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Surface-Associated Growth [and Discussion]

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Surface-associated growth

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[Plate 1]

In natural ecosystems, microbial activity is often associated with the presence of a surface, particularly in low-nutrient environments. The chemostat allows the study of such low-nutrient environments together with the precise control of other growth parameters. By using this system, enrichment cultures with inocula from two different river sources have been made. A more diverse community attached itself to surfaces placed in the chemostat when the cultures were carbon-limited than when the limiting nutrient was nitrogen. Further studies on a pseudomonad isolated from the carbon-limited enrichment cultures have shown that surface-associated organisms grow at approximately twice the rate of the same organism in the free surrounding medium. A hypothesis to explain this phenomenon based on the chemiosmotic theory is discussed.

Introduction

There are two basic life strategies to obtain food. The first is to stay in one place and collect the food as it passes by, the second is to seek out food and move around to find it. The major advantage of the first system is that there is no expenditure of energy in actually seeking out nutrients. For example, in the plant kingdom, given the ubiquitous distribution of carbon dioxide and light energy from sunlight, staying in one place is clearly an advantage. In contrast, in the animal kingdom the preferred system is the second strategy whereby food is sought by movement. These arguments are broad generalizations but they illustrate the two strategies employed by living systems.

In the microbial kingdom it was thought for a long time that the free movement of organisms to a nutrient source was the strategy most often utilized. Indeed, we know from the elegant studies of Adler and others that microbes have chemotactic systems that allow organisms to move along concentration gradients towards a food source (Adler 1975; Koshland 1977). More recently, it has become appreciated that microbes often exist in nature as a microbial film. Indeed, microbial films are the basis of a number of old established processes, for example in sewage treatment. Furthermore, surface adhesion of microbes to surfaces can lead to accelerated corrosion of many man-made structures, and the initial step in the induction of disease in animals and plants is the attachment of organisms to the host tissue.

This paper attempts first to review briefly the understanding of microbial growth at surfaces, secondly to report some recent studies in this area, and finally to speculate on the energetic advantages of microbes using this mode of growth.

If a microorganism is to divide on a surface it must be growing at the interface usually

between the bulk phase containing the microorganisms and the surface, be it solid or liquid (Marshall 1976). In physicochemical terms an interface is the boundary between two phases in a heterogeneous system. In nature there is a wide range of liquid–liquid, gas–liquid, solid–liquid and solid–gas interfaces. The phase differences, because of their different physical properties, show marked differences in their relations to microorganisms. A significant property of interfaces resides in their ability to concentrate and collect particles, since the deposition of microorganisms in most ecosystems has been shown to be concentration dependent.

Interfaces

Liquid–liquid

The oil-water phase boundary has importance in biological systems and most, if not all, oils give a distinct boundary with water. Microorganisms inhabit the interface between oils and water and as such have been shown to have a hydrophobic surface. These organisms are important in the degradation of oil spillages at sea and elsewhere. The growth of such organisms is clearly limited by the available interfacial area in the mixture, i.e. the smaller the droplet the greater the interfacial area, and hence the greater number of bacteria at the interface and the greater the utilization of the oil. Microbial activity at this interface has been suggested as a cause of the rapid degradation of these spillages (Marshall 1976).

Air–solid

This type of interface is apparently ubiquitous; indeed microorganisms grow on all such surfaces from the phylloplane of plant surfaces to the surfaces of stone buildings. It is important to recognize here that organisms growing on such a surface will have been derived from the air and hence will have been stressed by being aerosolized. Hence their ability to grow on such surfaces implies that they can recover from the aerosol stress. Furthermore, it should be noted that growth on such surfaces implies that some surface liquid, i.e. water, is present.

Solid–liquid–air (s–l–a)

Solid–liquid–air interfaces arise when a solid object is partly immersed in a fluid, as with the legs of drilling platforms in the North Sea where corrosion is a problem. Recently, evidence has been presented showing that material, including some bacterial species, accumulates preferentially at these interfaces (Leech *et al.* 1979), particularly when the contact angle of the meniscus is less than 90 ° (Rutter & Leech 1979). Small hydrophobic particles, such as bacteria, with densities similar to that of the liquid in which they are suspended, will tend to collect at liquid–air interfaces. If this liquid–air interface meets a surface, the particles will accumulate at the s–l–a contact zone. If the contact angle of the meniscus approaches 0°, the concentration of particles in this zone will be high and the surface area for deposition will increase, improving the chances of bacterial attachment. S–l–a interfaces will not necessarily be static; in many ecosystems, moving s–l–a interfaces occur, for example from the continual drainage of saliva over oral tissues. It has been argued that the accumulation of oral organisms at s–l–a interfaces may be important in the formation of dental plaque (Rutter & Leech 1979).

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Solid–liquid

The interface that has been most studied in terms of the growth of microorganisms is the solid–liquid. This is usually when a solid surface is exposed to an aqueous suspension of microorganisms. The charge on the solid surface will be important, and this is often negative. This may be due to the ionization of surface groups or by the adsorption of ions from the surrounding solution. This surface charge will also be dependent on the aqueous phase in that the pH of the phase will determine the ionization of the surface groups. Furthermore, once the surface has become charged it will attract ions of opposite charge. However, because of the effects of electrostatic attractions and thermal motion of the counterions, there will be a region next to the charged surface where the concentration of the counterions is greater than in the bulk aqueous phase (the Gouy–Chapman diffuse electric double layer). It is often found that both the surfaces of the solid and that of the bacterium are negatively charged and presumably this would lead to mutual repulsion. Studies on bacterial attachment to surfaces with different charges lend support to this suggestion (Fletcher & Loeb 1976). However, it has been suggested that divalent cations (such as Ca^{2+}) could act as a bridge between two negatively charged surfaces (Rölla 1976).

Surfaces of bacteria

The attachment of bacteria to a solid surface involves an interaction between the surface and the outer layers of the bacteria. The chemistry of the bacterial cell wall is now well understood but a detailed understanding of its function is lacking (for review see Ward & Berkeley 1980). The differentiation of bacteria by their reaction to the Gram stain is a traditional technique that correlates well with the structure of the bacterial cell wall.

Gram-positive bacteria

The cell walls of these organisms have a relatively simple structure and chemistry. The primary polymer found in Gram-positive bacterial cell walls is peptidoglycan, which makes up 40–50 % of the cell wall and confers rigidity on it. The glycan strand is composed of alternating residues of muramic acid and glucosamine in β-1-4 linkage. In most bacteria these residues are *N*-acetylated and the chain length can vary widely from several hundred to as low as 10–20 repeating disaccharide units. The carboxyl group of muramic acid is replaced by short peptides which contain both D- and L-amino acids. These peptides are often cross-linked with short chains of other peptides, e.g. pentaglycine residues in *Staphylococcus aureus*. The amount of cross-linking can also vary widely in walls of different organisms.

