

The Effects of Methanol, Ethanol, Propanol and Butanol on Bacterial Attachment to Surfaces

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The effects of methanol, ethanol, propanol and butanol, at concentrations of 0.2, 0.5, 1.0, 1.5 and 2.0% (v/v), on the attachment of a marine *Pseudomonas* sp. to polystyrene dishes (PD) and tissue culture dishes (TCD) were determined. When the bacteria attached in the presence of the alcohols, attachment to TCD was increased with certain concentrations of ethanol (0.2 and 0.5%), propanol (0.2, 1.5 and 2.0%) and butanol (1.0%), with either a decrease in attachment or no effect with the other concentrations tested. With PD, there was no increase in attachment, but the relationship between numbers of attached bacteria and alcohol concentration paralleled that obtained with TCD. Pre-incubation of the bacteria with the alcohols affected their subsequent attachment, but the resultant increases or decreases in attachment were not consistent with those obtained when attachment occurred in the presence of alcohols. Physicochemical properties of the attachment system were evaluated by measuring liquid surface tensions (γ_{LV}) and sessile drop (θ_{SD}) and air bubble (θ_B) contact angles on TCD and PD of the bacterial suspending medium with the various alcohol concentrations, both before and after incubation with the alcohols. There was a relationship between numbers of attached bacteria and medium γ_{LV} , with minimum attachment occurring at γ_{LV} values of 64–69 mN m⁻¹. The increase in attachment to TCD in the presence of ethanol, propanol and butanol was accompanied by an increase in respiration rate, which could reflect a modification of cell surface components.

INTRODUCTION

There are two basic ways in which bacteria can become attached to solid surfaces. First, attachment may be a spontaneous process determined by physicochemical adsorption and requiring no physiological activity on the part of the bacterium. Secondly, attachment may depend on a physiological contribution on the part of the bacterium. There are some experimental observations illustrating both types of attachment. For example, spontaneous physicochemical attachment has occurred when the attaching bacteria have been previously killed by UV radiation (Ørstavik, 1977), heat (Takakuwa *et al.*, 1979) or formaldehyde (Fletcher, 1980*a*). Sometimes attachment has been shown to be a time-dependent process, in which bacteria were easily washed off the surface during the initial attachment phase, but later became firmly attached and resisted washing (ZoBell, 1943; Marshall *et al.*, 1971). These two stages have been described as reversible and irreversible attachment (Marshall *et al.*, 1971), and firm attachment is believed to depend upon the production of an extracellular polymeric adhesive which binds the bacterium to the surface.

However, in most studies of bacterial attachment, it has not been possible to determine whether attachment was determined only by physicochemical adsorption or depended upon bacterial metabolism. Moreover, in studies on the effects of various environmental factors on adhesion, e.g. temperature (Fletcher, 1977), cation concentration (Marshall *et al.*, 1971;

Abbreviations: ASW, artificial seawater; PD, polystyrene dish; TCD, tissue culture dish.

Fletcher, 1980*b*), the addition of potential nutrients (Marshall *et al.*, 1971; Fletcher, 1976) or metabolic inhibitors (Takakuwa *et al.*, 1979; Fletcher, 1980*a*), it was rarely possible to confirm whether the factor influenced attachment by affecting the physicochemistry of adhesion, the physiology of the bacterium or both.

In a recent study on the effects of metabolic inhibitors on the attachment of a marine pseudomonad (Fletcher, 1980*a*), attachment to polystyrene tissue culture dishes was appreciably increased in controls containing 1.0% ethanol, as compared with ethanol-free controls. By contrast, ethanol did not increase attachment to polystyrene petri dishes, which are more hydrophobic than the tissue culture dishes. It has been unusual for an experimental treatment to increase attachment of this organism and most either have no effect or inhibit attachment.

The purpose of this study was to investigate the effect of four short-chain alcohols, methanol, ethanol, propanol and butanol, on the attachment of the *Pseudomonas* sp. and to determine how they affected attachment.

