The biofilm life cycle– expanding the portfolio of models

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Abstract

Bacterial biofilms are often defined as communities of surface attached bacteria. Biofilms are typically depicted with a classic mushroom-shaped structure that is a characteristic of *Pseudomonas aeruginosa*. However, it has become evident that this is not how all biofilms develop, especially *in vivo* and in the environment where biofilms often are observed as none surface attached aggregates. In this Review we describe the rationale behind the 5-step model and why it fails to capture many aspects of bacterial biofilm physiology, and aim to present an expanded developmental model for biofilm formation that is flexible enough to include all the diverse scenarios and microenvironments where biofilms are formed. With this new expanded inclusive model, we introduce a common platform for developing our understanding of biofilms and antibiofilm strategies that can be tailored to the microenvironment that is being investigated.

[H1] Introduction

In the past 40 years, microbiologists have categorized bacteria as displaying two life forms in nature. In one form, the bacteria appear as single, independent free-floating cells (planktonic). In the other form, the bacteria are organised in surface-attached sessile aggregates (biofilms). In addition aggregated bacteria have been divided into surface and non-surface attached, with the word biofilm originating from biomaterial on a surface(1, 2). In medicine until recently bacteria growing planktonically have been associated with acute infections that are generally treatable with antibiotics, though successful treatment largely depends on accurate and fast diagnosis. In cases where bacteria succeed in forming biofilms within the host, the infection is often untreatable and, sustained by low-grade inflammation, develops into a chronic state (3). However, this dogma has been challenged with the recent finding that the difference between bacteria in acute and chronic infections is the metabolic activity rather than aggregation(4), Bacterial biofilms predominate in both acute and chronic human lung infection. Chronic biofilm infections are further divided into surface associated, commonly observed in patients with implants or medical devices, and non-surface associated, such as respiratory tract infections where host mucociliary clearance is impaired (in viscous airway mucus in people with cystic fibrosis (CF)) or in persistent soft tissue infections, associated with comorbidities such as diabetes or impaired vascularization of the lower limbs predisposing to non-healing wounds.

In the environment, the functional consequences of bacterial life in biofilms have been associated with enhanced protection towards shear stress, desiccation, toxic compounds and protozoan grazing(5). Moreover, retainment of enzymes in the biofilm matrix was proposed to improve efficacy and diversity of organic matter decomposition, and biofilm formation on plant roots and fungal cells may promote bacterial nutrient acquisition and transport, respectively(6). While motile, planktonic cells are primarily found in water columns and soil pores, the predominant forms of microbial life in natural environments are linked to highly diverse biofilm communities in aquatic environments (including sediments, submerged surfaces, as free-floating flocs and on higher organisms), sediments and soil (e.g. on litter, plant roots and soil particles)(7). Likewise, biofilms dominate in industrial microbial applications, such as cleaning of wastewater and bioremediation of soil and water(8).

In industrial systems biofilms are a notorious challenge (9). Biofilms are associated with microbially induced corrosion in oil field pipelines, plugging pipes, fouling ship hulls creating drag and increased fuel costs, reducing heat transfer in cooling towers, and fouling manufacturing lines resulting in product contamination. In all these instances, the industrial system is not sterile, and so it is not necessarily an issue that bacteria are present, but more that the biofilm compromises a product or system performance. In these cases, biocide manufactures develop clean-in-place procedures to control biofilm growth, but in reality, the biofilm is never completely eliminated, and thus like dental biofilms routine cleaning maintenance is required to keep the biofilm biofouling in check.

A common denominator of bacterial biofilms is the distinction between surface-attached and non-surface-attached bacterial aggregates, despite new evidence showing that these share similar same phenotypes(10). A common denominator is that for both these phenotypes the bacteria create microenvironments which in turn influences bacterial community and behaviour in an interdependent dynamic manner (11, 12).

[H2] Growing bacteria and biofilms in the laboratory

While bacteria have been studied in the laboratory for well over 100 years, biofilms have been studied after surface-attached bacteria were observed attached to the pacemaker lead in a patient suffering from recurrent bacteraemia (13) and growing on glass slides inoculated with sea water (14). The bacteria attached to the pacemaker lead mark one of the first references to “biofilm growing bacteria” in medicine, with a subsequent explosion of interest in biofilm infections. Numerous *in vitro* systems have been devised to study

biofilm formation (15-17) and how biofilm bacteria differ from planktonic cells, including the hallmark property of increased antibiotic tolerance, or the presence of an extracellular polymeric (EPS) matrix, a hydrogel-like substance encasing biofilm cells(18). These initial findings supported the notion that microorganisms undergo significant changes in their phenotypic repertoire during the transition from planktonic to biofilm growth and revealed the potential for new ways to control or manipulate biofilms (Box 1). The *in vitro* systems commonly used shaken, well mixed cultures, and led to most biofilm experiments being initiated by using single cell planktonic cultures with one, controlled seeding event. Likewise, the transformation of single cells into sessile biofilm communities has been thoroughly studied in closed, surface-based *in vitro* systems without the influx of new cells during the biofilm formation and maturation process (19-21). Such studies led to a key publication in the field describing the developmental stages of *P. aeruginosa* (a nosocomial pathogen), presenting the current accepted "biofilm model"(Figure 1) (19). The biofilm developmental stages are referred to as reversible and irreversible attachment, biofilm maturation I and II involving cluster and microcolony formation, respectively, and dispersion (22)(19). Variations of this model have also been developed for other species such as *Staphylococcus aureus (23)* and the soil bacterium *Bacillus subtilis* (24) and for algal biofilms (25).

While the biofilm developmental model based on *P. aeruginosa in vitro* biofilm formation is easy to understand and has been grossly generalized to describe all biofilms, this model does not necessarily describe biofilms in real world industrial and natural settings, or in biofilm-linked infections or reflect relevant microenvironments that develop within these biofilms. Consider the substantial differences between the processes occurring in a laboratory flow cell and those leading to biofilm formation in the rhizosphere, a chronic wound, the respiratory tract, at an air-water interface (a pellicle), around a prosthetic joint, or in a wastewater granule. In these diverse systems, the processes of attachment, aggregation, interaction with biotic or abiotic materials and interfaces (e.g., roots, tissue, a gas phase, environmental polymers), growth and maturation, and detachment/dispersal are likely quite different. Given the variety of systems and conditions, we propose it would be useful to expand the existing model to include a wider spectrum of real-world scenarios.

In this Review, we describe the origin of the current biofilm model and its shortcomings, discuss differences in biofilm formation by diverse types of bacteria in varied experimental systems, both *in vitro* and *in vivo,* focussing on new findings that warrant amending the current model, such as lack of surface, difference in matrix properties and transcriptional profiles etc.. We suggest models that encompass additional pathways for biofilm and aggregate formation independent of surfaces and planktonic bacteria, and present a revised and expanded biofilm model that incorporates the range of real-world biofilm systems.

[H1] The origin of the biofilm model

Numerous studies support the notion that biofilm formation commences by initial surface attachment and that biofilm cells differ from their planktonic counterparts in the genes and proteins that they express (Box 1). Given the profound changes that microorganisms undergo during their transition from planktonic organisms to cells that are part of a complex, surface-attached community, it is not surprising that the transition from the planktonic to the biofilm mode of growth is a complex and highly regulated process, that is often referred to as a developmental process (26). However, while it was widely accepted that the transition to the surface is a highly regulated process, it remained unknown whether subsequent surface associated growth was simply an accumulation of cells due to growth or instead coincided with distinct events indicative of progressive or transitional changes over the course of biofilm formation. In an effort to better understand the progression of biofilm formation, in 2002, researchers (27) made use of a combination

of direct observation by microscopy, evaluation of biofilm morphology, matrix polymer production, and activation of quorum sensing-regulated genes, as well as quantitative analysis of protein abundance. The analysis led to the realization that over the course of biofilm formation, *P. aeruginosa* displays multiple phenotypes with distinct physiological characteristics (structural and metabolic changes) that can be correlated to distinct episodes or stages of biofilm development (Figure 1). These stages were referred to as reversible and irreversible attachment, maturation (maturation-I and -II stages), and dispersion , with each biofilm developmental stage corresponding to unique patterns of protein production and gene expression (28-33). The difference between reversible and irreversible attachment was based on the time scale of what happens over the next few minutes once a cell contacts a surface.

