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Specificity in the settlement – modifying response of bacterial biofilms towards zoospores of the marine alga *Enteromorpha*

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Summary

Previous studies have shown that the rate of settlement of zoospores of the green alga *Enteromorpha* is stimulated by mixed microbial biofilms and that the number of zoospores settling is positively correlated with the number of bacteria in the biofilm. In the present study the specificity of this relationship has been investigated. Ninety-nine strains of marine bacteria were isolated from natural biofilms on rocks and the surface of *Enteromorpha* plants. Isolates were screened by denaturing gradient gel electrophoresis (DGGE) to eliminate replicates and 16S rDNA sequencing identified a total of 37 unique strains. Phylogenetic analysis revealed that the isolated bacterial strains belonged to three groups γ-*Proteobacteria* (28 strains), *Cytophaga-Flavobacteria-Bacteroid* (CFB) group (six strains) and α-*Proteobacteria* (one strain). Two strains were unassigned, showing <93% sequence similarity with the CFB group. The main genera of γ-*Proteobacteria* were *Pseudoalteromonas* (14 strains), *Vibrio* (five strains), *Shewanella* (five strains), *Halomonas* (three strains) and *Pseudomonas* (one strain). Spore settlement experiments were conducted on single-species biofilms, developed for different times on glass slides. The effect of correcting spore settlement values for biofilm density was evaluated. Results showed that the effect of bacterial strains on spore settlement was strain- but not taxon-specific and activity varied with the age of the biofilm. However, most of the strains belonging to genera *Vibrio* and *Shewanella* showed stimulation. *Pseudoalteromonas* strains showed a range of effects including settlement-inhibiting, paralysing and lysing activities. Spatial analysis of bacterial density in the presence and absence of spores revealed a range of different types of association between spores and bacteria. Overall, the spatial association between spores and bacteria appears to be independent of the overall quantitative influence of bacterial cells on spore settlement.

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Introduction

Biofouling is the undesirable accumulation of organic molecules, microorganisms, plants and animals on surfaces exposed in an aquatic environment. It is a complex process and occurs in three main stages (Callow, 1996). The first stage is the rapid formation of a conditioning film by accumulation of organic molecules. During the second stage, microbial biofilms develop on the conditioning film. The final stage of biofouling involves settlement and growth of macrofouling organisms. Although the presence of a previous stage is not a prerequisite for a subsequent stage, the order reflects the length of time taken for attachment and the abundance of each component. As bacteria are primary colonizers, they can positively or negatively affect settlement of macrofouling organisms. Many studies have been carried out to explore how microbial biofilms and specific strains of bacteria affect settlement and growth of invertebrate larvae (Wieczorek and Todd, 1997; Maki, 1998; Holmström and Kjelleberg, 1999; Lau *et al*., 2002) but there have been relatively few studies on marine algae.

Enteromorpha is a cosmopolitan intertidal macroalga and a major contributor to marine biofouling (Callow, 1996). Reproduction is due mainly to motile, asexual zoospores that are produced at the distal end of a thallus. Zoospores settle through a process that involves exploration and sensing of a surface (Callow *et al*., 1997; Callow and Callow, 2000) followed by temporary and permanent phases of adhesion culminating in the withdrawal of flagella and the discharge of a glycoprotein adhesive (Stanley *et al*., 1999; Callow *et al*., 2000a). The attached spore subsequently develops into a new plant. Zoospores explore and settle temporarily on a surface, but will detach

if it is suboptimal, i.e. suitable surfaces appear to be selected for permanent attachment.

Many factors influence the attachment of zoospores including negative phototaxis, thigmotaxis, chemotaxis (Callow and Callow, 2000), surface chemistry and wettability (Callow *et al*., 2000b) and surface topography (Callow *et al*., 2002). Some or all of these may contribute to the location of a suitable surface on which to settle in the natural environment. Bacterial biofilms may be a source of some of these signals. Microbial biofilms are present on all submerged surfaces in the marine environment and thus change the properties of those surfaces. Dillon *et al.* (1989) found enhanced settlement of *Enteromorpha* spores on mixed microbial biofilms. In a preliminary study Thomas and Allsopp, 1983) showed that the biofilms of some strains ascribed to the *Pseudomonas/Alteromonas* group and *Coryneform,* enhanced the number of *Enteromorpha* germlings detectable after incubation with spores whereas other strains were inhibitory: spore settlement was not explored directly. A wider range of strains was tested on spores of the closely related green alga *Ulva lactuca* (Holmstrom *et al*., 1996). Sixteen out of 24 strains tested were inhibitory, three of which were darkly pigmented isolates that inhibited algal spore settlement by lysing spores. These strains showed a close phylogenetic affiliation with *Pseudoalteromonas tunicata* (Egan *et al*., 2002). In quantitative analyses of spore settlement in relation to biofilms, Joint *et al*. (2000) demonstrated a positive correlation between the number of bacteria in uncharacterized assemblages formed from natural seawater on glass slides, and the number of *Enteromorpha* spores that attached. Moreover, image analysis revealed that spores attached preferentially to bacteria or microcolonies suggesting they were attracted to certain bacteria present in the natural biofilms. At least one class of chemical signals used by bacteria during their quorum sensing processes, viz. the acylated homoserine lactones, appears to be recognized as settlement-enhancing signals by zoospores (Joint *et al*., 2002).

The present paper extends the studies of Joint *et al.* (2000) by examining the relationship between spore settlement and biofilms of specific strains of bacteria. Bacteria were isolated from surface biofilms of *Enteromorpha* plants and rock surfaces in close proximity to plants and identified by 16S rDNA sequencing. Biofilms of specific bacterial strains, and of different age, were examined for their ability to influence spore settlement. Results show that monospecies biofilms of specific strains of bacteria can stimulate spore settlement and that these effects are dependent on the age of the biofilm. Strains of *Pseudoalteromonas* showed inhibitory, lysing and paralysing activities. This study also explores the spatial relationships between biofilm bacteria and settled spores, revealing another aspect of the interaction between biofilms and spores.

Results

Morphological characteristics of bacterial isolates

A total of 99 bacterial isolates was obtained from the surface biofilms of rocks and *Enteromorpha* plants. To encompass seasonal variation, 47 strains were isolated during winter and 52 during summer. All isolates were Gram-negative, rod-shaped bacteria with a range of colony morphotypes: 54 isolates were motile in a liquid medium. Subsequent phylogenetic analysis (Table 1) showed that motile strains mainly belonged to *Shewanella*, *Vibrio* and *Pseudoalteromonas* genera, whereas all of the strains belonging to *Halomonas* genus and some of the strains of the *Cytophaga-Flavobacteria-Bacteroid* (CFB) group were non-motile. However, two strains showing similarity to *Zobellia galactanovora* were motile and 12 strains similar to *Cytophaga baltica* showed gliding motility on the agar plates. Isolates forming white colonies belonged to *Halomonas and Vibrio* genera, some of the isolates belonging to *Shewanella* and *Pseudoalteromonas* also formed white colonies. *Shewanella* strains mainly formed light orange colonies, whereas *Pseudoalteromonas* strains formed darkly pigmented colonies. Most of the strains belonging to the CFB group produced yellow to orange-pigmented colonies.

Phylogenetic characterization of isolates

The collection of isolates was screened for replicates by DGGE of the V9 region of 16S rDNA. For strains with similar migration positions on DGGE, colony morphotypes were also used. Selected isolates were sequenced using 27F and 1525R primers. As a result, the original 99 isolates were reduced to 37 unique strains and phylogenetic characterization was concentrated on single isolates representing each of these 37 strains (Table 1).

