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Biofilm formation and dispersal and the transmission of human pathogens

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Several pathogenic bacterial species that are found in the environment can form complex multicellular structures on surfaces known as biofilms. *Pseudomonas aeruginosa, Vibrio cholerae* and certain species of nontuberculous mycobacteria are examples of human pathogens that form biofilms in natural aquatic environments. We suggest that the dynamics of biofilm formation facilitates the transmission of pathogens by providing a stable protective environment and acting as a nidus for the dissemination of large numbers of microorganisms; both as detached biofilm clumps and by the fluid-driven dispersal of biofilm clusters along surfaces. We also suggest that emerging evidence indicates that biofilm formation conveys a selective advantage to certain pathogens by increasing their ability to persist under diverse environmental conditions.

Introduction

Most bacteria in oligotrophic environments grow in biofilms rather than as single planktonic cells. Biofilms, which provide a protective degree of homeostasis and stability in a changing environment, are structured, specialized communities of adherent microorganisms encased in a complex extrapolymeric substance (EPS) matrix. Local microenvironments within biofilms can be strikingly heterogeneous and organisms compete for space under varying conditions, such as nutrient limitation, fluid flow, desiccation, toxic chemical gradients, UV irradiation and pH and temperature fluxes. Therefore, biofilm formation represents a facile microbial survival strategy where microorganisms, including pathogens, exist in a dynamic equilibrium where cell clusters form, mature and detach to disseminate to new surfaces.

Biofilm development by pathogens in the environment

Some pathogens exist in the environment outside animal hosts as autochthonous inhabitants of aqueous systems, including freshwater, estuarine and marine environments and municipal water distribution systems. Subsequently, certain pathogens are transmitted from the environment, although biofilm formation is rarely considered as a mechanism of acquisition. Nevertheless, comparisons between planktonically-grown and biofilm-grown environmental pathogens might yield important information regarding virulence factors required for certain environmentally acquired infections.

Several mechanisms might make pathogens in biofilms more likely to cause disease than planktonic organisms. First, one obvious mechanism by which pathogens growing in biofilms could cause disease is through the seeding dispersal of large numbers of cells that subsequently initiate an infection. Time-lapse microscopy in our laboratories and others suggests biofilm organisms are indeed attached, but certainly not sessile (permanently attached or fixed; not free-moving), a term often used to differentiate biofilm cells from single planktonic cells [1]. We believe that dispersal is an integral part of the dynamic nature of life in surface-associated microbial communities [2–9]. Second, pathogens within biofilms are likely to be phenotypically heterogeneous, such that a virulent phenotype might survive and clonally expand within the biofilm. Third, high cell densities observed within biofilms might regulate quorum-sensing networks that also control virulence mechanisms [7,10,11]. These hypotheses might be tied to the probability that a sufficient number of virulent organisms will come into contact with a host and cause disease. However, studies within a model host that link biofilm formation with the expression of virulence genes and infection and pathogenicity are needed. We suggest that examples linking pathogenicity and biofilm formation in the natural environment exist for *Vibrio cholera* and *Pseudomonas aeruginosa* and are emerging for pathogenic nontuberculous mycobacteria (NTM). We propose that insights into pathogenicity and infective cycles might be gained by studying other environmental pathogens using the biofilm paradigm.

Detachment and infective dose

Biofilm clusters might be attached to a surface, suspended in fluid as flocs or exist as pellicles at air–liquid interfaces. Biofilms amass high numbers of organisms within a small scale and pathogen cell densities on a surface can reach 10^7 cells/cm^2 [12]. This is particularly significant for pathogen transmission from the environment. Biofilm development is a dynamic process of growth and detachment (or shedding) of bacterial cells and aggregates, which can lead to the ingestion or inhalation of a condensed infective dose. Detachment is particularly well-documented under fluid flow and is affected by variable hydrodynamic shear that occurs with changes in the flow rate. Data suggest that bacteria respond phenotypically to

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different hydrodynamic conditions; biofilms grown under low shear or static conditions dislodge clumps of bacteria more easily when the flow rate changes within a system [2,4]. Forces within biofilm EPS might be weaker in those biofilms that are grown under laminar shear and therefore more likely to detach when shear forces increase. The mechanical properties of biofilms might be an evolved trait for survival in aqueous environments [13].

