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Bacterial adhesion to inert thermoplastic surfaces

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The adhesion of four bacterial species, two strains of each, to four hydrophobic thermoplastics was observed. Image analysis of adherent cells stained with acridine orange provided a rapid, direct and objective means of measuring adhesion to clear, translucent and opaque surfaces by calculating the percentage area of a microscopic field covered by cells (percentage coverage). There was a highly significant correlation ($P > 0.05$) between percentage coverage value and adherent cell count (obtained manually) for both rods and cocci. Bacterial adhesion to thermoplastics appeared to be strain specific and was not related to polymer composition. Highest percentage coverage values were obtained using hydrophobic bacteria, and lowest using hydrophilic bacteria. There was no relationship between the origin of the organisms (culture collections or isolates from biomaterial-associated infections) and their ability to adhere, after cultivation in brain heart infusion broth. Many factors influence this ability: an awareness of all experimental variables is essential.

1. Introduction

Although there have been numerous studies on the adhesion of micro-organisms to inert surfaces such as plastics, glass and stainless steel [1–6] they tend to concentrate on specific applied areas of microbiology – oral, medical, environmental, industrial – and hence to particular micro-organisms.

Methods used for the quantification of adherent microorganisms *in situ* tend to use transparent surfaces and light microscopy for visualization [4] while restricting the study to one genus/species. Many surfaces *in vitro* are opaque: this paper describes and investigates the validity of a method for measuring the adhesion of rods and coccal bacteria to opaque surfaces.

Bacterial adhesion to a naked substratum is governed by an interplay of physicochemical interactions such as hydrophobicity [7, 8], surface free energy, surface charge [9, 10], zeta potential and electrostatic interactions [11].

The work described in this paper uses bacteria from a range of sources (culture collections and 'wild' isolates), and plastics with a variety of applications. Thus underlying similarities or differences in the adhesion process might be discerned.

2. Materials and methods

2.1. Micro-organisms

Four bacterial genera were selected to represent micro-organisms associated with the contamination of biomaterials *in vivo*: two coagulase negative staphylococci (*Staphylococcus hominis* from a catheter exit site, kindly provided by the Royal Manchester Children's Hospital, and *Staphylococcus epidermidis* NCTC 11047 [12]); *Streptococcus oralis* NCTC 11427 (kindly provided by Dr D Beighton, Kings College School of Medicine and Dentistry, London); *Streptococcus oralis* isolated from blood (kindly provided by Withington Hospital, Manchester); *Pseudomonas aeruginosa* isolated from a contact lens, associated with a corneal ulcer (kindly provided by The Manchester Royal Infirmary); *Pseudomonas fluorescens*, environmental isolate from an airport (kindly provided by the John Radcliffe Hospital); *Escherichia coli* ATCC 0157 [9] and *Escherichia coli* isolated from urine with a catheter *in situ* (kindly provided by the Royal Manchester Children's Hospital).

The cell surface hydrophobicities of test strains was determined using n-hexadecane adhesion [13,14] and contact angle measurement using the captive bubble technique [15, 16]. Both of these methods measure the avidity or otherwise of the cells (hexadecane adhesion), or dense layers of cells on a membrane filter (captive bubble technique), for water. All cultures were stored on beads (Microbank, Pro-Lab Diagnostics) at $-70\,^{\circ}C$. Cultures were grown to stationary phase on brain heart infusion (BHI) (Oxoid Ltd, Basingstoke, UK) slopes, except *Streptococcus oralis* which was maintained on 5% horse blood–BHI plates. Cultures on slopes were maintained at $+4\,^{\circ}C$ and replaced monthly with fresh cultures from beads, thus subculturing was kept to a minimum.

2.2. Thermoplastics

Four smooth surface thermoplastics of known composition were used. Polymethyl methacrylate (PMMA) was supplied by ICI (Acrylics Division, Darwen UK); polyethylene (PE) was manufactured by the

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Department of Materials Technology at the Manchester Metropolitan University; polyvinyl chloride with and without plasticizer (PVC ± P) was kindly provided by European Vinyl Corporation, Runcorn UK. Each material, 2 mm thick, was cut into 10 × 10 mm squares, dipped in 70% alcohol and stored dry at room temperature until use. Direct contact angle measurement (Cahn Instruments, California, USA) in water and bromonaphthalene were obtained to determine the advancing and receding contact angle and surface free energy for each material.

