

Mechanism of the Initial Events in the Sorption of Marine Bacteria to Surfaces

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SUMMARY

The sorption of two marine bacteria to surfaces involved an instantaneous *reversible* phase, and a time-dependent *irreversible* phase. Reversible sorption of the non-motile *Achromobacter* strain R8 decreased to zero as the electrolyte concentration decreased, or as the thickness of the electrical double-layer increased. The electrolyte concentration at which all bacteria were repelled from the glass surface depended on the valency of the cation. The reversible phase is interpreted in terms of the balance between the electrical double-layer repulsion energies at different electrolyte concentrations and the van der Waals attractive energies. Even at the electrolyte concentration of seawater, the bacteria probably are held at a small distance from the glass surface by a repulsion barrier. Reversible sorption often led to rotational motion of the motile *Pseudomonas* sp. strain R3 at a liquid-glass interface.

Pseudomonas R3 produced polymeric fibrils in artificial seawater; these may be concerned in the irreversible sorption of the bacteria to surfaces. Sorption and polymer production were stimulated by 7 mg./l. glucose but higher levels inhibited irreversible sorption. Omission of Ca^{2+} and Mg^{2+} from the artificial seawater prevented growth, polymer production, and sorption to surfaces by *Pseudomonas* R3.

INTRODUCTION

The sorption of bacteria to surfaces is a general phenomenon encountered in natural environments with important ecological implications (Wood, 1967; Marshall, 1971). Primary microbial film formation on surfaces immersed in seawater is considered by some investigators to be a prerequisite to fouling by larger organisms such as barnacles (Wood, 1967). The mechanism whereby marine bacteria sorb to surfaces has received scant attention. ZoBell (1943) suggested that, once bacteria are attracted to a surface, firm attachment requires incubation for several hours. He attributed this delay to the need for the synthesis of extracellular adhesive materials. Recently, Corpe (1970*a*) has reported the production of an extracellular acid polysaccharide by a primary film-forming bacterium, *Pseudomonas atlantica*. Glass slides coated with this polymer became fouled with micro-organisms more rapidly than uncoated slides. Corpe (1970*b*) has reviewed the literature on attachment of bacteria to surfaces immersed in marine environments.

The present investigation combines a study of some of the colloidal and biological properties of pure cultures of marine bacteria to obtain information on processes involved in the sorption to surfaces.

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Definition of phases of sorption

Our studies have confirmed ZoBell's (1943) suggestion that sorption consists of two phases. The bacteria were first attracted to a surface, and, after several hours, some became firmly attached. These phases are defined as follows.

Reversible sorption is an essentially instantaneous attraction of bacteria to a surface. Such bacteria are held weakly near the surface; they still exhibit Brownian motion and are readily removed by washing the surface with 2.5 % NaCl.

Irreversible sorption involves the firm adhesion of bacteria to the surface; they no longer exhibit Brownian motion and are not removed by washing with 2.5 % NaCl.

METHODS

Organisms. The bacteria used in this investigation were a motile *Pseudomonas* sp. strain R 3 and a non-motile *Achromobacter* sp. strain R 8. Both organisms were isolated from a glass coverslip that had been immersed in natural seawater for 1 h., rinsed several times in sterile 2.5 % NaCl, plated by smearing the coverslip on an artificial seawater agar, and incubated 24 h. at 25°. *Pseudomonas* R 3 required 2.5 % NaCl in the medium, while *Achromobacter* R 8 grew equally well in both high and low salt media. Logarithmic and stationary phase cultures of *Pseudomonas* R 3 refer to cultures incubated for 4 and 24 h., respectively.

Studies of reversible sorption. The behaviour of the motile *Pseudomonas* R 3 at liquid-glass interfaces was examined by preparing films (Kodak 4-X reversal film type 7277) of the bacteria viewed at the plane of a coverslip by phase-contrast microscopy. Detailed examination of these films made possible a reasonable interpretation of the curious gyratory motions observed with motile bacteria at such interfaces. The maximum velocity of *Pseudomonas* R 3 was determined by the motility tracking method of Vaituzis & Doetsch (1969).

The effect of electrolyte concentration on reversible sorption was investigated using *Achromobacter* R 8. This non-motile organism grew in low salt media and very low electrolyte concentrations did not cause lysis. *Achromobacter* R 8 was grown on nutrient agar (Difco) at 25° for 24 h. After washing twice in distilled water, portions of the suspension were mixed with equal volumes of a range of concentrations of NaCl or MgSO₄. Drops of these suspensions were run under coverslips supported above slides by broken coverslip pieces. The preparations were stood 30 min. to allow those organisms not sorbed at the liquid-glass interface to fall from view by gravity, so that these cells attracted to the coverslip surface could be counted. The same technique was employed to investigate the effect of divalent cations on the reversible sorption of *Pseudomonas* R 3.

