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Roles of bacterial community composition in biofilms as a mediator for larval settlement of three marine invertebrates

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ABSTRACT: This study investigated (1) the effects of salinity and temperature on the bacterial community composition of developing biofilms, and (2) the responses of marine invertebrate larvae (the polychaete *Hydroides elegans* and the barnacles *Balanus amphitrite* and *B. trigonus*) to these biofilms during settlement (i.e. attachment to a surface and metamorphosis into juveniles). Biofilms developed in a 3 × 3 array of salinity and temperature treatments resulted in different bacterial community compositions (revealed by DGGE and T-RFLP), bacterial densities and total biomasses. Larval settlement of *B. amphitrite* and *B. trigonus* was induced by biofilms developed at high temperatures (23 and 30°C), but was unaffected (*B. amphitrite*) or inhibited (*B. trigonus*) by those developed at a low temperature (16°C). The settlement response of these barnacles did not correlate with the biomass or the bacterial density of the biofilms, but did coincide with the marked differences in bacterial community composition between the biofilms developed at different temperatures. In contrast, larval settlement of *H. elegans* differed slightly among biofilms developed in different salinities, but not among those developed at different temperatures. This settlement response was moderately correlated with bacterial density but had no apparent relationship with bacterial community composition of the biofilms. Our results implied that the community composition and cell density of bacteria in biofilms, which can vary with local environmental conditions, may allow larvae of the 2 barnacles and *H. elegans*, respectively, to distinguish between habitats with different environmental conditions.

KEY WORDS: Bacterial community · Barnacle · Biofilm · DGGE · Larval settlement · Polychaete · T-RFLP

INTRODUCTION

Many sessile marine invertebrates have a pelagic larval stage specialized for dispersal and colonization of new habitats (Thorson 1950). After a period of development (from hours to weeks, depending on the species), larvae enter a competent stage wherein they have developed the ability to settle (i.e. attach to substrata and metamorphose into juveniles). Competent larvae can detect diverse arrays of environmental stimuli to determine whether an encountered habitat is suitable for settlement (Morse 1991). An important source of environmental stimuli for many marine invertebrates are biofilms — complex agglomerates of bacteria, diatoms, protozoa and fungi existing on virtually all submerged substrata (Wieczorek & Todd 1998).

Biofilms were once thought to simply act as a sticky layer that 'entraps' and 'forces' larvae to settle (Zobell & Allen 1935). In fact, larvae can have finely tuned responses to biofilms; they are able to distinguish between biofilms developed in different geographic locations and settle on those that are relevant to their adult habitats (Bonar et al. 1986, Le Tourneux &

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Bourget 1988, Pearce & Scheibling 1991, Keough & Raimondi 1996). This subtle response is believed to be mediated by the structural and/or physiological attributes of biofilms, which may vary spatially and temporally according to the biotic (e.g. availability and physiology of colonizing species, competition and cooperation among species, and grazing pressure) and abiotic factors (e.g. latitude, depth, illumination, exposure time, season, water chemistry, nutrient supply, flow shear and substratum characteristics) of the local environment (Wieczorek & Todd 1998).

Despite decades of study, the bioactive components of biofilms that mediate larval settlement are still largely unknown. Many investigations have nevertheless highlighted the bacterial component of biofilms as a major mediator for larval settlement of many invertebrates (Holmström et al. 1992, Harder et al. 2002, Qian et al. 2003). In laboratory assays, monospecies films of bacteria can influence larval settlement in a variety of ways (from inhibition to neutral to induction), depending on the species of bacteria and larvae of concern. The specificity of larval response to different species of bacteria as well as the sensitivity of biofilm bacterial community composition to environmental factors have led to the hypothesis that larvae might be able to distinguish between habitats by responding differentially to bacteria that prevail in particular environments (Strathmann et al. 1981, Thomason et al. 1998, Miron et al. 1999, Olivier et al. 2000).

The development and verification of this hypothesis have relied mostly on culture-dependent techniques and morphological characterization to describe bacterial community composition in the biofilms (Maki et al. 1988, Holmström et al. 1992, Avelin et al. 1993, O'Connor & Richardson 1998). However, these approaches generally suffer from the fact that <1% of bacterial species in the marine environment are culturable and that bacteria are largely indistinguishable by morphological characteristics (Eilers et al. 2000). Therefore, investigations based on conventional techniques are incomplete and often bear a strong speculative element. The objective of this study was to use a culture-independent approach (i.e. DNA fingerprinting techniques) to investigate the hypothesized role of bacterial community composition in biofilms as a signpost for larvae to recognize desirable habitats. Specifically, we tested the effects of 2 environmental parameters (salinity and temperature) on the bacterial community composition of developing biofilms (as characterized by 2 DNA fingerprinting techniques). We then investigated the effects of these biofilms on larval settlement of 3 marine invertebrates (the polychaete *Hydroides elegans*, and the barnacles *Balanus amphitrite* and *B. trigonus*) in laboratory assays.

MATERIALS AND METHODS

Biofilm development. Two batches of biofilms were developed in March and April 2003 on pre-sterile polystyrene dishes (base area ca. 20 cm^2; Falcon #1006) in a 3 × 3 array of salinity (20, 27, 34‰) and temperature (16, 23, 30°C) treatments. This array represented the range of salinities and temperatures normally encountered in Hong Kong waters throughout the year. We did not intend to investigate whether the biofilms developed in these treatments would allow larvae to distinguish between habitats of different salinity and/or temperature, but rather to use salinity and temperature as 'tools' to create biofilms of various bacterial communities and to use these biofilms to investigate whether larvae could distinguish between them.

To set up the treatments, seawater taken from the pier at the Hong Kong University of Science and Technology (HKUST) was diluted to the desired salinities (20, 27, 34‰) using autoclaved double-distilled water. Seawater at each salinity was transferred to 3 aquaria (10 l) each containing 24 dishes mounted vertically to the aquarium wall. The 3 replicate aquaria of each salinity were allocated to 16, 23, and 30°C treatments by placing the aquaria in temperature-controlled water baths. Biofilms were developed on the dishes over a 20 d incubation period on a 15:9 h light:dark cycle with aeration. The seawater in the aquaria was changed every 48 h.

