Effect of Growth Conditions and Surface Characteristics of Aquatic Bacteria on Their Attachment to Solid Surfaces

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The physico-chemical basis for the effects of nutrient conditions on the attachment of four freshwater isolates, *Pseudomonas fluorescens*, *Enterobacter cloacae*, *Chromobacterium* sp. and *Flexibacter* sp., to hydrophobic (PD) and relatively hydrophilic (TCD) polystyrene surfaces was investigated. Different nutrient conditions and growth rates resulted in changes in the physico-chemistry of the bacterial surfaces, measured either by liquid contact angles on lawns of cells or by hydrophobic and electrostatic interaction chromatography of cells, and in different levels of attachment to the substrata. The phenotypic changes in cell surfaces and the levels of attachment were different for each species. Levels of bacterial adhesion differed for the two substrata, indicating different adhesion interactions with PD and TCD surfaces. Treatment of attached cells with chloramphenicol did not cause detachment of any of the bacteria from PD or TCD, whereas periodate and protease treatment removed some attached cells, the degree of detachment depending on the species. The presence of complex organic molecules, in the liquid phase and conditioning the solid surface, influenced the extent of bacterial attachment, the effect depending on the substratum, organic concentration and bacterial species. The results suggest that changes in nutrient conditions in natural aquatic habitats will affect the attachment of individual bacterial species differently, thus influencing the population structure of developing biofilms.

INTRODUCTION

Bacterial attachment to solid surfaces in aqueous systems is a complex interaction between the bacterium, the solid substratum and the liquid phase. Each of these components can vary with changes in environmental conditions, which will in turn affect attachment processes. Bacteria react physiologically to fluctuations in environmental conditions, such as changes in nutrient concentrations. The macromolecular components of bacterial surfaces, e.g. lipopolysaccharide, protein and exopolymers, have been shown to vary in quantity and composition with growth conditions (Ellwood & Tempest, 1972; Jarman *et al.*, 1978) and with growth rate in continuous culture (Ellwood & Tempest, 1972). The effects vary with species. Differences in cell wall or polymer constitution appear to be reflected in the physico-chemical characteristics of the cells, e.g. through differences in electrophoretic mobility (Plummer *et al.*, 1962; Marshall, 1967) or hydrophobicity (Rosenberg *et al.*, 1982). The importance of cell surface chemical and physical properties in bacterial attachment interactions has been demonstrated both experimentally (Rosenberg, 1981; Hogt *et al.*, 1982) and theoretically (Lips & Jessup, 1979; Pethica, 1980) although the chemical bases for these interactions are still largely undetermined.

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Abbreviations: EIC, electrostatic interaction chromatography; HIC, hydrophobic interaction chromatography; PD, polystyrene Petri dish; TCD, tissue culture treated polystyrene Petri dish.

Organic substances can affect not only the bacteria (by serving as nutrients) but also the liquid and solid phases of the attachment interaction through their influence on thermodynamic parameters, e.g. surface tension. With a solid substratum, this involves the energetically-favoured irreversible adsorption of large macromolecules, sometimes called a conditioning film (Dexter, 1977). The amount and rate of adsorption of proteins is dependent on the solid surface involved (Baszkin & Lyman, 1980), the macromolecular species adsorbed (Norde & Lyklema, 1978), and the manner in which the macromolecule is anchored (Eirich, 1977). In general, high energy and high polarity substrata will adsorb macromolecules strongly, with a flattening of the macromolecules across the surface, while low energy, non-polar surfaces will bind macromolecules less strongly although a thicker layer may develop (Baier, 1981). A macromolecular conditioning film on a solid substratum presents a new set of surface characteristics to the bulk liquid phase. Thus, the electrostatic charge (Loeb & Neihof, 1975) and the critical surface tension (an indication of surface free energy) (Baier, 1981) may change with the accumulation of a conditioning layer. Accordingly, a variety of protein conditioning layers, on surfaces such as polystyrene and glass, may both increase and inhibit the attachment of different bacterial species (Fletcher, 1976).

The aim of this study was to determine the physico-chemical basis for the effects of growth conditions on bacterial attachment to surfaces, and to examine the role played by polysaccharide and protein cell surface components in the attachment interaction. The influence of organic molecules on bacterial attachment was also investigated, particularly in relation to cell surface changes induced by different growth conditions.

METHODS

Organisms. The bacteria, isolated from the River Sowe at Baginton Weir, Coventry (Pringle & Fletcher, 1983), were identified as *Pseudomonas fluorescens*, *Enterobacter cloacae*, *Chromobacterium* sp. and *Flexibacter* sp.