Studies of irreversible sorption. Glass slides were cleaned in chromic acid, rinsed in distilled water, and then supported in slots in a sheet of foam polystyrene over an evaporating basin (1 l. capacity) containing an appropriate medium inoculated with *Pseudomonas* R 3. Duplicate slides were removed at intervals, rinsed thoroughly with 2.5 % NaCl, and the previously immersed area of the slide covered with a coverslip. Organisms firmly sorbed (not exhibiting Brownian motion) were counted in at least ten fields on both slides. All counts are expressed as numbers sorbed/cm² of glass surface.

The media used included either 2.5 % NaCl or an artificial seawater (ASW), suggested by T. Waite, which contained (g./l.): NH₄Cl, 0.0007; NaCl, 24; KCl, 0.6; MgSO₄.7H₂O, 5; MgCl₂.6H₂O, 3.6; CaCl₂, 0.3; NaNO₃, 0.1; KH₂PO₄, 0.01; FeCl₃, 0.001; tris-HCl, 5.32; tris Base, 1.97; P₁₁ trace metals (below), 10 ml.; pH 7.8. The P₁₁ trace metal solution contained (g./l.): di-sodium ethylene-diaminetetracetic acid (EDTA), 1; FeCl₃, 0.01; H₃BO₃,

0.2; MnCl_2 , 0.04; ZnCl_2 , 0.005; CoCl_2 , 0.001. Modifications of these media are detailed in the Results section. The concentration of glucose added to artificial seawater is indicated by the number after ASWG, e.g. ASWG-30 indicates 30 mg./l. glucose.

Pseudomonas R3 was inoculated on large slopes in 100 ml. bottles of nutrient agar (Difco) containing 2.5 % NaCl, and normally incubated 24 h at 25°. The bacteria were washed once in 2.5 % NaCl before being inoculated into the test medium to give a final concentration of between 1 and 10×10^7 bacteria/ml.

For electron-microscope studies of irreversibly sorbed bacteria, nickel grids (formvar films) were immersed directly in the medium containing *Pseudomonas* R3; removed after 1 h., fixed in 2.5 % formaldehyde in 2.5 % NaCl (Hodgkiss & Shewan, 1968) for 30 min., dried, rinsed in distilled water, and dried prior to shadowing with gold-palladium alloy (60 % gold-40 % palladium).

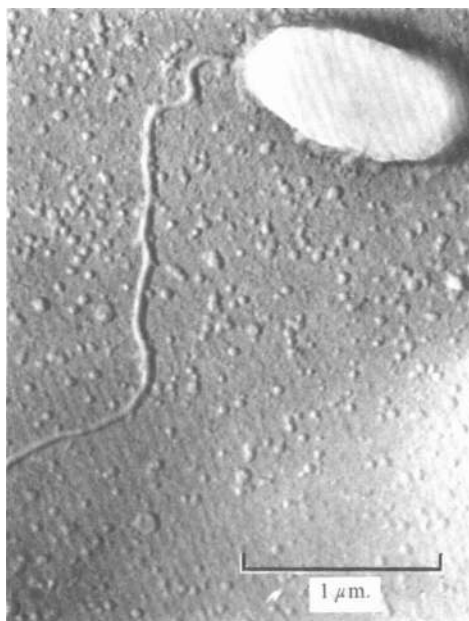


Fig. 1. Single polar flagellum of *Pseudomonas* strain R3.

RESULTS

Motility and reversible sorption of Pseudomonas R3. *Pseudomonas* R3 was a motile rod possessing a single polar flagellum (Fig. 1). In common with most motile bacteria, this organism exhibited a peculiar rotational behaviour at a liquid-glass interface. Cine films indicated that such bacteria sorbed at the pole (an edge-to-face association) and, by virtue of the motive force of the flagellum, rotated violently around the fixed pole (Fig. 2). If sorbed in a face-to-face position (Fig. 2), they rotated in either direction in a propeller-like fashion. The bacteria occasionally broke away from the surface and often sorbed at another point. Any non-motile cells were sorbed in a face-to-face position (Fig. 2) and showed only Brownian motion. Both motile and non-motile cells were readily desorbed by washing the glass surfaces in 2.5 % NaCl.

