Biofilms: A survival strategy of bacteria

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Bacterial biofilm is a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface, which constitutes a protected mode of growth that allows survival in hostile environment. The biofilm-forming microorganisms have been shown to elicit specific mechanisms for initial attachment to a surface, formation of microcolony leading to development of three-dimensional structure of mature biofilm. They differ from their free-living counterparts in their growth rate, composition and increased resistance to biocides, antibiotics and antibodies by virtue of up regulation and/or down regulation of approximately 40 per cent of their genes. This makes them highly difficult to eradicate with therapeutic doses of antimicrobial agents. A greater understanding of mechanism of their formation and survival under sessile environments may help in devising control strategies.

MICROORGANISMS have primarily been characterized as planktonic, freely-suspended cells and described on the basis of their growth characteristics in nutritionally-rich culture media. Microbiologists are always striving for pure cultures and rationally focused on free-floating bacteria growing in laboratory cultures. No bacterium is an island, i.e. nearly all bacteria live with, and depend on, other microorganisms for energy, carbon and other nutrients. Thus, most of the bacteria in the world live in microecosystems filled with hundreds of other microorganisms. Scientists have recently realized that in the natural world, more than 99% of all bacteria exists as biofilms[1]. Rediscovery of a microbiological phenomenon, the 'biofilms' exhibited a distinct phenotype with respect to gene transcription and growth rate, where bacteria undergo transition from a planktonic ('loner') existence to a community-based existence in which they must interact with many neighbours of various species in close proximity. Biofilms are defined as an assemblage of microbial cells that are irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material allowing growth and survival in sessile environment.

Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water-system piping, or natural aquatic systems. The water-system biofilm is highly complex, containing noncellular materials such as mineral crystals, corrosion particles, clay or silt particles, freshwater diatoms, and filamentous bacteria. Biofilms on medical devices, on the other hand, appear to be composed of a single, coccoid organism or blood components. The associated extracellular polymeric substance (EPS) matrix, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix[2]. Experts at the Centers for Disease Control and Prevention estimate that 65% of human bacterial infections involves biofilms[3].

Historical perspectives and biofilms in nature

Antoni van Leeuwenhoek, using his simple microscope, first observed 'animalcule' on tooth surfaces and can be credited with the discovery of microbial biofilms[4]. The bacterial growth and activity were substantially enhanced by the incorporation of a surface to which these organisms could attach[5], and the number of bacteria on the surfaces was dramatically higher than in the surrounding medium[6]. As early as 1973, Characklis[7] studied microbial slimes in industrial water systems and showed that they were not only tenacious, but also highly resistant to disinfectants such as chlorine. Costerton was instrumental in alerting the world about the importance of biofilms, coining the term in 1978. He put forth a theory of biofilms that explained the mechanisms whereby microorganisms adhere to living and nonliving materials and the benefits accrued by this ecologic niche. Much of the work in the last two decades has relied on tools such as microarray assays, scanning electron microscopy (SEM) or standard microbiological culture techniques for biofilm characterization. The two major thrusts in the last decade which dramatically impacted our understanding of biofilms are, the utilization of the confocal laser scanning microscope to characterize biofilm ultrastructure, and an investigation of the genes involved in cell adhesion and biofilm formation.

In nature, biofilms constitute a protected growth modality that allows the bacteria to survive in hostile environments. Bacterial biofilms colonize any humid surface, and one is already familiar with some biofilms: plaque on the teeth, slippery slime on river stones, gel-like film on the inside of a vase which held flowers for a week, and infected tissue[4]. Biofilms as slime sites thrive wherever there is water; in the kitchen, on contact lenses, in the gut linings of animals, etc. Occasionally, these bacterial aggre-

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gates release individual cells that disperse and rapidly multiply, thereby colonizing other places. Until recently, the slimy conglomerations of bacteria were recognized for their propensity to coat and corrode pipes, clogging water filters and harbouring bacteria that contaminate drinking water[8]. Researchers have made great strides in understanding the triggers that cause free-floating bacteria to form a biofilm and adjust to a sedentary existence. In the past few years, mounting evidence has shown that they cause a host of medical problems as well, where bacteria adopt a strategy of 'united we stand, divided we fall', allowing them to counter both the might of the body's immune system and the weapons that physicians use against them[4]. Biofilms are pervasive and problematic in medical, industrial and environmental settings because these communities express biofilm-specific properties such as increased resistance to antibiotics, UV light, and chemical biocides, increased rates of genetic exchange, altered biodegradability and increased secondary metabolite production[1,9].

