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Bioactivity of bacterial strains isolated from marine biofilms in Hong Kong waters for the induction of larval settlement in the marine polychaete *Hydroides elegans*

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ABSTRACT: In the present study, 38 bacterial isolates were obtained from a marine biofilm, identified by the comparison of 16S rRNA gene sequences, and investigated by laboratory bioassays for their effects on larval settlement of the marine polychaete *Hydroides elegans* (Haswell). The bacterial isolates belonged to 3 phylogenetic branches: γ-*Proteobacteria* (26 isolates), Gram-positive (8 isolates) and *Cytophaga-Flexibacter-Bacteroides* (4 isolates). Most of the isolates were affiliated to the genera *Vibrio* (7 isolates), *Alteromonas* (8 isolates) or *Pseudoalteromonas* (8 isolates), which are in the γ-*Proteobacteria* branch. According to their efficacy to induce larval settlement of *H. elegans* in laboratory bioassays, the isolates were categorized as strongly, moderately, and non-inductive for larval settlement. About 42% of the isolates were categorized as non-inductive and the rest of the isolates contained equal numbers of highly and moderately inductive strains. The results indicated that larval settlement of *H. elegans* could be induced by bacteria in a wide range of taxa. The isolates that induced high and moderate levels of larval settlement belonged to the genus *Cytophaga* in the *Cytophaga-Flexibacter-Bacteroides* branch; the genera *Bacillus*, *Brevibacterium*, *Micrococcus* and *Staphylococcus* in the Gram-positive branch; and the genera *Alteromonas*, *Pseudoalteromonas* and *Vibrio* in the γ-*Proteobacteria* branch. Results also showed that isolates affiliating to the same genus might have tremendously different activities for the induction of larval settlement. For example, isolates that were affiliated to the genus *Alteromonas* or *Pseudoalteromonas* distributed over the 3 categories of activity for induction of larval settlement.

KEY WORDS: *Hydroides elegans* · Larval settlement · Biofilm · Bacteria · 16S rRNA genes

INTRODUCTION

All surfaces, both animate and inanimate, exposed to the marine environment are under the pressure of colonization by marine organisms. This colonization is referred to as biofouling. The formation of a biofouling community is a dynamic and random process (reviewed by Clare et al. 1992). The primary driving force underlying this process is the relative abundance of each kind of foulers in the water column. The commonly observed sequential formation of biofouling communities (i.e. in the order of colonization: dissolved molecules, bacteria, diatoms, and invertebrates) is a consequence of the primary driving force. The secondary driving forces are physical, chemical and behavioral interactions between the foulers. The interactions between surface-associated bacteria and invertebrate larvae that seek suitable surfaces for settle-

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ment (i.e. attachment and metamorphosis) are well-known examples for the secondary driving force.

Surface-associated bacteria impose important influences over the settlement of many invertebrate larvae. Bacteria may stimulate (e.g. Szewzyk et al. 1991, Leitz & Wagner 1993, Lau & Qian 1997, Unabia & Hadfield 1999), inhibit (e.g. Holmström et al. 1992, Avelin et al. 1993, Maki et al. 1988, Lau & Qian 2000) or not affect (e.g. Brancato & Woollacott 1982, Maki et al. 1988, Avelin et al. 1993, Lau & Qian 1997, 2000, Unabia & Hadfield 1999) larval settlement, depending on the species of invertebrates and bacteria of concern. Although the presence of surface-associated bacteria is a prerequisite for larval settlement of certain invertebrates, larvae of these invertebrates may respond to individual bacterial isolates to different extents (Kirchman et al. 1982, Mitchell & Maki 1988, Lau & Qian 1997, Unabia & Hadfield 1999). Previous studies have suggested the effect of surface-associated bacterial communities on larval settlement to be a function of bacterial species composition (Keough & Raimondi 1996, Lau & Qian 1997, Rodriguez & Epifanio 2000). For example, invertebrate larvae settled at different rates in the field when exposed to surfaces that differed in the composition of microbial community (Keough & Raimondi 1996), and larvae of the mud crab *Panopeus herbstii* were induced to settle in laboratory conditions only by bacterial communities originated from sediments in the habitat of adult crabs (Rodriguez & Epifanio 2000).

The paramount importance of surface-associated bacteria for larval settlement of invertebrates makes finding the identity of these bacteria an interesting and important research goal. Larval settlement of the tubeworm *Hydroides elegans* is induced by surface-associated bacteria, but only few bacterial strains appeared to be inductive in laboratory bioassays (Lau & Qian 1997, Unabia & Hadfield 1999). Moreover, the species composition of artificially formed bacterial communities has been suggested to be an important factor governing their activity for the induction of larval settlement in *H. elegans* (Lau & Qian 1997). At present, virtually no information exists on the type(s) of bacteria that induce(s) larval settlement of *H. elegans*.

In the present study, through the isolation and genetic characterization of bacteria, we investigated the species diversity of bacteria occurring in the habitat of *Hydroides elegans* in Hong Kong waters. Subsequently, the bacterial isolates were investigated for their efficacies on the induction of larval settlement in *H. elegans*. Our aim was to answer the questions: (1) what are the species of bacteria that induce larval settlement of *H. elegans*?; and, (2) is the settlement induction activity limited to certain taxa of bacteria?