METHODS

Organism and culture conditions. The marine *Pseudomonas* sp. (NCMB 2021) was grown in 100 ml 0.1% (w/v) peptone plus 0.07% (w/v) yeast extract powder in artificial seawater (ASW; Kester *et al.*, 1967), pH 7.6, for 22–23 h. The inoculum for the culture was 1 ml of a stationary phase culture. After incubation, when the bacteria were in the early stationary phase, they were centrifuged and resuspended in ASW to a final concentration of $4.5\text{--}9.0 \times 10^8$ bacteria ml⁻¹.

Attachment in the presence of alcohols. A 20 ml volume of bacterial suspension was mixed with 40, 100, 200, 300 or 400 μ l methanol (A. R., Cambrian Chemicals), ethanol (A. R., J. Burrough), propan-1-ol or butan-1-ol (S. L. R., Fisons) to give final alcohol concentrations of 0.2, 0.5, 1.0, 1.5 and 2.0% (v/v), respectively. The corresponding molarities for these percentage concentrations were, respectively: methanol, 0.05, 0.13, 0.25, 0.38 and 0.50; ethanol, 0.03, 0.09, 0.17, 0.26 and 0.34; propanol, 0.03, 0.07, 0.13, 0.20 and 0.26; and butanol, 0.02, 0.06, 0.11, 0.17 and 0.22. Controls were prepared for each concentration by adding the same amount of sterile ASW. Some supplementary experiments used dimethyl sulphoxide (Hopkin and Williams) (see text). Samples (5 ml) of the mixtures were immediately placed in a hydrophobic polystyrene petri dish (PD; 3 cm diam., Sterilin; two replicates) and in a more hydrophilic polystyrene tissue culture dish (TCD; 3 cm diam., Costar; two replicates). After 2 h at room temperature (20–22 °C) without stirring, the suspension was tipped out of the dish, and bacteria not attached to the dish surface were removed by rinsing with sterile ASW from a washbottle. Attached bacteria were fixed with Bouin's fixative and stained with crystal violet. Comparative numbers of attached bacteria were evaluated with a spectrophotometer (Fletcher, 1976) by measuring the absorbance of four randomly selected areas of each dish surface at 590 nm, the absorbance maximum of crystal violet. The results were expressed as an index of attachment, I_a , which was the ratio of the A_{590} of the test surface to that of the same surface (PD or TCD) exposed to the alcohol-free control suspension. Thus I_a values near 1.0 showed no effect, whereas those appreciably below or above 1.0 indicated a decrease or an increase, respectively, in attachment to that particular surface.

Incubation with alcohols before attachment. In some experiments bacterial suspensions to be assessed for attachment were mixed with various concentrations of the four alcohols as before but were first incubated at room temperature (20–22 °C) for 1.5 h. Although this incubation period was somewhat shorter than the 2 h exposure to alcohols during attachment (above), it should have been long enough for any alcohol-induced metabolic changes to occur and it allowed for the further exposure of bacteria to alcohols after incubation when they were being centrifuged and prepared for the next experimental stage. Controls contained equivalent amounts of sterile ASW. The alcohols were removed by centrifuging and resuspending the bacteria in the same volume of ASW, and attachment experiments were then done as before. No attempt was made to ensure complete alcohol removal by repeated washing and centrifugation, as this in itself affects attachment (unpublished observations), presumably by removing cell surface components.

Results were expressed as an index of attachment, I_{aPD} , which differed from I_a in that I_{aPD} was the ratio of the A_{590} of the TCD or PD test substratum to that of the PD control. By relating all values to the PD control, numbers attached to both TCD and PD test surfaces could be compared.

Surface tension and contact angle measurements. Surface tension measurements (γ_{LV}) and sessile drop (θ_{SD}) and air bubble (θ_B) contact angles were measured by using two types of solution which were analogous to the suspending media for the two types of attachment experiments. First, to obtain a solution similar to the suspending medium used for attachment in the presence of alcohols, solutions were prepared by adding the alcohols in the concentrations given above to the supernatant from a bacterial suspension prepared by centrifuging the stationary phase culture, resuspending in 100 ml ASW, re-centrifuging and then collecting the supernatant. The supernatant was then diluted 1:6 with ASW, the volume dilution used to obtain the bacterial concentration in the attachment

