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Interactions between biofilms and the environment

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Abstract

The surfaces of bacteria are highly interactive with their environment. Whether the bacterium is Gram-negative or Gram-positive, most surfaces are charged at neutral pH because of the ionization of the reactive chemical groups which stud them. Since prokaryotes have a high surface area-to-volume ratio, this can have surprising ramifications. For example, many bacteria can concentrate dilute environmental metals on their surfaces and initiate the development of fine-grained minerals. In natural environments, it is not unusual to find such bacteria closely associated with the minerals which they have helped develop. Bacteria can be free-living (planktonic), but in most natural ecosystems they prefer to grow on interfaces as biofilms; supposedly to take advantage of the nutrient concentrative effect of the interface, although there must also be gained some protective value against predators and toxic agents. Using a *Pseudomonas aeruginosa* model system, we have determined that lipopolysaccharide is important in the initial attachment of this Gram-negative bacterium to interfaces and that this surface moiety subtly changes during biofilm formation. Using this same model system, we have also discovered that there is a natural tendency for Gram-negative bacteria to concentrate and package periplasmic components into membrane vesicles which bleb-off the surface. Since some of these components (e.g., peptidoglycan hydrolases) can degrade other surrounding cells, the vesicles could be predatory; i.e., a natural system by which neighboring bacteria are targeted and lysed, thereby liberating additional nutrients to the microbial community. This obviously would be of benefit to vesicle-producing bacteria living in biofilms containing mixed microbial populations.

Keywords: Biofilm; Fine-grain mineral development; Attachment to surface; Predatory membrane vesicle

Contents

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1. Introduction

To prosper, every creature requires a measurement of safety from predators, ample nutrition (and water), and a place within a healthy community. It is apparent from the study of natural environments that bacteria are found most commonly associated with surfaces. These can be solid and inanimate interfaces (e.g., the surfaces of rocks in fluid media, pipelines used to transport fluids, the surfaces of medical implants such as pace-makers and catheters, etc.) or softer animate interfaces (e.g., plant rootlets, intestinal tracts, etc.) [1]. Rich, diverse microbial communities are found on teeth (i.e., dental plaque [2]) as well as on the air-fluid interface of freshwater ponds. More recently, it has become apparent that those bacteria which inhabit the subsurface (whether the subsurface be in shallow sediments or soils, or in deep geological horizons) also prefer a 'surface mode' of growth [3–5]. Biofilms are found everywhere that life can exist.

Surfaces offer distinct advantages to prokaryotic cells which depend on the diffusiveness of nutrients and wastes for their well-being [6–8]. Most natural aqueous environments contain only dilute substances which can be used for metabolism and growth, and the interfacial effect of natural surfaces tends to collect and concentrate nutrients by charge-charge or hydrophobic interactions. This so-called conditioning of natural surfaces, then, provides bacteria with an opportunity to take advantage of more concentrated foodstuffs. Free-living, planktonic modes of growth, which we see so commonly in laboratory broth cultures, may be more infrequent in natural settings and could be a growth mode whereby individual cells leave unsuitable surfaces to seek out and find more advantageous niches. Once such surfaces have been identified, planktonic cells would attach (initially the attachment is readily reversed but, over time, it becomes strong and irreversible) and, through growth and division, biofilms eventually develop. Now, the initial interface has subtly been altered since biofilms can be one to hundreds of μm thick and the original surface is completely covered by the biofilm. But, the same nutrient concentrative process is still in effect and now a microbial community has been established (Fig. 1). Intermixed with cells are extracellular polysaccharides so that the entire biofilm is encased in a protective organic matrix. And, microbial hierarchies are established with different bacterial cells strategically placed with respect to one another; nutrient fluxes are established as are pH and redox gradients. Presumably, the size and thickness of a biofilm is dependent on the total availability of nutrients to the interface of the microbial community.

2. Physicochemistry of bacterial surfaces and the development of fine-grained minerals on them

Amongst eubacteria there are two obvious varieties of cell walls; those that are Gram-positive and those that are Gram-negative [9]. The former consists of a matrix, approximately 20–30 nm thick, composed of an intermingling of peptidoglycan and secondary polymers as typified by the *Bacillus subtilis* wall. Depending on the availability of phosphate in the growth medium, the secondary polymers can be either teichoic (plus PO_4) or teichuronic (minus PO_4) acids. Recent information has shown that this particular Gram-positive cell wall is not as simple as originally thought; subtle structural differentiation exists between the cylindrical walls, poles and wall-pole junctures, and active cell wall turnover during cell growth ensures that the outermost surface of the wall is pocketed with numerous depressions (Fig. 2) [10]. This substantially increases the surface area-to-volume ratio of the cell [11]. To compound the complexity further, the interpolymeric spaces throughout the wall fabric contain periplasmic components [12–14]. It is very possible that the walls of other Gram-positives could be at least as complex.

This structural complexity of Gram-positive walls suggests a coinciding chemical complexity. Even though the major ingredients of the wall are peptidoglycan and secondary polymers, there must be a full array of associated proteins (and other substances) which are constituent parts of the periplasm or are 'passing through' as secretion products. Yet, at pH $\sim$ 7 the dominant ionizable chemical groups are carboxylates (peptidoglycan, teichuronic acid and protein) and phosphates (teichoic acid) [15–17]. Because peptidoglycan hydrolases used during wall turnover are most active during growth [18], the outermost regions (region #3 in Fig. 2) of the wall