Heterogeneity of biofilm matrix and structures involved in biofilm formation

The structures which make up a biofilm contain canals through which nutrients circulate, and in different zones of the biofilm the cells express different genes, as if they were part of an organized structure. The microcolony continues to grow in volume, and the bacteria in proximity to the surface have difficulties in gaining access to nutrients from the external environment[10]. Only those located in the upper layers of the colony are able to continue multiplying – a situation that creates bacterial populations with metabolic differences. A logical assumption is that any given cell within the biofilm will experience a slightly different environment compared with other cells within the same biofilm, and thus be growing at a different rate. Gradients of nutrients, waste products and signalling factors contribute to this heterogeneity in biofilms. Heterogeneity has also been shown for protein synthesis and respiratory activity as DNA content remains relatively constant throughout the biofilm[11,12].

The term biofilm is in some ways a misnomer, since biofilms are not a continuous monolayer surface-deposit. Rather, biofilms are heterogeneous; components such as water, polysaccharides and other macromolecules will contribute not only for the heterogeneity of the matrix but also for its multicellular function. Microcolonies of bacterial cells encased in an EPS matrix are separated from each other by interstitial voids[13] (water channels). Liquid flow occurs in water channels, allowing diffusion of nutrients, oxygen, and even antimicrobial agents. This concept of heterogeneity is descriptive not only for mixed-culture biofilms (such as might be found in environmental biofilms), but also for pure-culture biofilms common on medical devices and those associated with infectious diseases.

The matrix will change considerably as equilibrium between the species is established and a balance between competition and commensalism is achieved within the microbial community. Bacteriocins, microcins and bacteriophages also provide specific tools for the selective attack of bacterial cells within the mixed biofilms[14].

Various structures such as flagella, fimbriae, outer membrane proteins (OMPs), curli (a proteinaceous surface structure) and EPS are involved in biofilm formation. These structures have distinct roles in different species and under different environmental conditions. Flagellar motility is important to overcome the forces that repel bacteria from reaching many abiotic materials. Once it reaches the surface, the nonflagellar appendages other than those involved in transfer of viral or bacterial nucleic acids (called pili), OMPs and curli are then required to achieve stable cell-to-cell and cell-to-surface attachment[15].

Flagella apparently play an important role in the early stages of bacterial attachment by overcoming the repulsive forces associated with the substratum. Korber *et al.*[16] used motile and nonmotile strains of *Pseudomonas fluorescens* to show that motile cells attach in greater numbers and against the flow more rapidly than do nonmotile strains. Nonmotile strains also do not recolonize or seed vacant areas on a substratum as evenly as motile strains, resulting in slower biofilm formation by the nonmotile organisms. Although motility by flagella is required, chemotaxis was not necessary for biofilm development when studied by microtitre dish system[15,17].

Fimbriae, contribute to cell-surface hydrophobicity. Most fimbriae that have been examined contain a high proportion of hydrophobic amino acid residues[18]. Fimbriae play a role in cell-surface hydrophobicity and attachment, probably by overcoming the initial electrostatic repulsion barrier that exists between the cell and substratum[19]. A number of aquatic bacteria possess fimbriae, which have also been shown to be involved in bacterial attachment to animal cells[19–21]. Genes encoding for both mannose-sensitive Type I (ref. 15) and type IV pili (ref. 22) are required for host-cell colonization and biofilm formation.

An Omp AG43 facilitates both cell-surface and cell-cell contacts when *Escherichia coli* cells are grown on minimal medium but apparently plays no role when the cells are grown in rich-medium (O'toole). The O-antigen component of lipopolysaccharide has been shown to confer hydrophilic properties to Gram-negative bacteria. For most strains tested, adhesion was greater on hydrophobic materials; for example, mutants of *P. fluorescens* lacking the O-antigen adhered in greater numbers to hydrophobic materials[23].

*Omp*R, a functional allele, was isolated and shown to increase the production of surface-adhesion curli. This increased production is required for biofilm formation in nonmotile strains[24,17].