For phylogenetic characterization, sequences of the 16S rDNA gene (approx. 1533 bp) of each of the 37 selected strains were compared with the GenBank database using the BLAST programme. The percentage sequence similarity of each bacterial strain to its closest phylogenetic neighbour is shown in Table 1, together with the number of times each strain was found amongst the original collection of 99 isolates. A greater proportion of sequences was found to belong to the γ-subdivision of *Proteobacteria*. Main genera comprised *Pseudoalteromonas (*14 strains), *Vibrio* (five strains), *Shewanella* (five strains) and *Halomonas* (three strains). Isolate E7 showed similarity with the *Pseudomonas* genus. Only one isolate, R11 grouped with α-*Proteobacteria*, showing similarity to *Roseobacter gallaeciensis*. A neighbour-joining tree demonstrating the 16S phylogenetic relationship between the isolates and well-characterized strains from GenBank

Table 1. Phylogenetic identification of bacterial strains by 16S rDNA and the effect of their biofilms on *Enteromorpha* spore settlement.

					1.5 h biofilms		3 h biofilms		48 h biofilms
Strains	No isolates	Closest matching strain in GenBank	Accession number	% sequence similarity	Spore settlement (S)	Density-adjusted settlement (S/Λ)	Spore settlement (S)	Density-adjusted settlement (S/Λ)	Spore settlement (S)
E4	11	*Cytophaga baltica*	AJ005972	99			+		
E46	1	*Cytophaga baltica*	AJ005972	98		–			
R43	1	*Cytophaga baltica*	AJ005972	91					+
E14	2	*Flexibacter uliginosum*	M62799	97					
E6	3	*Flexibacter uliginosum*	M62799	95					
RE2-13	2	*Zobellia galactanovora*	AF208293	97	–	–		–	++
E26	1	*Polaribacter irgensii*	M61002	96	++	+	++	+	
E42	1	*Psychroserpens burtonesis*	U62912	92	+	+			
E21	4	*Halomonas variabilis*	U85873	99					
E23	3	*Halomonas variabilis*	U85873	98					
R21	9	*Halomonas variabilis*	U85873	99					
R12	1	*Vibrio aestuarianus*	AF172840	98	++	++	++	+	
R9	1	*Vibrio pomeroyi*	AJ491290	97					
RE1–12a	3	*Vibrio pomeroyi*	AJ491290	98		+		+	
RE1-3	2	*Vibrio pomeroyi*	AJ491290	98	+	++	+		++
RE2-8	3	*Vibrio pomeroyi*	AJ491290	97					++
R2	7	*Shewanella baltica*	AF173966	98					+
RE2-10	1	*Shewanella baltica*	AF173966	98			+		++
R8	2	*Shewanella denitrificans*	AJ457092	96	+				+
E1	4	*Shewanella frigidimarina*	AJ491290	97					+
RE1-1	4	*Shewanella putrefaciens*	X81623	97					
E7	1	*Pseudomonas anguilliseptica*	AB021376	96			+		
R11b	1	*Roseovaris tolerans*	Y11551	93	+	+			++
R14	1	*Pseudoalteromonas agarovorans*	AJ417594	98			+		
E29	8	*Pseudoalteromonas citrea*	AF082563	99					
E47	2	*Pseudoalteromonas citrea*	AF082563	98					
E48	3	*Pseudoalteromonas citrea*	AF082563	99					
R44	3	*Pseudoalteromonas citrea*	AF082563	98	–	–	–	–	
R46	1	*Pseudoalteromonas citrea*	AF082563	99					
E30	1	*Pseudoalteromonas elyakovii*	AB000389	98					
E33	1	*Pseudoalteromonas elyakovii*	AB000389	99					– –[L]
E36	3	*Pseudoalteromonas elyakovii*	AB000389	98					+
R30	2	*Pseudoalteromonas elyakovii*	AB000389	99					
RE2–12b	3	*Pseudoalteromonas elyakovii*	AB000389	98	–				+
R6	2	*Pseudoalteromonas flavipulchra*	AF297958	96					
RE2–5b	1	*Pseudoalteromonas flavipulchra*	AF297958	99					– –[L]
RE2-11	1	*Pseudoalteromonas haloplanktis*	AF214729	97	–[P]		–[P]		– –[L]

For each of 37 representative strains the percentage nucleotide similarity strains in the GenBank database (with their accession numbers), together with numbers of times each strain was replicated among 99 isolates is shown. The level of spore settlement (S) on biofilms of each strain was compared with the glass control and categorized as significantly stimulating (+) or strongly stimulating (++) based on whether critical values after ANOVA and Tukey's *B*-test were 1 ×, or 2 × greater than the T statistic, respectively (inhibiting (–) or strongly inhibiting (– –) strains showed mean values 1 × or 2 × lower than the T statistic). For 1.5 and 3 h biofilms only, the level of spore settlement was adjusted for the density of each biofilm [(S/Λ) using Λ from Table 2] and the same statistical procedures applied. Absence of a symbol indicates no significant inhibition or stimulation. ––[L] indicates a strong inhibition of settlement but that some lysed spores were also present (lysed cells were not included in counts). –[P] indicates an inhibition of settlement but that some paralysed spores (i.e. immotile spores that had not settled) were also seen on the biofilmed surface (such cells were not included in settled spore counts).

(Fig. 1) shows that many of the bacteria isolated in this study have not been previously described.

Settlement of Enteromorpha zoospores on biofilms

The same 37 strains used for the phylogenetic analysis were used in detailed spore settlement studies in the form of 1.5 h, 3 h and 48 h biofilms (Fig. 2). There were significant differences in the effect of different bacterial strains on spore settlement and based on one-way ANOVA and the Tukey test, four groups could be recognized: no significant effect, inhibitory (–), stimulatory (+) and highly stimulatory (+ +) (Table 1). For most stimulatory strains, spore settlement was strongly influenced by the age of the biofilm but no general pattern applicable to all strains was apparent. For some stimulating strains (e.g. E26, R12, E42) the stimulatory effect declined with age of biofilm. Several strains (R43, RE2-13, R2, RE2-8, E1, E36) only stimulated with 48 h biofilms. Other strains (E7, E4, R14) only gave significant stimulation with 3 h biofilms. Spore settlement studies on each of the 37 representative strains were carried out at least twice and although absolute levels of stimulation or inhibition showed some variation between experiments, the overall pattern of effects was consistent.

