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Cooperative interactions within a marine bacterial dual species biofilm growing on a natural biodegradable substratum

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ABSTRACT: *Pseudoalteromonas* sp. S91 is a marine bacterium known to secrete chitinases and proteases, hydrolytic enzymes responsible for the degradation of chitin and protein, respectively, which enable access to nutrients contained in chitinous materials such as squid pen. In a dual species biofilm grown on squid pen, *Pseudoalteromonas* sp. S91 was able to support the accumulation of *Vibrio* sp. S141, which is unable to degrade squid pen but able to metabolise the chitin subunit *N′*-acetylglucosamine (GlcNAc), a product of squid pen hydrolysis. When grown on a glass substratum in the presence of a soluble carbon source that only *Pseudoalteromonas* sp. S91 could use, its biofilm provided no support to *Vibrio* sp. S141.

KEY WORDS: Chitin · Biofilm · Marine bacteria · Commensalism

INTRODUCTION

Bacterial populations within marine environments predominately exist as biofilms adhering to surfaces (Costerton et al. 1995). Within the dynamic biofilm environment, interspecies interactions directly influence biofilm morphology and species success (Nielsen et al. 2000, Christensen et al. 2002). Møller et al. (1997) found that nutrient concentration directly affects development of multispecies biofilms by influencing the location of species within the biofilm, while Rao et al. (2005) suggested that the formation of microcolonies by different species enhances their ability to persist under competitive conditions in mixed-species biofilms. Throughout the marine environment, biofilms predominately occur on biodegradable substrata (Costerton et al. 1994), such as marine snow, and usually consist of multiple species; analysis of species behaviour under such conditions has been little studied to date.

Past and current microbial research has concentrated on investigating the characteristics of pure species cultures supplemented with soluble carbon sources (Davies et al. 1998, O'Toole & Kolter 1998, Pratt & Kolter 1998, Sternberg et al. 1999). Studies of mixed-species biofilms have predominately used inert surfaces (Wolfaardt et al. 1994, Palmer et al. 2001, Rao et al. 2005). However, bacteria within natural environments exist in mixed-species biofilms exposed to heterogeneous sources of nutrients and nutrient-rich surfaces. In studies that have analysed biodegradable compounds exposed to single- or mixed-species biofilm consortia, data collected have principally reported the rate of substrate degradation, and how this correlates with numbers within a bacterial consortium (Kudo et al. 1987, O'Sullivan et al. 2005, Song et al. 2005).

Recent studies investigating multispecies microbial consortia have focused on bacterial interactions within biofilms degrading compounds of environmental or commercial significance (Møller et al. 1997, De Souza et al. 1998, Nielsen et al. 2000). This research commonly views bacteria as immobilised enzymes able to hydrolyse a specific substrate with efficiency dependent upon the identity of the species present in the biofilm. Increases in degradation rate of the specific substrate by the biofilm can often be related to the presence in the biofilm of a second species, which

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while unable to directly degrade the specific substrate itself, instead degrades a toxic or inhibitory metabolic by-product of the first species (De Souza et al. 1998). Although microbial metabolism of dissolved organic carbon sources has also been subject to extensive study, the investigation of microbial metabolism of particulate organic matter (POM) is much less common (Vetter & Deming 1999). It has been found that the mode of bacterial attachment, biofilm architecture and rate of biofilm development may be altered in response to the type of substratum it is grown upon (Dalton et al. 1994, Baty et al. 2000, Delpin et al. 2000). To better understand the interactions between different bacterial species within mixed-species biofilms when degrading the substratum to which they are attached, we used a model marine system, which combines a *Pseudoalteromonas* sp. with either a *Vibrio* sp. or a *Psychrobacter* sp. forming biofilms on a natural biodegradable substratum, squid pen, which is composed of 40% chitin and 60% protein (Gooday 1990). Chitin is composed of β-1-4-linked *N*′-acetylglucosamine (GlcNAc) subunits, to which it can be hydrolysed by chitinases (Keyhani & Roseman 1999); in squid pen, chitin has a β-crystalline structure (Gooday 1990). Chitin, a major component of oceanic POM, is among the most abundant organic carbon and nitrogen sources in the marine environment, with approximately 10^{11} t produced each year (Gooday 1990). The degradation of chitin and chitinous materials is predominately carried out by chitinase-producing micro-organisms (Gooday 1990), which are ubiquitous in the marine environment (Keyhani & Roseman 1999).