Biofilms are composed primarily of microbial cells and EPS. EPS may account for 50 to 90% of the total organic carbon of biofilms[25] and can be considered as the primary matrix material of the biofilm. Biofilm-associated EPS is distinct, both chemically and physically, from the bacterial capsule[26], but it is primarily composed of polysaccharides. EPS is also highly hydrated because it can incorporate large amounts of water into its structure by hydrogen-bonding. Different organisms produce differing amounts of EPS, and the amount of EPS increases with age of the biofilm. Chemically, EPS may be colonic acid in *E. coli* and alginate in *Pseudomonas aeruginosa*[9]. EPS may associate with metal ions, divalent cations and other macromolecules (such as proteins, DNA, lipids, and even humid substances)[25]. EPS production is known to be affected by nutrient status of the growth medium; excess available carbon and the limitation of nitrogen, potassium or phosphate promote EPS synthesis[27]. Slow bacterial growth will also enhance EPS production[27]. Because EPS is highly hydrated, it prevents desiccation in some natural biofilms. EPS may also contribute to the antimicrobial resistance properties of biofilms by impeding the mass transport of antibiotics through the biofilm, probably by binding directly to these agents[28]. Thus, cell-surface structures such as flagella, fimbriae, OMPs and EPS clearly play an important role in the attachment process. Cell-surface polymers with nonpolar sites such as fimbriae, other proteins, and components of certain Gram-positive bacteria (mycolic acid) appear to dominate attachment to the hydrophobic substrata, while EPS and lipopolysaccharides are more important in attachment to hydrophilic materials. Flagella are important in attachment also, although their role may be to overcome repulsive forces rather than to act as adsorbents or adhesives[2].

Regulation of biofilm formation

Factors such as availability of surface, nutrients and environmental cues regulate biofilm formation.

Surface

The surface could be a dead or living tissue, or any inert surface. The attachment of microorganisms to the surface is a complex process, with many variables affecting the outcome[15]. Further, growth requires a complex developmental pathway involving a series of events that are regulated in response to environmental- and bacterial-derived signals.

The surface may have several characteristics that are important in the attachment process. Microbial colonization appears to increase as the surface roughness increases[29]. This is because shear forces are less and surface area is more on rougher surfaces. Most investigators have found that microorganisms attach more rapidly to hydrophobic, nonpolar surfaces such as Teflon and other plastics than to hydrophilic materials such as glass or metals[30–32].

A material surface exposed in an aqueous medium becomes conditioned or coated by polymers from that medium, and the resulting chemical modification will affect the rate and extent of microbial attachment. Conditioning films are formed on surfaces exposed in sea water. These are organic in nature, formed within minutes of exposure, and continue to grow for several hours[33]. A prime example may be the proteinaceous conditioning film called 'acquired pellicle', which develops on tooth enamel surfaces in the oral cavity. Mittelman[34] noted that a number of host-produced conditioning films such as blood, tears, urine, saliva, intervascular fluid and respiratory secretions influence the attachment of bacteria to biomaterials.

Nutrients

Increase in nutrient concentration correlated with an increase in the number of attached bacterial cells[35]. However, nutrient concentrations too low to measure are sufficient for biofilm growth. Biofilm bacteria acquire nutrients by concentrating trace organics on surfaces by the extracellular polymer, using the waste products from their neighbours and secondary colonizers, and by pooling their biochemical resources with different enzymes to break down food supplies. Because the biofilm matrix is often negatively charged, many nutrients (particulary cations) are attracted to the biofilm surface. Besides, nutrients with negative charge can exchange with ions on the surface. This provides bacterial cells within the biofilm with plenty of food compared to the surrounding water.

Environmental cues

Other characteristics of the aqueous medium, such as pH, nutrient levels, iron, oxygen, ionic strength and temperature, may also play a role in the rate of microbial attachment to a substratum[9]. Several studies have shown a seasonal effect on bacterial attachment and biofilm formation in different aqueous systems[36,37]. This effect may be due to water temperature or other unmeasured, seasonally affected parameters. Fletcher[38,39] found that an increase in the concentration of several cations (sodium, calcium, lanthanum, ferric iron) affected the attachment of *P. fluorescens* to glass surfaces, presumably by reducing the repulsive forces between the negatively-charged bacterial cells and the glass surfaces.

Gene regulation

There is mounting evidence to show that both up- and down-regulation of a number of genes occurs in the attach-

ing cells upon initial interaction with the substratum. Combaret *et al.*[40] found that 22% of the genes was up-regulated and 16% down-regulated in biofilm-forming *P. aeruginosa*. Davies and Geesey[41] demonstrated *algC* up-regulation within minutes of attachment to a surface in a flow cell system. Genes encoding for enzymes involved in glycolysis or fermentation (phosphoglycerate mutase, triosephosphate isomerase, and alcohol dehydrogenase) are up-regulated in biofilm-forming *Staphylococcus aureus*[42]. The researchers surmised that the up-regulation of these genes could be due to oxygen limitation in the developed biofilm, favouring fermentation[42]. A recent study by Pulcini[43] also showed that *algD*, *algU*, *rpoS* and genes controlling polyphosphokinase synthesis were up-regulated in biofilm formation of *P. aeruginosa*.

