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Binary culture biofilm formation by *Stenotrophomonas maltophilia* and *Fusarium oxysporum*

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Binary culture biofilm formation by *Stenotrophomonas maltophilia* and *Fusarium oxysporum* was investigated using the recirculating modified Robbins device batch culture system. Sequential attachment studies were carried out in the Robbins device on PVC and glass surfaces, with each species as either the first or the second colonizer. Different surfaces had no significant effect on total numbers of *S. maltophilia* and *F. oxysporum* in the binary population biofilm. The attachment of the second colonizer was not influenced significantly by the previous attachment of the first colonizer. These results were confirmed using scanning electron micrographs. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 178–183.

Keywords: binary biofilms; Robbins device; *Stenotrophomonas maltophilia*; *Fusarium oxysporum*; bacterial and fungal adhesion

Introduction

Mixed species biofilms found in most natural and industrial environments are inherently complex and may contain a large number of species [7]. Most studies have been undertaken using pure cultures; however, to understand the interactions between species in biofilms, research should also concentrate on mixed species consortia. James *et al* [13] indicated that interspecies microbial interactions influence the biofilm during the initial stages of formation, bacterial attachment and surface colonization and continue to influence the structure and physiology of the biofilm as it develops. Adhesion is also affected by the properties of the substratum and numerous studies have shown bacterial attachment to different surfaces [1,3,9]. Dental plaque is a mixed community biofilm where ecology, physiology and structure have been investigated extensively [11,16,20]. In such a system coaggregation is thought to be involved in interspecies interactions [6,15].

Simpler interacting systems of two-membered or binary populations have been used to study species interactions. McEldowney and Fletcher [19] showed through attachment between pairs of bacterial species on different surfaces that the attachment of each species was either increased, decreased or not affected by simultaneous or sequential attachment of another species. Results depended upon the species combination, the surface composition and the sequence of attachment. Enhanced attachment and microcolony formation of *Listeria monocytogenes* was shown when the organism was grown with *Pseudomonas fragi* in a continuous flow slide chamber [27], whereas the adhesion of *Salmonella typhimurium* was significantly hindered by *Pseudomonas fluorescens* [18]. Distinct differences in biofilm structure were shown between *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* as single and binary culture biofilms [22,29].

A few studies have investigated the interactions between bacteria and yeast species [4,21], but there have been no reports on interactions between filamentous fungi and bacteria during adhesion to a surface.

This paper investigates the influence between two species, the bacterium *Stenotrophomonas maltophilia* and the fungus *Fusarium oxysporum*, on attachment to PVC and glass within a modified Robbins device (MRD) [17]. These species were selected from a previously studied seven-species model community isolated from industrial biofilms [10]. They were chosen: (i) for their predominance in these biofilms, (ii) a binary system of two very distinct species was amenable to quantitative analysis, and (iii) there has been no report of an interacting prokaryote/eukaryote system.

Material and methods

Organisms and culture conditions

S. maltophilia and *F. oxysporum* were isolated from biofilms formed within the wash tanks of industrial photoprocessors and were part of the seven-species model community selected [10]. Batch cultures of both species were grown in 100 ml R2A medium [25] in 250-ml shake-flasks for 18 h at 30°C for the inoculum. The species were enumerated by viable counts on nutrient agar (NA; Oxoid, Basingstoke, Hampshire, UK) with tetracycline (Sigma, Sigma-Aldrich Chemical, Poole, Dorset, UK; 50 μg ml^{-1}) for the fungus. Tetracycline was added to prevent growth of the bacterium allowing enumeration of the fungus. The cultures were stored and maintained as described [10].

The MRD and experimental design

MRDs were prepared with PVC (0.1 cm thick, 1 cm diameter) and glass (0.12 cm thick, 1 cm diameter) surfaces with backing discs of

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Table 1 Variables for each sequential attachment experiment within the MRD showing attachment surface, colonization sequence and corresponding figure for the results

Experiment	Attachment surface	Colonization sequence	Results in figure
i	PVC	*S. maltophilia* then *F. oxysporum*	Figure 1a
ii	Glass	*S. maltophilia* then *F. oxysporum*	Figure 1b
iii	PVC	*F. oxysporum* then *S. maltophilia*	Figure 2a
iv	Glass	*F. oxysporum* then *S. maltophilia*	Figure 2b

soft black rubber (0.16 cm thick, Esco Rubber, Bibby Sterilin, Aldershot, UK) fitted into removable studs and sterilized with ethylene oxide [10,17,26]. The glass surfaces were cut from microscope slides (BDH, Laboratory Supplies, Dorset, UK). Binary culture biofilm formation was established on these surfaces in a recirculating MRD batch culture system. Two flasks (A and B) each containing 1500 ml half-strength R2A medium for inoculation were placed on magnetic stirrers to maintain homogeneity and aeration of the culture. Flask A was attached to the MRD and the medium was pumped continuously around the system at a flow rate of 50 ml h^{-1} [Reynolds numbers (Re)=2.72]. Flask B remained as free-standing batch culture.

