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Assessment of a chemostat-coupled modified Robbins device to study biofilms

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The combination of a modified Robbins device (MRD) attached to the effluent line of a continuous cultivation vessel was assessed by the adhesion of planktonic bacteria maintained at a controlled growth rate. This combination of a chemostat and an MRD provides a large number of sample surfaces for monitoring both the formation and control of biofilms over extended periods of time. This apparatus was used to monitor the colonization of two soil isolates, *Pseudomonas fluorescens* (EX101) and *Pseudomonas putida* (EX102) onto silastic rubber surfaces. At a similar μ_{rel}, both bacteria attached to the silastic, however *P. fluorescens* formed confluent, dense biofilms in less than 24 h, whereas *P. putida* adhered as single cells or microcolonies after the same period. The metabolic activity, measured by INT-formazan formation, was similar for both organisms with a peak at 6 h of colonization and a subsequent decrease after 24 h. Long term colonization studies of *P. fluorescens* produced a population of greater than 9.5 log cfu cm^{-2} at 28 days demonstrating the advantages of the chemostat–MRD association. This technique proved to be successful for studying bacterial adhesion and biofilm formation in tubular devices by bacterial populations at controlled and low growth rates.

Keywords: chemostat; Robbins device; *Pseudomonas fluorescens; Pseudomonas putida;* biofilms; adhesion

Introduction

Bacterial adhesion and biofilm formation onto submerged surfaces is recognised as a major phenomenon in medical, industrial and environmental habitats [10]. Studies have been directed at monitoring adhesion processes, biofilm formation and the subsequent advantages of this mode of growth. Comparisons between planktonic and biofilm cells have demonstrated phenotypic differences which have been attributed to their respective growth state and the differing environmental conditions encountered in the bulk or biofilm phase [5,24].

Bacteria are able to adapt to many diverse environments. These adaptations result in phenotypic alterations that are frequently associated with the expression or loss of various cellular components [13]. The cell envelope, an interface between the bacterium and the external environment, is the main structure which interacts and reacts with the environment as a survival mechanism [6]. These phenotypic variations have a profound effect on bacterial attachment to surfaces and thus their response to antimicrobial agents [22]. Consequently, this emphasises the importance of controlling the growth environment of the planktonic bacteria used to colonize and form biofilms on surfaces [1,7,16,18,30]. These studies indicate that nutrients available in the bulk phase will have an effect on the physiological state of the planktonic cells and thus affect their tendency to attach to surfaces [25]. Furthermore, the difference between bacterial cells grown at different growth rates result in variations in phenotypes such as the expression of different cell wall proteins and production of extracellular polysaccharide [11,20,32,34]. Alterations in these characteristics have been implicated in an altered adhesion process of the bacteria [25].

Modelling biofilm formation and characteristics in the laboratory is difficult due to the complex interactions between bacteria and their environment. Initial studies consisted of using batch cultures to colonize surfaces, either by inserting surface material into bacterial suspensions [23] or by pumping the growing culture over the surfaces for colonization. The modified Robbins device (MRD), developed by Jim Robbins at the University of Calgary, has been used extensively in conjunction with batch cultures and used to study bacterial colonization and biofilm formation [28,31]. The device was constructed for the purpose of colonizing a large number of surfaces in a reproducible manner for multi-sampling of biofilms. It is used to model biofilm formation in tubular devices such as catheters and pipelines. However, the system has been criticised because of the lack of growth rate control of the planktonic bacteria colonizing the surfaces [7].

The use of continuous cultures allows defined growth conditions and offers greater control over the planktonic phase than batch culture growth. This permits the study of bacterial attachment by planktonic bacteria growing at a specific growth rate. Several research groups have developed models to use chemostats for biofilm studies [16,18,19,30]. Continuous culture has been used to study the adhesion of oral bacteria and the formation of dental plaque by placing acrylic surfaces into the chemostat reservoir [18]. The same system has also been used to monitor the corrosion of copper by biofilm bacteria [36] and is effective for studying mixed culture biofilms [37]. Another model was introduced to control the growth rate of the bac-

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teria in biofilms by collecting cells onto a filter membrane and thus maintaining a constant growth rate by perfusing the filtered cells with medium [16].