Process of biofilm formation

Biofilm-forming microorganisms have been shown to elicit specific mechanisms for initial attachment to a surface, microcolony formation, development of a three-dimensional community structure and maturation, and detachment (Figure 1).

Attachment

The bacterium approaches the surface so closely that its motility is slowed and its forms a transient association with the surface and/or other microbes previously attached to the surface. The solid–liquid interface between a surface and an aqueous medium (e.g. water, blood) provides an ideal environment for the attachment and growth of microorganisms[4].

In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic and coated by surface 'conditioning' films. An increase in flow velocity, water temperature or nutrient concentration may also equate to increased attachment, if these factors do not exceed critical levels. Properties of the cell surface, specifically the presence of fimbriae, flagella and surface-associated polysaccharides or proteins, are also important and may possibly provide a competitive advantage for one organism where a mixed community is involved[2].

Microcolony formation

After the bacteria adhere to the inert surface/living tissue, the association becomes stable for microcolony formation.

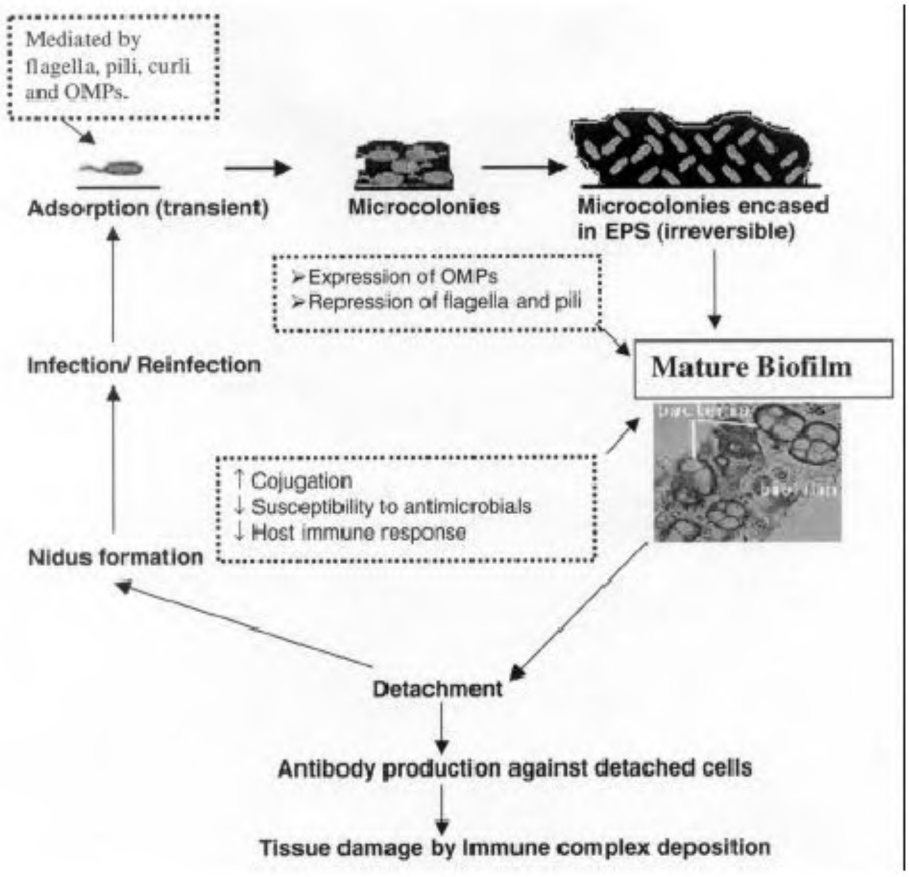

Figure 1. Schematic representation of steps in biofilm formation and its consequences.

The bacteria begin to multiply while emitting chemical signals that 'intercommunicate' among the bacterial cells. Once the signal intensity exceeds a certain threshold level, the genetic mechanisms underlying exopolysaccharide production are activated[4]. In this way, the bacteria multiply within the embedded exopolysaccharide matrix, thus giving rise to the formation of a microcolony[26].