Specificity in the stimulation of spore settlement was observed at the level of strains (44% stimulated at any of

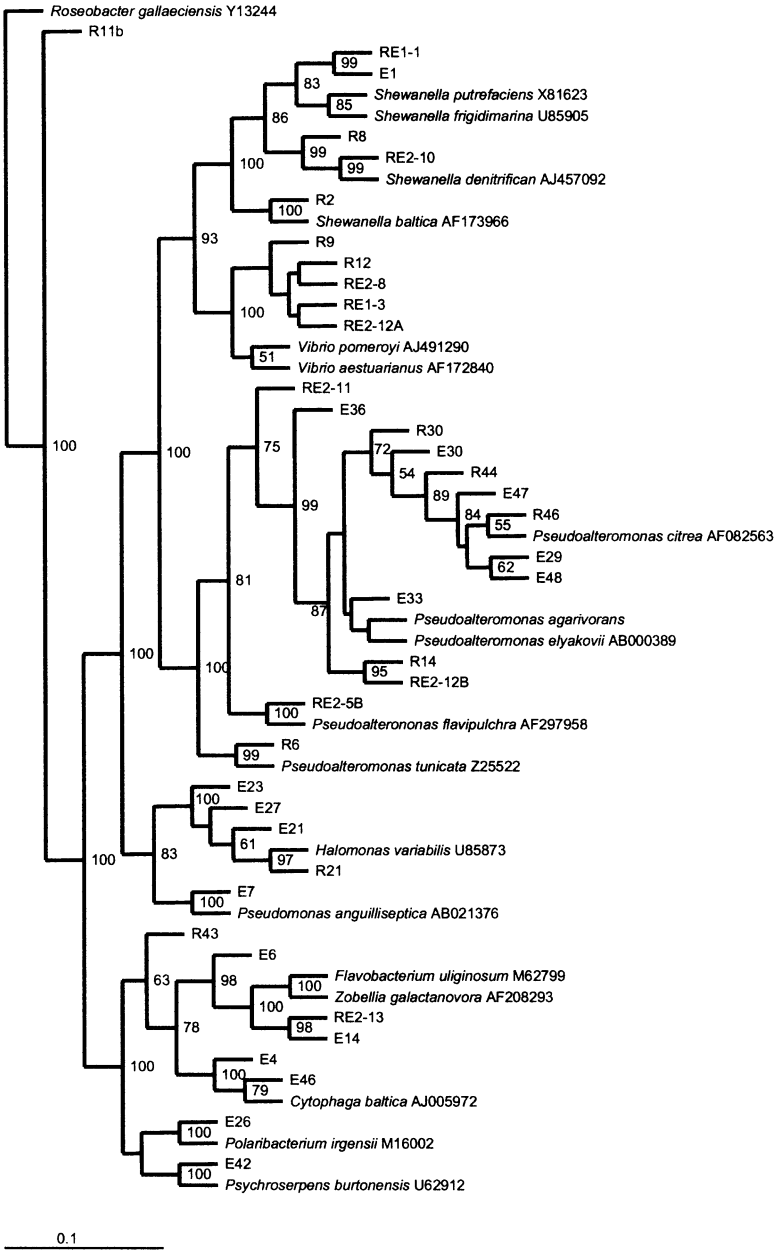

Fig. 1. Phylogenetic tree for representative isolates used in this study and various reference strains from GenBank, constructed by the neighbour-joining method. The accession numbers of the reference sequences are shown in parentheses. Bootstrap values are shown for nodes that had >50% support in a bootstrap analysis of 100 replicate trees. The scale bar represents 10% estimated sequence divergence.

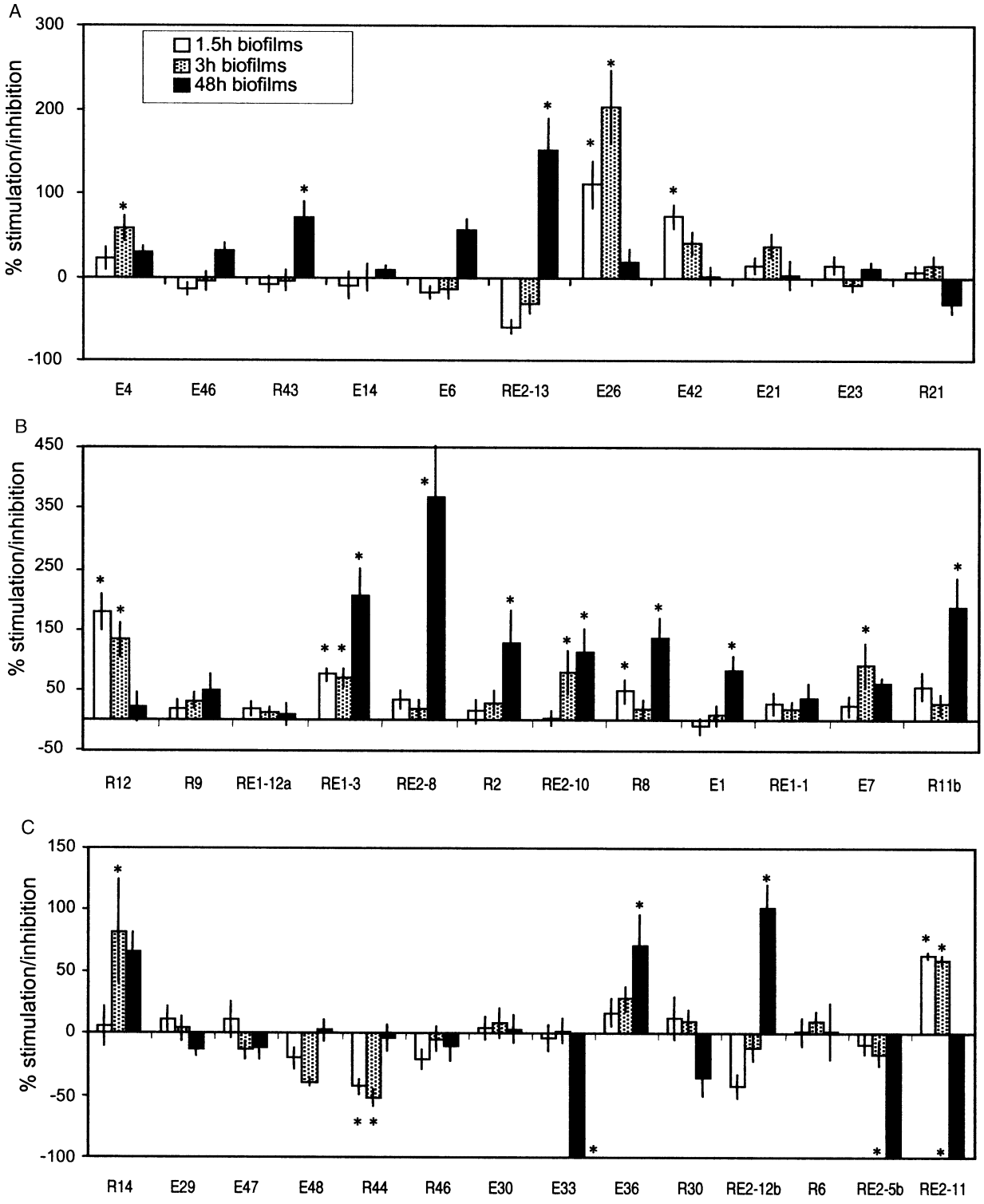

Fig. 2. *Enteromorpha* spore settlement on glass slides with biofilms of different age. Each bar represents 95% confidence intervals. The strains were tested in several batches, the absolute numbers of spores settled in controls (no biofilm) being 711 ± 69 per mm^2. Note that for clarity the y scale on each of the graphs is not the same. Asterisks show those values that are significantly different to controls (see Table 1).