The kinetic energy of motile *Pseudomonas* R 3 cells was estimated: the maximum velocity was $33 \mu\text{m./sec.}$; assuming an average mass of 10^{-12} g. , then the kinetic energy of a motile organism was $5.45 \times 10^{-18} \text{ ergs.}$

Effect of medium composition on irreversible sorption of Pseudomonas R 3. As reported below, the irreversible sorption of *Pseudomonas* R 3 was affected by divalent cations in ASW. However, the results in Table 1 show that deletion of divalent cations from ASW + glucose at 7 mg./l. (ASWG-7) did not influence the numbers of bacteria initially attracted to the glass surface.

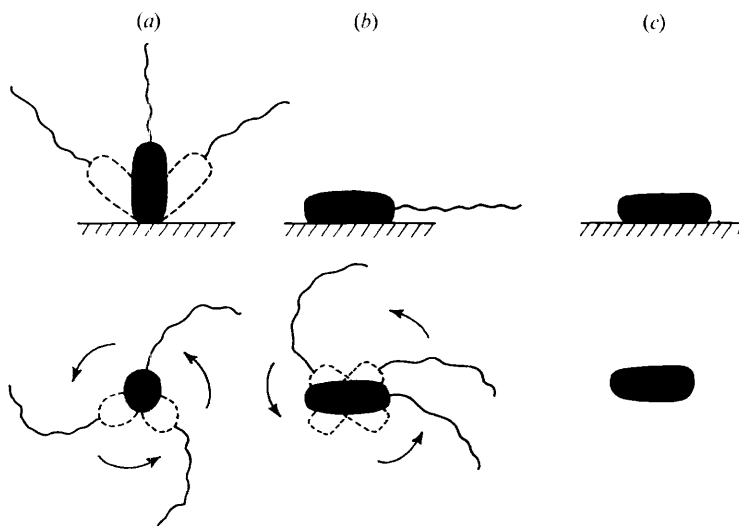


Fig. 2. A diagrammatic interpretation of the reversible sorption of *Pseudomonas* R 3 to a glass surface: (a) and (b) illustrate the rotational movements of motile bacteria in an edge-to-face and a face-to-face manner, respectively; (c) face-to-face sorption of non-motile bacteria.

Table 1. *Effect of divalent cations in artificial seawater on the reversible sorption of Pseudomonas R 3*

Medium	No. bacteria sorbed/cm ²
ASWG	5.35×10^3
ASWG-Ca ²⁺	5.27×10^3
ASWG-Mg ²⁺	5.58×10^3
ASWG-Ca ²⁺ , -Mg ²⁺	5.42×10^3

Effect of electrolyte concentration on reversible sorption of Achromobacter R 8. The number of achromobacteria reversibly sorbed increased with increasing electrolyte concentration or as the thickness of the electrical double layer decreased (Fig. 3). The theoretical thickness of the diffuse double layer ($1/K$) was calculated from the expression

$$K = 0.327 \times 10^8 Z\sqrt{c},$$

where Z = valency and c = molar concentration of electrolyte. This expression holds for aqueous solutions of symmetrical electrolytes at 25° (Shaw, 1966).

The bacteria were reversibly sorbed at lower concentrations of a divalent electrolyte (MgSO_4) than of a monovalent electrolyte (NaCl), an effect clearly related to the greater compression of the double-layer in the divalent system at comparable concentrations. In both electrolyte systems, all the bacteria were repelled from the surface when the value of $1/K$ exceeded about 200 \AA . This result suggests that the initial, reversible attraction of bacteria

to a surface depends on the magnitude of the double-layer repulsion energy at different electrolyte concentrations compared with the van der Waals attraction energy.

Effect of glucose on the irreversible sorption of Pseudomonas R 3. Sorption was negligible from NaCl, but appreciable from ASW (Fig. 4). Glucose (7 mg./l.) stimulated sorption from

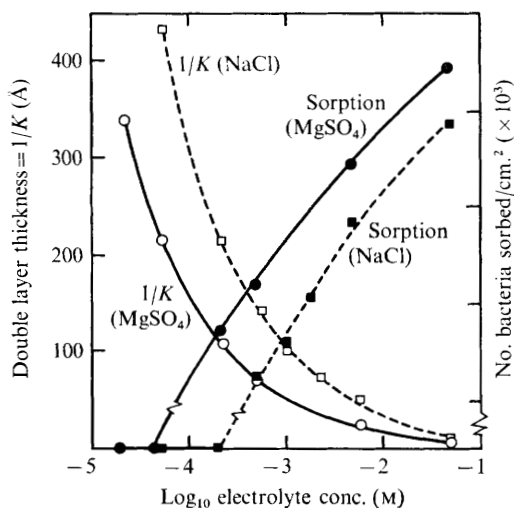


Fig. 3. Reversible sorption of *Achromobacter* R 8 and the theoretical double-layer thickness ($1/K$) in relation to electrolyte concentration and valency. $\square-\square$, $1/K$ for NaCl; $\circ-\circ$, $1/K$ for $MgSO_4$; $\blacksquare-\blacksquare$, sorption of *Achromobacter* R 8 from NaCl; $\bullet-\bullet$, sorption of *Achromobacter* R 8 from $MgSO_4$. The ionic strength of natural seawater is approximately 0.65 M.