Formation of three-dimensional structure and maturation

During the attachment phase of biofilm development, perhaps after microcolony formation, the transcription of specific genes takes place. These are required for the synthesis of EPS. Attachment itself can initiate synthesis of the extracellular matrix in which the sessile bacteria are embedded, followed by formation of water-filled channels. It has been proposed that these channels constitute primitive circulatory systems, delivering nutrients to and removing waste products from the communities of cells in the microcolonies.

Detachment

Occasionally, for purely mechanical reasons, some bacteria are shed from the colony, or (more frequently) some bacteria stop producing EPS and are thus 'released' into the surrounding environment. Biofilm cells may be dispersed either by shedding of daughter cells from actively-growing cells, or detachment as a result of nutrient levels or quorum-sensing, or shearing of biofilm aggregates (continuous removal of small portions of the biofilm) because of flow effects[44]. As the thickness of the EPS increases, anaerobic conditions develop within the biofilm with loci of the biofilm consisting of anaerobic bacteria. Because of film thickness and the activity of anaerobic species, the film detaches and sloughs-off from the surface of the substrate[45]. Polysaccharidase enzymes specific for the EPS of different organisms may possibly be produced during different phases of biofilm growth of the organisms and contribute to detachment[46].

It has been suggested that escape of *P. aeruginosa* cells from the biofilm matrix involves the action of an enzyme that digests alginate[4]. It is worth noting that in the non-pathogenic, photosynthetic bacterium *Rhodobacter sphaeroides*, an acylhomoserine lactone quorum-sensing signal is required for dispersal of individual cells from community structures. (The quorum-sensing genes in *R. sphaeroides* are called *cer* (community escape response) genes[47].)

The mode of dispersal apparently affects the phenotypic characteristics of the organisms. Eroded or sloughed aggregates from the biofilm are likely to retain certain biofilm characteristics, such as antimicrobial resistance properties, whereas cells that have been shed as a result of growth may revert quickly to the planktonic phenotype. Detachment of cells or biofilm aggregates may result in blood stream or urinary-tract infections or lead to the production of emboli[2].

Sequelae of detachment includes release of planktonic bacterial cells from biofilms, and evidence supports the notion that there is a natural pattern of programmed detachment of these cells, which can colonize on other surfaces or individuals to form new microcolonies[44]. Therefore, biofilms can act as 'niduses' of acute infection, if the mobilized host defences cannot eliminate the detached planktonic cells that are released at any one time during the infection[4].

Furthermore, the microcolony formed in the process of biofilm development or sessile bacterial cells under biofilm mode of growth, release antigens and stimulate immune response and induce antibody production. But the antibodies are not effective in killing bacteria within biofilms and may cause immune complex deposition and damage to surrounding tissues[48]. Even in individuals with excellent cellular and humoral immune responses, biofilm infections are rarely resolved by host defence mechanisms[49]. And in case of microcolonies, they are too large to be phagocytosed. Indeed, enzymes released by the phagocytes surrounding the colony may damage the host tissues in proximity to the biofilm – a phenomenon that in turn favours growth of the colony. Antibiotic therapy may typically reverse the symptoms caused by planktonic cells released from the biofilm, but fails to kill the biofilm[50]. For this reason, biofilm infections typically show recurring symptoms after cycles of antibiotic therapy, until the sessile population is surgically removed from the body[49].

During the lifetime of an animal, stressful situations are frequent and can lead to generalized immune depression. In such situations, the bacteria detached from the biofilm may overcome the inflammatory barrier and spread to other tissues. In fact, the continuous appearance of such acute outbreaks is what characterizes chronic processes. For example, in chronic mastitis due to *Staphylococcus aureus*, a characteristic observation is the appearance of acute outbreaks in the animal every few weeks or months, without eventual healing in any case. While in situations of streptococcal meningitis or in dermatitis, it is normal for the process to manifest on a recurrent basis in a given farm for years. Acute pathologies can often be resolved on a point basis by antibiotic therapy, and sometimes even disappear spontaneously, though this is not the case in chronic infectious disorders[4].

