BACTERIAL BIOFILMS: FROM THE NATURAL ENVIRONMENT TO INFECTIOUS DISEASES

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Biofilms — matrix-enclosed microbial accretions that adhere to biological or non-biological surfaces — represent a significant and incompletely understood mode of growth for bacteria. Biofilm formation appears early in the fossil record (~3.25 billion years ago) and is common throughout a diverse range of organisms in both the Archaea and Bacteria lineages, including the 'living fossils' in the most deeply dividing branches of the phylogenetic tree. It is evident that biofilm formation is an ancient and integral component of the prokaryotic life cycle, and is a key factor for survival in diverse environments. Recent advances show that biofilms are structurally complex, dynamic systems with attributes of both primordial multicellular organisms and multifaceted ecosystems. Biofilm formation represents a protected mode of growth that allows cells to survive in hostile environments and also disperse to colonize new niches. The implications of these survival and propagative mechanisms in the context of both the natural environment and infectious diseases are discussed in this review.

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What is of all things most yielding
Can overcome that which is most hard

Lao Tzu

Among the advances in microbiology that have taken place over the past 50 years, the realization of the extent to which microbial growth and development occurs on surfaces in complex communities has been one of the most subtle. Nevertheless, this behaviour has profound consequences for how prokaryotic survival is viewed in both nature and disease. By the mid-twentieth century, Claude Zobell[1] and others had noted that aquatic bacteria were more numerous on the solid surfaces of sample containers than as single, suspended cells. Since then, the combination of high-resolution three-dimensional imaging techniques, specific molecular fluorescent stains, molecular-reporter technology and BIOFILM-culturing apparatus[2,3] has shown that biofilms are not simply passive assemblages of cells that are stuck to surfaces, but are structurally and dynamically complex biological systems (FIG. 1).

In this review, we will discuss the role of biofilm formation in the survival of prokaryotes in the natural environment, and how these strategies are used for survival in the modern human world and in humans themselves.

Biofilm formation in the environment

Biofilms appear early in the fossil record. There is evidence of biofilm formation early in the fossil record, particularly in hydrothermal environments. Putative biofilm microcolonies have been identified by morphology in the 3.3–3.4-billion-year-old South African Kornberg formation[4] and filamentous biofilms have been identified in the 3.2-billion-year-old deep-sea hydrothermal rocks of the Pilbara Craton, Australia[5]. Similar biofilm structures can be found in modern hydrothermal environments, such as hot springs[6] and deep-sea vents[7]. Interestingly, biofilm formation is also a characteristic of prokaryotic 'living fossils' in the most ancient lineages of the phylogenetic tree in both the Archaea and Bacteria —

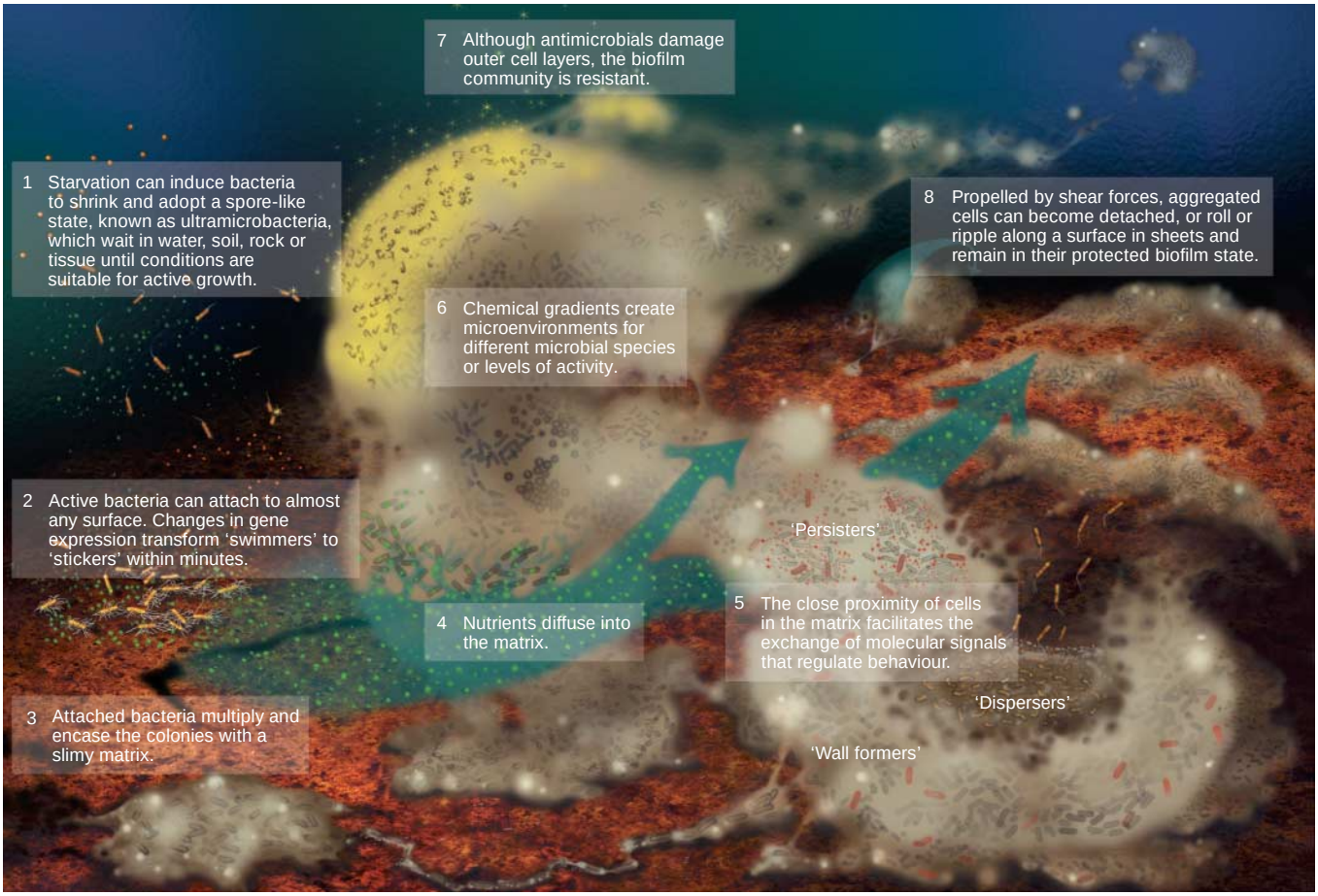

Figure 1 | **Conceptualization of biofilm development and dynamic behaviours.** The figure was compiled from laboratory and natural observations of pure culture (both Gram-positive and Gram-negative organisms), and mixed-culture biofilms. (For an interactive web-based version of Figure 1 and biofilm movies showing dynamic processes of growth and detachment, rolling and rippling, see the Online links). Image courtesy of P. Dirckx, Center for Biofilm Engineering, USA.

the Korarchaeota and Aquificales respectively[8,9]. Taken together, the data indicate that the ability to form biofilms is an ancient and integral characteristic of prokaryotes. In the context of evolution and adaptation it is likely that biofilms provided homeostasis in the face of the fluctuating and harsh conditions of the primitive earth (extreme temperatures, pH and exposure to ultraviolet (UV) light), thereby facilitating the development of complex interactions between individual cells and providing an environment which was sufficient for the development of signalling pathways and chemotactic motility[10]. In addition to facilitating cell–cell interactions that require close proximity, surfaces can also concentrate nutrients[11]. It is generally assumed that PLANKTONIC CELLS occurred before the development of more complex biofilm communities. However, we hypothesize that the catalytic and protective conditions offered by life on surfaces might have led to the concurrent development of both SESSILE and planktonic forms in biofilm cellular communities[10]. This concept of biofilms not only enhances our existing understanding of prokaryotic behaviour, but also our control strategies against the renowned tenacity of biofilms that has been acquired through billions of years of evolutionary adaptation.

Adaptation of biofilm structure for survival in varying environments. Intriguingly, the visual characteristics of biofilms growing in diverse environments are strikingly similar, indicating there are important convergent survival strategies that are conferred in part by structural specialization (FIG. 2).

