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Effect of Biofilm Formation by *Pseudoalteromonas spongiae* on Induction of Larval Settlement of the Polychaete *Hydroides elegans*[▽]

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The effects of culture conditions and chloramphenicol treatment on the induction of the marine bacterium *Pseudoalteromonas spongiae* to larval settlement of *Hydroides elegans* were investigated. The results showed that *P. spongiae* cells grown in the medium containing both yeast extract and peptone (YP-grown *P. spongiae*) was highly inductive to larval settlement, whereas *P. spongiae* cells grown in the medium containing only peptone (P-grown *P. spongiae*) or YP-grown *P. spongiae* cells treated with chloramphenicol at the onset of biofilm development (YPC-grown *P. spongiae*) did not induce larval settlement. Analysis of biofilm formation, biofilm structure, and the surface protein profile indicated that only the induction-capable YP-grown *P. spongiae* formed a well-developed biofilm, while the P-grown *P. spongiae* and the YPC-grown *P. spongiae* did not. We report here for the first time that bacterial biofilm formation was associated with its induction of larval settlement.

In the marine environment, natural and artificial substrata are readily colonized by micro- and macroorganisms in a process known as "biofouling" (4, 8, 52). The dioecious, free-spawning, tube-building polychaete *Hydroides elegans* (Haswell 1883) is one of the most troublesome fouling organisms, occurring widely in tropical and subtropical seawaters (41, 51). Larval settlement of *H. elegans* marks the turning point from a planktonic life stage to a sessile life stage and represents a crucial step in biofouling. Factors affecting larval settlement are therefore the focus of biofouling studies and antifouling control.

Competent larvae of *H. elegans* settled rapidly after induction by marine natural biofilms or certain monospecies bacterial films in the laboratory (26, 51). Larvae of *H. elegans* settle only in the presence of a metabolically active biofilm, however, not on a clean surface (21, 27, 51). Previous studies suggested that the bacterium-derived settlement cues were produced after bacteria attached to a surface and the cues were biofilm surface associated (11, 13, 19, 25). However, little attention was paid as to whether the bacterial biofilm formation was involved in bacterial induction of larval settlement. Bacteria undergo profound changes in physiological features during biofilm formation, a dynamic process wherein bacteria transform from planktonic (free-swimming) organisms to cells that are part of a complex, surface-attached community (5, 9, 15, 35, 36, 42, 47). Recent research revealed that many kinds of bacterial activity, such as infection or symbioses and production of bioactive compounds, were associated with biofilm formation (2, 12, 32, 53). Microbiologists have turned to biofilm formation for explanations of interesting microbial behaviors/bioactivities (37, 38).

In this study, employing the newly described marine bacterium *Pseudoalteromonas spongiae* (24, 28), we investigated the possible relevance of biofilm formation for its ability to induce larval settlement of *H. elegans*.

Effect of culture condition on bacterial induction of larval settlement. In a preliminary experiment, bacterial culture conditions including temperature (16°C and 30°C), salinity (17 ppt and 34 ppt), yeast extract (0 g lliter^{-1} and 3 g liter^{-1}), peptone (0 g liter^{-1} and 5 g liter^{-1}), and glucose (0 g liter^{-1} and 10 g liter^{-1}) were combined according to the Latin square design $L_8(2^7)$ in order to determine which factor(s) had a significant effect on bacterial biofilm's induction of larval settlement of *H. elegans*. Bacterial cells from different culture conditions were harvested from the broth by centrifugation (3,000 × *g*, 10 min), washed with autoclaved filtered seawater (AFSW), and then resuspended in AFSW (optical density at 600 nm of 0.15). Four milliliters of bacterial suspension was added to a polystyrene petri dish (diameter, 50 mm; Falcon no. 1006), incubated at 24°C for 3 h under a static condition to develop a single-layer bacterial film (26). Larval settlement bioassays were then performed according to references 26 and 39. The results showed that among the five factors, temperature, salinity, glucose, and peptone had no significant effect ($P > 0.05$), while yeast extract was the only factor having significant effect on the induction of the biofilm: $P < 0.05$; one-way analysis of variance (ANOVA); $F = 77.65 > F_{0.05}(1, 2) = 18.51$.