Several other polymers are found in the walls of Gram-positive bacteria. The most widely studied of these has been the class of compounds known as teichoic acids, which may account for over 60 % of the wall in some Gram-positive bacteria. These polymers have been defined as polymers of glycerol and ribitol phosphate, carrying sugars and ester-linked D-alanine substituents. The presence of these polymers in the bacterial wall may be changed by growth of the bacterium in a phosphate-limiting system. In such situations another acidic polymer replaces the teichoic acid and is composed of alternating *N*-acetyl galactosamine and glucuronic acid residues (teichuronic acid). By studying the rate of change from one polymer to another when the organism was switched from phosphate-limited to phosphate-excess growth it was found that turnover of teichoic acid to teichuronic acid had occurred. This together with

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studies of peptidoglycan turnover has produced the concept that the composition of the wall is related to the environmental conditions in which the bacteria grows. This suggests that the outer coat of bacteria is in dynamic equilibrium with its environment (Ellwood & Tempest 1972).

Another important point arises here in considering the charge on bacterial walls. Walls are often found to be negatively charged and this reflects the presence of anionic polymers, e.g. teichoic and teichuronic acids. However, the net charge of teichoic acids at least is modified by the presence of ester-bound D-alanine groups. Furthermore, the amount of ester-bound groups is known to vary with the pH of growth (Ellwood & Tempest 1972). Thus it is tempting to speculate that the charge distribution on the surface walls of Gram-positive bacteria (i.e. the outer surface of the bacteria) may be altered to reflect the environment of growth.

A second type of teichoic acid seems to be widespread in Gram-positive bacteria. This is a classical glycerol teichoic acid, which may be substituted with sugar or D-alanine residues or both, but the end of which is attached to a glycolipid. This type of teichoic acid is not covalently attached to the peptidoglycan, and it is secreted continuously by the membrane into the space between the wall and membrane, through the wall and outside into the environment surrounding the cell. The material secreted may still have its lipid moieties attached, in which case micelles of large molecular mass are formed. However, this lipoteichoic acid (LTA)may be deacylated to give simple solutions of teichoic acid (Wicken & Knox 1975).

The walls of some Gram-positive bacteria also contain specific polysaccharides and proteins, e.g. the type-specific polysaccharide and the M-proteins of group A streptococci.

Outer membrane of Gram-negative bacteria

These outer membranes are complex, with a basal layer of peptidoglycan and outer layers with protein, lipoprotein, phospholipid and lipopolysaccharide moieties. The lipopolysaccharides have received a lot of attention, as their specific chemical components have been used in the chemotyping of many groups of Gram-negative bacteria. The structure consists of a complex lipid (Lipid A) linked via a C_8 sugar acid, KDO, to a core polysaccharide, which is in turn substituted by side chains of oligosaccharides. However, it is interesting to note that substitution with 4-aminoarabinose and O-phosphorylethanolamine residues also takes place. Thus the overall charge of LPS is due to the acidic phosphorus diesters, KDO, and the two positively charged bases: these could be variable and as such might be analogous to the teichoic acid–alanine complex of Gram-positive bacteria. The outer membrane proteins have had a number of functions ascribed to them. For example, specific mannose lectins have been isolated from outer membrane proteins of *Escherichia coli* and *Pseudomonas aeruginosa*. They also provide the attachment points for a number of phages and appear to be involved in specific uptake systems for a number of nutrients (for review see Ward & Berkeley 1980).

Capsules and slimes

Many bacteria produce layers of material outside their cell wall. The material may be diffuse and found free in the culture fluids, or more closely attached to give distinct capsules. Strains of *Streptococcus pneumoniae* that produce capsules have been shown to be pathogenic, whereas those that do not are non-pathogenic. These capsules are polysaccharide in nature and there are over 70 different types of capsular polysaccharide produced. Interestingly some of these types

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are classified as teichoic acids, because of the polyol phosphate diester residues present in them. Other strains of streptococci secrete enzymes capable of producing polysaccharide from sucrose, and these strains have been implicated in the aetiology of dental caries (Gibbons & van Houte 1975). Water-soluble α-1–6 glucans, water-insoluble α-1–6, 1–3 glucans, and fructans are produced. It is clear how the water-insoluble glucans could play a role in the adhesion of these streptococci to the tooth surface. However, the role of the water-soluble glucan is not yet understood but it is suggested that this glucan can interact with secreted LTA (also produced by these strains) to give an insoluble matrix.

Many other types of bacteria also produce extracellular polysaccharides that are often acidic. They contain, for example, O-acetyl groups, pyruvic acid in ketal linkage, and often uronic acid residues.

Flagella and fimbriae

Bacteria also often produce filamentous appendages. There are two types, the flagella, which are rotated to move the bacteria passing them through the environment, e.g. in the direction of attractants ('food'). The second type, fimbriae, are somewhat smaller, and are found distributed over all the surfaces of the bacteria. Fimbriae have been implicated in the adhesion of bacteria. For example, the K88 and K99 antigens of *E. coli* are fimbrial proteins that bind the strain of *E. coli* possessing them to the intestine of the pig and cow, respectively (for review see Lingwood & Porter 1980).

Adhesion of microorganisms to a surface

There are three stages in the adhesion of microorganisms to a surface (Marshall 1976).

1. The deposition of the organism on to the surface (often referred to as the adsorption step). This can also be a reversible process and as such deposition studies give equilibrium data.

2. The permanent attachment to the surface. This often involves polymers acting as bridges between the two surfaces.

3. Colonization of the surface by growth of the organism.

Studies have been carried out on the deposition of organisms to a wide range of surfaces including glass, wire, polystyrene and hydroxyapatite. It is important to recognize that surfaces exposed to aqueous solution, particularly in natural systems, very rapidly adsorb any polymers that may be present. For example, in the mouth, a tooth surface (hydroxyapatite) which has been carefully cleaned rapidly acquires a protein layer (the acquired pellicle) on reimmersion into saliva, which will influence the pattern of deposition. Thus data on the deposition of bacteria onto clean surfaces is difficult to relate to natural systems.

The major surface forces that operate in particle deposition are (*a*) the London–van der Waals forces, (*b*) double-layer electrostatic interactions, and (*c*) bridging interactions (Rutter 1980).

Once a particle has been brought into the close proximity of a surface, these surface–particle interactions determine whether or not the particle is captured. The best described treatment of the interaction of small particles at close separation distances is attributable to the Derjaguin & Landau and the Verwey & Overbeek (D.L.V.O.) theory of colloid stability. This states that the total interaction energy, V_t, of two smooth particles is determined solely by the sum of the van der Waals attractive energy (V_a) and the usually repulsive electrostatic energy (V_r). Curves

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showing the variation of V_t with separation distance (h) can show two values of h at which a net attraction occurs (figure 1). These are referred to as the primary minimum (h very small) and the secondary minimum (h = 5–10 nm). These minima are separated by a repulsive maximum. Bacteria captured by a surface in a weak secondary minimum are in equilibrium with the remaining bacteria suspended in the bulk phase. The number of captured cells depends upon