This paper reports a study that investigates the combination of an MRD and a chemostat culture where the planktonic bacteria are maintained at growth rates below μ_{max} for biofilm formation. In this manner biofilm formation was studied using planktonic bacteria growing at low growth rates to monitor attachment and biofilm formation by two soil isolates, *Pseudomonas fluorescens* EX101 and *Pseudomonas putida* EX102. No growth rate control of the biofilm bacteria is implied.

Materials and methods

Organisms and growth medium

Pseudomonas fluorescens EX101 and *Pseudomonas putida* EX102 were isolated from soil and identified using the Biolog Identification System (Biolog, Inc, Hayward, CA, USA) [27]. *Pseudomonas fluorescens* produced a mucoid colony morphology on Pseudomonas isolating agar (Difco, West Molesey, Surrey, UK) while *Pseudomonas putida* maintained a non-mucoid morphology. The bacteria were cultured in sodium citrate medium (SCM) containing trisodium citrate (6.45 g L^{-1}), $(NH_4)_2SO_4$ (0.198 g L^{-1}), KH_2PO_4 (2.72 g L^{-1}), K_2HPO_4 (5.23 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.246 g L^{-1}) and $FeCl_2$ (0.0082 g L^{-1}) in distilled water. The maximum specific growth rate (μ_{max}), determined in SCM by batch culture was 0.395 h^{-1} for *P. fluorescens* EX101 and 0.321 h^{-1} for *P. putida* EX102.

Chemostat growth conditions and experimental design

The bacteria were grown separately in pure cultures in a BioFlow Model C30 chemostat (New Brunswick, Hatfield, Herts, UK) at 30° C, agitated at 300 rpm and aerated with sterile air at 0.75 L min^{-1} in a culture volume of 1.37 L. Nutrient flow was maintained at 15.5 ml h^{-1} for EX101 (dilution rate of 0.011 h^{-1}) and 14.0 ml h^{-1} for EX102 (D = 0.010 h^{-1}. The MRD, described by Nickel *et al* [28], was prepared by affixing silastic rubber surfaces (SAMCO Silicone Materials, St Albans, UK) to the stubs before ethylene oxide sterilisation. Silastic was chosen as a model surface as it is much used in attachment studies and is easy to use. The chemostat was considered to have reached steady-state conditions when constant population sizes were established, that is 8.88 log cfu ml^{-1} for EX101 and 8.77 log cfu ml^{-1} for EX102. The MRD was attached to the effluent line of the chemostat. The μ_{rel} (μ/μ_{max}) [15,33] for *P. fluorescens* EX101 was 0.029 and for *P. putida* EX102 it was 0.032. Sample studies were removed at regular time intervals over a 24-h period to monitor biofilm formation.

For the long term colonization study, *P. fluorescens* EX101 at μ_{rel} of 0.029, was used to colonize the silastic rubber over a 28-day period. Colonization was monitored by removing studs at approximately 7-day intervals and viable cell counts were assessed.

The colonization of *P. putida* at the three different growth rates was undertaken using the parameters listed in Table 1. *P. putida* was grown initially at the lowest specific growth rate and brought to steady state before attaching the MRD to the effluent line for colonization. Biofilm formation was monitored by viable cell count. *P. putida* was then grown at a higher specific growth rate and the experimental procedure repeated until each growth rate had been studied.