Batch cultures. The bacteria were cultured in a selection of minimal media containing different carbon sources and carbon:nitrogen ratios. The minimal media consisted of 0.544 g KH_2PO_4 , pH 7.4, and 0.6 ml of a salt solution consisting of (l^{-1}) 10 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.4 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 100 ml distilled water with the addition of one of the following: (i) 0.05 g glucose, 0.38 g NH_4Cl (carbon-limited); (ii) 0.2 g glucose, 0.02 g NH_4Cl (nitrogen-limited); (iii) 0.2 g glucose, 0.38 g NH_4Cl (carbon- and nitrogen-sufficient); (iv) 0.2 g glucose, 0.38 g NH_4Cl , 10% (v/v) glycerol; (v) 1% (w/v) lactose, 0.38 g NH_4Cl ; (vi) 1% (w/v) galactose, 0.38 g NH_4Cl ; (vii) 1% (w/v) mannose, 0.38 g NH_4Cl ; (viii) 1% (w/v) sucrose, 0.38 g NH_4Cl .

After inoculation with 1 ml of a stationary phase culture, cultures were grown in the test media for 24 h, at 15 °C on a rotary incubator at 150 r.p.m., before sampling. *Chromobacterium* sp. was sampled after both 24 and 48 h incubation.

The organisms were also grown to late stationary phase before sampling in a broth medium consisting of 0.1% (w/v) bacteriological peptone and 0.07% (w/v) yeast extract (PYE) at pH 7.4 at 15 °C on a rotary incubator at 150 r.p.m.

Continuous culture. The organisms were grown in continuous culture in PYE buffered at pH 7.4 (KH_2PO_4 , 5.44 g l^{-1}) at a constant temperature of 15 °C (± 2 °C) maintained by a Churchill Chiller Thermocirculator and at a steady aeration rate. For each bacterial culture the dilution rate (D) was changed successively from 0.05 h^{-1} to 0.1 h^{-1} to 0.15 h^{-1} to 0.2 h^{-1} . At each dilution rate the cultures were sampled only after a minimum of three complete volume changes.

Attachment assays. The batch cultures or 100 ml samples from the continuous cultures were harvested by centrifugation at 11 000 g. The cells were washed once in 0.01 M-HEPES buffer (Sigma), pH 7.4, and resuspended in (i) 0.01 M-HEPES buffer; (ii) 0.05% (w/v) peptone and 0.035% (w/v) yeast extract powder ($\frac{1}{2}$ PYE); or (iii) PYE broth, to an optical density of 0.1 at 540 nm in a colorimeter (Corning Colorimeter 252).

Samples (5 ml) of the suspensions were then transferred to 5 cm polystyrene Petri dishes (PD) (Sterilin), a hydrophobic surface, or tissue culture treated polystyrene dishes (TCD) (Costar), a relatively hydrophilic surface; these served as the attachment substrata. Duplicate PD and TCD for each type of cell suspension were incubated for 5 and for 60 min at 15 °C and then washed gently three times with 0.01 M-HEPES buffer (pH 7.4) to remove loosely attached bacteria. The remaining attached bacteria were fixed with Bouin's fixative (71% v/v, saturated aqueous picric acid solution; 24% v/v, formalin; 5% v/v, acetic acid) and stained with crystal violet (5 g l^{-1}) (Fletcher, 1976). Bacterial attachment was estimated indirectly by measuring the A_{590} of the stained attached bacteria. Four readings of randomly selected areas were taken from each of the duplicate dishes.

Results were expressed either as $A_{590} (\times 10^3)$ of the attached cells or, for those cells attached in $\frac{1}{2}$ PYE and PYE, as an index of attachment (I_a). I_a was calculated as the ratio of the $A_{590} (\times 10^3)$ of the test substratum to that

of the corresponding control substratum, e.g. cells attached in PYE to PD/cells attached in buffer to PD. I_a values of 1 were recorded for treatments whose 95% confidence limits of the mean ($n = 8$) overlapped with that of the controls; any value of I_a outside these limits was considered to represent an effect.

Detachment assays. Cells grown to late stationary phase in PYE broth were centrifuged, washed once in 0.01 M-HEPES buffer (pH 7.4) and resuspended to an OD_{540} of 0.1. Samples were then placed in PD and TCD plates and incubated for 1 h as described for the attachment assay. One set of duplicate plates for each bacterial species was then washed (three times with 0.01 M-HEPES, pH 7.4) and stained, whereas the remaining duplicate plates were washed as above and then exposed to 5 ml samples of one of the following in 0.01 M-HEPES buffer (pH 7.4): (i) control; (ii) 5 μ g chloramphenicol ml^{-1} ; (iii) 1% (w/v) sodium periodate; (iv) 1 unit per 5 ml bacterial protease. The duplicate plates were incubated at 15 °C for a further 1 h before washing and staining as in the attachment assay.