To confirm the importance of yeast extract for the bacterial induction of larval settlement, *Pseudoalteromonas spongiae* was grown in media containing 8 g liter^{-1} yeast extract (Y) and peptone (P) at different ratios, namely, Y/P ratios of 0:8, 1:7, 3:5, 5:3, 7:1, and 8:0. Bacteria grew well in all media; bacterial biomasses in different media after cultivation were not significantly different from each other (one-way ANOVA; $P < 0.05$).

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However, biofilms of bacteria from different culture media showed remarkable difference in induction of larval settlement. *P. spongiae* cells grown in the medium containing only peptone (designated P-grown *P. spongiae*) or only yeast extract (designated Y-grown *P. spongiae*) showed no induction (19% of larvae settled) or only moderate induction (46% of larvae settled), respectively, whereas *P. spongiae* cells grown in media containing both Y and P (YP-grown *P. spongiae*), no matter at what ratio, were highly inductive to larval settlement (more than 63% of larvae settled). The highest settlement rate, 78%, was recorded in response to the bacterium grown in the medium with a 3:5 Y/P ratio (YP-grown *P. spongiae*). In all cases, bacterial densities in biofilms before the larval settlement bioassay were around 3×10^5 cells mm^{-2} and were not significantly different from each other (one-way ANOVA; $P < 0.05$).

These results indicated that presence of yeast extract in the culture medium was essential for the bacterial induction of larval settlement. Nutrient availability could be the cause for the difference, since peptone consists of only protein hydrolysis products, while yeast extract contains different types of nutrients such as trace metals, vitamins, etc. The key element in the yeast extract for the biofilm's inductivity deserves further investigation in the future.

Marine *Pseudoalteromonas* spp. are capable of producing bioactive compounds (18). Several studies of the relationship between marine *Pseudoalteromonas* spp. and invertebrate larval settlement suggested that *Pseudoalteromonas* spp. might provide settlement cues for invertebrate larvae (18, 20, 33). In our study, supernatants from planktonic culture and biofilm-conditioned seawater of the YP-grown *P. spongiae* and P-grown *P. spongiae* cells had no inductive effects, suggesting that the inductive cues should be surface-associated small compounds or macromolecules, which was consistent with previous studies (19, 25, 27).

Effect of chloramphenicol treatment on bacterial induction of larval settlement. The highly inductive biofilm of YP-grown *P. spongiae* was treated with chloramphenicol (100 μg ml^{-1}; Sigma) and tested for larval settlement. Chloramphenicol served here as the protein synthesis inhibitor (14). In a preliminary experiment, we found that incubating the bacterial cells in chloramphenicol (100 μg ml^{-1} in AFSW) for 3 h did not significantly ($P < 0.05$) reduce the number of viable bacteria (3-h-treated cultures, 1.06×10^8 CFU ml^{-1}; untreated culture, 1.28×10^8 CFU ml^{-1}), while in a disk diffusion assay, chloramphenicol did exert an inhibitive effect on protein synthesis, since no bacteria grew around the paper disk.

Development of bacterial films was performed the same as described before. Chloramphenicol was added at the onset (0 h) of biofilm formation or after 10 h of biofilm formation to a final concentration of 100 μg ml^{-1}. In both cases, after 3 h of treatment, chloramphenicol solution was removed before larval settlement bioassay; parallel dishes without the chloramphenicol treatment served as controls. The larval settlement bioassay showed that chloramphenicol treatment at the onset of biofilm formation significantly reduced the biofilm's level of induction (one-way ANOVA; $P < 0.05$), and only 16% of larvae settled on the treated biofilm, whereas 72% of larvae settled on the control biofilm. In contrast, treatment at the 10th hour of biofilm formation did not affect the biofilm's ability to induce.