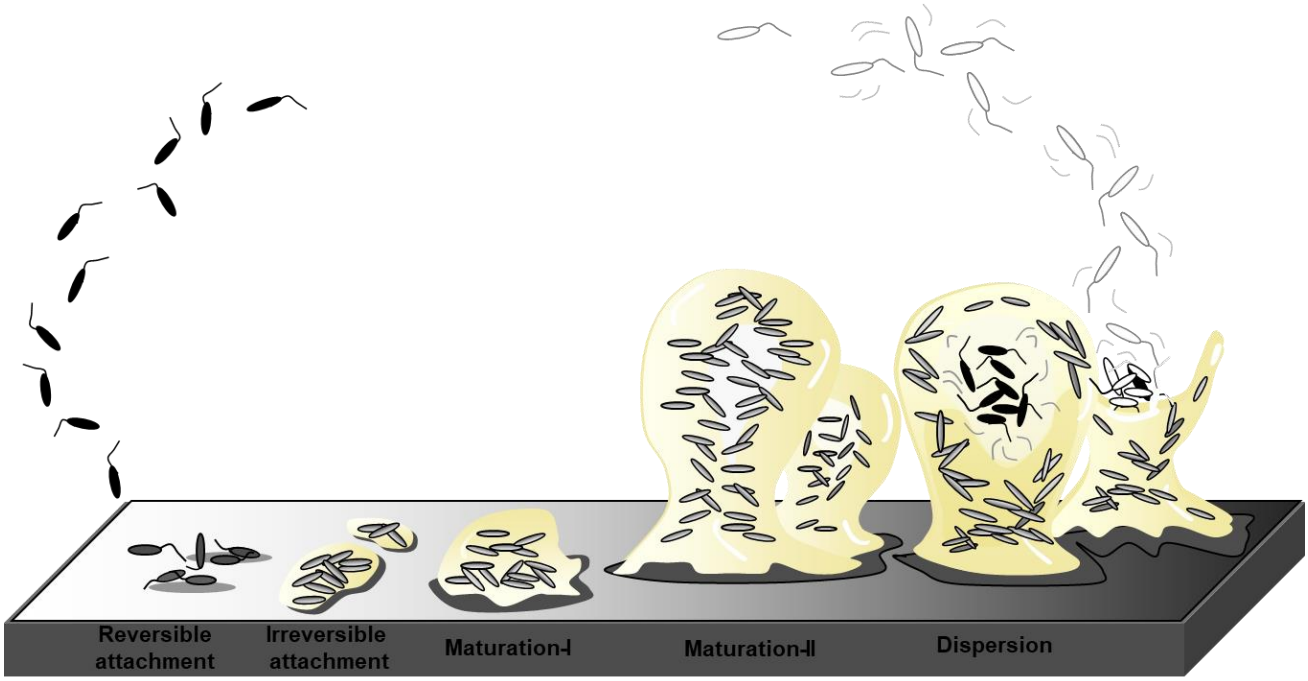

Figure 1 The stages of biofilm development as diagrammed in (19). The formation of biofilms is a cyclic process that occurs in a stage-specific and progressive manner. The process is initiated following surface contact by single planktonic cells. Several developmental steps are discernable as reversible attachment, irreversible attachment and biofilm maturation (maturation-I and -II)(19, 34). During reversible attachment, bacteria attach to the substratum via the cell pole or via the flagellum (step I), followed by longitudinal attachment. Transition to the irreversible coincides with a reduction in flagellar reversal rates, reduction in flagella gene expression and the production of biofilm matrix components. This stage is also characterized by attached cells demonstrating drug tolerance(35). Biofilm maturation stages are characterized by the appearance of cell clusters that are several cells thick and are embedded in the biofilm matrix (maturation-I stage) which subsequently fully mature into microcolonies (maturation-II stage)(19, 34). Dispersion has been reported to coincide with the decrease in and degradation of matrix components, with dispersed cells being motile and demonstrating increased drug susceptibility relative to biofilm cells. Biofilm matrix is shown in beige.

In *P. aeruginosa* the reversible attachment stage is characterized by cells attaching to a surface by a single pole (Fig. 1). Most surface contact is unstable, and cells are often seen returning to the bulk phase. Once rod-shaped cells commit to a more stable surface existence, cells attach via their longitudinal axis. This phenomenon is referred to as 'irreversible attachment' (Fig. 1). Reports furthermore suggest irreversible

attachment initiates a cascade of changes in the bacterial cells. Apparent changes following bacterial attachment include cessation of flagella-mediated motility while at the molecular levels, changes include surface-induced gene activation of *P. aeruginosa algC*, a gene involved in lipopolysaccharide core biosynthesis and in the biosynthesis of the exopolysaccharide alginate (36, 37), induction of genes involved in the biosynthesis of the Psl matrix polymer (38), as well as genes linked to antibiotic resistance, including β-lactamase (39), phenazine (40), SagS and BrlR (35). The findings suggest that committing to the surface associated mode of growth not only coincides with the production of biofilm matrix components that enable cells to more firmly cement themselves to the surface, but also with biofilm antimicrobial tolerance, a hallmark characteristic of biofilms, as an early adaptative response to the sessile lifestyle. Once attached, cells will grow into a more complex multicellular mature form, which in some bacterial species including *P. aeruginosa* is characterized by the presence of differentiated, mushroom- or pillar-like structures or microcolonies interspersed with fluid-filled channels (41) (Fig. 1). The structuring of biofilms in microcolonies with water channels has been shown to be dependent on intercellular small messenger molecules (acylated homoserine lactones, AHLs) that are used for bacterial communication (42), rhamnolipids (43, 44), and regulatory proteins, mostly 2-component regulatory systems (45-47). However in *P. aeruginosa* even cell signaling knockout mutants have been shown to form such channels and so the structure is likely determined by the interplay between intrinsic bacterial regulation as well as the environmental conditions (48). As the biofilms develop three-dimensional structure, resident bacteria near the base will become increasingly separated from the bulk liquid interface and essential sources of energy or nutrients, with biofilm cells experiencing an everchanging micro-environment. Changes are driven by cellular crowding, chemical gradients, and nutrient competition, leading to stratification within the biofilm and the creation of subpopulations (49, 50), with bacteria residing at different locations within the biofilm structure experiencing concentration gradients of nutrient resources, oxygen and waste products (such as acids produced by fermentation in oxygen-depleted zones) as well as extracellular signaling molecules (49-51). This is supported by resident biofilm cells having been shown to express genes linked to oxygen deprivation, general stress and stationary phase conditions, nutrient stress, and slow growth (49-53). Importantly, cells can leave the biofilm structure and return to the planktonic mode of growth by a process referred to as dispersion (54). Dispersion is an active event in which sessile, matrix-encased biofilm cells actively escape from the biofilm, leaving behind eroded biofilms and biofilms with central voids (27, 28, 55, 56). Not surprisingly, dispersion is not only referred to as seeding dispersal (57), but also as a next stage of biofilm formation that is an active event leading to bacterial dissemination and the colonization of new locations.

The above described findings led to an expanded model of biofilm development by *P. aeruginosa* that detailed progression of biofilm formation and stage-specific formation of biofilms (28). While the model represented developmental stages specifically for *P. aeruginosa* biofilms, the model became widely used to represent biofilm formation by diverse biofilm-forming microorganisms in various settings. For example biofilms growing in extreme environments (58) and microalgal biofilms (59).

