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Green fluorescent protein as a novel species-specific marker in enteric dual-species biofilms

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Green fluorescent protein (GFP) was used as a tool to examine the interactions between pairs of bacterial species and their effects on subsequent biofilm development over 24 h. A plasmid encoding GFP from *Aequorea victoria* was transformed into strains of *Enterobacter agglomerans* and *Escherichia coli* ATCC 11229. The development of dual-species biofilms, containing one fluorescent and one non-fluorescent partner, was examined using viable counts. UV illumination of plates enabled both species to be identified in a mixture. The spatial distribution of each species was examined by UV microscopy, simultaneously staining the non-fluorescent strain with propidium iodide. GFP fluorescence was measured to quantify the adhesion of the strains to other cells or cell constituents or the invasion into pre-existing biofilms. Co-operation between *Ent. agglomerans*/GFP and *Klebsiella pneumoniae* G1 resulted in a 54 and a 23% increase in biofilm formation, respectively, compared with single-species biofilms. *E. coli*/GFP and *Serratia marcescens* 87b stably co-existed in biofilms but did not affect the growth of each other. The other bacterial partnerships examined were competitive, with the end result that one species dominated the biofilm. The methods described provide a convenient technique for the examination of mixed-species biofilm communities where the unique interactions between species determine the true properties of the resultant biofilms.

Keywords: green fluorescent protein (GFP), mixed biofilm, adhesion, co-operation, competition

INTRODUCTION

Green fluorescent protein (GFP) was originally isolated from the jellyfish *Aequorea aequorea* (Shimomura *et al.*, 1962) and the cDNA of GFP was cloned from *Aequorea victoria* by Prasher *et al.* (1992). The use of GFP has expanded and it has rapidly become a widely used reporter of gene regulation (Cubitt *et al.*, 1995; Crameri *et al.*, 1996). GFP is a 238 aa protein which emits green light (508 nm) when excited by blue light (395 nm). It can be detected by irradiation with near-UV or blue light and requires no substrate or cofactors for activity. Chalfie *et al.* (1994) first described the use of cloned GFP to monitor gene expression in both prokaryotes and eukaryotes. Vectors for use in Gram-negative bacteria other than *Escherichia coli* have now been constructed (Matthysse *et al.*, 1996). GFP has been used to investigate a range of diverse systems from host–pathogen interactions to activated sludge communities (Valdivia *et al.*, 1996; Eberl *et al.*, 1997; Leff & Leff, 1996; Niswender *et al.*, 1995; Albano *et al.*, 1996). More recently, Bloemberg *et al.* (1997) have reported the use of GFP plasmids to identify *Pseudomonas* species in a biofilm and on seedling roots.

In most natural and industrial environments, biofilms are complex communities consisting of more than one microbial species. A two-species system is simple enough to allow quantitative analysis and *in situ* speciation (Camper *et al.*, 1996; Stewart *et al.*, 1997; Banks & Bryers, 1991; Siebel & Characklis, 1991). Diversity in microbial communities leads to a variety of complex relationships involving interspecies and intraspecies interactions. The interactions between organisms within

Abbreviations: GFP, green fluorescent protein; PI, propidium iodide.

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plaque have been comprehensively studied and found to be both beneficial and antagonistic (Hasty *et al*., 1992; Marsh *et al*., 1997; Whittaker *et al*., 1996). McEldowney & Fletcher (1987) studied the sequential attachment of a number of bacteria and found that biofilm development depended on species composition, surface composition and the sequence of attachment. Brading *et al*. (1997) investigated the challenge of a monoculture biofilm of one organism with another. Although *Pseudomonas fluorescens* had advantages for initial colonization over *Pseudomonas putida*, neither species precluded the incorporation of the other species into the biofilms. Thus specific surface interactions between different species are thought to play a major role in controlling succession during the development of a microbial community. Specific, direct interactions may involve adhesins on the bacterial cell surface and receptors on the surface of other bacterial cells of the same or different species (James *et al*., 1995). Investigation of the specific adhesins on the bacterial cell surface, on which the inhibition or reversal of some coaggregations occurs with the addition of simple sugars, suggests lectin-like proteins can be involved (Kinder & Holt, 1994).