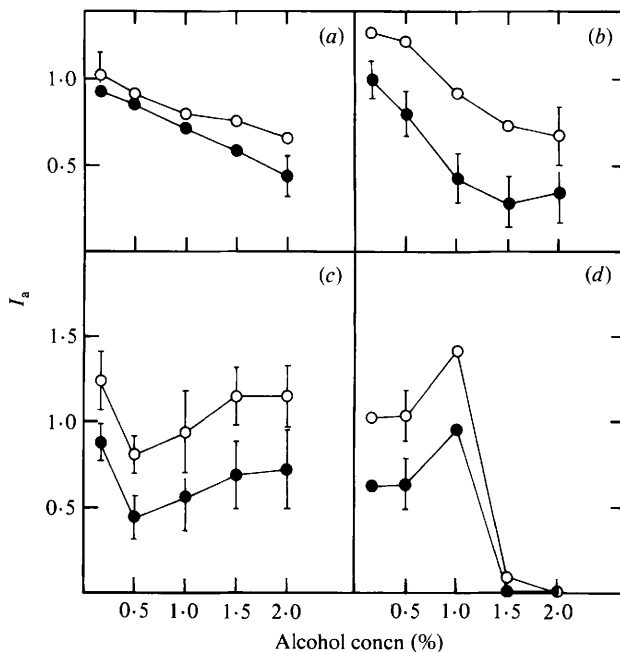


Fig. 1. The attachment of bacteria, expressed as the index of attachment, I_a (see text), to TCD (○) and PD (●) in the presence of (a) methanol, (b) ethanol, (c) propanol and (d) butanol. S.E.s are ≤ 0.1 unless otherwise indicated (bars).

experiments. Secondly, the suspending media for bacterial suspensions incubated with the alcohols for 1.5 h before allowing attachment (above) were collected after removal of the bacteria by centrifugation.

γ_{LV} values were measured using a Pt loop and torsion balance (White Electrical Instruments). θ_{SD} values were measured by placing drops (≈ 1 mm diam.) of the appropriate solution using a 1 ml syringe on either TCD or PD and measuring the contact angles (Fletcher *et al.*, 1980) using a vernier microscope with a goniometer eyepiece (Precision Tool and Instruments, Thornton Heath, U.K.). Values were recorded for both edges of 8–12 drops for each solution. θ_B values on TCD and PD were measured as described by Fletcher & Marshall (1982). Briefly, an air bubble (≈ 2 mm diam.) was injected from a syringe into a perspex chamber containing the liquid, so that the bubble rose 6–7 mm from the point of release at the bottom to rest against the TCD or PD placed horizontally at the surface of the liquid. Both edges of five bubbles were measured as above.

Bacterial respiration. Oxygen uptake by bacterial suspensions containing the different concentrations of the alcohols (see above) was measured at 15 °C with an oxygen electrode (Rank Brothers).

RESULTS

Attachment of bacteria in the presence of alcohols

Methanol, ethanol, propanol and butanol all affected bacterial attachment, but the nature and extent of the effect depended on the alcohol, its concentration and the substratum (Fig. 1). With methanol there was a progressive decrease in numbers of bacteria attached to both TCD and PD with an increase in methanol concentration. With the other alcohols, however, the relationship between numbers of attached bacteria and alcohol concentrations was more complicated. Numbers of bacteria attached to TCD increased, with respect to the control, in the presence of ethanol (0.2 and 0.5%), propanol (0.2, 1.5 and 2.0%) and butanol (1.0%), with either a decrease in attachment or no change at the other alcohol concentrations tested. With PD, there was no increase in numbers of attached bacteria, as compared with the control, but the relationship between numbers of attached bacteria and the different alcohol concentrations paralleled that which occurred with TCD.