The strains chosen for this study represent commonly culturable Gram-negative marine bacteria that can be genetically manipulated. *Pseudoalteromonas* sp. S91, a Gram-negative motile marine bacterium, is capable of recycling nutrients and energy contained in organic macromolecules and polymers via the production and subsequent action of hydrolytic enzymes, such as chitinases and proteases (Albertson et al. 1996). Squid pen can support *Pseudoalteromonas* sp. S91 growth as the sole carbon source (Techkarnjanaruk et al. 1997). *Vibrio* sp. S141, a Gram-negative motile marine bacterium (Albertson et al. 1996), also produces chitinases and proteases. *Vibrio* sp. S141 can grow on solid, non-crystalline chitin (Angles 1997), but in contrast to *Pseudoalteromonas* sp. S91, grows poorly with squid pen as the sole source of carbon. *Psychrobacter* sp. SW5H is a Gram-negative non-motile marine bacterium (Angles et al. 1993, Poulsen et al. 1997), which grows well at laboratory temperatures of 20 to 30°C, and its affiliation to the genus *Psychrobacter* is based on 16srRNA gene sequencing (Poulsen et al. 1997). This strain does not produce chitinases or proteases and cannot grow on squid pen, chitin or GlcNAc as sole sources of carbon. *Vibrio* sp. S141 and *Psychrobacter* sp. SW5H, although unable to metabolise squid pen, are both capable of biofilm formation and plasmid transfer on the squid pen surface in the presence of a suitable soluble nutrient source (Angles 1997). A chitinase-negative mutant *Pseudoalteromonas* sp. S91CGFP was also used to investigate any role that chitinases may have during the interactions between *Pseudoalteromonas* sp. S91 and other species in dual-species biofilms on squid pen.

We analysed the interactions between different species in dual-species biofilms growing on squid pen as a biodegradable substratum, which acted as the sole source of carbon. To study dual-species biofilms, green fluorescent protein (GFP) was employed as a species-specific marker. Tagging of 1 strain enabled the spatial analysis of that strain within the biofilm by 2-photon laser scanning microscopy (TPLSM), and facilitated the real time qualitative and quantitative analysis of living cells within hydrated biofilms (Yoshida & Kuramitsu 2002). Following specific inoculation regimes, one of the species was localised and quantified.

We found that the marine bacterium *Pseudoalteromonas* sp. S91 was able to support the accumulation of *Vibrio* sp. S141 when grown in dual-species biofilms on squid pen in the absence of a soluble carbon source. This relationship was altered when the capability of *Pseudoalteromonas* sp. S91 to produce chitinases was eliminated and when the biodegradable substratum was replaced with an inert substratum. In contrast, we found that *Pseudoalteromonas* sp. S91 was unable to provide similar support to *Psychrobacter* sp. SW5H when grown in dual-species biofilms on squid pen.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. *Pseudoalteromonas* sp. Strain S91SB is a derivative of Strain S91 (Techkarnjanaruk et al. 1997) into which a *gfp* gene has been inserted. *Pseudoalteromonas* sp. Strain S91CGFP, also a derivative of Strain S91, was constructed by transposon mutagenesis of the *chiA* gene with a promoterless transposon cassette containing a *gfp* gene (Matthysse et al. 1996). The plasmid p519*ngfp* is derived from the broad host range IncQRSF1010 derivative PDSK519 (Keen et al. 1988) and has an *npt2* promoter constitutively expressing the *gfp* gene (Matthysse et al. 1996).

Growth conditions. Marine bacteria were grown in batch at 30°C in either tryptone soy broth (TSB; Oxoid) containing NaCl (0.26 M), $MgCl_2$ (1 mM) and $CaCl_2$

Table 1. Strains and plasmids used in the study. GFP: green fluorescent protein; Sm^R: streptomycin resistant; Km^R: kanamycin resistant; +: positive; –: negative

Bacterial strain or plasmid	GFP	Chitinase	Characteristic(s)	Source
Bacterial strains				
Pseudoalteromonas sp.	–	+		
Strain S91	–	+	Sm^R	Albertson et al. (1996)
Strain S91CX	–	–	Sm^R, Km^R	Techkarnjanaruk et al. (1997)
Strain S91CGFP	+	–	Sm^R, Km^R	Stretton et al. (1998)
Strain S91SB	+	+	Sm^R, Km^R	E. M. Schaffer (unpubl.)
Vibrio sp. Strain S141	–	+	Sm^R	Östling et al. (1991)
Psychrobacter sp.	–	–	Sm^R	Angles et al. (1993)
Strain SW5#19	–	–	Sm^R Km^R	This study
Escherichia coli SM10	–	–	Km^R	Miller & Mekalanos (1988)
Plasmids				
p519*gfp*	+		Km^R *mob*$^+$	Matthysse et al. (1996)
p519*ngfp*	+		*npt*-2 promoter in front of *gfp*	Matthysse et al. (1996)
pDSK519	–		Km^R *mob*$^+$, nonconjunjugative	Keen et al. (1988)
pLOFKm*gfp*	+		Promoterless *gfp*, Km^R	Stretton et al. (1998)

(0.33 mM); Luria broth (LB) (Miller 1972) containing NaCl (0.26 M), $MgCl_2$ (1 mM) and $CaCl_2$ (0.33 mM); or artificial seawater minimal medium (Östling et al. 1991) supplemented with 20 mM glutamate (MMM glt_{20}) for *Psychrobacter* sp. SW5H strains, 40 mM glutamate (MMM glt_{40}) for *Pseudoalteromonas* sp. S91 strains or 20 mM glucose (MMM $gluc_{20}$) for *Vibrio* sp. S141 strains. *Pseudoalteromonas* sp. S91 and *Psychrobacter* sp. SW5H strains are able to use glutamate, but not glucose, as a sole carbon source, whereas *Vibrio* sp. S141 can use glucose but not glutamate. *Escherichia coli* was grown in batch at 37°C in LB supplemented with 5 $g l^{-1}$ NaCl (LB5). Kanamycin (Km) and streptomycin (Sm) were both used at 100 µg ml^{-1}; Km was included for plasmid maintenance where appropriate.