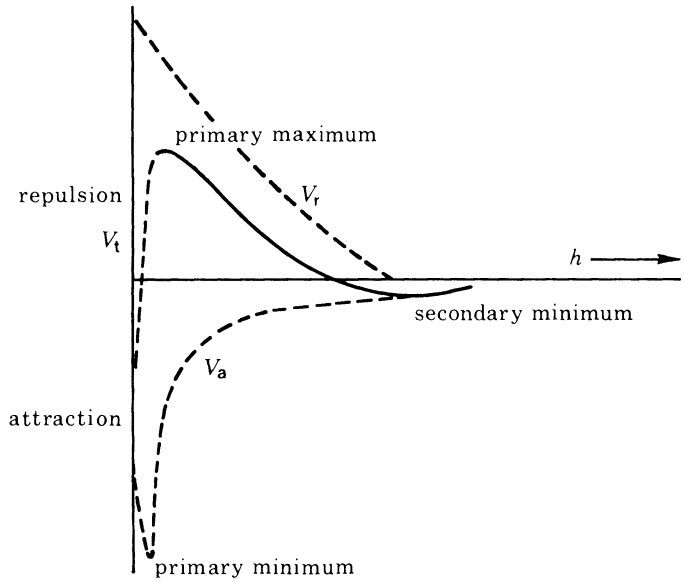

FIGURE 1. Variation of the total interactive energy, V_t, with h, the separation distance between a particle and a surface. The total interactive curve is obtained by the summation of an attractive curve, V_a, and a repulsive curve, V_r.

the concentration of bacteria in the suspension and the depth of the secondary minimum. The D.L.V.O. theory can be a useful guide to the interpretation of data on adhesion, but care should be taken when considering bacterial systems since many of the parameters required to calculate key functions cannot be determined accurately (Rutter 1980).

In addition to D.L.V.O. interactions, surface wettability and polymer interactions are important at very short and at large finite separations, respectively. It has been mentioned earlier that the outer layers of bacteria contain a wide range of polymeric material with a spectrum of properties and that most surfaces in Nature have an adsorbed conditioning film or possess specific surface receptors. The presence of solvated layers of macromolecules on both the approaching particle and the collector surface can lead to either repulsion or attraction.

Thus, in general, as bacteria approach a surface the van der Waals attractive forces can hold a cell in the weak D.L.V.O. secondary minimum for a short time. During this period, cell-surface polymers are either able to adsorb non-specifically to the surface or interact specifically with complementary polymers adsorbed to or forming part of the surface (polymer bridging). This increases the probability of attachment until enough links or bridges have been formed to hold the cell irreversibly. The presence of a secondary minimum is not an absolute requirement for this process but its presence does facilitate bridge formation. Further consolidation of attachment would occur by the synthesis of insoluble macromolecules, such as the mutan produced by *S. mutans*, that are extruded into the local environment, or by gelation of existing polymers (Morris *et al.* 1977), or even by precipitation of the polymers (Ash 1979), perhaps by

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neutralization with divalent cations (Rutter 1980). Growth of the attached cells would lead to the production of microcolonies and eventually to film formation.

Experimental studies on microbial communities and surface attachment

The second part of this paper describes some recent results from the collaborative research programmes of our two laboratories. The work in Scotland was concerned primarily with microbial attachment in an estuarine environment, while that at Porton dealt with the adhesion of bacteria to surfaces in the oral cavity. Such apparently diverse habitats share some properties and a common feature of both ecosystems is the absolute necessity for the indigenous micro-organisms to be attached to a surface if they are to remain in their particular habitat. The oral cavity provides a clear illustration of this point: saliva contains, on average, 10^8 micro-organisms per millilitre, which are derived from the surfaces of oral tissues. The flow rate of saliva through the mouth produces an effective dilution rate greater than 3.0 h^{-1}. In order for these organisms to stay in saliva their doubling times would have to be less than 20 min. The division rate of oral bacteria *in vivo* is not known with certainty, but values lower than 1–2 h have never been reported. Thus, unless the organisms adhere to a surface, saliva flow and swallowing will rapidly result in their removal ('wash-out') from the mouth. Similar arguments could be proposed for the growth of bacteria in a flowing stream.

The importance of surfaces for microbial growth was recognized early when Zobell & Anderson (1936) reported that the growth of bacteria in stored sea water was proportional to the surface to volume ratio of the containers. In work of a similar nature, Heukelekian & Heller (1940) showed that the growth of *Escherichia coli* in glucose–peptone water at glucose concentrations of less than 25 mg l^{-1} was possible only in the presence of glass beads. More recently, Jannasch (1958) found that the growth of *Bacillus subtilis* in dilute media occurred only in the presence of chitin particles. Furthermore, in a series of studies, Corpe (1970, 1974) showed that bacterial growth in supplemented sea water was restricted to a glass surface when very low nutrient concentrations were used. Enteric bacteria adsorbed to glass surfaces were metabolically more active than organisms in free suspension (Hendricks 1974) and a wide spectrum of bacteria exhibited an enhanced respiratory rate when adsorbed to clay particles (Stotzky & Rem 1966). A rise in the respiration rate of *B. subtilis* was also recorded when cells were adsorbed on bentonite and attapulgite particles (Lahav & Keynan 1962). Finally, lactic acid production by oral streptococci was found to be enhanced when hydroxyapatite crystals were added to the medium (Berry & Henry 1977). When considered collectively, the evidence presented above could be interpreted as suggesting that surfaces have, intrinsically, a positive influence on the growth of microorganisms isolated from a range of habitats, and that the subsequent behaviour of an organism on a surface may be different from that observed in liquid culture.

In our collaborative studies, we have used continuous culture techniques to study the effect of different variables, e.g. nutrient concentration, nutrient limitation, and sodium chloride concentration, on the composition and subsequent kinetics of surface growth.

In our initial experiments using enrichment techniques with river water systems, a carbon-limited and a carbon-excess (nitrogen-limited) culture was compared. The rationale for the experiment was that there was considerable evidence that extracellular polysaccharide

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production played an important role in bacterial attachment to a surface (Fletcher & Floodgate 1973). Furthermore, it was also known that carbon-excess cultures produced copious amounts of extracellular polysaccharide. Thus we expected that in these enrichment cultures there would be greater bacterial growth on the surfaces placed in the glucose-excess cultures. To our surprise we found that although there was a deep polysaccharide film on the surfaces in the glucose-excess culture, very few organisms were associated with the surface. In contrast, the film associated with the surfaces placed in the glucose-limited culture had only small amounts of

TABLE 1. ENRICHMENTS FROM THE RIVER TAY IN ACETATE-LIMITED CHEMOSTAT CULTURES

day 16 slide		day 18 liquid		day 19 slide	
		(*a*) *with no* NaCl *addition*			
entero. 2	95%	pseud. 1	28%	pseud. 4	53%
pseud. 1	5	pseud. 2	23	aer./vib.	24
		pseud. 3	22	acin.	21
		entero. 1	17	pseud.	2
		entero. 2	10		
population density					
9.5×10^6 cm^{-2}		6.0×10^3 cm^{-3}		5.0×10^5 cm^{-2}	
		(*b*) *with* 0.2 M NaCl *addition*			
pseud. 5	66%	entero. 3	93%	entero. 3	85%
entero. 3	34	entero. 4	7	entero. 5	15
population density					
7.0×10^4 cm^{-2}		4.4×10^3 cm^{-3}		5.0×10^5 cm^{-2}	
		(*c*) *with* 0.4 M NaCl *addition*			
acin. 1	91%	entero. 6	66%	acin. 1	44%
acin. 2	9	pseud. 6	28	pseud. 6	34
		entero. 6	6	acin. 2	18
				entero.	3
				acin.	1
population density					
2.6×10^3 cm^{-2}		1.5×10^7 cm^{-3}		8.3×10^3 cm^{-2}	

Abbreviations: entero., enterobacterium; pseud., pseudomonad; aer./vib., aeromonad/vibrio; acin., acinetobacter.

polysaccharide present, but a wide variety of different-shaped bacteria were attached to the surface. Cultural studies of the communities in the liquid phase of each chemostat showed significant differences. A single organism, *Aeromonas* sp., dominated (more than 90% of the cultivable flora) the glucose-limited chemostat at all growth rates tested. In contrast, although this organism was present in large numbers in the nitrogen-limited culture (75–80% of the cultivable flora), other populations including *Pseudomonas* sp., *Flavobacterium* sp. and *Vibrio* sp. were isolated.