Enumeration of bacteria. Biofilms in 3 replicate dishes of each treatment were fixed with 4% formaldehyde in 0.22 mm filtered seawater (FSW). After staining with 4,6-diamidino-2-phenylindole, bacterial density was enumerated using an epifluorescence microscope at a magnification of 1000 in 10 haphazardly chosen fields of view on each biofilm. At least 1000 cells were counted for each biofilm.

Biofilm biomass measurement. Biofilms were scraped from 3 replicate dishes of each treatment using sterile glass coverslips, lysed by ultrasonic pulses (Branson Sonifier 450) and analyzed for total organic carbon (TOC) using high temperature catalytic oxidation at 680°C in a Shimadzu TOC-$V_{CPH/CPN}$ analyzer (Shimadzu Corporation) according to the manufacturer's protocol.

DNA extraction. Biofilms scraped from 3 replicate dishes of each treatment using sterile glass coverslips were combined and then suspended in 0.8 ml of lysis buffer (100 mM Tris-HCl, 100 mM sodium EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1% hexadecylmethylammonium bromide; pH = 8.0). Total DNA was extracted and purified according to Zhou et al. (1996).

Polymerase chain reaction (PCR). The 16S rRNA genes of bacteria in the DNA were PCR amplified

using the primer pairs 968F-GC (5′-GAACGCGAA-GAACCTTAC-3′) and 1346R (5′-TAGCGATTCCGAC-TTCA-3′) (Nübel et al. 1996) for DGGE, and 341F-HEX (5′-CCTACGGGAGGCAGCAG-3′) and 907R (5′-CCGTCAATTCCTTTRAGTTT-3′) (Ishii et al. 2000) for T-RFLP analysis. The primers 968F-GC and 341F-HEX carry a 39-base GC-clamp (5′-CGCCCGGGGCGCG-CCCCGGGCGGGGCGGGGGCACGGGGG-3′) and a fluorescent label (HEX, hexachlorofluorescein) at their 5′ ends, respectively. PCR was performed in a total volume of 50 ml containing 2 ml of DNA template, 250 mM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP; Pharmacia Biotechnology), 1 U of AmpliTaq Gold™ DNA polymerase (Applied Biosystems) in 1 buffer, 1.5 mM $MgCl_2$, and 1 mM of each primer. PCR was performed at 95°C for 10 min; 25 cycles of 95°C for 1 min, 56°C for 3 min, 72°C for 3 min; and 72°C for 10 min. The amount and size of amplified DNA was verified by electrophoresis in agarose.

Denaturing gradient gel electrophoresis (DGGE). DGGE of PCR products was performed in a DGGE-1001 system (C.B.S. Scientific). The PCR products were resolved in a 1 mm thick vertical gel containing 8% (w/v) polyacrylamide (37.5:1 acrylamide:bisacrylamide) and a linear gradient of 45 to 60% chemical denaturants, whereas 100% denaturants refers to 7 M urea and 40% (v/v) formamide. Electrophoresis (100 V for 17 h) was performed in 1 TAE buffer maintained at 60°C. After that, DNA was visualized using silver stain according to Bassam et al. (1991).

Terminal restriction fragment length polymorphism (T-RFLP) analysis. Fluorescently labeled PCR products were purified using the Wizard® PCR Preps DNA purification system (Promega) and subsequently digested with 20 U *Msp*I (Boehringer Mannheim Biochemicals) at 37°C for 6 h. Aliquots of digested products (10 ml) were mixed with 0.5 ml of internal size standard (ET550-R, Amersham Biosciences), denatured (2 min at 95°C) and snap-cooled on ice before capillary electrophoresis on a MegaBACE™ genetic analyzer (Amersham Biosciences) operating in Genotyping mode. After electrophoresis, the length of fluorescently labeled terminal restriction fragments was determined using the Genetic Profiler software package (Amersham Biosciences).

Larval culture. Broodstocks of the polychaete *Hydroides elegans* and the barnacles *Balanus amphitrite* and *B. trigonus* were collected from Port Shelter, Hong Kong. For *H. elegans*, gametes released from adults were fertilized in FSW; larvae were raised to the competent stage (i.e. a physiological stage in which larvae are able to settle) in batch culture on a diet of the flagellate *Isochrysis galbana* (Tahitian strain) at 25°C according to the procedures described in Lau & Qian (2001). For *B. amphitrite* and *B. trigonus*, larvae released from adults were raised to the competent stage in batch cultures on a diet of the diatom *Chaetoceros gracilis* at 25°C according to Thiyagarajan et al. (2003). The competent larvae of *B. amphitrite* were allowed to age for 2 d before the assays, whereas those of *B. trigonus* were used in the assays within 12 h upon transformation to the competent stage. The power of assays with *B. amphitrite* can be greatly improved by using competent larvae aged for 2 d because the larvae become more 'desperate' to settle due to the depletion of energy reserve and yet not loosing their ability to distinguish between substrata (Rittschof et al. 1984, Harder et al. 2001). In contrast, competent larvae of *B. trigonus* usually suffer from high mortality during aging (Thiyagarajan et al. 2003). Therefore, newly transformed competent larvae of *B. trigonus* were used in the assays.