The results were expressed as PD I_d and TCD I_d , i.e. indices of detachment. I_d values were determined in an analogous way to I_a values.

Measurement of contact angle (θ) of lawns of cells. A method was developed based on that of Absolom *et al.* (1983). Bacteria were harvested from batch minimal media cultures by centrifugation, washed once in 0.15 M-NaCl, and resuspended to a concentration of approximately 10^{10} cells ml^{-1} in 2 ml 0.15 M-NaCl. Drops of the cell suspension were then spread over an agar-covered slide (2%, w/v, Ionagar + 10%, w/v, glycerol in 0.15 M-NaCl), and allowed to dry until the cells took on a matt appearance.

The advancing contact angles of 10 μ l drops of 0.15 M-NaCl (θ_s) were then measured every 10 min, using a vernier microscope with a goniometer eyepiece (PTI). Duplicate measurements were made at each interval and were considered reliable if they did not vary by more than $\pm 0.5^\circ$. No two measurements were made on the same area of cells. When a plateau value of θ_s was reached ($\pm 1^\circ$) it was taken to represent the saline contact angle of the lawn of cells.

Measurements of contact angle of clean and treated PD and TCD surfaces. To evaluate the effect of adsorbed medium components on the substratum surface, θ values were measured on medium-coated surfaces. PD and TCD materials were incubated in 5 ml samples of PYE or $\frac{1}{2}$ PYE at 15 °C for 60 min before being washed three times with 0.01 M-HEPES (pH 7.4). The treated surfaces were then allowed to air-dry for a minimum of 2 h at room temperature. The advancing water contact angle (θ_{H_2O}) of a 10 μ l drop of distilled water on clean and treated PD and TCD surfaces was measured as above except that 10 separate measurements were made for each surface, and a mean value was calculated.

Measurement of liquid surface tension. The liquid surface tension (γ_{LV}) of 0.01 M-HEPES buffer, $\frac{1}{2}$ PYE and PYE were measured using a platinum loop and torsion balance (White Electrical Instruments).

Hydrophobic, cationic and anionic chromatography of cells. The method for investigating the hydrophobic, cationic and anionic interactions of different bacterial species was adapted from the methods of Dählback *et al.* (1981) and Pederson (1980).

Glass wool-plugged Pasteur pipettes were washed with 95% (v/v) ethanol followed by 0.1 M-NaCl. Phenyl-Sepharose CL-4B gel (Sigma) for hydrophobic interaction chromatography (HIC) was diluted 1:1 with 0.1 M-NaCl (w/v), degassed and packed into the pipettes. Similarly, for electrostatic interaction chromatography (EIC), 1 ml quantities of anion exchange resin (Dowex 1 $\times$ 8 mesh size 100/200, 80–150 μ l) (Sigma) or cation exchange resin (Dowex 50W $\times$ 8 mesh size 100/200, 80–150 μ l) (Sigma) were suspended in 1 ml 0.1 M-NaCl, degassed and packed into the Pasteur pipettes. These two types of column were used for EIC.

Cells were harvested from continuous culture samples (100 ml) by centrifugation, resuspended in 20 ml KH_2PO_4 buffer (5.4 g l^{-1} , pH 7.4) and incubated at 15 °C for 2 h after the addition of 1 μ Ci L-[3H]leucine ml^{-1} (specific activity 46 Ci $mmol^{-1}$; 1702 GBq) (Amersham). The 3H -labelled bacteria were then washed three times by centrifuging with 0.1 M-NaCl to remove unmetabolized label, and resuspended in 0.1 M-NaCl to a volume of 10 ml, giving a final cell density of approximately 10^9 cells ml^{-1} . Samples (1 ml) of the suspensions were applied to duplicate HIC and EIC columns, followed by 3 ml 0.1 M-NaCl. Two further 4 ml samples of 0.1 M-NaCl were run through the columns. The eluates from all these columns were collected separately.

Four replicate 100 μ l samples from each eluate and the original cell suspension were individually counted for radioactivity, using 10 ml of a scintillation cocktail consisting of toluene/Triton X-100 (2:1, v/v) (scintillation grade) and 0.6% (w/v) of the scintillator butyl PBD (Fisons). Counts were made with a liquid scintillation counter (System LS 7000, Beckman Instruments) and the extent of quenching was determined by the H number system (Horrocks, 1977). Counts were repeated if there was a discrepancy $> \pm 5\%$ between replicate values. The final eluate had counts normally not much above background level. The extent of leakage from labelled cells was determined by counting 100 μ l duplicate samples of the final wash supernatant; 10% leakage was the maximum considered acceptable.