Biofilms growing in fast-moving water tend to form filamentous STREAMERS regardless of whether they occur in the drainage run-off from acid mines[12], in hydrothermal photosynthetic mats (algal or bacterial)[6] or as PERIPHYTON in rivers (FIG. 2). In quiescent waters, biofilms tend to form mushroom or mound-like structures that are similar to those of STROMATOLITES. The overall patterns are isotropic with no obvious indication of flow direction. The structure of biofilms also changes with nutrient conditions[13,14]. The ability of prokaryotes to adopt different biofilm structures in response to environmental conditions — owing to genetic regulation[15], selection[13], or both[16], or to localized growth patterns determined by MASS TRANSFER[17] — gives them the flexibility to rapidly adapt to an extent that is not possible in multicellular eukaryotic organisms. The proclivity of bacteria to adhere to surfaces and form biofilms in so many environments is undoubtedly related to the selective advantage that surface association offers.

BIOFILM
Microbial biofilms are populations of microorganisms that are concentrated at an interface (usually solid–liquid) and typically surrounded by an extracellular polymeric substance (EPS) matrix. Aggregates of cells not attached to a surface are sometimes termed 'flocs' and have many of the same characteristics as biofilms.

PLANKTONIC CELLS
Planktonic (or suspended) cell cultures are those grown primarily as single cells in suspension, either in a chemostat or a shake flask.

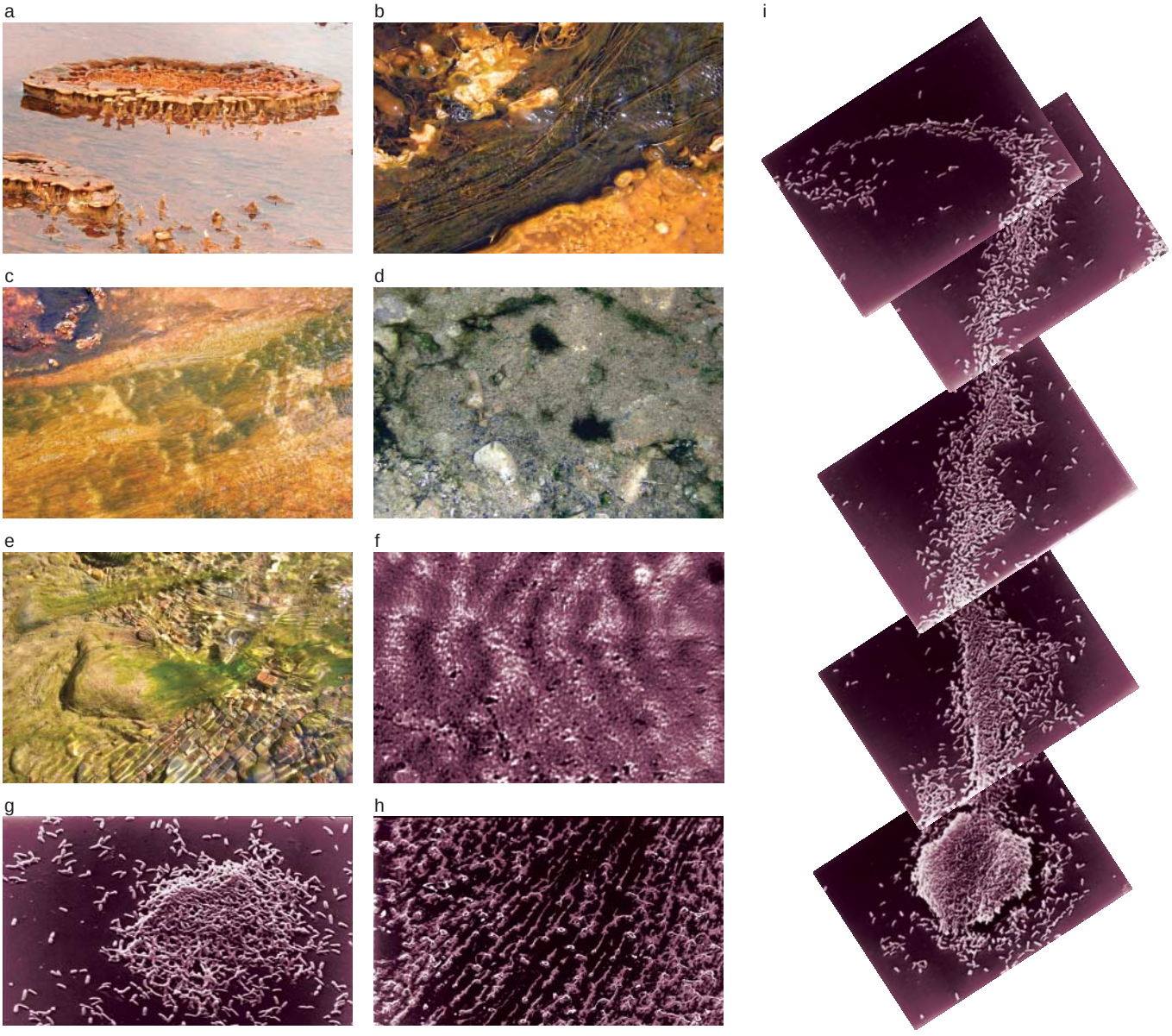

Figure 2 | **Structural similarity of biofilms growing in hydrothermal hot springs, freshwater rivers and laboratory flow cells.** Similar structures are seen in biofilms growing in hydrothermal hot springs (**a**–**c**), biofilms growing in freshwater rivers (**d**,**e**) and laboratory flow cells (**f**–**h**). Biofilms growing in quiescent or low-shear environments tend to form circular structures, such as 'mushrooms' or mounds (**a**,**d**,**g**). Biofilm streamers (**b**,**e**,**h**,**i**) and ripple structures (**c**,**f**) form in faster, high-shear flows. Biofilms are from the Biscuit Basin thermal area, Yellowstone National Park, USA (**a**–**c**), Gardener River, Yellowstone National Park, USA (**d**), Hyalite Creek, Bozeman, Montana, USA (**e**), mixed species biofilm grown at a flow velocity of 1 m s^{-1} (REF. 14) and a *Pseudomonas aeruginosa* PAN067 biofilm grown in a flow cell with a flow of 0.03 m s^{-1} (**g**) or 1 m s^{-1} (**h**, **i**) (REF. 43). Panel **c** is modified with permission from REF. 53 © (2004) ASM Press.

SESSILE
In an ecological context, a stationary organism such as a plant or a barnacle. In biofilm microbiology, it is used to distinguish planktonic (free-floating) prokaryotic cells from those attached to surfaces. However, new evidence shows that these 'sessile' cells are often dynamic, at least on the microscopic scale.

Biofilm development

The developmental sequence. The structural and developmental complexity of biofilms, and its significance in both natural and man-made environments, has been increasingly appreciated over the past two decades owing to the concomitant development of sophisticated imaging and molecular techniques that have identified the mechanisms that are involved in biofilm development. *In situ* observations of biofilm structure using confocal laser microscopy showed sessile bacteria growing in heterogeneous matrix-enclosed microcolonies interspersed with open water channels[18,19]. This complex architecture was one of the first indications that biofilm development was not simple and uniform, but rather more complex and differentiated. The ability of channels to facilitate efficient nutrient uptake by infusing fluid from the bulk phase into the biofilm[20], thereby optimizing nutrient and waste-product exchange, provided the first link between form and function[19].

More recently, proteomic studies have indicated that biofilm formation in *Pseudomonas aeruginosa* proceeds as a regulated developmental sequence, and five stages have been proposed[10,15]. Stages one and two are generally identified by a loose or transient association with the surface, followed by robust ADHESION. Stages three and four involve the aggregation of cells into

microcolonies and subsequent growth and maturation. Biofilm structures can be flat or mushroom-shaped depending on the nutrient source, which seems to influence the interactions between localized clonal growth and the subsequent rearrangement of cells through TYPE IV PILUS-mediated gliding motility in response to the nutritional cues[13]. Stage five is characterized by a return to transient motility where biofilm cells are sloughed or shed.