The species investigated in this study are all members of the *Enterobacteriaceae*; three were isolated from an industrial biofilm and identified as *Enterobacter agglomerans*, *Klebsiella pneumoniae* and *Serratia marcescens*. They show between 60 and 7 % genetic relatedness, with *Ent. agglomerans* and *K. pneumoniae* G1 the most closely related and *S. marcescens* 87b and *Ent. agglomerans* the most distant. The presence of enteric bacterial species in industrial environments may present considerable problems of hygiene and monitoring. This is accentuated when the bacteria are present in biofilms because of their increased resistance to disinfection. The aim of this work was to compare interactions between these species and to correlate them with increased or decreased biofilm formation, using plasmid-encoded GFP to label and subsequently identify *Ent. agglomerans*/GFP and *E. coli*/GFP.

METHODS

Bacterial strains. *Enterobacter agglomerans*, *Klebsiella pneumoniae* G1 and *Serratia marcescens* 87b were isolated from biofilms on industrial surfaces and typed using the API 20E identification system (bioMérieux). *E. coli* ATCC 11229 is a strain commonly used for industrial testing purposes. The organisms were cultured in yeast extract (YE) medium (Sutherland & Wilkinson, 1965). The strains had doubling times (ln2/μ) of: 90 min, *K. pneumoniae*; 75 min, *Ent. agglomerans*/GFP; 60 min, *S. marcescens*; and 55 min, *E. coli*/GFP. Non-fluorescent and fluorescent strains were coupled to allow subsequent differentiation using viable counts, microscopy and fluorimetry.

Transformation by electroporation. The strains *Ent. agglomerans* and *E. coli* ATCC 11229 were grown up in 100 ml YE to early exponential phase, transferred to a chilled tube and incubated with ice for 15 min. The cells were pelleted at 2700 ***g*** for 10 min at 4 °C, washed twice in 10 ml 1 mM HEPES, pH 7·0, and resuspended in 0·5 ml cold 10 % glycerol. Suspension (0·2 ml) was mixed with 1 µl pBAD DNA (Crameri *et al*., 1996) in TE buffer (1× TE is 10 mM Tris/HCl, 1 mM EDTA). This was electroporated in a cold 0·2 cm cuvette applying one pulse (2·5 kV, 25 µF, 200 Ω). The cells were resuspended in SOC (recovery) buffer (2 % Bacto-tryptone, 0·5 % Bacto yeast extract, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2·5 mM KCl, 20 mM glucose), incubated at 30 °C for 1 h and plated out onto agar containing ampicillin and 0·5 % arabinose. pBAD-GFP transformants could then be selected on the basis of their ampicillin resistance and GFP expression could be induced with 0·5 % arabinose and visualized using UV illumination. The resultant transformants will be referred to as *Ent. agglomerans*/GFP and *E. coli*/GFP and their growth rates were the same as the non-transformed strains.

Plasmid stability. YE broth (without arabinose or ampicillin; 100 ml) was inoculated with 1×10^5 *Ent. agglomerans*/GFP (or *E. coli*/GFP) cells and incubated, shaking at 30 °C for 24 h. One millilitre was then dispensed into fresh broth and incubated for a further 24 h. This was repeated for a total of 72 h. At each time interval, samples were removed, serially diluted and plated out. Loss of the plasmid could be calculated by counting the non-fluorescent colonies. To further monitor plasmid stability, a 1 l chemostat vessel containing 700 ml YE + 1 % glucose was inoculated with *Ent. agglomerans*/GFP. Different flow rates were used to assess plasmid loss at different doubling times. Samples were taken at steady state after three residence times ($T_R = 1$/dilution rate), diluted and plated out.

Properties of GFP. Sphaeroplasts (prepared as outlined by Osborn *et al*., 1972) were sonicated to assess the effect of cell lysis on fluorescence. The sphaeroplasts were progressively broken by sonication for 4 × 1 min bursts. The fluorescence of sonicated samples was measured on a Perkin Elmer LS 50B fluorimeter. A crude extract of GFP was prepared by centrifuging an overnight culture of *Ent. agglomerans*/GFP at 5000 ***g*** for 10 min. The pellet was resuspended in 10 ml PBS (Oxoid), 50 µl of a protease inhibitor (PMSF) was added and it was sonicated for 4 min. It was recentrifuged at 15 000 r.p.m. for 20 min to remove membranes and other cell debris, resulting in a crude protein preparation. The protein was then used to investigate the effect of pH and temperature on protein denaturation and fluorescence. Twenty microlitres of protein (1 mg ml^{-1}) or whole cells was mixed with 80 µl PBS at the appropriate pH and fluorescence was determined.

