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The effects of pH, ionic strength and organic phase on the bacterial adhesion to hydrocarbons (BATH) test

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Summary

The effects of pH, ionic strength of suspending buffer and choice of organic phase upon the subsequent adherence of *E. coli* to organic phase in the bacterial adherence to hydrocarbons (BATH) test were investigated. It was found that both pH and ionic strength altered adherence of *E. coli* and the organic phase employed for the BATH test influenced the classification of adherence. Generally, *E. coli* showed pronounced affinity for chloroform and dichloromethane, and only intermediate affinity for xylene. Lowering pH and increasing the ionic strength of the suspending buffer resulted in increased adherence.

Introduction

The biological implications of hydrophobic interactions involving bacteria have been highlighted in a wide range of adhesion phenomena including: adhesion to hydrocarbons (Rosenberg et al., 1980), adhesion to glass (Absolom et al., 1982; Strong et al., 1982), adhesion to plastics (Hogt et al., 1985, 1986; Van Pelt et al., 1985), phagocytosis (Neumann et al., 1982) and adhesion to mineral surfaces (Satou et al., 1988).

It is widely agreed that bacterial adhesion to mucosal surfaces is the initial event in the pathogenesis of most infectious diseases (Martin et al., 1986; Galdiero et al., 1987; Romano et al., 1987; Weerkamp et al., 1990). The adhesion of pathogens to mucous membranes enables the bacteria to successfully avoid the flushing mechanisms which cleanse mucous membranes.

Bacterial and host cell surfaces, for example, epithelial tissue, have a net negative charge. However, the resultant electrostatic repulsion may be overcome by the van der Waals attractive forces of the hydrophobic groups on the surface of the bacterial and epithelial cells leading to adhesion. This is termed the DLVO (Deryagin, Landau, Verwey and Overbeek) theory. Having attached loosely to the epithelium this reversible association may be rendered irreversible via interactions by means of type 1 fimbriae, mannose-resistant haemagglutination-promoting fimbriae and glycocalyces on the microbial cell and corre-

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sponding receptors on the epithelial cell surface (Beachey, 1981; Harber, 1985; Jones et al., 1991).

Therefore, bacterial adherence to surfaces may be described as consisting of two phases: the initial phase (reversible) due to an attraction between bacteria and the surface, whereas the second phase (irreversible) involves adhesion of bacteria to the surface by means of specific structures on the surface of the bacteria (Marshall et al., 1971).

Various methods have been employed to investigate the cell surface hydrophobicity (CSH) of bacteria: hydrophobic interaction chromatography (Romano et al., 1987) and electrostatic interaction chromatography (Pedersen, 1980; Stenstrom, 1989), salt aggregation test (Przondo-Hessek et al., 1987), contact angle measurement (Minagi et al., 1986), and bacterial adhesion to hydrocarbons (BATH) (Rosenberg et al., 1980). However, there are inherent limitations of some of these methods which reduce their reproducibility (Van Loosdrecht et al., 1987).

Much work has investigated factors such as growing conditions of organisms (Bandin et al., 1989; Malouin et al., 1991) or pretreatment of organisms with various agents such as chlorhexidine diacetate and benzalkonium chloride (El-Falaha et al., 1985), amphotericin B, miconazole, ketoconazole and azalomycin F (Miyake et al., 1990), piperacillin, cefotaxime, ceftrixone, netilmicin and aztreonam (Savoia et al., 1990), taurolidine, chlorhexidine acetate and povidone-iodine (Jones et al., 1991) before investigating CSH. However, few studies have looked at the physical properties of the BATH test while determining CSH. Nesbitt et al. (1982) have studied the effect of temperature, ionic strength, pH and ion species present, whereas other authors have reported the effect of ionic strength (Ofek et al., 1983; Rosenberg, 1984; Galdiero et al., 1987). However, a detailed investigation of the effect of physical parameters on the BATH test has yet to be performed.

The aim of this investigation was to examine the effects of pH, ionic strength and choice of organic phase on the BATH test by means of a factorial design and appropriate statistical analysis.