Transposon mutagenesis and isolation of strain *Psychrobacter* sp. SW5#19. The vector pLOFKm*gfp* carrying a mini-Tn-*10*:*gfp*:*kan* transposon (Stretton et al. 1998) was transferred from the donor strain *Escherichia coli* SM10 to the recipient (*Psychrobacter* sp. SW5H) using the filter mating protocol described by Albertson et al. (1996). From log phase cultures (OD595 approximately 0.35) of donor and recipient cells, 50 µl were gently mixed and spotted on a sterile 0.2 µm Millipore membrane filter placed on an LB plate supplemented with 15 g l^{-1} NaCl (LB15). Controls of each strain were spotted on separate filters on individual plates. After overnight inoculation, filters were separately resuspended in 1 ml LB15 and then spread onto LB15 Sm Km plates to enable counter selection of donors and recipients. Seventy transconjugants, selected at appropriate dilutions on LB15 Sm Km plates, were screened for GFP fluorescence as colonies on the same medium (Stretton et al. 1998). Twenty-five positive strains were purified on MMM glt_{20} Sm Km plates; wet mounts of each were screened for constitutive GFP production by epifluorescence microscopy. Six positive strains were grown in liquid MMM glt_{20} Sm Km, and their growth rates were compared to *Psychrobacter* sp. SW5H. Transconjugant S *Psychrobacter* sp. SW5#19 was selected for further use, as its growth rate was similar to that of *Psychrobacter* sp. SW5H (data not shown).

Squid pen. Squid pen pieces (Stretton et al. 1998) were cut into approximately 100 mm^2 pieces (between the ridges) for use in experiments.

Squid pen growth test. *Pseudoalteromonas* sp. S91SB, *Vibrio* sp. S141(p519*ngfp*) and *Psychrobacter* sp. SW5#19 were inoculated into flasks, each containing a single piece of squid pen and 10 ml of MMM with no carbon supplement. Shake flasks were incubated at 30°C for up to 15 wk. By 15 wk *Pseudoalteromonas* sp. S91SB produced thick visible biofilm on the pen within a few days and had completely degraded the pen piece, whereas the chitinase-negative mutant, *Pseudoalteromonas* sp. S91CX, did not fully degrade the pen, although it produced visible thick biofilm on it. In contrast, the *Vibrio* and *Psychrobacter* strains neither produced any visible biofilm on the pen nor planktonic growth, and squid pen pieces showed no degradation after 15 wk of incubation.

Flow chamber experiments. Experiments were carried out using flow chambers based on the design of Hale & Mitchell (2001) as used by Delpin et al. (2000) and Fitch et al. (2002). A rubber o-ring placed between 2 glass cover slips sealed the chamber and provided a viewing port. Two needles perforated the o-ring; one

served as the inlet and the other as the outlet channel. The chamber volume was approximately 1 ml. At approximately 10 and 15 cm upstream from the chamber, 2 T-junctions were created in the silicone tubing, and these provided capped, sterile injection ports. Each injection port was used for the inoculation of 1 strain. Prior to assembly, an autoclaved sterile piece of squid pen was placed in each chamber. Assembled flow chambers and all tubing were sterilised by autoclaving and cleaned by pumping 70% ethanol through them for 15 min followed by sterile MMM for 20 min. An 8-roller Cole-Palmer Masterflex peristaltic pump (7521-57) was used to pump sterile MMM through the chamber, at a flow rate of 1 ml min^{-1} (Delpin et al. 2000); the outlet of the chamber went to waste. Flow chambers containing squid pen were inoculated with 3 ml of a 10^{-1} diluted overnight culture, grown with the appropriate carbon source at 30°C on a gyratory shaker. Where more than 1 species was inoculated at the same time, 1.5 ml of a 10^{-1} dilution of each overnight culture was injected into the chamber at the same time through different injection ports. Chambers were left for 1 h without flow to allow cells to attach to the squid pen. After 1 h, the flow was started and maintained at 1 ml min^{-1}. Flow was stopped and chambers were disconnected after 72 h, unless stated otherwise, and examined using a TPLSM.

To determine whether time or order of inoculation would affect the success of strains, 3 inoculation regimes were tested and are labelled as follows: 0 h means that the strain was inoculated at time 0 (T_0), and allowed to develop a biofilm for 72 h; 24 h means that the strain was inoculated 24 h after T_0 and allowed to develop for a further 48 h. Each experiment was run for a total of 72 h.