Although there is understandably intense interest in the investigation of the initial stages of biofilm formation, more detailed investigation into biofilm DETACHMENT as a discrete process that is important to structural development and dispersal is also warranted. Dispersal mechanisms are discussed below. There is also evidence for developmental sequences in *Escherichia coli*[21–23] and *Vibrio cholerae*[24] biofilms. Such behaviour parallels the more social behaviour that is observed in myxobacteria[25]. Until recently, it was thought that highly regulated social behaviour in prokaryotes was an unusual feature of the myxobacteria. Now, the more subtle structures and behaviours of biofilms indicate that it might be more common within the proteobacteria. Conceptual 'motility-based' models that have been derived from observations of the Gram-negative proteobacteria are often generalized as universal biofilm development cycles. However, many of the same dynamic processes — moving over surfaces, growth cycles and detachment and reattachment — can also be observed in non-motile species, such as the human pathogen *Staphylococcus aureus*[26] or *Mycobacterium* spp.[27]. It is interesting that the stages of biofilm development seem to be conserved among a remarkable range of prokaryotes. Similar convergent behaviour in eukaryotes might be seen in the cellular slime moulds, which can live either as single motile cells or as multicellular colonies that are held together in an extracellular matrix[28].

Determinants of biofilm structure

There is a continuing debate among biofilm researchers concerning the relative contributions of genetics (active response) and environmental conditions (passive response) to the development of biofilm structure and development[29,30]. These are not mutually exclusive and the relative contributions of each genetic response to a specified set of growth conditions requires more multifactorial experiments to determine how genetic and environmental responses interact.

Prediction of complex biofilm structure using models and genetic determination of biofilm structure and redundancy. Structural and temporal complexity can also be successfully modelled using simple rules that are based on localized growth patterns determined by the distribution of nutrients and FLUID SHEAR[17]. Models also predict that biofilm heterogeneity can be maintained through the production of diffusible 'detachment factors', which cause localized detachment[31].

Molecular techniques, such as random transposon mutagenesis and knockout mutant studies, have been used extensively to identify 'biofilm-specific' genes. In these studies, biofilm formation by a mutant strain is compared with that of wild-type bacteria to assess the influence of a particular gene. To adapt these studies for high-throughput screening, biofilms are often grown in microtitre wells. However, this limits the growth conditions to those of a poorly mixed batch culture, with little shear and no nutrient exchange. Also, comparative biofilm growth is usually assessed after short periods, which limits interpretation to the early stages of biofilm development. These studies have identified numerous genes or factors as being 'essential' or 'required' for biofilm formation[32–39]. Such genes include those that regulate or express surface-adhesion proteins, appendages such as pili and flagella, and extracellular polymeric substance (EPS) matrix materials. However, in many cases, on closer inspection biofilm formation is not prevented by 'knocking out' a specific gene, but is only retarded or reduced. The redundancy of the pathways that are involved in biofilm differentiation could reflect the fundamental importance of biofilm formation.

Also, cell signalling has been shown to control biofilm differentiation[40]; however, this influence can be negated or mitigated by growth under different nutrient[41] or fluid shear conditions[42,43]. Taken together, observations from laboratory experiments and biofilms growing in nature (FIG. 2) show that both the environment and the genome significantly influence biofilm formation.

Biofilm dispersal strategies

Traditionally, laboratory experiments focus on the attachment of planktonic batch-cultured or chemostat-cultured cells to surfaces and the subsequent biofilm growth. The detachment and dispersal of cells from biofilms has received less attention. Detachment can be caused by external perturbations, such as increased fluid shear[44], by internal biofilm processes, such as endogenous enzymatic degradation, or by the release of EPS or surface-binding proteins[45–47]. Detachment is normally viewed from the perspective of control (biofilm removal strategies), or the contamination of food and water production facilities[48,49], or medical and dental devices[50–52]. In some species, dispersal from biofilms seems to be an active process, presumably adapted to allow colonization of new niches[15]. Three distinct biofilm dispersal strategies can be identified: 'swarming/seeding dispersal', in which individual cells are released from a microcolony into the bulk fluid or the surrounding substratum; 'clumping dispersal', in which aggregates of cells are shed as clumps or emboli; and 'surface dispersal', in which biofilm structures move across surfaces. The survival advantages and disadvantages of active (motility-driven) and passive (shear-mediated) dispersal strategies are shown in TABLE 1.

Swarming dispersal. Swarming dispersal has been best described in non-mucoid *P. aeruginosa* biofilms. After initial biofilm growth, the microcolonies differentiate to form an outer 'wall' of stationary bacteria (of biofilm

STREAMERS
Filamentous biofilm microcolonies that form in flowing water. The streamers are atached to the surface by an upstream 'head', while the downstream 'tail' can oscillate in the current.

PERIPHYTON
An assemblage of organisms attached to and living on submerged solid surfaces in natural environments such as rivers.

STROMATOLITE
Mushroom- and tower-shaped structures formed by layers of cyanobacteria and entrapped sediments that grow in quiescent (calm) saline and hydrothermal waters.

MASS TRANSFER
In the context of biofilms, mass transfer refers to the process by which dissolved and particulate substances (such as nutrients) are moved into and out of the biofilm by the surrounding fluid.

ADHESION
A stable interaction of a cell with respect to a surface. Living cells actively excrete chemicals from their surface to anchor themselves to a substratum. This is referred to as adhesion or attachment.

TYPE IV PILUS
An elongated structure extending from the surface of Gram-negative cells that is independent of flagella and which can retract and pull the cell forward.

DETACHMENT
The loss of single cells or aggregates of cells from the biofilm, usually into an overlying flow of fluid. Detachment can be an active process (dispersal), a passively induced mechanical process (for example, through fluid shear) or a chemical process (by adding agents that 'dissolve' the EPS matrix).

Table 1 | Hypothetical dispersal mechanisms for biofilms compared with those for single cells

Cellular state	Medium	Dispersal mechanism	Advantages and disadvantages
Single cells	Fluid	Swimming motility	Active directional chemotactic motility, but vulnerable
		Sporulation	Passive fluid-directed dispersal, but protected
	Surface	Gliding or twitching motility	Active directional chemotactic motility, but vulnerable
Biofilm	Fluid	Clumping fluid-borne dispersal	Passive fluid-directed dispersal, but protected
	Surface	Shear-mediated rippling or rolling	Passive fluid-directed dispersal, but protected

phenotype), while the inner region of the microcolony 'liquefies', which allows motile cells (of planktonic phenotype) to 'swim' out of the microcolony, leaving a hollow mound[15,53,54]. Liquification has been attributed to lysis of a subpopulation due to prophage-mediated cell death[55]. The lysing population can be regarded as a third phenotype, whereas the remaining swarming cells might be a surviving, apoptosis-negative, 'persister' phenotype[56]. Hollow microcolonies have been seen in *Staphylococcus epidermidis* growing on agar plates (P. Stewart, personal communication), and transmission electron micrographs (TEM) indicate that the hollowing occurs through localized lysis. Bacteriophages have also been shown to reduce the viscosity of purified *P. aeruginosa* ALGINATE[57]. The authors of this study concluded that this increased the transport of bacteriophage through the biofilm to enhance infection. However, it is possible that this phenomenon is also important in swarming/seeding dispersal. A similar phenomenon has been reported in other species, including the non-motile dental pathogen *Actinobacillus actinomycetemcomitans*[45,58]. Instead of active swimming dispersal, it was hypothesized that the released cells took advantage of convective flow currents in the overlying fluid for a fluid-borne dispersal strategy[59].