Table 1 Parameters for three different growth rates of *P. putida* EX102 continuous cultures used for colonization of silastic rubber

Properties	1	2	3
Flow rates (ml h^{-1})	7.5	48	84
D (h^{-1})	0.005	0.035	0.061
μ_{rel}	0.02	0.10	0.19
Viable counts Log (cfu ml^{-1})[a]	9.34 ± 0.08	9.31 ± 0.02	8.85 ± 0.02

[a]This is the average steady state planktonic population in the chemostat during colonization under the three different specific growth rates

Monitoring bacterial attachment

Some of the surfaces were withdrawn from the MRD at different time intervals, washed with 10 ml of 1/4 Ringers solution to remove any non-attached bacteria from the surface and monitored for viable counts, total bacterial counts, total carbohydrate, metabolic activity by the reduction of INT to INT-formazan and scanning electron microscopy. The surfaces monitored for viable counts and total carbohydrate were scraped with a sterile scalpel blade and the bacteria were dispersed by sonication (5 min) and vortexed in 1 ml of 1/4 Ringers solution. Viable counts were determined by serially diluting the bacterial scrapings in 1/4 Ringers solution, plating them onto nutrient agar and incubating at 30° C overnight. The total carbohydrate of the biofilm scrapings was determined spectrophotometrically using the method described by Dubois *et al* [12] against a standard of glucose. For total bacterial counts, bacteria were scraped from the silastic and resuspended in 10 ml of 0.5% glutaraldehyde in cacodylate buffer (1.0 M, pH 7.4) by sonicating and vortexing the suspension to disperse the microcolonies. The cells were filtered onto Nuclepore filters, stained with acridine orange and bacteria were counted from a minimum of 10 fields using an Olympus BH-2 epifluorescence microscope [14].

The metabolic activity of *P. fluorescens* EX101 and *P. putida* EX102 was measured photometrically by extracting the reduced INT-formazan crystals from the cells with methanol [3]. The studs were removed from the MRD at different intervals and, together with the adherent bacteria, were incubated in 0.02% INT (Aldrich, Gillingham, Dorset, UK) solution at 30° C for 6 h. The surfaces were washed with 10 ml of 1/4 Ringers solution and the INT-formazan formed by the metabolically active cells was extracted by scraping the cells into 3 ml of chilled methanol, and placed in a freezer for 1 h. The suspensions were filtered and the absorbance read at 480 nm. The concentrations were determined against a standard curve of INT-formazan (Aldrich) in methanol.

Specimens for scanning electron microscopy were gently washed with 10 ml of 1/4 Ringers solution and fixed in 5.0% v/v glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 2 h at room temperature. The surfaces were washed

twice with cacodylate buffer, dehydrated in a 10, 20, 30, 50, 70, 90 and 100% aqueous ethanol series and critical point dried in ethanol. The specimens were subsequently mounted, sputter coated with gold and viewed on a Stereoscan 100 scanning electron microscope (Leica, Cambridge, UK) at 25 kV.

Results and discussion

Colonization of P. fluorescens *and* P. putida *over 24 h*

The attachment of the MRD to the chemostat effluent line was successfully used to permit colonization of silastic surfaces by *P. fluorescens* EX101 and *P. putida* EX102. Both organisms were able to attach and grow on the silastic to above 8.0 log cfu cm^{-2} by 24 h (Figure 1). The adherent population of *P. putida* remained relatively constant from 12 to 24 h whereas the *P. fluorescens* numbers continued to increase over the same period. The variations in attached populations between the two organisms may be due to the production of excess exopolysaccharide by *P. fluorescens* under these growth conditions. The production of polysaccharide has been observed previously for other pseudomonads during surface attachment and different nutrient conditions [29,35]. Scanning electron microscopy (Figure 2) allowed observations of surface attachment of the two organisms. *P. putida* did not form biofilms over a 24-h period but instead the adherent cells produced microcolonies while the *P. fluorescens* produced confluent biofilm formation over large areas of the silastic surface. From the micrographs it was apparent that *P. fluorescens* was surrounded by an extracellular material, possibly polysaccharide, identifying the structure as a biofilm [9].