Biofilm growth and invasion studies. Biofilms were grown on borosilicate glass beads; 25 g 4-mm-diameter beads was placed in a 250 ml flask with 100 ml YE and 1 % glucose. Flasks were inoculated in triplicate to obtain approximately 10^5 bacteria ml^{-1}, consisting of one species or a 1 : 1 mixture of the two species. The flasks were incubated at 30 °C with shaking at 80 r.p.m. Six beads were removed from the three flasks, and each bead was rinsed and transferred to an Eppendorf tube containing 1 ml PBS, vortexed for 30 s to remove the biofilm cells, diluted and plated out. Fluorescent colonies (*Ent. agglomerans*/GFP and *E. coli*/GFP) were identified in a mixture by UV illumination of the plates. For microscopy, glass coverslips were used for biofilm growth in a batch culture system consisting of a box containing YE broth (250 ml) and 1 % glucose. The coverslips were supported by a metal holder. A small magnetic stirring bar was also incorporated in the system and a magnetic stirrer set at 100 r.p.m. provided thorough mixing of available nutrients. Each system was inoculated with overnight bacterial cultures to obtain approximately 10^5 bacteria ml^{-1}. The invasion of

non-fluorescent biofilm monolayers by a second fluorescent partner was investigated using fluorimetry. Aliquots (100 µl) of *K. pneumoniae* or *S. marcescens* overnight cultures were added to each well of a microtitre plate and incubated at 30 °C for 16 h. The liquid phase was removed and 100 µl overnight cultures of *E. coli*/GFP or *Ent. agglomerans*/GFP were added and incubated at 30 °C. At 1, 2, 4 and 24 h, the excess liquid was removed, the wells were washed once with PBS and 100 µl PBS was added. The fluorescence of the biofilms in the wells was measured on a Perkin Elmer LS 50B fluorimeter. Adhesion to plastic surfaces was lower than adhesion to glass for all strains. This did not affect enhanced dual-species biofilm formation by *Ent. agglomerans*/GFP and *K. pneumoniae*.

Microscopy. Sixteen hour dual-species biofilms on glass coverslips were immersed in a detergent (10 mM cetylpyridinium chloride) for 5 min, then immersed in propidium iodide (PI; 25 µg ml^{-1}, Sigma). Pretreatment with the detergent caused cell membrane damage and access of PI to the cell interior. GFP was not masked by the presence of PI in the cell, therefore *Ent. agglomerans*/GFP cells appeared green (or yellow) and *K. pneumoniae* cells appeared red under UV illumination. The biofilms were observed on a Polyvar microscope with a tungsten bulb attachment (Reichert-Jung) with a violet-blue excitation filter (395–446 nm). This method gave no indication of cell viability but was effective for examining spatial distribution.

Production of inhibitors. Stationary phase liquid cultures of *E. coli*/GFP and *S. marcescens* were centrifuged at 10 000 ***g*** for 10 min and the supernatant was filter-sterilized. Aliquots (50 µl) of the *S. marcescens* or *E. coli*/GFP supernatants were added to 50 µl YE in each well of a microtitre plate. *K. pneumoniae* or *Ent. agglomerans*/GFP stationary phase liquid culture supernatant (50 µl) was used for controls. Stationary phase *K. pneumoniae* (OD_{570} 0·25; 25 µl) was added to *E. coli*/GFP supernatant and 25 µl *Ent. agglomerans*/GFP was added to *S. marcescens* supernatant. The increase in turbidity was measured after 24 h incubation at 30 °C on a Beckman MR 5000 plate reader. Inhibition of growth indicated the presence of a growth inhibitor in the supernatant.