Clumping dispersal. In this dispersal strategy, whole aggregates are continually shed from the biofilm[60]. The aggregates consist of biofilm cells that are surrounded by EPS and which might be more similar physiologically to the attached biofilm than to planktonic cells. The tendency to shed clumps containing hundreds of cells by *S. aureus*, a non-motile human pathogen, contrasts with the detachment pattern for *P. aeruginosa* biofilms, in which the loss of single cells and small clumps predominates (P.S. and S. Wilson, unpublished observations). Moreover, the antibiotic resistance of detached *S. aureus* clumps is similar to the resistance that is associated with attached biofilms. The reattachment of detached, protected emboli from *S. aureus* (FIG. 3) might explain the high frequency of infectious metastasis that is associated with this organism[50]. Although the dispersal direction is not actively controlled, it is possible that the biofilm uses the fluid flow in a similar manner to the wind-borne seed-dispersal strategies used by dandelions and other plants.

Surface dispersal. Another strategy for biofilm dispersal is movement across surfaces. Although it is known that in some species single cells can actively move across surfaces through GLIDING OR TWITCHING MOTILITY[61], there is evidence that whole biofilms can also move across surfaces through shear-mediated transport. Migratory ripple structures travelling at velocities of up to 1 mm hour^{-1} have been reported in laboratory studies on *P. aeruginosa* and mixed-species biofilms[42,62], and similar structures have also been seen in natural biofilms (FIG. 2). Rippling transport might have consequences in medicine. Ripple structures have been reported in biofilms in endotracheal tubes and it has been hypothesized that the flow of biofilms down the tubes is related to dissemination into the lungs and subsequent cases of ventilator-associated pneumonia[63]. Similar mechanisms might be important for dissemination from infected catheters. This type of dispersal might be more clinically challenging than planktonic dispersal because it can be more difficult to detect and can be more resistant to antibiotics[64]. In addition to rippling migration, we have recently found that *S. aureus* biofilm microcolonies can 'roll' along the lumen of a glass tube in an *in vitro* central venous catheter model (C.J. Rupp, S. Wilson and P.S., unpublished observations). VISCOELASTIC tethers were observed between the rolling *S. aureus* microcolony and the glass surface. The tethers stretched until breaking at the upstream edge, which allowed the microcolony to roll forward and create a new tether at the new downstream point of contact.

Biofilm viscoelasticity and adaptation to survival on surfaces. So far, we have made the case that biofilm formation is an important factor in the survival of prokaryotes. We have also discussed aspects of multicellularity in biofilms and how dynamic biofilm behaviour could be related to dispersal. The mechanical properties of biofilms might also explain the tenacity of biofilms that are associated with solid surfaces while, at the same time, allowing enough flexibility to flow or move over those surfaces. Sessile plants and animals have developed several different strategies to remain attached in moving fluids. Generally, very little is known about the mechanical properties of intact biofilms, but biochemical analysis of the EPS slime matrix indicates that biofilms are HYDROGELS. Recently, it has been reported that a wide range of biofilms that are grown under flow, either *in vitro* or in hot spring environments, show classic viscoelastic behaviour[44,65–68]. These properties allow the biofilms to withstand the transient periods of rapidly changing shear stresses that are expected in many marine and river environments due to seasonal variation, run-off due to

FLUID SHEAR
The mechanical force that is exerted by a fluid as it moves past a surface. Although shear exists throughout the fluid in biofilms, 'shear stress' is usually used in the context of the shear exerted at the solid surface — for example, where the biofilm is growing. The shear stress will tend to 'wash away' the attached biofilm from the surface on which it is growing, and it increases as the flow rate is increased.

EPS
Extracellular polymeric substance. Polymers of varying chemical composition that are excreted by the cells in the biofilm. The EPS is the slime matrix that gives the biofilm stability and helps it to adhere to a surface. Although generally assumed to be primarily composed of polysaccharides, the EPS can also contain proteins and nucleic acids.

ALGINATE
An exopolysaccharide produced by *P. aeruginosa*, which is believed to contribute to the antibiotic resistance of *P. aeruginosa*.

GLIDING OR TWITCHING MOTILITY
Movement, predominantly by Gram-negative cells, that is dependent on type IV pili.

VISCOELASTIC
A material that has both elastic (solid-like) and viscous (liquid-like) properties.

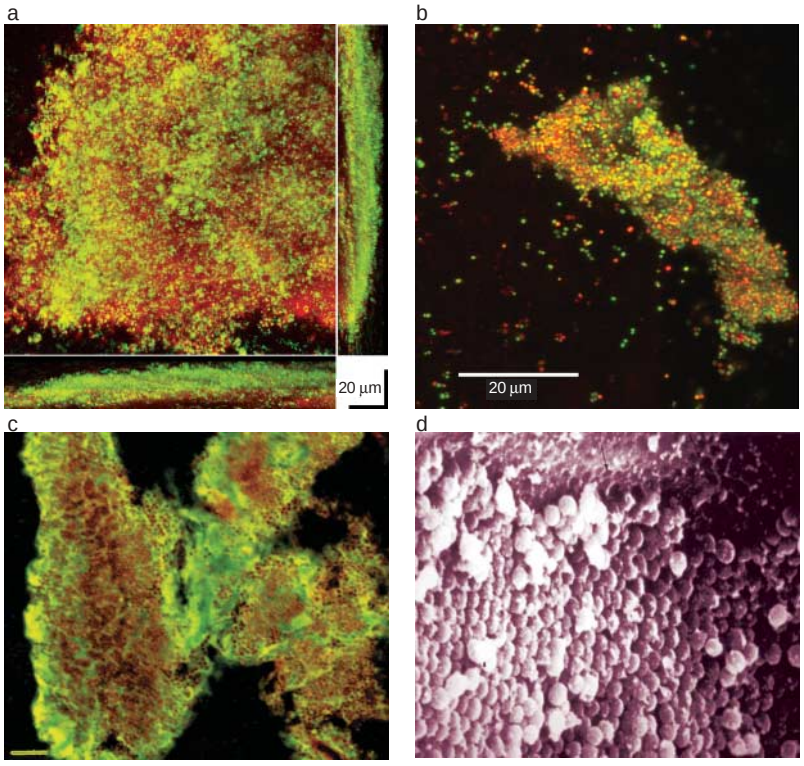

Figure 3 | ***Staphylococcus aureus* biofilms. a** | Confocal laser microscopy image of a biofilm growing in a flow cell with a flow rate of 1 ml min^{-1}. The square panel is a plan view and the side panels are vertical and horizontal cross sections, respectively. **b** | Detached cells from the biofilm shown in part (**a**) captured on a polycarbonate filter. The detached fraction consisted of single cells and large clumps containing thousands of cells. Biofilms in parts **a** and **b** were stained with Molecular Probes Live/Dead™ kit so that live cells appeared green and dead cells appeared red. Images courtesy of Suzanne Wilson, Center for Biofilm Engineering, USA. **c** | Biofilm grown in the skin cut of a neutropoenic mouse. The extracellular polymeric substance (EPS) of the biofilm was stained with FITC-ConA (green) and safranine (red). Reproduced with permission from REF. 87 © (2002) Blackwell Publishing. **d** | Scanning electron micrograph of *S. aureus* biofilm that developed on the distal tip of a cardiac pacemaker lead in a patient. Reproduced with permission from REF. 85 © (1982) American Heart Association.

rapidly changing weather conditions and changes in local currents due to variations in bed morphology or flow channel. Over short periods (seconds), biofilms can absorb elevated shear by behaving elastically, but over longer periods loading stresses in the biofilm can be dissipated through viscous flow, so that instead of detaching, the biofilm can either flow over the surface[62] or become streamlined to reduce drag[43] (FIG. 2G).

Although the viscoelastic response of biofilms is similar, the magnitudes of the viscoelastic parameters are highly variable. For biofilms that are grown *in vitro*, the shear modulus (G, which is a measure of rigidity) is 10^{-1}–10^{3} Pa, and the viscosity (η) is 10^{5}–10^{8} Pa, whereas for biofilms from natural hot springs, G is 10^{3}–10^{5} Pa and η is 10^{7}–10^{8} Pa (P.S., T. Shaw, M. Winston and I. Klapper, unpublished observations). The variability in the absolute magnitude of the viscoelastic parameters might reflect the diversity of biofilm organisms and the growth environment. Viscoelasticity might be not only an adaptation for survival on surfaces in flowing water, but might also allow an adaptive response to different shear environments through EPS modification. A better characterization of the mechanical properties of biofilms will elucidate the role of biofilm mechanics for sessile survival in flowing environments, explain observed shear-mediated dynamic behaviour and allow the development of more effective mechanical removal strategies for biofilm control.