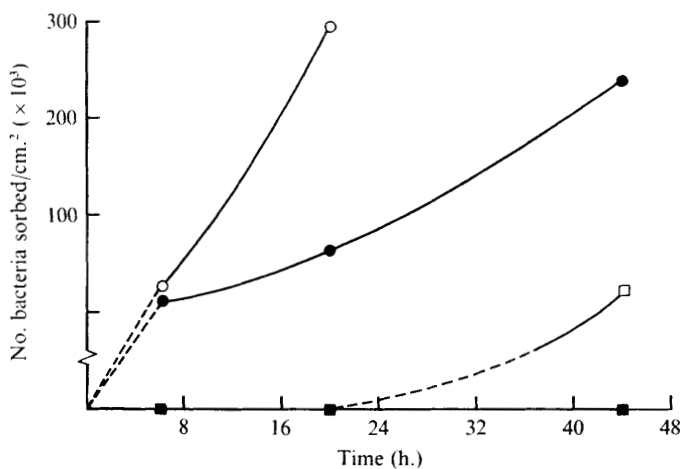


Fig. 4. Irreversible sorption of *Pseudomonas* R 3 from NaCl and artificial seawater (ASW). $\blacksquare-\blacksquare$, 2.5% NaCl; $\square-\square$, 2.5% NaCl + glucose (7 mg./l.); $\circ-\circ$, ASW + glucose (7 mg./l.); $\bullet-\bullet$, ASW.

both media. Electron micrographs of grids suspended in ASW or ASWG-7 showed very fine extracellular polymeric fibrils on bacteria sorbed from ASW (Fig. 5); bacteria sorbed from ASWG-7 had many more of such fibrils (Fig. 6).

Although the growth of *Pseudomonas* R 3 was stimulated by higher levels of glucose

irreversible sorption of the bacteria from ASWG-30 and ASWG-70 was inhibited completely (Fig. 7). Sorption was rapid from ASWG-7 and moderate from ASW. The irreversible sorption of *Pseudomonas* R3 was lowered dramatically even at glucose levels of 14 and 21 mg./l. (Table 2). Flocculation of bacteria in the bulk suspension was observed in conditions in which sorption was greatest.

Electron micrographs of grids immersed in ASWG-7 revealed large numbers of bacteria all with extracellular polymeric fibrils. Sometimes it appeared that some sorbed bacteria had been sheared from the grid surface by washing, leaving polymer 'footprints' on the grid surface (Fig. 8). Very few bacteria were found on grids from ASWG-14 and ASWG-21. Some bacteria showed little evidence of polymer production (Fig. 9), while others appeared to produce abundant polymer (Fig. 10).

Although ASW contained both NH_4Cl and NaNO_3 as nitrogen sources, the individual salts at equivalent nitrogen levels did not influence growth or the irreversible sorption of