Similar enrichments were carried out on water and sediments derived from the estuarine mudflats of the River Tay, Scotland. To favour the slow-growing bacterial populations likely to be present in such habitats, the enrichment cultures were carried out in a chemostat at low dilution rates ($D = 0.035$ h^{-1}, which corresponds to a community doubling time of 21 h) at 15 °C (Brown *et al.* 1978). The cultures were made carbon-limited with a variety of carbon sources and, to represent the spread of salinity found in this area of the estuary, the sodium chloride concentration in the medium was varied over the range 0–0.4 M.

Acetate-limited cultures were run for 19 days. Over the first 3 days the culture density

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increased from $3–4 \times 10^4$ to $10^7–10^9$ colony-forming units per millilitre. After day 5, the initial complex flora settled down to a steady-state community consisting of only a small number (usually less than 6) of bacterial types. After day 11, the community became stable and the data given in table 1 for day 18 are representative of the climax community. The composition of this final flora, however, did vary as a function of the sodium chloride concentration. A more complex community was obtained with the medium containing no added sodium chloride (table 1).

TABLE 2. ENRICHMENTS FROM THE RIVER TAY IN GLYCEROL-LIMITED CHEMOSTAT CULTURES

day 14 slide		day 14 liquid	
(*a*) *with no* NaCl *addition*			
pseud. 7	62%	entero. 7	52%
entero. 7	34	pseud. 7	28
pseud.	4	acin.	11
		pseud.	9
population density			
9.1×10^7 cm^{-2}		5.4×10^3 cm^{-3}	
(*b*) *with* 0.2 M NaCl *addition*			
entero. 8	48%	pseud. 8	60%
pseud. 8	48	entero. 8	34
pseud. 9	3	entero.	3
		pseud. 9	3
population density			
3.98×10^5 cm^{-2}		9.4×10^6 cm^{-3}	
(*c*) *with* 0.4 M NaCl *addition*			
pseud. 9	89%	pseud. 9	82%
entero. 9	7	entero. 9	10
pseud.	1	acin.	5
entero.	1	entero.	2
		pseud.	1
population density			
2.88×10^2 cm^{-2}		1.4×10^6 cm^{-3}	

Abbreviations as in table 1.

Sterile glass microscope slides were placed in the chemostats on day 1 and were removed on day 16. The data presented in table 1 show that only a limited number of bacterial populations were attached to these surfaces but, interestingly, that these adherent organisms were markedly different from those isolated from the liquid phase of the chemostat. Furthermore, when slides were inserted into the cultures on day 16 and removed on day 19, more complex communities were recovered from the surfaces, which again showed differences from those found in the liquid media.

When glycerol was used as the limiting nutrient, the liquid phase and surface-associated communities bore strong resemblances, although their composition was influenced by the sodium chloride concentration of the medium (table 2).

The number of organisms found on the surface of a glass slide appeared to be related to the cell density of the liquid phase, although no direct correlation could be discerned. This might be due to the morphology of the organisms on the slide (chains of cells, and filaments), which suggested that they were growing as microcolonies, whereas in the liquid phase the same organism grew as single cells. This variation of cell morphology when growing on a surface may be an

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important point. In their work on holdfast bacteria, Dow & Whittenbury (1980) have shown that changes in the nutrient composition of the media can induce variation in the structure of these prosthecate bacteria.

In enrichment cultures derived from human dental plaque (P. D. Marsh *et al.*, unpublished results) it was found that the composition of the microbial community associated with the glass surface of the splatter-zone of the chemostats was different from that in the liquid phase. Interestingly, the microbial film that developed in the glucose-limited chemostat harboured spirochaetes that could not be isolated at any time from the liquid phase or from the original inocula. As with the river water experiments, the community from the surface of the glucose-limited culture was different from that of the glucose-excess chemostat.

The results of these enrichment experiments suggested, firstly, that the surface provided a different growth environment than did the free solution and, secondly, that carbon-limited cultures resulted in a more diverse surface-associated microbial community that apparently did not depend on the production of copious amounts of extracellular polymers for attachment.

These conclusions could be rationalized on the basis that under carbon-limited conditions there might be an increased concentration of the limiting substrate at the surface. Organisms grown under glucose-limited conditions are known to have very efficient uptake systems for this sugar (Herbert & Kornberg 1976; Ellwood *et al.* 1979). Thus, organisms with a high-affinity uptake system might recognize molecules of the limiting nutrient concentrated at a surface, so that an interaction leading to adhesion could occur. This type of docking procedure has been suggested by Marshall (1976) to explain his results with marine bacteria. However, the problem with this proposal is whether there would be a constant supply of the limiting substrate to the surface to maintain growth. In an attempt to answer this question, experiments with pure cultures to study the kinetics of bacterial growth on an artificial surface were carried out in a chemostat.

Studies on pure cultures

One of the organisms (*Pseudomonas* sp. GAG7) isolated from a surface in the River Tay experiments was chosen for further study in pure culture. The organism was grown in the same media as those previously used (Brown *et al.* 1977) but with no added sodium chloride. The growth rate was fixed at $D = 0.06\ h^{-1}$ but the limiting-substrate concentration was varied to give different culture densities. Both carbon- and nitrogen-limiting conditions were used and by using banks of chemostats it was possible to study the effect of culture density on the colonization of surfaces under otherwise identical experimental conditions.

Clean glass surfaces were immersed for fixed time periods of 5 h and 1–5 generation times of the liquid culture. On removal from the culture vessel the slides were rinsed in sterile 0.2 M NaCl

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Description of plate 1

Figure 2. Scanning electron micrograph of a glass surface exposed for five generation times in a carbon-limited chemostat culture of a *Pseudomonas* sp. ($D = 0.06\ h^{-1}$, $T = 15\ °C$, $S_r = 50\ \mu M$ glycerol), showing micro-colony development.

Figure 3. Scanning electron micrograph of a glass surface exposed for five generation times in a carbon-limited chemostat culture for a *Pseudomonas* sp. ($D = 0.06\ h^{-1}$, $T = 15\ °C$, $S_r = 5$ mM glycerol), showing extensive 'microfibril' development.

Figure 4. Scanning electron micrograph of a glass surface exposed for five generation times in a nitrogen-limited chemostat culture of a *Pseudomonas* sp. ($D = 0.06\ h^{-1}$, $T = 15\ °C$, $S_r = 300\ \mu M$ NH_4Cl), showing short microfibrils and cell–cell adhesion.

