 200 μl of 95% ethanol was added to each well to solubilize the stained biofilm. The absorbance of the stained dye was determined by a spectrophotometer (InfiniteM200 Pro; Tecan) at 600 nm. Here, we chose strains which produced biofilms with an optical density at 600 nm (OD_{600}) of >1.0 for further study.

Identification of isolated strains with strong biofilm-forming capacity. The chosen strains were tentatively identified based on sequencing 1.5 kbp of the 16S rRNA genes using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (10, 25). The 16S rRNA gene sequences were analyzed with the BLAST program of the GenBank database at the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/).

Larval culture of *M. sallei*. Gravid adults of *M. sallei* were collected from the submerged ropes of an oyster farm in Maluan Bay, Xiamen, China (24°33′N, 118°01′E). Spawning induction and larval culture were carried out in the laboratory following the method described by He et al. (17). Pediveligers which were capable of settlement and metamorphosis were used for the bioassays.

Effects of monospecific bacterial biofilms on larval settlement and metamorphosis. Monospecific bacterial biofilms were prepared following the method described by Yang et al. (25), with some modifications. Each isolated strain with strong biofilm-forming capacity was cultured in ZoBell 2216E broth for 48 h (28°C, 180 rpm) and harvested by centrifugation (4,000 rpm, 10 min). Bacterial cells in the pellet were resuspended in sterile seawater (20 practical salinity units [PSU]) and the density adjusted to ~10^8 cell/ml. The bacterial suspension (4 ml) was added to each well of sterile 6-well polystyrene plates. After incubating at 28°C for 24 h, the medium in the wells was removed, the wells were rinsed three times gently with sterile seawater to remove unattached bacteria, and then 10 ml fresh sterile seawater and 30 to 40 pediveliger larvae of *M. sallei* were added to each well. Three replicates were set up for each treatment. FSW was used as a control. The plates were cultured at 28°C. Larval settlement, metamorphosis, and mortality of *M. sallei* were observed at 12, 24, and 48 h by using a DM IL light-emitting diode (LED) Leica microscope. Settlement is defined by the following behavior: the pediveliger larvae stop swimming, descend to the substratum, and crawl with an extended foot or attach by byssus while the velum is absorbed. Mussel larval metamorphosis includes loss of the swimming and feeding organ velum, development of the gills, and the production of a juvenile/adult shell dissoconch (16, 23).

Bioassay-guided isolation of the inducing cue(s) from the strain *V. owensii* MS-9. Strain *V. owensii* MS-9 showed the highest inducing activity on larval settlement and metamorphosis of *M. sallei* and was therefore analyzed further for the presence of chemical cues. Bioassay-guided isolation of the cue(s) from *V. owensii* MS-9 was carried out as described by Tebben et al. (10), with some modifications. Three hundred ZoBell 2216E agar plates were inoculated with a stock culture of strain *V. owensii* MS-9 and grown for 96 h at 28°C. Bacterial colony biomass (130 g) was carefully scraped off with glass slides, and the pellet was extracted twice with ethanol at 1:10 (wt/vol). The extract was evaporated to dryness under reduced pressure. Then, the combined ethanol extract was subjected to chromatography on a

reversed-phase ODS column (25 by 1,500 mm; Amersham, Piscataway, NJ) and sequentially eluted with water-acetonitrile (7:3), acetonitrile, methanol, and dichloromethane. The resulting eight fractions (F1 to F8) were tested in the bioassay described below. Fraction F2 showed significant inducing activity on larval settlement and metamorphosis. Accordingly, F2 was further purified via a Sephadex LH-20 column (25 by 1,500 mm; Amersham) eluted with 70% methanol at a flow rate of 0.1 ml min^{-1} to yield three fractions (F2-1, F2-2, and F2-3). Bioassays were again carried out in the same manner. The inductive fraction F2-2 was analyzed by LC-MS (Q Exactive; Thermo Fisher, USA) with electrospray ionization in positive-ion mode in the 100 to 1,000 atomic mass unit (AMU) range. The solvents were water with 10 mM ammonium acetate (A) and methanol (B). With the column temperature maintained at 30°C and the solvent flow at 1.0 ml/min, a 10-μl sample was separated on a reversed-phase C_{18} SB-Aq column (250 mm by 4.6 mm, 5 μm). Elution was carried out at a constant gradient solvent with a composition of 98:2 (A:B) for 30 min. UV absorption was monitored at 254 nm. Five nucleobases, namely, uracil (Ura), guanine (Gua), thymine (Thy), xanthine (Xan), and hypoxanthine (Hyp), were detected in the inductive fraction F2-2 by LC-MS. These compounds were identified on the basis of their retention time, absorbance spectrum, MS fragmentation pattern, and cochromatography with standard compounds.

Bioassays with the extract and fractions from the inducing bacterium on larval settlement and metamorphosis. Bioassays were conducted in sterile 6-well polystyrene plates. The ethanol extract and fractions obtained as described above were dissolved in dimethyl sulfoxide (DMSO) or FSW. A volume of 50 μl of each solution was added to each well containing 9.95 ml FSW. Approximately 30 to 40 pediveligers of *M. sallei* were added to each well containing 10 ml of a test solution. FSW and a 0.5% (vol/vol) solution of DMSO in FSW were used as controls. The pretest showed that there were no significant differences between the FSW control and the 0.5% DMSO control for larval response (data not shown). The plates were cultured at 28°C. Three replicates were set up for each treatment. Larval settlement, metamorphosis, and mortality of *M. sallei* were observed at 24 h through a Leica microscope (DM IL LED).