The developmental model of microbial biofilm formation was adopted quickly by the scientific community to serve as the major conceptual framework for biofilm research on which to base empirical research and scientific inference due to its elegant simplicity. As discussed below, the ability to extrapolate this model to biofilms outside the laboratory - in nature, engineered systems, and medicine - is limited by the diversity and complexity of the structures and processes in real-world system

[H1] Limitations of the biofilm life cycle model

However, there are at least four limitations of the model described above: 1) it is not yet resolved that the formation of a biofilm can be described as a true developmental process outside of the flow cell and *P. aeruginosa* and the growing *in vitro* literature on *Staphylococcus aureus* as model biofilm species; 2) the model does not capture the wide variety of biofilm architectures observed in real world systems such as

microbial mats which can be highly stratified along horizontal layers (60); 3) the model does not incorporate the diversity of aggregation (see below) and detachment mechanisms now recognized in the field by both motile and non-motile organisms such as *Staphylococcus aureus*, although the model has been adapted to accommodate this organism (23); and 4) the model does not consider the succession of events in biofilms formed in open systems with a continuous influx of new colonizers. Even for dental biofilms where it is recognized that biofilm progression proceeds as an ecological succession with new species proliferating in different parts of the biofilm as the microenvironment develops the single species model is commonly depicted (61). Likewise, applying the biofilm model to industrial systems is limited. These systems are so large and complex that it is likely that all stages of growth and detachment are simultaneously occurring at various points in the system. The tidy description of how biofilm forms in a simple laboratory system in rich media does not necessarily capture the complexity of biofilm in most industrial or environmental systems, where surface characteristics - for instance surfaces coated in scale or corrosion, the chemical properties of the bulk fluid and the fluid dynamics all influence how the biofilm attaches, grows, and detaches to impact the system. This also applies to infectious sites, as we do not know whether the site is seeded with single cells or aggregates or whether the bacteria are trapped within host material in a complex environment, rather than just forming aggregates by clonal expansion. Further, there are no *in situ* sensors that can be incorporated into these complex systems that directly monitor biofilm on surfaces, in fluid suspensions or associated with host materials. Sections of the system can be sampled during upgrades or replacement but these only give a snapshot in time at specific locations. While sampling fluids can give clues that biofilms might be present through capturing releasing shed cells or aggregates all that is known is that these originated from somewhere upstream in the system.

The paradigmatic value of this model began to be challenged by the research community as early as 2009 (62), not only questioning the validity of the model, but also the concept of biofilm formation being a developmental process. Based on the definitions by several researchers (63, 64), and reviewed elsewhere (62), development coincides with changes in form and function that are part of the normal life cycle of the cell. This is regulated by a dedicated hierarchical ordered genetic pathway and stage-specific transitions in response to environmental cues. If biofilm formation is indeed a regulated developmental process, the formation of biofilms would require genetic pathways that evolved to facilitate cooperation among members of the biofilm. However, although it is undeniable that a community of cells form a biofilm, with biofilm formation coinciding with changes in an overall surface associated structure over time, and while several regulators affecting biofilm formation had been identified (65-67), no such genetic pathway regulating these morphological changes and stage-specific transitions in a hierarchical ordered manner had been identified (62) at this time. However, in the same year, another group (29) reported a previously uncharacterized signal transduction network regulating committed biofilm developmental steps by *P. aeruginosa* following attachment, in which phospho-relays and response regulators appeared to be key components of the regulatory machinery that coordinates gene expression during *P. aeruginosa* biofilm development in response to environmental cues. More specifically, the signaling network is composed of several two-component regulatory systems (TCS) named SagS, BfiSR, BfmRS, and MifRS (29). Activation of these four TCSs occurred in a sequential manner (SagS<BfiSR<BfmSR<MifSR) over the course of biofilm formation, while inactivation of these systems arrested biofilm formation at distinct developmental stages, with *ΔsagS* and *ΔbfiS* biofilms being arrested at the irreversible attachment stage, while biofilms formed by *ΔbfmR* and *ΔmifR* were found to be arrested at the maturation-1 and -2 stages of biofilm development, respectively (29, 46, 68-70).

While the discovery of the signal transduction network strongly supported the idea that formation of biofilms was a biologically regulated developmental process, at least for *P. aeruginosa* grown under laboratory conditions, other concerns remained, including the validity of the biofilm structure or architecture being composed of mushroom-like microcolonies. In fact, several reports demonstrated that even in *P. aeruginosa,* the biofilm architecture varied with growth conditions as well as the growth medium. For instance, Klausen

et al. (71) demonstrated that while *P. aeruginosa* PAO1 biofilms grown on glucose minimal medium demonstrated the typical mushroom-shaped multicellular biofilm structures, growth in minimal medium containing citrate, casamino acids or benzoate as carbon source led to the formation of flat unstructured biofilms by the same strain (Figure 2). In multispecies biofilms, different medium composition impacts not only biofilm morphology, but also species composition (72). In addition to growth medium and nutrient sources, other variations in growth conditions have been reported to influence the biofilm architecture. While *P. aeruginosa* forming mushroom-shaped biofilms has been associated with growth under relatively low flowing conditions (73), static growth conditions favor the formation of pellicles that form at the air–liquid (74). However, at higher flows, structures such as streamers and ripples can form, demonstrating the remarkable ability of biofilms to adapt to the physical conditions under which they are growing (48, 75).

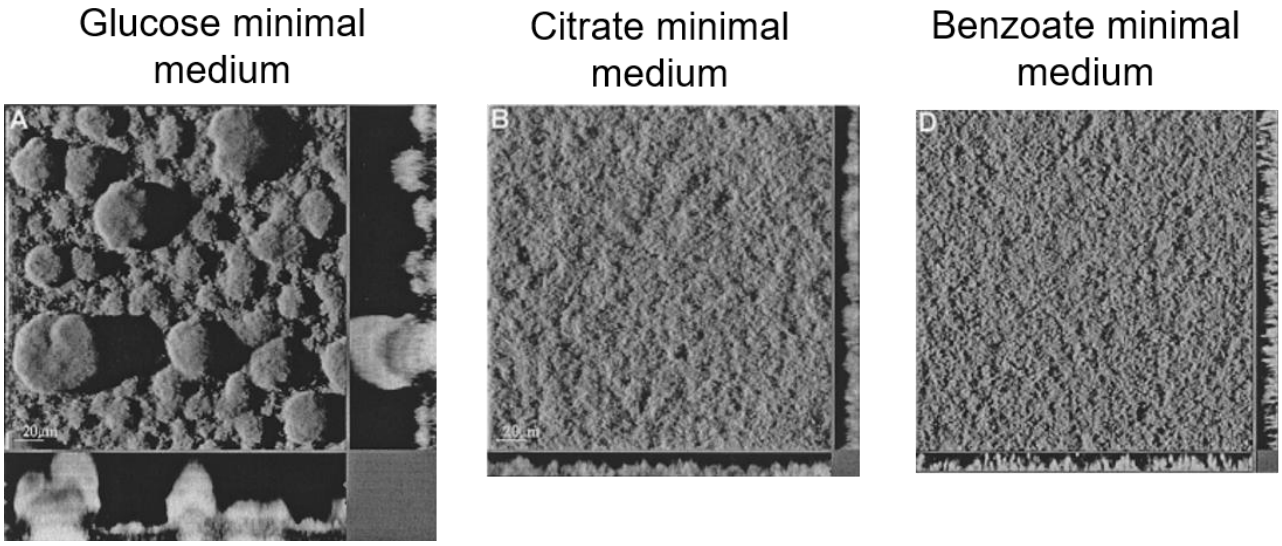

Figure 2, P. aeruginosa grown in flow cells under flow conditions but with different carbon sources shows remarkedly different three-dimensional architecture (Sauer, K 2021)

Additionally, the organisms composing the biofilm also have a marked effect on the biofilm structure (Figure 2) (73). For example, in comparison to pure cultures of laboratory grown biofilms of either *K. pneumoniae* or *P. aeruginosa*, biofilms containing both species were thicker (76, 77). Moreover, in a mixed species biofilm composed of four bacterial soil isolates, removal of one biofilm member completely changed biofilm morphology, species structural organization and relative abundance, even when the species removed was initially low abundant and intrinsically weak in biofilm formation capability (78). Biofilms by Gram-positive bacteria *S. aureus* (79) and *Streptococcus pneumoniae* (80), while having a heterogenous appearance indicative of the presence of water channels, lack the distinct microcolonies that had become an iconic feature of the biofilm architecture. In contrast, studies with pneumococcal biofilms formed under static conditions were used to investigate chronic otitis media with effusion, since fluid/flow is severely disrupted in the middle ear during infection. Biofilm structures were also dependent on bacterial strains but were smaller (5-15µm) recapitulating the appearance of pneumococcal biofilms from *ex vivo* middle ear mucosa samples from children with chronic otitis media (81, 82). Similarly, non-typeable *Haemophilus influenzae* (NTHi), a Gram-negative bacterium, also formed biofilm aggregates in these otitis media samples, which are recapitulated in a chinchilla model of OM (83). NTHi biofilms also formed on differentiated airway epithelial cultures from patients with primary ciliary dyskinesia (PCD)(84). These smaller aggregated structures suggest that biofilms with highly complex architectures are less likely to form in host microenvironments even in hosts with defective immune responses. Importantly, such biofilm aggregates are still able to induce inflammation and tissue destruction that leads to sustained chronic infection because they display tolerance to antibiotic therapy and persist despite host innate immune responses.