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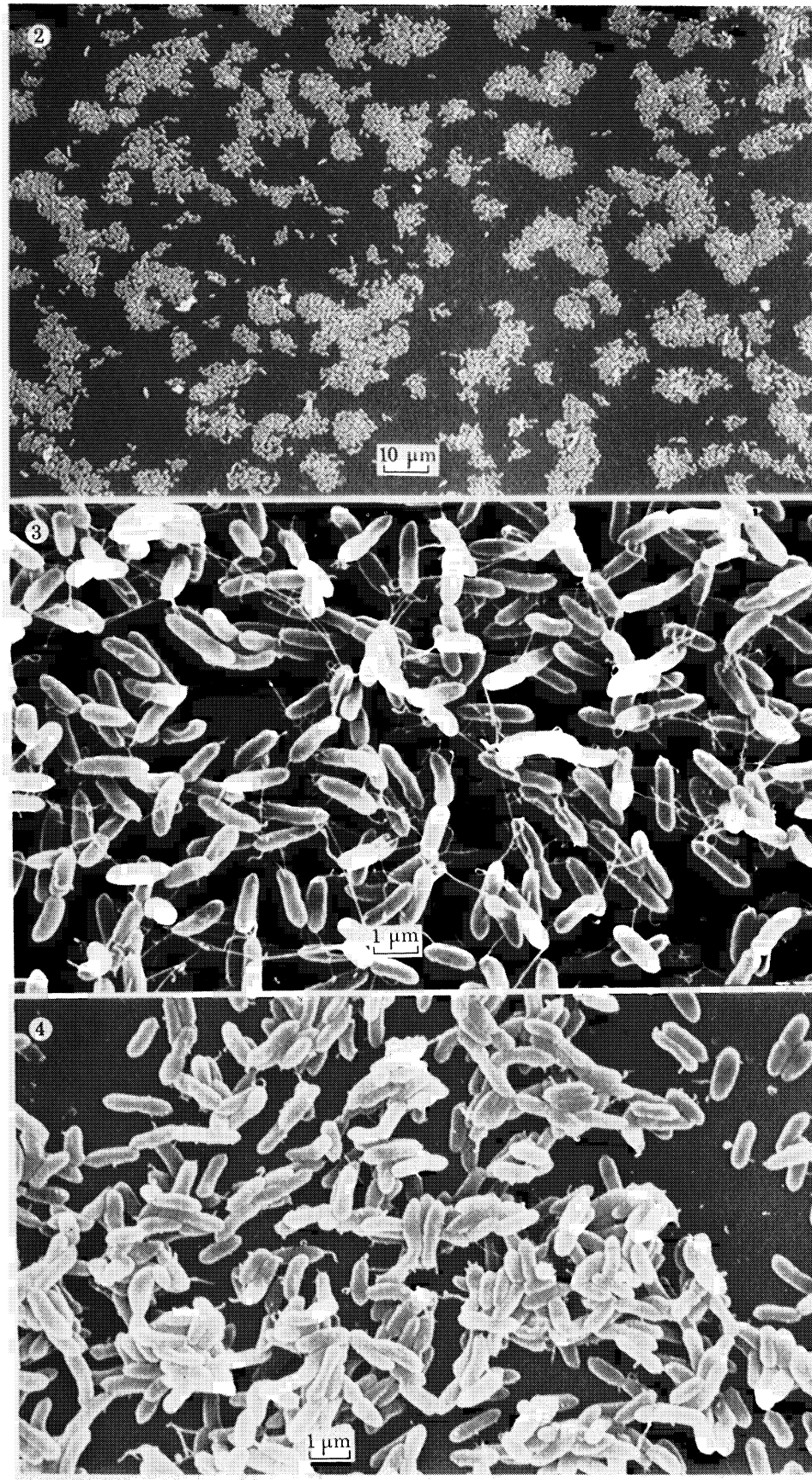

FIGURES 2–4. For description see opposite.

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and swabbed to remove adhered bacteria, which were counted, after serial dilution, on Tryptone Soya Agar (Oxoid) plates. In duplicate experiments, exposed surfaces were prepared for scanning electron microscopy by rinsing with distilled water, fixing in glutaraldehyde (25 g l^{-1}) in cacodylate buffer, and drying at the critical point under liquid CO_2. Specimens were then mounted, coated with gold and examined with an ISI-60 scanning electron microscope.

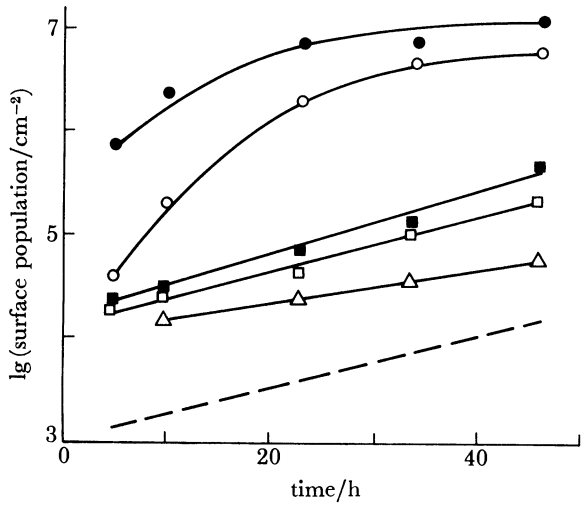

FIGURE 5. Rate of increase in the surface population density of a *Pseudomonas* sp. grown in carbon-limited chemostat culture (D = 0.06 h^{-1}, T = 15 °C). △, S_r = 50 nM glycerol, liquid population density (l.p.d.) = 3×10^5 ml^{-1}; □, S_r = 500 nM glycerol, l.p.d. = 7×10^5 ml^{-1}; ■, S_r = 5 μM glycerol, l.p.d. = 4×10^5 ml^{-1}; ○, S_r = 50 μM glycerol, l.p.d. = 4×10^6 ml^{-1}; ●, S_r = 5 mM glycerol, l.p.d. = 7×10^7 ml^{-1}; - - -, rate of growth of liquid population.

The examination of stained films and the scanning electron micrographs showed that surface growth occurred as microcolonies that eventually coalesced into relatively uniform films after five generations of the liquid population. This development was somewhat masked at high culture densities by the adsorption of single cells, which in turn developed into new microcolonies. At low culture densities, however, where this 'background scatter' was less obvious and the development of microcolonies could be clearly seen, the scanning electron micrograph (figure 2, plate 1) shows the development of microcolonies. At higher magnification, a second feature of considerable interest is seen (figures 3 and 4, plate 1).

Fibrils that connect cell to cell and also connect cells to the surface are clearly apparent. These fibrils are well developed in films derived from carbon-limited cultures but are also present in films prepared from nitrogen-limited cultures. It should be noted that the presence of these fibrils was only revealed by the use of critical-point drying techniques. The use of more conventional techniques showed no fibrils present.

The results of the time-course studies are shown in figures 5 and 6 and both carbon and nitrogen limitation gave essentially similar results. Each point on the graphs represents the average value obtained from five separate experiments.

At low liquid culture densities (low S_r) the rate of increase of organisms on the surface was similar to that of the doubling time of the liquid population. However, as the liquid population increased so did the rate of increase of the surface population until at a liquid population density of *ca.* 10^8 ml^{-1} (S_r = 5 mM) under carbon (glycerol) limitation the rate of increase

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was logarithmic until the surface became saturated at *ca.* 7×10^6 colony-forming units per square centimetre, after which the surface population density fluctuated. A similar pattern of results was obtained with nitrogen-limited cultures.