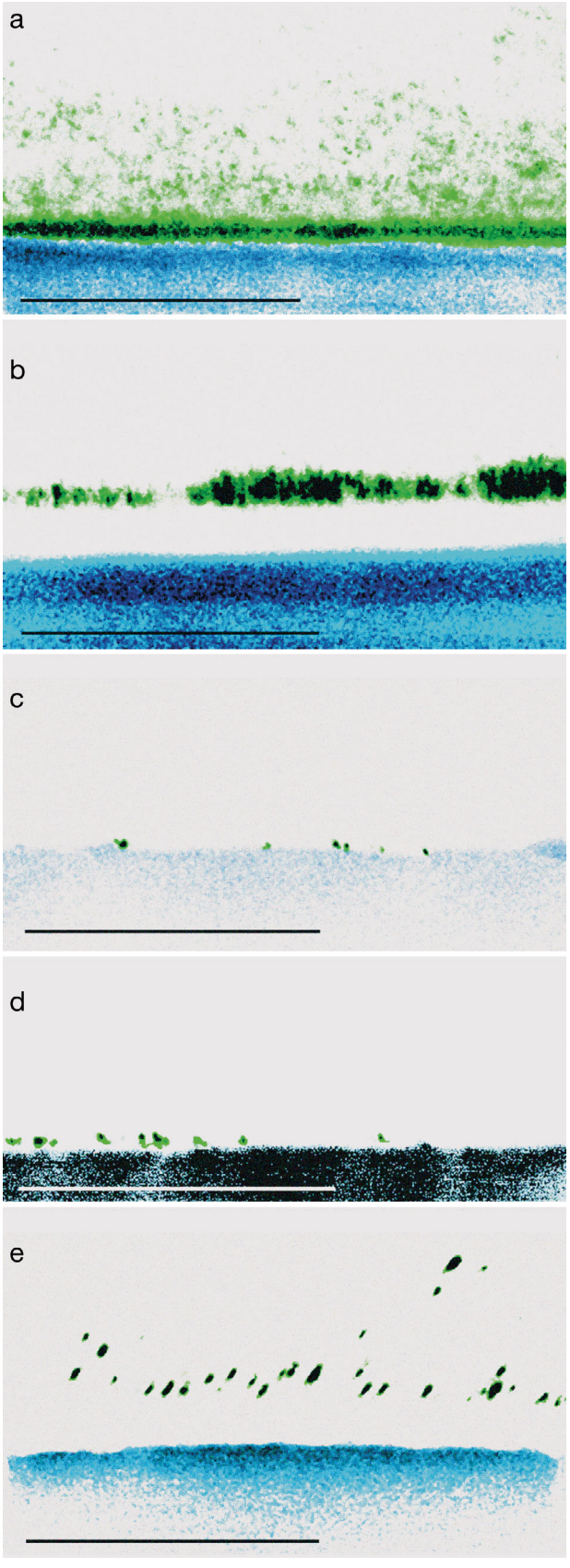
TPLSM. Following completion of flow chamber experiments, images of the bacterial biofilms within the chambers were collected using a Bio-Rad Radiance 2000MP visualising system in conjunction with a Nikon Eclipse TE300 inverted microscope. The microscope was equipped with a 60× water immersion lens with a numerical aperture of 1.2 and a Coherent Mira900-F titanium:sapphire ultra fast laser, which has an excitation spectrum of pulsed 800 nm light equivalent to 1 photon of 400 nm light, suitable for exciting GFP without damaging cells (Lim et al. 2007). An excitation wavelength (λ) of 2 photons of 800 nm and an emission λ of 515 to 530 nm was used to visualise GFP. An emission λ of 450 to 480 nm was used to visualise squid pen autofluorescence. Each experiment was repeated at least once, so that a minimum of 2 flow chambers were analysed for each experiment. Images of the bacterial biofilms were collected as highest resolution horizontal (*xy*) and vertical (*xz*) optical sections and recorded as digital computer files. For each chamber, 9 *xz* images were randomly captured and recorded. Images were converted using Confocal Assistant 4.02 (Todd Clarke Brelje, University of Minnesota). Image analysis was performed using ImageJ 1.32j (Abramoff et al. 2004) computer image analysis software.