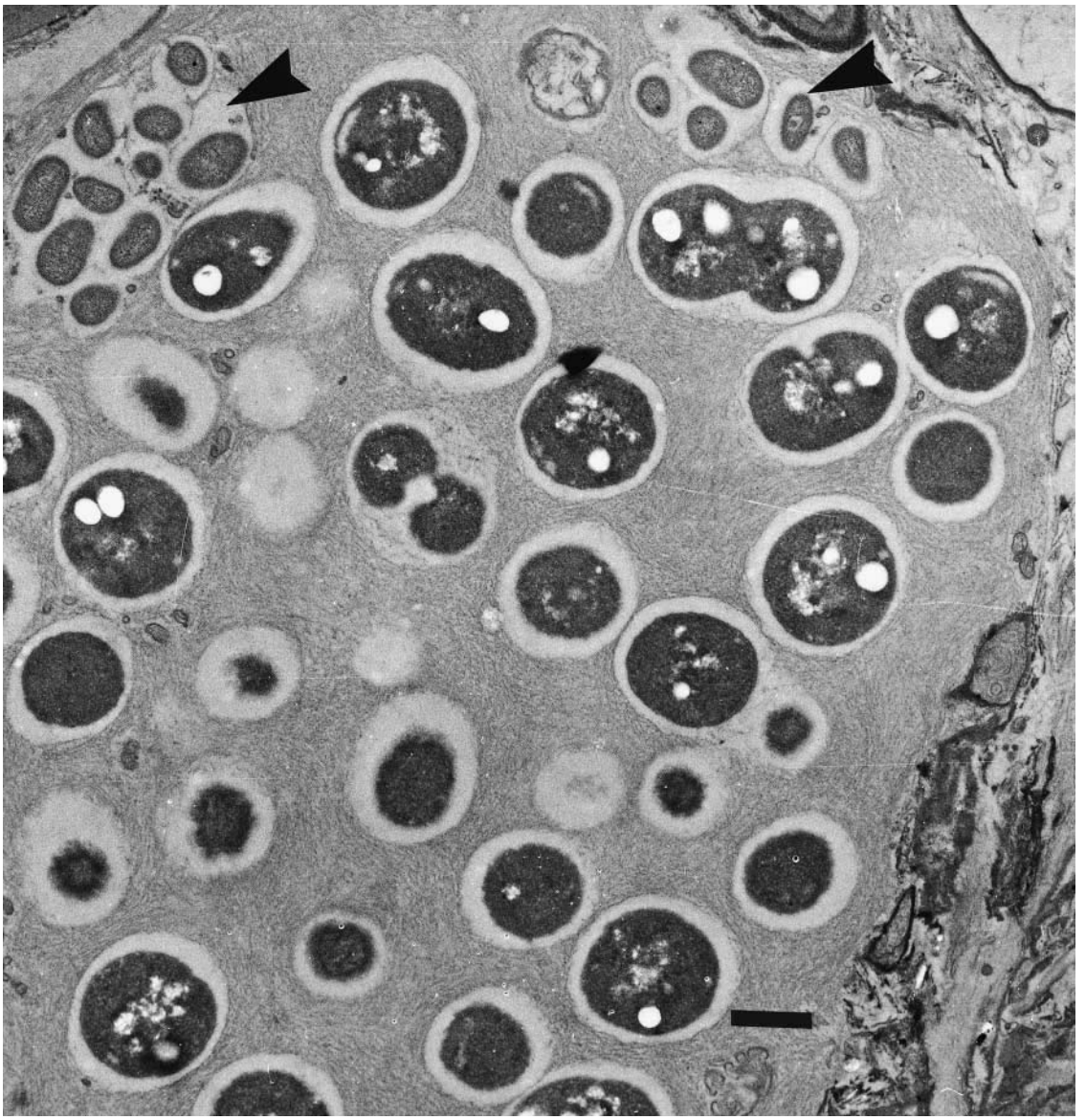

Fig. 1. Thin section of a bacterial biofilm showing distinct microcolonies (arrowheads) scattered throughout a dominant family of Gram-negative bacteria (their general appearance suggests they are purple sulfur bacteria). Extracellular polysaccharide can be seen adhering the cells together, possibly preserving the cells' localized microenvironment necessary for metabolism. The inert substratum which the biofilm is attached to can be seen at the bottom right-hand corner. This biofilm is part of the community found in a microbial mat taken from a saline alkaline lake in British Columbia. The figure was kindly provided by S. Schultze-Lam of our laboratory. Bar = 1 μm.

(where peptidoglycan is being cleaved) have more COO^- groups available than the immediately underlying or 'stress-bearing' region (region #2 in Fig. 2). Because region #1 of the wall (Fig. 2) directly above the plasma membrane (which is also directly below the 'stress-bearing' region) is receiving large quantities of newly synthesized peptidoglycan (which is not stress-bearing), it is the most compacted region and, accordingly, has more electronegative sites per unit volume than the other regions. Consequently, this cell wall turnover which is dependent on growth and division rates can have a profound effect on

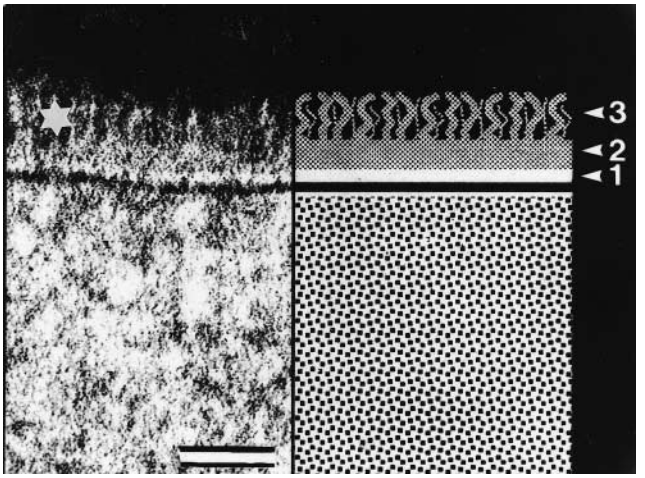

Fig. 2. Thin section of a freeze-substituted cell envelope ('star') of *Bacillus subtilis* (left-side) and an artist's rendering of the three separate structural regions within the cell wall which correspond to the dynamic aspects of wall turnover. The fibers of region #3 drastically increase the surface area-to-volume ratio of the cell. The thin section is a darkfield image so that the cellular substance appears white and the background black and this is reflected in the drawing. A similar image appeared in an article by Graham and Beveridge [10]. Bar = 50 μm.

the overall charge capacity of Gram-positive cell walls.