The results were expressed in terms of the percentage of counts remaining in the column. The results from duplicate columns were considered consistent if they differed by no more than 5%. The higher the percentage of cells remaining in the HIC and EIC columns, the greater the hydrophobic or electrostatic interactions, respectively.

Fig. 1. Effect of carbon:nitrogen ratio and carbon source on the attachment of (a) *P. fluorescens*, (b) *E. cloacae*, (c) *Chromobacterium* sp. after 24 h growth, (d) *Chromobacterium* sp. after 48 h growth and (e) *Flexibacter* sp. The nutrient conditions were glucose and nitrogen sufficient (GN), glucose-limited (G), nitrogen-limited (N), glucose and glycerol (GG), lactose (L), galactose (GA), mannose (M) and sucrose (S). □, Cells attached to the PD surface; ▨, cells attached to the TCD surface; error bars define the 95% confidence limits of the mean ($n = 8$).

RESULTS

Effect of carbon source and carbon:nitrogen ratio on bacterial attachment and cell surface characteristics

Bacterial growth in media with different carbon:nitrogen ratio and carbon source had a marked effect on both the attachment of bacteria and the bacterial cell surface characteristics.

The four bacterial species investigated, *P. fluorescens*, *E. cloacae*, *Chromobacterium* sp. and *Flexibacter* sp., all reacted differently, with respect to attachment ability, to changes in culture conditions (Fig. 1). The variations in adhesion could be very large, e.g. *P. fluorescens* attached in high numbers after growth in glucose with glycerol and in relatively low numbers with the other culture conditions (Fig. 1a). Such differences were found for all species; however, the strains did

Table 1. *Effect of carbon:nitrogen ratio and carbon source on the cell surface characteristics (measured by saline contact angles) of P. fluorescens, E. cloacae, Chromobacterium sp. and Flexibacter sp.*

Carbon:nitrogen ratio or carbon source	Saline contact angle on lawns of cells (θ_s)				
	<i>P. fluorescens</i>	<i>E. cloacae</i>	<i>Chromobacterium sp.*</i>		<i>Flexibacter sp.</i>
			24 h	48 h	
Glucose + nitrogen excess	17.5	14.5	20	38	19.5
Glucose-limited, nitrogen excess	10.0	10.0	59	40.5	13.0
Nitrogen-limited, glucose excess	16.5	12.5	32.5	30	21.0
Glucose + glycerol	9.5	10.5	52.5	29	13.0
Lactose	11.5	9.0	10.5	20	11.0
Galactose	9.5	14.0	75	42	13.0
Mannose	8.5	19.0	29	16.5	12.5
Sucrose	17.0	13.0	44.5	27.5	11.5

* θ_s of *Chromobacterium sp.* after 24 h or 48 h growth in batch culture.

not all show the same attachment response to growth with a given medium composition. The organism least susceptible to large changes in attachment after growth in different nutrient conditions was *Flexibacter sp.* (Fig. 1e). *Chromobacterium sp.* showed considerable differences in attachment after growth for 24 h and for 48 h in most conditions tested; for example, after growth for 24 h in glucose- and nitrogen-sufficient medium, attachment was approximately a third of that after 48 h growth (Figs 1c and 1d).

The extent of attachment to the PD and TCD surfaces was independently variable with the culture conditions, e.g. *E. cloacae* after growth in galactose attached to TCD in numbers approximately fourfold higher than those obtained with PD whereas after growth in sucrose medium there was only a slight difference between the two surfaces (Fig. 1b).

Differences in bacterial cell surface characteristics were demonstrated by saline contact angle (θ_s) measurements on lawns of cells after growth in the different media (Table 1). Thus, *P. fluorescens* showed a range of θ_s from 17.5° after growth in glucose- and nitrogen-sufficient media to 8.5° when mannose was the carbon source (Table 1). The largest differences were shown by *Chromobacterium sp.*, which had θ_s of 75° after 24 h growth in galactose and 10.5° after 24 h growth in lactose (Table 1). This bacterium was, in general, the most hydrophobic. The θ_s for *Chromobacterium sp.* varied not only with growth condition, but also with culture age (i.e. after 24 h and 48 h growth; Table 1).