Psychrobacter sp. SW5#19, *Pseudoalteromonas* sp. S91SB and *Vibrio* sp. S141(p519*ngfp*) grown in batch monoculture were each viewed as wet mounts using the TPLSM. Horizontal *xy* sections of *Psychrobacter* sp. SW5#19, *Pseudoalteromonas* sp. S91SB and *Vibrio* sp. S141(p519*ngfp*) biofilm cells were collected in the emission spectra λ 515 to 530 nm. The mean area covered by a single GFP^+ cell was determined by measuring the collective area of 50 random cells. The mean area was 0.6, 0.56 and 1.86 μm^2 for a single cell of *Psychrobacter* sp. SW5#19, *Pseudoalteromonas* sp. S91SB and *Vibrio* sp. S141(p519*ngfp*), respectively. Numbers of GFP^+ cells within *xz* images in the green spectra were calculated from the total area showing fluorescence using ImageJ 1.32j (Abramoff et al. 2004). Images used for analysis with ImageJ 1.32j (Abramoff et al. 2004) were computer manipulated; a median filter of 2.0 was applied to all images to reduce noise, and the histogram was modified to exclude background pixels, focusing on bacteria. Presented TPLSM images were manipulated using Adobe Photoshop. The histogram was optimised and a colour hue applied separately to squid pen and GFP-positive cells where appropriate. For *xz* sections, the negative image was falsely coloured.

Statistical analysis. To compare numbers of GFP-tagged cells in biofilms between experiments, Student's *t*-test assuming unequal variances was used to compare means from each experiment. Formulae used were as described by Zar (1996). A standardised area of *xz* sections was used when quantifying cell numbers: 96 µm in length (*x*) and 52 µm in height (*z*). For each experiment, 18 or more images, collected from chambers run in duplicate, were used to calculate mean cell numbers. Tables 2 to 4 show the numbers of GFP-tagged cells present in biofilms from each experiment.

RESULTS

Monoculture biofilms were grown on squid pen for 72 h. *Pseudoalteromonas* sp. S91 and derivative strains heavily colonised the pen and formed thick biofilms (Fig. 1a,b, Table 2). *Vibrio* sp. S141(p519*ngfp*) and *Psychrobacter* sp. SW5#19 strains showed patchy colonisation of the pen, with single cells and microcolonies situated directly on the pen surface (Fig. 1c,d, Table 2).



Pseudoalteromonas sp. S91(pDSK519) and *Psychrobacter* sp. SW5#19 interactions

When *Pseudoalteromonas* sp. S91(pDSK519) colonised the pen first or both strains were inoculated together, squid pen pieces were completely colonised by *Pseudoalteromonas* sp. S91(pDSK519) only, with no *Psychrobacter* sp. SW5#19 cells detected; when *Psychrobacter* sp. SW5#19 cells were inoculated first, numbers detected after the addition of *Pseudoalteromonas* sp. S91(pDSK519) were significantly lower ($p < 0.05$) than when *Pseudoalteromonas* sp. S91(pDSK519) was absent (Table 2). These data showed that the *Psychrobacter* SW5#19 strain was not supported, and was in fact outcompeted by, *Pseudoalteromonas* sp. S91(pDSK519) on squid pen.

Pseudoalteromonas sp. S91(pDSK519) and *Vibrio* sp. S141(p519*ngfp*) interactions

Biofilm numbers of *Vibrio* sp. S141(p519*ngfp*) were significantly higher ($p < 0.05$) in the presence of *Pseudoalteromonas* sp. S91(pDSK519) than when grown in monoculture, independent of inoculation order (Table 2). Following all 3 inoculation regimes, squid pen pieces were heavily colonised by *Pseudoalteromonas* sp. S91(pDSK519) cells (seen by light microscopy, data not shown) which were always positioned between the *Vibrio* sp. S141(p519*ngfp*) cells and the pen (Fig. 1e).

Following the addition of either glucose or GlcNAc, the monomer subunit of chitin, for the first 24 h of a 72 h experiment, *Vibrio* sp. S141(p519*ngfp*) monoculture numbers were significantly greater ($p < 0.05$) than when no soluble carbon source was added for the same time period (Table 2). This experiment was repeated except that at 24 h, when the feed of the soluble carbon source was discontinued, *Pseudoalteromonas* sp. S91(pDSK519) was inoculated. The numbers of *Vibrio* sp. S141(p519*ngfp*) under these conditions were significantly higher ($p < 0.05$) than those when *Vibrio* sp. S141(p519*ngfp*) was grown in monoculture or dual-species biofilms without the addition of a soluble carbon source for the first 24 h (Table 2). The results also showed that an increased accumulation of *Vibrio* sp.

Fig. 1. Artificially coloured *xz* profiles of representative areas of green fluorescence protein $(GFP)^+$ biofilm cells (green) on squid pen (blue) after 72 h. (a) GFP^+ *Pseudoalteromonas* sp. S91SB in monoculture; (b) GFP^+ *Pseudoalteromonas* sp. S91CGFP in monoculture; (c) GFP^+ *Vibrio* sp. S141(p519*ngfp*) in monoculture; (d) GFP^+ *Psychrobacter* sp. SW5#19 in monoculture; (e) GFP^+ *Vibrio* sp. S141(p519*ngfp*) and *Pseudoalteromonas* sp. S91(pDSK519) mixed species. Scale bars = 50 µm

Table 2. Experimental inoculation regimes and numbers of GFP$^+$ cells after 72 h (mean ± SD) in biofilms on squid pen