Larval settlement assays. All assays were conducted twice, once using the biofilms developed in March 2003 (Batch 1) and once using biofilms developed in April 2003 (Batch 2). On each occasion, 5 replicates were used per treatment. For barnacles, the assays were conducted in a 'double-dish' setup constructed by connecting (using parafilm) a dip-rinsed biofilmed dish (as treatment) and an unfilmed dish (as control) into a single unit along with 20 to 30 larvae in 5 ml autoclaved FSW (35‰) (Harder et al. 2001). Subsequently, the paired dishes were then completely filled with autoclaved FSW using a syringe. After 24 h incubation at 25°C in a vertical position, the number of barnacle larvae settled on each dish was determined using a dissecting microscope. Traditionally, assays with barnacle larvae were conducted in a single dish format as for *Hydroides elegans* (see below). However, due to the surface property of barnacle larvae, the air-water interface entraps significant amount of larvae and causes severe bias. In contrast, the lack of air-water interface in the double-dish setup can improve the reliability of the assays (Harder et al. 2001). Since the larvae of *H. elegans* are never trapped at the air-water interface, the bioassays for *H. elegans* were conducted in a conventional, single dish format for simplicity (Lau & Qian 2001). Briefly, the biofilmed dishes were dip-rinsed 10 times in autoclaved FSW (35‰) and filled with 5 ml of autoclaved FSW (3‰) along with 20 to 30 competent larvae. After 12 h of incubation at 25°C, the number of settled larvae was determined using a dissecting microscope. A positive control was dip-rinsed, 5 d old biofilms developed at the HKUST pier; the ambient seawater temperature and salinity were ca. 23 to 25°C and 33 to 35‰, respectively. Unfilmed dishes served as a negative control.

Statistical analysis. ***Biomass and bacterial abundance in biofilms:*** Data for biomass and bacterial abundance were checked for normality using the Shapiro-Wilk's test and for homogeneity of variance using the Cochran's *C*-test. Since a 3-way ANOVA revealed no significant batch temperature salinity interaction, data from the 2 batches of assays were pooled and analyzed using a 2-way ANOVA (temperature × salinity).

Bacterial community composition: Band patterns in DGGE gels and peak patterns in T-RFLP chromatographs were transformed to binary character tables (1 or 0 corresponding to the presence or absence of a given band/peak in a biofilm sample) using the GelCompar II program package (Applied Maths) and the Genetic Profiler package, respectively. A similarity matrix was constructed based on the total number of bands/peaks observed in all biofilm bacterial communities and the presence or absence of these bands/peaks in each community. Agglomerative hierarchical clustering was performed using UPGMA (unweighted pair group method using arithmetic averages) and the distances (i.e. similarity) among communities were displayed as a dendrogram.

Larval settlement: The mean percent settlement data for *Hydroides elegans* were arcsine-transformed and tested for normality and homogeneity of variance. Since a 3-way ANOVA revealed no significant batch temperature salinity interaction, data from the 2 batches of assays were pooled and analyzed using a 2-way ANOVA (temperature salinity). For the assays with *Balanus amphitrite* and *B. trigonus*, the number of larvae that settled on each of the paired dishes was compared to the null hypothesis of 1:1 distribution among the 2 dishes using replicated *G*-tests for goodness of fit. The *G*-value was calculated as a measure of heterogeneity among the replicate paired dishes within an assay as well as between the 2 batches of assays. Homogenous data sets were pooled and corresponding *G*-values were transformed by the Williams' correction. Furthermore, the relationship between percent larval settlement and bacterial density/biomass of the biofilms was determined using the Spearman rank order correlation analysis.

RESULTS

Bacterial density of biofilms

After 20 d development, bacterial densities in the 2 batches of biofilms ranged from 12.5×10^3 to 29.1×10^3 cells mm^{-2} (Table 1); total biomass ranged from 0.06 to 0.27 mg mm^{-2} (Table 1). Salinity had a significant effect on bacterial density ($F = 13.027$, $p < 0.001$), but not on total biomass ($F = 0.575$, $p = 0.567$). Conversely, temperature had a significant effect on biomass ($F = 4.654$, $p = 0.015$), but not on bacterial density ($F = 0.079$, $p = 0.924$). Salinity and temperature had no interactive effect on bacterial density ($F = 2.110$, $p = 0.095$) or on biomass ($F = 1.431$, $p = 0.239$).

Table 1. Bacterial density and biomass of biofilms developed in different salinity and temperature treatments for 2 batches of the assays. Data presented are mean ± 1 SD of 3 replicates in each batch of assays without transformation

Salinity (%)	Temp. (°C)	Bacterial (10^3 cells mm^{-2})	Biomass (mg mm^{-2})
Batch 1			
34	30	21.2 ± 4.9	0.16 ± 0.01
27	30	18.5 ± 4.6	0.15 ± 0.01
20	30	18.8 ± 5.1	0.15 ± 0.03
34	23	25.6 ± 2.8	0.14 ± 0.02
27	23	22.8 ± 4.5	0.17 ± 0.03
20	23	15.8 ± 1.6	0.15 ± 0.01
34	16	29.1 ± 1.8	0.07 ± 0.03
27	16	26.8 ± 0.6	0.08 ± 0.01
20	16	14.3 ± 1.4	0.07 ± 0.01
Batch 2			
34	30	23.1 ± 5.9	0.24 ± 0.05
27	30	17.7 ± 2.8	0.27 ± 0.09
20	30	19.2 ± 4.2	0.12 ± 0.04
34	23	18.9 ± 1.8	0.06 ± 0.01
27	23	19.6 ± 2.3	0.08 ± 0.04
20	23	12.5 ± 1.2	0.15 ± 0.11
34	16	19.2 ± 2.4	0.20 ± 0.07
27	16	15.6 ± 2.6	0.17 ± 0.05
20	16	12.8 ± 1.7	0.14 ± 0.03

Bacterial community composition

Visual inspections revealed that biofilms from the same treatment had different DGGE profiles within each of the 2 experimental batches (Fig. 1). Nevertheless, cluster analysis indicated that, in both batches of biofilms, the lowest temperature treatment (16°C) resulted in bacterial communities highly distant (<35% similarity) from those developed in the higher temperature treatments (23 and 30°C) (Fig. 2a,b). Bacterial communities in the higher temperature treatments (23 and 30°C) were mostly segregated into small clusters according to salinity.