Thus at low culture densities the rate of increase of bacterial numbers on surfaces occurs at a rate very close to the growth rate of the liquid culture. At high culture densities, however, the

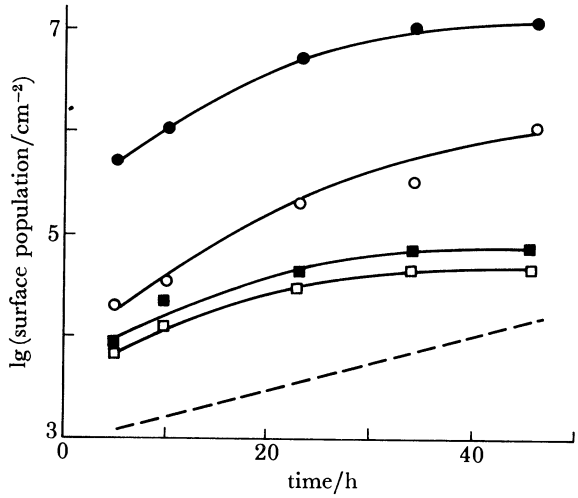

FIGURE 6. Rate of increase in the surface population density of a *Pseudomonas* sp. grown in nitrogen-limited chemostat cultures ($D = 0.06\ h^{-1}$, $T = 15\ °C$). □, S_r = 300 pM NH_4Cl, l.p.d. = $4 \times 10^5\ ml^{-1}$; ■, S_r = 30 nM NH_4Cl, l.p.d. = $3 \times 10^5\ ml^{-1}$; ○, S_r = 3 μM NH_4Cl, l.p.d. = $1 \times 10^6\ ml^{-1}$; ●, S_r = 300 μM NH_4Cl, l.p.d. = $2 \times 10^7\ ml^{-1}$.

rate of increase of bacterial numbers is greatly in excess of this value. At high culture densities collisions between bacteria and the surface will occur more frequently and this must in part account for the apparent increased rate of development of the surface film. However, it is apparent from the microscopic evidence that large numbers of bacteria on the surface are the result of microcolony development rather than non-specific attachment and it seems likely that the increased rate of numbers of organisms on the surface is due in large part to an increased rate of growth at the surface compared with that of the liquid population.

DISCUSSION AND SPECULATION

The results obtained with the chemostat enrichment studies showed clearly that the surface provided a different environmental niche from that in the free fluid culture. Furthermore, organisms that grew on the surface seemed to have a specific morphology when growing in this situation.

Further evidence to support our proposal that microbial behaviour at a surface is different from that in liquid culture was obtained from a study of a pure culture of a pseudomonad isolated from a surface enrichment. This organism grew on the surface as microcolonies, which developed into a film of organisms over the whole surface. Scanning electron microscopy of critical-point dried films showed that, particularly when growing in carbon-limited conditions, there appeared to be fine fibrils that attached the cells to the surface. Finally, when the growth rate on the surface was compared with the known fixed growth rate of the culture it was found

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that surface growth was faster, particularly at the higher cell densities. This could have resulted from increased deposition, but as growth on the surface was in colony form this explanation does not seem likely. In addition, studies of the deposition of *Streptococcus sanguis* to a glass surface showed that saturation coverage was only 30 % of the total area available (Rutter & Leech 1980). Film formation was only achieved by cell growth when fresh medium was pumped over the deposited cells. The generally accepted explanation of surface-enhanced growth is that as increased concentrations of molecules occur at an interface there will be an increased concentration of the limiting nutrient at the surface (for review see Marshall 1976). However, unless the mass transfer of the limiting substrate to a surface from the bulk phase is continuous and rapid, the increased concentration will soon be consumed.

We therefore wish to propose an alternative interpretation based on ideas derived from the chemiosmotic hypothesis of energy conservation. Microorganisms derive energy for growth either by ATP synthesis from substrate-level phosphorylation by central metabolism or by the generation of ion gradients across the cell membrane. In particular, protons are translocated out of the cell to generate a membrane potential (inside negative) between the bulk phase and intracellular phase. This process has been described by Mitchell's chemiosmotic hypothesis (1961, 1966) as a proton motive force (Δp). If $\Delta\psi$, the electrical potential difference, is expressed in millivolts and ΔpH, the pH gradient difference, in pH units, then the electrochemical activity gradient for protons (Δp) is defined as

$$\Delta p = \Delta\psi - 2.3\ (RT/F)\ \Delta\mathrm{pH},$$

where R is the gas constant, T is the absolute temperature and F is the Faraday constant. At 30 °C, $2.3\ RT/F = 60$. Hence Δp at 30 °C can be expressed in millivolts as

$$\Delta p = \Delta\psi - 60\Delta\mathrm{pH}.$$

The potential energy developed by chemiosmotic processes can be used to drive such apparently diverse functions as flagellar motion, the uptake of essential ions and substrates, the excretion of ions and metabolic by-products against their concentration gradients, and the generation of ATP. Implicit in this last function is the requirement of an ATPase in the proton-impermeable cell membrane that catalyses the synthesis of ATP from ADP and inorganic phosphate against the inward flow of protons through the enzyme complex.

At present there is considerable controversy as to whether protons are lost to the surrounding medium or are conducted through channels at the cell surface, which prevents their loss (see Mitchell 1966; Williams 1981; Kell *et al.* 1981). Nevertheless, it is experimentally true that pH changes are detectable in the surrounding milieu, suggesting loss of protons from the cell's influence at some stage in the chemiosmotic process (figure 7*a*).

By contrast, localized proton concentrations at the cell surface will tend to increase in the restricted zone established when the cell domain interacts with a surface (figure 7*b*). Because these protons will not diffuse so easily the probability of their re-uptake by chemiosmotic process is greatly increased. As a consequence, the cell's membrane will be more energized on the side nearest the surface. A polarity across the cell will be established and it is possible that this informs the cell of its position in space and provides the driving force for adhering processes on the surface side of the cell, enabling it to attach to the surface (figure 7*c*). With the increased efficiency of proton re-uptake established, more energy is available to the cell, which will encourage growth, division and the build-up of a colony or film of cells (figure 7*d*), as we

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observed in our experiments. It is then apparent that cells will not only interact with the surface but also with their neighbours. Proton gradients will therefore be shared within the community, and actively metabolizing cells will fuel their weaker neighbours.

This could be true for all aerobic organisms, in that protons are passed through the membrane by the respiratory chain and extra ATP is generated by these protons moving back into the cell via the ATPase.

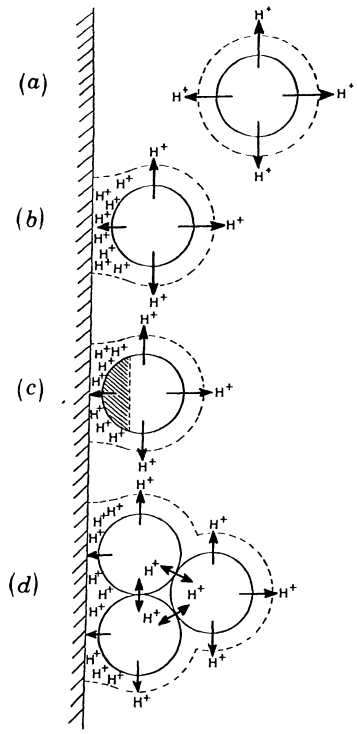

FIGURE 7. Postulated chemiosmotic interactions of a cell at a surface. The diagram describes (*a*) a cell in free suspension generating a proton gradient, (*b*) the interaction of the cell's domain with a surface, establishing a localized higher concentration of extruded protons, (*c*) localized Δp and ATP synthesis leading to increased metabolic activity and polarity of the cell to drive adhering processes in this region, and (*d*) the establishment of a microcolony with sharing of proton gradients between members.