Strain inoculation at time 0 h	Strain and time of inoculation (h)	GFP$^+$ cell numbers per 5000 µm^2 after 72 h
Pseudoalteromonas sp. S91SB	–	1.5×10^3 ($\pm 5.7 \times 10^2$)
Vibrio sp. S141(p519*ngfp*)	–	2.2×10^0 ($\pm 1.7 \times 10^0$)
Pseudoalteromonas sp. S91CGFP	–	5.9×10^2 ($\pm$ 1.3×10^2)
Pseudoalteromonas sp. S91(pDSK519)	*Vibrio* sp. S141(p519*ngfp*) (24 h)	2.5×10^1 ($\pm 1.4 \times 10^1$)
Vibrio sp. S141(p519*ngfp*)	*Pseudoalteromonas* sp. S91(pDSK519) (24 h)	1.8×10^1 ($\pm 0.7 \times 10^1$)
Vibrio sp. S141(p519*ngfp*)	*Pseudoalteromonas* sp. S91(pDSK519) (0 h)	2.9×10^1 ($\pm 2.1 \times 10^1$)
Vibrio sp. S141(p519*ngfp*) (Gluc for 24 h)	–	1.6×10^1 ($\pm 1.1 \times 10^1$)
Vibrio sp. S141(p519*ngfp*) (Gluc for 24 h)	*Pseudoalteromonas* sp. S91(pDSK519) (24 h)	6.3×10^1 ($\pm 4.7 \times 10^1$)
Vibrio sp. S141(p519*ngfp*) (GlcNac for 24 h)	–	2.3×10^1 ($\pm 1.3 \times 10^1$)
Vibrio sp. S141(p519*ngfp*) (GlcNac for 24 h)	*Pseudoalteromonas* sp. S91(pDSK519) (24 h)	2.3×10^1 ($\pm 1.3 \times 10^1$)
Vibrio sp. S141(p519*ngfp*) (GlcNac for 24 h)	*Pseudoalteromonas* sp. S91(pDSK519) (24 h)	7.1×10^1 ($\pm 3.1 \times 10^1$)
Psychrobacter sp. SW5#19	–	7.7×10^1 ($\pm 4.1 \times 10^1$)
Psychrobacter sp. SW5#19	*Pseudoalteromonas* sp. S91(pDSK519) (24 h)	1.0×10^1 ($\pm 0.8 \times 10^1$)
Pseudoalteromonas sp. S91SB	*Vibrio* sp. S141wt (0 h)	1.6×10^3 ($\pm$ 4.0×10^2)

S141(p519*ngfp*) was supported during dual-species biofilm development on squid pen, since cell numbers were again significantly increased ($p < 0.05$) by the presence of *Pseudoalteromonas* sp. S91(pDSK519) (Table 2). In both mono- and dual-species biofilms, *Vibrio* sp. S141(p519*ngfp*) numbers were similar when either glucose or GlcNAc was used as the soluble carbon source for the initial 24 h.

Vibrio sp. S141(p519*ngfp*) did not appear to affect the growth of *Pseudoalteromonas* sp. S91SB as its numbers were similar ($p < 0.05$) in both mono- and dual-species biofilms (Table 2).

Do the major *Pseudoalteromonas* sp. S91 chitinases play a role in supporting *Vibrio* sp. S141?

When grown in the presence of the chitinase negative strain (*Pseudoalteromonas* sp. S91CX), the numbers of *Vibrio* sp. S141(p519*ngfp*) were significantly lower ($p < 0.05$; Table 3) following all 3 inoculation regimes than those grown in the presence of *Pseudoalteromonas* sp. S91(pDSK519) (Table 2) and similar to numbers grown in monoculture (Table 2). These data suggested that accumulation of the *Vibrio* sp. S141(p519*ngfp*) on squid pen was dependent on the chitinase-degrading ability of the *Pseudoalteromonas* sp. S91; therefore, the effect of biofilm growth on an abiotic surface was investigated.

Effect of substratum on *Vibrio* sp. S141(p519*ngfp*)–*Pseudoalteromonas* sp. S91 interactions in dual-species biofilms

To investigate the effect of the substratum on dual-species biofilm development and species behaviour, the 2 species were grown on glass in the presence of glutamate, which *Vibrio* sp. S141(p519*ngfp*) is unable to use as a carbon source. After 72 h, glass cover slips were heavily colonised by either *Pseudoalteromonas* sp. S91(pDSK519) or *Pseudoalteromonas* sp. S91CX, with *Vibrio* sp. S141(p519*ngfp*) cells predominately localised at the outer edge of the biofilms (data not shown). *Vibrio* sp. S141(p519*ngfp*) numbers were similar to those attached to the glass in monoculture biofilms in the presence of glutamate (Table 4). There was no significant difference in numbers when cells were grown with either *Pseudoalteromonas* sp. S91(pDSK519) or *Pseudoalteromonas* sp. S91CX, indicating that *Vibrio* sp. S141(p519*ngfp*) was able to co-exist with, but was not supported by, *Pseudoalteromonas* sp. S91 biofilms growing on an inert substratum.