HYDROGELS
An extremely hydrated polymer gel. The polymer chain holds many times its weight in trapped water.

Why do prokaryotes form biofilms?

What are the advantages of biofilm formation and surface association that make it such a widespread phenomenon? At present, there are several hypotheses. First, surfaces provide a space to be occupied and, as discussed previously, they provide a degree of stability in the growth environment and might have catalytic functions through localizing cells in close proximity. Second, biofilm formation affords protection from a wide range of environmental challenges, such as UV exposure[69], metal toxicity[70], acid exposure[71], dehydration and salinity[72], phagocytosis[73] and several antibiotics and antimicrobial agents[64,74,75].

Three mechanisms have been proposed to explain the general resistance of biofilms to biocidal agents. The first is the barrier properties of the slime matrix. This mechanism might be more relevant for reactive (bleach or superoxides), charged (metals) or large (immunoglobulin) antimicrobial agents that are neutralized or bound by the EPS and are effectively 'diluted' to sublethal concentrations before they can reach all of the individual bacterial cells within the biofilm. The barrier properties of the EPS hydrogel might also protect against UV light and dehydration, and might localize enzymatic activity. For example, extracellular β-lactamase enzymatic activity against *P. aeruginosa* occurs within the matrix[76].

The second protective mechanism could involve the physiological state of biofilm organisms. Although many antibiotics can freely penetrate the EPS, cells within the biofilm are often still protected. The creation of starved, stationary phase dormant zones in biofilms seems to be a significant factor in the resistance of biofilm populations to antimicrobials[56,77,78], particularly against antibiotics such as β–lactams, which are effective against rapidly dividing Gram-positive bacteria by interruption of cell-wall synthesis. However, arguably all antibiotics require at least some degree of cellular activity to be effective, because the mechanism of action of most antibiotics involves disruption of a microbial process. Therefore, pockets of cells in a biofilm in stationary phase dormancy might represent a general mechanism of antibiotic resistance.

A third mechanism of protection could be the existence of subpopulations of resistant phenotypes in the biofilm[79], which have been referred to as 'persisters'[56]. Persisters comprise a small fraction of the entire biomass, whether in planktonic or biofilm culture, but as distinct phenotypes have yet to be cultured, it remains unclear if these organisms do indeed represent a distinct phenotype or are simply the most resistant cells within a population distribution. Although the relative contribution of each of these mechanisms (and possibly others) varies with the

type of biofilm and the nature of the environmental stress, the result is one of general protection.

We have hypothesized that biofilm formation is likely to be an ancient adaptation of prokaryotic life. Paradoxically, many of the impacts of the proclivity of bacteria to stick to surfaces have become more obvious with ever-increasing technological developments. Industrial pipelines, nuclear power stations, space stations, air conditioning systems, water distribution systems and one of the fastest technologically advancing settings, the hospital, are all susceptible to colonization by microorganisms growing in biofilms.

Biofilm infections

The first part of this review concentrated on the importance of biofilm formation for the survival of prokaryotes in the natural environment. Some of the developmental processes and observed dynamic phenomena of biofilm formation were outlined and interpreted in the context of convergent survival strategies. In the second part, we discuss biofilm formation as a fundamental consequence of bacterial adhesion and biofilm growth in a host. Many of the physiological characteristics of biofilm formation — for example, localized clusters of bacteria adhering to a substratum that are more resistant to antibiotic therapy — are similar whether in the natural environment or in an animal host. Fundamental knowledge of biofilm formation from environmental studies helped to characterize biofilms growing on medical devices in the earliest studies, and continues to provide insights into biofilm infections. However, we argue that the complex interaction between the biofilm pathogen and the host inflammatory response modifies the host environment, and that successful biofilm parasites respond accordingly by altering their phenotype to the biofilm mode of growth.

Matrix-enclosed microbial communities adherent to non-biological and biological surfaces. A distinguishing feature of biofilms from that of other colonizing infections is the presence of aggregated microcolonies of cells that are attached to a surface. Importantly, biofilm formation as a protective mechanism could have profound implications for the host, because the microorganisms that are growing in these matrix-enclosed aggregates are more resistant to antibiotics and host defences. The biofilm model proposes that microbial cells growing in biofilms are clustered. It fundamentally challenges the assumption that infectious agents are evenly distributed and therefore equally susceptible to the host immune response or antibiotic therapy. It might further account for several problematic clinical challenges, such as symptomatic, but unculturable, inflammation, antibiotic resistance, recurrence or persistence, and metastasis or the spread of infectious emboli.

However, a problem with assessing the contribution of biofilms in human disease is the lack of defined criteria with which to characterize biofilm-induced pathogenesis. Parsek and Singh[80] propose four criteria for defining a biofilm aetiology of an infection: the pathogenic bacteria are surface associated or adherent to a substratum; direct examination reveals bacteria in clusters, encased in a matrix of bacterial or host constituents; the infection is localized; and the infection is resistant to antibiotic therapy despite the antibiotic sensitivity of the constituent planktonic organisms.

The infections discussed in this review were chosen because they illustrate consistencies between biofilm growth in the environment and published literature investigating clinical infections. Owing to a great increase in the number of medical biofilm papers, however, space does not allow a comprehensive review of the medically relevant biofilms and the readers are referred to several reviews[80–82]. Device-related infections were the first clinical infections to be identified as having a biofilm aetiology and show that biofilm formation can be facilitated by the host inflammatory response because host inflammatory molecules facilitate adhesion to the surface of the device. Bacterial endocarditis shows how microorganisms on the skin or in the oral cavity that transiently enter the bloodstream can colonize abnormal or implanted valves, or altered endothelial surfaces in the heart (FIG. 4). Surface attachment within vegetations occurs as a result of interactions between microbial cells and host products. Cystic fibrosis (CF) illustrates how the opportunistic pathogen *P. aeruginosa* exploits the unique environment of the CF lung and responds to environmental cues by altering its phenotype.

Device-related infections. Intravenous catheters, prosthetic heart valves, joint prostheses, peritoneal dialysis catheters, cardiac pacemakers, cerebrospinal fluid shunts and endotracheal tubes save millions of lives, but all have an intrinsic risk of surface-associated infections. Biofilms associated with medical devices were first noted in the early 1980s when electron microscopy revealed bacteria deposited on the surface of indwelling devices, such as intravenous catheters and cardiac pacemakers[83–85].

The microorganisms that are most frequently associated with medical devices are the staphylococci (particularly *S. epidermidis* and *S. aureus*), followed by *P. aeruginosa* and a plethora of other environmental bacteria that opportunistically infect a host who is compromised by invasive medical intervention, chemotherapy or a pre-existing disease state. Biofilm formation on medical implants has even led to the characterization of a new infectious disease called chronic polymer-associated infection[82,86]. Staphylococci commonly colonize the skin and are frequently found in wounds and implants[87]. Interestingly, *S. epidermidis* was not considered an opportunistic pathogen until the widespread use of medical devices. Biofilm formation, then, can be thought of as a virulence factor — a bacterial strategy that contributes to its ability to cause an infection.