Talysurf (Rank-Taylor Hobson) equipment was used to obtain roughness (R_a) data [17].

2.3. Adhesion assay [18]

Bacteria were incubated for 18 h in BHI broth at 37°C without agitation. Cells were harvested and washed three times in phosphate buffered saline (PBS), pH 7.2, containing (g/l): NaCl, 19.29; KCl, 0.224; Na_2HPO_4, 1.192; KH_2PO_4, 0.218. Bacteria were resuspended in the same buffer to an optical density of 1.0 (540 nm), corresponding to the following colony forming units (cfu) $\times 10^8$ per ml, as determined by culturing and plate counting: streptococci 3.67 ± 0.98; staphylococci 3.33 ± 0.66; *E. coli* 2.3 ± 1.07; pseudomonads 2.15 ± 1.03. Test plastic squares were each incubated with 2 ml of cell suspension for 1 h at 37 °C without agitation, and were then removed from the suspension, rinsed twice in 5 ml of PBS and sonicated for 1 min in an ultrasonicating water bath (Dawe Instruments Ltd., USA) to remove any loosely bound cells. The test squares with adherent bacteria were air dried lying horizontally. Three replicates of each type of thermoplastic were used in each assay, and each adhesion assay was repeated twice for all bacteria used in the study, using organisms cultured under identical conditions.

2.4. Epifluorescent staining of bacteria

The test squares were dipped in methanol for 1 min to fix adherent cells, air dried and stained for 2 min in 0.03% acridine orange in 2% acetic acid [19]. They were then dipped into distilled water to remove excess stain, air dried and attached to microscope slides using petroleum jelly. Adherent bacteria were observed using epifluorescent incident beam microscopy (Leitz) at × 1000 magnification (oil immersion).

2.5. Image analysis

The system utilized a Victor 286S computer (Digithurst Ltd., Royston, Herts.) and a Vista low light monochrome CCD camera (Saville Group Ltd., Salford) to display images onto a separate monitor. Once cells are visible on the monitor screen (area of 0.0063 mm^2), the image is grabbed.

Thresholding of the image instructs the system on the features to be measured, whereby cells are extracted from a monochrome image because their grey-shades lie between predetermined ranges.

The coverage of material by bacteria on each test square, given as a percentage area, was measured for 20 microscopic fields selected at random. This was taken as an indication of the adhesive ability of the organism.

2.6. Validity of percentage coverage

To relate percentage area coverage figures to cell number for each of the four microbial genera used in this study, adhesion experiments were performed using PMMA only and one strain of each genus. Adherent cells were counted for each field for which a percentage area coverage figure was obtained. One hundred measurements were taken and a scatter plot constructed. Differences between treatments were tested via a one-way analysis of variance with the student Newman–Keule procedure for a multiple range test.

3. Results

As expected the percentage coverage data correlated with counts ($p < 0.05$) of adherent cells (Fig. 1a–d) for all genera. The concentrations of all standardized cell suspensions did not vary significantly. Correlation coefficients were as follows: streptococci 0.97, staphylococci 0.96, *E. coli* 0.87, pseudomonads 0.83. Cocci adhered in much higher numbers than rods, and also gave overall higher percentage coverage values. However, for the same number of adherent cells, rods tended to give a higher percentage value, accounted for by the larger cell size. Thus the importance of standard curves for test species is emphasized. Of the strains used in this study, the Gram positive cocci were more hydrophobic than the Gram negative rods (Table I). The medical strain of *E. coli* was the least hydrophobic/most hydrophilic. The two methods used to measure hydrophobicity gave similar results: captive bubble contact angle measurements followed a sequence of relative hydrophobicity (higher contact angle, lower hydrophobicity) comparable ($p < 0.05$), but not identical to hexadecane partition data. Contact angle measurements were similar for the four thermoplastics used (Table II). Surface roughness (R_a) values for polyethylene, which exhibited an undulating surface profile, were highest. For the other materials R_a values were broadly comparable for the purpose of this study, since differences in contact angle measurements for materials with $R_a < 0.1$ μm are believed to be due to differences in polymer composition rather than difference in surface roughness [20]. The overall percentage coverage of eight strains of bacteria (Gram positive/Gram negative cocci/rods) to four thermoplastics (Fig. 2) varied more between bacteria than between surfaces.