T-RFLP analysis resulted in 12 to 29 terminal restriction fragments (TRFs) in Batch 1 biofilms (Table 2), and 11 to 28 TRFs in Batch 2 biofilms (Table 3). Similar to that of the DGGE profiles, cluster analysis of T-RFLP profiles also distinguished bacterial communities developed at 16°C from those at 23 or 30°C (<45% similarity; Fig. 2c,d).

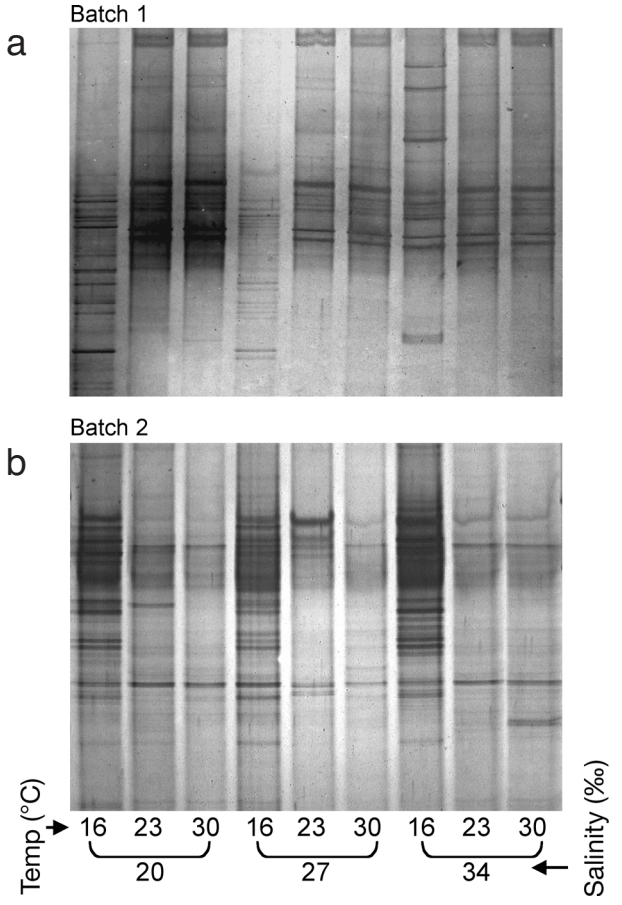

Fig. 1. DGGE profile of bacterial communities in biofilms developed in different salinity and temperature treatments in each batch of larval settlement assays. The range of denaturant gradient in the gel images was (a) 45 to (b) 60%. Electrophoresis was conducted at 100 V for 17 h

Larval settlement

Balanus amphitrite

A goodness of fit *G*-test performed with pooled data from Batch 1 and 2 assays indicated that *Balanus amphitrite* larvae did not distinguish between an unfilmed surface and any of the biofilms developed at 16°C (Fig. 3, Table 4). However, larvae settled at a significantly higher proportion on all biofilms developed at 23 and 3°C than on the unfilmed surface (Fig. 3, Table 4).

Balanus trigonus

A goodness of fit *G*-test performed with pooled data from Batch 1 and 2 assays indicated that larvae of *Balanus trigonus* settled preferentially on biofilms developed at either 23 or 30°C over unfilmed surfaces, except for biofilms developed in the 34‰/30°C treatment (Fig. 4, Table 4). However, the larvae preferred unfilmed surfaces over biofilms developed in the 16°C treatments (Fig. 4, Table 4).

Hydroides elegans

In both batches of assays, mean percent larval settlement of *Hydroides elegans* after 12 h was ≥91.7% in the positive control (dish with natural biofilm) and ≤4.2% in the negative control (unfilmed dish) (Fig. 5). The mean percent larval settlement on biofilms developed in different salinity and temperature treatments ranged from 80.0 to 96.7%. Generally, larval settlement varied among biofilms developed at different salinities ($F = 19.240$, $p < 0.001$), but not among those developed at different temperatures ($F = 0.050$, $p = 0.953$). The 2 factors (i.e. biofilms developed at different salinity and temperature) had no interactive effect on larval settlement ($F = 0.210$, $p = 0.930$).

Correlation of larval settlement with biomass and bacterial density

The Spearman rank order correlation analysis indicated that the percent larval settlement of *Hydroides elegans* was positively correlated with bacterial density but not with biomass of the biofilms (Table 5). In contrast, the percent larval settlement of *Balanus amphitrite* and of *B. trignous* were unrelated to either bacterial density or biomass of the biofilms (Table 5).

DISCUSSION

The 3 × 3 array of salinity and temperature treatments resulted in biofilms that differed qualitatively in terms of bacterial community composition (Figs. 1 & 2, Tables 2 & 3) and quantitatively in terms of bacterial density and total biomass (Table 1). Particularly, the 2 DNA fingerprinting techniques DGGE and T-RFLP demonstrated substantial differences in bacterial community composition between the biofilms developed at high (i.e. 23 and 30°C) and low (i.e. 16°C) temperatures; salinity seemed not to have any profound effect.

The evaluation of T-RFLP and DGGE data in concert provided some possible explanations as to what might have caused the temperature-induced shift in bacterial community composition. T-RFLP analysis detected a higher number of operational taxonomic units (OTUs; i.e. bands/peaks detected by DNA fingerprinting techniques) for biofilms developed at higher temperatures (both batches), suggesting that the indigenous