the three stages of biofilm development) but these strains were distributed between several genera and species and no specificity at these levels was detected (Table 1). Of the eight strains belonging to the CFB group (E4, E46, R43, E14, E6, RE2-13, E26, E42), four showed stimulatory effects, two with younger biofilms and two with the 48 h biofilms. The *Zobellia* strain showed both inhibitory (as distinct from paralysing and lysing effects, see later) and stimulatory effects, depending on biofilm age. Most (8/10) of the *Vibrio* and *Shewanella* strains were stimulatory, several strongly so, especially with 48 h biofilms. *Halomonas* strains had no statistically significant effect on settlement. Of the 14 strains of *Pseudoalteromonas* studied, seven strains had no significant influence on spore settlement; two showed some stimulation, but four strains showed inhibitory effects that were particularly strong with 48 h biofilms. Closer examination of the inhibitory strains revealed a range of different effects. For example, strain RE2-11 (97% similar to *Pseudoalteromonas haloplanktis*) caused many zoospores to be paralyzed on 1.5 h and 3 h biofilms (i.e. flagella were retained but the spores were immotile) and at 48 h many spores were lysed. However, at all three time points some spores had settled and these spores could be counted using direct, manual counting rather than image analysis. Hence Table 1 records a net inhibitory effect in terms of the reduced level of genuinely settled spores rather than unsettled, paralysed or lysed spores residing on the surface but not truly adhered. Two other *Pseudoalteromonas* strains, RE2–5b and E33 showed no paralysing effect with 1.5 h and 3 h biofilms but still caused lysis with 48 h biofilms. Strains not classified amongst the above groups were α-*Proteobacteria* strain R11b that showed stimulation with 1.5 h biofilms and very strong stimulation with 48 h biofilms, and *Pseudomonas* strain E7 which showed stimulation only with a 3 h biofilm.

Settlement as a function of biofilm density

Assessment of spore settlement-modifying activity described above is a simple measurement of differences in spore settlement compared with controls. However, it could be argued that the effects of some bacterial strains might be greater simply because higher numbers of bacteria were present in the biofilms. Conversely, the apparent activity of some strains could be low because of the fact they form biofilms of low density. To assess the potential for such effects the spore settlement data for 1.5 h and 3 h biofilms were corrected for biofilm areal density (all 48 h biofilms and 3 h biofilms of *Pseudomonas* strain E7 were too dense for accurate estimation of areal density by image analysis). Results (Table 1) indicated that for 1.5 h biofilms this correction made relatively little difference: most of the stimulatory strains were still recorded as stimulatory after the correction was applied. However, one strain RE1–12a formed weak biofilms and the correction had the effect of raising it to the stimulatory category. In the case of the older, 3 h biofilms, corrections for bacterial density had the effect of reducing the number of stimulatory strains to three but again, only one of the strains not recorded as stimulatory (RE1–12a), was raised to the stimulatory category. Regressions of stimulation against bacterial density gave R^2 values of 0.0011 for 1.5 h biofilms and 0.0028 for 3 h biofilms. Overall it appears that for most strains that significantly stimulate spore settlement, the ability to form dense biofilms is an important, but not sufficient feature because some strains that form dense biofilms did not stimulate settlement.

Spatial association between bacteria and Enteromorpha *spores*

To examine the spatial relationships between settled spores and bacteria, bacterial density was compared on replicate slides exposed and unexposed to spores, the hypothesis being that a similar bacterial density would indicate random spore settlement in relation to bacterial cells and colonies. However, a significant positive difference between the two would result from an apparently reduced density of visible bacteria caused by preferential settlement of spores on top of bacterial assemblages (Joint *et al*., 2000). Similarly, a significant negative relationship between the two counts would indicate that spores were avoiding bacteria. Comparisons of spore settlement (Table 1) and bacterial coverage data (Table 2) shows that of the 10 strains that significantly stimulated spore settlement on either or both of the 1.5 h and 3 h biofilms, eight strains (E4, E42, R12, RE2-10, R8, E7, R11 and R14) significantly covered more bacteria than would be expected from random settlement alone, i.e. spores showed a preference for settlement on bacterial cells and colonies of these strains. The eight strains showing this effect were found in various taxa. In some cases the effect was very strong, for example, strain R12, which strongly promoted spore settlement, also strongly promoted preferential settlement on bacteria, between 60 and 67% of bacteria being covered by spores. Two strains that significantly stimulated spore settlement, E26 and RE1-3, did not give significant bacterial coverage by spores. However, preferential association (Table 2) was also observed for 19 strains that did not significantly stimulate spore settlement. For example, strain RE2-8 gave 90% bacterial coverage but was not a significant stimulator of spore settlement at 1.5 or 3 h.

Discussion

Most of the bacterial strains isolated in this study showed affiliation with the CFB group and γ-*Proteobacteria* and

Table 2. Bacterial biofilm areal density (1.5 and 3 h) in the absence (Λ) and presence (λ) of *Enteromorpha* spores.

	1.5 h biofilms			3 h biofilms		
	% bacterial density on control slides (Λ)	% visible bacterial density + spores (λ)	'preferential settlement' (Λ − λ/Λ)	% bacterial density on control slides (Λ)	% visible bacterial density + spores (λ)	'preferential settlement' (Λ − λ/Λ)
E4	3.95 ± 0.64	2.37 ± 0.51	+	11.54 ± 1.00	2.75 ± 0.71	+++
E46	0.48 ± 0.04	0.67 ± 0.08	–	0.66 ± 0.06	0.79 ± 0.11	
R43	0.93 ± 0.09	0.71 ± 0.12		0.62 ± 0.06	0.78 ± 0.10	–
E14	0.94 ± 0.10	0.66 ± 0.07	+	0.79 ± 0.14	0.60 ± 0.07	
E6	2.18 ± 0.21	1.28 ± 0.39	+	1.55 ± 0.11	1.37 ± 0.19	
RE2-13	1.00 ± 0.19	0.49 ± 0.34	+	0.77 ± 0.21	0.20 ± 0.04	++
E26	4.89 ± 0.97	5.58 ± 0.47		6.30 ± 1.03	5.63 ± 0.52	
E42	2.47 ± 0.20	2.24 ± 0.24		3.73 ± 0.25	1.95 ± 0.17	+
E21	2.06 ± 0.34	0.88 ± 0.10	+	2.72 ± 0.46	0.86 ± 0.09	++
E23	0.88 ± 0.11	0.83 ± 0.08		1.01 ± 0.09	0.78 ± 0.07	
R21	1.17 ± 0.12	0.85 ± 0.08		1.08 ± 0.27	0.96 ± 0.14	
R12	4.45 ± 0.74	1.46 ± 0.27	++	3.77 ± 0.84	1.53 ± 0.20	++
R9	2.60 ± 0.26	2.08 ± 0.36		1.98 ± 0.48	2.53 ± 0.34	–
RE1–12a	1.75 ± 0.14	1.75 ± 0.12		3.22 ± 0.30	2.57 ± 0.17	
RE1-3	4.85 ± 0.77	6.10 ± 0.64	–	6.89 ± 0.45	7.36 ± 0.72	
RE2-8	2.32 ± 0.19	0.64 ± 0.05	++	9.41 ± 0.72	0.95 ± 0.12	+++
R2	1.95 ± 0.29	1.03 ± 0.13	+	1.55 ± 0.23	1.07 ± 0.17	+
RE2-10	17.85 ± 0.77	3.78 ± 0.26	++	10.51 ± 1.33	5.65 ± 0.62	+
R8	4.54 ± 0.67	1.90 ± 0.26	++	4.13 ± 0.65	1.70 ± 0.44	++
E1	8.42 ± 0.99	1.72 ± 0.24	++	9.29 ± 0.67	3.35 ± 0.20	++
RE1-1	0.77 ± 0.07	0.85 ± 0.11		0.50 ± 0.05	0.81 ± 0.08	–
E7	10.62 ± 1.54	6.78 ± 1.04	+	11.02 ± 1.06	–	
R11b	1.75 ± 0.33	1.59 ± 0.26		2.72 ± 0.62	2.04 ± 0.48	+
R14	3.76 ± 0.76	2.50 ± 0.40	+	4.03 ± 0.84	3.92 ± 0.41	
E29	0.97 ± 0.15	0.97 ± 0.09		1.12 ± 0.12	0.91 ± 0.12	
E47	6.32 ± 1.21	1.98 ± 0.26	++	6.65 ± 0.52	4.18 ± 0.59	+
E48	1.09 ± 0.11	1.33 ± 0.29		4.35 ± 0.60	2.75 ± 0.25	+
R44	2.10 ± 0.27	0.70 ± 0.08	++	2.05 ± 0.25	1.06 ± 0.20	+
R46	5.27 ± 0.93	3.63 ± 0.60	+	5.03 ± 0.31	4.26 ± 0.56	
E30	1.61 ± 0.23	1.05 ± 0.07	+	2.01 ± 0.14	1.12 ± 0.11	+
E33	11.76 ± 2.16	4.63 ± 0.78	++	8.72 ± 1.18	3.27 ± 0.47	++
E36	7.15 ± 1.10	4.00 ± 0.41	+	6.74 ± 0.85	4.03 ± 0.76	+
R30	4.25 ± 0.29	1.27 ± 0.11	++	3.25 ± 0.23	1.19 ± 0.15	++
RE2–12b	14.81 ± 1.37	1.63 ± 0.97	+++	16.36 ± 0.97	1.16 ± 0.15	+++
R6	6.64 ± 0.83	3.52 ± 0.63	+	5.19 ± 1.42	3.43 ± 0.84	+
RE2–5b	1.97 ± 0.24	0.50 ± 0.06	++	1.39 ± 0.09	0.34 ± 0.05	+++
RE2-11	4.36 ± 0.58	0.96 ± 0.14	++	4.94 ± 0.64	0.54 ± 0.06	+++