Effect of growth rate on bacterial attachment and cell surface characteristics

The effect of dilution rate in continuous culture on bacterial attachment in buffer varied with the organism as did its effect on bacterial cell surface characteristics, as measured by HIC and EIC (Table 2). The extent of attachment after both 5 and 60 min was affected by bacterial growth rate, although the differences were more pronounced at 60 min. *E. cloacae* and *Chromobacterium sp.* (both of which often flocculated in PYE) showed the largest variations in cell surface characteristics, while the cell surface characteristics of the two non-flocculating species, i.e. *P. fluorescens* and *Flexibacter sp.*, were more stable (Table 2a, d).

These two categories of flocculating and non-flocculating bacteria could be further subdivided, as one member of each category attached in large numbers, while relatively few cells of the other member attached to the solid surfaces. Thus, *P. fluorescens* and *E. cloacae* (Table 2a, b) attached in relatively low numbers to both surfaces at all dilution rates. Yet the fluctuations in these low attachment levels were accompanied by changes in HIC and EIC values, indicating changes in cell surface characteristics. By contrast, *Chromobacterium sp.* and *Flexibacter sp.* showed increased attachment to both surfaces with increases in dilution rate, reaching quite high attachment numbers at the two highest dilution rates. HIC and EIC values, and thus cell surface characteristics, also varied with dilution rate (Table 2c, d).

Table 2. *Effect of growth rate on cell surface characteristics, measured by interaction chromatography and attachment of the isolates*

Species	Chemostat dilution rate (h ⁻¹)	Interaction chromatography (percentage of counts retained in columns)			10 ³ × A ₅₉₀ of cells attached to:*			
		Hydrophobic chromatography column	Anion exchange resin	Cation exchange resin	PD		TCD	
					5 min	60 min	5 min	60 min
(a) <i>P. fluorescens</i>	0.05	16.1	16.5	83.0	12 (± 2)	25 (± 3)	21 (± 3)	27 (± 3)
	0.1	17.9	10.2	85.9	6 (± 3)	30 (± 7)	14 (± 0.8)	25 (± 3)
	0.15	12.0	2.3	77.5	9 (± 0.8)	20 (± 2)	19 (± 2)	32 (± 3)
	0.2	14.6	8.9	84.1	6 (± 2)	32 (± 4)	13 (± 2)	21 (± 3)
(b) <i>E. cloacae</i>	0.05	45.0	33.1	80.1	8 (± 2)	31 (± 3)	19 (± 3)	43 (± 4)
	0.1	79.9	79.0	92.9	7 (± 0.8)	18 (± 2)	17 (± 0.8)	31 (± 0.5)
	0.15	86.2	1.8	62.8	3 (± 3)	5 (± 3)	17 (± 3)	24 (± 3)
	0.2	6.4	1.8	54.4	5 (± 0.8)	8 (± 0.5)	17 (± 2)	22 (± 4)
(c) <i>Chromobacterium</i> sp.	0.05	10.0	32.3	6.7	14 (± 3)	34 (± 6)	16 (± 3)	13 (± 4)
	0.1	32.4	24.0	21.6	14 (± 0.8)	37 (± 2)	25 (± 2)	39 (± 3)
	0.15	61.4	54.7	94.9	21 (± 4)	70 (± 3)	23 (± 4)	59 (± 5)
	0.2	30.0	44.4	94.1	27 (± 3)	84 (± 8)	29 (± 0.8)	56 (± 4)
(d) <i>Flexibacter</i> sp.	0.05	73.1	87.6	62.4	9 (± 2)	15 (± 3)	7 (± 3)	7 (± 3)
	0.1	60.2	91.5	87.0	13 (± 2)	51 (± 5)	13 (± 2)	76 (± 7)
	0.15	44.2	99.9	100	10 (± 0.8)	101 (± 17)	17 (± 3)	90 (± 15)
	0.2	55.7	62.7	73.5	32 (± 3)	108 (± 23)	25 (± 3)	111 (± 10)

* Values in parentheses are 95% confidence limits of the mean ($n = 8$).

As with attachment after growth in different nutrient conditions (above), the attachment to PD and TCD surfaces after growth at different dilution rates was independently variable. For example, after 60 min attachment *Chromobacterium* sp. showed consistently higher attachment values to PD than to TCD at most dilution rates; however, at $D = 0.1 \text{ h}^{-1}$ there was no apparent difference between the two surfaces (Table 2c).

Effect of chloramphenicol, sodium periodate and protease on bacterial detachment from solid surfaces

The inclusion of chloramphenicol ($5 \mu\text{g ml}^{-1}$), an inhibitor of protein synthesis, in the detachment suspension did not cause removal of attached cells (Table 3). This was true for all four species and for both PD and TCD surfaces. Indeed, with *P. fluorescens* and *E. cloacae* the presence of chloramphenicol seemed to stabilize the film, reducing detachment compared to the control (Table 3).