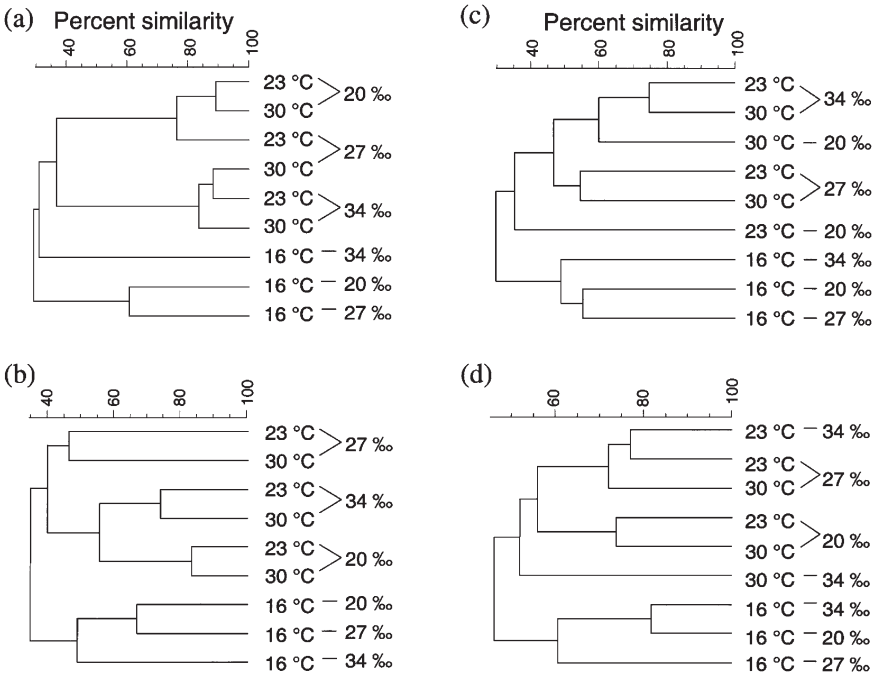

Fig. 2. Similarity of DGGE (a: Batch 1; b: Batch 2) and T-RFLP (c: Batch 1; d: Batch 2) profiles among the bacterial communities developed in a 3 × 3 array of salinity and temperature treatments in each batch of larval settlement assays

bacterial colonizers in the seawater taken for the experiments might be mainly mesophilic. In contrast, DGGE detected a large number of OTUs exclusive to biofilms of the 16°C treatments (both batches), suggesting that the observed community shift might be due not only to the suppression of mesophilic bacteria by low temperature, but also to the opening of niches to bacteria that were retarded to attach and/or grow at higher temperatures. The observed bacterial community shift was consistent among the 2 batches of biofilms, although the position and abundance of OTUs in each treatment differed between each batch. In general, bacterial communities in Batch 1 biofilms were more diverse, as indicated by the generally higher number of OTUs detected by both DNA fingerprinting techniques. The batch-to-batch varia-

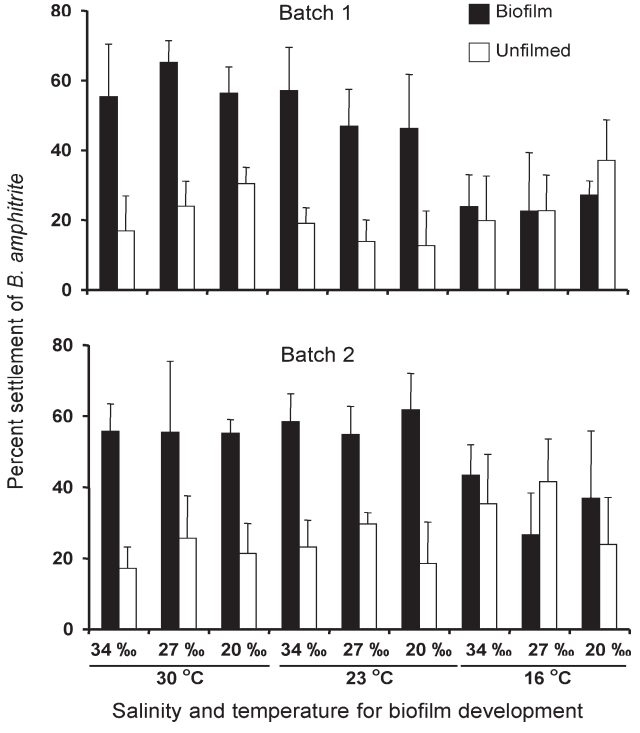

Fig. 3. *Balanus amphitrite*. Percent larval settlement after 24 h incubation in a double-dish setup containing a biofilmed dish and an unfilmed dish. Biofilms were developed in a 3 × 3 array of salinity (20, 27 and 34‰) and temperature (16, 23 and 30°C) treatments. Data presented are mean + 1 SD of 5 replicates in each batch of assays without transformation

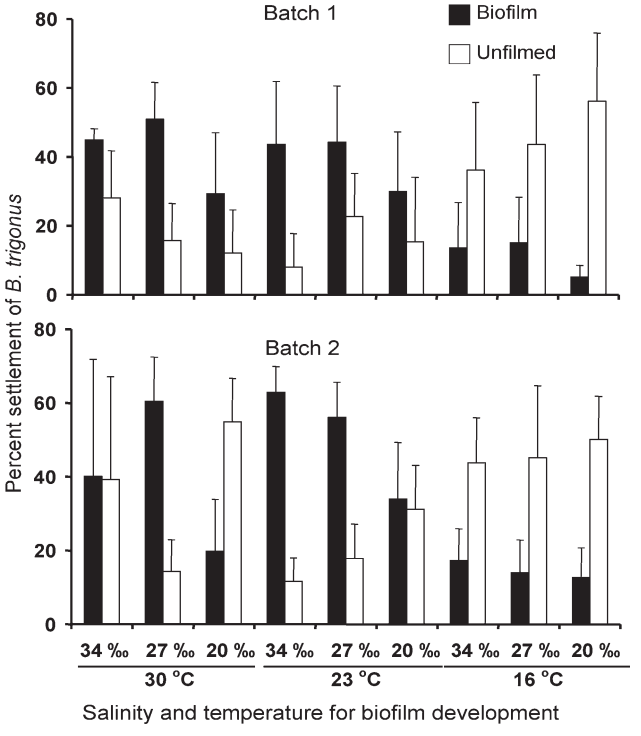

Fig. 4. *Balanus trigonus*. Percent larval settlement after 24 h incubation in a double-dish setup containing a biofilmed dish and an unfilmed dish. Biofilms were developed in a 3 × 3 array of salinity (20, 27 and 34‰) and temperature (16, 23 and 30°C) treatments. Data presented are mean + 1 SD of 5 replicates in each batch of assays without transformation