All mean values are shown ±2 × SE. 'Preferential settlement' is a measure of the proportion of bacteria covered by spores, determined as (λ − λ/Λ) grouped on a +/++/+++ scale, based on whether critical values after ANOVA and Tukey's *B*-test were 1×, 2× or 3× greater than the T statistic respectively. A similar procedure was applied to strains showing minus (–) values (i.e. where λ > Λ). Absence of a symbol indicates no significant effect. No value for λ is recorded for strain E7 due to loss of sample.

there was only one strain belonging to α-*Proteobacteria*. Several studies have reported that culturable diversity underestimates true environmental diversity of microorganisms. γ-*Proteobacteria* were shown to be most abundant culturable bacteria in the marine environment (Mullins *et al*., 1995; Bowman *et al*., 1997; Suzuki *et al*., 1997), whereas the molecular approach showed that significant fractions of total bacterioplankton were α-*Proteobacteria* (Fuhrman *et al*., 1993; Hagström *et al*., 2000). The phylogenetic affiliation of most of the isolates recovered in the present study, to γ-*Proteobacteria* or the CFB group, is in agreement with previous findings by Delong *et al*. (1993) who showed that most macroaggregate-associated rRNA clones were related to the *Cytophaga-Flavobacterium* cluster and the γ-*Proteobacteria*, whereas rRNA genes recovered from free-living plankton were related to the α-*Proteobacteria.* The high abundance of γ-*Proteobacteria* and CFB among isolate collections might be due to the tendency of these groups to form biofilms.

This study provides clear evidence that *Enteromorpha* zoospores respond in different ways to monospecific biofilms of different bacterial strains. The effect of bacterial biofilms on the settlement of spores could not be assigned to species or even genus level as strains within species and genera showed dissimilar effects. However, within the γ-*Proteobacteria*, the genera *Vibrio* and *Shewanella* contained a greater proportion of more stimulatory strains than *Pseudoalteromonas* and *Halomonas.* The CFB group

also contained more stimulatory strains than *Pseudoalteromonas* and *Halomonas*. Thomas and Allsopp (1983) did not study the influence of bacterial biofilms on the settlement of *Enteromorpha* zoospores directly but a number of marine biofilm isolates generally ascribed to the *Pseudomonas/Alteromonas* group affected the growth of *Enteromorpha* germlings. No account of biofilm conditions was recorded and without phylogenetic identification it is difficult to compare their results with those of the present work. In the present study the only strain identified as *Pseudomonas* (E7) showed stimulation of spore settlement with 3 h biofilms, but not at 1.5 h or 48 h. In studies on the effect of bacterial biofilms on the settlement of *Hydroides elegans* larvae, Lau *et al*. (2002) also showed that stimulatory strains from biofilms were distributed across several taxa and strains within the same taxon showed different activities.

In the present study, 5/14 strains of *Pseudoalteromonas* showed inhibitory effects on spore settlement. Many members of this genus produce compounds that inhibit growth and settlement of other bacteria as well as a range of higher organisms including *Enteromorpha/Ulva* (Holmström and Kjelleberg, 2000). Inhibitory bacterial strain D2 (subsequently identified as *Pseudoalteromonas tunicata*) produces an active <3 kDa peptide, which lyses *Ulva lactuca* spores (James *et al*., 1996): the production of inhibitory components is related to pigment production (Egan *et al*., 2002). Lovejoy *et al*. (1998) showed that a yellow-pigmented *Pseudoalteromonas* strain Y had an algicidal effect on *Chattonella, Gymnodinium* and *Heterosigma*. The algicidal compound of strain Y was secreted into the seawater. In the present study a bacteria-free supernatant of stationary phase culture of RE2-11 (a brown-pigmented strain 97% similar in 16S sequence to *Pseudoalteromonas haloplanktis*) had a paralysing effect on spores (data not shown). The potential application of *Pseudoalteromonas* strains in antifouling measures is discussed by Holmström *et al*. (2000).

The age of the biofilm had a marked effect on quantitative and qualitative aspects of spore settlement. Although it could be that biofilm density of some of the strains at earlier time-points was not sufficiently high to give a statistically significant effect on spore settlement, the greater stimulatory effects with increased age observed for many strains may also suggest different underlying mechanisms for promoting spore settlement. For example, if spores are responding to EPS produced by bacteria then this effect might only become evident as the biofilm bacteria produce more EPS with time. Wieczorek and Todd, 1997) reported that the settlement of ascidian *Ciona intestinalis* larvae on natural biofilms was affected by biofilm age. They concluded that the increasing settlement of larvae with increasing biofilm age was due to the combined effect of active habitat selection and physical entrapment of larvae onto the biofilm extracellular polymeric substance (EPS).

Although age of the biofilm is clearly important, and has been recognized as such in several other studies on the effect of microbial biofilms on settlement of higher organisms (Keough and Raimondi, 1995; Holmström *et al*., 1996; Wieczorek and Todd, 1997; Maki *et al*., 2000) the actual density of the biofilm is rarely recorded. The present study clearly illustrates the very different rates of biofilm formation and how analyses of spore settlement are influenced by biofilm density. Particularly interesting is the *Vibrio* strain RE1–12a which formed very weak biofilms but was revealed as a promoter of spore settlement when biofilm density was taken into account.