In contrast mixed species biofilms taken from the environment are structurally very diverse. As an example, microbial mats are thick and layered, whereas bacterial aggregates on sand grains are thin and small (60). In addition, environmental biofilms often form on biodegradable material, and thus the nutrients are provided not only from 'above', potentially impacting growth zones and structure of the biofilm. Likewise, bacterial communities lacking surface-association altogether, such as microbial flocs found in water treatment facilities or biofilms in the gut, as well as in bacterial aggregates entrapped by polymeric substances are surface independent (85).

[H2] Biofilms in the absence of an attachment surface

As reviewed by several groups (86, 87), many of the chronic bacterial infections linked to biofilms that involve aggregated bacteria and antimicrobial recalcitrance, may not involve hard surface attachment, even if a surface is present. (Figure 3). Likewise, biofilms in the environment are often free-floating, including diverse bacterial aggregates (granules) formed in wastewater treatment plants(88) or those in marine, lake and river habitats, commonly referred to as 'marine-snow'(7).

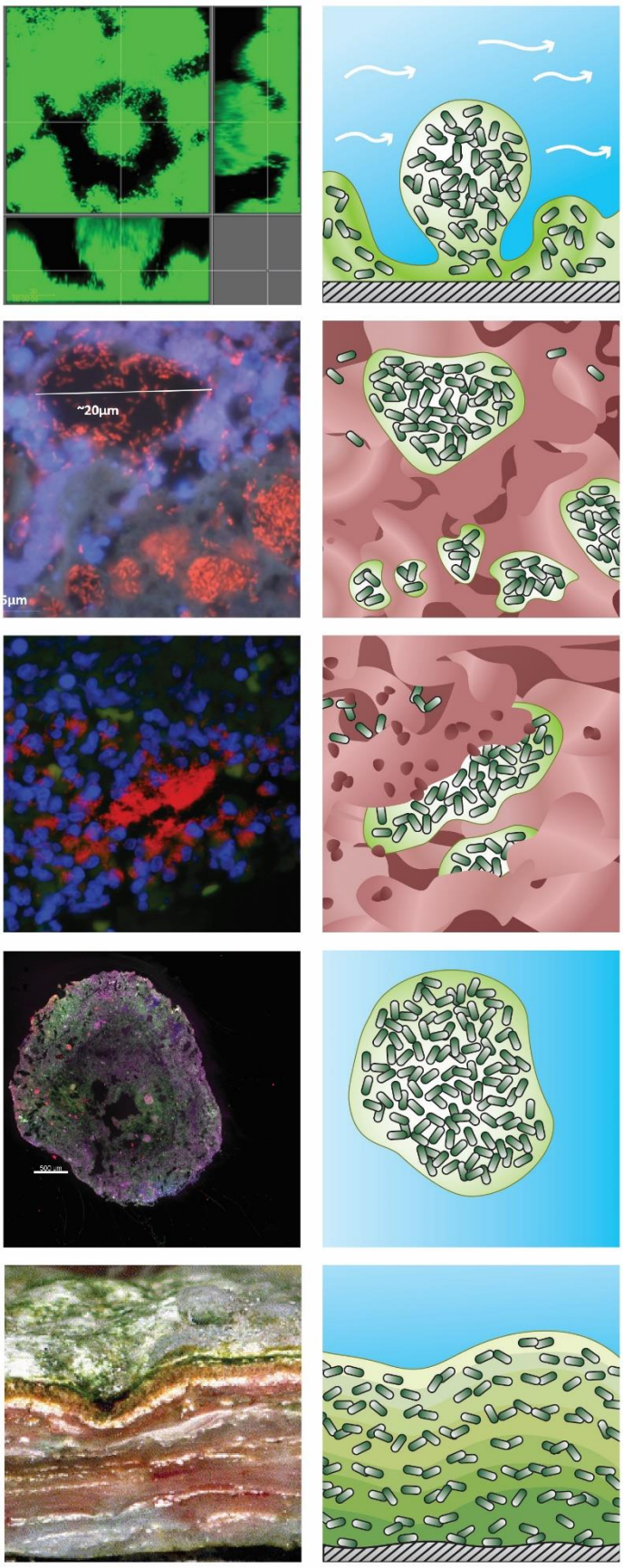

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Figure 3. *Variety of biofilm structures underscores differences between in vitro and in vivo or environmental biofilms. Original images are shown in the left column and a schematic drawing of the structure and its organization in the right column with shading denoting water (blue), aggregated microbial cells (dark green) and their extracellular polymeric substances (light green), host cells and other material including mucus or tissue (red), and attachment surface (hatched grey). A: Mushroom structure of Pseudomonas aeruginosa biofilm in vitro in a flow cell. B: Mucus embedded aggregates of P. aeruginosa surrounded by polymorphonuclear leucocytes in a cystic fibrosis lung (89) C: Wound-embedded aggregates of P. aeruginosa surrounded by polymorphonuclear leucocytes(90). D: Aerobic granules from a full-scale AquaNereda® wastewater treatment process (image courtesy of Kylie Bodle and Cat Kirkland). E: Striated microbial mat from a Brazilian lake(91). (Jill Story assisted with figure preparation)*

Two "classic" chronic infections linked to aggregated rather than surface-associated bacteria include the infection of soft tissues such as the chronic lung infection of people with cystic fibrosis (CF) (89) and chronic dermal wounds (92). Similarly, in other biofilm associated respiratory infections, such as chronic otitis media, rhinosinusitis, or biofilms on differentiated ciliated cells from people with Primary ciliary dyskinesia (PCD), aggregates (~10-20 μm) may adhere to mucosal epithelia or grow as aggregates in effusion, mucus, and airway surface liquid. The bacterial aggregates seen in these infections are not necessarily modeled well by flow biofilm experimental systems, although shear is present in the airways (93).

Osteomyelitis with and without an implant also belongs to this category. In the case of osteomyelitis with implants, it is generally assumed that the bacteria are attached to the implant surface, and so can be described by the current biofilm model. This has led to much research into designing antibacterial and anti-adhesive surfaces. However current studies show that even though the bacteria can be associated with the surface, the implant does not have to be colonized to cause a persisting infection (94, 95). Samples from implant-associated infections show that bacteria can be present both in peri-implant tissue and on the implant, but not necessarily both places (96). Importantly, detached aggregates recapitulated the antibiotic tolerance observed in biofilms that were surface attached(97).

Aggregates have also been reported for non-infectious biofilms. Consider the microbiota in the oral cavity, such as on the teeth or on the skin where the majority of bacteria are organized as small aggregates (98, 99). On the teeth, the bacteria attach to the enamel surface, however not in three-dimensional mushroom structures (100). Similarly, on the skin bacteria are scattered in small heterogenous distributed aggregates and as single cells.

In addition to clinical and infectious biofilms, bacteria in the environment are present both as surface attached colonies as well as free floating or embedded aggregates. In biological wastewater treatment processes, dense multispecies aggregates of microorganisms self-assemble in both aerobic and anaerobic processes. The overarching observation is that the environmental microbiota is dominated by heterogeneous patterns of aggregated bacteria (7) rather than continuous films of bacteria over large (centimeter) areas, however to a certain extent this is a scalar issue. Algal biofilms on ship hulls may appear macroscopically continuous and in localized areas as a uniform flat layer but can also appear patchy (101).

[H1] Aggregate formation

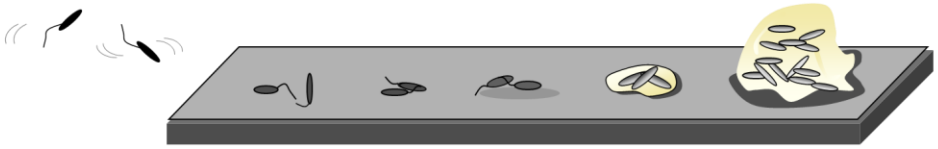
As outlined above a shortcoming of the current biofilm model is that it does not account for non-surface-attached aggregates that are often observed in clinical or environmental settings. While aggregates had been observed when the model was first published in 2002, little was known about aggregates at the time. Since

then, several publications have reported on bacterial aggregation independent of surfaces, with bacteria in aggregates displaying similar phenotypes as bacteria present in surface-attached communities, such as increased antibiotic and host defense tolerance as well as matrix production and slow growth (102, 103).