The situation for organisms growing anaerobically and producing ATP by glycolysis of glucose is similar. It has been shown by ten Brink & Konings (1980) that the export of lactate from a cell occurs with extrusion of protons across the cell membrane. Thus it is possible that these protons could re-enter the cell via the ATPase and produce more ATP and hence increased cell metabolism and growth.

A possible problem for microorganisms utilizing lactate gradients for energy generation results from the accumulation of the anion outside the cell, thereby dissipating the gradient. However, this problem is overcome in certain complex communities where it is becoming increasingly apparent that lactate-consumers are intimately related with the lactate-producers. For example, lactate utilizers are major colonizers of the gastrointestinal tract (Savage 1977) and the rumen (Latham & Jayne-Williams 1978). Furthermore, acetobacteria, desulphovibrios and veillonellae are regularly isolated from marine sediments considered to be energy-limited (Laanbroek & Veldkamp, this symposium), whereas mixed communities of methanogens are very successful in removing lactate from the environment (Mah, this symposium). As a conse-

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quence, lactate accumulation is reduced and proton recirculation is encouraged. Such beneficial interactions have also been demonstrated in the laboratory between *S. mutans* and *Veillonella alcalescens* (Mikx & van der Hoeven 1975); the yield of the streptococcus increased markedly when grown in continuous culture with the veillonella. Indeed, organisms can be seen in sections of dental plaque to be intimately associated with each other and their morphology is distorted to maximize surface contact (van Houte & Saxton 1971).

These speculations suggest (1) an alternative interpretation for the increased metabolic activity associated with growth at a surface, (2) that it is energeticallv advantageous for organisms to be attached to and interact on a surface, which might explain the ubiquitous nature of microbial films, and (3) mechanisms whereby organisms might detect the presence of a surface. The extra energy generated by cells at a surface could drive the synthesis of extracellular polymers that enable permanent attachment to that surface.

In summary, we feel that our experiments have demonstrated that surfaces provide unique environments that profoundly affect microbial growth. Our speculations, with respect to the chemiosmotic hypothesis, on energetics of microbial growth at a surface provide an explanation as to why microbial films and the interactions of different species therein are ubiquitous in Nature.

We would like to thank Dr Tony Williams of Western General Infirmary, Edinburgh, for his help in the preparation of the scanning electron micrographs.

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Discussion

N. Le Roux (*Warren Spring Laboratory, Stevenage, U.K.*). When bacteria grow on surfaces, do they divide *in situ* or do they loosen from the surface to divide?

D. C. Ellwood. There is no strong evidence either way, but I consider that they divide *in situ*.

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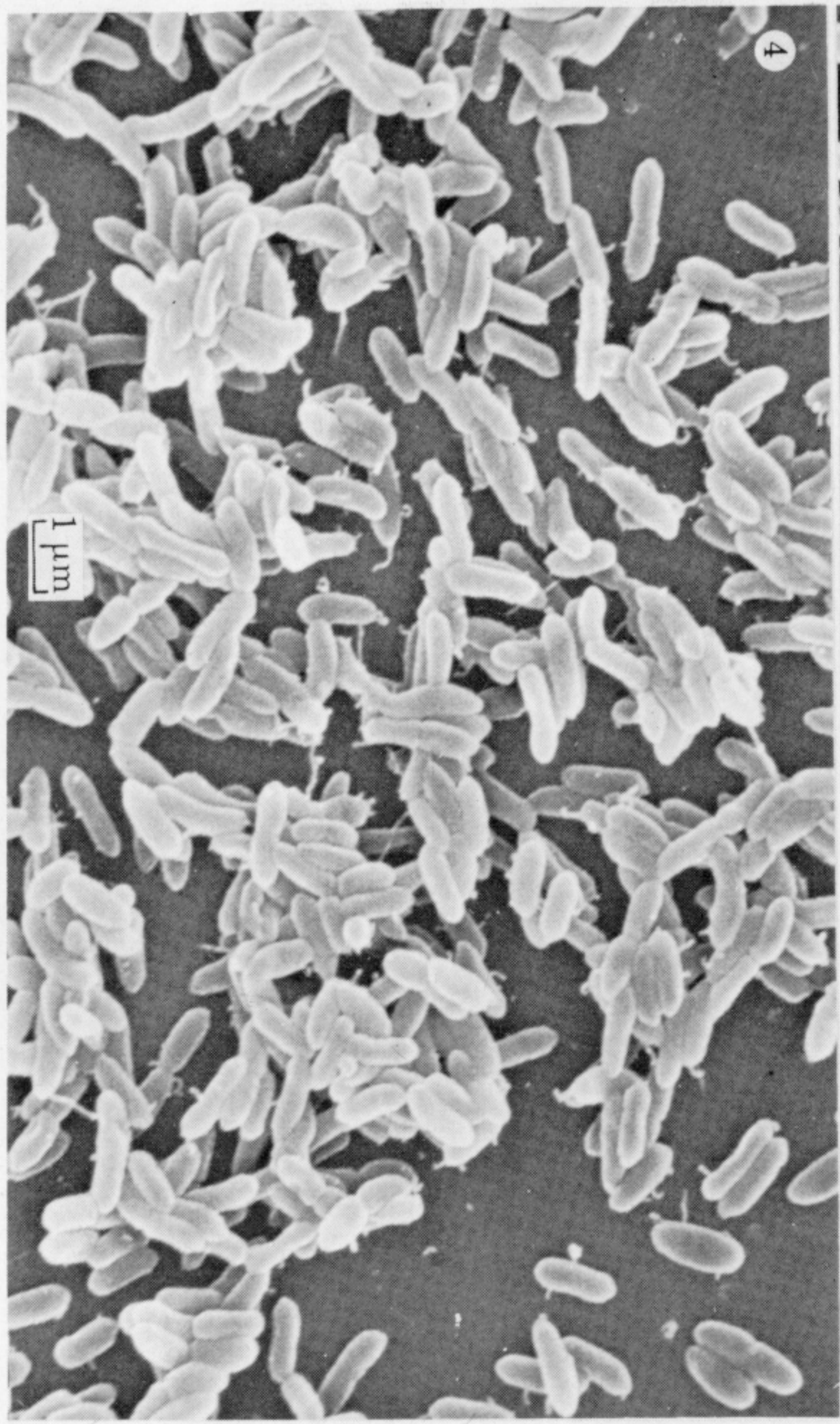