Effects of five nucleobases on larval settlement and metamorphosis. To determine whether the nucleobases detected in the inductive fraction F2-2 had inducing activity, we tested the activities of Ura, Gua, Thy, Xan, and Hyp. These standard compounds were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO, USA). These five nucleobases were dissolved in FSW and assayed at 1, 5, 10, 20, and 50 μM, respectively, with pediveligers of *M. sallei* following the procedures described above. FSW was used as a control. Throughout this work, experiments were carried out in triplicate.

Test for activity of BCS of the strain *V. owensii* MS-9, quantification of nucleobases in BCS, and determination of the synergistic effects of nucleobases. Since the five nucleobases (Ura, Gua, Thy, Xan, and Hyp) could be dissolved in FSW, we hypothesized that the inducing cues from strain *V. owensii* MS-9 could be released into seawater. To test this, we investigated larval responses to the BCS of the strain *V. owensii* MS-9. A monospecific bacterial biofilm of the strain *V. owensii* MS-9 was prepared as described above. Bacterial suspensions (4 ml, ~10^8 cell/ml) were added to each well of the sterile 6-well plates incubating at 28°C for 24 h. After the biofilm formed, 10 ml fresh sterile seawater was added to each well, which was incubated at 28°C for 24 h, after which the BCS was obtained. The BCS was filtered through a 0.22-μm sterile filter unit with a Durapore polyvinylidene difluoride (PVDF) membrane (Millex-HV; Merck Millipore Corporation). The filtered BCS was assayed with pediveligers of *M. sallei* in new sterile 6-well plates, following the procedures described above. FSW was used as a negative control and the biofilm of *V. owensii* MS-9 as a positive control.

The result showed that the BCS of the *V. owensii* MS-9 biofilm induced larval settlement and metamorphosis. To examine whether the nucleobases were responsible for the inducing activity of BCS, we first determined the concentrations of nucleobases in the BCS of *V. owensii* MS-9 biofilm by HPLC. Quantification was performed by external calibration with standards. A standard curve was obtained by plotting the peak area versus concentration (2, 5, 10, 20, and 50 μg ml^{-1}) by HPLC using a C_{18} SB-Aq column (250 mm by 4.6 mm, 5 μm). The elution solvents were 7.5 mM potassium phosphate buffer (A) and methanol (B). Constant gradient elution with a solvent composition of 98:2 (A:B) for 30 min was performed. The eluent was monitored by UV detection at 254 nm. Quantification of nucleobases was achieved based on regression analysis of the peak area against concentration (Table S3). BCS and standard solutions were analyzed in triplicate.

Since the concentrations of Thy, Hyp, and Gua in the BCS of strain *V. owensii* MS-9 were found to be much lower than the lowest effective concentration of each compound when tested individually, the synergistic effect of a mixture of nucleobases was suspected. We tested all the possible combinations of the five nucleobases at concentrations near to their concentrations in BCS (Table S2). The responses of larvae to the combinations of the nucleobases were assayed with pediveligers of *M. sallei* following the procedures described above. The biofilm of strain *V. owensii* MS-9 was used as a positive control.

Quantification of nucleobases in BCS of three inducing strains and three noninducing strains and test for activity of synthetic nucleobase mixture similar to the natural nucleobase mixture present in the BCS. Since bacteria are known to produce nucleobases (39), to examine whether nucleobases play an important role in the inducing activity in other bacterial biofilms, we chose three inducing strains (*P. profundi* MS-3, *P. celer* MS-14, and *A. palladensis* MS-24) and three noninducing strains (*Pseudoalteromonas* sp. MS-1, *Pseudoalteromonas* sp. MS-2, and *V. fluvialis* MS-10) and determined the concentrations of Ura, Gua, Thy, Xan, and Hyp in the BCS of these strains. The BCS of each strain was prepared. The kinds and concentrations of nucleobases in each BCS were determined by HPLC, as described above. Then, we prepared nucleobase mixtures with each compound at concentrations similar to those observed in the BCS of each strain. The effects of the nucleobase mixtures for the six strains on larval settlement and metamorphosis of *M. sallei* were tested following the procedures described above.

Statistical analysis. The results were analyzed with the SPSS 17.0 software. Prior to analysis by one-way analysis of variance (ANOVA) with a Tukey *post hoc* test, all data were expressed as percentages of larval settlement, metamorphosis, and mortality and were arcsine transformed. The significance level was set at a P value of <0.05.

Data availability. The sequences for all 16S rRNA genes are publicly available at the NCBI. The accession numbers are MK995596 (MS-1), MK995597 (MS-2), MK995598 (MS-4), MK995599 (MS-20), MK995600 (MS-12), MK995601 (MS-21), MK995602 (MS-16), MK995603 (MS-23), MK995604 (MS-5), MK995605 (MS-17), MK995606 (MS-6), MK995607 (MS-18), MK995608 (MS-19), MK995609 (MS-24), MK995610 (MS-22), MK995611 (MS-13), MK995612 (MS-14), MK995613 (MS-7), MK995614 (MS-8), MK995615 (MS-9), MK995616 (MS-3), MK995617 (MS-10), MK995618 (MS-15), and MK995619 (MS-11).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01039-19.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We declare no competing interests.

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