MATERIALS AND METHODS

Obtaining natural biofilms. Marine biofilms (i.e. conglomerate of organic molecules, bacteria, diatoms and fungi) were collected from the habitat of adult *Hydroides elegans*, according to the methods stated in Lau & Qian (1997) with modifications. We opted for the pier of the Hong Kong University of Science and Technology (HKUST), Clear Water Bay, as the collection site at which colonies of *H. elegans* are present year round (Lau & Qian unpubl. data). Briefly, 5 acid-washed, autoclaved glass microscope slides were submerged in the seawater for the formation of biofilms. The slides were maintained at 2 m below the lowest water level for the subsequent submersion period. After 3 d, the slides were retrieved, individually encased in 50 ml centrifuge tubes filled with autoclaved, 0.45 µm filtered seawater (FSW) and transported to the laboratory immediately. To protect the slides from temperature fluctuation during transportation, the centrifuge tubes were held in a container of seawater collected on site.

Isolation of bacterial strains. In the laboratory, the glass slides were thoroughly rinsed with autoclaved FSW and scraped with an autoclaved nylon paintbrush. The biofilm detached from each slide was individually suspended in 40 ml of autoclaved FSW, mixed vigorously by vortexing and diluted 10 and 100 times in autoclaved FSW. From each biofilm suspension, 200 µl aliquots were spread on plates of nutrient agar for bacterial growth (0.3% yeast extract, 0.5% peptone, 1.5% agar, FSW) in triplicate. The inoculated agar plates were incubated at 30°C on a 15:9 h light: dark cycle for 24 h. All subsequent incubations for bacterial growth were performed under the same conditions.

The bacterial colonies that grew on the agar plates were examined under a dissecting microscope for morphological characteristics such as color, shape, size, surface topography and the presence of granules. Conspicuous colony types were isolated, purified, and further examined for cellular morphology and growth form in nutrient broth (0.3% yeast extract, 0.5% peptone, FSW). The cell shape and Gram-stain property of the isolates were examined under a light microscope after the procedures for Gram stain (Murray et al. 1994). The isolates' growth form in nutrient broth was examined after growing pure cultures of isolates to stationary phase in nutrient broth in a static condition (i.e. no agitation or aeration). The motility of the isolates in wet-mount was examined under a light microscope. To establish stock cultures, the isolates were grown to the stationary phase in nutrient broth, mixed with equal volume of autoclaved glycerol and stored at –80°C in 1 ml aliquots.

Table 1. Primers used in this study. The primers are universal for the domain *Bacteria*. This primer pair amplifies the region between the 27th and 784th nucleotides (*Escheria coli* numbering) of the 16S rRNA gene

Primer	*E. coli* numbering	Sequence	Source
26F	8–26	5′-AGAGTTTGATCCTGGCTCA-3′	Hicks et al. (1992)
785R	785–804	5′-CTACCAGGGTATCTAATCC-3′	Lee et al. (1993)

Extracting DNA from the isolates. For each isolate, a loopful of pure colony grown on agar plate was suspended in 1 ml of autoclaved double-distilled water (ddH_2O), boiled for 15 min (Valsecchi 1998) and centrifuged at 5000 × *g* for 2 min. While the pellets were discarded, the supernatants were saved as crude DNA extracts and stored at –20°C until use.

PCR amplification of 16S rRNA gene. The crude DNA extracts were subject to PCR for the amplification of 16S rRNA gene (rDNA). The primers used in the PCR were 26F and 785R (Table 1), which are specific for the domain *Bacteria*. Each PCR mixture contained 1 U of AmpliTaq Gold™ DNA polymerase (Applied Biosystems, USA), GeneAmp® PCR buffer (Applied Biosystems), 1.5 mM $MgCl_2$, 0.8 µM of each primer, 250 mM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP and dTTP) (Pharmacia Biotechnology, USA) and 1 µl of crude DNA extract in a total volume of 25 µl. PCR was performed on a PTC-100™ programmable thermal controller with a heated lid (MJ Research, USA) under the following conditions: 95°C for 3 min; 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min; and a final PCR at 72°C for 1 min. Subsequently, 7 µl of each PCR product was subject to electrophoresis performed in a 2% agarose gel in 1 × Tris-borate-EDTA (TBE) buffer. PCR products on the gel were visualized with UV illumination after staining with ethidium bromide.

Purification and quantification of PCR products. Desired PCR products were subject to purification using Wizard® PCR preps DNA purification system (Promega, USA), according to the manufacturer's instructions. The quantity of DNA in the purified PCR products was determined by using a PicoGreen® double-stranded DNA quantitation kit (Molecular Probes, USA), according to the manufacturer's instructions.

Sequencing of rDNA fragments. The purified PCR amplicons were sequenced bidirectionally using an ABI PRISM™ big-dye terminator cycle-sequencing ready-reaction kit (Applied Biosystems) and the primer pair mentioned above. The products were resolved on an ABI PRISM™ 310 genetic analyzer (Applied Biosystems), according to the manufacturer's instructions.

Nucleotide sequence accession numbers. Obtained rDNA sequences were submitted to GenBank under the accession numbers AF343922 to AF343959.