In general, attachment to the TCD surface appeared to be more stable than that to the PD surface. Only *P. fluorescens* (Table 3) showed increased detachment from TCD for only protease

Table 3. *Effect of chloramphenicol, sodium periodate and protease on detachment of the isolates*

I_d values were calculated as the ratio of the A_{590} ($\times 10^3$) of the test substratum to that of the relevant control substratum. I_d values of 1 were recorded for treatments whose 95% confidence limits of the mean ($n = 8$) overlapped with that of the controls.

Treatment	<i>P. fluorescens</i>		<i>E. cloacae</i>		<i>Chromobacterium</i> sp.		<i>Flexibacter</i> sp.	
	PD	I_d	TCD	I_d	PD	I_d	TCD	I_d
Chloramphenicol (5 $\mu\text{g ml}^{-1}$)	1.5		1.1		1.35		1	
Sodium periodate (1%, w/v)	1		1		0.59		1	
Protease (1 unit per 5 ml)	0.4		0.6		0.29		1	

Table 4. *Effect of nutrients on the attachment of P. fluorescens and Chromobacterium sp. to PD and TCD after growth in continuous culture at various dilution rates*

I_d values were calculated as the ratio of the A_{590} ($\times 10^3$) of the test substratum to that of the relevant control substratum. I_d values of 1 were recorded for treatments whose 95% confidence limits of the mean ($n = 8$) overlapped with that of the controls.

Species	Dilution rate (h^{-1})	$\frac{1}{2}$ PYE				PYE			
		PD I_d		TCD I_d		PD I_d		TCD I_d	
		5 min	60 min	5 min	60 min	5 min	60 min	5 min	60 min
(a) <i>P. fluorescens</i>	0.05	1.3	1.76	1	2.4	1	1	1	1.9
	0.1	2.7	1	1.4	2.36	1	1	1.86	2.48
	0.15	1.22	1	1	1	1.67	0.55	1.42	1
	0.2	1	0.18	1	1	1	0.16	1.54	1
(b) <i>Chromobacterium</i> sp.	0.05	1.64	2.2	1	1	1	2.53	0.56	0.38
	0.1	1	1.89	1	1	1	1.46	0.72	0.54
	0.15	1	1.18	1	1.29	0.43	0.54	1	0.41
	0.2	0.74	0.75	0.86	1.21	0.33	0.6	0.66	0.86

treatment. Protease also removed attached cells of *P. fluorescens*, *E. cloacae* and *Chromobacterium* sp. from the PD surface. Sodium periodate (1%, w/v), which would denature exopolysaccharide at cell surfaces, detached both *E. cloacae* and the *Chromobacterium* sp. (Table 3) from the PD surface. The detachment of the *Flexibacter* sp. (Table 3) did not increase with any of the treatments or surfaces.

Effect of bacterial growth rate and the presence of complex nutrients on bacterial attachment

Two concentrations of PYE medium were used to determine the effect of complex nutrients on bacterial attachment. There was a large variation in the effects depending upon surface, organism and bacterial growth rate (Table 4) (NB the data in Tables 4 and 2 were obtained using the same culture and attachment procedures and can thus be directly compared).

The PD I_d and TCD I_d values indicated that either an increase or a decrease in attachment could result from the presence of nutrients, depending on the solid substratum and the concentration of nutrients present. Moreover, the 5 min and 60 min I_d values for the two surfaces did not necessarily show the same effect, e.g. the I_d value for attachment of *P. fluorescens* to PD ($D = 0.15 \text{ h}^{-1}$) with PYE and 5 min attachment showed an increase in attachment over the control, but at 60 min there was a reduction in I_d with PD (Table 4a). An example of the effect of growth rate on the type of effect was observed in *Chromobacterium* sp. attached for 60 min in PYE, which showed an increase in attachment to PD at a growth rate of 0.05 h^{-1} but a decrease at $D = 0.15 \text{ h}^{-1}$ (Table 4b). The treatments also affected the rate of increase in attachment from 5 min attachment to that at 60 min, e.g. *Chromobacterium* sp. ($D = 0.05 \text{ h}^{-1}$) showed a far larger rate of attachment in PYE than in the buffer control (Table 4b). *E. cloacae* showed similar types of variation in attachment in the presence of PYE media.

The only species to show a consistent relationship between attachment and nutrient concentration was *Flexibacter* sp. The presence of nutrients at all but the lowest growth rate, where there was no apparent effect, caused a decrease in attachment to both PD and TCD surfaces after 60 min attachment.