Traditionally, detachment from biofilms has been considered a passive behavior, largely dependant on fluid shear or starvation [14]. However, detachment might also be a strategy by which bacteria proactively colonize new niches before space and nutrients become limited. Hunt *et al.* [5] used a computer model to demonstrate that the production of a hypothetical signaling-like detachment factor resulted in a similar detachment pattern of clumps as observed *in vitro* with mixed and axenic *Staphylococcus aureus* biofilms [6]. In both pure and mixed cultures of bacteria, biofilms exhibit dynamic behaviour, migrating in ripples or as discrete cell clusters that detach, move or roll over the surface, sometimes reattaching downstream [3]. We suggest three different dispersal strategies that can be observed for biofilm bacteria: swarming dispersal, clumping dispersal and surface dispersal [1] (Figure 1). All of these dynamic detachment events could succeed in dispersing biofilm bacteria to new surfaces or to a susceptible host. For *V. cholerae*, this might be a strategy that is used to persist in diverse marine and freshwater environments where it is transmitted to the human intestine [15–17]. Similarly, *P. aeruginosa* and environmental mycobacteria also are capable of surviving in vastly different environmental niches as well as opportunistically infecting a human host.

V. cholerae causes cholera epidemics and is a well-studied example that links bacterial biofilms, environmental transmission and pathogenesis. Pathogenic species of *V. cholerae* inhabit diverse ecological niches and readily form biofilms on biotic surfaces. Because the concentration of *V. cholerae* required to induce symptomatic cholera is estimated to be approximately 10^4–10^6 total cells [8], the accumulation on biotic surfaces of bacteria within biofilms, which can achieve concentrations of this magnitude, increases the risk of cholera infection [12].

Similarly, many NTM are autochthonous inhabitants of soil and water. Environmental pathogenic species include the NTM-members *Mycobacterium avium, M. marinum, M. ulcerans* and also members of the rapidly growing mycobacteria (RGM), such as *M. fortuitum* and *M. chelonae;* these mycobacteria have been shown to form biofilms *in vitro* [3,12,18–20]. Biofilm formation might specifically contribute to NTM transmission by facilitating a sufficient infective dose to be ingested, inhaled or abraded into the skin when clumps of bacilli are sloughed from the biofilm.

NTM were present in 90% of the water distribution systems in one European study, and mycobacteria were observed in biofilms when water from a distribution system was inoculated into silicone tubing [21,22]. Pathogenic RGM are intrinsically capable of biofilm development even under extremely oligotrophic conditions and are not passive or secondary surface colonizers