The anionic nature of Gram-positive walls can be aptly seen by their enormous capacity to sequester dilute metal ions from natural environments [19,20]. This is at least a two-stage process whereby the initial interaction between a multivalent metal ion and the reactive chemical sites within the wall is stoichiometric (e.g., Fe^{3+} could bind to three COO^- groups). Once the initial complexation to reactive sites within the wall has proceeded, these same sites initiate the deposition of larger amounts of metal [21,22]. Precipitates formed of metal and environmental counterions grow rapidly and, over time, dehydrate into bona fide fine-grained, crystalline minerals [23]. Ultimately, the rate of the reaction and the size of the reaction deposit depend on the abundance of anionic sites in the wall and the concentration of environmental metal ions. For cells which have a high electronegative surface charge, it is not unusual for the entire cell surface to be encrusted with minerals (Fig. 3).

The structure of Gram-negative walls is much different from that of Gram-positive bacteria [9]. Here, we have a thin peptidoglycan layer surrounded by the dense periplasm of the periplasmic space [14,24,25]. Overlying this resides an outer membrane which is an asymmetric lipid bilayer with (mostly) lipopolysaccharide (LPS) on its outer face. Intermingled with the lipids are outer membrane proteins (omps) some of which form small aqueous channels through the bilayer (porins), others which are lipoproteins and help cement the outer membrane and peptidoglycan layer together, and others which aid the transport of essential substances (e.g., Fe) into the periplasm and cell [9]. Even though the peptidoglycan of Gram-negative bacteria is capable of interacting strongly with metal [26], it is shielded from the external environment by the outer membrane. It is the LPS on this membrane's outer face which dictates the essential physicochemistry of Gram-negative surfaces [27]. LPSs which are devoid of highly charged, long side chains (such as those of *Escherichia coli* K12) are dominated by their inner phosphoryl groups of the core and lipid A regions and not by the carboxyl functions of the 2-keto-3-deoxyoctonate (3-deoxy-D-*manno*-2-octulosonate) residues [28,29]. When long, charged LPS side chains are attached to the LPS molecule (attachment is at the 'core' region), they dominate surface physicochemistry and readily bind heavy metal cations [22] so that they can be easily visualized by freeze-substitution electron microscopy [25,30]. With Gram-negative surfaces, the two-step metal deposition outlined for

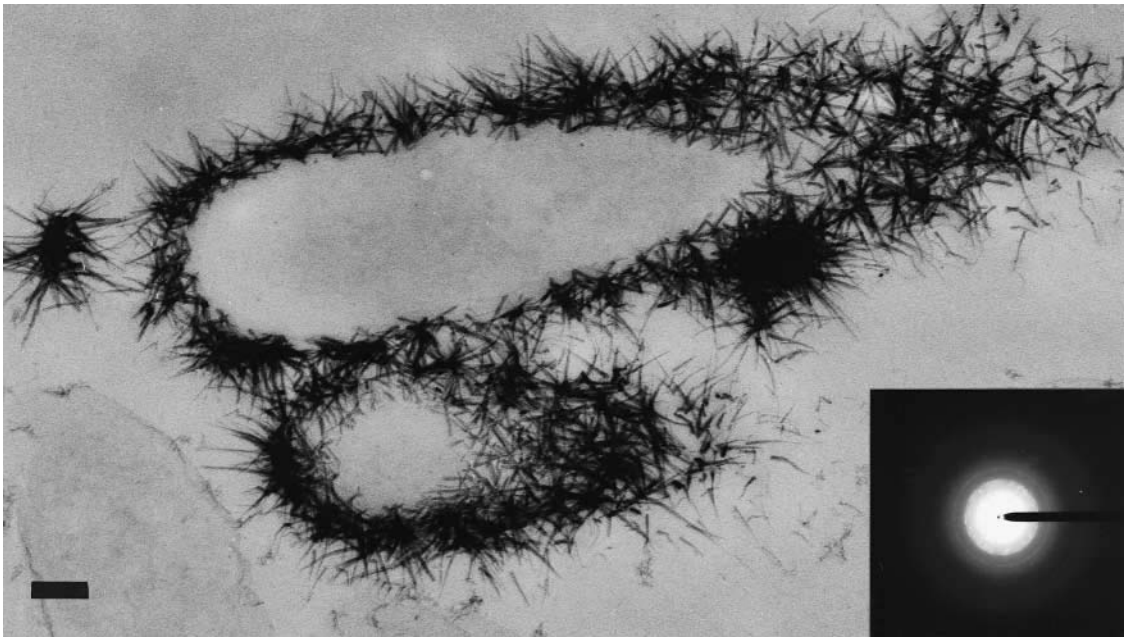

Fig. 3. Like the surfaces of Gram-positive bacteria (such as *B. subtilis*), the surfaces of Gram-negative cells can also be highly charged so that they interact with environmental ions and form fine-grained minerals. This is a thin section of *Pseudomonas aeruginosa* after the cells were reacted with 1 mM lanthanum nitrate at 20°C for 10 min and washed free of unbound ions. The selected area electron diffraction pattern seen in the lower right-hand of the figure reveals that the electron dense material surrounding each of the two cells is a crystalline fine-grained lanthanum mineral. This image is from Mullen et al. [22].