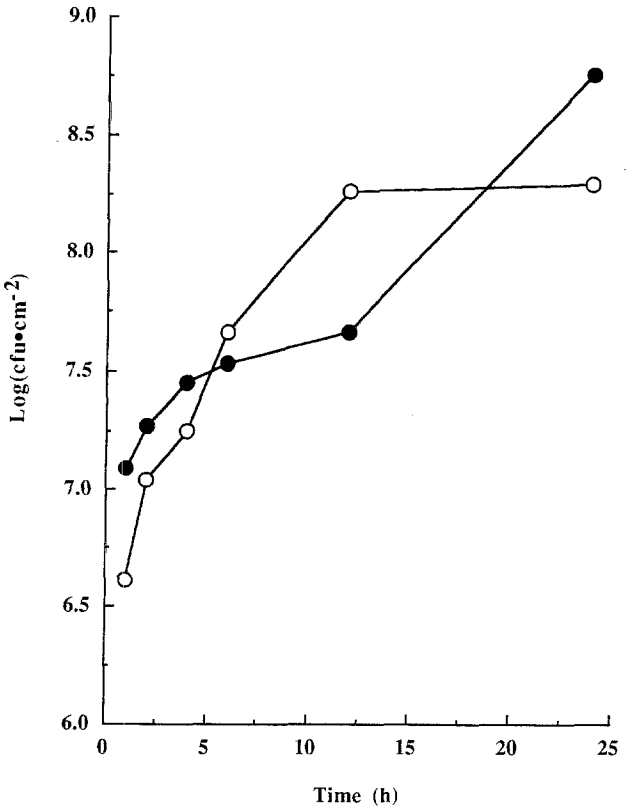

Figure 1 Kinetics of bacterial attachment by *P. fluorescens* EX101 (●) and *P. putida* EX102 (○) to silastic discs colonized with cultures growing at 0.03 μ_{rel}

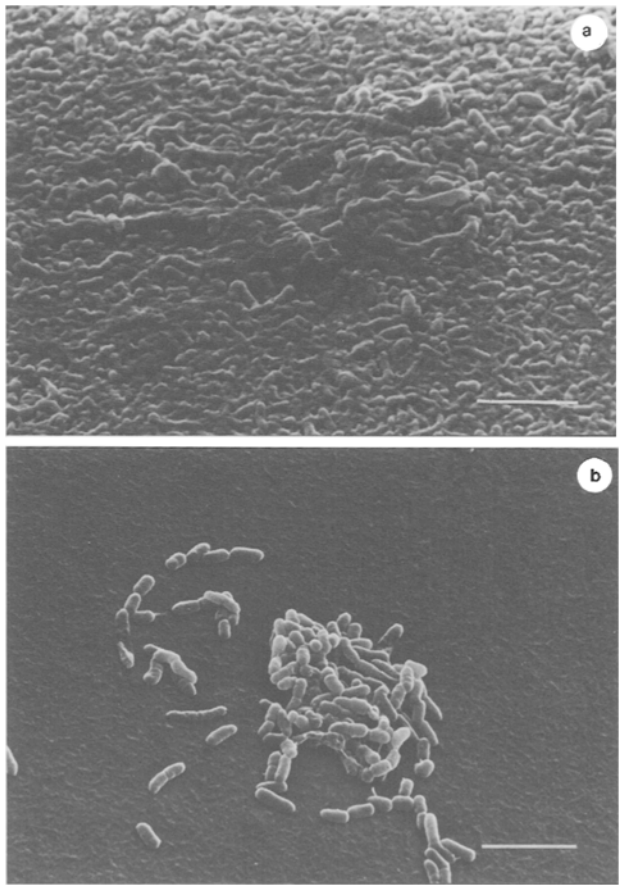

Figure 2 Scanning electron micrograph of (a) *P. fluorescens* EX101 biofilms and (b) adherent populations of *P. putida* EX102 as single cells and microcolonies after colonization for 24 h. Bar = 5.0 μm