Joint *et al*. (2000) used image analysis to explore the spatial relationship between bacterial cells in mixed species biofilms and attached zoospores. Preferential spore settlement on or in close proximity of some bacterial clumps was observed, suggesting that these microcolonies represent direct cues for spore settlement. The present study extended this analysis to biofilms of individual strains but the results indicate a more complex situation as several different categories of association can be recognized and overall, the spatial association between spores and bacteria appears to be independent of the overall quantitative influence of bacterial cells on spore settlement. The first category is represented by 19 strains showing non-random, preferential settlement on bacterial cells but without significantly enhancing the level of spore settlement overall. Analysis of biofilm density shows that this was not due to the fact that biofilms of these strains were not sufficiently dense to give a statistically significant stimulation of settlement since some strains in this category formed quite dense biofilms.

The second category of strains that both enhance spore settlement and show preferential settlement on bacterial cells was represented by eight strains distributed across a number of taxa. This close association may suggest that the spores are responding chemotactially to local cues with restricted potential for diffusion. Bacterial EPS would fall into this category and was suggested by Dillon *et al.* (1989) in their study of mixed biofilms and Maki *et al*. (2000) in their study on barnacle cyprid settlement, although point sources of other, more diffusible signals could also give the same effect. In this context (Joint *et al*., 2002) recently showed that *Enteromorpha* spore settlement in stimulated by the acylated homoserine lactones used by bacteria in quorum sensing processes.

The third category of strains that enhance spore settlement but on a random spatial basis, was only represented by two strains (E26, RE2-13). This loose form of association may suggest that these strains are producing molecules that have the effect of enhancing spore settlement through non-chemotactic mechanisms.

The final category of strains that both inhibit settlement and show preferential association between spores and bacteria, is much more difficult to understand, but could be the result of such strains producing both positive, chemotactic-type cues and inhibitors. Chemical analysis of the responsible cues may eventually elucidate the basis of this and other effects described.

In conclusion, this paper has demonstrated that there is specificity in the settlement-stimulating activity of individual, phylogenetically characterized strains of bacteria, towards spores of *Enteromorpha*, but the relationship is not taxon-specific and is complicated by strain-specific influences of biofilm age. Analysis of spatial relationships between the settling spores and the biofilm bacteria suggest that more than one mechanisms or type of cue may be involved. Whereas the ecological relevance of cues from bacterial biofilms in potentiating the settlement of invertebrate larvae is increasingly understood (Kirchman *et al*., 1982; Lau and Qian, 1997; Maki, 1998; Lau *et al*., 2002) the relevance of specific settlement-inducing strains to algal spore settlement on natural biofilms requires further exploration through community DNA analysis and fluorescent *in situ* hybridization. Nakanishi *et al*. (1996) have shown that a wide range of strains of marine bacteria isolated from several marine algae, exhibited morphogenesis-inducing activity when added to axenic cultures of *Ulva*, a close-relative of *Enteromorpha*. One possibility therefore is that preferential settlement of spores on biofilms of specific bacteria may facilitate a close association of the developing plant with these important bacterial epi-symbionts. These aspects are currently under investigation for *Enteromorpha*.

Experimental procedures

Bacterial strains

Enteromorpha plants and neighbouring small rocks of the same geological type as those colonized by the alga were collected from rockpools on Wembury Beach, Devon, England (50°18′ N; 4°02′ W) and shaken overnight with 150 ml of sterile seawater at 20°C. From each sample 100 μl of suspension were spread on three types of agar medium: seawater agar (filtered seawater with 1.5% agar), marine agar (Difco medium 2216) and VNSS agar (Marden *et al*., 1985). Marine agar and VNSS plates were incubated for 3–4 days and seawater agar plates were incubated for 10–12 days at 20°C. Individual colonies were picked and streaked on marine agar or VNSS agar accordingly to the medium they were isolated from, to obtain single colonies. Colonies from seawater agar were streaked on marine agar to accelerate the growth of single colonies. Isolations were carried out twice during the winter of 2000 and the summer of 2001. Isolated colonies were subcultured at least four times before examination of colony morphology, colour, cell shape, Gram-staining properties and motility.

DNA extraction

Bacterial colonies from agar plates were lysed using 500 μl of prewarmed lysis buffer (0.5% SDS and 20 $\mu g l^{-1}$ proteinase K) by incubating at 55°C for 30 min After a further 10 min incubation at 65°C, 100 μl of hexadecyltrimethyl ammonium bromide (10% in 0.7 M NaCl) and 80 μl of 5 M NaCl were added. Proteinaceous material was separated by adding 500 μl of chloroform:isoamyl alcohol (24:1) and centrifuging at 13 000 r.p.m. for 10 min. The upper aqueous layer was added to 0.6 volumes of isopropanol and DNA was precipitated by centrifugation at 13 000 r.p.m. for 5 min. The DNA pellet was washed with 70% ethanol and resuspended in Tris-EDTA buffer, pH 8 (Bailey, 1995).

PCR amplification of 16S gene for DGGE

The variable V9 region of 16S rRNA genes was amplified from DNA extracted from single isolates. One primer was based on conserved region among domain *Bacteria* 5′-ATGGCTGTCGTCAGCT-3′; *Escherichia coli* position 1055 to 1070) and other primer complimented universally conserved region (primer sequence 5′-ACGGGCGGTGTGTAC-3′; *E. coli* positions 1392–1406) with a GC clamp were used in the reaction (Amann *et al*., 1995). Reaction mixtures contained 1 × PCR buffer, 5 mM $MgCl_2$, 100 ng of each forward and reverse primer, 20 mM of each deoxynucleoside triphosphates [dATP, dCTP, dGTP and dTTP (Bioline, UK)], 1 μg μl^{-1} bovine serum albumin, 1 unit of *Taq* polymerase (Bioline, UK) and ~10 ng of template DNA in a total of 50 μl. The temperature cycle for PCR was 20 s denaturation at 94°C, 30 s of annealing at 52°C and 30 s of primer extension at 72°C. The 30 cycles were followed by the final extension for 10 min at 72°C using a HYBAID Touchdown thermocycler (Hybaid, UK). Purity and quantity of PCR products were checked on 1% agarose gel before DGGE analysis.

Denaturing gradient gel electrophoresis (DGGE) analysis

Denaturing gradient gel electrophoresis was performed on a D-Gene Mutation Detection System (Bio-Rad). The gel was made with 8% (w/v) polyacrylamide gels (40:1, acrylamide:bisacrylamide) in 0.5 × Tris-acetate-EDTA (TAE) and denaturant containing 7 M urea and 40% (v/v) formamide in 0.5 × TAE. A gel with a 30–60% denaturant gradient was cast using a Model 475 Gradient Delivery System (Bio-Rad) according to manufacturer's instructions. The PCR products containing similar amounts of DNA were loaded on the gel and run at 200 V for 3 h at 60°C. Gels were stained with ethidium bromide for 30 min and destained in 1 × TAE for 10 min before visualizing on a UV transilluminator.