B. subtilis [21], is (presumably) also at work because there is the same progression of stoichiometric metal binding, to hydrous precipitates and to anhydrous, crystalline mineral phases (Fig. 3).

Clearly, bacterial surfaces do not always have to end at the cell wall surface; other structures such as capsules, S-layers and sheaths can lie on top [9,25]. In most cases, these overlying structures are also anionic at pH ~ 7. Although they may be constructed out of different polymeric substances, the same chemical reactive sites are involved. Capsules and sheaths are usually constructed of acidic mucopolysaccharides or polypeptides and are excellent candidates for metal deposition [31–33] as are those exopolymers found in biofilms [34]. It is not so straightforward for S-layers which are paracrystalline surface arrays composed of either protein or glycoprotein [35,36]. These are self-assembly (entropy-driven) systems and, as the proteins fold and arrange themselves on the bacterial surface, the polar residues of each subunit often become internalized leaving the non-polar residues exposed [37]. Usually, S-layered bacteria are more hydrophobic than their 'naked' counterparts. Therefore, active interaction of S-layers with environmental metals is often rare in natural settings (unless metal cations such as Ca^{2+} are used in the self-assembly process [38]). There is one major exception to this statement. Even though the S-layer on *Synechococcus* GL24 is relatively hydrophobic [39], enough electronegative sites are properly arranged on the protein array to initiate the mineralization of gypsum ($CaSO_4 \cdot 2H_2O$) or calcite ($CaCO_3$) from natural waters (Fig. 4) [40]. This bacterium drives the solid mineral field of the waters of Fayetteville Green Lake, New York and is responsible for the lake's marl sediment and calcitic bioherms [41]. The photosynthetic and metabolic processes of the cyanobacterium control its microsurface environment and this dictates which mineral phase is produced; i.e., pH ≈ 7 is the solid field for gypsum and pH ≈ 8 for calcite.

Remarkably, bacterial surfaces also contribute to

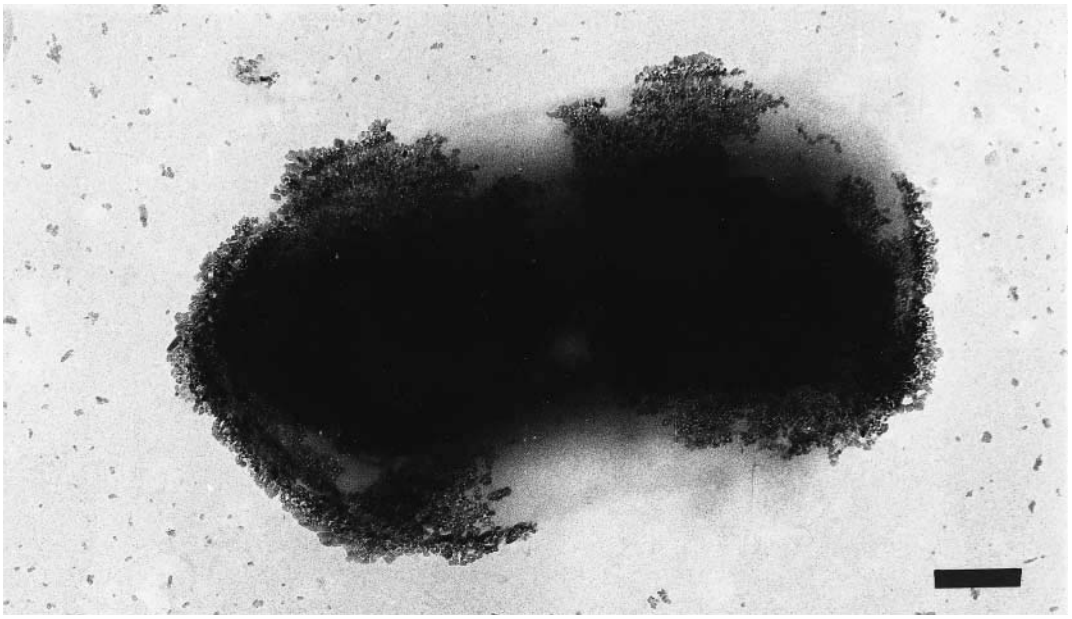

Fig. 4. Unstained whole mount of a *Synechococcus* GL24 cell whose S-layer (seen as dark patches on the cell surface) has begun to mineralize into gypsum ($CaSO_4 \cdot 2H_2O$) from the natural ions found in Fayetteville, Green Lake, New York water. Gypsum is formed under dark conditions, whereas calcite ($CaCO_3$) is formed in the light since photosynthetic metabolism extrudes OH^- ions from the cell thereby forming a high pH microenvironment around the cell which promotes calcite development. Gypsum is produced in more pH-neutral environments such as this cell encountered. The S-layer is so electron-opaque because it is mineralized. This figure was kindly supplied by S. Schultze-Lam of our laboratory. Bar = 100 nm.

the production of fine-grained silicates [42], the most abundant mineral in the Earth's crust. Although most bacteria have a net negative charge, localized positive charges can also be found in cell walls and are due to amine groups [43]. These can interact with SiO_2^{2-} and nucleate mineral development. Yet, most bacterially derived silicates stem from a complex chemical interaction with multivalent metal cations which salt-bridge the initial silica to the wall [43].

Clearly, the physicochemistry of bacterial surfaces favours complex and far-reaching biogeochemical interactions with environmental metal and silicate ions. Since prokaryotes have inhabited the Earth for at least 3.6 billion years and since their surviving ancient remains are completely mineralized (i.e., microfossils; [44]), these biogeochemical reactions have been proceeding since the dawn of life. If microbes of the archean shared the same high reactivity with metal ions as their present-day ancestors, the total microbially-produced mineral, over time, is staggering and is equal to the weight of all continental matter above sea level [45].