Attachment of S. maltophilia to PVC or glass already colonized by F. oxysporum

Flask A was inoculated with 30 ml *F. oxysporum* allowing 12 h colonization of the surfaces within the MRD at ambient temperature (22°C). At the same time, flask B was inoculated with 30 ml *S. maltophilia*, which was grown as a batch culture for 12 h at 22°C. After 12 h the MRD was sampled for viable counts and then the flasks were exchanged to allow colonization of *S. maltophilia* onto the *F. oxysporum* biofilm. This will be referred to as "sequential attachment." Surfaces were sampled at regular intervals for 48 h for viable counts on NA and 2% MEA with tetracycline and for scanning electron microscopy (SEM). This was repeated in duplicate on PVC and glass surfaces.

Attachment of F. oxysporum to PVC or glass already colonized by S. maltophilia

The procedure was same as above except the order in which the species colonized the Robbins device was reversed. Flask A was inoculated with 30 ml *S. maltophilia* allowing 12-h colonization of the surfaces within the MRD. Flask B was inoculated with 30 ml *F. oxysporum*, which was grown as a batch culture for 12 h. This was repeated in duplicate on PVC and glass surfaces. The variables for all the experiments are summarized in Table 1.

Scanning electron microscopy

Surfaces were removed and prepared for SEM following the procedures described previously with the secondary fixation in 1% osmium tetroxide [10,14]. All samples were mounted and sputter coated with gold–palladium for viewing on a Stereoscan 100 scanning microscope (Leica, Cambridge, UK) at 25-kV acceleration voltage.

Statistical analysis

The data were analysed by analysis of variance (ANOVA). The ANOVAs were conducted in MINITAB (release 10 for Windows) using a restricted model with treatment and time as fixed effects and replicate as a random effect nested within treatment. Differences were considered significant at P value ≤ 0.05. Experiments were conducted in duplicate and means, standard deviations and standard error of the mean were calculated for all results where appropriate.

Results

Effect of attachment surface

Attachment of F. oxysporum to PVC or glass already colonized by S. maltophilia: The influence of attachment surface on binary culture biofilm formation, where *S. maltophilia* is the first colonizer and *F. oxysporum* the second colonizer is shown for PVC (Figure 1a) and glass (Figure 1b). Numbers of *S. maltophilia* in the 12-h biofilm (time=0) on PVC and glass were 3.2×10^{7} cfu cm^{-2}. Over 48 h numbers of bacteria in the biofilm showed small changes but generally remained at this level. Numbers of *F. oxysporum* after 2-h colonization onto the *S. maltophilia* biofilm were 1.3×10^{3} cfu cm^{-2} on PVC and 4.0×10^{2} cfu cm^{-2} on glass. There was a gradual increase in numbers over 48 h to 3.6×10^{5} and 6.0×10^{4} cfu cm^{-2} on PVC and glass, respectively. Statistical analysis using a restricted model ANOVA showed no significant difference between numbers of *S. maltophilia* and *F. oxysporum* in the binary biofilm on PVC and glass (P=0.767 and P=0.277, respec-