Examples of different types of aggregates include cells embedded in host material such as mucus in CF lungs, slough in the chronic wound bed or external material flocs in wastewater treatment and soil. Host fluids including synovial fluid and human serum can induce rapid (within minutes) aggregation in both Gram positive and negative bacteria *in vivo* (103-105), suggesting that host components such as fibronectin are forming bridging connections. Such planktonic aggregates have also been seen *ex vivo* (106), and in shaken *in vitro* cultures (107-109). Bacteria in a shaken, liquid culture have until recently been assumed to be entirely planktonic single cells (or short chains or clusters) independent of each other. However, recent publications challenge the conceptual separation between planktonic and biofilm bacteria by showing that *S. aureus* and *P. aeruginosa* grow as a mixture of planktonic and aggregates in liquid batch cultures (108-110).

Based on several laboratory studies, literature currently points to five mechanisms for the formation of free-floating aggregates (Figure 4) which are discussed in the order they have been recognized. The first is the detachment of pieces of attached biofilm due to changes in hydrodynamic shear, nutrient reduction, physical abrasion or exogenously added or endogenously produced dispersal agents (111). The loss of biofilm bacterial cells due to this process has often been referred to as sloughing. The second is through growth in the planktonic phase (110). As cells divide, the daughter cells remain with the mother cells rather than dispersing, presumably through interactions of self-recognizing surface adhesion molecules or simultaneous production of EPS. The presence of surface adhesins may also contribute to the co-aggregation of cells in the planktonic state, leading to the formation of aggregates in the absence of growth. More recently, it has been proposed that aggregation can occur in the liquid phase mediated by host polymers such as mucin and DNA (112). One potential mechanism is depletion aggregation, which occurs as entropic forces between uncharged or like-charged polymers forces particles (single bacterial cells in the case of our discussion) in the suspension to “push out” polymers between the cells as they come close together forcing the formation of aggregates (113). Another possible aggregation mechanism is that bacteria bind to molecules in host fluids through surface adhesion interactions. For example, staphylococci have been shown to aggregate in synovial fluid, which has been a proposed mechanism for initiating periprosthetic joint infection (103). This aggregation is a binding interaction between bacterial factors such as adhesin proteins and host factors such as fibrinogen, fibronectin, and hyaluronic acid (114, 115). Notably, aggregate formation in liquid or in response to host polymers includes both co-aggregation without bacterial growth as well as clonal growth of trapped bacteria, coinciding with continued increasing aggregate size. Moreover, very little is known about how cells disembark aggregates, including whether aggregates disassemble by dispersion or sloughing, or simply revert into single cells.

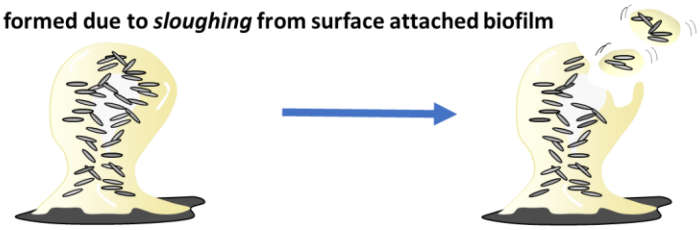
SURFACE-ASSOCIATED BIOFILM FORMATION, based on 2002 biofilm model (attachment followed by clonal growth and EPS production)



BIOFILM-LIKE AGGREGATE FORMATION IN THE LIQUID PHASE

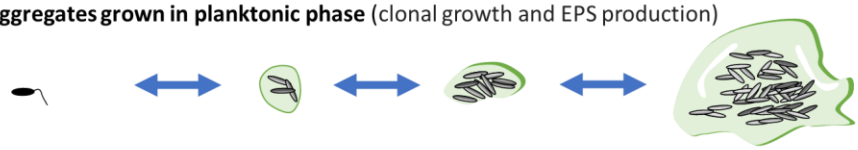
BIOFILM-LIKE AGGREGATES ORIGINATING FROM THE SURFACE

1. Aggregates formed due to *sloughing* from surface attached biofilm

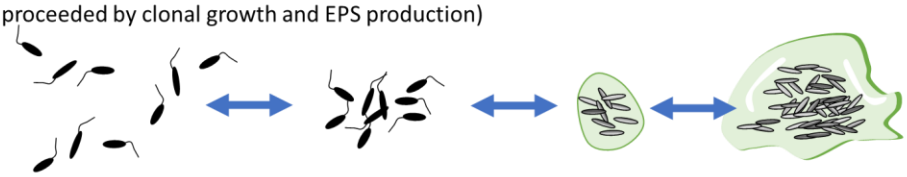


BIOFILM-LIKE AGGREGATES ORIGINATING FROM IN THE LIQUID

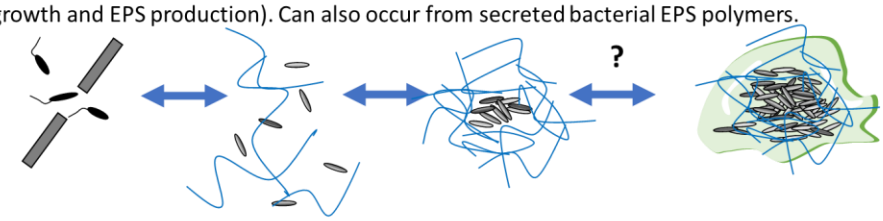
2. Aggregates grown in planktonic phase (clonal growth and EPS production)



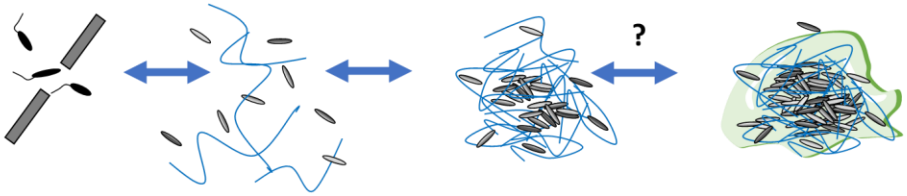
3. Aggregate formation initiated through cell surface components of single cells - *autoaggregation* for same species or ***coaggregation*** for multiple species - possibly proceeded by clonal growth and EPS production)



4. Aggregate formation initiated through host or environmental fluid polymers - *Polymer dispersion* - phase separation between cells and polymers, possibly proceeded by clonal growth and EPS production). Can also occur from secreted bacterial EPS polymers.



5. Aggregate formation initiated through host or environmental fluid polymers - *Polymer bridging* - cells and polymers are integrated, possibly proceeded by clonal growth and further EPS production).



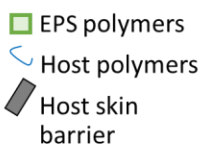


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Figure 4. Microbial aggregate formation mechanisms. The top panel shows the "standard" model for biofilm formation prceeding from the attachment of single planktonic cells to a smooth surface followed by cell division and production of EPS to form 3D surface attached aggregate structures. Below are different mechanisms for generating free floating biofilm-like aggregates. The first is detachment of aggregates from attached biofilms. The second is from clonal growth (division) in the liquid which can be facilitated with or without and EPS matrix. The third is aggregation of individual cells in a process called autoaggregation for a single species or coaggregation for multiple species, in which bacteria attach to each other through mutual attraction of surface molecules such as adhesins or EPS bridging interactions. Bridging aggregation can also be mediated by host polymers such as appears to be the case in synovial fluid (116). Another mechanism of aggregation is "polymer depletion aggregation" when bacteria are in the prescence of non-absorbing polymers (117) due to entropic ordering of the colloidal system. Depeletion aggregation can be through bacterially produced EPS or host derived polymers (112).

[H1] Expanding the biofilm model

Visualization of biofilms and bacterial aggregates in other *in vitro* experimental systems, in the environment and in infections, reveal major disparities with the original model (figure 1). A major difference is the microenvironment of the individual biofilms and aggregates and the access to substrates and oxygen as well as exposure to secreted products. This varies depending on whether bacteria are directly adjacent to the growth medium or entrapped in some sort of biological (mucus, tissue, wound bed within infections) or non-biological (as within corrosion or hard water deposits) material, not to be confused with a self-produced biofilm matrix. The microenvironment plays a dominant role in determining the metabolism and behavior of the bacteria, including such characteristics as antibiotic tolerance, growth rate, and expression of virulence factors (12, 118-120).