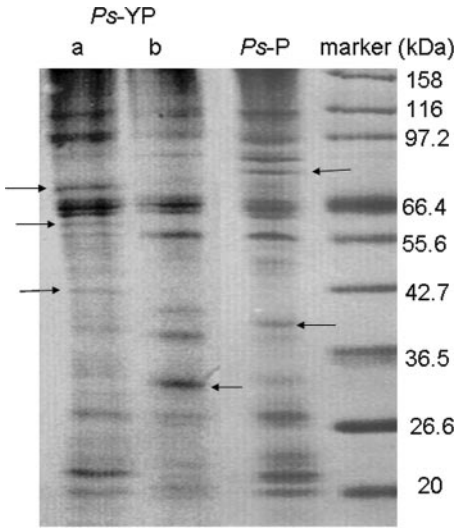

FIG. 1. Surface protein profiles of YP-grown *P. spongiae* (*Ps*-YP) and P-grown *P. spongiae* (*Ps*-P) as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane a, the surface protein profile after attachment; lane b, surface protein profile before attachment. Samples were analyzed in a 12% separation gel and stained with Coomassie blue. Arrows indicate the unique bands in each sample.

These results indicated that the initial 10 h of biofilm development was essential for the biofilm's ability to induce. O'Toole and Kolter reported that protein synthesis was required for initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 (36). Treatment of chloramphenicol at the onset of biofilm formation might affect the protein synthesis or the activity of some regulatory proteins, which in turn would affect its biofilm development and produce induction cues for larval settlement, while after 10 h of development, those proteins that were required for presenting larval settlement induction might have already been synthesized (50), which in turn would maintain the biofilm's ability to induce larval settlement. The surface protein profiles of YP-grown *P. spongiae* and P-grown *P. spongiae* before attachment and after 10 h of attachment were then further investigated.

Bacterial surface protein profile. Surface proteins were extracted by increasing the pH value (34) and analyzed in 12% separating gel using standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis protocols (23). After electrophoresis, protein bands were stained with Coomassie blue.

The results showed that the surface protein profile of YP-grown *P. spongiae* changed remarkably after attachment (Fig. 1). Some bands, such as those around 40 and 32 kDa in lane b, were unique in the YP-grown *P. spongiae* before attachment, whereas some bands, such as those around 70, 60, and 42 kDa in lane a, were unique in the YP-grown *P. spongiae* after attachment, suggesting that some specific surface proteins were either newly presented or shed during bacterial biofilm formation. It was also observed that some protein bands, such as bands around 97 and 22 kDa in lane a, were up-regulated, while some protein bands, such as bands around 55 and 38 kDa in lane b, were down-regulated after biofilm formation. These results indicated that the surface protein profile of YP-grown *P. spongiae* changed remarkably during the first 10 h of biofilm

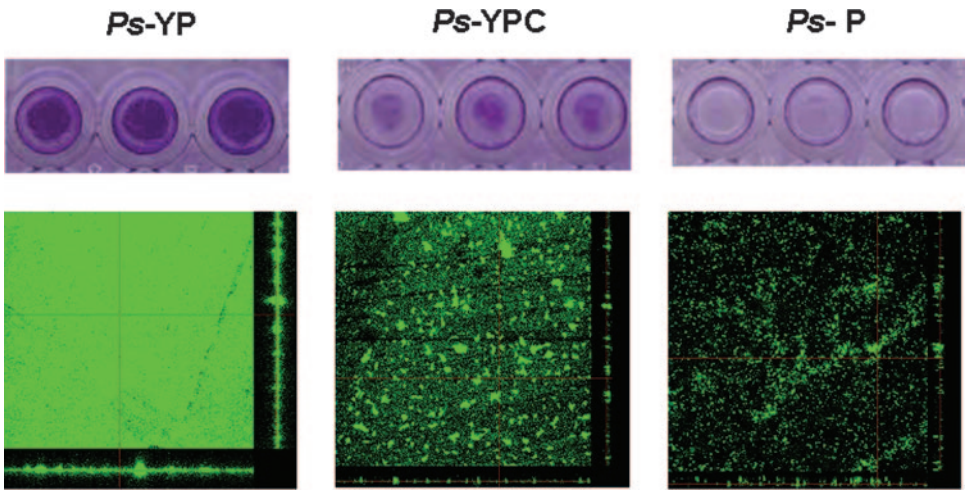

FIG. 2. Biofilm formation of *Pseudoalteromonas spongiae* under different conditions. (Top panels) Biofilm formation analysis in 96-well polystyrene plates with three replicates. The extent of biofilm formation was determined by a crystal violet assay. (Bottom panels) CLSM images of biofilms. Biofilms were stained with fluorescein isothiocyanate-conjugated concanavalin A and were viewed at a ×400 magnification. *Ps*-YP, *Pseudoalteromonas spongiae* grown in medium containing yeast extract and peptone; *Ps*-YPC, YP-grown *P. spongiae* treated with chloramphenicol at the onset of biofilm formation; *Ps*-P, *Pseudoalteromonas spongiae* grown in medium containing peptone.

formation. Sauer et al. reported that more than 30 genes and 40 proteins were altered within 6 h following the attachment of *Pseudomonas putida* (44).

