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Notes

The effects of pH, ionic strength and polyvalent ions on the cell surface hydrophobicity of *Escherichia coli* evaluated by the BATH and HIC methods

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Abstract

The effects of pH, ionic strength and polyvalent ions upon the subsequent adherence of *E. coli* to octyl-sepharose in hydrophobic interaction chromatography (HIC), and the effect of polyvalent ions on adherence to dichloromethane in the bacterial adherence to hydrocarbons (BATH) tests were investigated. Ionic strength, pH and polyvalent ions were found to influence adhesion. Lowering pH and increasing ionic strength increased adherence of *E. coli* to octyl-Sepharose and dichloromethane. However, polyvalent ions increased adherence to octyl-Sepharose while reducing adherence to dichloromethane, indicating the possibility of different mechanisms of interaction between *E. coli* and these substrates.

Keywords: Adherence; BATH; Hydrophobic interaction chromatography; pH; Ionic strength; Polyvalent ion; *Escherichia coli*

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).

Bacterial adhesion to mucosal surfaces is widely agreed to be 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 that cleanse mucous membranes.

Bacterial and host cell surfaces, for example, epithelial tissues, have a net negative charge. However, van der Waals attractive forces of the hydrophobic groups on the surface of the bacterial and epithelial cells may overcome the resultant electrostatic repulsion leading to adhesion. This is termed the DLVO (Deryagin, Landau,

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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 corresponding receptors on the epithelial cell surface (Beachey, 1981; Harber, 1985).

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).

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 cell surface hydrophobicity (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 all., 1983; Rosenberg, 1984; Galdiero et al., 1987). We have recently shown that the pH and ionic strength of the suspending buffer and the choice of organic phase will significantly influence the evaluation of CSH by the BATH test (Bunt et al., 1993). CSH was found to be significantly lower with high pH (7.4) and low ionic strength (0.5 M).

The bacterial adherence to hydrocarbons (BATH) test has been described as "a turbid, aqueous suspension of washed microbial cells is mixed by vortexing in the presence of a test hydrocarbon under controlled conditions" (Rosenberg, 1984). A major limitation of the BATH test for evaluating bacterial adherence is that quantitative criteria have not been well established. It has been shown that bacterial adherence to organic phases approaches a plateau with increasing volumes of the organic phase (Rosenberg et al., 1980; Nesbitt et al., 1982; Rosenberg, 1984; El-Falaha et al., 1985; Minagi et al., 1986; Jones et al., 1991). This has led to the use of such vague terms as 'pronounced', 'intermediate' or 'slight' adherence (Lichtenberg et al., 1985).

Hydrophobic interaction chromatography utilises the hydrophobic characteristics of proteins. Columns are essentially hydrophobic ligands, which interact with hydrophobic portions of proteins, attached to a supporting gel matrix (Arakawa and Narhi, 1991). Hydrophobic interaction chromatography has been used to describe the CSH of bacterial organisms, as well as *Bacillus* spores and staphylococcal exotoxins (Galdiero et al., 1987; Eriksson et al., 1989; Ronner et al., 1990).

Reliability in evaluating CSH may be improved by combining other tests (Van Loosdrecht et al., 1990), therefore the aim of this investigation was the evaluation of CSH by HIC and the BATH test and compare the effects of pH, ionic strength and the presence of polyvalent ions on CSH by means of appropriate statistical analysis.

Growth and collection of bacteria (*Escherichia coli* ATCC 29214) were as previously reported (Bunt et al., 1993). Bacteria were suspended in the relevant buffer to the required cell density (10^9 cells/ml).

A modified version of the BATH test by Rosenberg et al., (1980) was employed to investigate the effect of polyvalent ions. In brief, 6 ml of a suspension of *E. coli* in Tris-HCl buffer at pH 7.4 containing Ca^{2+} or Al^{3+} (either 0, 40 or 80 mM) and adjusted to ionic strength 0.5 M with KCl, was added to six different volumes (0.05–0.7 ml) of dichloromethane in test tubes (internal diameter 10 mm, tapered bottom). One tube was designated a control and consisted of buffer and dichloromethane. 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, relative to the control. 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.

Pasteur pipettes were packed with 1 ml of octyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) suspension, and washed with 10×1 ml of either McIlvaine buffer at pH 2.2, 4.0 or 7.4 and adjusted to ionic strength (either 0.5 or 1.0 M) with KCl, to investigate the effects of pH and ionic strength. A 1 ml volume of *E. coli* suspension was placed on the column and eluted with 4×1 ml of the suspending buffer. In addition 1 ml of buffer, eluted with 4×1 ml of buffer acted as a reference control. The eluent was assayed at 400 nm, relative to the control, 15 min after the final addition of the buffer and calculated as a fraction of the applied cell suspension concentration. All experiments were performed in triplicate.

Pasteur pipettes were packed with 1 ml of octyl-sepharose CL-4B suspension, and washed with 10×1 ml of Tris-HCl buffer at pH 7.4 containing Ca^{2+} or Al^{3+} (either 0, 40 or 80 mM) and adjusted to ionic strength 0.5 M with KCl, to investigate the effects of pH and ionic strength. Experiments were performed as for assessment of the effect of pH and ionic strength.

For the purpose of statistical analysis of the BATH results the mean fraction remaining with 0.45 ml of organic phase was calculated. Analyses of the HIC results were performed using the mean fraction remaining of the applied cell suspension concentration after passing through the column. All statistical analyses were performed using Minitab 8.2 (Minitab Inc. State College, PA, U.S.A.).