Materials and Methods

Bacteria

Escherichia coli ATCC 29214 was obtained from the Dunedin Public Hospital, New Zealand, and maintained on nutrient agar (Difco, MI, U.S.A.) slopes at 4°C.

Bacterial growth and collection

One loopful of the organism was transferred into nutrient broth (Difco, MI, U.S.A.) for incubation at 37°C in a shaking water bath for 18 h at 100 oscillations/min. Cultures were centrifuged at $3300 \times g$ for 15 min and the deposit washed three times with and resuspended in the relevant buffer to the required cell density (10^9 cells/ml).

Assessment of bacterial hydrophobicity

A modified version of the BATH test by Rosenberg et al. (1980) was employed to investigate the effect of pH, organic phase and ionic strength by means of a complete factorial design. In brief, 6 ml of a suspension of *E. coli* in McIlvaine buffer at pH 2.2, 4.0 or 7.4 and adjusted to ionic strength (either 0.5 o 1.0 M) with KCl, was added to six different volumes (0.05–0.7 ml) of organic phase (either chloroform, dichloromethane or xylene) in test tubes (internal diameter 10 mm, tapered bottom). The resulting mixture was vortex-mixed on an MT19 auto vortex mixer (Chiltern, Wellington, New Zealand) at constant speed for 5 min (setting 8). After equilibration for 15 min at room temperature (25°C), to allow for phase separation, the aqueous phase was removed and the absorbance measured at 400 nm. Cell concentration was expressed as a fraction of the initial concentration of the aqueous layer and plotted against the volume of the organic phase, corrected for solvent dissolution into the aqueous phase. All experiments were performed in triplicate.

Statistical analysis

For the purpose of statistical analysis the average fraction remaining with 0.45 ml of organic phase was calculated. Statistical analysis were performed using Statview (Abacus Concepts Inc., CA, U.S.A.). Testing for significant differences

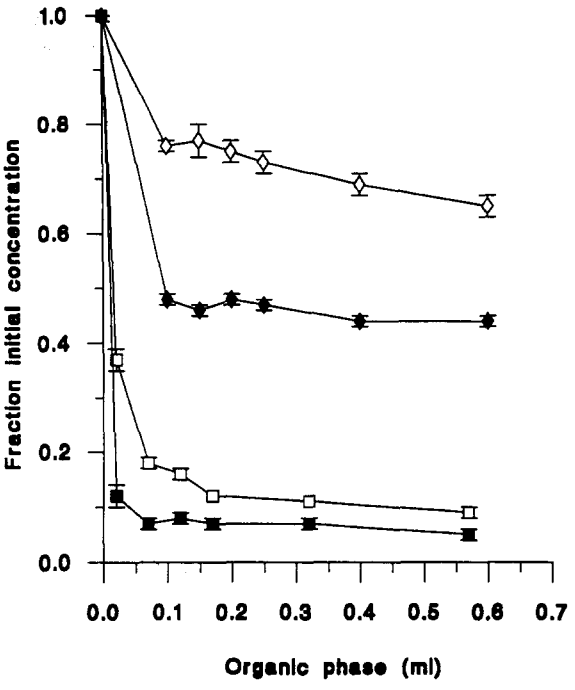

Fig. 1. Effect of organic phase volume on the adhesion of *E. coli* to xylene (diamonds) and chloroform (squares), using a pH 7.4 buffer at 0.5 M (open) and 1.0 M (filled) ionic strength. Standard error bars ($n = 3$) are shown.

between individual pairs of means was performed using Tukey's HSD test.

Results

Typically it was found that *E. coli* adherence approached a plateau with increasing volumes of the organic phase (Fig. 1).

TABLE 1

Analysis of variance on fraction of E. coli remaining in the aqueous phase after mixing with 0.45 ml of organic phase

Source	df	Sum of squares	Mean square	*F*-test
Ionic strength (A)	1	0.064	0.064	9.14 [a]
pH (B)	2	0.206	0.103	14.71 [a]
AB	2	0.018	0.009	1.29
Solvent (C)	2	0.243	0.122	17.43 [a]
AC	2	0.054	0.027	3.86
BC	4	0.083	0.021	3.00
ABC	4	0.030	0.007	

[a] Significant at $p = 0.05$.