Phylogenetic assignment. The rDNA sequence of each isolate was compared to the DNA sequences in the non-redundant nucleotide database in GenBank using BLAST (Basic Local Alignment Search Tool).

Larval culture. Adult *Hydroides elegans* were obtained from a laboratory brood stock. The procedures for obtaining gametes and raising larvae followed those in Lau & Qian (1997). Briefly, adult *H. elegans* were placed in a Petri dish containing FSW and stimulated to release gametes by gently probing the anterior ends of the worms with a needle. Newly released oocytes and sperm were mixed and transferred to 500 ml of FSW for fertilization and hatching. After hatching, the larvae were transferred to 2 l of FSW and fed with the chrysophyte *Isochrysis galbana* (Tahitian strain) at a concentration of approximately 6×10^5 cells ml^{-1}. The culture was aerated and maintained at 25°C on a 15:9 h light:dark cycle. Larvae became competent for settlement after 4 to 5 d of culture. The larval competency was checked according to the morphological characteristics described in Wisely (1958) and a rapid test with 3-isobutyl-1-methylxanthine (IBMX) according to the methods in Qian & Pechenik (1998). When over 90% of the larvae in a culture were competent, the culture was gently filtered through a 90 µm nylon mesh to harvest the larvae. Those retained on the mesh were immediately transferred into autoclaved FSW and then, within 5 min, to test vessels for larval settlement bioassays.

Formation of bacterial films for larval settlement bioassays. The effects of bacterial isolates on larval settlement of *Hydroides elegans* were investigated by bioassays performed in polystyrene Petri dishes (Falcon #1006) containing monospecies bacterial films. The formation of bacterial films followed the procedures stated in Lau & Qian (1997) with modifications. Briefly, the isolates were grown to stationary phase in nutrient broth and harvested by centrifugation (3000 × *g*). The cell pellets were washed and suspended in autoclaved FSW. The density of bacterial cells in suspension was adjusted to 10^8 cells ml^{-1} by turbidimetry. From each bacterial suspension, 4 ml aliquots were transferred into pre-sterile Petri dishes and incubated at room temperature for 3 h to allow the bacteria to attach onto the dish surface. After incubation, Petri dishes were emptied and dipped 10 times into 500 ml of autoclaved FSW to remove unattached cells.

Larval settlement bioassays. The bacterial isolates were arbitrarily distributed over 5 individual bioassays, each performed in 3 repeats. There were 2 sets of controls in each bioassay: (1) Petri dishes without

Table 2. Morphological characteristics of bacterial isolates. For cell morphology, motility and Gram stain property are designated with '+' and '–' signs: '+' for motile and Gram-positive; '–' for non-motile and Gram-negative. For colony morphology, 'Diam.' and 'Granule' represent diameter in mm and the presence of granular deposition, respectively. Diameters of colony were measured after 24 h of growth on nutrient agar at 30°C. For growth in broth, flocculent refers to bacteria that aggregate to form a wooly appearance whereas membranous refers to the formation of a suspending membranous structure