Adhesion. *Ent. agglomerans*/GFP and *K. pneumoniae* co-operated, forming biofilms more successfully when together than apart, indicating specific interactions. The interactions were investigated by measuring adhesion and its inhibition, using fluorescence (*Ent. agglomerans*/GFP) and turbidity (*K. pneumoniae*). *Ent. agglomerans*/GFP cells were treated with a protease [bromelain; 103 units (µg protein)$^{-1}$ h^{-1}] and a polysaccharide-degrading enzyme obtained from an *Enterobacter*-specific bacteriophage to inhibit the adhesion. Phage-free enzyme was prepared by filtration using a 100 kDa filter (Vivaspin; Vivascience). The preparation had a specific activity of $3·78 \times 10^{-3}$ µmol glucose h^{-1} (mg protein)$^{-1}$. EDTA was used to determine the effect on adhesion of ion sequestration. *Ent. agglomerans*/GFP overnight culture (10 ml) was added to 10 ml sterile distilled H_2O (control), 10 ml 2 mg EDTA ml^{-1} or 200 µg EDTA ml^{-1}. One hundred microlitres of each was added to each *K. pneumoniae* monolayer and allowed to attach for 5, 15, 30, 45, 60, 90 and 120 min. The liquid phase was removed and the wells were rinsed once with PBS. The fluorescence of adhering *Ent. agglomerans*/GFP was then measured on a Perkin Elmer LS 50B fluorimeter.

In addition to the ability to measure *Ent. agglomerans*/GFP adhesion using fluorescence measurements, a method has been developed to enable the adhesion of *K. pneumoniae* (a non-fluorescent strain) to be quantified. The technique relies on the ability of *K. pneumoniae* to re-grow into the nutrient-rich liquid phase above the biofilm. To prevent re-growth of the base species (*Ent. agglomerans*/GFP), it was killed using UV irradiation for 120 min prior to *K. pneumoniae* addition. Aliquots (100 µl) of washed stationary phase *Ent. agglomerans*/GFP cells were added to each well of a transparent microtitre plate and incubated at 30 °C for 24 h. The *Ent. agglomerans*/GFP monolayers were killed by exposing to UV for 120 min. *K. pneumoniae* cells (100 µl) were added for the required time. The wells were then rinsed once with PBS, which was replaced with 100 µl YE (+ glucose). The re-growth of *K. pneumoniae* into the liquid phase was proportional to the number of attached cells. The adhesion of *K. pneumoniae* could thus be measured using a Dynatech MR 5000 plate reader to quantify the increase in turbidity. To inhibit *K. pneumoniae* adhesion to *Ent. agglomerans*/GFP, 10 ml stationary phase *K. pneumoniae* cells was treated with bromelain and various commercial polysaccharase mixtures. In addition, 0·5 % mannose was added during a 30 min attachment period of *K. pneumoniae* to *Ent. agglomerans*/GFP monolayers. Re-growth after 20 h was measured on a Dynatech 5000 plate reader.

RESULTS AND DISCUSSION

Use of GFP as a species-specific marker

Usually the plasmid encoding GFP can be selected for by including a gene encoding antibiotic resistance. In this system, plasmid maintenance in the absence of ampicillin selection is an important factor because when mixed cultures are used, the antibiotic may adversely affect the growth of the second species. After 72 h in batch culture, 0·5 % of *Ent. agglomerans*/GFP cells had lost their plasmid. The plasmid loss in *E. coli*/GFP was higher: after 72 h 14 % of cells had lost the plasmid. In continuous culture, plasmid loss increased with increasing specific growth rate (Table 1). The length of biofilm experiments was therefore kept to a minimum, i.e. below 24 h with a maximum plasmid loss of 4·6 % (*E. coli*/GFP) and 0·2 % (*Ent. agglomerans*/GFP). Sonication of *Ent. agglomerans*/GFP sphaeroplasts leading

Table 1. Loss of the GFP plasmid from *Ent. agglomerans*/GFP in chemostat culture in the absence of ampicillin selection

Samples were plated out onto YE agar containing arabinose (0·5 %) and UV illumination of the plates enabled non-fluorescing colonies to be identified. SE values are presented where $n = 4$.

Doubling time (h)	Plasmid loss (%)	SE
0·5	28·6	1·90
2·5	21·8	1·50
4·5	16·2	2·10
6·5	12·2	1·80
8·5	4·3	0·50
10·5	0·5	0·05