On the other hand, the surface protein profile of the P-grown *P. spongiae* did not change distinguishably after attachment. In comparison to the surface protein profile of the YP-grown *P. spongiae* before attachment, the P-grown *P. spongiae* did share some common protein bands, such as the bands around 116, 97, 66, and 56 kDa, representing the identical surface proteins for this bacterial species (Fig. 1). However, overall they were different, especially these bands around 70, 60, and 42 kDa: these bands, which appeared only in the YP-grown *P. spongiae* after attachment, did not appear in the P-grown *P. spongiae*, indicating that these proteins may be biofilm-specific proteins.

The "biofilm phenotype" is loosely defined by the patterns of protein and gene expression associated with biofilm cultures in comparison to those associated with planktonic culture (37). Therefore, an alteration in the protein profile is expected in bacteria after biofilm formation (43, 45). Here, we investigated only the surface protein profile, since surface proteins were considered to be important in the interaction between bacteria and surfaces (6, 16, 40) and only surface proteins could have any direct interaction with settling larvae. In the present study, the surface protein profile of the YP-grown *P. spongiae* changed remarkably after attachment, whereas changes in that of the P-grown *P. spongiae* were not distinguishable, suggesting that the YP-grown *P. spongiae* underwent a biofilm formation process, whereas the P-grown *P. spongiae* did not. Biofilm formation of YP-grown *P. spongiae*, YP-grown *P. spongiae* treated with chloramphenicol at the onset of biofilm formation (YPC-grown *P. spongiae*), and P-grown *P. spongiae* was further investigated.

Bacterial biofilm formation analysis. Analysis of bacterial biofilm formation was performed in a 96-well polystyrene plate (10). The results showed that the YP-grown *P. spongiae* cells attached well to the surface of the well, whereas a treatment with chloramphenicol significantly reduced the biomass of YPC-grown *P. spongiae* cells (Fig. 2). The P-grown *P. spongiae* cells could hardly attach to the surface of the well (Fig. 2). These results suggested that the P-grown *P. spongiae* was not capable of biofilm formation, whereas the YP-grown *P. spongiae* was capable of biofilm formation, yet chloramphenicol treatment inhibited its biofilm formation.

Biofilm structures of YP-grown *P. spongiae*, YPC-grown *P. spongiae*, and P-grown *P. spongiae* were further investigated by confocal laser scanning microscopy (CLSM). A bacterial suspension (optical density at 600 nm of 0.5) in AFSW was incubated in a petri dish (diameter, 90 mm; Sterilin, United Kingdom) containing a glass slide for 10 h in order to develop a biofilm. Biofilms were then stained with 1 mg ml^{-1} solution of fluorescein isothiocyanate-conjugated concanavalin A (Sigma) and visualized under a CLSM at a magnification of ×40 (dry, NA, 0.75; Nikon C1, Japan). Two replicate biofilms were formed for each treatment. For each biofilm, three image stacks were taken. In total, there were six image stacks for each treatment. The three-dimensional CLSM image of a biofilm was quantified using the computer program COMSTAT (15). Biofilm biomass, mean thickness, maximum thickness, surface coverage, and roughness were chosen to characterize the biofilm structure.