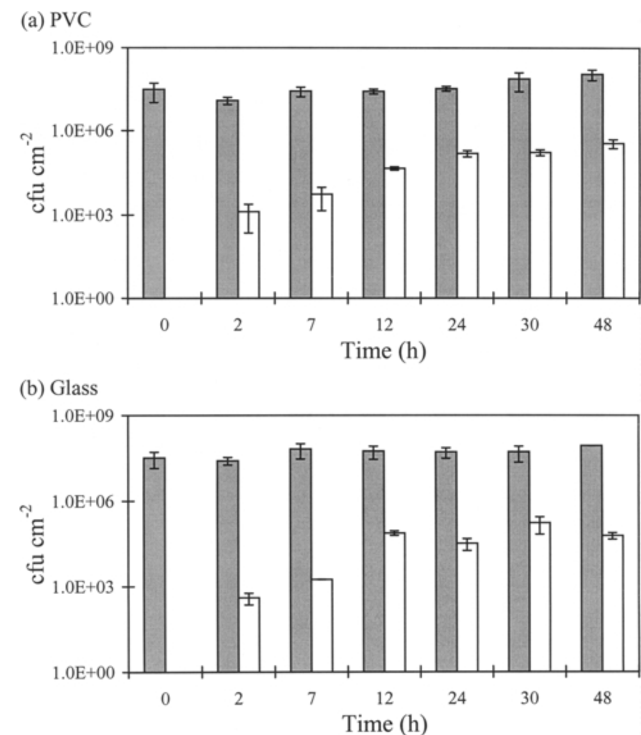

Figure 1 Binary culture biofilm formation by *S. maltophilia* (■) as the first colonizer followed by attachment of *F. oxysporum* (□) as the second colonizer to PVC (a) and glass (b) at 22°C. At $t=0$, bar shows numbers (cfu cm^{-2}) of *S. maltophilia* in a 12-h biofilm, and time in hours shows attachment of the second colonizer. Bars = 1 SE. $n=2$.

tively). Surface type did not affect numbers of either bacteria or fungus in the binary population biofilms when *F. oxysporum* was allowed to attach to a surface already colonized by *S. maltophilia*.

Attachment of S. maltophilia to PVC or glass already colonized by F. oxysporum: The influence of attachment surface on binary culture biofilm formation, where *F. oxysporum* is the first colonizer and *S. maltophilia* the second colonizer is shown for PVC (Figure 2a) and glass (Figure 2b). Numbers of *F. oxysporum* in the 12-h biofilm (time=0) on PVC and glass were 1.5×10^3 and 5.3×10^2 cfu cm^{-2}, respectively. Over 48 h numbers of fungi in the biofilm on both PVC and glass showed a gradual increase to 10^5 cfu cm^{-2}. Numbers of *S. maltophilia* after 2-h colonization onto the *F. oxysporum* biofilm were 2.8×10^7 cfu cm^{-2} on PVC and 3.6×10^6 cfu cm^{-2} on glass. There was a small increase in numbers over 48 h to 10^8 cfu cm^{-2} on both PVC and glass. Statistical analysis using a restricted model ANOVA showed no significant difference between numbers of *F. oxysporum* in the binary biofilm on PVC and glass (P=0.947). There was a significant difference in numbers of *S. maltophilia* in the binary biofilm (P=0.035), however, it is proposed that this difference was negligible and was a result of greater fluctuation in numbers of *S. maltophilia* in the biofilm at 12 h. Surface type did not affect numbers of either bacteria or fungus in the binary population biofilms when *S. maltophilia* was allowed to attach to a surface already colonized by *F. oxysporum*.

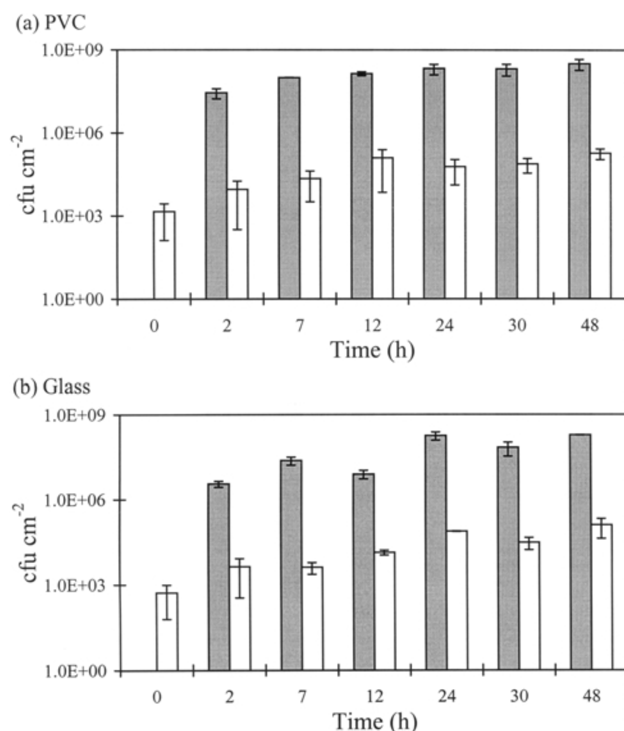

Figure 2 Binary culture biofilm formation by *F. oxysporum* (□) as the first colonizer followed by attachment of *S. maltophilia* (■) as the second colonizer to PVC (a) and glass (b) at 22°C. At t = 0, the bar shows numbers (cfu cm^{-2}) of *F. oxysporum* in a 12-h biofilm, and time in hours shows attachment of the second colonizer. Bars = 1 SE. n = 2.