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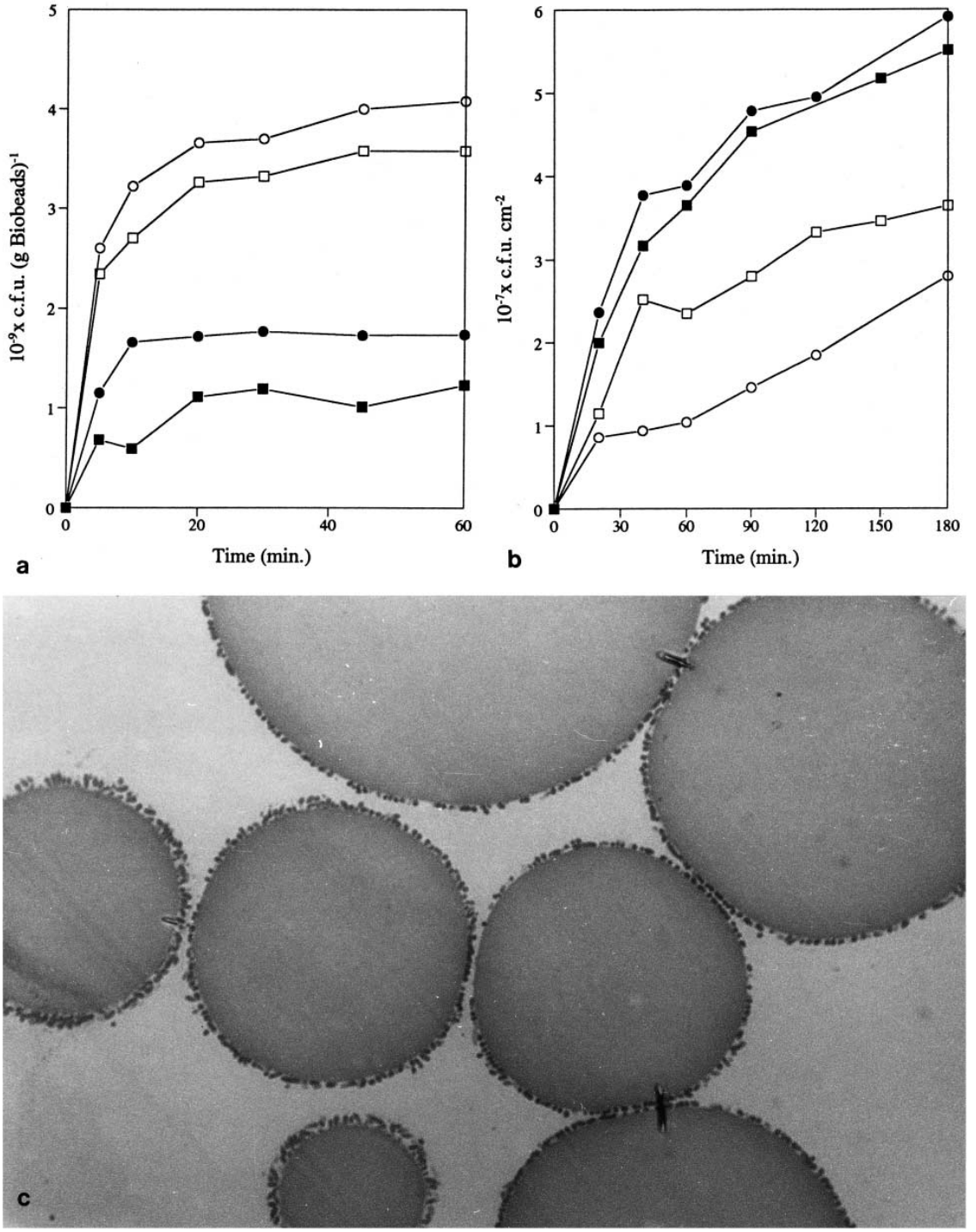
Fig. 5. a: Adhesion to polystyrene of *Pseudomonas aeruginosa* PAO1 (A^+B^+) (●) and its LPS derivatives AK1401 (A^+B^-) (○), dps89 (A^-B^+) (■) and rd7513 (A^-B^-) (□). Washed cell suspensions (ca. 5×10^8 cfu ml^{-1}) were incubated (37°C) with hydrophobic SM2 Biobeads. At regular intervals, 1 ml aliquots were taken and the Biobeads removed by filtration. The number of attached cells was calculated from estimating the viable counts of suspended cells, relative to a suspensions without Biobeads. The experiment was performed three times and a representative result is shown. b: Adhesion to glass of *Pseudomonas aeruginosa* PAO1 (A^+B^+) (●) and its LPS derivatives AK1401 (A^+B^-) (○), dps89 (A^-B^+) (■) and rd7513 (A^-B^-) (□). Cleaned glass coverslips were placed in washed cell suspensions (ca. 5×10^8 cfu ml^{-1}) and incubated at 37°C. At regular intervals, coverslips were removed and the cell number adhering determined by vortexing with glass beads and viable counting. The experiment was performed three times and a representative result is shown. c: *P. aeruginosa* rd7513 (A^-B^-) attached to the surface of positively charged DEAE Sepharose beads. Beads with attached cells were fixed, dehydrated, embedded in resin, and sectioned. Sections were stained with toluidine blue and examined using light microscopy. Cells which are 1–3 μm long can be seen to form uniform layers on the surface of the beads.