There was no significant difference between the percentage coverage of *S. hominis*, *P.fluorescens* or *E.coli* (medical isolate) on any surface ($p < 0.05$), but the streptococci (most hydrophobic of strains tested) gave percentage coverage values which were significantly higher than those for any other bacteria ($p > 0.05$). The use of different plastics had no significant effect on adhesion ($p < 0.05$), although adhesion to polyethylene was often highest, which may have been due to the increased surface roughness [13] (or greatest hydrophobicity).

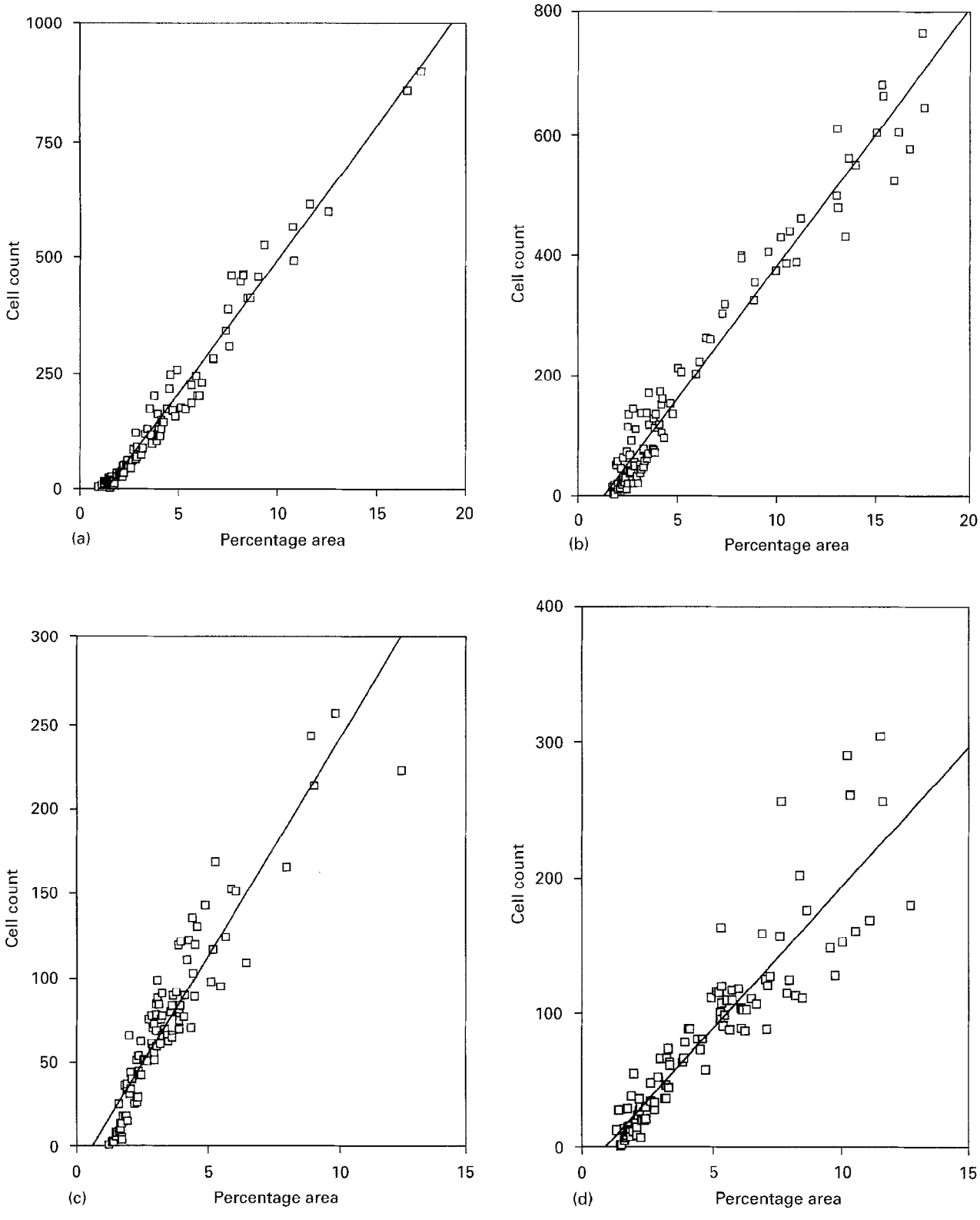

Figure 1 The relationship between percentage area coverage (%) of microscopic field by cells and the number of adherent cells in the same field ($n = 100$) (a) *Streptococcus oralis* NCTC 11427; (b) *Staphylococcus epidermidis* NCTC 11047; (c) *Escherichia coli* ATCC 0157; (d) *Pseudomonas aeruginosa* (medical strain).

4. Discussion

Image analysis has provided a rapid, objective way to measure the amount of microbial adhesion on transparent surfaces [19, 21, 22]. Acridine orange staining of adherent cells viewed under incident beam epifluorescence microscopy allows the use of opaque substrata, and the method is validated by excellent correlation with counts of adherent cells, as used in independent studies [4]. It has been suggested that the colour of bacteria (orange-green) stained with acridine orange is associated with their viability, but experimental variables also exert an effect [23–25]. In this study the stain was used to indicate the presence of cells: the complex issue of cell viability–or otherwise–was not taken into consideration [12]. Calibration curves do not pass through zero because the image analysis system may detect debris giving very low percentage coverage figures, while counting cells