Influence of colonization sequence

Effect of sequential attachment on adhesion of S. maltophilia and F. oxysporum to PVC with each species as the first and second colonizer: The attachment of *S. maltophilia* to uncolonized PVC followed by attachment of *F. oxysporum* was compared to attachment of *S. maltophilia* to PVC already colonized by *F. oxysporum*. Thus the colonization sequence was reversed and experiments (i) and (iii) were compared (Table 1; Figures 1a and 2a). Numbers of *S. maltophilia* as the first colonizer in the 12-h biofilm on PVC were 3.2×10^7 cfu cm^{-2} (Figure 1a). As the second colonizer, numbers of *S. maltophilia* after 2 h attaching to the *F. oxysporum* biofilm were 2.8×10^7 cfu cm^{-2} (Figure 2a). Viable counts of *S. maltophilia* after 48 h were 10^8 cfu cm^{-2} irrespective of the initial colonization order. Statistical analysis showed no significant difference (P=0.755) between numbers of *S. maltophilia* in the binary biofilm as first or second colonizer to PVC.

Numbers of *F. oxysporum* as the first colonizer in the 12-h biofilm on PVC were 1.5×10^3 cfu cm^{-2} (Figure 2a). As the second colonizer, numbers of *F. oxysporum* after 2 h attaching to the *S. maltophilia* biofilm were 1.3×10^3 cfu cm^{-2} (Figure 1a). Viable counts of *F. oxysporum* after 48 h were 10^5 cfu cm^{-2} irrespective of the initial colonization order. Statistical analysis showed no significant difference (P=0.432) between numbers of *F. oxysporum* in the binary biofilm as first or second colonizer to PVC.

Effect of sequential attachment of S. maltophilia and F. oxysporum to glass with each species as the first and second colonizer: The attachment of *S. maltophilia* to uncolonized glass followed by attachment of *F. oxysporum* was compared to attachment of *S. maltophilia* to glass already colonized by *F. oxysporum*. Thus the localization sequence was reversed and experiments (ii) and (iv) were compared (Table 1; Figures 1b and 2b). Numbers of *S. maltophilia* as the first colonizer in the 12-h biofilm on glass were 3.2×10^7 cfu cm^{-2} (Figure 1b). As the second colonizer, numbers of *S. maltophilia* were approximately 1 log lower after 2-h attachment to the *F. oxysporum* biofilm (Figure 2b). Viable counts of *S. maltophilia* after 48 h were 8.5×10^7 cfu cm^{-2} as the first colonizer and 1.9×10^8 cfu cm^{-2} as the second colonizer, approximately 0.4 log difference. Statistical analysis showed a significant difference (P=0.001) between numbers of *S. maltophia* in the binary biofilm as first or second colonizer to glass.

Numbers of *F. oxysporum* as the first colonizer in the 12-h biofilm on glass were 5.3×10^2 cfu cm^{-2} (Figure 2b). As the second colonizer numbers of *F. oxysporum* after 2 h attaching to the *S. maltophilia* biofilm were 4.0×10^2 cfu cm^{-2} (Figure 2a). Viable counts of *F. oxysporum* after 48 h were approximately 10^5 cfu cm^{-2} irrespective of the initial colonization order. Statistical analysis showed no significant difference (P=0.120) between numbers of *F. oxysporum* in the binary biofilm as first or second colonizer to glass.