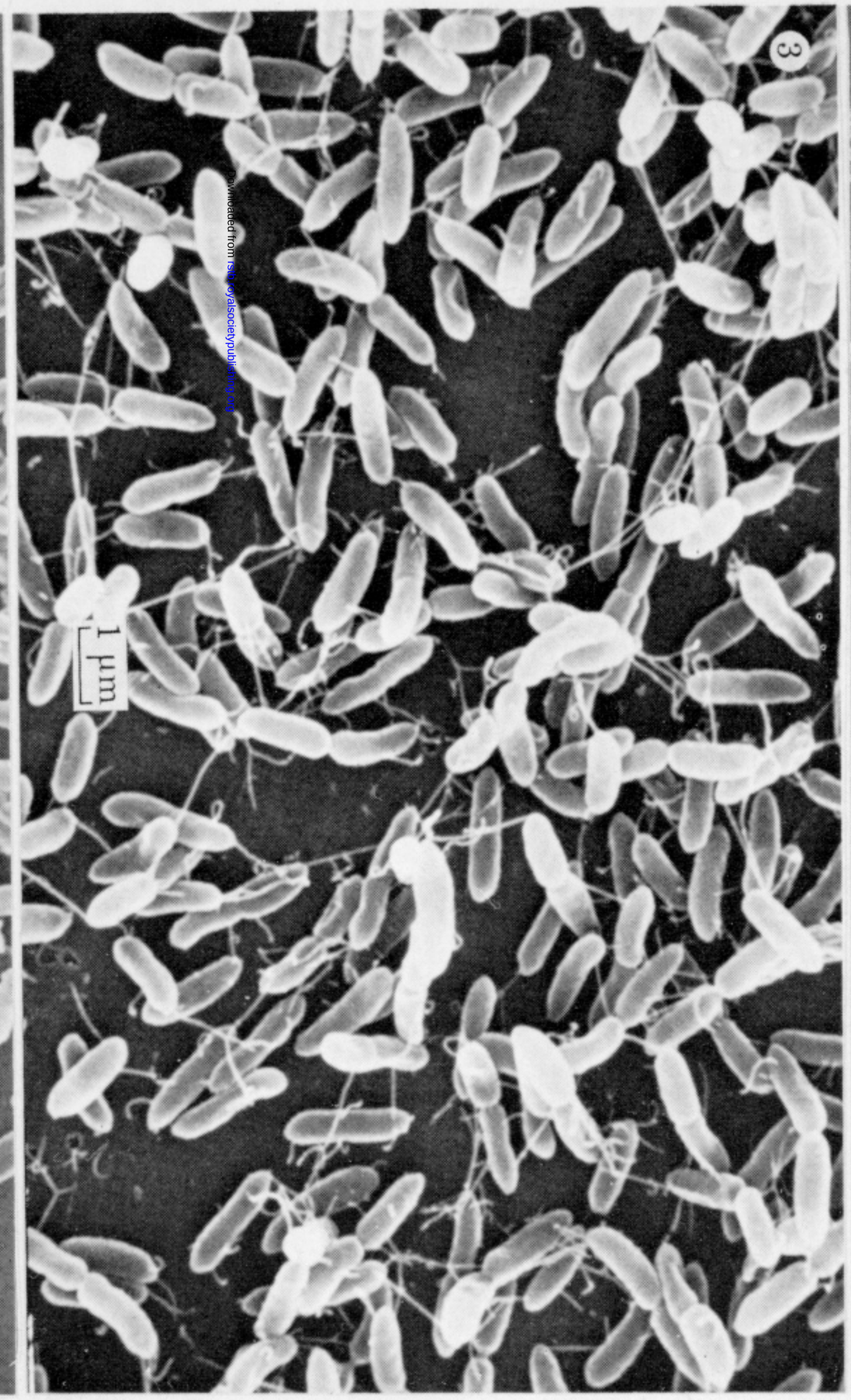

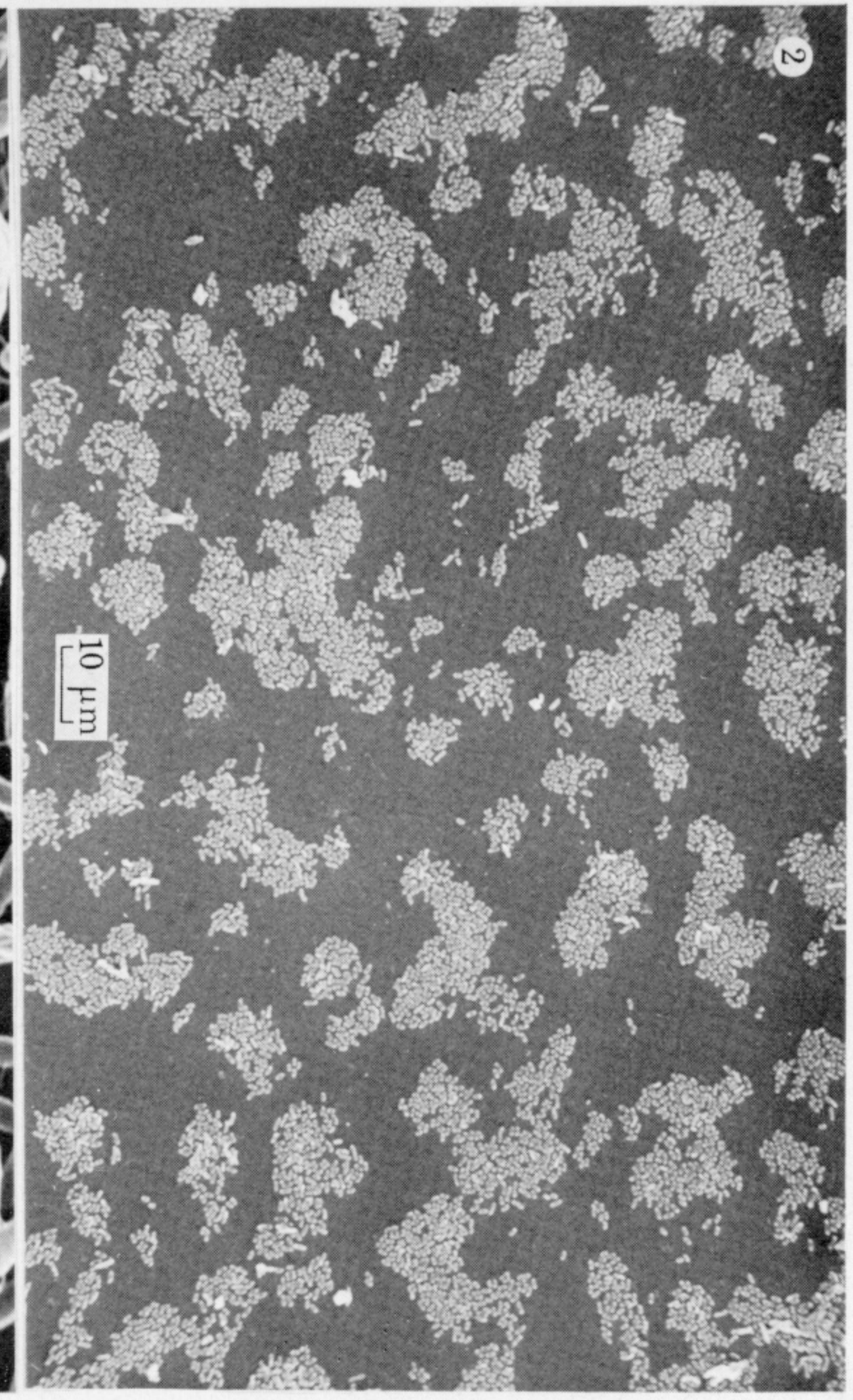

FIGURES 2–4. For description see opposite.