For these reasons, we have constructed an updated, more encompassing model describing different aggregation and interaction fates that bacteria can follow in different macro- and microenvironments (Figure 5).

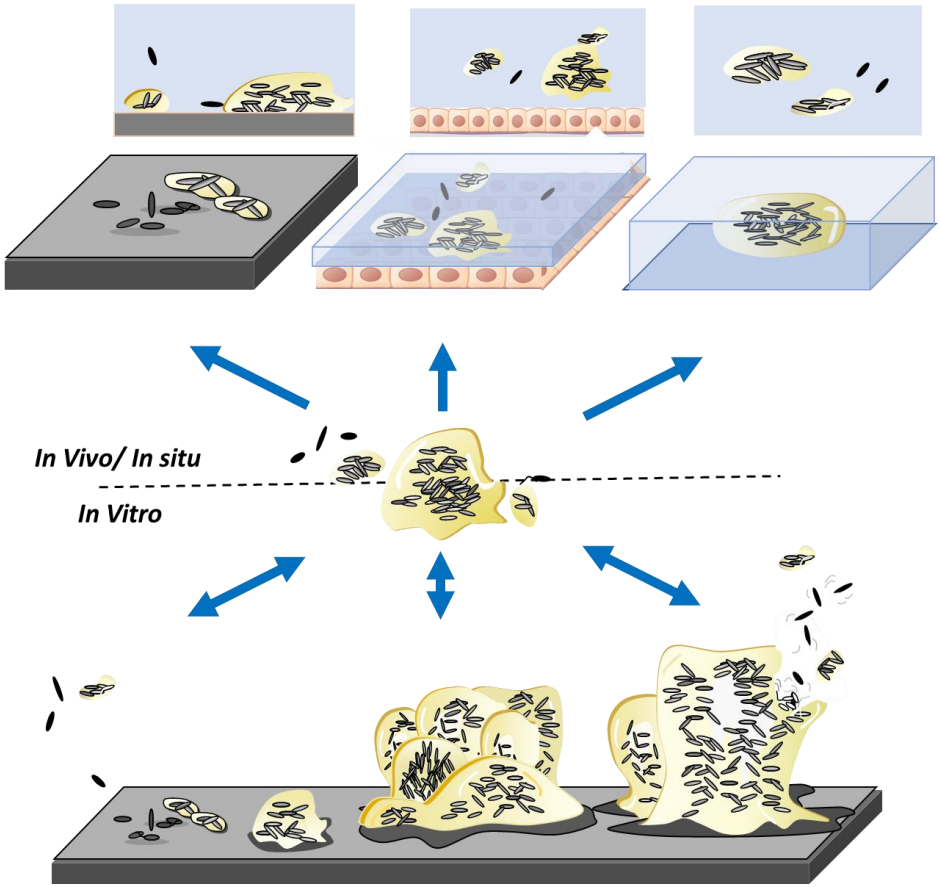

Figure 5, Expanded model showing different biofilm scenarios in vitro, in situ and in vivo, where one pathway does not exclude the other. Middle: Bacteria can exist as both single cells and biofilm depending on the growth and microenvironment. Top: In vivo/in situ, bacteria can be present as both single cells and in aggregates, and depending on the focus, the bacteria can be either suspended in fluids (urine, synovial fluids, blood, marine and freshwater systems, wastewater treatment plants), present within a matrix of host material (chronic wounds and mucus in the CF lung) or attached to biotic or abiotic surfaces such as tissue (native heart valves, gut epithelium, middle ear mucosal epithelium), implants, calcifications within the body, plant roots and leaves, soil particles, submerged surfaces. Bottom: In vitro bacteria can be present as single cells and aggregates both in liquid cultures and attached to surfaces. On surfaces under flowing conditions, some bacterial species may also form the famous mushroom shaped structures.

While the new model lacks the simplicity of the previous model, it bridges and combines the different possibilities and pathways of biofilm aggregate development in an inclusive model. We acknowledge that this is a work in progress, based on what we know to date. Thus, the model is not final, but will likely be revised in the years to come.

With this model we embrace the microenvironment that governs the developmental processes by which bacteria behave and organize themselves. In contrast to the initial model, the present model considers open systems that may be encountered in the environment or the human gut, where a continuous influx of new biofilm members must be considered. Importantly, there are no known correlations suggesting a particular biofilm structure is either “better” or “worse” in any given situation. *Ex vivo* and *ex situ* observations suggest mushroom structures and surface-attached three-dimensional structures are just as real as the aggregates observed in chronic infections and the natural environment with and without surface association. What is

important is that they are diverse microbial communities, shaped and influenced by different environmental cues, that represent different microenvironments.

The original model is largely derived based on data from *in vitro* flow cells experiments, however snapshots of biofilms from environmental systems and from *in vivo* and *ex vivo* studies suggest this development is not always supported, which led to questioning of the original model. From *in vitro* investigations we know that flow and nutrients are important in the experimental systems to shape the three-dimensional architecture of the surface attached biofilms (19, 121). The question is how much do we really know about the microenvironment and biofilm development in the environmental and *in vivo* and *ex vivo* examples? Photosynthetic mats are well described relatively flat biofilms where the penetration of sunlight and metabolic activity of the organisms leads to stratified species distribution and micoenvironments (122). Suspended biofilm aggregates used for wastewater treatment such as aerobic granules are another example of a stratified biofilm. In this case the aggregates are generally spherical. While direct measurements of the microenvironment is difficult because they are free floating, stratification showing aerobes on the outside and anaerobes on the inside provide evidence of oxic and anoxic zones (123). These microenvironments allow simultaneous aerobic digestion and anaerobic denitrification of wastewater, as well as in industrial systems patchy aggregates of bacteria and corrosion products in tubercules (124). In iron and steel industrial pipes biofilms can cause microbially induced corrosion due to the development of microenvironments (125). These biofilms tend to be present as mound shaped aggregates on metal surfaces and consist of bacteria and corrosion products. Stratification of organisms such as iron-oxidizing and sulfur-reducing bacteria create anoxic zones within the tubercule, which become anodic relative to the surrounding metal causing pitting corrosion below the tubercule and rust deposition at the surface. These examples illustrate how the interplay between the original external environmental conditions and physiology of biofilm microorganisms lead to the creation of different biofilm structures and microenvironments *in situ*. Mechanical forces can also shape biofilm architecture, microbial community, and microenvironment development. Samples from river biofilms growing under higher turbulence were thinner, more compact and formed more homogenous layers than those growing under lower hydrodynamic shear (126). In a medical context in the CF lung bacteria can be present and form aggregates independent of the epithelial surface (127). Thus, the expanded model includes a variety of conditions and biofilm developmental pathways to embrace multiple diverse habitats and microenvironments from the environment, industry and in medicine. What we do know is that the microenvironment depends on the immediate milieux surrounding a single cell, next the aggregate itself and finally the close proximity of the aggregate(128).

The different pathways and structures are most likely governed by the microenvironment, for example flow/shear conditions, nutrient availability, and the genetic traits and phenotypic capabilities of the bacterial species(121, 129, 130). The surface can be a niche for attachment and growth or can trap aggregates from suspension, however bacteria can auto-aggregate without a surface suggesting that surface attachment also depends on the bacterial phenotypic capabilities as shown in the present conceptual model. Moreover, the current model indicates that aggregation, developmental processes, attachment, structure, and tolerance towards antibiotics and host defenses are reversible, depending on the microenvironmental conditions(10). As for the hallmark mushroom-shaped structures of the original developmental model, these appear to be dependent on the flow conditions, surface attachment, and carbon source of principally *P. aeruginosa*, where the mushroom structure forms during flow conditions on a surface with glucose as the carbon source(20). For most other species, even under flow conditions and in the presence of glucose, mushroom structures do not form. In the environment outside of stromatolites and some hot spring structures, mushroom structures appear to be uncommon. Thus, in some ways dissecting the *P. aeruginosa* mushroom model by designing

experimental conditions to study to show how mushrooms formed led to its over-generalization to all biofilms, including *Pseudomonas* species.