Isolate	Cell			Colony							Broth
	Gram	Shape	Motility	Color	Shape	Diam.	Surface	Elevation	Edge	Granule	
NBF1	–	Rod	–	Yellow	Circular	1	Smooth	Raised	Entire	Yes	Membranous
NBF2	–	Short rod	–	White	Circular	1	Smooth	Flat	Entire	No	Flocculent
NBF3	–	Rod	–	Yellow	Circular	3	Smooth	Raised	Entire	No	Membranous
NBF4	–	Rod	–	Tan	Circular	2	Smooth	Flat	Entire	Yes	Membranous
NBF5	–	Rod	–	Tan	Circular	3	Wrinkled, dry	Raised	Entire	No	Membranous
NBF6	–	Rod	–	Yellow	Circular	1	Smooth	Convex	Entire	Yes	Membranous
NBF7	–	Rod	–	Yellow	Circular	1	Smooth	Convex	Entire	No	Membranous
NBF8	–	Rod	–	Brown	Circular	1	Smooth	Convex	Entire	No	Membranous
NBF9	+	Coccus	–	Yellow	Circular	4	Smooth	Convex	Entire	No	Flocculent
NBF10	–	Rod	–	Light brown	Circular	3	Smooth	Raised	Entire	No	Membranous
NBF11	–	Short rod	Swarm	Tan	Irregular	>5	Smooth	Flat	Curled	No	Flocculent
NBF12	–	Rod	–	Yellow	Circular	3	Smooth	Raised	Entire	Yes	Membranous
NBF13	–	Rod	–	Tan/transparent	Circular	4	Wrinkled, dry	Flat	Entire	No	Membranous
NBF14	–	Rod	–	Yellow	Oval	1 × 2	Smooth	Raised	Entire	No	Flocculent
NBF15	–	Rod	–	Light brown	Circular	2	Smooth	Flat	Entire	No	Membranous
NBF16	–	Rod	–	Tan	Circular	2	Wrinkled, dry	Raised	Curled	No	Membranous
NBF17	–	Short rod	+	Tan/transparent	Circular	2	Smooth	Raised	Entire	No	Membranous
NBF18	–	Rod	–	White	Circular	1.5	Smooth	Raised	Entire	No	Membranous
NBF19	–	Rod	+	Light brown	Circular	2	Smooth	Raised	Entire	No	Membranous
NBF20	–	Rod	–	Tan	Circular	2	Wrinkled, glistening	Raised	Entire	No	Membranous
NBF21	–	Short rod	–	Tan	Circular	2	Wrinkled, dry	Umbonate	Undulate	No	Membranous
NBF22	–	Rod	–	Tan	Circular	2	Wrinkled, dry	Umbonate	Undulate	No	Membranous
NBF23	–	Rod	+	Tan	Circular	1	Wrinkled, dry	Raised	Curled	No	Membranous
NBF24	–	Rod	–	Tan	Circular	2	Smooth	Convex	Entire	No	Membranous
NBF25	–	Rod	–	Tan	Circular	2	Wrinkled, glistening	Raised	Curled	No	Membranous
NBF26	–	Short rod	Swarm	Tan	Irregular	>8	Smooth	Flat	Curled	No	Flocculent
NBF27	–	Rod	+	Tan	Circular	2	Smooth	Raised	Entire	No	Membranous
NBF28	–	Rod	+	Yellow	Circular	2	Smooth	Raised	Entire	No	Membranous
NBF29	–	Rod	+	Orange	Circular	1	Smooth	Convex	Entire	No	Membranous
NBF30	–	Rod	–	Tan	Circular	1	Smooth	Umbonate	Entire	No	Flocculent
NBF31	–	Rod	Swarm	White	Irregular	>5	Smooth	Flat	Curled	No	Flocculent
NBF32	–	Rod	Swarm	Tan	Irregular	>5	Smooth	Flat	Curled	No	Flocculent
NBF33	+	Rod	–	Yellow	Circular	2	Smooth	Raised	Entire	Yes	Membranous
NBF34	–	Rod	+	Light brown	Circular	1	Smooth	Raised	Entire	No	Flocculent
NBF35	+	Coccus	+	Light brown	Circular	1.5	Smooth	Flat	Entire	Yes	Flocculent
NBF36	–	Coccus	–	White	Circular	1	Smooth	Raised	Entire	No	Membranous
NBF37	+	Coccus	–	Brown	Circular	1	Smooth	Raised	Entire	No	Membranous
NBF38	–	Short rod	–	Green	Circular	0.5	Smooth	Convex	Entire	No	Membranous

bacterial films; and (2) Petri dishes that were coated with a 3 d old natural biofilm after submersion at 2 m depth at HKUST pier. For each treatment 5 replicate dishes, each receiving 20 competent larvae and 4.5 ml of autoclaved FSW, were used. All the dishes were incubated at 25°C on a 15:9 h light:dark cycle for 24 h and the number of settled individuals was recorded after incubation. Individuals that attached on the dish surface, and developed branchial radioles and calcareous tubes were recorded as settled. Mortality and abnormality of larvae were also recorded.

Statistical analysis. Data in the form of percentage of larval settlement were arcsine-transformed prior to statistical analysis. To improve the transformation, a value of ¼n (n = number of larvae used in a replicate) was given to the replicates in which no larvae settled (Zar 1996). The normality of the data was checked with Shapiro-Wilk's test (Shapiro & Wilk 1965). Data that met the normality assumption of parametric tests were analyzed using 1-way ANOVA followed by Dunnett's test. Those that did not meet the normality assumption were analyzed using non-parametric statistics. This was done by transforming the values to ranks and then applying the above-mentioned statistics (Conover & Iman 1981). Type 1 rank transformation was used, by which the entire set of data was ranked from the smallest to the largest, with the smallest value having Rank 1, the second smallest having Rank 2, and so on; average ranks were assigned in the event of ties in the ranking (Conover & Iman 1981). The data presented in all the figures are not transformed.

RESULTS

Morphological characteristics of bacteria isolated from natural biofilms

Thirty-eight bacterial morphotypes were isolated from natural biofilms (Table 2). In terms of cell morphology, the bacterial isolates were dominated by Gram-negative rods. Only 4 isolates showed a positive result in Gram stain; 3 were cocci and 1 was rod. Twenty-six isolates appeared to be non-motile and 12 isolates appeared to be motile (including 4 swarming isolates). In terms of colony morphology, tan colored circular colonies were the dominant type (Table 2). Brownish and yellowish colonies were also common. Most of the isolates formed colonies with smooth surfaces; only few had wrinkled surfaces. Some isolates formed colonies with granular depositions. In terms of growth form in nutrient broth without aeration or agitation, 28 isolates showed membranous aggregation while the rest were flocculent.

Identification of bacterial isolates by comparisons of rDNA sequences

The PCR products obtained with the primers 26F and 785R were approximately 800 bp, spanning from the 8th to the 804th nucleotide (*Escheria coli* numbering). Comparison of the 16S rDNA sequences of the isolates to the DNA sequences in the EMBL/GenBank database is given in Table 3. Three isolates were affiliated to some unidentified strains; 35 isolates were affiliated to the 9 genera, including *Alteromonas* (8 isolates), *Pseudoalteromonas* (8 isolates), *Pseudomonas* (1 isolate), *Vibrio* (7 isolates), *Bacillus* (2 isolates), *Brevibacterium* (1 isolate), *Micrococcus* (2 isolates), *Staphylococcus* (2 isolates) and *Cytophaga* (4 isolates). These genera distribute over 3 phylogenetic branches: γ-*Proteobacteria*, Gram-positive and *Cytophaga-Flexibacter-Bacteroides*.