Biofilm formation may be monitored by various techniques which could be facilitated by using the MRD and sampling more surfaces from the same biofilm experiment. The two cultures produced different amounts of total carbohydrate during surface attachment (Figure 3). The total carbohydrate per 10^6 viable cells of *P. fluorescens* showed that during the early stages of biofilm formation, the carbohydrate was first detected at 4 h, increased up to 0.35 μg at 12 h and then declined as the biofilm matured. The increased presence of carbohydrate in *P. fluorescens* biofilms suggests that the extracellular material observed in the electron micrographs is polysaccharide in nature. Nonspecific attachment involving exopolymer-associated adhesion of bacteria to surfaces is a time-dependent process limited by the time required to synthesize the exopolymer [8]. The decrease in carbohydrate may be due to the growth and cell division which occurs after the initial bacterial attachment [21,35]. Consequently, the number of bacterial cells may be increasing more rapidly than the synthesis of polysaccharide, which can be in the form of exopolymer, resulting in a decrease in total carbohydrate per cell as the biofilm matures. Furthermore, the presence of large

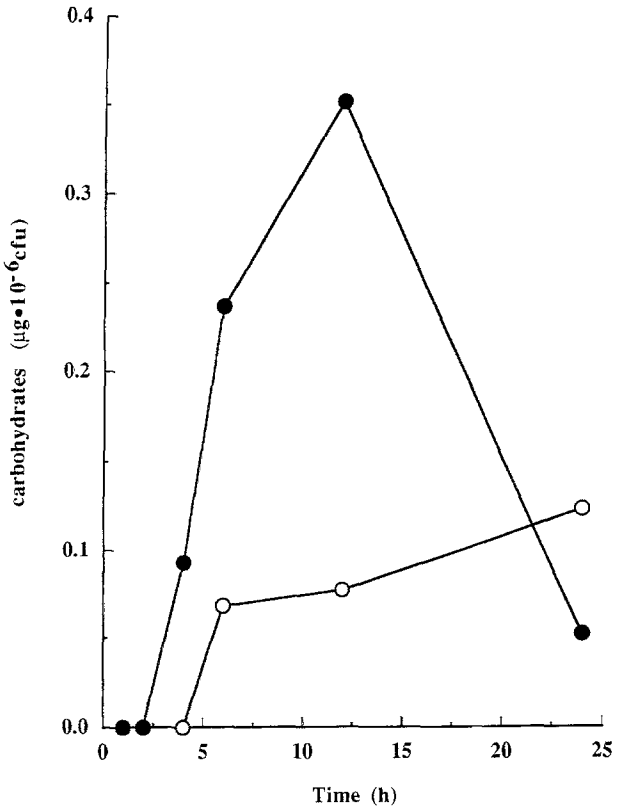

Figure 3 The total carbohydrate associated with the adherent bacterial population on the silastic surfaces colonized over 24 h by *P. fluorescens* EX101 (•) and *P. putida* EX102 (○)