Scanning electron microscopy

Scanning electron micrographs show the structural heterogeneity of the binary culture biofilm of *S. maltophilia* and *F. oxysporum* to PVC (Figure 3) and glass (Figure 4). There were no visual differences between biofilms formed by the fungus or bacteria as the first or second colonizers. The biofilms were composed of a nonuniform distribution of attached conidia and hyphae surrounded

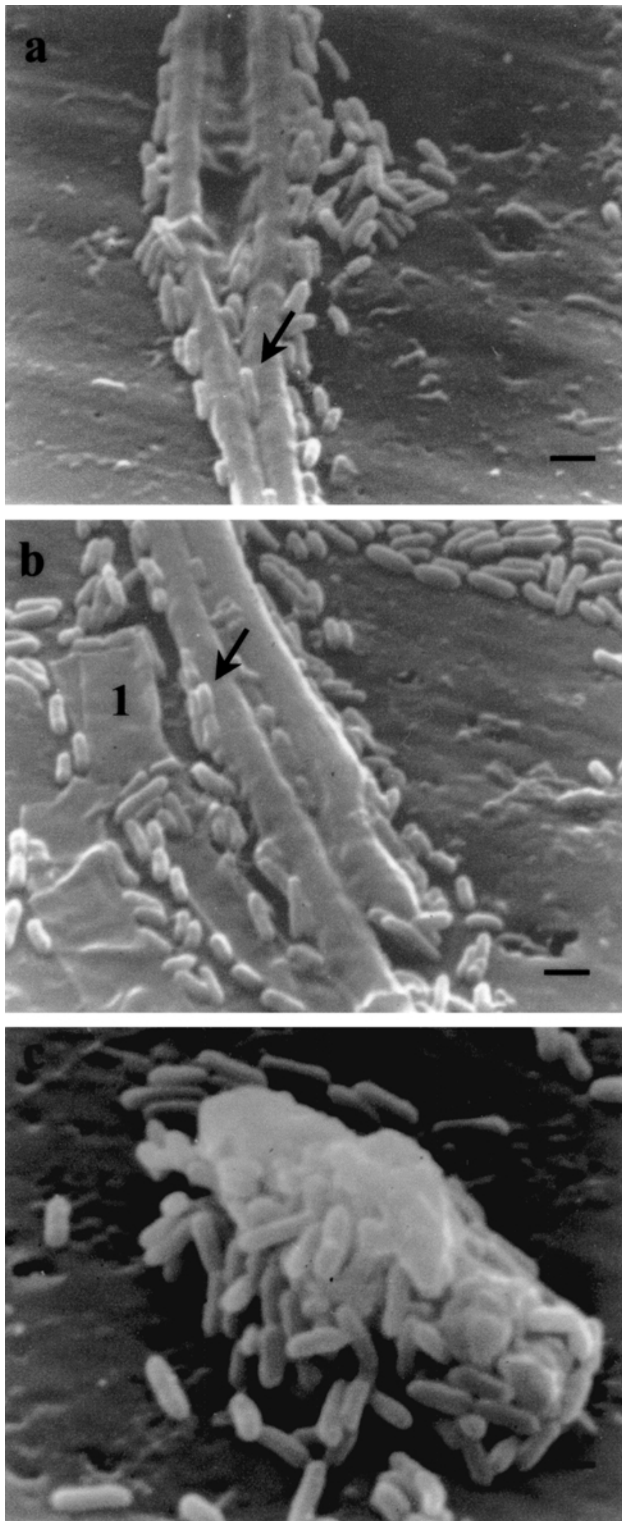

Figure 3 Scanning electron micrographs of binary culture formation by *S. maltophilia* and *F. oxysporum* on PVC within the Robbins device after 48 h. (a and b) Fungal hyphae surrounded by bacteria. Some of the bacteria are attached to the hyphae and others are lying between two hyphal branches (indicated by narrows). "1" indicates hyphae that have collapsed during SEM preparation, bar = 2 μm and (c) a single conidium almost covered by bacterial cells, bar = 1.5 μm.