Effect of the isolates on larval settlement

All bacterial strains formed films that were visible under a low-power dissection microscope and all films appeared to be confluent. In all bioassays, natural biofilms (positive control) elicited the highest amount of larval settlement in *Hydroides elegans* (30 to 70%) and clean surface (negative control) had the lowest (0 to 25%) (Fig. 1a to e). In all bioassays, the amounts of larval settlement on the films of individual isolates spanned between the 2 controls (Fig. 1a to e). Since the isolates induced highly variable amounts of larval settlement in different experimental repeats (e.g. isolates NBF1, 5 and 8 in Fig. 1a), the larval settlement inductive effect of each bacterial isolate was determined according to the summation of results from 3 repeats and was categorized as having strong, moderate or no activity for the induction of larval settlement (Fig. 2, Table 4). Strongly inductive isolates were the ones that had percentages of larval settlement equal to or higher than natural biofilm ($\alpha = 0.05$, Dunnett's test) in at least 2 of the 3 trials. Non-inductive isolates are the ones that had percentages of larval settlement equal to or less than a clean surface ($\alpha = 0.05$, Dunnett's test) in at least 2 of the 3 trials. Isolates that do not fall into these 2 categories were classified as moderately inductive. Under these criteria, 58% of the isolates were categorized as strongly or moderately inductive, and the remaining 42% of isolates were classified as having no effect on larval settlement.

DISCUSSION

Microbial diversity often appears to be overwhelming, as demonstrated by the occurrence of several thou-

sand independent bacterial genomes in 1 soil sample (Torsvik et al. 1990). However, in many ecosystems, bacterial numbers estimated by counts of colony forming units (CFU) are orders of magnitude lower than those by epiflourescence direct counts (Hobbie et al. 1977, Kogure et al. 1979). Although a large portion of cells detected by direct counts on natural samples is empty, referred to as 'ghost cells' (Zweifel & Hagström 1995, Heissenberger et al. 1996, Karner & Fuhrman 1997, Vosjan & van Noort 1998), culturable bacteria normally represent less than 1% of living bacteria extant in the environment (Pace 1996). This discrepancy is referred to as the great plate count anomaly (Staley & Konopka 1985). The current dogma in microbial ecology is that culturable bacteria are not the representatives of bacteria extant in the environment.

During the last decade, culture independent methods have emerged to describe the microbial diversity in different environments (Olsen et al. 1986). This has mainly been done by extracting and analyzing community

Table 3. Phylogenetic affiliation of bacterial isolates. The 16S rRNA gene sequences of individual bacterial isolates were compared to the nucleotide sequences in GenBank. The closest matching nucleotide sequence for each bacterial isolate is indicated by the strain name and accession number. 'Larval settlement' indicates the activity for the induction of larval settlement in *Hydroides elegans* (refer to Table 4 for details)

Isolate	Closest match at GenBank			Larval settlement
	Strain	Genbank accession number	Sequence similarity (%)	
γ-*Proteobacteria* division				
NBF13	Uncultured DCM-ATT-24	AF114598	94	Moderate
NBF22	Uncultured γ-*Proteobacterium* Isolate 400M ATT	AF114495	98	None
NBF19	*Alteromonas* sp.	AB015135	95	Moderate
NBF21	*Alteromonas* sp. SCB33	U64010	93	Strong
NBF16	*Alteromonas macleodii* DSM 6062	Y18228	99	Moderate
NBF17	*Alteromonas macleodii* DSM 6062	Y18228	99	Moderate
NBF18	*Alteromonas macleodii* DSM 6062	Y18228	98	Strong
NBF20	*Alteromonas macleodii* DSM 6062	Y18228	97	Moderate
NBF34	*Alteromonas macleodii*	AF025957	95	Strong
NBF38	*Alteromonas macleodii*	AF025957	97	None
NBF5	*Pseudoalteromonas* sp. Isolate PRLIST2	Y15323	99	Moderate
NBF3	*Pseudoalteromonas* sp. Strain S9	U80834	98	Moderate
NBF8	*Pseudoalteromonas* sp. Strain S9	U80834	97	Moderate
NBF11	*Pseudoalteromonas* sp. Strain S9	U80834	96	None
NBF15	*Pseudoalteromonas* sp. Strain S9	U80834	99	Strong
NBF24	*Pseudoalteromonas* sp. Strain S9	U80834	98	None
NBF25	*Pseudoalteromonas* sp. Strain S9	U80834	99	None
NBF28	*Pseudoalteromonas piscicida*	AF297959	97	Strong
NBF23	*Pseudomonas stutzeri* DNSP21	U26414	99	None
NBF26	Uncultured *Vibrio*	AF108137	96	Strong
NBF31	*Vibrio* strain NAP-4	AF064637	97	None
NBF10	*Vibrio alginolyticus* ATCC 17749T	X74690	97	Strong
NBF32	*Vibrio haloticoli* IAM14599	AB000393	91	None
NBF4	*Vibrio mediterranei* CIP 103203T	X74710	97	None
NBF2	*Vibrio nigripulchritudo* ATCC 27043T	X74717	94	None
NBF27	*Vibrio tubiashi* ATCC 19109T	X74725	100	Strong
Gram-positive division				
NBF29	Benzene mineralizing bacterium	AF029046	97	None
NBF1	*Bacillus halmapalus* DSM 8723	X76447	97	Moderate
NBF33	*Bacillus halmapalus* DSM 8723	X76447	97	None
NBF30	*Brevibacterium casei* NCDO 2048	X76564	96	Moderate
NBF9	*Micrococcus luteus*	AB023371	98	None
NBF35	*Micrococcus luteus*	AF057289	96	Strong
NBF36	*Staphylococcus haemolyticus*	L37600	97	Strong
NBF37	*Staphylococcus aureus* ATCC 12600T	D83358	99	None
***Cytophaga-Flexibacter-Bacteroides* division**				
NBF6	*Cytophaga* sp. F12	AF125325	90	Strong
NBF7	*Cytophaga* sp. F12	AF125325	93	None
NBF12	*Cytophaga* sp. F12	AF125325	93	None
NBF14	*Cytophaga* sp. F12	AF125325	92	Moderate