Table 2. T-RFLP profile of bacterial communities in Batch 1 of biofilms developed in different salinity and temperature treatments. Individual TRFs are indicated by fragment size (bp). Entries without a dash (–) indicate TRFs of particular sizes occurring in a given biofilm; values therein represent the number of discernible TRFs occurring in a given fragment size range. Total number of TRFs in each biofilm is in **bold**

TRF (bp)	16°C			23°C			30°C		
	20‰	27‰	34‰	20‰	27‰	34‰	20‰	27‰	34‰
36–40	–	–	2	–	–	4	–	2	1
41	–	–	–	–	–	–	1	–	1
42	1	1	–	1	–	1	–	–	–
43	–	–	–	–	–	1	–	–	–
64	–	–	–	–	1	–	1	–	–
71	–	–	–	–	1	–	–	–	–
74–78	–	1	–	–	1	–	–	–	–
83–85	–	–	–	1	–	1	–	–	–
99–104	3	–	–	–	–	–	–	–	–
123–146	–	–	–	8	4	7	2	1	6
155–157	–	–	–	2	–	–	–	–	–
160–167	–	–	–	1	2	2	1	2	2
179	–	–	–	1	–	–	–	–	–
184	–	–	–	–	–	1	–	–	–
194	–	1	–	–	1	–	–	–	–
195	–	1	1	–	–	–	–	–	–
196	–	–	–	–	1	–	–	–	–
197	–	–	–	1	–	–	–	–	–
206	–	1	–	–	–	–	–	–	–
207	1	–	–	–	–	–	–	–	–
209–214	3	3	3	3	2	2	2	2	3
215	1	1	–	–	–	–	–	–	–
216	–	1	–	–	–	–	–	–	–
223	–	–	–	–	–	–	–	1	–
225	–	–	–	–	–	1	1	–	–
226	–	–	–	–	–	–	–	–	1
234–241	3	6	3	4	3	4	4	5	4
254	–	1	–	–	–	–	–	–	–
270	1	1	–	–	–	–	–	–	–
310–316	–	–	–	1	2	2	2	2	3
322–324	–	–	–	–	–	–	–	1	1
332–340	–	–	–	3	3	3	2	4	3
405–407	–	3	–	–	–	–	–	–	–
523	1	–	1	–	–	1	–	–	–
524	–	–	–	1	–	–	–	–	–
526	–	–	–	–	–	–	–	1	–
528–530	–	–	–	–	1	–	2	2	1
562	1	–	–	–	–	–	–	–	–
563	–	1	–	–	–	–	–	–	–
564	1	1	–	–	1	–	–	–	–
565	–	1	–	–	–	–	–	–	–
566	–	–	–	–	–	–	–	1	–
590	–	1	–	–	–	–	–	–	–
570	–	–	1	–	–	–	–	–	–
572–575	–	–	–	–	–	–	2	–	2
582–583	1	2	–	–	–	–	–	–	–
585–588	–	2	1	–	–	–	–	–	–
595	–	–	–	–	–	–	–	–	1
Total	**17**	**29**	**12**	**28**	**26**	**27**	**19**	**24**	**29**

tions in bacterial community composition may be due to possible intrinsic temporal variation of the indigenous bacterial population in the seawater taken for the experiments.

DNA fingerprinting techniques are valuable tools for the characterization of environmental bacterial communities, but different techniques have different limitations and biases. Therefore, we used 2 different techniques (DGGE and T-RFLP) to reach a presumably more reliable description of bacterial community composition (Hoffmann et al. 2002). T-RFLP cannot differentiate closely related DNA sequences, which are likely to have the same terminal restriction site, and thus may reduce the number of detectable OTUs. However, T-RFLP is also prone to overestimation of bacterial diversity due to pseudo-TRF formation (i.e. single stranded amplicons that are recalcitrant to restriction enzyme digestion) (Egert & Friedrich 2003). In contrast, DGGE can resolve closely related DNA sequences better than T-RFLP (Casamayor et al. 2002), but is more affected by heteroduplexe formation and rRNA operon heterogeneity (Muyzer 1998), which can increase the number of OTUs artificially. It is not known to what extent our analysis was affected by the limitations of the 2 techniques, and also by possible biases due to differential extraction of DNA from different bacterial populations (Martin-Laurent et al. 2001) as well as preferential PCR amplification of numerically dominant DNA templates (Polz & Cavanaugh 1998). Nevertheless, the bacterial community composition obtained by the 2 techniques, each using primers targeting a different region of the 16S rRNA gene (V3 to V5 for T-RFLP, and V6 to V8 for DGGE), clearly indicated a temperature-induced shift in bacterial community composition in both batches of biofilms. In addition to bacteria, a small population of diatoms was present in all biofilms (data not shown); however, the cell density (<100 cell mm^{-2}) was lower than those having known effects on larval settlement of the 2 barnacles and *Hydroides elegans* (Harder et al. 2002, authors' unpubl. data). Therefore, these biofilms were deemed appropriate for investigating whether the larvae of *H. elegans*, *Balanus amphitrite* and *B. trigonus* responded differentially to biofilms composed of different bacterial communities.