The most notable characteristic of the adherent staphylococci colonizing medical implants is the copious amount of EPS (also known as glycocalyx or 'slime') that encases and protects cells from host defences and antibiotic treatment (FIG. 3c). Biofilm

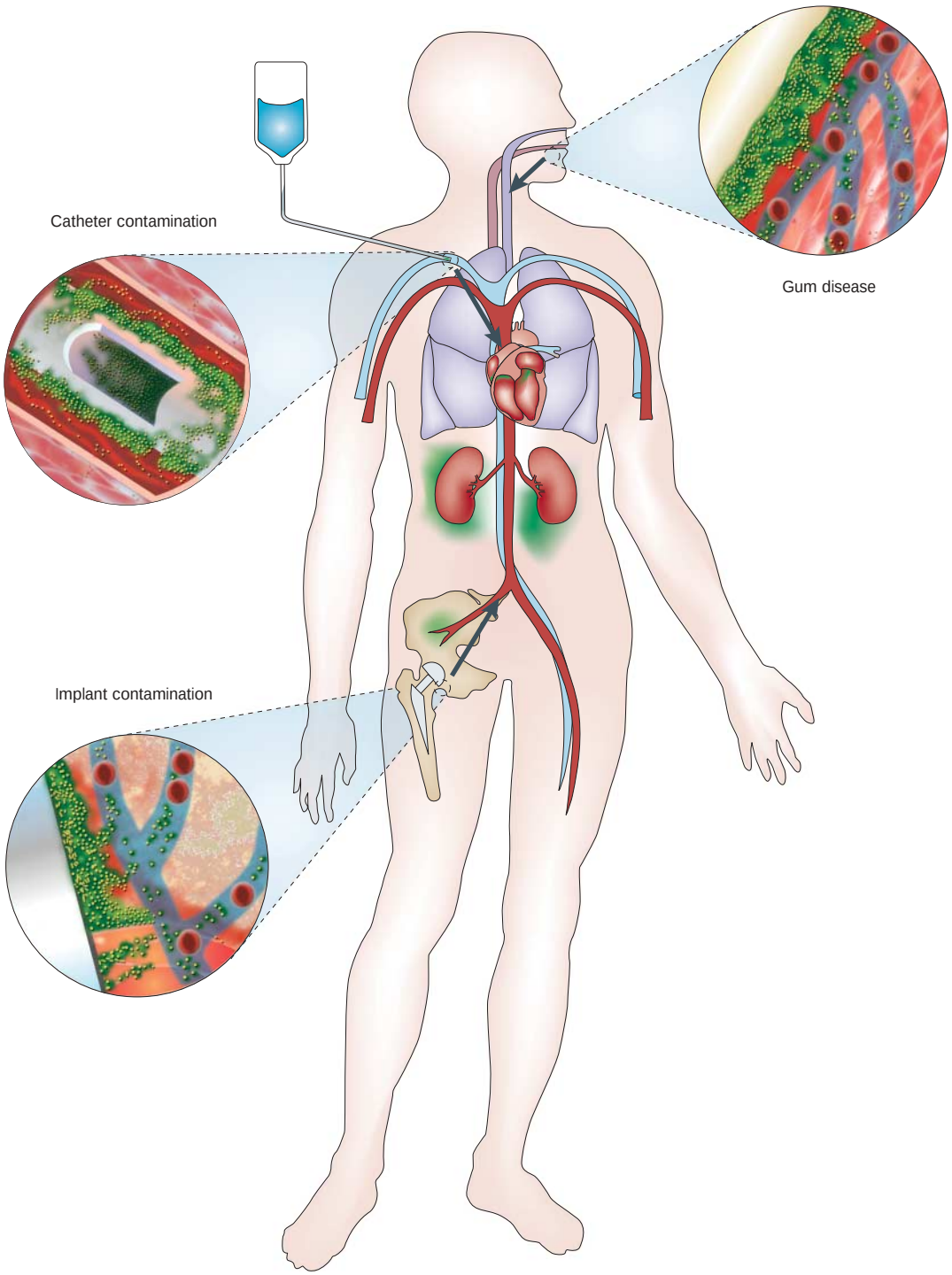

Figure 4 | **Schematic showing three examples of possible points of entry into the body for infectious biofilms; catheter, hip replacement, and periodontal disease.** Arrows show how the biofilm (green) might be disseminated around the body, either by single cells or clumps of protected emboli, using the example of native or artificial heart valve infective endocarditis as a common central location for embolization. Sporadic detachment could lead to cycles of bacteraemia. Image courtesy of P. Dirckx, Center for Biofilm Engineering.

formation is characterized by two principal stages in staphylococci: adhesion of bacteria to a solid surface, followed by growth-dependent accumulation of cells which generates multiple layers of cell clusters. In *S. epidermidis*, the formation of multiple cell layers has been attributed specifically to cell-to-cell adhesion mechanisms that are associated with a β-1,6-linked glycosaminoglycan polysaccharide known as polysaccharide intercellular adhesin (PIA)[88]. Proteins that are involved in the synthesis of matrix polysaccharides are regulated by the *ica*

gene locus in *S. epidermidis*[89], a locus that is conserved in *S. epidermidis*, *S. aureus* and other phylogenetically-related staphylococci[34]. Mutations in this locus disrupt biofilm formation primarily by disrupting cell aggregation and accumulation[90,91]. However, strains with PIA and *ica* might still fail to form biofilms if they are defective in initial adherence. The extensive literature on *S. epidermidis* and *S. aureus* shows that the adhesion stage alone is multifactorial, and depends on both the physiochemical properties of the biomedical polymer material and the nature of the bacterial cell surface. In particular, the hydrophobicity and the electrostatic charge of the material will influence interactions between the polymer and the surface of the bacterial cell.

Bacterial surface proteins contribute significantly to adhesion, and several key proteins have been identified as being important in staphylococcal biofilm formation. *S. epidermidis* adherence to polystyrene is mediated by AtlE, the major autolysin. An AtlE mutant that is defective in forming biofilms on polystyrene, but not on glass surfaces, is also less hydrophobic and forms large cell clusters compared with the wild type[89]. This protein also mediates binding to vitronectin, a component of the host extracellular matrix. In *S. aureus*, mutants lacking D-alanine in teichoic acid (*dltA*) exhibited a change in surface charge that compromised their ability to adhere to polystyrene or glass, although the production of PIA was intact[92]. The adhesion step of this defect in biofilm formation was re-established by the addition of Mg^{2+}. Additionally, *S. epidermidis* biofilm formation was enhanced by Mg^{2+} and inhibited by EDTA[93], illustrating the role of environmental factors on biofilm development. Other surface proteins, including the biofilm-associated protein (Bap) and the accumulation-associated protein (AAP), are important in biofilm formation. Mutations in Bap affect biofilm formation and pathogenesis in a mouse foreign body infection model[33].

Finally, the host can contribute significantly to adhesion in device-related infections, particularly with staphylococci[94]. Multiple specific receptors on the cell surface, called adhesins, bind to host molecules (for example, protein/glycoprotein components in plasma or platelets or components of the host extracellular matrix). Many of these proteins belong to a family of microbial surface components that recognize adhesive matrix molecules (MSCRAMMs), which mediate adhesion to various host cell types as well as to polymer surfaces coated with host plasma proteins[82,95]. Several bacteria have adhesins for fibronectin, which is a host protein that is frequently associated with bacterial attachment to surfaces[94], followed by fibrinogen/fibrin, collagen, laminin and vitronectin. Fibronectin also participates in adhesion by bridging associations with fibrin, collagen, heparin and other host cell surface glycosaminoglycans. Two fibronectin adhesins have been identified in *S. aureus* — FnBPA and FnBPB.

A collagen-binding protein (Cna) and two fibrinogen-binding proteins, known as clumping factors A and B (ClfA and ClfB) also belong to the MSCRAMM family. ClfA has also been shown to be important in the binding of *S. aureus* in adhesion to both polyethylene and polyvinyl surfaces[96]. So, certain bacteria seem to have the ability to exploit host proteins that are produced in wound healing or inflammation, which indicates that bacterial adhesins provide a mechanism by which colonization of the host can occur on viable, but damaged tissues, and on devices in conditions where these host inflammatory molecules are present and might be characterized by biofilm-like infections.

Infective endocarditis. The biofilm matrix is most frequently referred to as being of bacterial origin. Although this applies to biofilms grown on an abiotic surface in the laboratory, biofilm infections within the body, which are characterized by adherent bacteria on tissue, might also include host cells and molecules as part of the surface-associated infection. An interesting example is bacterial endocarditis.