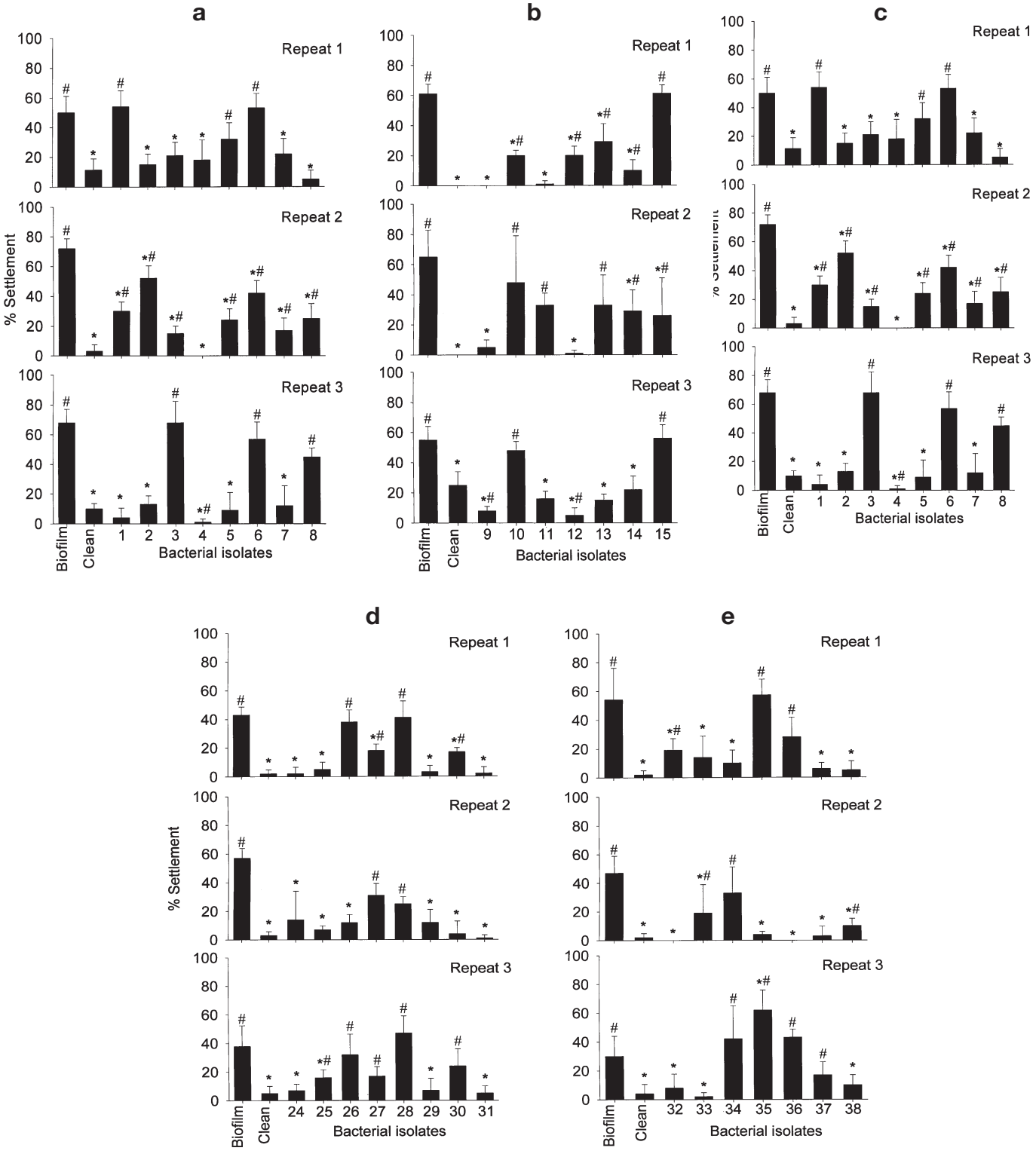

Fig. 1. *Hydroides elegans*. Larval settlement in response to the bacterial isolates after 24 h of incubation. The bacterial isolates were arbitrarily distributed over 5 individual bioassays (a–e), each performed in triplicate (denoted as Repeats 1, 2 and 3). The numbers on the *x*-axis indicate individual bacterial isolates: 1 for isolate NBF1, 2 for isolate NBF2, and so on. Control dishes containing 3 d old natural biofilms and control dishes with clean surfaces are indicated as Biofilm and Clean, respectively. Data are expressed as mean ± 1 SD of 5 replicates. Data that are significantly different from natural biofilm at $\alpha = 0.05$ in Dunnett's test are indicated by* whereas data that are significantly different from clean surface at $\alpha = 0.05$ in Dunnett's test are indicated by #

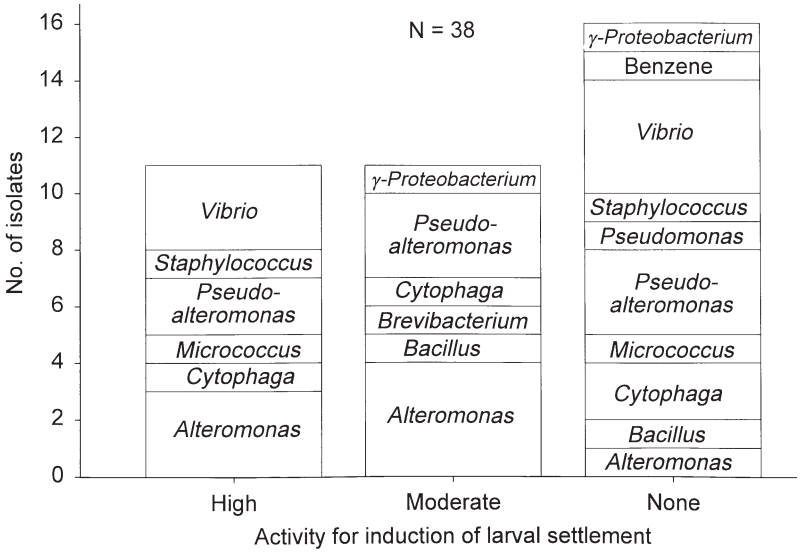

Fig. 2. A summary for Tables 3 & 4. Information shown is the number of bacterial isolates being assigned to each category of settlement induction activity. The isolates in each category are grouped into the genera that the isolates were affiliated

rDNA sequences from environmental samples, and has revealed a profound diversity of microbes not described previously (Giovannoni et al. 1990, Amann et al. 1991, Fuhrman et al. 1994). Although the culture-dependent method has long been known to underestimate bacterial diversity in natural samples, it was taken as an important approach in this study. Since the investigation of larval settlement induction activity of bacteria requires the bacteria to be cultured for bioassays, the direct analysis of community rDNA for bacterial diversity does not fulfill the need of this study. On the other hand, there has been emerging evidence showing that, in