PCR amplification and sequencing of whole 16S rRNA genes

DNA from isolates with unique melting behaviour was subjected to PCR for the amplification of nearly complete (approx. 1533 bp) 16S rRNA genes using primers set pA (5′-AGAGTTTGATCCTGGCTCAG-3′; *E. coli* positions 8–27) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′; *E. coli* posi-

tions 1541–1522). The PCR reaction mixture contained 1 × PCR buffer, 5 mM $MgCl_2$, 100 ng of each forward and reverse primer, 40 mM of each deoxynucleotide triphosphate, 1 μg μl^{-1} bovine serum albumin, 1 unit of *Taq* DNA polymerase (Bioline) and ~ 50 ng of template DNA in a total of 50 μl. The temperature cycle for PCR was 5 min initial denaturation at 95°C with a pause at 80°C to add *Taq* DNA polymerase, then 25 cycles of 30 s denaturation at 94°C, 30 s of annealing at 52°C and 45 s of primer extension at 72°C, followed by the final extension for 10 min at 72°C and storage at 4°C. Excess dNTPs and primers were removed using a PCR purification kit (Qiagen) according to manufacturer's instructions. A 3700 DNA Analyzer (Applied Biosystems) automated sequencer was used for DNA sequencing. Reactions were carried out using ABI PRISM Big Dye Terminator Cycle Sequencing Kit (version 2.0) supplied by Applied Biosystems. Similarity between DNA sequences and with previously published sequences was detected using the BLAST program available at http://www.ncbi.nlm.nih.gov/. All sequences and selection of closely related sequences extracted from the GenBank were aligned with CLUSTAL W (Thompson *et al*., 1994). All sequence analysis was implemented in PHYLIP software (Felsenstein, 2001). Distance matrix analysis was carried out with Jukes and Cantor model and tree construction was done by the neighbour-joining method. Bootstrapping was conducted with 100 replicate trees with the program Seqboot. rDNA sequences have been submitted to GenBank under the accession numbers AF539753-539789.

Biofilm development

Glass microscope slides were cleaned by immersion in concentrated HCl overnight followed by extensive washing with distilled water. Slides were sterilized by flaming after dipping in ethanol. Bacterial strains were grown in marine broth (Difco medium 2216) or VNSS medium by inoculating a loopful of bacteria from an agar plate into 150 ml of marine broth in a 250 ml Ehrlenmeyer flask at 25°C. Growth curves were determined for a sample of 12 strains selected on the basis of difference of colony morphology and colour. In all cases stationary phase was reached 12–15 h after inoculation, hence this was the standard culture age used in subsequent experiments. To make biofilms, six replicate slides for each time point and each strain were held vertically in an early stationary phase culture and incubated for 1.5 and 3 h at 20–22°C. In order to develop older biofilms, slides were held vertically in a gently shaking culture for 48 h and the growth medium was changed every 12 h. After incubation, loosely attached bacteria were removed by washing the slides in sterile seawater. Three replicate slides were used for the spore attachment assay and the other three replicates were incubated in sterile seawater for 1 h and fixed in 2% (v/v) glutaraldehyde in seawater for bacterial counts. Control slides for spore settlement assays were prepared in the same way using sterile marine broth (Difco medium 2216).

Spore settlement assay

Enteromorpha linza was collected from Wembury beach a few days before spring tides. Spores were released from fertile pieces of thallus using the method described by Callow *et al*. (1997). Control and biofilm slides were washed in sterile seawater before use in assays. The spore concentration was adjusted to 10^6 spores ml^{-1} using artificial seawater. Each glass slide was placed in a 5-cm diameter Sterilin Petri dish and 5 ml of spore suspension added. Three replicates of each treatment were incubated in the dark for 1 h. The slides were washed in seawater to remove unattached spores before fixing in 2% glutaraldehyde (v/v) in seawater for 15 min followed by washing in seawater, 1:1 (v/v) seawater/distilled water and distilled water and allowed to dry.

Settled spores were counted by chlorophyll autofluorescence using a Zeiss Kontron 3000 image capture analysis system attached to a Zeiss epifluorescence microscope (Callow *et al*., 2002). Counts were made for 30 fields of view on each of three replicate slides. As biofilms were tested against different batches of zoospores, the absolute level of zoospore settlement on control slides varied. Therefore, to compare the effect of different biofilms, results are presented as percentage stimulation or inhibition of spore settlement, defined by:

$$S_i = [(E_i - C)/C]*100$$

where S is the percentage stimulation/inhibition, E is the number of settled spores on biofilm slides, C is the mean number of settled spores on control slides and i = 1–30 (10 fields of view counted on each of three replicate slides). The means of 30 values of S ± 95% confidence limits were calculated.

Measurement of bacterial density

Slides were stained with 1% methylene blue and images were captured by transmitted light using an Olympus UM2 light microscope (40 × objective) connected via a video camera to an image analysis system. Images were captured for 10 fields of view for each of three replicates and subjected to image analysis using a Ziess Kontron KS300 system. The areal density of bacteria per unit area of slide (Λ) is determined by $\Lambda = x/A$ where x is the area covered by bacteria and A is the area of the total field of view. The area of bacteria was the total count of pixels brighter than an established threshold level. The level of spore settlement on biofilms was then corrected for Λ.

Parallel measurements of bacterial density on slides settled with spores enabled the spatial relationship between the spores and bacteria in the biofilms to be determined as described by Joint *et al*. (2000). In this method the measured bacterial density on slides not exposed to spores is compared with the equivalent estimate of bacterial density on slides exposed to spores. In the latter case the area of interest is the total field of view minus the area which is not visible below the spores; bacterial density (λ) being determined by $\lambda = y/A\text{-}z$, where y is the area covered by visible bacteria and the visible surface area is A-z (the total field of view, minus the portion covered by spores (z)); see Joint *et al*. (2000) for further details on the theory behind this approach.

Statistical analysis

Analysis of all data on spore settlement and bacterial density was carried out using one-way ANOVA using SPSS 10.0 after

checking the data for normality by the method of Shapiro and Wilk (1965). Most data conformed to normality assumptions but a small proportion of the data had a minor level of negative skewness. It is common practice with data that show any degree of non-normality to resort to non-parametric tests. The major concern here would be skewness not kurtosis. However, Model I ANOVA, as used here, is extremely robust to non-normality as far as comparisons between means and their confidence intervals are concerned (Kendall *et al*., 1983). Therefore, the small amounts of skewness of the size detected here are very unlikely to result in spurious significance and certainly not where the *P*-values are <<1%. Resort to non-parametric tests in such situations would lead to considerable and unnecessary loss of statistical power. After ANOVA, comparisons between means and groups were expressed on a semi-quantitative (+/++/+++) scale, based on whether critical values after Tukey's *B*-test were 1×, 2× or 3× greater than the T statistic respectively. A similar procedure was applied to means showing minus (–) values.

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References

Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995) Phylogenetic Identification and in-situ detection of individual microbial cells without cultivation. *Microbiol Rev* **59:** 143–169.

Bailey, M.J. (1995) Extraction of DNA from the phyllosphere. In *Nucleic Acids in the Environment, Methods and Applications.* Trevors, J.T., and van Elsas, J.D. (eds). pp. 89–110.

Bowman, J.P., McCammon, S.A., Brown, M.V., Nichols, D.S., and McMeekin, T.A. (1997) Diversity and association of psychrophilic bacteria in Antarctic sea ice. *Appl Environ Microbiol* **63:** 3068–3078.

Callow, M.E. (1996) Ship-fouling: The problem and method of control. *Biodeterioration Abstracts* **10:** 411–421.

Callow, M.E., and Callow, J.A. (2000) Substratum location and zoospore behaviour in the fouling alga *Enteromorpha*. *Biofouling* **15:** 49–56.

Callow, M.E., Callow, J.A., Pickett-Heaps, J.D., and Wetherbee, R. (1997) Primary adhesion of *Enteromorpha* (Chlorophyta, Ulvales) propagules: quantitative settlement studies and video microscopy. *J Phycol* **33:** 938–947.