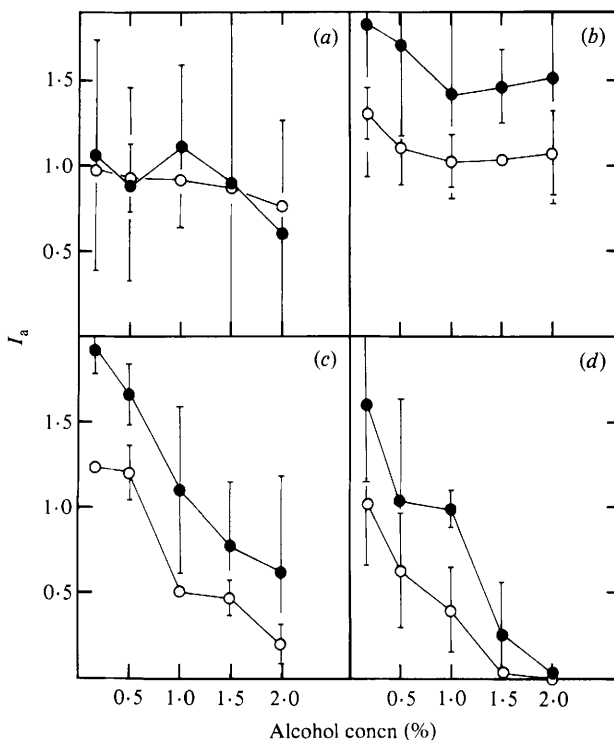


Fig. 2. The attachment of bacteria, expressed as the index of attachment, I_a (see text), to TCD (○) and PD (●) after 1.5 h incubation with (a) methanol, (b) ethanol, (c) propanol and (d) butanol. S.E.s are ≤ 0.1 unless otherwise indicated (bars).

Attachment of bacteria which were pre-incubated with alcohols

Pre-incubation with methanol, ethanol, propanol or butanol affected subsequent bacterial attachment, but the nature and extent of the effect depended on the alcohol, its concentration and the surface (Fig. 2), and these effects were not consistent with those obtained when attachment occurred in the presence of the alcohols (Fig. 1). With methanol, there was a progressive decrease in attachment to TCD with increase in methanol concentration, but attachment to PD was variable, there being either an increase or decrease in attachment in replicate experiments. There was an increase in attachment with ethanol (0.2% with TCD; 0.5 and 1.5% with PD), propanol (0.2 and 0.5% with TCD and PD) and butanol (0.2% with PD). At other alcohol concentrations, attachment was either reduced or not affected. Unlike attachment in the presence of alcohols, pre-incubation in alcohols tended to result in higher numbers of bacteria attached to PD than to TCD, with respect to controls.

Effect of alcohols on liquid surface tension (γ_{LV}) and on sessile drop (θ_{SD}) and air bubble (θ_B) contact angles

The values for γ_{LV} obtained with the bacterial supernatant containing various alcohol concentrations and with supernatants from bacterial cultures incubated with the alcohols are given in Table 1. Surface tension measurement was the most sensitive method used for detecting differences in the properties of the supernatants with different alcohol concentrations. With all alcohols, there was a progressive decrease in γ_{LV} with increase in alcohol concentration. γ_{LV} values for supernatants from cultures incubated with the alcohols were generally lower than γ_{LV} values for the corresponding bacterial supernatants with added alcohols, particularly at the highest alcohol concentrations.

Table 1. *Effect of alcohols on liquid surface tension (γ_{LV})*

Treatment	Concn (%)	γ_{LV} (mN m ⁻¹)*	
		Alcohol added to supernatant from bacterial culture	Measurements made with supernatants from bacterial cultures incubated with alcohols
No addition	—	73.5	73.2
Methanol	0.2	73.3	73.3
	0.5	72.7	72.4
	1.0	71.6	71.3
	1.5	70.7	69.3
	2.0	70.3	69.5
Ethanol	0.2	72.8	72.6
	0.5	71.4	71.2
	1.0	69.8	69.3
	1.5	68.3	67.4
	2.0	67.2	65.8
Propanol	0.2	72.2	71.5
	0.5	68.9	68.4
	1.0	65.2	64.2
	1.5	61.8	61.3
	2.0	59.6	58.6
Butanol	0.2	67.6	68.2
	0.5	61.6	61.8
	1.0	55.6	54.7
	1.5	50.9	49.4
	2.0	47.5	46.2

* Measured values were generally reproducible.