Analysis of variance on fraction remaining with 0.45 ml of organic phase was performed. Interactions were nonsignificant, however, the effects of ionic strength, pH or organic phase were significant (Table 1).

Suspension of *E. coli* in buffers of pH 2.2, 4.0 or 7.4 resulted in maximum adhesion at pH 2.2 and 4.0, and minimal at pH 7.4 (Fig. 2). The affinity of *E. coli* for organic phases at pH 7.4

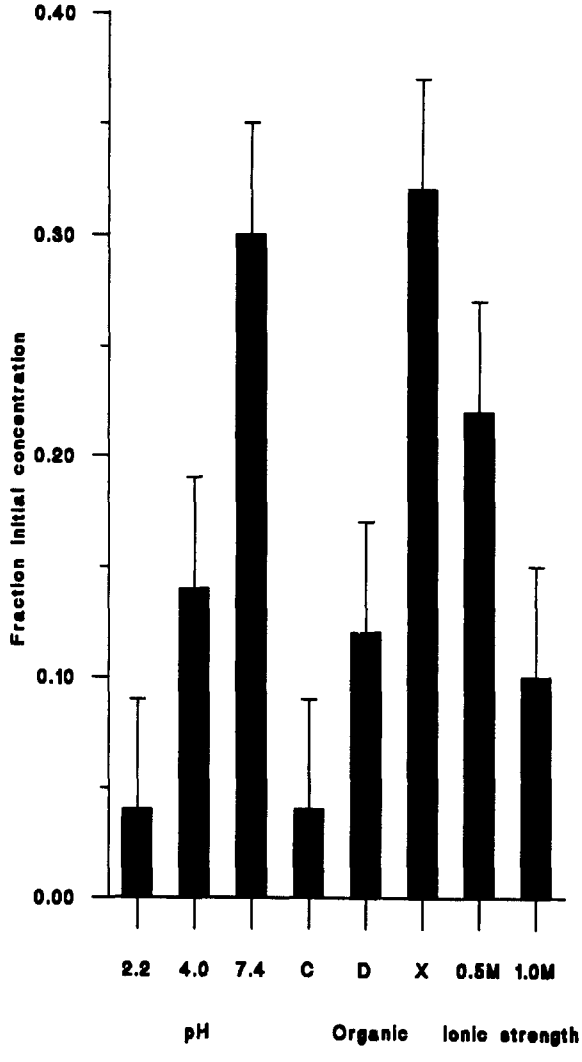

Fig. 2. Effect of pH (pH 2.2, 4.0 and 7.4), organic phase (chloroform (C), dichloromethane (D) and xylene (X)) and ionic strength (0.5 and 1.0 M) on the adhesion of *E. coli* to organic phases. Standard error bars were determined using the pooled error mean square ($n = 3$).

was significantly less than ($p = 0.05$) at pH 4.0 or 2.2. Exposure of *E. coli* to buffers of different ionic strength resulted in an increase of adhesion when the ionic strength was increased from 0.5 to 1.0 M. *E. coli* displayed a similar affinity for the organic phases chloroform and dichloromethane, with slightly greater affinity for chloroform. However, the affinity of *E. coli* for xylene was significantly ($p = 0.05$) less than for chloroform or dichloromethane.

Discussion

A major limitation of the BATH test for evaluating bacterial adherence is that quantitative criteria have not been well established. This had led to the use of such vague terms as 'pronounced', 'intermediate' or 'slight' adherence (Lichtenberg et al., 1985). Therefore, for the purpose of statistical analysis the fraction remaining in the aqueous phase with 0.45 ml of organic phase was used to describe adherence, as at this volume saturation appeared to occur. It has been shown previously that bacterial adherence to organic phases approaches a plateau with increasing volumes of the organic phase (Rosenberg et al., 1980; El-Falaha et al., 1982; Nesbitt et al., 1982; Rosenberg, 1984; Minagi et al., 1986; Jones et al., 1991).