Streptococci are the aetiological agents in more than half of infective endocarditis cases, with staphylococci accounting for another quarter of the cases[81]. Many strains are common commensals of the skin and the oral cavity. Clinically, bacterial endocarditis lesions are referred to as vegetations and they comprise aggregates of bacterial cells, platelets and fibrin which are adherent to the damaged epithelium of cardiac valves. Endocarditis is associated with congenital heart defects, prosthetic heart valves and vascular grafts, and is most likely caused by clots of platelet and fibrin, which amass where turbulent flow is aggravated by abnormal tissue, pre-existing heart disease or an indwelling vascular catheter. Damaged endothelium exposes the underlying basement membrane, which consists of collagens, laminin, vitronectin and fibronectin, thereby providing a substratum for bacterial adherence (the initial stage in the pathogenesis of endocarditis). In addition, after endothelial damage and turbulent blood flow, inflammatory processes stimulate the clotting system, leading to the deposition of fibrin and the creation of an insoluble clot of fibrin and platelets.

Durack showed that *Streptococcus sanguis* adhered to the surface of sterile vegetations within 30 minutes of injection into catheterized rabbits and began to replicate[97]. Streptococcal microcolonies developed in the thrombus and were surrounded by strands of fibrin 'capsules' that seemed to retard leukocyte interactions. When the vegetations were examined four weeks post-infection, viable streptococci were present within a calcified lesion surrounded by fibroblasts underlying the endothelium. The metabolic activity of organisms in the vegetation seemed reduced compared with bacteria at the periphery — consistent with biofilm formation in the vegetation. Over time, the vegetations grew by the addition of layers of fibrin and platelets, with bacterial colonies 'sandwiched' between them, indicating cycles of thrombosis and further bacterial colonization of the layers[97].

However, using the rabbit model, Höök and Sand showed that bacterial colonization occurred even when vegetations were prevented with anticoagulant treatment, which indicated that the presence of a clot

is sufficient, but not necessary, for endocarditis. Interestingly, the course of the disease was remarkably different. In rabbits that were treated with anticoagulants, disease was more fulminant and animals exhibited higher bacteraemia and a lower survival rate. By contrast, animals without anticoagulant treatment exhibited a subacute, chronic infection that was more recalcitrant to antibiotic therapy. The ultrastructure of the resulting vegetations was comprised of large bacterial colonies, densely packed with fibrin and platelets and surrounded by a fibrin mesh. The authors speculated that the bacteria within the vegetation were metabolically less active[98]. Similar structures were observed in human vegetations[99]. Therefore, the appropriate local conditions exist for colonization of underlying host tissues by bacteria growing as microcolonies, and the pathogenesis of endocarditis as a biofilm disease is consistent with bacteria growing in biofilms. However, these reports also indicate that the presence of platelets and fibrin owing to the host inflammatory response confers biofilm characteristics that are observed in endocarditis — a persistent, recurring infection that is more resistant to antibiotic treatment.

Several studies have examined the ability of bacteria to adhere to tissue surfaces and establish a localized site of infection by specific interactions between bacterial adhesins and host tissue. Ramirez-Rhonda examined streptococcal species for their ability to adhere to cardiac valves and found that strains that produce EPS consisting of glucans and dextrans adhered better to damaged heart valves[100]. More recently, in *Streptococcus parasanguis*, a colonizer of the human tooth surface and an opportunist that is found in both native and prosthetic heart valve endocarditis, a gene encoding PERITRICHOUS FIMBRIAE, *fap1*, was shown to be associated with biofilm formation on plastic. A *fap1* mutant showed limited adherence, but primarily failed to aggregate and form microcolonies[36]. Fey *et al*. showed a correlation between haemagglutination and biofilm formation in *S. epidermidis*, which indicated a link between PIA, intercellular adhesion and adhesion to erythrocytes. Defective PIA strains were found to be less virulent in a rabbit endocarditis model[101,102]. Similarly, *S. aureus* mutants that are defective in adherence to platelets correlated with reduced virulence in a rabbit model of endocarditis, which is characterized by fewer bacteria within vegetations and reduced embolization[103].

An *S. aureus* mutant defective in fibronectin binding also showed decreased binding to damaged heart valves[104]. The clumping factor ClfA, a fibrinogen-binding protein in *S. aureus* which also mediates adhesion of *S. aureus* to plastic, seems to have a s pecific role in the rat model of endocarditis. Mutant and complementation studies showed that *clfA* was important in both adherence to surfaces and virulence; however, endocarditis still occurred with a larger innoculum of *clfA* mutants[105]. However, when ClfA and FnbA (another adhesin) were co-expressed in a non-virulent organism — *Lactococcus lactis* — endocarditis in the rat model was comparable to that observed for pathogenic species[106]. ClfA has also been shown to bind *S. aureus* to human platelets directly by a previously uncharacterized platelet-membrane receptor[107]. These specific interactions show that, similar to the example of biofilms in device-related infections, biofilm development is a multistep process of initial adherence between specific bacterial adhesins and host molecules, followed by intercellular accumulation of bacterial cells and host components that generate multiple cell layers of the biofilm vegetation.

Bacterial endocarditis might also illustrate how turbulent flow contributes to the formation of vegetations. Although turbulence has been traditionally thought to induce clot formation and tissue damage, it is conceivable that clumps of biofilm cells respond to the turbulent flow of the cardiac environment by producing more EPS. Large vegetations are particularly friable, and amplify the risk of embolization (detachment through clumping dispersal) that might cause infarcts and septic abscesses in other tissues.

Importantly, antibiotic therapy in the treatment of endocarditis is also consistent with a role for biofilms. *In vivo* studies in a rabbit model with *E. coli* as the infectious agent required sustained antibiotic concentrations that were 220 times the serum minimum bacteriocidal concentrations. Even when the vegetations were treated with antibiotic *ex vivo*, antibacterial effects within the vegetation required 150 times the minimal bacteriocidal concentration[108].

Cystic fibrosis pneumonia. CF is an autosomal recessive disease that is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that results in dysfunctional electrolyte secretion and absorption. Although multiple, complex physiological dysfunctions are present, the primary site of morbidity is the respiratory system. Reduced hydration of the airway surface fluid that renders the respiratory mucous more viscous and impairs mucociliary clearance, leads to the main clinical feature of CF — chronic endobronchial bacterial infection and airway inflammation — which leads to airway obstruction, progressive destruction of the airway epithelium and, ultimately, respiratory failure.

Pulmonary colonization of the lower respiratory tract of CF patients begins in infancy or early childhood, most commonly by *S. aureus* and *Haemophilus influenzae*. However, by adolescence and early adulthood most CF patients have become colonized with *P. aeruginosa*[109,110]. The shift from colonization with other bacteria to chronic infection with *P. aeruginosa* seems to be the result of the peculiar environment of the CF lung, which includes asialylated receptors on epithelial cells that facilitate pseudomonal attachment in addition to impaired mucociliary clearance[109–111].

There are two salient features regarding colonization of the CF lung with *P. aeruginosa*. First, *P. aeruginosa* grows in biofilms within the CF lung[110,112–115]. Microscopic analysis of sputum from CF patients showed that *P. aeruginosa* forms biofilm-like structures consisting of clusters of bacteria surrounded by a dense matrix[113,114]. A similar structural morphology

PERITRICHOUS FIMBRIAE
The many short extracellular pili appendages that protrude from the surface of some prokaryotic cells. Fimbriae are used for attachment to surfaces.

has been observed in CF lung specimens[116]. Moreover, homoserine lactone (HSL) QUORUM SENSING (QS) signals measured in CF sputum were consistent with the QS profile of *P. aeruginosa* grown in biofilms, not the profile from planktonically grown *P. aeruginosa* cultures[115].

The second salient feature of *P. aeruginosa* colonization of the CF lung is the selection of mucoid variants of *P. aeruginosa*, which are characterized by overproduction of the exopolysaccharide alginate, and a resistance to antibiotic therapy[110,111]. Initially, *P. aeruginosa* isolated from the lungs of CF patients is non-mucoid. However, mucoid isolates typically coincide with persistent chronic infection. Interestingly, mucoid variants are absent among environmental isolates of *P. aeruginosa*, although non-mucoid strains seem to have the genotype for mucoidy. Research indicates that the host inflammatory response contributes to mucoid conversion. Mathee *et al.* grew *P. aeruginosa* in biofilms and subjected these biofilms to either exogenous hydrogen peroxide (H_2O_2) or activated human polymorphoneutrophils (PMNs) *in vitro*[117]. They observed that mucoid conversion was consistent with a deletion in the *mucA* open reading frame; the same deletion was also observed in 25% of mucoid isolates from CF patients. Therefore, *P. aeruginosa* responds to the microenvironment of the CF lung by modifying its phenotype.