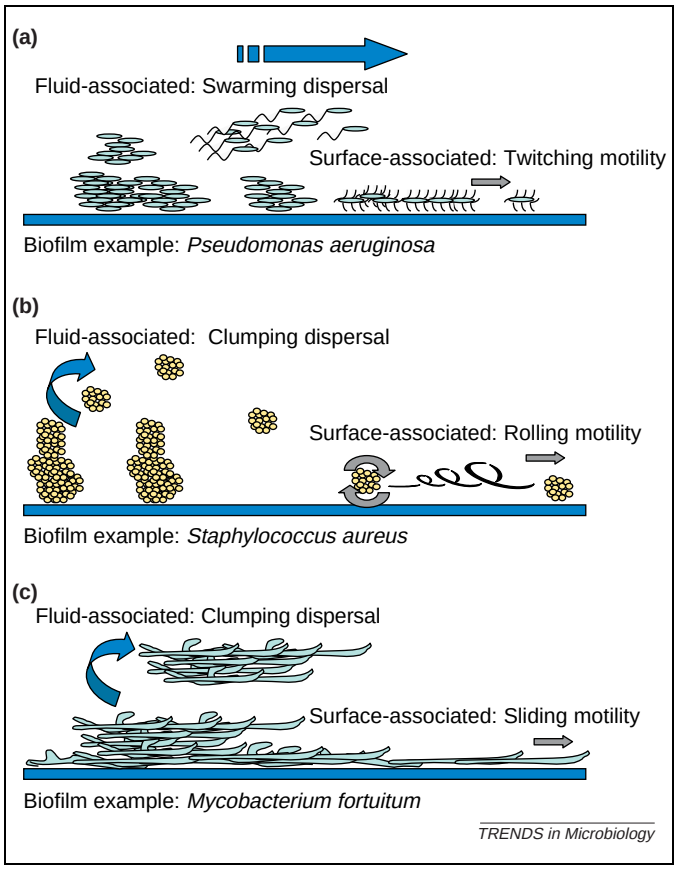

Figure 1. Various dispersal mechanisms displayed by biofilm-forming bacteria. Time-lapse microscopic imaging of biofilms grown *in vitro* in biofilm flow cells demonstrates that biofilms use various strategies for the dispersal of cells. This is illustrated by three different species; **(a)** *Pseudomonas aeruginosa,* **(b)** *Staphylococcus aureus,* and **(c)** *Mycobacterium fortuitum.* These dispersal strategies can be split into two distinct modes: (i) dispersal through detachment into an overlying fluid and (ii) dispersal over a solid surface. Additionally, in each of these modes dispersal can be through self-propelled locomotion (swimming, sliding or twitching motility) or through fluid-driven dispersal (clumping, rippling or rolling). Although swimming or twitching motility has the advantage that the motile bacterium is self-propelled and directional (i.e. chemotactic) the single cells do not have protective advantage of being in a biofilm. Conversely, cells in clumps rely on non-directed fluid driven dispersal, but are in the protective biofilm.

restricted only to hydrophobic surfaces [12,18]. Importantly, *M. fortuitum* and non-pathogenic mycobacterial biofilms also exhibit dynamic behaviour, detaching as clumps, sliding over a surface and reattaching downstream [3,23]. Such observations suggest how NTM biofilm development could lead to the dispersal of large clumps of mycobacteria into water or aerosols. NTM hypersensitivity pneumonitis and infections associated with spas, swimming pools, hot tubs, water baths and machine-working fluids might originate from biofilms [24–26].

Persistence of pathogens within biofilms

Evidence suggests that biofilm development by pathogenic *V. cholerae* facilitates its persistence in the environment where adherence to surfaces in aqueous environments plays a fundamental role in the epidemic cycles of this pathogen [15–17]. After investigating *V. cholerae* biofilm formation over time under different nutrient conditions, Moorthy and Watnick [27] suggest a model for biofilm development in the environment. Stage I is characterized by the attachment of flagellated *V. cholerae* to surfaces,

including the exoskeletons of crustaceans, aquatic insects and cell walls of aquatic plants. In Stage II, a monolayer formed on these surfaces degrades the polysaccharide polymers, providing a carbohydrate-rich environment and probably signals for *V. cholerae* to progress to Stage III, a phase characterized by decreased flagellar gene expression and increased transcription of genes required for intercellular adhesion and EPS production. Therefore, *V. cholerae* growing in Stage III biofilms, and sloughed as aggregated cells and ingested, would be expected to deliver a significantly higher concentration of bacteria than single-cell organisms. Evidence to support this was provided when the number of cholera cases in a Bangledeshi village dramatically declined following a crude method of water filtration through sari cloth [8].

Another example of biofilm formation and putative environmental transmission was recently provided by the emerging pathogen *M. ulcerans*, which colonizes aquatic plants in regions where the disease Buruli ulcer (BU) is endemic [28]. *M. ulcerans* grew as dense clusters on the surface of a species of algal filaments indigenous to BU regions, and algal extracts were found to halve the doubling period for this organism. Not only does colonization of aquatic plants enable persistence, but it also probably contributes to the necessary growth conditions for amassing an infective dose that might be abraded into the skin by this slow-growing organism.

Do biofilms select for different phenotypes that have increased virulence?