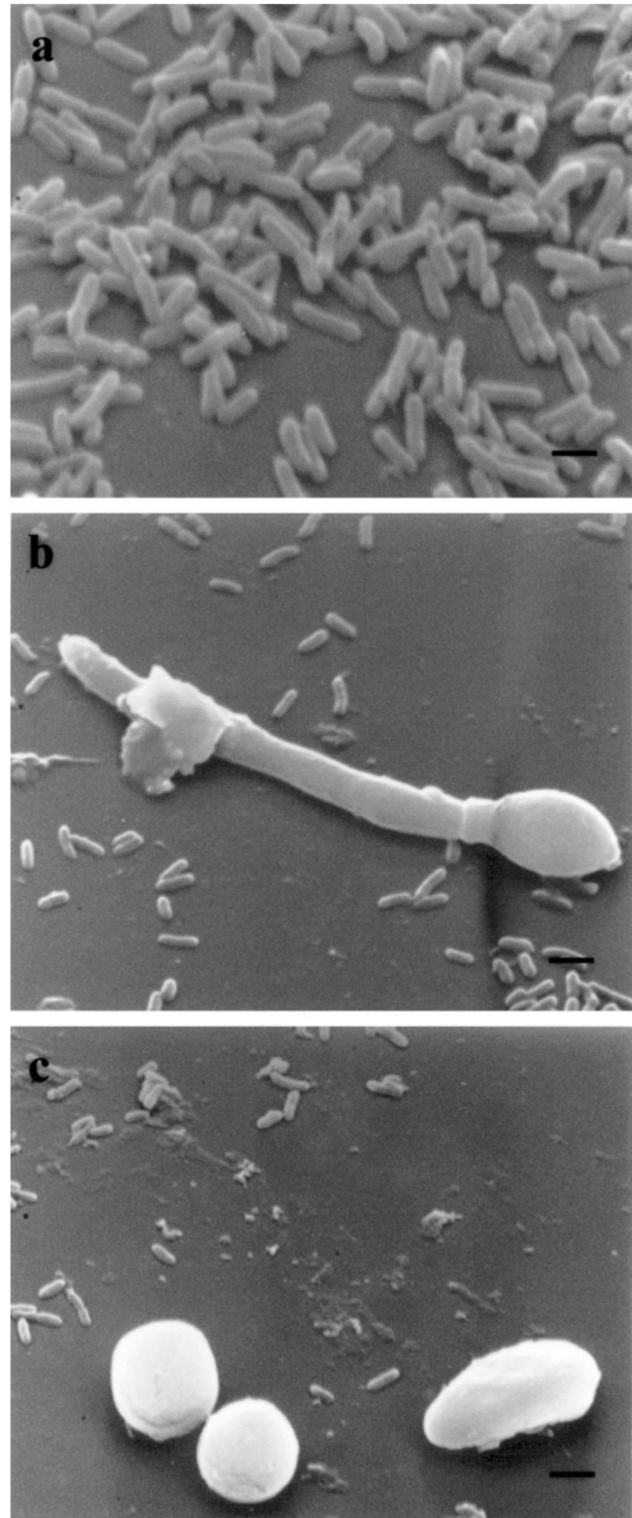

Figure 4 Scanning electron micrographs of binary culture formation by *S. maltophilia* and *F. oxysporum* on glass within the Robbins device after 48 h. (a) Cluster of bacterial cells, bar = 2 μm; (b) germinating conidium closely associated with bacterial cells, bar = 3.5 μm and (c) three conidia closely associated with bacterial cells, bar = 3.5 μm.

by bacterial cells and clusters of bacteria. It appeared that conidia germinate forming branched hyphae following surface attachment. Some areas of the biofilm showed the bacteria were closely associated with the fungus, and other areas where the bacteria were attached to the fungus and also areas where each species attached separately. There were also areas of bare substratum. This structure was similar for biofilms formed on PVC or glass.

Discussion

Use of the MRD with SEM allows qualitative and quantitative data to be obtained for binary culture biofilm formation. Most biofilm studies concentrate on adhesion of single species; however, this work describes a two-membered population of a bacterium and filamentous fungus. Results showed both the effect of surface on adhesion and the influence of colonization sequence. *S. maltophilia* and *F. oxysporum* coexisted in a stable, reproducible biofilm on PVC and glass. Numbers of *F. oxysporum* within the biofilm were <5% of the total bacterial numbers that may result from the slower growth rate of the fungus [10], differences in cell size and attachment mechanisms. However, there was no significant difference between glass and PVC for total numbers of *S. maltophilia* and *F. oxysporum* in binary population biofilms. The type of surface did not effect adhesion of the bacterium and fungus under the conditions described. The surface may not have influenced adhesion because biofilm formation was only monitored from introduction of the second colonizer onto the biofilm of the first colonizer. Events occurring at the surface within the first hours such as absorption of a conditioning film are most likely to be influenced by the surface properties of the substrate [9]. Thus attachment could be dependent on cell to cell adhesion between the bacterium and fungus and not on cell surface attachment. However, although some electron micrographs showed bacteria attached to the fungal hyphae, a direct association between cells was not necessary for the promotion of adhesion. The surfaces were also shown to be colonized by each species separately. A number of processes may have occurred during adhesion such as growth and cell division in the planktonic phase and at the surface combined with the attachment and detachment processes.