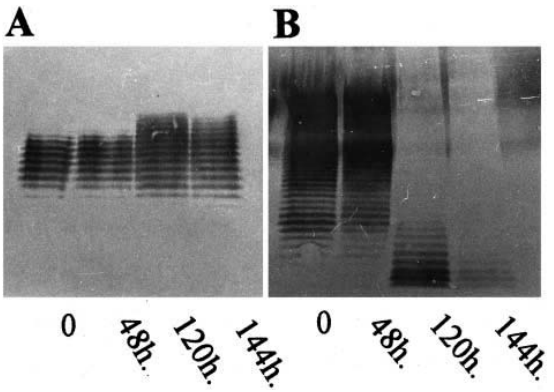


Fig. 6. Western immunoblots of LPS isolated from *Pseudomonas aeruginosa* PAO1 populations which have been growing as biofilms for various times on the surface of DEAE beads. LPS was fractionated by SDS-PAGE, transferred to nitrocellulose, and reacted with MAbs specific for A-band (A) or B-band (B) LPS. Notice that as the biofilm matures over time, the A^+B^+ LPS phenotype is displaced by A^+B^-.

3. Initial attachment of *Pseudomonas aeruginosa* to inert surfaces

The establishment of a biofilm requires a complex set of events ··· from initial, reversible attachment of a cell to a surface, to irreversible attachment, and to growth and development of the mature biofilm. After attachment, a set of genes is activated so that each bacterium's physiology is better refined for a sessile mode of growth [46,47]. During maturation of the film, subtle cellular changes continue to be expressed [6,7,48] but we will not dwell on these in this review. Instead, we will concentrate on the influence of LPS on the initial binding of *P. aeruginosa* to both hydrophobic and hydrophilic surfaces in an effort to understand preliminary binding forces.

P. aeruginosa PAO1 is a Gram-negative bacterium which expresses two separate LPSs on its outer membrane; these are termed A- and B-band LPSs [49,50]. The high molecular mass B-band defines the serotype of each strain (PAO1 is serotype 05) and the side chain consists of a multimeric repeat of a trisaccharide consisting of two uronic acid derivatives and one *N*-acetylfucosamine residue [51]. A-band is the common antigen and this side chain consists of shorter polymeric units of α1→2-, α1→3-, α1→3-linked D-rhamnose and low levels of 2-keto-3-deoxyoctonate [52]. In this way, the surface of *P. aeruginosa* PAO1 is studded with a mosaic of A- and B-band LPSs, the former having charge-neutral, shorter side chains and the latter, long, highly anionic chains. Since the side chains of B-band can extend ~40 nm from the face of the outer membrane [30], this LPS dominates surface physicochemistry of nonmucoid stains [53,54]; i.e., it out-reaches both the A-band side chains and omps.

For our adhesion study, we used the PAO1 strain (A^+B^+) and its isogenic derivatives (which were A^+B^-, A^-B^+ and A^-B^-) to determine their attachment to glass (hydrophilic) and polystyrene (hydrophobic) surfaces. The relative hydrophobicities of the strains were $A^+B^- > A^-B^- > A^+B^+ > A^-B^+$ and these characteristics mirrored the cells' ability to attach to polystyrene beads (Fig. 5a). The exact oppo-

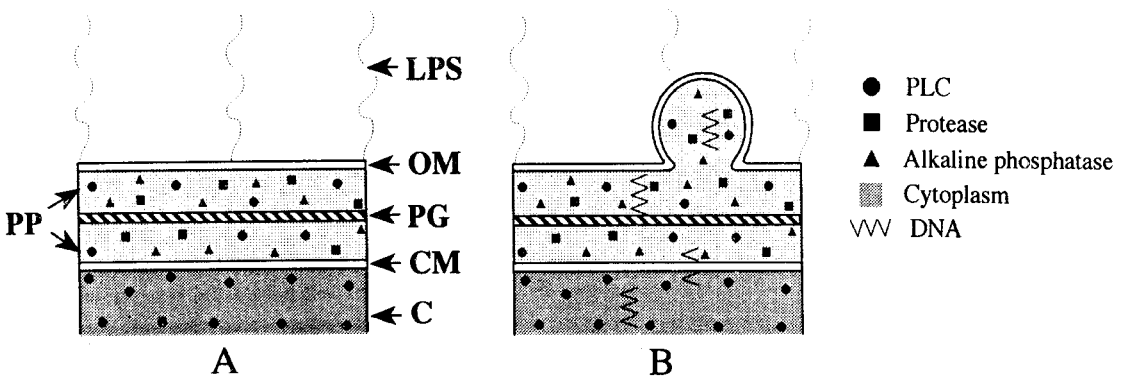


Fig. 7. A model leading up to the packaging of periplasm into outer membrane vesicles (MVs) of *Pseudomonas aeruginosa* from the intact, smooth envelope surface (A) to bleb formation (B). This diagram is a modified version of the one previously published in Kadurugamuwa and Beveridge [61].

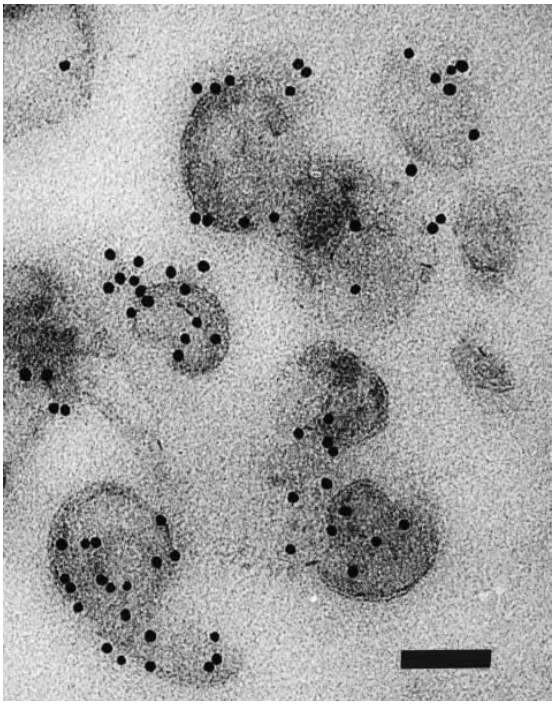


Fig. 8. Thin section of membrane vesicles (MVs) from *Pseudomonas aeruginosa* that have been immunogold labelled for the 26 kDa peptidoglycan hydrolase (autolysin) of the microorganism. This figure was originally published in Li et al. [64]. Bar = 100 nm.