Role of biofilms in chronic infections

Modern-day acute infections can often be resolved effectively with antibiotics (except for cases of infections by an antibiotic-resistant strain) and are not considered to involve biofilms. However, more than half of the infec-

tious diseases that affect mildly compromised individuals involve bacterial species that are commensals are common in our environments. For example, *S. aureus*, *S. epidermidis* or *S. hyicus*, which colonize the skin; *E. coli, Salmonella*, *Streptococcus suis* and *S. agalactiae*, which colonize the mucosal membranes; *Pasteurella multocida*, *P. haemolytica*, *Actinobacillus pleuropneumoniae*, Mycoplasma spp. or *Haemophilus parasuis*, which are found in the upper airways, etc.[4,8]. It is difficult to eradicate such infections from a farm, and in many cases they may even cause chronic infections in livestock, which may show the presence of biofilm bacteria surrounded by an exopolysaccharide matrix. These biofilm-associated infections do share clinical characteristics such as growing slowly in one or more locations, slow in producing overt symptoms[51] besides biofilm-associated Gram-negative bacteria producing endotoxins.

Mechanisms of antimicrobial resistance

The bacteria enclosed within the biofilm are extremely resistant to antibiotic treatments. Such resistance can be explained by hypotheses, not necessarily limited to the following ones (Figure 2).

First, the EPS secreted by biofilm bacteria, acts as a physical/chemical barrier, thus preventing penetration by antibodies or many antibiotics[10,52,53]. Moreover, EPS is negatively charged and functions as an ion-exchange resin which is capable of binding a large number of the antibiotic molecules that are attempting to reach the embedded biofilm cells.

Second, embedded biofilm bacteria are generally not actively engaged in cell division, are smaller in size and less permeable to antibiotics. Virtually all antimicrobials are more effective in killing rapidly-growing cells. Further, transition from exponential to slow/no growth is generally accompanied by expression of antibiotic-resistant factors[10,54,55]. Slow growth activates the RelA-dependent synthesis of ppGpp, which inhibits anabolic processes in bacterial cells[56]. Interestingly, ppGpp suppressed the activity of a major *E. coli* autolysin, SLT[57], which would make the cells more resistant to autolysis and could explain the mechanism of tolerance to antibiotics in slowly-growing cells. ppGpp inhibits peptidoglycan synthesis, which would explain the decreased levels of activity of cell-wall synthesis inhibitors under starvation conditions.

Third, antibiotic degrading enzymes such as β-lactamase may also be immobilized in the EPS matrix, so that the incoming antibiotic molecules can be inactivated effectively. It is interesting to note that biofilm cells of the *P. aeruginosa* have been shown to produce 32-fold more β-lactamase than cells of the same strain grown planktonically[3,58].

Fourth, up to 40% of the cell-wall protein composition of bacteria in biofilms is altered from that of its planktonic brethren[3,9]. The membranes of biofilm bacteria might be better equipped to pump out antibiotics before they can cause damage, or even antibiotics targets may disappear.

Fifth, the antimicrobial agent is deactivated in the outer layers of the biofilm, faster than it diffuses. This is true for reactive oxidants such as hypochlorite and H_2O_2 (refs 10, 59–61). These antimicrobial oxidants are products of the oxidative burst of phagocytic cells and poor penetration of these may partially account for the inability of phagocytic cells to destroy biofilm microorganisms.

Biofilms also provide an ideal niche for the exchange of extrachromosomal DNA responsible for antibiotic resistance, virulence factors and environmental survival capabilities at accelerated rates, making it a prefect milieu for emergence of drug resistance pathogens[2,62,63]. Plasmid-carrying strains have also been shown to transfer plasmids

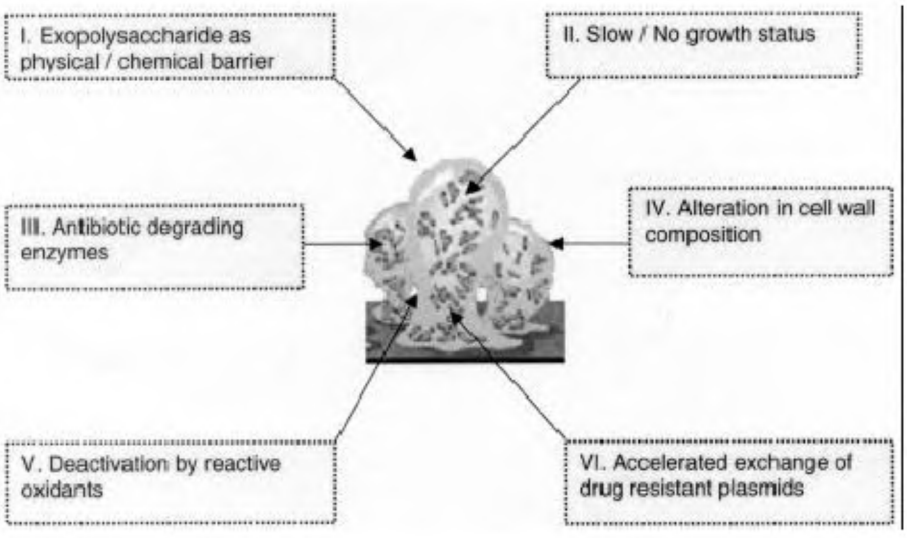

Figure 2. Illustration of mechanisms of antimicrobial resistance by a mature biofilm.