Conclusion

The most cited and used model (Figure 1) for biofilm development is extremely intuitive, which explains in part why it has become the preferred model to describe all kinds of biofilm formation. However, as we discussed in this Review, and present in the expanded model, one single model does not fit all. In the expanded model the possibility of aggregation is presented, and one pathway does not exclude another. Biofilms do not necessarily form a mushroom shaped structure as the final culminating structure, nor is there an absolute dependence on a surface. Currently no developmental model accurately depicts biofilm formation of all microorganisms, habitats, and all microenvironments. Biofilms are communities of bacterial cells, in the absence or presence of a surface, that respond to their environmental growth conditions in different ways, evidenced by the observations that biofilms formed by the same species but in different environments exhibit different gene expression profiles and display different behaviors. Specifically, this was shown by differences in gene expression of *P. aeruginosa* relating to antibiotic resistance, metabolism, transportation, and extracellular secreted molecules between different microenvironments, *in vitro* vs. *in vivo,* shaken culture vs. surface biofilms, rich vs. minimal growth media etc. (131).

Growing evidence indicates that biofilms do not require an attachment surface to form. Aggregates formed in fluids, due to clonal growth, co-aggregation, or aggregates induced by bacterial EPS or host fluids, demonstrate many of the characteristics previously attributed only to surface-associated biofilms. These aggregates are not limited to laboratory conditions but may be found as part of the human microbiota, and in several chronic infection sites (85, 132-135). Two decades of biofilm research indicates that the model depicted in Figure 1 was incomplete because it did not capture multiple biofilm phenotypes that can form with different bacteria and in different microenvironments. This has implications for how we study biofilms specifically and bacteria in general, as different biofilm experimental systems *in vitro* or experimental animals *in vivo* cannot encompass all the factors important for different microenvironments (15). We propose that it is imperative that the research question drives the study and interpretation of the results, not the experimental system used to do the research. This is also important for how we extrapolate from the experimental situation to the native scenario. We need to understand biofilms in the context of the relevant microenvironment.

Given that aggregates are now accepted as sharing similarities to surface-associated biofilms, several questions remain to be addressed. For example, it is not known what drives aggregate formation in the absence of a surface — that is, does bacteria-bacteria attachment involve the same mechanisms as attachment of single cells to surfaces? Also do aggregates interact with surfaces and can aggregates attach to surface biofilms, and if so how? Are the same surface properties commonly associated with initial cell-surface adhesion (stiffness and surface energy, which in turn is a function of electrostatic charge, wettability, surface tension and roughness) as important for attachment as macroscale topographical features such as edges, screw holes, expansions and contractions, threads, etc, which may physically entrap aggregates.

It is well established that *in vitro* biofilms actively disperse but do aggregates actively disassemble and/or disperse cells to the surroundings? These questions could be investigated by analyzing gene-expression profiles during the different stages of biofilm development in the absence and presence of a surface. How do

the transcriptional profiles of bacteria in aggregates that have developed though chemical/physical interaction or growth differ from each other and from biofilms formed on surfaces? Furthermore, are successional dynamics and community assembly processes similar for aggregates and surface-associated biofilms?

Finally, does aggregation protect bacteria from antimicrobials, long considered a hallmark phenotype of biofilm formation for medically relevant species? We know that it is not the aggregation alone that promotes tolerance towards antimicrobial agents and host defenses, but gradients of oxygen and nutrients that become pronounced in aggregates as they increasing in size (136). The aggregate size also seems to determine how easy phagocytes engulf the aggregates. In flow cells and as depicted in the original 5 step model, this results in stratified growth with a fast growing exterior and a dormant inner subpopulation(137). In infections it is often host material surrounding microbial aggregates which causes gradients, thus the original 5 step model does not accurately represent the microenvironment around these aggregates, and likely also fails to capture the reality of biofilms in complex environmental and industrial systems. Concentration gradients influence and regulate bacterial physiology and metabolism, which is reciprocally controlled by the microenvironment as well as by matrix components. However, is there a threshold aggregate size for tolerance to manifest? It probably depends on the microenvironment and access to nutrients and electron acceptors. The sizes of biofilms have been shown to vary much between *in vitro* and *in vivo* biofilms(85). The questions of tolerance and matrix production and physiology in general need to be addressed by controlling the microenvironment possibly in three dimensional experimental models to move beyond the attachment surface as the main constraint controlling immediate access to nutrients and electron acceptors.

The current biofilm model becomes especially problematic when used to describe clinical manifestations and devise new *in vitro* test methods that evaluate medical implants, drugs and treatments, as these fail due to lack of extrapolation. Crucially, differences in the microenvironment likely underpin why direct extrapolation is not possible (138). Additionally, relying on the current biofilm model (Figure 1) healthcare professionals may have a conceptual framework that markedly differs from clinical findings and observations, leading to the conclusion that a biofilm is not present in a given clinical sample and thus in treatment regimens that will not effectively treat infections (139).

In summary, we suggest an expanded developmental model for biofilm formation that does take the most inclusive recent insights into account but does not necessarily depict the formation and maturation process of all biofilms in the various contexts; instead it demonstrates the possible fates of microbes as single cells or aggregates in different conditions and habitats. Our intent is that this unified model will alleviate some of the misconceptions of how biofilms form ranging from industrial systems, to environmental habitats and medical settings. We hope that as a scientific community, we can expand on this model to facilitate an inclusive, less controversial interdisciplinary discussion on biofilms and biofilm formation.

New proposed text box 1: Before the biofilm model

Biofilm research in the early years primarily focused on engineering applications and observational descriptions of biofilms. However, biofilm research changed with the observation of surface-attachment specific gene regulation *in vitro* and the introduction of *in vitro* systems to study biofilm formation and phenotypes in the laboratory. This facilitated the study of specific and differential gene expression upon surface attachment *in vitro* (36, 37, 140) including the role of cell signaling as genetic regulation from a population (42) as well as the use of genetic tools to identify genes required for *in vitro* surface and subsequent biofilm formation (141-143).

The idea that biofilms are amenable to molecular genetic studies (141, 142) also opened the door to the exploration of factors beyond early surface attachment, to those contributing to biofilm architecture, metabolic interactions, phylogenetic groupings, and competition and cooperation. Molecular genetic applications furthermore led to exciting progress in the development of new technologies for studying biofilm communities, advanced our understanding of the ecological significance of surface-attached bacteria, and provided new insights into the molecular genetic basis of biofilm development (32).

What followed was extensive research on genes that are required for bacteria to associate with surfaces, and investigations of differences in the transcriptional abundance of bacterial genes when growing planktonically and as biofilms. While some studies failed to detect differences in the transcriptomes of planktonic and surface associated cells (144), the majority of studies confirmed planktonic and sessile biofilm cells to display distinct transcriptomic profiles, with the number of genes changing in transcript abundance upon surface-associated growth ranging from less than twenty to several hundred (145, 146). Moreover, transcriptome analyses of *in vitro* grown biofilms suggested that biofilm cells experience various stresses including hypoxia or oxygen deprivation, nutrient stress, and slow growth, which increase as the biofilm grows in size, but also coincide with the presence of chemical gradients and the formation of subpopulations within the biofilm structure (147, 148). Additionally, changes in cell-to-cell signaling, virulence gene expression and the biosynthesis of matrix components have been reported (149, 150). Notably, many of these findings have been confirmed using *in vivo* (animal models) grown biofilms, although not in human infections (151, 152).