When the solutions comprised alcohols added to the bacterial supernatant there was little change in either θ_{SD} or θ_B on PD, except with butanol at 1.5% and 2.0% ($\gamma_{LV} < 55$ mN m⁻¹) where θ_{SD} was reduced from 85° (control) to 78° and 75°, respectively, and θ_B was reduced from 95° (control) to 76° and 69°, respectively. However, with TCD there was a progressive decrease in θ_{SD} with an increase in alcohol concentration, with a reduction from 59° (control) to 44° for methanol, 53° for ethanol, 50° for propanol and 39° for butanol.

When the solutions comprised supernatants from bacteria incubated with alcohols, with PD there was little change in θ_{SD} with a change in alcohol concentration, except with butanol, where θ_{SD} values were appreciably smaller (<80° as compared with a control of 88° for all concentrations). There was little change in θ_B values with PD, except for butanol at concentrations >1.0%, when it was also possible at $\times 10$ magnification to see undissolved butanol being adsorbed at the surface as very small droplets. With TCD, θ_{SD} and θ_B changed considerably with different alcohol concentrations. Although there was generally a decrease in θ_{SD} and θ_B with an increase in alcohol concentration, with propanol there was an increase in θ_B at concentrations of 0.5, 1.0 and 1.5%. The decrease in θ_{SD} was proportionally greater, with respect to the corresponding γ_{LV} , with butanol (from 57° for the control to 40°) than with the other alcohols (to 53° for ethanol, 51° for methanol and 49° for propanol).

When the numbers of bacteria which attached in the presence of alcohols were plotted against the γ_{LV} values for the different alcohol-supernatant solutions (Table 1), a curve with a minimum between ≈ 67 and 69 mN m⁻¹ was obtained with both surfaces. However, with TCD, a separate linear relationship was obtained with methanol and dimethyl sulphoxide. A better curve fit (Fig. 3) was obtained if the γ_{LV} values were those for supernatants from bacterial cultures incubated with the alcohols (Table 1). A separate linear relationship was still obtained with methanol and TCD, but the curve minima altered slightly to ≈ 66 and ≈ 66 –68 mN m⁻¹ for TCD and PD, respectively. Further experiments with butanol (at concentrations between 0.1% and 1.1% at 0.1% intervals) and ethanol (at concentrations between 0.5% and 3.5% at 0.5% intervals)

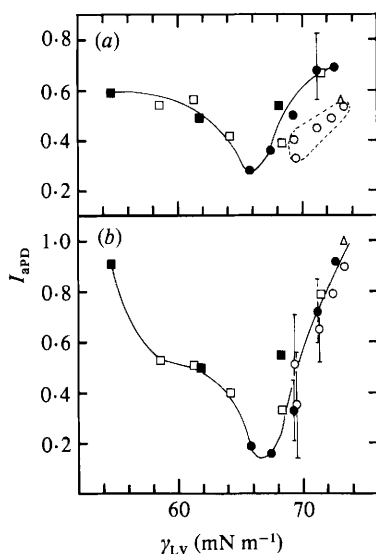


Fig. 3

Fig. 3. The relationship between numbers of bacteria which attached to (a) TCD and (b) PD, in the presence of methanol (○), ethanol (●), propanol (□) or butanol (■) and in the alcohol-free control (△), and the surface tensions (γ_{LV}) of the bacterial supernatants after incubation with the different alcohol concentrations. Numbers of attached bacteria are expressed as the index of attachment, I_{aPD} (see text). S.E.s are ≤ 0.1 unless otherwise indicated (bars). Note the separate linear relationship obtained with methanol (points surrounded by dashed line).

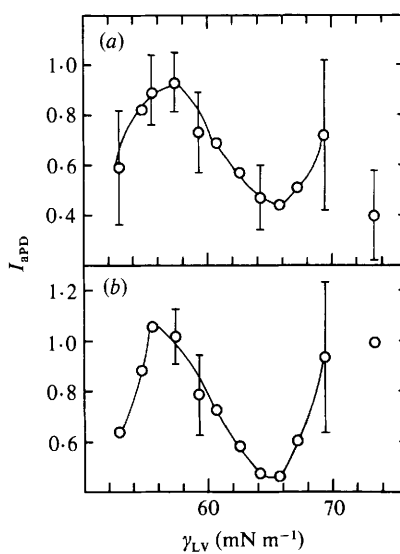


Fig. 4

Fig. 4. The relationship between numbers of bacteria which attached to (a) TCD and (b) PD, in the presence of butanol, and the surface tensions (γ_{LV}) of the different concentrations of butanol in bacterial supernatant. Numbers of bacteria are expressed as the index of attachment, I_{aPD} (see text). S.E.s are ≤ 0.1 unless otherwise indicated.