to recipient organisms, resulting in biofilm formation; without plasmids, the same organisms produce only microcolonies without any further development. The probable reason for enhanced conjugation is that the biofilm environment provides minimal shear and closer cell-to-cell contact. Since plasmids may encode for resistance to multiple antimicrobial agents, biofilm association also provides a mechanism for selecting and promoting the spread of bacterial resistance to antimicrobial agents.

It can be misleading to draw conclusions regarding the performance of antibacterial agents *in vivo* based on data obtained with cells cultivated in a complex laboratory medium. Therefore, it is of paramount importance to identify the growth parameters that are likely to affect the physiology of bacterial cells *in vivo*, so that they can be incorporated into protocols designed to test the efficacy of the antimicrobial agents more realistically *in vitro*. One such protocol being investigated by researchers is identifying a mutation in a gene called *rpos* in *P. aeruginosa* strain by microarray assay. It was found that the gene gets repressed in biofilms. The researchers concluded that biofilms formed by the mutant bacteria grew faster and thicker than those of normal strains, and were even more resistant to tobramycin, a frontline antibiotic used to treat *P. aeruginosa* infections. The microarray studies also provided other insights into antibiotic resistance mechanisms used by biofilms.

Phase variation

Bacteria do not express the same antigens *in vitro* as *in vivo* – a mechanism known as phase variation. When bacteria grow *in vivo*, they must cope with a hostile environment in which certain nutrients are not abundant, and where the host attempts to eliminate them in different ways. In such situations, the bacteria use certain survival strategies that are entirely useless when growing *in vitro*, particularly in a 'favourable' medium. For example, under *in vivo* conditions, iron and certain oligoelements are scarce, and bacteria must produce proteins that bind or capture such elements. Also, bacteria must express adhesion factors (fimbriae, pili, adhesins, etc.) to avoid being eliminated with the body fluid. Moreover, some bacteria produce a thick exopolysaccharide capsule that protects them from phagocytosis[64]. These structures are lost *in vitro* because in energy terms, the production of exopolysaccharides, adhesins, iron-sequestering proteins, etc. is costly and unnecessary. In fact, the bacteria sometimes lose the genetic information needed to produce these structures, and are never again able to express them. The way in which phase variation occurs is simple: under *in vitro* conditions, variants appear among the bacterial population that no longer express these antigens, and since these cells do not waste energy on other activities, their growth rate increases considerably and in only a few generations, they become predominant. Under *in vivo* conditions, the opposite applies. However, the variants that produce such antigens are able to proliferate within the host, while the bacteria that do not are eliminated[1,10].

During the formation of a biofilm, approximately 40% of the genes is altered by at least twofold[9,65]. An exception is *P. aeruginosa*, where only 73 of 5500 genes have different activation pattern when they exist as biofilms[66]. Genes required for flagellin synthesis are down-regulated, while those responsible for production of EPS and OMPs are up-regulated approximately fourfold in biofilm associated cells[41,67]. Thus, to become a productive member of a biofilm community, the bacterium must differentiate into a biofilm-associated cell by repressing synthesis of the flagellum that might destabilize the biofilm, and producing EPS that will reinforce the biofilm structure. The gene required for synthesis of EPS, alginate (*algC*) is up-regulated 3-to 5-fold in recently attached cells vs planktonic counterparts[41,67].

Prevention and control

One is still able to eradicate bacterial biofilm infection if antibiotic therapy is implemented as early as possible, and if sufficiently high concentration of antibiotics is used. Any delay in implementing therapy may result in treatment-failure.

Usage of signalling molecules to block the adhesion processes that are mediated by pili and flagella or interfering with the cell-to-cell communication systems involved in biofilm development is the obvious possible approach that may be taken to combat the existence of biofilms. For example, the organism in the seaweed *Delisea pulchra* produces a number of halogenated furanones and enones that interfere with the formation of biofilms[65]. Researchers have also discovered that chemical signalling controls the behaviour of biofilms, and they have begun to manipulate both biofilm formation and detachment using these signals and their analogues.