contrast to other ecosystems, the marine environment may host a higher proportion of culturable bacteria (Rehnstam et al. 1993, Fuhrman et al. 1994). Therefore, culture-dependent analysis of microbial diversity in marine samples may be more meaningful than for samples from other ecosystems.

The results of this study revealed that the culturable bacterial portion in natural biofilms was low in diversity with most of the isolates belonging to the genera *Alteromonas*, *Pseudoalteromonas* and *Vibrio* in the γ-*Proteobacteria* branch (Table 3). Previous studies have shown by the analysis of community rDNA sequences that bacterial community in the aquatic environment is markedly different for cells that are free living and those associated with substratum. While the free-living bacterial compartment is dominated by members of α-*Proteobacteria*, the attached bacterial community is mainly composed of strains in γ-*Proteobacteria* branch (DeLong et al. 1993, Acinas et al. 1999). On a global scale, *Alteromonas*, *Pseudoalteromonas* and *Vibrio* are the dominant genera in attached bacterial communities (see Pinhassi et al. 1997 for a review).

Comparison of 16S rRNA showed that 8 isolates belonged to the Gram-positive branch (Table 3), but some of these isolates appeared to be negative in Gram stain, for example isolates NBF1 (*Bacillus*), NBF30 (*Brevibacterium*) and NBF36 (*Staphylococcus*) (Table 2). The discrepancy may be due to over-decolorizing in the destaining process. *Brevibacterium* is known to be prone to decolorization in Gram stain (Holt et al. 1984). In addition, some *Bacillus* strains might give a positive result in Gram stain only when in early stage of growth and some *Bacillus* strains may never appear to be Gram-positive (Holt et al. 1984).