site binding affinities were seen for glass (Fig. 5b) and the initial attachment to this hydrophilic surface thus correlated well with the overall cell surface charge of the strains as determined by the strength of binding to an anion exchange resin (DEAE sepharose; Fig. 5c). Interestingly, once A^-B^- cells were attached to positively charged beads, their attachment was so strong they were the most difficult of all strains to displace [53]. This suggests that even though B-band side chains dominate initial interactions with inanimate surfaces, the major charge-determining sites of the LPS reside in core and lipid A regions. This supports our initial metal binding results using *E. coli* K12 [27–29].

In more recent work, using positively charged agarose beads as 'growth supports' in columns for continuous biofilm growth of the PAO1 strain, we have been able to detect significant changes in LPS expression as biofilms develop and mature on the beads. A^+B^+ gradually becomes A^+B^- (Fig. 6); yet, if cells are scraped from the biofilm and grown in broth (i.e., forced into a planktonic mode of growth), they rapidly convert to A^+B^+ again. It therefore appears that A^+B^- is somehow a physiological advantage for the cells once the biofilm mode of growth has commenced, even though B-band LPS was necessary for initial attachment to the hydrophilic interface. This ability of *P. aeruginosa* to alter its surface expression of LPS to fit environmental requirements is remarkable, and may have broad implications for the pathogenesis (e.g., cystic fibrosis isolates are A^+B^-; [49]) and certain antibiotic resistance strains (A^+B^- strains are not as sensitive to aminoglycoside antibiotics such as gentamicin [54]) of this microorganism. Interestingly, the PAO1 strain undergoes a similar LPS conversion when grown at high temperature [55]. It is reasonable to assume that other biofilm-forming bacteria can also modulate their surface physicochemistry under such environmental influences.

4. Surface blebbing of *P. aeruginosa* and the production of predatory membrane vesicles

A number of Gram-negative bacteria form outer membrane vesicles (MVs) as they grow [56–62]; some of these have been implicated in the transfer of DNA to recipient strains [62] and virulence factors during infection [57,58,61]. MVs from *P. aeruginosa* PAO1 have been well characterized and, interestingly, possess only B-band LPS [61]. They also contain a general protease, alkaline phosphatase, phospholipase C, and small quantities of DNA. These MVs appear to be a way in which the PAO1 strain packages periplasmic material for efficient transport to various interfaces. For example, virulence factors are concentrated and transported in MVs to tissue culture cell-lines where they fuse into the tissue and release their contents (Kadurugamuwa, unpublished). The packaging of periplasm by MVs was confirmed by following the secretion of elastase, a virulence factor that is synthesized in the cytoplasm as a large inactive enzyme, 'pre-pro-elastase', and translocated across the plasma membrane. During translocation a small segment of the enzyme is clipped-off so that in the periplasm it is in the 'pro-