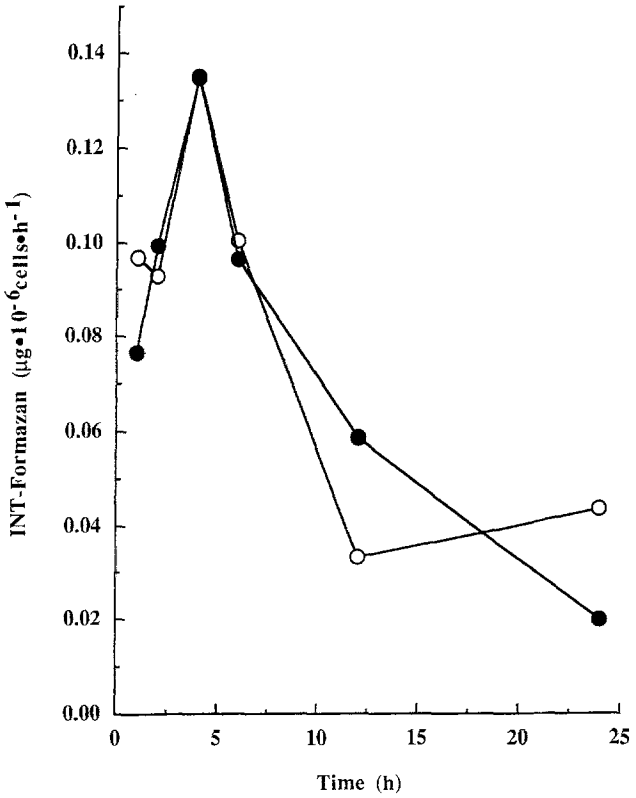

Figure 4 The metabolic activity of adherent populations of *P. fluorescens* EX101 (•) and *P. putida* EX102 (○) measured by the reduction of INT to INT-formazan by the cellular electron transport system

amounts of polysaccharide may be necessary for the initial attachment of *P. fluorescens* but as the biofilm develops, large amounts of extracellular polysaccharide may not be required, consequently the cell would reduce exopolysaccharide synthesis. The total carbohydrate for *P. putida* was much lower than for *P. fluorescens* (Figure 3) and it was not detected in the first 6 h. These results may indicate that a lesser amount of polysaccharide is necessary for adhesion of *P. putida*, reflecting microcolony formation instead of biofilm formation. Mengistu *et al* [26] using *Klebsiella pneumoniae* K1 demonstrated that polysaccharide production was not only dependent on the nutrient conditions (such as carbon and nitrogen limitation) but also temperature, dilution rate and pH which could then be controlled by a chemostat. These studies suggest that by altering the growth conditions of the planktonic bacteria, initial colonization may be influenced.

The metabolic activity of the surface-attached bacteria was determined by monitoring the reduction of INT to INT-formazan by the electron transport system of metabolically active cells. The metabolic activity profile of the adherent cells was similar for both bacteria, with an initial increase per 10^6 cells h^{-1} followed by a decrease as the microcolonies or biofilm matured (Figure 4). In nutrient-rich environments, it has been suggested that surface-attached bacteria are less metabolically active than the batch culture cells, due to a reduced diffusion of nutrients through the biofilm. Under such circumstances, in the initial stages of attachment the bacteria will have a higher metabolic activity than when the biofilm thickens and matures. An increased activity of the electron transport chain was observed just prior to the increase in the production of carbohydrate. Thus, the sequence of events suggested by the study involving *P. fluorescens* colonization includes initial bacterial adhesion to the surface requiring high metabolic activity, followed by an increase in exopolymer production. These processes may continue during cell division and growth to eventually form a biofilm. Although *P. putida* was surrounded by less exopolymer (Figure 2a), it had a similar metabolic activity to *P. fluorescens* over the 24 h. This leads us to conclude that the above hypothesis is not the only reason for the metabolic activity profile but that it is a surface-associated phenomenon with other mechanisms involved. Consequently, the initial increase in metabolic activity of the adherent bacteria may be a short term response to surface association which subsequently decreases to a steady metabolic state as the biofilm thickens and matures. This shows that there may be differences in the mechanisms of adhesion between the two pseudomonads.

Long term colonization by P. fluorescens

Batch cultures have been traditionally used to study surface attachment and biofilm formation but such studies are limited by the length of the growth cycle of the bacteria. This has been overcome by using the chemostat to provide a continuous source of bacteria and to maintain the culture at the same specific growth rate throughout the experiment. Ultimately a long term study of the colonization of *P. flu-*