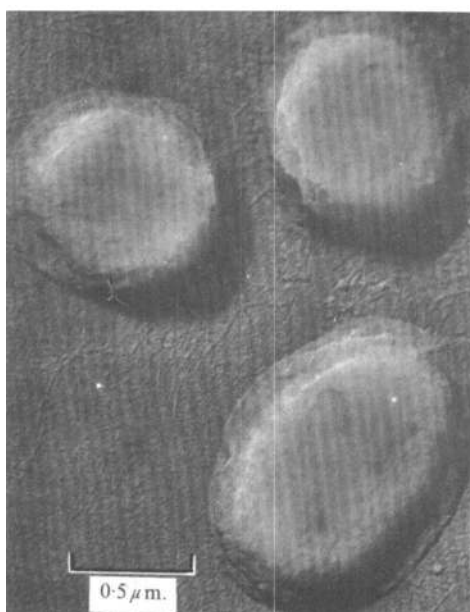


Fig. 5

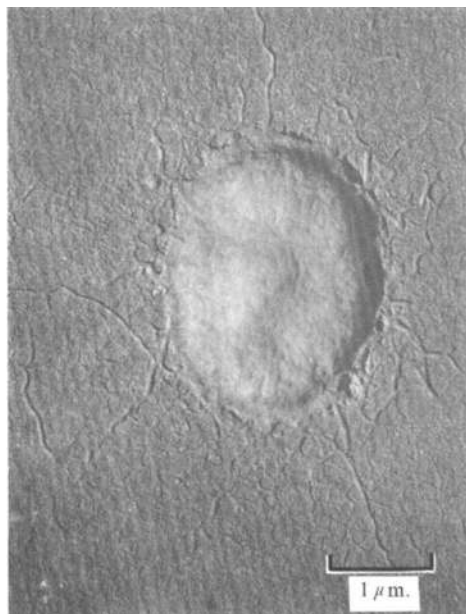


Fig. 6

Fig. 5. Extracellular polymeric fibrils on bacteria sorbed on grid immersed in artificial seawater.

Fig. 6. As in Fig. 5, but grid immersed in artificial seawater + glucose (7 mg./l.).

Table 2. *Effect of glucose levels in artificial seawater on growth and irreversible sorption of Pseudomonas R3*

Glucose (mg./l.)	No. bacteria/ml. after 24 h*	No. bacteria sorbed/cm. ² ($\times 10^3$)	
		20 h	4 days
0	14.8×10^7 †	44.6	488
7	26.1×10^7 †	65.8	514
14	33.2×10^7	2.3	78
21	44.1×10^7	2.1	31

* Initial inoculum = 10.0×10^7 bacteria/ml.

† Flocculation evident in the bulk suspension.

Pseudomonas R3 to glass surfaces. Since all the nitrogen in the basic ASW medium was available to *Pseudomonas* R3, then the C/N ratios ranged from 0.17 in ASWG-7 to 1.70 in ASWG-70. Consequently, all of these media were carbon deficient, so N-limitation was not the reason for production of extracellular polymeric materials in the media.

Effect of divalent cations on irreversible sorption of Pseudomonas R3. The omission of Ca^{2+} and Mg^{2+} (with Na_2SO_4 added to provide SO_4^{2-}) from ASWG-7 prevented the irreversible sorption of *Pseudomonas* R3 (Fig. 11). Mere addition of Ca^{2+} and Mg^{2+} to 2.5 % NaCl + glucose did not stimulate the sorption of *Pseudomonas* R3 from such a medium. Total numbers of *Pseudomonas* R3 in the suspension remained constant in all but ASWG-7, where rapid sorption appeared to be related to the limited growth of the bacteria. Few bacteria adhered to electron microscope grids immersed in ASWG-7 lacking Ca^{2+} and Mg^{2+} , and those that did showed no evidence of polymer production (Fig. 12).

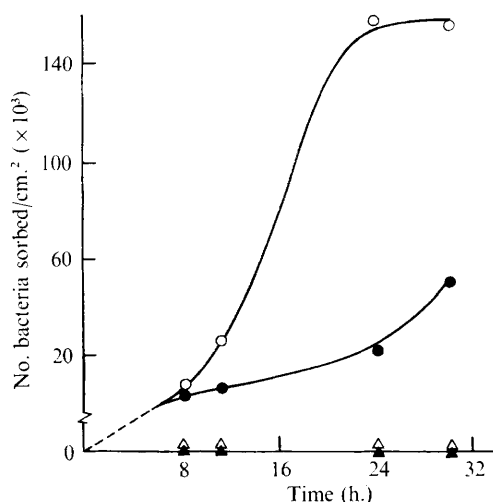


Fig. 7

Fig. 7. Effect of glucose concentration on the irreversible sorption of *Pseudomonas* R3 from ASW. ●—●, ASW; ○—○, ASW + glucose (7 mg./l.); △—△, ASW + glucose (30 mg./l.); ▲—▲, ASW + glucose (70 mg./l.).

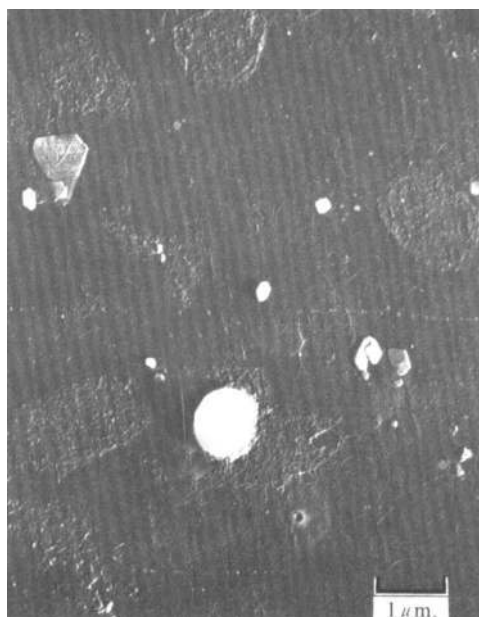


Fig. 8

Fig. 8. Polymer 'footprints' remaining after bacteria were sheared from the grid surface.