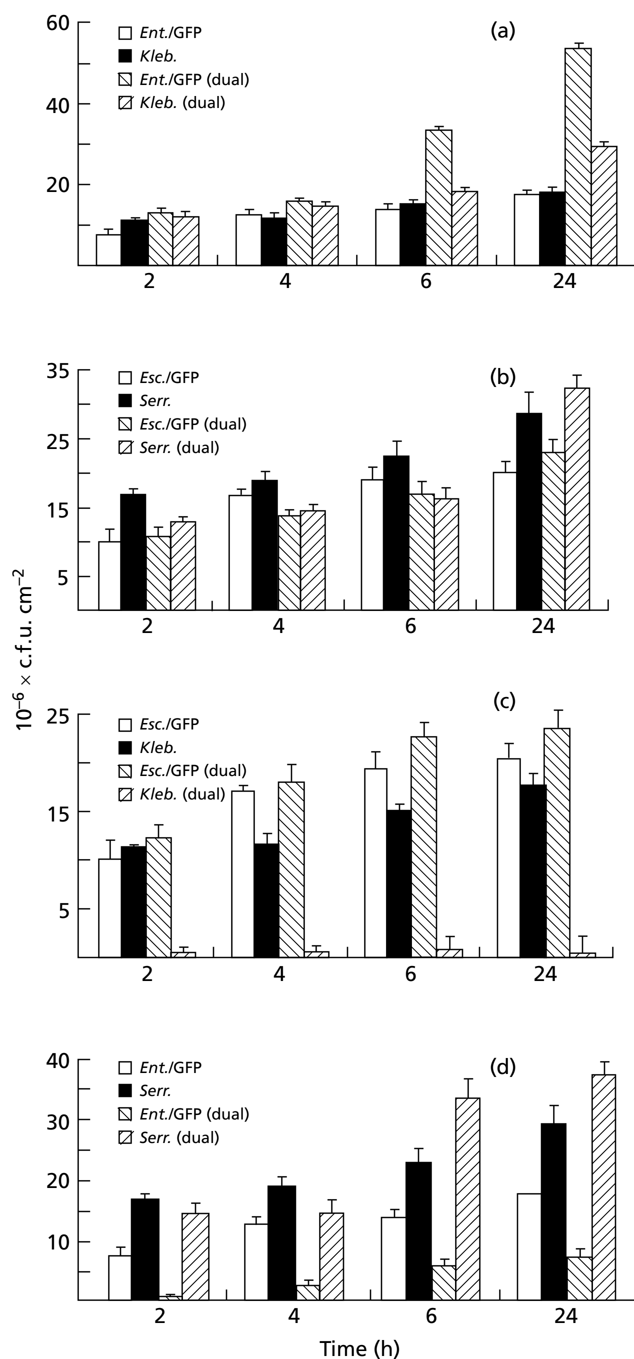

Fig. 1. Comparison of single-species and dual-species biofilm development on glass beads using viable counts. Two strains, *E. coli*/GFP and *Ent. agglomerans*/GFP, were transformed with a plasmid encoding GFP. Colonies in a mixture could then be identified using UV illumination of plates. Results are presented as surface coverage (cm^{-2}). (a) *Ent. agglomerans*/GFP + *K. pneumoniae*; (b) *E. coli*/GFP + *S. marcescens*; (c) *E. coli*/GFP + *K. pneumoniae*; (d) *Ent. agglomerans*/GFP + *S. marcescens*. Bars represent SE, where $n = 6$.

to progressive cell lysis did not result in a large decrease in fluorescence. A larger decrease in fluorescence was observed between 10 and 30 min after sonication, possibly due to cellular enzyme degradation. This has important implications because fluorescence can only be related to the presence of GFP, not intact cells. In addition, dead cells will still fluoresce if they are killed after synthesizing GFP.

A crude extract of the protein was isolated from *Ent. agglomerans*/GFP and compared with intact cells to determine the stability of the protein. GFP was more sensitive to low pH than to high pH, with maximum fluorescence occurring at pH 8–10. The effect of pH was muted in whole cells. In addition, the protein was thermostable up to 60 °C. Because the GFP is so stable and persists after cell death, fluorescence per se cannot be used to indicate viability. However, fluorescence can be used to assess adhesion of *Ent. agglomerans*/GFP cells and microscopic localization is possible. GFP is a cytoplasmic protein with low toxicity and should therefore have a minimal influence on bacterial cell surface dynamics. The interactions between bacterial species should not be adversely affected.