Water contact angle measurements (θ_{H_2O}) on clean PD and TCD were 90° and 59° respectively. Nutrient-treated PD and TCD had reduced θ_{H_2O} ; after $\frac{1}{2}$ PYE treatment θ_{H_2O} was PD 72.5° and TCD 18.5° and after PYE treatment θ_{H_2O} was PD 80° and TCD 13° . The liquid surface tension of HEPES, $\frac{1}{2}$ PYE and PYE was 7.0, 6.6 and 6.4 J cm⁻² respectively.

DISCUSSION

There was a wide variation in levels of bacterial attachment to the solid substrata PD and TCD for *P. fluorescens*, *E. cloacae*, *Chromobacterium* sp. and *Flexibacter* sp. with changes in carbon source and growth rate. The composition of the growth medium has been found previously to be important in subsequent levels of bacterial adhesion, though the basis for the effect was largely unexplained (Zvyagintsev, 1959). Differences in the attachment of *Chromobacterium* sp. were found after growth for 24 h and 48 h in batch cultures with different carbon source and carbon:nitrogen ratio. This illustrates the significance of growth phase in the attachment process, an effect previously demonstrated (Fletcher, 1977).

The variations in levels of attachment for the isolates were accompanied by changes in physico-chemical properties of the bacterial cell surfaces detected by changes in θ_s and HIC and EIC interactions. Thus, phenotypic changes in surface properties may lead to changes in attachment ability, but these responses can vary with species. There was no general relationship between growth conditions, cell surface adhesives and attachment ability that was applicable to all species. The phenotypically-induced changes in the cell surface physico-chemistry of the four bacterial species were almost certainly related to changes in macromolecular composition and structure of the cell surfaces. Differences in cell surface macromolecular composition have been shown previously to affect bacterial attachment (Hogt *et al.*, 1982; Pringle *et al.*, 1983).

Depending on the type (e.g. hydrogen bonding, charge and hydrophobic interactions) and number of physico-chemical interactions between the solid surface and a bacterial surface, attachment may be increased or decreased by changes in the bacterial surface if adhesive interactions are increased or reduced, respectively. TCD has a higher negative charge and is more hydrophilic than PD and, therefore, adhesive interactions between the two substrata and a particular cell surface would be different. Thus, changes in bacterial surface characteristics could be expected to affect differentially the levels of attachment to the PD and TCD surfaces, as was observed.

The synthesis of protein did not appear to be a significant feature in maintaining adhesion for any of the freshwater isolates investigated once they had attached to the substratum, as the presence of chloramphenicol caused no detachment. Chloramphenicol, however, by inhibiting the production of enzymes, may interfere with the synthesis of other potentially adhesive molecules, such as polysaccharides. This suggests that the synthesis of fresh cell wall material is not required to maintain adhesion over short periods, i.e. 1 h. Elsewhere, inhibitors of protein synthesis have been shown both to have no effect on bacterial adhesion (Marshall, 1974) and to inhibit adhesion (Paul, 1984).

The significance of exopolysaccharides in adhesion was evaluated by treatment with sodium periodate, which denatures polysaccharides by oxidizing and cleaving vicinal hydroxyl groups characteristic of carbohydrate molecules. The possibility of a protein adhesive was tested by treatment with a non-specific protease. The effects of periodate and protease on bacterial detachment differed between the three non-gliding bacteria and *Flexibacter* sp., and with the solid substratum. Generally, *Flexibacter* sp. detached least. The effect of proteolytic enzymes has been found previously to vary with bacterial species and substratum (Fletcher, 1980). Periodate treatments have been used to detach successfully several bacterial types (Corpe, 1974a; Fletcher, 1980).

Apparently, both proteins and exopolysaccharides were involved in the attachment of *E. cloacae* and *Chromobacterium* sp. to the PD surface because both protease and sodium periodate treatments increased their detachment. *P. fluorescens* showed detachment from both surfaces in the presence of protease but not with periodate, suggesting that proteins and not exopolysaccharides may have been significant contributors to the attachment of this species. However, the lack of bacterial detachment after protease or periodate treatment, e.g. for *Flexibacter* sp., need not indicate necessarily that proteins or polysaccharides were not involved in the bacterial attachment interactions. The particular configuration adopted by cell surface proteins interacting with a substratum, and the closeness of the interaction may have caused differences in the efficacy of degradative enzymes. The type of proteolytic enzymes used in desorption studies has been found to affect detachment differently (Corpe, 1974*a*), as has the temperature of incubation during the desorption (Fletcher & Marshall, 1982). Further, periodate does not degrade all carbohydrates, requiring the presence of vicinal hydroxyl groups, and it can also be excluded by highly charged polymers (Rambourg, 1971).