Table 3. Effect of divalent cations in artificial seawater on the irreversible sorption of *Pseudomonas* R3

Medium	No. bacteria sorbed/cm ²	
	24 h.	48 h.
ASWG	242×10^3 *	464×10^3 *
ASWG- Ca^{2+}	155×10^3	400×10^3
ASWG- Mg^{2+}	126×10^3 *	280×10^3 *
ASWG- Ca^{2+} , - Mg^{2+}	0	0

* Flocculation evident in the bulk suspension.

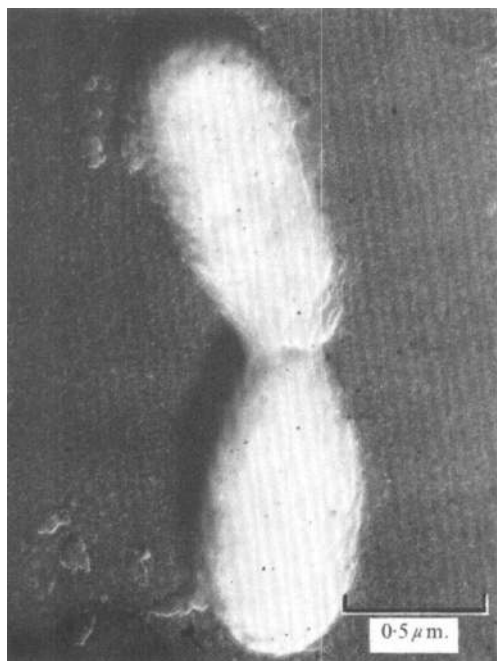


Fig. 9



Fig. 10

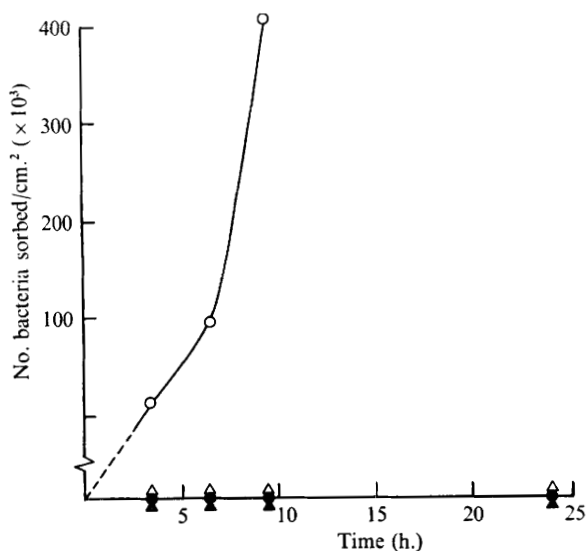


Fig. 11

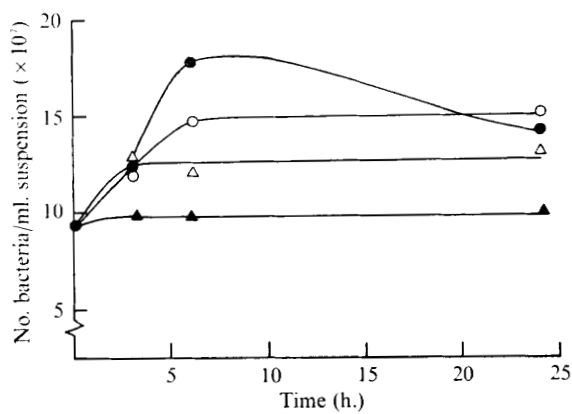


Fig. 12

Fig. 9. Bacterium lacking extracellular polymeric fibrils. From ASWG without Ca^{2+} and Mg^{2+} .

Fig. 10. Grid immersed in artificial seawater + glucose (14 mg./l.). Bacterium showing abundant polymer production.

Fig. 11. Effect of divalent cations on the irreversible sorption of *Pseudomonas R3* from NaCl and ASW. \blacktriangle — \blacktriangle , 2.5% NaCl + glucose; \triangle — \triangle , 2.5% NaCl + glucose + Ca^{2+} + Mg^{2+} ; \circ — \circ , ASW + glucose; \bullet — \bullet , ASW + glucose - Ca^{2+} , - Mg^{2+} .