The specific mechanism of *P. aeruginosa* colonization of the CF lung is not known. One hypothesis proposes that airway inflammation leads to the attachment of *P. aeruginosa* to denuded airway epithelium. One study found that CF bronchial secretions possess proteolytic activity against fibronectin that is associated with the respiratory mucosa[118], which provides a mechanism that might favour *P. aeruginosa* colonization over *S. aureus* colonization. Another study indicated that *P. aeruginosa* binding to nasal polyp primary cultures was due to modification of epithelial cells by bacterial exoproducts that exposed asialoganglioside-binding sites which facilitate pseudomonal adherence[119]. Using a similar *ex vivo* model, *P. aeruginosa* was found to adhere to undifferentiated epithelial cells undergoing repair, specifically between $\alpha_v\beta_1$ integrins and the fibronectin RGD (Arg-Gly-Asp) receptor on epithelial cells and a *P. aeruginosa* outer-membrane protein[120]. *P. aeruginosa* has several adhesins and binds to a broad range of receptors and cell types in the respiratory tract[121].

The QS molecule 3-oxo-C_{12}-HSL that is produced by *P. aeruginosa* also seems to have modulatory effects on the respiratory epithelium. Cultured bronchial epithelial cells produced interleukin (IL)-8, an inflammatory cytokine, in response to *P. aeruginosa*, and it was later shown that this effect was due to 3-oxo-C_{12}-HSL, not to other signalling molecules[122]. Additionally 3-oxo-C_{12}-HSL induced the expression of other inflammatory cytokines and chemokines, such as IL-1, IL-6 and interferon (INF)-γ, and several macrophage inflammatory proteins. 3-oxo-C_{12}-HSL also induced cyclooxygenase 2 (COX-2) and prostaglandin E_2 (PGE_2) in human lung fibroblasts[123]. These results indicate that the presence of *P. aeruginosa* induces the release of inflammatory mediators and leukocyte infiltration into lung tissue that is not associated with clearance of the pathogen, but rather with continuing inflammation and lung pathology.

Another hypothesized mechanism of bacterial colonization in CF implicates the mucous layer. In this model, the increased viscosity of CF airway mucous acts as a matrix scaffold and is important in decreased clearance. Recently, Worlitzsch *et al.*[124] studied CF patients with chronic lung disease directly using electron microscopy and tissue explants, and found that *P. aeruginosa* was present in mucopurulent hypoxic 'macrocolonies' of 100-μm diameter in the airway lumen, rather than attached to the epithelium. Both *in vivo* and *in vitro* experiments using microelectrodes showed that oxygen was depleted in these mucoid macrocolonies. Furthermore, motile *P. aeruginosa* penetrated the hypoxic mucous layers and responded to anaerobic conditions by producing more alginate. These results argue that the local environment in CF lungs, which is characterized by thick mucous plaques and depleted O_2 in the respiratory epithelium, leads to colonization by *P. aeruginosa* that further exacerbates the pathology of CF pneumonia.

Yoon *et al.*[125] have also shown that anaerobic growth might be an important feature of *P. aeruginosa* growing in biofilms in CF patients. *P. aeruginosa* formed vigorous biofilms under anaerobic conditions, leading to the build-up of toxic nitrogen metabolites. Proteomic analysis identified an outer-membrane porin, OprF, the concentration of which increased 40-fold in anaerobic culture. OprF was also detected in secretions from CF lungs and circulating antibodies against OprF were found in chronically infected CF patients. This study indicates a mechanism that explains several clinical aspects of CF *P. aeruginosa* pneumonia: the ineffectiveness of phagocytes against *P. aeruginosa*, the mucoid *P. aeruginosa* phenotype and the resistance of *P. aeruginosa* biofilms to tobramycin[78,126].

Interestingly, *P. aeruginosa* antibiotic resistance and biofilm formation seem to be induced at the same time[127]. When antibiotic resistance was studied in a clinical isolate of *P. aeruginosa*, a different phenotype that is associated with both an enhanced ability to form biofilms and increased antibiotic resistance was observed. The phenotype was observed both *in vitro* and in CF patients undergoing antibiotic therapy, but not in untreated patients. Experimentally, resistance variants also arose more frequently in response to environmental cues, such as alterations in salt concentration. It was speculated that antibiotic-resistant phenotypic variants of *P. aeruginosa* observed in CF infections were either selected within biofilms by sub-lethal antibiotic treatment, or by the specific environment of the CF lung (characterized by osmotic and oxidative stress). This study showed that both are responsible for the resistant phenotype[127].

In the complex environment of the CF lung, it is unlikely that there is an exclusive mechanism of pathology, and therefore the host inflammatory response

QUORUM SENSING
A system by which bacteria communicate. Signalling molecules — chemicals similar to pheromones that are produced by an individual bacterium — can affect the behaviour of surrounding bacteria.

undoubtedly induces changes in the local microenvironment to which *P. aeruginosa* responds. What is clear is that in CF pneumonia, the complex interactions between bacteria and the local environmental cues in the host owing to the inflammatory response contribute to the complex pathology of this disease.

From planktonic to biofilm microbiology

Although it is accepted that biofilms are found ubiquitously in natural environments, the significance of biofilms in infectious disease is often not recognized or still debated. For those with experience of the problematic nature of biofilms in the clinic, this might be seen as academic. However, to those who have been studying planktonic bacterial cultures to investigate fundamental aspects of microbial physiology, pathogenesis and control — such as minimum inhibitory concentrations (MIC) of antibiotics — this is a matter of intense importance. The lag in studying surface-attached bacteria rather than the planktonic complement is understandable given the difficulties of working with surface-attached populations compared with homogenous batch-culture planktonic populations. Organisms are more laborious to culture as biofilms; the inherent heterogeneity of spatial distribution[18] leads to the creation of localized zones that vary widely in both physiological conditions[19] and cellular physiologies[128] over distances of only tens of micrometres. Another complexity when dealing with culturing on surfaces is that mass transfer (diffusion and flow through the biofilm), which controls nutrient exchange, becomes an important consideration[129], as do the fluid forces (shear and drag) that act on the biofilm[14]. Also, because biofilm cells stick together and to surfaces, simple volume-independent concentration-based manipulations, such as dilutions or concentration series that are used to calculate inhibitory and bacteriocidal concentrations (which are routinely used in batch cultures) become difficult. Calculating the concentration of antibiotic per cell in planktonic cultures is trivial when each cell is assumed to encounter the same level of antibiotic. In biofilms this is difficult given local heterogeneities and uncharacterized growth parameters, such as the surface area:volume ratio, residence time (the time a volume of fluid is in the system) or the nutrient loading rate (the concentration of nutrients per unit area per time).

The lack of standard methods for growing, quantifying and testing biofilms in continuous culture results in incalculable variability between laboratory systems. So far there is only one ASTM (American Society for Testing and Materials) standard method for growing biofilms (E-2196-02), which uses a rotating disc reactor (BioSurface Technologies, Bozeman, Montana), although the Centers for Disease Control (CDC) have also recently developed a biofilm growth reactor[130]. Quantification is being addressed with the development of image analysis packages, such as COMSTAT[131] and ISA[132].

Biofilm microbiology is complex and not well represented by flask cultures. Although homogeneity allows statistical enumeration, the extent to which it reflects the real, less orderly world is questionable. Arguably, it is the complexity of biofilms that helps make them so resilient. Biofilms present the next challenge in microbiology — to confront this complexity and devise more relevant testing protocols to deal with demanding microbial problems in industry and medicine.

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Competing interests statement
The authors declare that they have no competing financial interests.

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