For the induction of larval settlement, the isolates in this study exhibited highly variable activities (Fig. 1). The isolates were categorized as strongly, moderately, and non-inductive for larval settlement by comparing the amounts of larval settlement occurring on the films of individual bacterial isolates to those on natural biofilms and clean surfaces, respectively (Fig. 2). Under these criteria, 42% of the isolates belonged to the non-inductive category and the rest of the isolates contained equal numbers of highly and moderately inductive strains (Fig. 2). Our results are similar to those by Unabia & Hadfield (1999), who isolated 34 bacterial strains from biofilms developed in Hawaiian waters and showed that only a small portion of the isolates was as active as natural biofilms in terms of induction of larval settlement in *Hydroides elegans*. In addition, Lau & Qian (1997) suggested that the larval settlement inductive effect of a bacterial community could be due to a small portion of settlement-inductive bacteria present in the community.

Results here revealed that larval settlement of *Hydroides elegans* could be induced by a variety of bacteria belonging to the genus *Cytophaga* in the *Cytophaga-Flexibacter-Bacteroides* branch; the genera *Bacillus*, *Brevibacterium*, *Micrococcus* and *Staphylococcus* in the Gram-positive branch; and the genera *Alteromonas*, *Pseudoalteromonas* and *Vibrio* in the γ-*Proteobacteria* branch (Fig. 2). Results also showed that isolates that were affiliated to the same genus had different activities for the induction of larval settlement (Fig. 2). For the γ-*Proteobacteria* branch, isolates that belong to the genus *Alteromonas* or *Pseudoalteromonas* distributed all over the 3 categories of activity for induction of larval settlement; isolates belonging to *Vibrio* were either strongly or non-inductive for larval settlement. Isolates belonging to the Gram-positive or the *Cytophaga-Flexibacter-Bacteroides* branch also distributed across the 3 categories.

In many studies, the effects of individual bacterial strains on larval settlement have varied tremendously among trials (for example, isolates NBF1 and 24 in the present study; see also Lau & Qian 1997, 2000, Unabia & Hadfield 1999). The cause of this variation is not clear, but the most probable explanation seems to be that there are unavoidable variations in the physiological conditions of the larvae and bacteria that are involved in the bioassays (Wieczorek et al. 1996, Wieczorek & Todd 1998, Lau & Qian 2001). Importantly, this variation in larval response towards a bacterial strain revealed a complex and dynamic interaction between larvae and bacteria. The amount of larval settlement on natural biofilms is comparatively consistent. It may be due to the fact that natural biofilms are comprised of bacterial strains that are of different species and physiological status. It was suggested that the settlement of larvae on a biofilm might be to a result of the overall stimuli (facilitation and/or inhibition) from the constituent bacteria (Lau & Qian 1997, Maki 1999).

Table 4. The bacterial isolates' activity for the induction of larval settlement in *Hydroides elegans*. Data shown are the number of repeats in which individual isolates induced a particular level of larval settlement. The isolates were categorized as strongly, moderately and non-inductive for larval settlement. 'Strong' refers to the isolates that were as inductive as natural biofilms (≥ Biofilm) in at least 2 of the 3 repeats; 'No effect' indicates isolates that were inactive (≤ Clean) in at least 2 of the 3 repeats. Isolates that do not fall into these 2 categories are shown as 'Moderate'

Isolate	No. of repeats			Induction of larval settlement
	≥ Biofilm[a]	< Biofilm & > Clean[b]	≤ Clean[c]	
NBF1	1	1	1	Moderate
NBF2	0	1	2	None
NBF3	1	1	1	Moderate
NBF4	0	0	3	None
NBF5	1	1	1	Moderate
NBF6	2	1	0	Strong
NBF7	0	1	2	None
NBF8	1	1	1	Moderate
NBF9	0	0	3	None
NBF10	2	1	0	Strong
NBF11	1	0	2	None
NBF12	0	1	2	None
NBF13	1	1	1	Moderate
NBF14	0	2	1	Moderate
NBF15	2	1	0	Strong
NBF16	0	3	0	Moderate
NBF17	1	1	1	Moderate
NBF18	2	1	0	Strong
NBF19	1	1	1	Moderate
NBF20	0	3	0	Moderate
NBF21	2	1	0	Strong
NBF22	0	0	3	None
NBF23	1	0	2	None
NBF24	0	0	3	None
NBF25	0	1	2	None
NBF26	2	0	1	Strong
NBF27	2	1	0	Strong
NBF28	3	0	0	Strong
NBF29	0	0	3	None
NBF30	1	1	1	Moderate
NBF31	0	0	3	None
NBF32	0	1	2	None
NBF33	0	1	2	None
NBF34	2	0	1	Strong
NBF35	2	0	1	Strong
NBF36	2	0	1	Strong
NBF37	1	0	2	None
NBF38	0	1	2	None

[a]Percentages of larval settlement that are higher than or equal to those on natural biofilms

[b]Percentages of larval settlement that are in-between those on natural biofilms and clean surfaces

[c]Percentages of larval settlement that are equal to or lower than those on clean surfaces

In conclusion, the results of our experiments indicate that competent larvae of *Hydroides elegans* respond to bacteria in a wide range of taxa and that bacterial isolates belonging to the same genus may have tremendously different activities for the induction of larval settlement. Therefore, the settlement response of larvae to bacteria is not likely to be taxon specific. At this point, it is unclear whether this broad range of inductive bacteria produces the same larval settlement cue for *H. elegans* or that different bacteria produce different cues. Resolution of this question awaits the results of ongoing studies on the isolation and characterization of the larval settlement cue.

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