Fig. 12. Effect of divalent cations on growth of *Pseudomonas R3* in ASWG (glucose at 7 mg./l.). \bullet — \bullet , ASWG; \circ — \circ , ASWG - Ca^{2+} ; \triangle — \triangle , ASWG - Mg^{2+} ; \blacktriangle — \blacktriangle , ASWG - Ca^{2+} , - Mg^{2+} .

Although irreversible sorption was inhibited when both Ca^{2+} and Mg^{2+} were omitted from ASWG-7, sorption did occur when either was omitted (Table 3). The degree of sorption from the various media (Table 3) appeared to be related to the growth of *Pseudomonas* R3 in the media (Fig. 12). Flocculation was observed consistently in the ASWG and ASWG-7 without Mg^{2+} , but not in ASWG without Ca^{2+} or ASWG-7 with neither Ca^{2+} nor Mg^{2+} . Thus, flocculation in the bulk suspension did not necessarily involve the same mechanisms as sorption.

Effect of age of inoculum on irreversible sorption of Pseudomonas R3. Sorption of young (log phase) bacteria from ASWG was much faster than that of older bacteria (Fig. 13), but within 24 h. the originally turbid bacterial suspension had completely lysed. Neither growth, lysis, nor sorption were observed with young bacteria in ASWG without Ca^{2+} and Mg^{2+} .

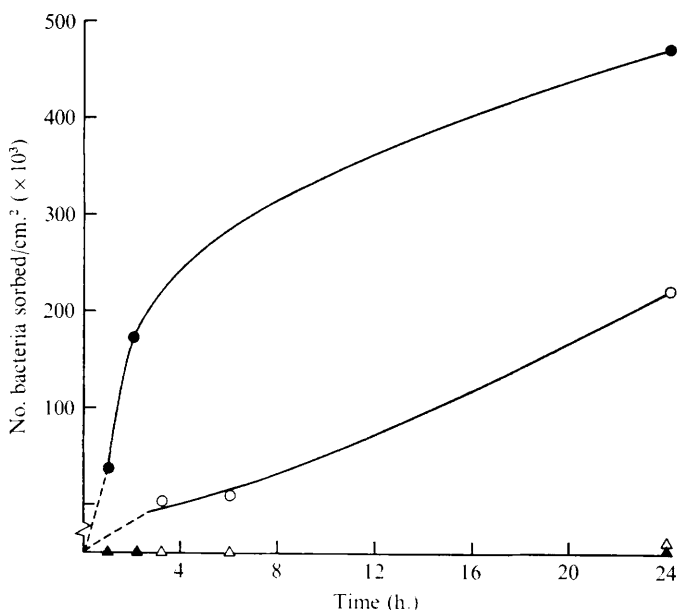


Fig. 13. Effect of age of inoculum on reversible sorption of *Pseudomonas* R3 from ASWG. ●—●, Log phase bacteria in ASWG, all bacteria lysed; ▲—▲, log phase bacteria in ASWG - Ca^{2+} , - Mg^{2+} , no lysis; ○—○, stationary phase bacteria in ASWG, no lysis; △—△, stationary phase bacteria in ASWG - Ca^{2+} , Mg^{2+} , no lysis.

DISCUSSION

In the sorption of marine bacteria to surfaces, different mechanisms must be involved in the essentially instantaneous reversible phase and in the time-dependent irreversible phase of sorption.

Reversible sorption. The effects of different concentrations of monovalent and divalent electrolytes on the reversible sorption of *Achromobacter* R8 suggest that this phenomenon may be explained in terms of the Derjaguin-Landau and Verwey-Overbeek theory (Shaw, 1966; Weiss, 1968). This theory involves an estimation of the magnitude, and variation with interparticle distance, of the London-van der Waals attractive energies between two surfaces and the electrical repulsive energies resulting from the overlapping ionic atmospheres (diffuse double-layers) around the surfaces.

The energies of interaction between *Pseudomonas* R3 and a glass surface at different values for the double-layer thickness have been computed (Weiss & Harlos, 1971) with a programme based on a rearranged version of the formula derived by Hogg, Healy & Fuerstenau (1966):

$$V_T = V_R + V_A,$$

where

$$V_R = \text{repulsion energy} = \frac{\epsilon}{4} \left(\frac{a_1 a_2}{a_1 + a_2} \right) \left[(\psi_1 + \psi_2)^2 \ln(1 + e^{-KH}) + (\psi_1 - \psi_2)^2 \ln(1 - e^{-KH}) \right],$$

$$V_A = \text{attraction energy} = -\frac{A}{6} \frac{a_1 a_2}{a_1 + a_2} \frac{1}{H},$$

where a is the radius of curvature of the particle, $a_1 = 10^4$ nm. (glass) and $a_2 = 0.4 \mu\text{m}$. (bacterium); ψ is the surface potential of the particle, $\psi_1 = -15$ mV (glass), $\psi_2 = -25$ mV (bacterium); K is the inverse Debye-Huckel length, $1/K$ varies from 0.645 nm. at 2×10^{-1} M-NaCl to 20 nm. at 2×10^{-4} M-NaCl; H is the distance of closest approach of the 2 particles; ϵ is the dielectric constant (of water); A is Hamaker's constant (5×10^{-15} ergs).