The biofilm environment exhibits remarkable heterogeneity, which might select for distinct phenotypes. For example, within biofilms highly aerated zones can border anaerobic zones by distances of only tens of microns. Phenotypes exhibiting virulence factors or adaptive traits to a particular host environment might arise within these microenvironments. Different phenotypes observed in biofilms often involve EPS biosynthesis and correlate with a certain colony morphology. Examples include the rugose morhpotype of *V. cholerae* [16] and the mucoid phenotypes and small-colony variants of *P. aeruginosa* [29–33].

A rugose morphotype of *V. cholerae* that arose after prolonged incubation during a biofilm assay conferred increased growth and optimal biofilm development for this organism, even when this morphotype was present as a small percentage of the seeded population [10]. These data suggest a selective pressure for biofilm formation in *V. cholerae* that might facilitate its survival during stress-inducing environmental changes.

P. aeruginosa, a model biofilm former, is ubiquitous in the environment and is the principal pathogen in people who have cystic fibrosis (CF), where it colonizes the lung and resists antibiotic therapy [31,32]. Quorum-sensing molecule expression observed in CF patients parallels *P. aeruginosa* growing in biofilms, not planktonic cultures [34]. Multiple phenotypic mutants of *P. aeruginosa* have been isolated from CF patients. A common variant is a mucoid phenotype that is characterized by the overproduction of the exopolysaccharide aliginate. Over time, mucoid isolates become more prevalent and coincide with persistent chronic infection. Non-mucoid *P. aeruginosa* primarily disseminates through the dispersal of single cells, although larger clumps are also shed by fluid-driven rippling over a surface [7,8]. It is not yet known how conversion to mucoidy affects these dispersal mechanisms.

The host environment appears to contribute to mucoid conversion in *P. aeruginosa*: deletions from specific genes regulating mucoidy are observed directly in isolates from CF patients and in the presence of exogenous H_2O_2 or neutrophils *in vitro*, suggesting that the CF lung microenvironment selects for this phenotype [30]. Although the mucoid phenotype is rarely reported in environmental isolates of *P. aeruginosa*, this might be due to differences in clinical and environmental culturing techniques [35]. Small-colony variants are another *P. aeruginosa* morphotype that is isolated from CF patients. This morphotype exhibits increased antibiotic resistance, enhanced autoaggregative and adherent behavior, and biofilm formation, which correlates with the increased expression of type III secretion system genes and increased cytotoxicity in macrophages and virulence in a mouse lung infection model [33]. What is apparent is that certain microenvironments provide the necessary conditions for the persistence and growth of sufficient numbers of organisms that are capable of producing disease.

Concluding remarks

Biofilm formation is not itself necessarily a virulence factor, because many non-pathogenic organisms produce biofilms that do not cause disease. However, biofilm formation by certain pathogens appears to facilitate the survival of these pathogens in the environment and the host (Box 1). This might be due to the accumulation and dispersal of a sufficient number of pathogens for an infective dose, which is not typically found in a bulk fluid. Additionally, the heterogeneous microenvironments that occur within biofilms might promote a differentiated population of phenotypic and genotypic variants of microorganisms that promises survival in the face of changing environmental conditions and might also facilitate infection. We think that the investigation of biofilm development will yield insights into pathogenicity, virulence and the prevention of certain infections. A better

Box 1. Mechanisms by which biofilms could facilitate pathogen survival, transmission and/or evolution

Accumulation and dispersal of high numbers of microorganisms

- Accumulation and detachment of pathogenic biofilm bacterial clusters [2–9].
- Quorum sensing [motility, biofilm formation, extrapolymeric substance (EPS) production and virulence gene expression]. Recently biofilm development and virulence for *Vibrio cholerae* has been linked by the identification of intersecting quorum-sensing circuits that regulate virulence and biofilm formation [10,11].
- Coaggregation and autoaggregation [10,33].

Diverse microenvironments in biofilms facilitate multiple selection pressures

- Changes in EPS biosynthesis [16,30,31].
- Selection of different metabolic pathways (anaerobic versus aerobic; evidence in literature, but not discussed).
- Increased adherence [16,30].
- Genetic exchange (horizontal gene transfer; evidence in literature, but not discussed).

understanding of environmental pathogens will come with the investigation of the complete 'lifecycle' of these pathogens, including the investigation of pathogenic phenotypes attached to surfaces.

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