The CLSM pictures showed that biofilm structures of YP-grown *P. spongiae* (with or without chloramphenicol) and P-grown *P. spongiae* differed from each other (Fig. 2). Characterization of biofilm structures by COMSTAT (15) showed that the total biomass, substratum coverage, and maximum/mean thickness of the YP-grown *P. spongiae* biofilm were significantly higher ($P < 0.05$; *t* test) than those of the treated YP-grown *P. spongiae* biofilm and P-grown *P. spongiae* biofilm, whereas the roughness coefficient of the YP-grown *P. spongiae* biofilm was significantly lower ($P < 0.05$; *t* test) (Table 1). These values indicated that the YP-grown *P. spongiae* could effectively build up a biofilm with three-dimensional architecture in 10 h and tent to form a thick and even biofilm, while the

TABLE 1. Total biomass, maximum thickness, mean thickness, roughness coefficient, and substratum coverage of biofilms of YP-, YPC-, and P-grown *P. spongiae*[a]

P. spongiae biofilm	Total biomass ($\mu m^3/\mu m^2$)	Thickness (μm)		Roughness coefficient	Substratum coverage
		Maximum	Mean		
YP grown	6.94 ± 0.87	19.00 ± 2.35	8.03 ± 1.06	0.25 ± 0.02	0.99 ± 0.02
YPC grown	1.83 ± 0.09	7.60 ± 0.96	1.49 ± 0.20	0.74 ± 0.08	0.65 ± 0.05
P grown	0.76 ± 0.04	11.40 ± 1.19	0.99 ± 0.06	1.63 ± 0.03	0.11 ± 0.02

[a] Values are means ± standard deviations of data from six CLSM image stacks.

chloramphenicol treatment inhibited biofilm development. As for the P-grown *P. spongiae*, few bacteria attached to the surface; the substratum coverage and mean thickness were very low, suggesting that the P-grown *P. spongiae* cells were unable to effectively attach to the surface and build up a biofilm.

A five-stage process is generally accepted to describe the development of biofilm formation. This process includes initial reversible surface attachment, irreversible attachment by producing extracellular polymeric substance (EPS), early development of biofilm architecture, maturation of biofilm architecture, and dispersion of single cells from the biofilm (47). The first three stages can happen in hours (35, 43). In the present study, biofilm formation of *P. spongiae* was performed under static conditions and for a short period of time (10 h) as compared to biofilm formation performed in a flow system. It is not surprising that no complex biofilm architecture such as the mushroom shape or water channels were observed in our biofilms (47). Our data on biofilm structure suggested that biofilm formation of the YP-grown *P. spongiae* had progressed to the third stage where bacteria attached irreversibly to the surface and started developing the biofilm architecture, whereas treatment with chloramphenicol arrested biofilm development at the first or second stage. On the other hand, biofilm formation in the P-grown *P. spongiae* was unlikely to happen. The bacterium seemed to stay at the first stage, where the reversibly attached cells were easily washed off in the 96-well plate analysis.

Bacterial biofilm formation is believed to be influenced by environmental signals and regulatory pathway (46). Nutrient availability is a common environmental signal that affects biofilm formation in different bacterial species. For example, phosphate, iron, and glucose were reported to affect biofilm formation of a *Citrobacter* sp., *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, respectively (1, 3, 29). Molecular genetic analyses have also begun to reveal regulatory proteins and, through them, the environmental signals that affect biofilm formation (3, 29, 46). In this study, yeast extract in the medium provided necessary nutrients that enable YP-grown *P. spongiae* to develop a biofilm subsequently, whereas chloramphenicol treatment at the onset of biofilm formation might inhibit certain regulatory proteins and hence inhibit the biofilm formation.

The importance of bacterial biofilms in inducing larval settlement of certain benthic invertebrates in the marine environment has long been realized (11, 17, 22, 52). Previous studies on the interaction between a bacterial biofilm and a settling larva had pointed out that EPS of the biofilm might play an important role in inducing larval settlement (19, 25, 30, 31, 49). In fact, EPS also plays an important role in bacterial biofilm formation (7, 47, 48). EPS consists not only of polysaccharides but also considerable amounts of proteins, nucleic acids, and lipids (48). More detailed investigation of EPS with consideration of the protein activity would help to elucidate the mechanism underlying the induction of larval settlement by bacterial biofilm.

In conclusion, our data showed that the ability of the marine bacterium *P. spongiae* to induce larval settlement of *H. elegans* was associated with its biofilm formation. This study is the first one to investigate the effect of biofilm formation on bacterial induction of larval settlement. However, the question remains open as to how biofilm formation affects bacterial induction of larval settlement. A biofilm formation-deficient mutant would be necessary in the future to address this question.

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