Table 3. Experimental inoculation regimes and numbers of GFP$^+$ cells (mean ± SD) in biofilms on squid pen

Strain inoculation at time 0 h	Strain and time of inoculation (h)	GFP$^+$ cell numbers per 5000 µm^2 after 72 h
Pseudoalteromonas sp. S91CX	*Vibrio* sp. S141(p519*ngfp*) (24)	1.7×10^0 ($\pm 1.2 \times 10^0$)
Vibrio sp. S141(p519*ngfp*)	*Pseudoalteromonas* sp. S91CX (24)	1.0×10^0 ($\pm 1.2 \times 10^0$)
Vibrio sp. S141(p519*ngfp*)	*Pseudoalteromonas* sp. S91CX (0)	0.9×10^0 ($\pm 0.8 \times 10^0$)

Table 4. Experimental inoculation regimes and numbers of GFP$^+$ cells (mean ± SD) in biofilms with glutamate on glass

Strain inoculation at time 0 h	Strain and time of inoculation (h)	GFP$^+$ cell numbers per 5000 µm^2 after 72 h
Vibrio sp. S141(p519*ngfp*)	–	3.7×10^1 ($\pm 1.3 \times 10^1$)
Vibrio sp. S141(p519*ngfp*)	*Pseudoalteromonas* sp. S91(pDSK519) (0)	3.3×10^1 ($\pm 1.4 \times 10^1$)
Vibrio sp. S141(p519*ngfp*)	*Pseudoalteromonas* sp. S91CX (0)	4.4×10^1 ($\pm 2.8 \times 10^1$)

DISCUSSION

Our results indicated that the production of chitinases by *Pseudoalteromonas* sp. S91(pDSK519) enabled *Vibrio* sp. S141(p519*ngfp*) cells to accumulate in numbers in biofilms on squid pen, since no increase in numbers was found when *Vibrio* sp. S141(p519*ngfp*) was grown with *Pseudoalteromonas* sp. S91CX, a chitinase-negative mutant. The simplest explanation for these findings is that *Vibrio* sp. S141(p519*ngfp*) was able to use products of squid pen hydrolysis (such as GlcNAc) released into the biofilm by the *Pseudoalteromonas* sp. S91(pDSK519) chitinases. This was consistent with the results found for dual-species biofilms grown on glass in the presence of glutamate, a soluble carbon source only utilisable by *Pseudoalteromonas* sp. S91(pDSK519). Under these conditions, *Vibrio* sp. S141(p519*ngfp*) cell numbers did not accumulate. In contrast, *Psychrobacter* sp. SW5#19, which is unable to use products of chitin degradation, was unable to accumulate in mixed-species biofilms and may have been outcompeted by *Pseudoalteromonas* sp. S91(pDSK519), since its numbers were significantly decreased in the presence of *Pseudoalteromonas* sp. S91(pDSK519).

Vetter & Deming (1999) found that released bacterial extracellular enzymes could remain functional for extended periods of up to 6 h time and at distances of up to 100 µm from enzyme-producing cells. These findings suggest the non-specific activity of hydrolytic enzymes produced by bacterial cells does not assure that the catabolic by-products released following the enzymes' action will be metabolised by the enzyme-producing cells, possibly even being metabolised by cells of different species (Vetter & Deming 1999). This behaviour can be described as commensal and does not inhibit the fitness of the species being exploited, providing that the resources procured through this process do not become a limiting factor for growth or development of the exploited species. In our study, the ability of *Vibrio* sp. S141(p519*ngfp*) to increase in the presence of *Pseudoalteromonas* sp. S91(pDSK519) in biofilms on squid pen indicated that *Vibrio* sp. S141(p519*ngfp*) was able to scavenge or metabolise by-products of squid pen degradation. This would appear to satisfy the definition of a commensal relationship, since *Pseudoalteromonas* sp. S91SB did not appear to suffer reduced fitness from *Vibrio* sp. S141 scavenging nutrients. As the majority of bacteria within the marine environment persist as biofilm-associated mixed microbial consortia attached to surfaces (Costerton et al. 1995), interactions of this nature may be common among many species.

Many examples of cross-feeding within mixed-species biofilms have been published (Moller et al. 1997, Okabe et al. 2005), but few studies have investigated interspecies cross-feeding within biofilms attached to biodegradable substrata. Studies by Baty et al. (2000) found that single-species biofilms of *Pseudoalteromonas* sp. S91, growing on a solid chitin surface, contained 2 physiologically different populations. It was observed that a relatively small proportion of surface-associated *Pseudoalteromonas* sp. S91 cells were chitinase positive and primarily responsible for the production of extracellular chitinase enzymes that liberated nutrients from the chitin surface. The second biofilm subpopulation of *Pseudoalteromonas* sp. S91 did not produce detectable levels of extracellular chitinase. This population also remained associated with the chitin surface but instead took up liberated nutrients and proliferated, producing cells that entered the aqueous phase ready to colonise new surfaces (Baty et al. 2000). This interaction could be viewed one of 2 ways: as social cheating (Velicer 2003) or as a division of labour within a biofilm community resulting in both nutrient liberation and species proliferation (Baty et al. 2000). During our experiment, the *Vibrio* sp. S141 that scavenged similarly liberated nutrients from the *Pseudoalteromonas* sp. S91 biofilm may have exploited this intraspecies altruistic behaviour.