Surface roughness, type, hydrophobicity and attaching medium influence adhesion. It is generally acknowledged that increasing surface roughness increases adhesion through the production of more attachment sites and by providing a degree of protection to the developing biofilm [23,24]. Blackman and Frank [3] demonstrated increasing percentage coverage by *Listeria monocytogenes* biofilms in the order of nylon to Teflon to stainless steel. Changing the attaching menstrum resulted in significantly less biofilm accumulation on all surfaces with greatest attachment on Teflon. Similar numbers of *L. monocytogenes* and *Salmonella typhimurium* attached to stainless steel and Bua-nitryl rubber [12]. However, attachment was significantly reduced for both surfaces in the presence of milk and individual milk components. Absolom *et al* [1] showed greater adhesion to hydrophilic than to hydrophobic polymers when the surface tension of the bacteria was larger than that of the suspending medium. When the surface tension of the suspending medium was larger than that of the bacteria, the opposite behaviour was observed. Earlier work also showed the importance of wettability with much greater attachment of marine bacteria to glass than to nickel, polystyrene, polypropylene, polyvinyl fluoride, teflon and copper [9].

Attachment of the second colonizer was not significantly influenced by the previous attachment of the first colonizer on either PVC or glass. Thus the first colonizer does not significantly limit the availability of attachment sites on the surface for the second colonizer as biofilm numbers are the same for each species irrespective of which colonized the surface first. If attachment sites were scarce as a result of adhesion of the first colonizer, competition for the remaining sites would be indicated by the more successful species colonizing the free sites resulting in lower numbers of the second colonizer in the biofilm. However, scanning electron micrographs also showed areas of bare substratum; thus at this stage of attachment, sites appeared not to be limited.

Other researchers have shown one species was influenced by the simultaneous or previous attachment of another species to form a binary population biofilm in one of three ways: (i) decreased attachment, (ii) increased attachment and in agreement with this study (iii) no effect. McEldowney and Fletcher [19] demonstrated all three effects between simultaneous and sequential attachment of *Acinetobacter calcoaceticus, Staphylococcus aureus*, a *Staphylococcus* sp. and a coryneform. The results depended on the species combination, the surface and the sequence of attachment. Decreased attachment of a *Hyphomicrobium* sp. was shown when it did not become successfully established in a *Pseudomonas putida* biofilm; however, in the reverse situation *P. putida* became the dominant species in the biofilm [2]. *P. putida* was also the dominant species in the simultaneous deposition studies. These results were attributed to the relative growth rates of the two species with the faster growing species having the competitive advantage.

P. fluorescens was shown to significantly hinder adhesion of *S. typhimurium* to polycarbonate in binary biofilms. However, no significant difference in percentage of attached cells of *P. fluorescens* was shown between single and binary species suspensions [18]. Cowan *et al* [8] showed that interactions between bacterial species may allow attachment of a poor surface colonizer, *Aeromonas hydrophilia* to be enhanced by the presence of *P. fluorescens*. They also showed that a strong adhesive species such as *Xanthomonas maltophilia* influenced biofilm coverage by promoting attachment of a mixed species biofilm formed by *A. hydrophilia*, *P. fluorescens* and a coryneform. Brading *et al* [5] showed that during simultaneous colonization of *P. fluorescens* and *P. putida*, *P. fluorescens* maintained an advantage over *P. putida* for initial attachment. During sequential attachment *P. fluorescens* became incorporated into a *P. putida* biofilm faster than *P. putida* was detected in *P. fluorescens* biofilms. However, one species did not preclude the other from being incorporated into the biofilms.

No species interaction was observed in binary biofilms of *Klebsiella pneumoniae* and *P. aeruginosa* as neither the specific cellular product formation rate nor the glucose–oxygen stoichiometric ratio was affected by the other species [28].

The results demonstrate that an understanding of biofilm formation cannot be based solely on pure culture studies or only on populations of bacteria. Stable, reproducible biofilms clearly could consist of bacteria and fungi on PVC and glass. Previously, specific studies on interactions and biofilm formation of a prokaryote and eukaryote have not been carried out. This study was particularly concerned with species isolated from fouling biofilms in the photoprocessing industry. Implications for control of these biofilms, for example through dosing with biocide, includes

targeting the biocide against both bacteria and fungi as removal of one species could allow persistence of the remaining species. Understanding of biofilm dynamics and interactions between species in binary and multispecies biofilms requires further research to develop better control strategies.

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