Nesbitt et al. (1982) reported the effect of pH, with a maximum adherence to hexadecane at pH values between 3 and 4. They reported that raised temperature, dilute sodium chloride and low pH increased the adherence of *Staphylococcus sanguis* to hexadecane. While maximal adherence occurred at low pH, the effect of various ionic species was also noted. It was found that K^+ had little effect on adherence, whereas Na^+, Li^+, SCN^-, sodium dodecyl sulfate (SDS), urea and tetramethylurea (TMU) had an inhibitory effect on adherence. The ability of the ionic species Li^+ and SCN^- to inhibit adherence is expected as they have hydrophobic bond-disrupting natures, whereas SDS, urea and TMU can disrupt macromolecules. Stenstrom (1989) found that bacterial adhesion to resins or minerals was not affected by changes in pH over a 4–9 pH range; however, it was suggested that at pH values below 4 greater charge effects may result in increased adherence as the isoelectric point in this range. The results of this investigation support the findings of other studies that maximum adherence to the organic phases occurs at pH values below 4.

Previous investigations of CSH have reported decreased adherence at lower ionic strength of the suspending buffer (Nesbitt et al., 1982; Ofek et al., 1983; Rosenberg, 1984; Galdiero et al., 1987). Conversely, Stenstrom (1989) reported that at higher ionic strengths charge effects on the bacterial cell surface may be more pronounced, resulting possibly in greater adhesion. Greater ionic strength may increase adherence by suppressing the thickness of the diffuse double layer. It was found in this investigation that *E. coli* suspended in buffer of 1.0 M ionic strength resulted in a significantly greater adherence to the organic phase (chloroform, dichloroform and xylene), compared with *E. coli* suspended in 0.5 M ionic strength buffer.

Investigations using the BATH test have employed various organic solvents, for example, hexadecane, octane, xylene (Rosenberg 1984), cyclohexane (Jones et al., 1991), isopropyl myristate (El-Falaha et al., 1985) and toluene (Nesbitt et al., 1982). It has been observed that cells adhere to octane and xylene to a greater degree than hexadecane (Rosenberg et al., 1980). However, reasons for differences in affinity of organisms for various organic solvents remain unclear. The findings of the investigation have noted similar trends, with the affinity of *E. coli* for chloroform and dichloromethane being similar and greater than that for xylene. Rosenberg (1984) has suggested that the different affinities for organic phases may be due to the viscosity of the test solvent and the size of droplets formed during the BATH test. However, as there was a plateau approached as solvent volume was increased, it is proposed that organic phase surface area remains constant, regardless of volume used, due to the method of mixing used by the BATH test. This plateau has also been reported by other authors (Rosenberg et al., 1980; Nesbitt et al., 1982; El-Falaha et al., 1985; Minagi et al., 1986; Jones et al., 1991). As a vortex mixer is employed to mix the organic and aqueous phases for the BATH

test, the resulting vortex formation may only allow partial mixing of the two phases.

The results from this study indicate that pH and ionic strength play an important role in determining the CSH of *E. coli* and evaluation of the degree of CSH by the BATH test is influenced by the choice of organic phases. It has previously been suggested that considerable care should be taken in the choice of technique to evaluate CSH, for example, BATH, salt aggregation test and hydrophobic interaction chromatography (Jones et al., 1991). Therefore, having chosen to evaluate CSH by the BATH method, attention must be given to the nature of the suspending buffer, specifically pH and ionic strength. Furthermore, the solvent employed must be considered, for example, the use of chloroform with a suspending buffer of pH 7.4 and ionic strength 0.5 or 1.0 M would give a CSH of 10 and 6%, respectively, for *E. coli* (Fig. 1), i.e., and 94% of bacteria adhering to the organic phase, leading to the classification of pronounced adherence. However, xylene, at pH 7.4, would give a CSH of 68 and 44%, i.e.., and 56% of bacteria adhering to the organic phase, with suspending buffer of 0.5 and 1.0 M, respectively (Fig. 1), resulting in the classification of intermediate adherence.

Evaluating bacterial hydrophobicity using contact angle measurement is accepted as the best method, with reliability improved by combining it with other tests (Van Loosdrecht et al., 1990). However, the choice of buffer, specifically pH and ionic strength, must be considered as this will greatly influence the cell surface hydrophobicity of bacteria.

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