TABLE I The hydrophobicity of bacteria measured via bacterial adherence to hexadecane, and captive bubble contact angle measurement in water ($n = 3$)

Bacteria	Percentage adhesion to hexadecane		Air contact angle (°)	
	Mean	SD	Mean	SD
S. oralis NCTC 11427	95	0.35	81	1
S. oralis (medical isolate)	94	0.5	84	3
S. epidermidis NCTC 11047	76	4.6	90	5
S. hominis (medical isolate)	81	3.7	117	8
P. aeruginosa (medical isolate)	54	0.5	127	3
E. coli 0157	57	0.5	132	2
P. fluorescens (environmental isolate)	39	1.7	140	3
E. coli (medical isolate)	30	0.15	149	5

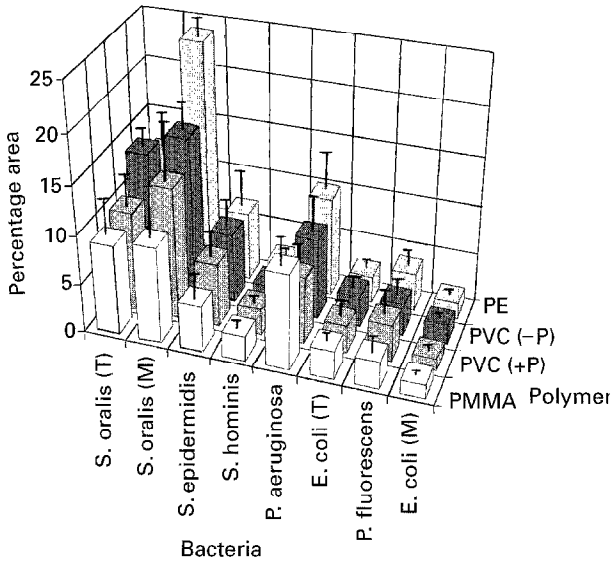

Figure 2 Bacterial adhesion to thermoplastics expressed as percentage area coverage (% coverage). Mean deviations are shown in the figure. Bacteria are listed in order of decreasing hydrophobicity (air contact angle measurement) from left to right. (Abbreviations: M - medical strain; T - type strain; E - environmental stain; S. ora - *S. oralis*; S. hom - *S. hominis*; S. epi - *S. epidermidis*; P. aeru - *Ps. aeruginosa*; P. fluor - *Ps. fluorescens*

gives zero. The effect of different cell area of rods compared with cocci is difficult to account for, because rods also adhered lengthwise (giving rod shaped area) or end on (giving coccal shaped area). Accordingly, the correlation coefficients for Gram negative rods are slightly lower than those for the cocci. Also, when clumping (three-dimensional) was observed for rods or cocci, the area covered was less than that which would have been obtained by a monolayer (two-dimensional) of well separated cells. Nevertheless, image analysis is providing rapid and useful measurements.