confirmed that both alcohols produced attachment minima at γ_{LV} values between 62 and 68 mN m^{-1} . With butanol, there was a second decrease in numbers of attached bacteria at γ_{LV} values below $\approx 58 \text{ mN m}^{-1}$ (Fig. 4), but this could be related to the effect of butanol on the bacteria or on the substratum surface.

Effect of alcohols on bacterial respiration, growth and motility

Although the addition of methanol at concentrations of 0.5–2.0% was followed by an increase in respiration rate, this fell back to the base rate within 10 min and remained stable for 20 min, when measurement was discontinued. However, the addition of ethanol, propanol and butanol all resulted in a sustained increase in oxygen uptake rate. With these alcohols, the initial increases in rates were directly related to the concentration added, but after ≈ 10 min, all rates stabilised at $\approx 0.5\text{--}0.09 \text{ ml O}_2 \text{ l}^{-1}$ above base rate for a 5 ml bacterial suspension at $\approx 6 \times 10^8 \text{ ml}^{-1}$. However, turbidity measurements of bacterial cultures containing each alcohol showed that none of the alcohols supported growth. Microscopic observation of bacterial suspensions at alcohol concentrations between 0.2 and 1.0% showed that only butanol at 1.5% and 2.0% inhibited motility.

DISCUSSION

There are three components in the attachment process: the solid surface, the bacterial surface and the medium. For adhesion to occur, some component(s) of the bacterial surface must become adsorbed on the solid surface, and this may require displacement of adsorbed medium from either or both surfaces. This displacement will tend to become more difficult with an increase in interaction between the solid or bacterial surface and the medium.

The adhesion process is thermodynamically described by the equation:

$$\Delta F^{\text{adh}} = \gamma_{\text{BS}} - \gamma_{\text{LS}} - \gamma_{\text{BL}} \quad (1)$$

where ΔF^{adh} is the change in free energy of adhesion and γ_{BS} , γ_{LS} and γ_{BL} are the bacterium/solid, liquid/solid and bacterium/liquid interfacial energies, respectively (Neumann *et al.*, 1979). Adhesion is favoured by a reduction in free energy, i.e. a negative value for ΔF^{adh} . The interfacial energies in each case are determined by the surface free energies of the two phases. In general, the more disparate the two surface free energies, the greater the interfacial energies, whereas the interfacial energy between two phases of the same surface free energy is theoretically zero. The significance of each of these phases, the solid and bacterial surfaces and the medium, in the attachment process is illustrated by data from this investigation. Moreover, the physiological activity of the bacteria is shown to be significant, presumably because of its influence on bacterial surface energy and/or medium surface tension.

The polystyrene substrata, TCD and PD, differ in surface free energy and water wettability. TCD has a higher surface free energy and is more hydrophilic, as was shown by lower θ_{SD} and θ_{B} values, as compared with PD. Accordingly, the numbers of bacteria which attached to the two surfaces differed, and in controls considerably more bacteria attached to PD than to TCD (see I_{aPD} in Figs 3 and 4; Fletcher, 1980*a*). Substratum surface energies have frequently been found to affect numbers of bacteria which become attached (Dexter *et al.*, 1975; Fletcher & Loeb, 1979; Gerson & Scheer, 1980).

The significance of substratum surface properties in attachment may also have been illustrated by the lack of attachment in the presence of butanol at 1.5% and 2.0%, as the adsorption of butanol to PD was seen while making θ_{B} measurements. Thus, attachment to PD may have been prevented in part by an adsorbed layer of butanol, and adsorbed substances, e.g. proteins, bacterial medium components, have been shown to inhibit bacterial attachment (Fletcher & Loeb, 1979; Fletcher & Marshall, 1982). However, the bacteria were also affected at these butanol concentrations and became non-motile, which probably contributed to the decrease in attachment. Previous experiments demonstrated that the removal of flagella from this bacterium by treatment in a blender reduced attachment (Fletcher, 1979).