Attachment of the four freshwater bacteria was less disrupted by protease and periodate on the TCD than on the PD surface. The more hydrophilic TCD surface may have produced firmer bacterial adhesion than the relatively hydrophobic PD substratum since hydrophilic surfaces, i.e. high energy high polarity surfaces, tend to absorb macromolecules more strongly than low energy hydrophobic surfaces (Baier, 1981). It is possible that different bonding types or different macromolecular constituents were involved in attachment interactions with the different solid substrata. Separate attachment mechanisms for PD and TCD have previously been suggested (Fletcher & Marshall, 1982). Alternatively, adhesion to the hydrophilic TCD surface may have resulted in a molecular configuration of cell surface groups less accessible to disruption.

The presence of nutrients modified levels of bacterial attachment to the solid surfaces, the effect varying with the nature of the substratum and the particular bacterial species involved. Inhibition of attachment has been found with undefined macromolecules (Fletcher & Loeb, 1979) and with several different proteins (Fletcher, 1976). In some cases protein has promoted adhesion (Meadows, 1971).

The presence of PYE in the liquid phase would have two physico-chemical effects. First, the liquid surface tension changes because of the interaction between water molecules and solutes, which varies with the solute and its concentration; e.g. $\frac{1}{2}$ PYE and PYE had different surface tensions. The influence of liquid surface tension on bacterial attachment has been discussed by several workers (Fletcher & Pringle, 1985), particularly in relation to thermodynamic predictions of bacterial attachment (Absolom *et al.*, 1983). Second, a conditioning layer will form on the substrata, an interaction favoured by a reduction in interfacial free energy, dictating the surface characteristics presented to the bulk phase.

Conditioning film formation on PD and TCD by PYE was indicated by θ_{H_2O} measurements, though the θ_{H_2O} values cannot be directly related to surface energies since the films were air-dried. The θ_{H_2O} changes on TCD were greater than those on PD and both varied with the concentration of PYE solutions, possibly indicating differences in the conditioning film.

The decreased and increased levels of bacterial adhesion for some of the PYE treatments may be explained, partially, by the effects of a conditioning film. For example, a reduction of interfacial free energy (Fletcher & Marshall, 1982) and osmotic effects (Lips & Jessup, 1979) caused by a conditioning film would tend to lower attachment, while polymer bridging (Lips & Jessup, 1979) and a change in substratum bonding capacity might increase attachment. The effect of PYE on bacterial adhesion, differed between 5 min and 60 min attachment. Conditioning films increase in thickness with time (up to a limiting thickness) (Baier, 1980), and this may have resulted in the differences in attachment with incubation time.

If physiological activity was involved in bacterial adhesion (Fletcher & Marshall, 1982), then the presence of nutrients in the attachment suspension and the increased growth rate in the chemostat cultures may have affected bacterial adhesion through changes in bacterial physiological processes and activity. However, increased bacterial physiological activity did not necessarily increase subsequent bacterial attachment. The major role of physiological activity in bacterial adhesion, for the species investigated, appeared to be indirect, through changes in cell

surface characteristics. As the cell surface characteristics of the bacteria changed with growth rate so did the attachment interactions when PYE was present in the suspending medium (bacterial cell surface characteristics shown in Table 2 were those of the cells undergoing adhesion in the PYE media, Table 4). Physiological activity, however, may have been more closely related to the attachment of the *Flexibacter* sp., the gliding bacterium, since its levels of attachment did increase with growth rate.

As the surface characteristics of the isolates changed there was a subsequent change in their adhesion to solid surfaces. However, no one physico-chemical cell surface property correlated with attachment numbers nor thus appeared dominant in the attachment interactions. This suggests a balance of physico-chemical factors were probably involved in net attachment (or detachment). Observations on the adhesion of other bacterial species have also suggested the involvement of more than one type of adhesive interaction (Feldner *et al.*, 1983; Kjelleberg & Hermansson, 1984).

The different patterns in bacterial adhesion to a substratum shown by *P. fluorescens*, *E. cloacae*, *Chromobacterium* sp. and *Flexibacter* sp. in response to changes in growth rate, nutrient conditions, the presence of organic molecules during attachment and the substratum underlines a major difficulty in the extrapolation of laboratory data to environmental conditions. However, it is probable that the changes in nutrient conditions in natural aquatic habitats will affect the attachment of individual bacterial species differently, resulting in differences in the species composition of a newly developed biofilm. There is some evidence for this both in marine and in freshwater environments (Corpe, 1974*b*; Baker, 1984) and in river water chemostat enrichment cultures (Ellwood *et al.*, 1982; Wardell *et al.*, 1980).

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