Surface hydrophobicity has been generally acknowledged to be one of the factors contributing to a cell's ability to adhere to another surface [7,26]. In this study, and others [27,28] the degree of preference of strains for substrata of similar hydrophobicity was strain dependent. As has been shown in other studies, smooth thermoplastics of different chemical composition but comparable hydrophobicities [9,28] behaved similarly as inert substrata for bacterial adhesion. It would have been useful to have included more widely differing surfaces. Slightly elevated levels of adhesion to polyethylene were probably due to an increased surface roughness [17]. Increases in surface roughness of a substratum would also interfere with contact angle measurement. Other studies in our laboratories indicate that adhesion to hydrophilic material using the same strains is minimal [29].

For the bacteria, Gram positive cocci were more hydrophobic than Gram negative rods. The most hydrophobic of all strains tested (the streptococci) adhered to the plastics in highest numbers (and highest percentage coverage). Hydrophobic strains of oral streptococci have been shown to adhere in higher numbers to hydrophobic rather than to hydrophilic surfaces [30], but very few studies have compared genera as well as strains/species and substrata. The use of a number of variables provides an interesting overview, but limits the number of strains which can be

TABLE II Properties of thermoplastics

Thermoplastic	Advancing contact angle (distilled water) [a] (°)	Receding contact angle (distilled water) [a] (°)	Surface energy[b] (Dynes/cm)	R_a^c (μm)
PMMA	63	41	54	0.012
PVC (with plasticizer)	78	60	48	0.110
PVC (without plasticizer)	72	61	50	0.035
Polyethylene	86	61	46	0.620

[a] Contact angle measurements measured in distilled water using a dynamic contact angle.
[b] Surface energy measures in distilled water and bromonaphthalene using a dynamic contact angle.
[c] R_a = centre line average. The arithmetic mean of departures of the roughness profile from a mean line. The value given by automated surface measurement is an average of five measurements.

tested. The existence of a relationship between the hydrophobicity, surface structure and adhesive ability of type cultures and more recent isolates has been explored more fully in other studies using more strains of a given species, but few trends have been identified, [28, 31–34].

For two of the genera used in this study, *Staphylococcus* and *Pseudomonas*, the hydrophobicity of test strains used were very different. If hydrophobicity were a dominant feature dictating adhesion, then the identity of the organism would be of less significance. This does not appear to be the case [8, 28, 33, 35]. Clearly other factors such as habitat and physiology are prime determinants for surface colonisation. Additionally *in vivo* inert substrata can become conditioned with biological molecules [37] thus facilitating more specific cell–surface adhesive interactions. *In vitro* for adhesion work, cells should also be cultured in media more representative of their *in vivo* environment [28]. Even the ionic strength of the suspending fluid can dictate the degree of electrostatic repulsion and thus alter adhesion both to thermoplastics and hexadecane [11]. The adhesion of washed cells to naked inert substrata has often been termed non-specific, since the cell–surface interactions are not entirely biological. Nevertheless, from this relatively simple system, more complex variables can be introduced.

The methods described above provide a valid, reproducible and accepted means for exploring variables affecting adhesion in diverse environments. The hydrophobicities of micro-organisms would appear to be one of the most variable factors, and perhaps one of lesser significance.

5. Conclusions

A method has been described which provides a valid, reproducible and accepted means for exploring variables affecting adhesion in diverse environments.

The adhesion of eight strains of four genera of bacteria of varying hydrophobicities to four different thermoplastics with similar hydrophobicities was compared. Although the more hydrophobic bacteria adhered in higher numbers than less hydrophobic strains, other factors such as strain and roughness of substratum surface appeared to exert effects. The hydrophobicities of micro-organisms would appear to be one of the most variable factors, and perhaps one of lesser significance. Differences observed in the behaviour of strains provides further evidence against overgeneralizations concerning microbial adhesion.

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