Callow, J.A., Stanley, M.S., Wetherbee, R., and Callow, M.E. (2000a) Cellular and molecular approaches to understanding primary adhesion in *Enteromorpha*: an overview. *Biofouling* **16:** 141–150.

Callow, M.E., Callow, J.A., Ista, L.K., Coleman, S.E., Nolasco, A.C., and Lopez, G.P. (2000b) The use of self-assembled monolayers of different wettabilities to study surface selection and primary adhesion processes of green algal (*Enteromorpha*) zoospores. *Appl Environ Microbiol* **66:** 3249–3254.

Callow, M.E., Jennings, A.R., Brennan, A.B., Seegert, C.E., Gibson, A., and Wilson, L. (2002) Microtopographic cues for settlement of zoospores of the green fouling alga *Enteromorpha*. *Biofouling* **18:** 237–245.

Delong, E.F., Franks, D.G., and Alldredge, A.L. (1993) Phylogenetic diversity of aggregate-attached vs free-living marine bacterial assemblages. *Limnol Oceanogr* **38:** 924–934.

Dillon, P.S., Maki, J.S., and Mitchell, R. (1989) Adhesion of *Enteromorpha* swarmers to microbial films. *Microb Ecol* **17:** 39–47.

Egan, S., James, S., Holmström, C., and Kjelleberg, S. (2002) Correlation between pigmentation and antifouling compounds produced by *Pseudoalteromonas tunicata*. *Env Microbiol* **4:** 433–442.

Felsenstein, J. (2001) *PHYLIP: Phylogeny Inference Package Version* 3.6.*:* University of Washington, Seattle.

Fuhrman, J.A., McCallum, K., and Davis, A.A. (1993) Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific Oceans. *Appl Environ Microbiol* **59:** 1294–1302.

Hagström, A., Pinhassi, J., and Zweifel, U.L. (2000) Biogeographical diversity among marine bacterioplankton. *Aquat Microbial Ecol* **21:** 231–244.

Holmström, C., and Kjelleberg, S. (1999) Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiol Ecol* **30:** 285–293.

Holmström, C., and Kjelleberg, S. (2000) Bacterial interactions with marine fouling organisms. In *Biofilms: Recent Advances in Their Study and Control*. Evans, L.V. (ed). The Netherlands: Harwood Academic, pp. 101–115.

Holmström, C., James, S., Egan, S., and Kjelleberg, S. (1996) Inhibition of common fouling organisms by marine bacterial isolates with special reference to the role of pigmented bacteria. *Biofouling* **10:** 251–259.

Holmström, C., Steinberg, P., Chrisov, V., Christie, G., and Kjelleberg, S. (2000) Bacteria immobilised in gels: improved methodologies for antifouling and biocontrol applications. *Biofouling* **15:** 109–117.

James, S.G., Holmström, C., and Kjelleberg, S. (1996) Purification and characterization of a novel antibacterial protein from the marine bacterium D2. *Appl Environ Microbiol* **62:** 2783–2788.

Joint, I., Callow, M.E., Callow, J.A., and Clarke, K.R. (2000) The attachment of *Enteromorpha* zoospores to a bacterial biofilm assemblage. *Biofouling* **16:** 151–158.

Joint, I., Tait, K., Callow, M.E., Callow, J.A., Milton, D., Williams, P., and Camara, M. (2002) Cell-to-cell communication across the prokaryote–eukaryote boundary. *Science* **298:** 1207.

Kendall, M., Stuart, A., and Ord, J.K. (1983) *The Advanced Theory of Statistics*. London and High Wycombe: Charles Griffin & Company.

Keough, M.J., and Raimondi, P.T. (1995) Responses of settling invertebrate larvae to bioorganic films – effects of different types of films. *J Exp Mar Biol Ecol* **185:** 235–253.

Kirchman, D., Graham, S., Reish, D., and Mitchell, R. (1982) Bacteria induce settlement and metamorphosis of *Janua*

(*Dexiospira*) Grube (Polychaeta: Spirorbideae). *J Exp Mar Biol Ecol* **56:** 153–163.

Lau, S.C.K., and Qian, P.Y. (1997) Phlorotannins and related compounds as larval settlement inhibitors of the tube-building polychaete *Hydroides elegans*. *Mar Ecol Progr Series* **159:** 219–227.

Lau, S.C.K., Mak, K.K.W., Chen, F., and Qian, P.Y. (2002) Bioactivity of bacterial strains isolated from marine biofilms in Hong Kong waters for the induction of larval settlement in the marine polychaete *Hydroides elegans*. *Mar Ecol Progr Series* **226:** 301–310.

Lovejoy, C., Bowman, J.P., and Hallegraeff, G.M. (1998) Algicidal effects of a novel marine *Pseudoalteromonas* isolate (class Proteobacteria, gamma subdivision) on harmful algal bloom species of the genera *Chattonella, Gymnodinium*, and *Heterosigma*. *Appl Environ Microbiol* **64:** 2806–2813.

Maki, J.S. (1998) The influence of marine microbes on biofouling. In *Recent Advances in Marine Biotechnology*. Fingerman, M., Nagabhushanam, R., and Thompson, M.-F. (eds). Horizon Scientific Press, pp. 147–171.

Maki, J.S., Ding, L., Stokes, J., Kavouras, J.H., and Rittschof, D. (2000) Substratum/bacterial interactions and larval attachment: Films and exopolysaccharides of *Halomonas marina* (ATCC 25374) and their effect on barnacle cyprid larvae, *Balanus amphitrite* Darwin. *Biofouling* **16:** 159–170.

Marden, P., Tunlid, A., Malmcronafriberg, K., Odham, G., and Kjelleberg, S. (1985) Physiological and morphological-changes during short-term starvation of marine bacterial isolates. *Arch Microbiol* **142:** 326–332.

Mullins, T.D., Britschgi, T.B., Krest, R.L., and Giovannoni, S.J. (1995) Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. *Limnol Oceanogr* **40:** 148–158.

Nakanishi, K., Nishijima, M., Nishimura, M., Kuwano, K., and Saga, N. (1996) Bacteria that induce morphogenesis in *Ulva pertusa* (Chlorophyta) grown under axenic conditions. *J Phycol* **32:** 479–482.

Shapiro, S.S., and Wilk, M.B. (1965) An analysis of variance test for normality (complete samples). *Biometrika* **52:** 591–611.

Stanley, M.S., Callow, M.E., and Callow, J.A. (1999) Monoclonal antibodies to adhesive cell coat glycoproteins secreted by zoospores of the green alga *Enteromorpha*. *Planta* **210:** 61–71.

Suzuki, M.T., Rappe, M.S., Haimberger, Z.W., Winfield, H., Adair, N., Strobel, J., and Giovannoni, S.J. (1997) Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. *Appl Environ Microbiol* **63:** 983–989.

Thomas, R.W.S.P., and Allsopp, D. (1983) The effects of certain periphytic marine bacteria upon the settlement and growth of *Enteromorpha*, a fouling alga. *Biodeterioration* **5:** 348–357.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL-W – improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22:** 4673–4680.

Wieczorek, S.K., and Todd, C.D. (1997) Inhibition and facilitation of bryozoan and ascidian settlement by natural multi-species biofilms: effects of film age and the roles of active and passive larval attachment. *Mar Biol* **128:** 463–473.