Biological control by phage and its associated specific polysaccharide depolymerases could be used to selectively eliminate particular species of bacteria from mixed-community biofilms[68]. In another study, it was found that bacteriophage active on biofilm of Gram-negative bacteria is widespread in nature[69].

The combination of polysaccharide-hydrolysing enzymes and oxidoreductases caused both removal and inactivation of bacterial biofilms[70]. Another interesting possibility for biofilm elimination comes from the observation of biofilm self-destruction. As the oxygen gets depleted by the growing biofilm mass, a specific exopolysaccharide lyase is induced which digests the biofilm matrix, liberating the cells[71].

Arnold[72] found that surface finishing treatments such as polishing, sandblasting, and grinding all, reduced the

build-up of bacterial biofilms. But eletropolishing seemed to work the best. It involves placing steel in an acid bath, and then running an electric current through the solution. The process may change the electrical charge on the metal. Bacteria are negatively-charged, and the charge on a given surface can affect how well they attach to it.

However, biofilms do serve some beneficial functions; for example, in the environment certain bacterial species, e.g. *P. fluorescence* can colonize plant roots and act as bio-control agents. Rhizobia species can often colonize roots, living in symbiosis with certain legumes by fixing nitrogen[73]. Sewage-treatment plants, for instance, rely on biofilms to remove contaminants from water.

Designing of biofilm-based vaccines

When a killed or attenuated vaccine is injected into an animal, an immune response occurs. However, this response may not afford protection, because the vaccine may not contain adequate immuno-dominant antigens, or because the induced immune response does not present the characteristics required for protection[74]. In the manufacture of conventional vaccines, laboratories often work with bacteria that no longer express such antigens, since the capacity has been lost through successive replications *in vitro*. Hence to mimic *in vivo* conditions, *in vitro*, bacteria have to be grown in liquid media by providing surface (such as bentonite clay, microsperes, chitin flakes, etc.)-depleting nutrients and adding iron chelators and growing for longer periods. This leads to slow growth of the organisms forming biofilms, which can express some novel proteins and ensure maximum EPS production.

The capsular exopolysaccharide layer is thick, yet sufficiently permeable to allow the passage of nutrients, antibiotics or even large proteins. Antibodies are therefore able to penetrate without difficulty, reaching and binding to the bacterial wall in their Fab region. However, due to the thickness of the bacterial exopolysaccharide layer, the bound antibodies are effectively masked or hidden, and their Fc region may be unable to establish contact with the corresponding phagocyte receptors. For this reason, antibodies directed against the bacterial wall are often unable to eliminate exopolysaccharide-producing bacteria, and the infectious process continues despite the existence of prior vaccination[75]. In order to effectively phagocytose a bacterium surrounded by an exopolysaccharide capsule, the produced antibodies must be targetted to the exopolysaccharide component rather than to the bacterial wall itself. Or, alternatively, vaccines can also be directed against bacterial adhesion factors such as (for example, vaccines against K88, K99, 987P of *E. coli*) flagella and pili, which are important in attachment to host cell receptor prior to colonization, thereby avoiding the establishment of these microcolonies and obviating the relapsing of infections.

Prospects for future research

Research on microbial biofilms is proceeding on many fronts, with particular emphasis on elucidation of the genes specifically expressed by biofilm-associated organisms, evaluation of various control strategies (including medical devices treated with antimicrobial agents and antimicrobial locks) for either preventing or remediating biofilm colonization of medical devices, and development of new methods for assessing the efficacy of these treatments. Research should also focus on the role of biofilms in antimicrobial resistance and in chronic diseases, and biofilms as a reservoir for pathogenic organisms. The field of microbiology has come to accept the universality of the biofilm phenotype. Researchers in the fields of clinical, food and water, industrial and environmental microbiology have begun to investigate microbiological processes from a biofilm perspective. As the pharmaceutical and healthcare industries embrace this approach; novel strategies for biofilm prevention and control will undoubtedly emerge. The key to success may hinge upon a more complete understanding of what makes the biofilm phenotype so different from the planktonic phenotype.

The authors are currently working on designing various *in vitro* techniques to grow the bacteria in biofilm mode, which simulate natural *in vivo* conditions to express novel proteins. Such biofilm-grown bacteria are being exploited as potential vaccine candidates against important bacterial pathogens of poultry. Experimental trials using these vaccines showed promising results, necessitating further field trials.

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