Dual-species biofilm formation

Single- and dual-species biofilm formation between the four enterobacterial species was monitored by viable counts over 24 h (Fig. 1). A total inoculum of 1×10^5 cells ml^{-1} of one species or a 1:1 mixture of the two organisms was used. Viable cell numbers in single-species biofilms were greatest for *S. marcescens*, followed by *E. coli*/GFP, *K. pneumoniae* and *Ent. agglomerans*/GFP. The biofilm growth of the four dual-species biofilms, comprising one fluorescent and one non-fluorescent partner, differed according to the species involved. In dual-species biofilms of *K. pneumoniae* and *Ent. agglomerans*/GFP, both showed increased adherence and growth when compared with single-species biofilms: *Ent. agglomerans*/GFP showed a 54 % increase and *K. pneumoniae* a 23 % increase in 24 h dual-species biofilms. The co-operation could be the result of specific adhesion mechanisms increasing their attachment. This correlates with another study on dual-species biofilms containing *Salmonella enteritidis* and *K. pneumoniae*, which also showed synergistic biofilm formation, with increased attachment and metabolic activity (Jones & Bradshaw, 1997). *E. coli*/GFP and *S. marcescens* did not affect the attachment or growth of each other: cell numbers were the same whether or not the other partner was present. They did not compete and this could indicate that they have separate binding sites or mechanisms of attachment. Stewart *et al.* (1997) have also reported a system where *K. pneumoniae* and *Pseudomonas aeruginosa* stably coexisted in biofilms despite differing growth rates under planktonic conditions. Both *Ent. agglomerans*/GFP + *S. marcescens* and *E. coli*/GFP + *K. pneumoniae* showed competitive interactions. *S. marcescens* out-competed *Ent. agglomerans*/GFP, with *Ent. agglomerans*/GFP comprising a maximum 14·7 % of the biofilm. *E. coli*/GFP out-competed *K. pneumoniae*, with *K. pneumoniae* comprising between 0·6 and 3·1 % of the biofilm.

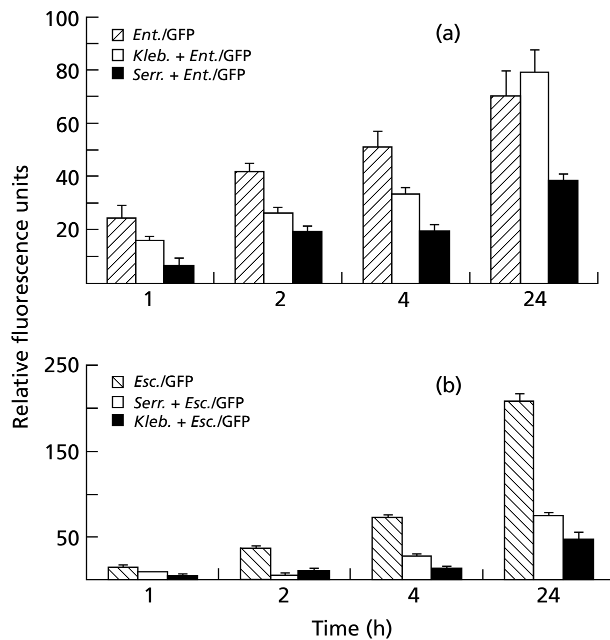

Fig. 2. Invasion of bacterial monolayers of non-fluorescent strains (*K. pneumoniae* and *S. marcescens*) by fluorescent strains labelled with a plasmid encoding GFP. The incorporation of *E. coli*/GFP and *Ent. agglomerans*/GFP into the monolayers was measured, after various contact times, on a Perkin Elmer LS 50B fluorimeter. (a) *Ent. agglomerans*/GFP invasion into *K. pneumoniae* and *S. marcescens* monolayers; *Ent. agglomerans*/GFP adhesion to blank wells was included as control. (b) *E. coli*/GFP invasion into *K. pneumoniae* and *S. marcescens* monolayers; *E. coli*/GFP adhesion to blank wells was included as control. Bars represent SE, where $n = 84$.