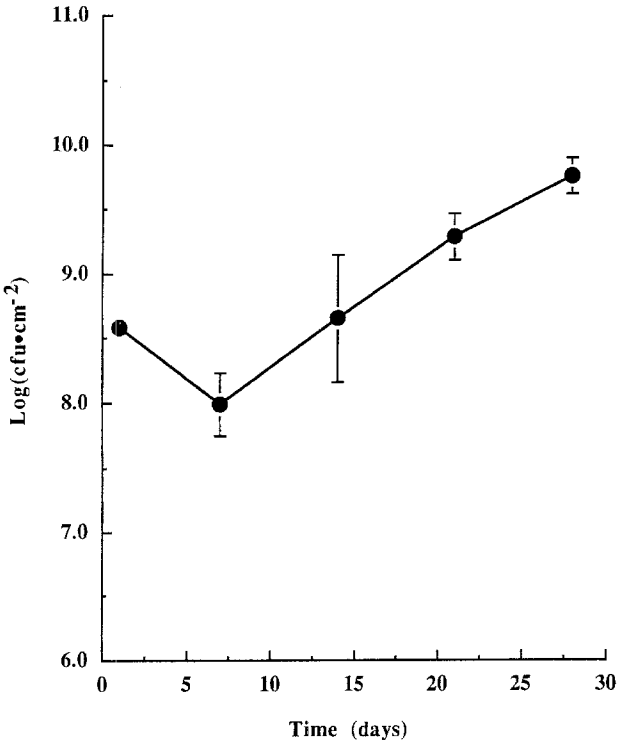

Figure 5 *P. fluorescens* EX101 biofilm formed on the silastic surface over 28 days of colonization

orescens was possible over a 28-day interval (Figure 5). During this period, the bacterium colonized the surface to greater than 9.0 log cfu cm^{-2}. Scanning electron micrographs revealed the structure of the biofilm changed from the more confluent, thin biofilm as reported in the short term study (Figure 2a) to become large, thicker microcolonies that were not confluent by the 5th day (Figure 6). As biofilms mature and thicken, sloughing of the biofilm occurs due to the fluid dynamics and shear effects of the bulk fluid [2]. Consequently, large microcolonies and sections of the biofilm are displaced from the surface giving it the characteristic patchy appearance found after longer term colonization (Figure 6). Anwar *et al* [1] determined that old *P. aeruginosa* biofilms were more resistant to tobramycin than young biofilms. The experimental system described here would be suitable for testing antimicrobial agents against biofilms formed over different lengths of time. Furthermore, the large number of sample surfaces provides the possibility to monitor other parameters, such as those carried out in this study, to help determine why the biofilm age would affect the bacterial cell sensitivity to antimicrobial agents.

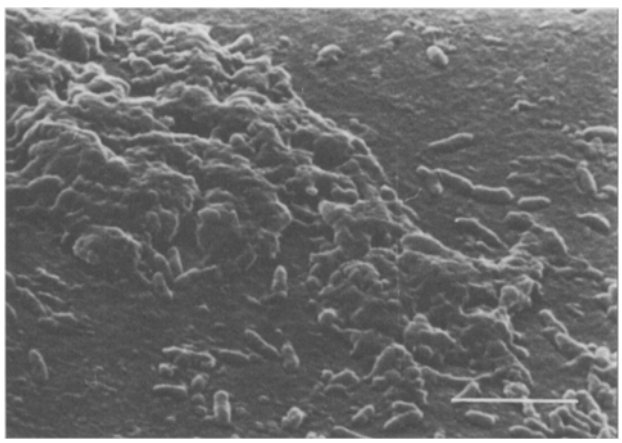

Figure 6 Scanning electron micrograph of *P. fluorescens* EX101 adhering to silastic discs by 5 days of colonization. Bar = 5.0 μm

Colonization by P. putida *at different growth rates*

The growth rate of a bacterium had been implicated in altering its physiology and thus the response of the organisms to surfaces [4,6]. The chemostat–MRD model provides a system to study the effect of different specific growth rates of the planktonic population on the colonization of *P. putida* EX102. Effectively, little difference was apparent in the colonization profiles for the three different growth rates chosen. The adherent bacterial populations formed using chemostat cultures grown at μ_{rel} 0.02 and 0.10 had similar colonization profiles over the 24-h period (Figure 7). When grown at μ_{rel} 0.19 the initial adherent populations were low but by 24 h the cell numbers surpassed the populations found for the lower growth rates. To determine if this trend continues, a long term study would have to be undertaken. While the adherent populations of the three growth rates over the 24-h period were similar for *P. putida*, other parameters such as metabolic activity and exopolymer production may be significantly different.