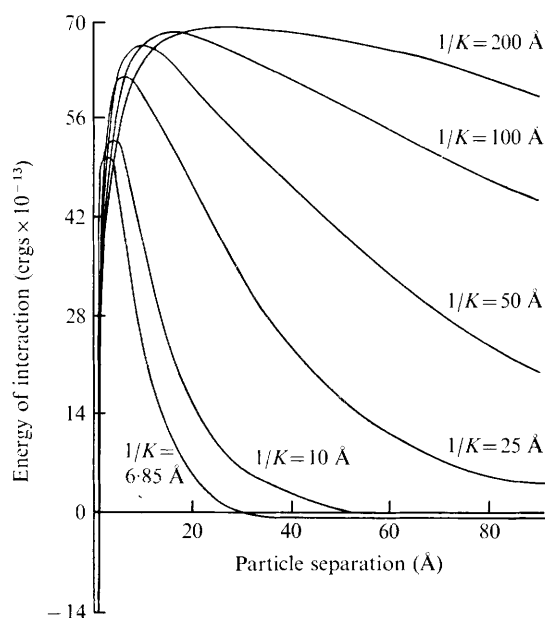


Fig. 14. Computed curves of the energy of interaction between glass and bacterial (*Achromobacter* R8) surfaces at varying electrolyte concentrations (varying double-layer repulsion) using a value of the Hamaker constant (A) of 5×10^{-15} ergs.

The series of curves in Fig. 14 demonstrate the increase in magnitude of the resultant repulsion curves and the increase in particle separation with decreasing electrolyte concentration (increasing values of $1/K$). At high electrolyte concentrations, a secondary minimum ('attractive trough') is apparent. The progressive increase in repulsion energy with decreasing electrolyte concentration closely parallels the observed decrease in reversible sorption of *Achromobacter* R8 (Fig. 3).

In the reversible phase of sorption in seawater or 2.5% NaCl it is likely that *Pseudomonas* R3 organisms are attracted to the point of the secondary minimum, a small but finite distance from the glass surface. It is unlikely that thermal motion would provide sufficient energy

for the bacteria to overcome the repulsion barrier. In fact, the kinetic energy of motile *Pseudomonas* R3 (5.45×10^{-18} ergs) is not sufficient to overcome the repulsion barrier at any value for $1/K$ shown in Fig. 14. The magnitude of the attractive energy at the secondary minimum may not be sufficient to hold the bacteria against the shearing effect of the rinsing process. The cine films clearly showed that motile *Pseudomonas* R3 could break away from the surface, although the attractive energy held them against violent rotational movements resulting from flagellar activity.

Irreversible sorption. The irreversible phase of sorption implies a firmer adhesion of bacteria to a surface. Polymeric bridging between the bacterial surface and that of the test surface might overcome the repulsion barrier between such surfaces. Such a mechanism has been proposed for the adhesion of tissue cells (Moscona, 1962) and sponge cells (Humphreys, 1965), and for the flocculation of microbial cells (Tenney & Stumm, 1965; Busch & Stumm, 1968). The electron micrographs presented provide evidence for the production of polymeric fibrils by *Pseudomonas* R3. In ASWG without Ca^{2+} and Mg^{2+} , polymer was not detected and irreversible sorption did not occur. The observation that polymer 'footprints' were left behind when bacteria sheared from the surface suggests that this polymer bound more tightly to the formvar coated surface than to the bacteria.

The fact that extremely low levels of available carbon stimulated irreversible sorption while higher levels inhibited this process may be relevant to microbial ecology. In natural seawaters the available carbon levels are usually very low, and such conditions probably favour the firm adhesion of micro-organisms to surfaces immersed in such environments.

Other factors are probably involved in both phases of sorption of bacteria to surfaces. The sorption to a surface of monolayers of various macromolecules present in seawater or even in bacterial cultures might drastically alter such physical characteristics of the surface as wettability and surface charge properties (Baier, Shafrin & Zisman, 1968).

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