Pre-incubation of the bacteria with the alcohols affected their subsequent attachment (Fig. 2) probably either by directly affecting cell surface properties or by affecting physiological processes involved in attachment and/or determining cell surface properties. When the numbers of attached bacteria from pre-incubated suspensions were plotted against the γ_{LV} values of the supernatants from those suspensions (excluding the control and erratic methanol values for PD), a positive linear relationship was obtained ($r = 0.90$ for TCD, and 0.95 for PD). However, this effect alone cannot account for the differences in numbers of attached bacteria in the presence of the alcohols, as the relationships between the numbers of attached bacteria and alcohol concentrations were not the same in the two treatments (Figs 1 and 2).

The significance of the bacterial surface in the attachment mechanism is also indicated by the increase in numbers of bacteria attached to TCD in the presence of certain concentrations of ethanol, propanol and butanol. As these alcohols also increase respiration rate, increase in attachment is probably due to a modification in cell physiology and/or surface characteristics. Moreover, in similar experiments with dimethyl sulphoxide, which like methanol does not affect respiration rate, there was no increase in attachment to TCD. It is not clear, however, why the increase in attachment was achieved with TCD, but not with PD.

The increase in respiration rate was not accompanied by an increase in growth. Thus, the increased activity may reflect an attempt to repair damage or compensate for some modification of cell envelope structures caused by the alcohols. For example, a modification of membrane structure would in turn affect membrane associated enzyme activity such as electron transport and oxidative phosphorylation. Growth in the presence of ethanol (Berger *et al.*, 1980) and other short-chain alcohols (Ingram, 1976) produced a change in the fatty acid composition of *Escherichia coli* membranes. This change was possibly an adaptive response to physicochemical changes in membrane fluidity caused by the alcohols, and was accompanied by an initial cessation of growth followed by resumed growth at a slower rate (Ingram, 1976). The ability of

an alcohol to interact with membranes may also be reflected in γ_{LV} values for the alcohol solutions, as both are a function of polar and non-polar groups and molecular size. Thus the linear relationship between the numbers of pre-incubated attached bacteria and the γ_{LV} values of the suspending media could be an indirect indication of the effect of the alcohols on membrane structure.

The γ_{LV} of the medium had a considerable effect on the number of bacteria which attached to both TCD and PD. The values of the curve minima (Figs 3 and 4), which were between ≈ 64 and 69 mN m^{-1} , may in some way be related to the surface free energy of the bacteria. The minima cannot be a function of the solid surface as they were approximately the same for both TCD and PD, and the minima values are similar to values for bacterial surface free energy obtained by contact angle measurements (van Oss *et al.*, 1975). Moreover, equation 1 predicts that attachment will tend to be reduced as the surface free energies of the medium and the bacterium approach the same value, thus reducing the value of their interfacial energy, γ_{BL} . However, some variations in the bacterial surface energy would be expected with the different alcohol treatments, as each alcohol probably has a somewhat different effect on physiology and/or surface properties. Thus the significance of the curve minima is still not clear. Attempts were made to measure the surface free energies of the alcohol-treated bacteria by contact angle measurements on lawns of bacteria (van Oss *et al.*, 1975), but it was impossible, as aqueous solutions were adsorbed by the bacteria and did not form drops, whereas non-polar liquids gave nonsensical results.

With θ_{SD} and θ_B measurements, changes produced by the addition of alcohols were detected more often with TCD than with PD. As TCD is the higher energy surface, substances would tend to be adsorbed more readily than they would on PD, and an increase in liquid adsorption with an increase in alcohol concentration in the bacterial supernatant was suggested by the corresponding decrease in θ_{SD} . However, θ_{SD} and θ_B measurements were not very helpful in determining the way in which alcohols affected attachment, and these measurements are probably more useful in evaluating the influence of macromolecules (Fletcher & Marshall, 1982).

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