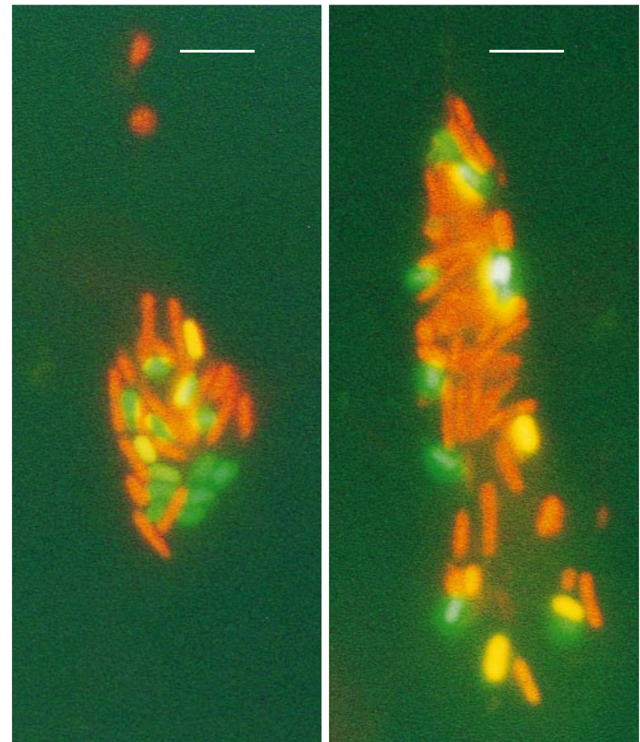

Fig. 3. Microcolonies of a 16 h co-operative dual-species biofilm of *Ent. agglomerans*/GFP and *K. pneumoniae*. Biofilms were grown on glass coverslips, and treated with 10 mM cetylpyridinium chloride (5 min) and 25 µg PI ml^{-1} (5 min) before visualizing on a Polyvar microscope with a tungsten bulb attachment and violet-blue excitation filter (395–446 nm). *Ent. agglomerans*/GFP cells appeared green (or yellow) and *K. pneumoniae* cells appeared red under UV illumination. Bars, 3 µm.

Invasion and interactions

Incorporation of the fluorescent strains into pre-existing biofilms was quantified after various time intervals (Fig. 2). *E. coli*/GFP was better at binding to blank wells than *Ent. agglomerans*/GFP. *Ent. agglomerans*/GFP successfully invaded the *K. pneumoniae* biofilm, with numbers almost as high (or higher at 24 h) as for uncolonized surfaces. *Ent. agglomerans*/GFP was incorporated at lower levels into *S. marcescens* monolayers, representing on average 19% of the cells bound to blank wells. *E. coli*/GFP did not invade biofilms of *S. marcescens* or *K. pneumoniae* very successfully, representing on average 35 and 21% of cells bound to blank wells, respectively. This demonstrated that invasion was not dependent on biofilm-forming ability; for example, when inoculated simultaneously, *E. coli*/GFP out-competed *K. pneumoniae* and *S. marcescens* out-competed *Ent. agglomerans*/GFP. The resultant biofilms were dominated by the best competitor of the pair. When a monolayer of *K. pneumoniae* was already present, the competitive partner (*E. coli*/GFP) showed poor integration into the biofilm. In contrast, where co-operative associations between *Ent. agglomerans*/GFP and *K. pneumoniae* occurred, bacteria could more easily invade a monolayer of their partner. This suggests that co-operation is beneficial in terms of invasion. If a bacterial species possesses the ability to adhere specifically to another species it could gain a foothold in a new environment, enhancing its survival. If the co-operation is beneficial, it could in turn help its partner.

GFP is extremely stable and persists up to 60 °C and within the pH range 2–12 and also through treatment with formaldehyde, allowing the detection of GFP even in fixed samples (Eberl *et al.*, 1997). This allowed the visualization of *Ent. agglomerans*/GFP (or *E. coli*/GFP) cells in dual-species biofilms, even following the detergent treatment necessary to label the second species, *K. pneumoniae* (or *S. marcescens*), with PI. However, this also prevented the use of GFP as a marker of viability. Microscopic examination revealed that *Ent. agglomerans*/GFP and *K. pneumoniae* are often closely associated in microcolonies (Fig. 3). The close proximity suggests that surface-associated macromolecules form the basis of the interactions. This may involve adhesins and receptors on the surface of one or both species. In contrast, *E. coli*/GFP and *S. marcescens* mainly formed discrete microcolonies. As *S. marcescens* + *E. coli*/GFP formed biofilms in equal numbers, irrespective of

Table 2. *Inhibition of the adhesion of Ent. agglomerans/GFP cells to monolayers of K. pneumoniae, and K. pneumoniae cells to Ent. agglomerans/GFP monolayers in microtitre plates*

Percentage reduction of *Ent. agglomerans*/GFP adhesion was calculated after measuring the fluorescence of attached cells. Percentage reduction of *K. pneumoniae* adhesion was calculated after turbidometric measurement of the re-growth of adhering cells. SE values are presented where $n = 84$.