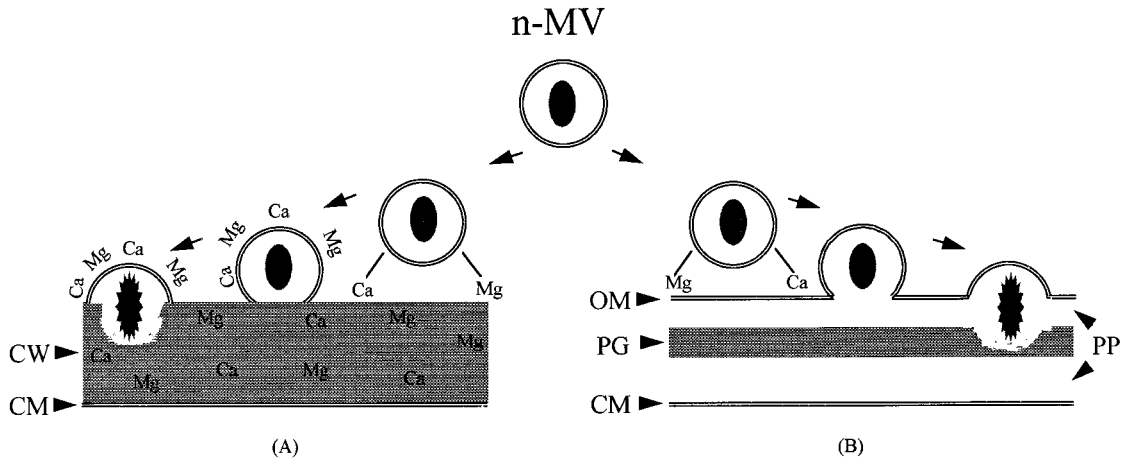


Fig. 9. Diagram explaining how MVs could interact with Gram-positive (A) and Gram-negative (B) surfaces. MVs are high-curvature vesicles ~80 nm in diameter, and the LPS which coats their outer bilayer surface is B-band LPS. For high curvatures, the acyl chains of this LPS would be tightly packed in the hydrophobic domain of the bilayer and the O side chains would be loosely packed and separated from one another. For the loose packing of the latter, there could be little salt-bridging by Ca^{2+} or Mg^{2+} between adjacent O side chains; i.e., they would be Ca and Mg deplete. Because Gram-positive walls (CW in panel A) are rich in these two metal ions, when MVs contact Gram-positive surfaces, there should be immediate adherence by means of salt-bridging with the ions. As more and more Ca^{2+} or Mg^{2+} becomes available, electronegative sites on the O side chains of the MVs thereby pull adjacent side chains together and break apart the high curvature of the MVs. This would break open the MVs and liberate peptidoglycan hydrolases so that they could begin digestion of the Gram-positive wall. For Gram-negative walls (B), the MVs must break through the outer membrane (OM) before the peptidoglycan hydrolases can attack the peptidoglycan layer (PG). Because the outer membrane, like Gram-positive walls, is also rich in Ca^{2+} or Mg^{2+}, MVs would be readily salt-bridged to the surface of Gram-negative bacteria. But in this case, because the MVs possess a compatible bilayer surface to the outer membrane bilayer (each has LPS, phospholipid, and protein), the MVs would fuse into the outer membrane. This fusion would liberate the peptidoglycan hydrolases into the periplasm (PP) of the Gram-negative envelope, where they would be free to act on the peptidoglycan layer. This diagram was originally published in Kadurugamuwa and Beveridge [66].

elastase' form where it is still inactive. The enzyme is only activated as it is transported across the outer membrane, a translocation that requires further proteolytic cleavage to the 'elastase' form [63]. MVs from the PAO1 strain possess only the inactive, periplasmic pro-elastase [61].

The recognition that *P. aeruginosa* is capable of concentrating and packaging periplasm into MVs (see Fig. 7) was important, especially since it is recognized that peptidoglycan hydrolases (autolysins) are periplasmic enzymes used for peptidoglycan turnover during cell expansion and division [64]. One of these enzymes is the major 26-kDa autolysin and it is excreted as the cell grows [65], almost entirely in the lumen of MVs (Fig. 8 [64]). Obviously, this autolysin must have additional function beyond assisting the bacterium to grow. Since MVs containing the enzyme are highly interactive with other types of bacteria and quickly lyse them [66], we believe that MVs not only benefit the parent strain by conveying virulence factors to tissue during infection, but that they also can be used to attack and lyse competing bacteria within natural settings. In this way, MVs would not only destroy the competition, they would also liberate complex nutrients to the parent cells to feed on from the lysed bacteria. These are predatory MVs [66]. It is interesting to note that MVs from the PAO1 strain lyse both Gram-negative and Gram-positive bacteria. In the former, MVs fuse into the outer membrane of a competing Gram-negative cell and liberate the peptidoglycan hydrolase into the periplasm where it attacks the peptidoglycan layer (Fig. 9B). In the latter, MVs bind to the Gram-positive wall, break open, and hydrolyze the wall substance immediately below them (Fig. 9A). Surrounding cells of the parent PAO1 strain do not lyse, even if MVs fuse into them, because the autolysin is 'self' and can therefore be regulated.

These observations of predatory MVs could be very important in the context of biofilms containing

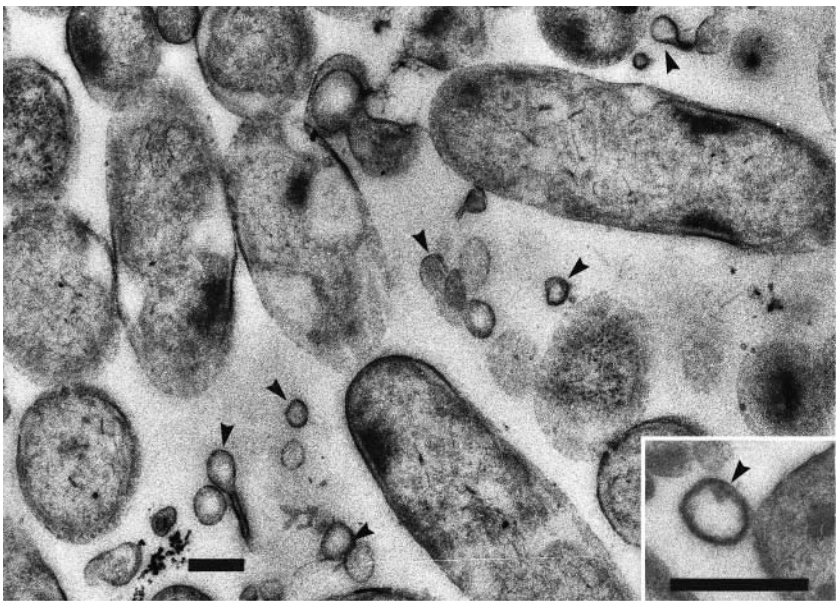


Fig. 10. Thin section of a *Pseudomonas aeruginosa* biofilm in which several membrane vesicles (arrowheads) can be seen. The inset at the lower right-hand of the figure shows a vesicle at higher magnification coming off a mother cell. Bars = 250 nm.

mixed populations of bacteria. The biofilm mode of growth dictates that complex amalgams of bacteria live in close proximity to one another. Often, metabolic processes must be shared between different bacterial types in biofilms and these bacteria become dependent on one another. For example, methanogens and methylotrophs will live in close proximity to each other as one group (methanogens) generates CH_4 for the other (methylotrophs). Yet, we also know that discrete populations within biofilms can undergo serious decline or rapid growth. This may not only be due to nutritional modulation or environmental fluctuation (e.g., O_2 tension, pH, redox potential, etc.) within the film; it is also possible that more specific factors (such as predatory MVs) are responsible. It is not unusual to see MVs being liberated from Gram-negative constituents within natural biofilms, nor is it unusual to see lysed cells (Fig. 10). It is not yet possible to determine if the former is actually responsible for the lysis in these natural settings and we are currently experimenting with artificial mixtures of bacteria in biofilms to simulate such natural conditions. It is possible that the extracellular matrix in biofilms would impede the migration of MVs to susceptible cells.

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