Table 1
Effect of pH and ionic strength (μ) on the fraction (mean ± sd) of *E. coli* remaining in the aqueous phase after mixing with 0.45 ml of organic phase (BATH) (from Bunt et al., 1993) or passage through a 1 ml octyl-Sepharose column (HIC)

Buffer pH, μ	Hydrophobicity assays (mean ± SD)			
	BATH ($n = 3$)			HIC ($n = 3$)
	Dichloro-methane	Chloroform	Xylene	Octyl-Sepharose
2.2, 0.5	0.01 ± 0.00	0.00 ± 0.00	0.15 ± 0.01	0.47 ± 0.02
4.0, 0.5	0.08 ± 0.01	0.05 ± 0.01	0.53 ± 0.02	0.59 ± 0.02
7.4, 0.5	0.37 ± 0.01	0.10 ± 0.00	0.68 ± 0.01	0.63 ± 0.01
2.2, 1.0	0.01 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	0.41 ± 0.01
4.0, 1.0	0.09 ± 0.03	0.02 ± 0.00	0.08 ± 0.03	0.53 ± 0.01
7.4, 1.0	0.14 ± 0.01	0.06 ± 0.01	0.44 ± 0.02	0.58 ± 0.01

Table 2
Effect of polyvalent ions on the fraction (mean ± SD) of *E. coli* remaining in the aqueous phase after mixing with 0.45 ml of organic phase (BATH) or passage through a 1 ml octyl-Sepharose column (HIC)

Buffer Ion, concentration	Hydrophobicity assays (mean ± SD)	
	BATH ($n = 3$) Dichloromethane	HIC ($n = 3$) Octyl-Sepharose
Nil	0.33 ± 0.03	0.68 ± 0.05
Ca^{2+} 40 mM	0.67 ± 0.02	0.63 ± 0.01
Ca^{2+} 80 mM	0.65 ± 0.02	0.59 ± 0.01
Al^{3+} 40 mM	0.66 ± 0.02	0.53 ± 0.02
Al^{3+} 80 mM	0.63 ± 0.03	0.51 ± 0.00

Typically it was found that CSH was greater at pH 2.2, whether evaluated by the BATH test or HIC (Table 1).

Suspending *E. coli* in buffer containing either Ca^{2+} or Al^{3+} resulted in a decrease in CSH when evaluated by BATH, whilst CSH increased when HIC was employed (Table 2).

Analysis of variance on fraction remaining of *E. coli* suspended in pH 2.2, 4.0 or 7.4 and ionic adjusted to 0.5 or 1.0 M buffer, after passing through the 1 ml octyl-Sepharose column (HIC), was performed. Interactions were nonsignificant, however, the effect of pH or ionic strength was significant ($p < 0.05$).

One-way analysis of variance was performed to evaluate the addition of polyvalent ions to the bacterial suspension, both concentrations of Ca^{2+} and Al^{3+} (40 and 80 mM) significantly ($p = 0.000$) decreased CSH as evaluated by the BATH test.

Evaluation of CSH by HIC with the addition of polyvalent ions was assessed by one-way analysis of variance and showed no significant change in CSH with Ca^{2+} at 40 mM, however, a significant ($p = 0.000$) increase in CSH was found with the higher concentration of Ca^{2+} (80 mM). Both 40 and 80 mM Al^{3+} significantly increased ($p = 0.000$) CSH greater than Ca^{2+}.

We have previously reported that pH and ionic

strength significantly influence CSH when evaluated by the BATH test. Greatest adhesion was found at pH 2.2 and 4.0 and ionic strength 1.0 M. Adhesion to chloroform and dichloromethane was found to be similar, whilst adhesion to xylene was significantly less (Bunt et al., 1993). Investigation of the effects of pH and ionic strength upon CSH by HIC has shown similar results with maximum adherence at pH 2.2 and ionic strength 1.0 M.

Husmark and Ronner (1990), have reported that the adhesion of *Bacillus cereus* spores is influenced by pH and ionic strength, with maximum adherence at around pH 3 when the spore surface was uncharged, and increased adhesion with increased ionic strength. The effect of increased ionic strength was suggested to be due to the suppression of a solvation barrier. (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 is in this range. It has also been reported by Nesbitt et al. (1982) that maximum adherence to hexadecane occurs at pH values between 3 and 4. They also reported that raised temperature, dilute sodium chloride and low pH increased the adherence of *Staphylococcus sanguis* to hexadecane.

Previous investigations of CSH have reported decreased adherence at low 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.

The effects of salts on the binding of proteins in HIC may be explained in terms of the cavity theory (Arakawa and Narhi, 1991). Work against the surface tension of water is required for the formation of a cavity for the accommodation of macromolecules in aqueous solution. The requirement for work is decreased when the protein is bound to a hydrophobic ligand due to a reduction in surface area. The binding between protein and ligand may be further increased when the surface tension of water is increased by the addition of salts, thereby increasing the energy for cavity formation. The difference in free energy between unbound and bound protein drives the system to a more thermodynamically favourable state, i.e. protein bound to ligand.

If *E. coli* interacts with the HIC by means of proteins on the cell surface, the observed effects of ionic strength and polyvalent ions may be explained in terms of the cavity formation theory. At low ionic strength and in the absence of polyvalent ions intermediate adhesion was observed, whilst at higher ionic strength or in the presence of polyvalent ions an increase in adhesion was shown.

While the effects of pH and ionic strength are similar when CSH is evaluated by either the BATH test or HIC, the lack of agreement between the BATH test and HIC when evaluating CSH may indicate different mechanisms of interaction.

Due to the possible different mechanisms of interaction results obtained by HIC might not be applied to findings by the BATH test. Reliability in evaluating CSH may be improved by combining 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 cell surface hydrophobicity of bacteria, as shown by both the bacterial adherence to hydrocarbons test and hydrophobic interaction chromatography.

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