Table 3. T-RFLP profile of bacterial communities in Batch 2 of biofilms developed in different salinity and temperature treatments. Individual TRFs are indicated by fragment size (bp). Entries without a dash (–) indicate TRFs of particular sizes occurring in a given biofilm; values therein represent the number of discernible TRFs occurring in a given fragment size range. Total number of TRFs in each biofilm is in **bold**

TRF (bp)	16°C			23°C			30°C		
	20‰	27‰	34‰	20‰	27‰	34‰	20‰	27‰	34‰
30–32	3	–	–	–	–	3	3	3	–
33	–	–	–	–	–		1	1	–
39–40	2	1	3	1	1	1	–	1	1
41	–	–	–	–	1	1	–	–	1
42	1	1	1	–	1	–	1	1	1
43	–	–	–	1	1	–	1	1	–
45	–	–	–	1	–	–	–	–	–
61	–	–	–	1	–	–	–	–	–
62–64	2	–	–	1	1	2	2	2	1
65	–	–	–	–	1	–	–	–	–
67–68	1	1	1	1	1	1	1	1	1
71	–	–	–	–	–	1	1	1	–
72	–	–	–	1	–	–	–	–	–
76–79	–	1	–	1	–	–	1	–	1
80	–	–	–	–	1	–	–	1	–
85–86	–	–	–	1	1	1	1	–	–
94–104	–	–	–	–	–	–	2	–	3
118	–	–	–	–	–	–	–	–	1
127	–	–	–	1	1	–	1	1	–
129	–	1	–	–	–	–	–	–	–
135	1	–	1	–	–	–	–	–	–
136	–	1	–	–	–	–	–	–	–
137	1	–	1	–	1	1	1	1	1
139	–	1	–	–	–	–	–	–	–
140	1	–	1	–	–	1	1	1	1
141	–	1	–	–	–	–	–	–	–
149	–	–	–	–	–	–	–	1	1
150	–	–	–	1	–	–	–	–	–
159	–	1	–	–	–	–	–	–	–
161	–	–	–	–	–	–	–	–	1
163–166	3	3	2	1	2	2	1	1	3
190	–	–	–	–	1	–	–	1	–
191	–	–	–	–	–	–	–	–	1
194	–	–	–	–	–	–	–	–	1
195	–	–	–	–	–	–	1	–	1
196	–	1	–	–	–	–	–	–	–
216	–	–	1	–	–	–	–	–	–
223	–	–	–	1	1	–	–	1	–
244	–	1	–	–	–	–	–	–	–
273	1	–	–	1	–	1	1	1	1
275	–	–	–	–	–	1	–	–	–
324	–	–	–	–	–	–	–	–	1
378–379	–	–	–	1	–	1	1	1	1
434	–	–	–	–	–	–	–	–	1
562–564	–	–	–	–	–	3	–	–	2
569–570	–	–	–	–	–	–	–	–	2
587	–	1	–	–	–	1	–	–	1
589–590	–	–	–	–	–	2	–	–	1
Total	**16**	**15**	**11**	**15**	**15**	**23**	**21**	**23**	**28**

Larvae of the barnacles *Balanus amphitrite* and *B. trigonus* can settle with or without the presence of a biofilm (Wieczorek & Todd 1998). The assays in this study demonstrated that biofilms could attract, repel or not affect settlement of the 2 barnacle species, depending on the biofilm being tested (Figs. 3 & 4, Table 4). Biofilms developed at 23 and 30°C attracted larval settlement of both barnacle species. However, those developed at 16°C either repelled (*B. trigonus*) or did not affect (*B. amphitrite*) larval settlement. This settlement response did not correlate with the biomass or bacterial density of the biofilms, but coincided well with the clearly observable temperature-induced shift in bacterial community composition in the biofilms. These results suggest that the bacterial community composition of biofilms has strong influences on larval settlement of these 2 barnacle species.

The preferential barnacle settlement on biofilms developed at higher temperatures may be due to the presence of one or a few settlement-inducing bacterial species. Conversely, the avoidance of biofilms developed at 16°C by *Balanus trigonus* larvae may be due to the presence of settlement-inhibiting bacterial species. The different responses of *B. amphitrite* (neutral) and *B. trigonus* (avoidance) larvae to the same biofilms developed at 16°C suggest that either the 2 barnacle larvae responded to diverse bacterial species in the same biofilm or the same bacteria elicited varying responses in the 2 larvae. Although our data suggest a possible role of bacterial community composition as a mediator of barnacle larval settlement, we cannot rule out the possible role(s) of gene expression, physiological response and production of compounds by bacteria and other biofilm components, and especially that the biofilms might be faced with possible stresses due to sudden changes in salinity and temperature during the setting up of the assays (e.g. from 16°C/20‰ for biofilm development to 25°C/33‰ for the larval settlement assay).

In contrast to the 2 barnacles, the presence of a biofilm is a pre-requisite for larval settlement of *Hydroides elegans* (Lau & Qian 1997, Beckmann et al. 1999). This is shown by the clearly contrasting larval response to the natural biofilm and unfilmed controls (Fig. 5). *H. elegans* larval settlement varied among the biofilms developed under

Table 4. Results of log-likelihood ratio analysis used to test the preference of barnacles *Banalus amphitrite* and *B. trignous* larvae to settle on unfilmed surfaces or biofilms developed in different salinity and temperature treatments. Data for the analysis were pooled from the 2 batches of assays. The critical value for this log-likelihood test is G_{adj} (0.05, 1) = 3.841. p-values are denoted with the degree of freedom (df). A significant p-value (indicated by *) means the ratio of larval settlement on the biofilm and the pre-sterile surface deviated from the expected 1:1 ratio

Treatment Salinity (‰)	Temp. (°C)	Log-likeihood ratio test G_{adj}	p (df)
B. amphitrite			
34	30	188.23	<0.001 (1)*
27	30	150.94	<0.001 (1)*
20	30	93.93	<0.001 (1)*
34	23	94.65	<0.001 (1)*
27	23	61.91	<0.001 (1)*
20	23	110.21	<0.001 (1)*
34	16	3.57	>0.050 (1)
27	16	2.81	>0.050 (1)
20	16	0.25	>0.050 (1)
B. trigonus			
34	30	1.69	>0.050 (1)
27	30	98.21	<0.001 (1)*
20	30	9.45	<0.001 (1)*
34	23	170.29	<0.001 (1)*
27	23	66.17	<0.001 (1)*
20	23	5.41	<0.050 (1)*
34	16	74.11	<0.001 (1)*
27	16	141.95	<0.001 (1)*
20	16	50.95	<0.001 (1)*