Inhibitor or treatment	Reduction in adhesion (%)	SE
Ent. agglomerans*/GFP adhesion to *K. pneumoniae		
Mannose	0·0	–
Protease (bromelain)	46·3	8·0
Specific phage depolymerase	38·6	4·1
EDTA	17·5	3·3
***K. pneumoniae* adhesion to *Ent. agglomerans*/GFP**		
Mannose	5·0	0·6
Protease (bromelain)	0·0	–
Polysaccharase mixtures:		
Cellulase TV	22·3	1·1
Driselase	10·1	1·2
β-Glucuronidase	0·0	–
Mutanolysin	0·0	–
Hemicellulase	2·1	3·2
Cellulase	2·6	2·1

whether the other was present, they interacted 'neutrally' and therefore were not investigated further.

If one species can initially out-compete another species, then it could exploit that particular microenvironment. The production of inhibitors by *S. marcescens* and *E. coli*/GFP, the competitive partners, has also been investigated.

To determine whether the competitive partners (*E. coli*/GFP and *S. marcescens*) produced bacteriocins, the supernatants were tested for growth inhibition of *K. pneumoniae* and *Ent. agglomerans*/GFP, respectively. There was no evidence that either species produced any growth inhibitors as growth remained unchanged and cross-streaking on agar plates had no effect. Their dominance in dual-species biofilms was probably due to their higher growth rates. As *K. pneumoniae* and *Ent. agglomerans*/GFP formed biofilms co-operatively, their specific interactions were investigated in more detail (Table 2). Protease treatment caused a 46 % reduction in *Ent. agglomerans*/GFP adhesion to *K. pneumoniae*. Degrading the extracellular polysaccharide with a specific phage depolymerase also caused a 39 % reduction in adhesion. The fluorescence measurements used to follow the adhesion of *Ent. agglomerans*/GFP to *K. pneumoniae* imply that the interactions are both protein- and polysaccharide-dependent. The extracellular polysaccharides may act as receptors to the adhesins, not only coating the bacterial outer surface but also adsorbing to inert surfaces. This may influence intercellular contacts and accumulation at surfaces. Alternatively, production of extracellular polysaccharide or other cell products by one species may coat the surface and indirectly affect the adhesion of another species. Quantification of *K. pneumoniae* adhesion has also permitted the evaluation of treatments to prevent the adhesion of *K. pneumoniae* to *Ent. agglomerans*/GFP. Addition of mannose caused a small reduction (4·5 %, SE 0·2) in adhesion, which may indicate specific adhesin–carbohydrate interactions, although other components must also be important in the interactions between these two species. Bromelain (protease) treatment caused no significant reduction in adhesion. This contrasts with *Ent. agglomerans*/GFP adhesion, where protease treatment caused a 50 % reduction in adhesion. This may suggest that only *Ent. agglomerans*/GFP possesses protein adhesins; alternatively, the protein components in *K. pneumoniae* may be protected from degradation. The polysaccharase mixtures marketed as cellulase TV (Sigma) and driselase (Sigma) also resulted in a decrease in *K. pneumoniae* adhesion. This suggests that more than one cell component is responsible for the interactions between *K. pneumoniae* and *Ent. agglomerans*/GFP, but both appear to contribute to the beneficial partnership.

Concluding remarks

Plasmid-borne GFP was used to investigate dual-species biofilm development. *E. coli*/GFP and *S. marcescens* stably co-existed in biofilms but did not benefit or antagonize each other. The other pairs, *E. coli*/GFP + *K. pneumoniae* and *Ent. agglomerans*/GFP + *S. marcescens*, competed for the surface. The ability of *E. coli*/GFP and *S. marcescens* to out-compete *K. pneumoniae* and *Ent. agglomerans*/GFP, respectively, may be a result of their higher growth rates. *K. pneumoniae* and *Ent. agglomerans*/GFP co-operated, resulting in enhanced biofilm formation. Both species were involved in the interactions, which may involve adhesin–receptor interactions as they were partially protein and partially carbohydrate mediated. The methods described provide a convenient technique for the examination of mixed-species biofilm communities where the unique interactions between species within biofilms determine the true properties of the resultant biofilms.

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