In common with several other studies, the chemostat–

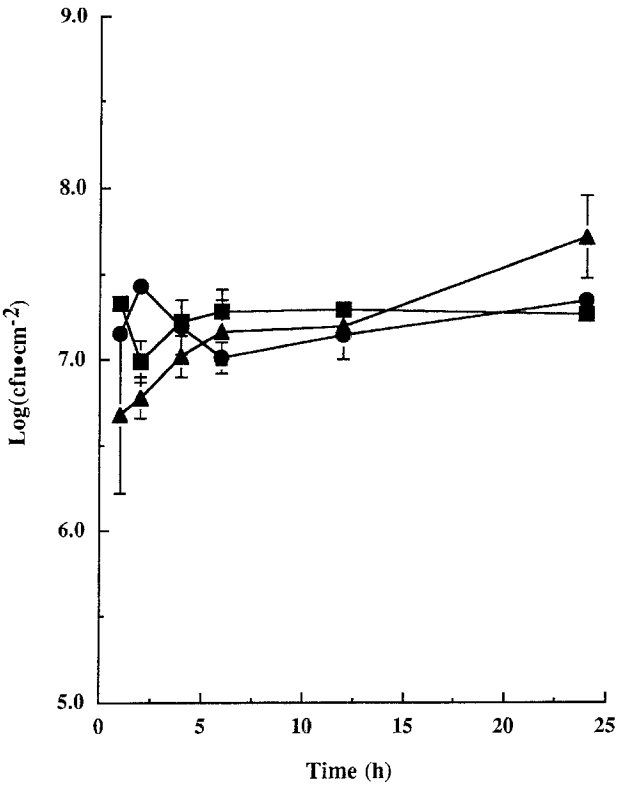

Figure 7 Effect of different growth rates on the colonization by *P. putida* EX102 of silastic discs. The chemostat was brought to steady state at 0.02 (●), 0.10 (■), and 0.19 (▲) μ_{rel} and colonized for 24 h. The standard error bars were of an average of three stubs at each sample time

MRD system involves investigating surface attachment by bacteria growing at specific growth rates and does not involve controlling the growth rate of the biofilm itself. Biofilms are not homogeneous structures and gradients of nutrients, oxygen and other substances exist producing different microenvironments within the biofilm which lead to different physiological responses and bacterial growth rates [17]. Models which use bacteria grown in batch culture and collected onto a filter paper to study biofilm formation have provided interesting information about phenotypic response of bacteria to growth rate [16] but they do not reflect the heterogeneous nature of biofilms. The novel combination of a chemostat and a MRD offers various advantages: the availability of a large number of sample surfaces to monitor both the formation and control of biofilms, connecting the device onto the culture effluent line reduces the risk of contaminating the chemostat while providing a continuous source of bacterial culture at growth rates below μ_{max} and monitoring biofilm formation and control over extended periods of time. The bacterial population in the chemostat must reach steady state before attaching the biofilm sampler and thus the surfaces can be colonized for extended periods of time at different specific growth rates. Furthermore, the experimental apparatus minimises opportunities to contaminate the culture during sampling as in the case of some of the other chemostat systems that immerse the colonization surfaces directly into the reservoir [18]. Although the microbial activity in some environmental processes can be monitored *in situ* many colonization studies cannot. Such systems require a controlled laboratory model that resembles the microbial ecosystem of interest. A similar environment found in tubular flow systems is achieved by attaching the Robbins device to a chemostat where the growth rate of the cells used to colonize the surface is controlled. Furthermore, by controlling the essential nutrients available for growth, the organism can be grown in low nutrient conditions prevalent in natural environments. This study demonstrated that attaching an MRD onto the chemostat effluent line can be useful for studying bacterial attachment and biofilm formation by planktonic populations growing at a low specific growth rate.

Acknowledgements

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