A study by Sandoz et al. (2007) found that mutant populations arose, carrying a mutation in the *lasR* gene, within *Pseudomonas aeruginosa* biofilms from cystic fibrosis patients. These mutant *P. aeruginosa* stopped producing proteases and instead consumed nutrients that were liberated by the proteolytic activity of their wild type neighbours. This is similar to the report by Baty et al. (2000) of the sub-population of *Pseudoalteromonas* sp. S91 that did not to produce chitinase enzymes, instead consuming the nutrients that were produced by their biofilm neighbours. However, in our study, the ability of *Vibrio* sp. S141 to accu-

mulate in numbers within a biofilm of *Pseudoalteromonas* sp. S91 is an example of an interspecies interaction that resulted in the success of a genetically unrelated population.

It should be noted that, like the subpopulation of proliferating non-enzyme-producing *Pseudoalteromonas* sp. S91 observed by Baty et al. (2000), *Vibrio* sp. S141 and *Psychrobacter* sp. SW5#19 may have exploited *Pseudoalteromonas* sp. S91 for growth and detachment, but our experiments were not designed to detect this.

Zhang & Bishop (2003) found that some biofilm-associated microorganisms were capable of degrading extracellular polymeric substances, produced during biofilm formation, when under conditions of starvation. Additionally, in mixed-species consortia, a species not responsible for extracellular polymeric substance production was seen to benefit from its consumption (Zhang & Bishop 2003). However, if extracellular polymers produced by *Pseudoalteromonas* sp. S91(pDSK519) were the source of metabolic support obtained by *Vibrio* sp. S141(p519*ngfp*), then polymer production, or the ability of *Vibrio* sp. S141(p519*ngfp*) to access this resource, was directly affected when *Pseudoalteromonas* sp. S91(pDSK519) had lost its ability to produce chitinases or when *Pseudoalteromonas* sp. S91(pDSK519) was grown on glass. The production of extracellular polymers by biofilm-associated bacteria in mixed-species consortia requires further analysis to determine its role in mediating interspecies interactions.

On squid pen in mixed-species biofilms, *Vibrio* sp. S141(p519*ngfp*) cells were localised at the outer edge of the *Pseudoalteromonas* sp. S91(pDSK519) biofilm (Fig. 1e), whereas in monoculture, *Vibrio* sp. S141 (p519*ngfp*) cells were localised directly on the pen surface (Fig. 1c), indicating that *Pseudoalteromonas* sp. S91(pDSK519) displaced *Vibrio* sp. S141(p519*ngfp*), or *Vibrio* sp. S141(p519*ngfp*) migrated to a preferred location within the dual-species biofilm. *Psychrobacter* sp. SW5#19 cells also attached directly to the pen surface in monoculture (Fig. 1d), and in a dual-species biofilm, remaining *Psychrobacter* sp. SW5#19 cells were also localised directly on the squid pen surface. *Psychrobacter* sp. SW5#19 is a non-motile marine bacterium, and its inability to be displaced by *Pseudoalteromonas* sp. S91(pDSK519) or actively migrate within the biofilm may have been a contributing factor as to why it did not persist within dual species.

Few studies have examined changes in biofilm structure or species localisation within the biofilm when challenged with a second species. Fitch et al. (2002) found that *Vibrio* sp. S141(p519*ngfp*), provided with a soluble carbon source, is able to displace *Psychrobacter* sp. SW5H to multiply preferentially on a glass surface in mixed-species biofilms, and Sturman et al. (1994) found that a developed *Pseudomonas aeruginosa* biofilm attached to an inert pellet was displaced and outcompeted by *Klebsiella pneumoniae* over a 21 d period.

Future work employing a non-motile *Vibrio* sp. S141 mutant (Delpin et al. 2000) will investigate whether motility plays a role in the behavioural interaction between this species and *Pseudoalteromonas* sp. S91 forming biofilms on squid pen. Furthermore, a non-motile *Pseudoalteromonas* sp. S91 mutant may be unable to displace the motile *Vibrio* sp. S141, which may also change the behavioural interaction between them in mixed-species biofilms.

In conclusion, our results showed that microbial interactions in biofilms growing on biodegradable substrata may be cooperative or competitive and completely differed from those grown on inert substrata. Little information is available in the literature about mixed-species biofilms grown on biodegradable substrata, although this is a major component of natural ecosystems. This represents an important area for further research.

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