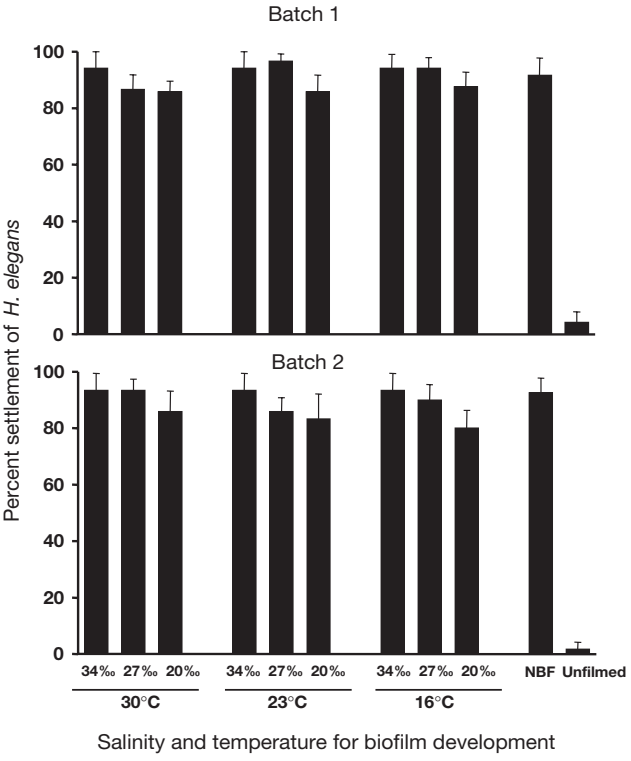

Fig. 5. *Hydroides elegans*. Percent larval settlement after 12 h incubation in dishes containing biofilms developed in different salinity (20, 27 and 34‰) and temperature (16, 23 and 30°C) treatments. Controls were unfilmed dishes and 5 d old biofilms developed naturally in the sea (NBF). Data presented are mean + 1 SD of 5 replicates in each batch of assays without transformation

Table 5. Correlations between percent larval settlement (*Hydroides elegans*, *Balanus amphitrite and B. trigonus*) and 2 quantitative biofilm attributes (bacterial density and total biomass). Data were pooled from all treatments in the 2 batches of assays and tested by the Spearman rank order correlation analysis. p < 0.050 indicates a significant correlation

Biofilm attribute		Percent larval settlement	r^2	p-value
Bacterial density	vs.	*H. elegans*	0.74	<0.001
		B. amphitrite	−0.13	0.604
		B. trigonus	−0.08	0.735
Total biomass	vs.	*H. elegans*	0.03	0.901
		B. amphitrite	0.04	0.868
		B. trigonus	0.19	0.443

different salinities but not among those developed at different temperatures. This settlement response appeared to be moderately correlated with bacterial density but not with biomass of the biofilms (Table 5). More importantly, this settlement response had no apparent relationship with the temperature-induced shift in bacterial community composition in the biofilms, suggesting that bacterial community composition had no apparent effect on larval settlement of *H. elegans*. Our finding is the opposite of what has been commonly hypothesized — bacterial species composition in biofilms can influence larval settlement of *H. elegans* (Lau & Qian 1997, Huang & Hadfield 2003). This hypothesis is largely based on the observation that monospecies biofilms derived from different bacterial species can induce diverse levels of larval settlement in *H. elegans*, varying from none to as high as natural biofilm (Unabia & Hadfield 1999, Lau & Qian 2001). However, our data cannot rule out the possibility that larval settlement of *H. elegans* was induced by one or a few bacterial species that commonly occurred in all the tested biofilms. It is also uncertain whether the commonly observed bacterial cell density dependent larval settlement was mediated by the total bacterial population or one or a few inductive bacterial species (Hadfield et al. 1994, Beckmann et al. 1999, Huang & Hadfield 2003, the present study).

The observed differences in biofilm selectivity by the 2 barnacles and *Hydroides elegans* may be explained by disparities in their adult distribution in the field. The

occurrence of the 2 barnacles is limited to particular tidal zones (mid to lower intertidal for *Balanus amphitrite* and subtidal for *B. trigonus*) (Harder et al. 2001, authors' unpubl data,). Since bacterial community composition in biofilms varies substantially among tidal zones, the differential response of barnacle larvae to biofilms with different bacterial community composition may allow the larvae finely evaluate substrata and hence precisely settle in appropriate tidal zones (Strathmann et al. 1981, Miron et al. 1999, Olivier et al. 2000, Qian et al. 2003). In contrast to the 2 barnacles, *H. elegans* occurs in a wider range of habitats and is often a pioneer colonizer of newly exposed surfaces. The larvae of *H. elegans* only appeared to settle in response to the bacterial density of the biofilms (Table 5). These results concur with those in a previous study that a low percentage (<15%) of *H. elegans* larvae can readily settle on natural biofilms as young as 1 d old (Qian 1999), and that the intensity of larval settlement on biofilms of different ages was strongly correlated with the age and hence the bacterial density of the biofilms. This strategy may allow the widest spread of populations by having a small portion of larvae to rapidly colonize any open substrata. Since bacterial density is generally a function of biofilm age and thus a good indicator of substratum longevity, bacterial density dependent settlement may allow the majority of *H. elegans* larvae to settle on substrata that have been reasonably stable.

By coupling DNA fingerprinting techniques and larval settlement assays, our study exemplified the impact of environmental factors on the bacterial community composition of biofilms and the ability of marine invertebrate larvae to recognize biofilms with varying bacterial community composition. These results suggest that the composition and cell density of bacterial communities in biofilms may serve as signposts for larvae of the 2 barnacles and *Hydroides elegans*, respectively, to distinguish between habitats with different environmental conditions.

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