578 **References**

579 1. Costerton JW, Geesey GG, Cheng KJ. How bacteria stick. Sci Am. 1978;238(1):86-95.
580 2. McCoy WF, Bryers JD, Robbins J, Costerton JW. Observations of fouling biofilm formation.
581 Can J Microbiol. 1981;27(9):910-7.
582 3. Hoiby N, Bjarnsholt T, Moser C, Bassi GL, Coenye T, Donelli G, et al. ESCMID guideline for the
583 diagnosis and treatment of biofilm infections 2014. Clin Microbiol Infect. 2015;21 Suppl 1:S1-25.
584 4. Kolpen M, Kragh KN, Enciso JB, Faurholt-Jepsen D, Lindegaard B, Egelund GB, et al. Bacterial
585 biofilms predominate in both acute and chronic human lung infections. Thorax. 2022.
586 5. Raghupathi PK, Liu W, Sabbe K, Houf K, Burmølle M, Sørensen SJ. Synergistic Interactions
587 within a Multispecies Biofilm Enhance Individual Species Protection against Grazing by a Pelagic Protozoan.
588 Frontiers in Microbiology. 2018;8(2649).
589 6. Jass J, Roberts SK, Lappin-Scott HM. Microbes and enzymes in biofilms. Enzymes in the
590 Environment Activity, Ecology and Applications Marcel Dekker Inc, New York, USA. 2002:307-26.
591 7. Flemming HC, Wuertz S. Bacteria and archaea on Earth and their abundance in biofilms. Nat
592 Rev Microbiol. 2019;17(4):247-60.
593 8. Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, et al. Bacterial biofilms
594 in nature and disease. Annu Rev Microbiol. 1987;41:435-64.
595 9. Vishwakarma V. Impact of environmental biofilms: Industrial components and its
596 remediation. Journal of Basic Microbiology. 2020;60(3):198-206.
597 10. Alhede M, Kragh KN, Qvortrup K, Allesen-Holm M, van Gennip M, Christensen LD, et al.
598 Phenotypes of non-attached Pseudomonas aeruginosa aggregates resemble surface attached biofilm. PLoS
599 One. 2011;6(11):e27943.
600 11. Bjarnsholt T, Whiteley M, Rumbaugh K, Stewart PS, Jensen PO, Frimodt-Moller N. The
601 importance of understanding the infectious microenvironment. lancet Infect Dis. 2021;In Press.
602 12. Cornforth DM, Diggle FL, Melvin JA, Bomberger JM, Whiteley M. Quantitative Framework for
603 Model Evaluation in Microbiology Research Using Pseudomonas aeruginosa and Cystic Fibrosis Infection as
604 a Test Case. mBio. 2020;11(1).
605 13. Marrie TJ, Nelligan J, Costerton JW. A scanning and transmission electron microscopic study
606 of an infected endocardial pacemaker lead. Circulation. 1982;66(6):1339-41.
607 14. Zobell CE. The Effect of Solid Surfaces upon Bacterial Activity. J Bacteriol. 1943;46(1):39-56.
608 15. Thaarup IC, Bjarnsholt T. Current In Vitro Biofilm-Infected Chronic Wound Models for
609 Developing New Treatment Possibilities. Adv Wound Care (New Rochelle). 2021;10(2):91-102.
610 16. Sternberg C, Bjarnsholt T, Shirtliff M. Methods for dynamic investigations of surface-attached
611 in vitro bacterial and fungal biofilms. Methods Mol Biol. 2014;1147:3-22.
612 17. Azeredo J, Azevedo NF, Briandet R, Cerca N, Coenye T, Costa AR, et al. Critical review on
613 biofilm methods. Crit Rev Microbiol. 2017;43(3):313-51.
614 18. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment
615 to infectious diseases. Nat Rev Microbiol. 2004;2(2):95-108.
616 19. Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. Pseudomonas aeruginosa displays
617 multiple phenotypes during development as a biofilm. J Bacteriol. 2002;184(4):1140-54.
618 20. Klausen M, Heydorn A, Ragas P, Lambertsen L, aes-Jorgensen A, Molin S, et al. Biofilm
619 formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. Mol Microbiol.
620 2003;48(6):1511-24.
621 21. Pamp SJ, Sternberg C, Tolker-Nielsen T. Insight into the microbial multicellular lifestyle via
622 flow-cell technology and confocal microscopy. Cytometry A. 2009;75(2):90-103.
623 22. Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as complex differentiated
624 communities. Annu Rev Microbiol. 2002;56:187-209.
625 23. Moormeier DE, Bayles KW. Staphylococcus aureus biofilm: a complex developmental
626 organism. Mol Microbiol. 2017;104(3):365-76.

24. Vlamakis H, Chai Y, Beauregard P, Losick R, Kolter R. Sticking together: building a biofilm the Bacillus subtilis way. Nature Reviews Microbiology. 2013;11(3):157-68.
25. Hu Y, Xiao Y, Liao K, Leng Y, Lu Q. Development of microalgal biofilm for wastewater remediation: from mechanism to practical application. Journal of Chemical Technology & Biotechnology. 2021;96(11):2993-3008.
26. O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annual Reviews in Microbiology 2000;54:49-79.
27. Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. Journal of Bacteriology. 2002;184(4):1140-54.
28. Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as complex differentiated communities. Annual Reviews in Microbiology. 2002;56(1):187-209.
29. Petrova OE, Sauer K. A novel signaling network essential for regulating *Pseudomonas aeruginosa* biofilm development. PLoS Pathogens. 2009;5(11):e1000668.
30. Petrova OE, Gupta K, Liao J, Goodwine JS, Sauer K. Divide and conquer: the *Pseudomonas aeruginosa* two-component hybrid SagS enables biofilm formation and recalcitrance of biofilm cells to antimicrobial agents via distinct regulatory circuits. Environmental Microbiology. 2017;19(5):2005-24.
31. O'Toole GA, Kolter R. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Molecular Microbiology. 1998;28(3):449-61.
32. Davey ME, O'Toole G A. Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev. 2000;64(4):847-67.
33. Characklis WG. Attached microbial growths-II. Frictional resistance due to microbial slimes. Water Research. 1973;7(9):1249-58.
34. Petrova OE, Sauer K. A novel signaling network essential for regulating Pseudomonas aeruginosa biofilm development. PLoS Pathog. 2009;5(11):e1000668.
35. Gupta K, Marques CNH, Petrova OE, Sauer K. Antimicrobial tolerance of *Pseudomonas aeruginosa* biofilms is activated during an early developmental stage and requires the two-component hybrid SagS. Journal of Bacteriology. 2013;195(21):4975-87
36. Davies DG, Charabarty AM, Geesey GG. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by Pseudomonas aeruginosa. Appl Environ Microbiol. 1993;59:1181 - 6.
37. Davies DG, Geesey GG. Regulation of the alginate biosynthesis gene *algC* in *Pseudomonas aeruginosa* during biofilm development in continuous culture. Appl Environ Microbiol. 1995;61(3):860-7.
38. Colvin KM, Irie Y, Tart CS, Urbano R, Whitney JC, Ryder C, et al. The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. Environmental Microbiology. 2012;14(8):1913-28.
39. Bagge N, Hentzer M, Andersen JB, Ciofu O, Givskov M, Hoiby N. Dynamics and spatial distribution of β-lactamase expression in *Pseudomonas aeruginosa* biofilms. Antimicrob Agents Chemother. 2004;48(4):1168-74.
40. Wood DW, Gong F, Daykin MM, Williams P, Pierson LS. N-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. Journal of Bacteriology. 1997;179(24):7663-70.
41. Wood SR, Kirkham J, Marsh PD, Shore RC, Nattress B, Robinson C. Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy. J Dent Res. 2000;79(1):21-7.
42. Davies D. G. PMR, Pearson J. P., Iglewski B. H., Costerton J. W., Greenberg E. P. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science. 1998;280(5361):295-8.
43. Lequette Y, Greenberg EP. Timing and localization of rhamnolipid synthesis gene expression in Pseudomonas aeruginosa biofilms. J Bacteriol. 2005;187(1):37-44.
44. Espinosa-Urgel M. Resident parking only: rhamnolipids maintain fluid channels in biofilms. J Bacteriol. 2003;185(3):699-700.

45. Kuchma SL, Connolly JP, O'Toole GA. A three-component regulatory system regulates biofilm maturation and type III secretion in *Pseudomonas aeruginosa*. Journal of Bacteriology. 2005;187(4):1441-54.
46. Petrova OE, Schurr JR, Schurr MJ, Sauer K. Microcolony formation by the opportunistic pathogen *Pseudomonas aeruginosa* requires pyruvate and pyruvate fermentation. Molecular Microbiology. 2012;86:819–35.
47. Sriramulu DD, Lünsdorf H, Lam JS, Römling U. Microcolony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. J Med Microbiol. 2005;54(7):667-76.
48. Purevdorj B, Costerton JW, Stoodley P. Influence of hydrodynamics and cell signaling on the structure and behavior of Pseudomonas aeruginosa biofilms. Appl Environ Microbiol. 2002;68(9):4457-64.
49. Stewart PS, Franklin MJ. Physiological heterogeneity in biofilms. Nature Reviews Microbiology. 2008;6(3):199.
50. Serra DO, Hengge R. Stress responses go three dimensional–the spatial order of physiological differentiation in bacterial macrocolony biofilms. Environmental Microbiology. 2014;16(6):1455-71.
51. Williamson KS, Richards LA, Perez-Osorio AC, Pitts B, McInnerney K, Stewart PS, et al. Heterogeneity in *Pseudomonas aeruginosa* biofilms includes expression of ribosome hibernation factors in the antibiotic-tolerant subpopulation and hypoxia-induced stress response in the metabolically active population. Journal of Bacteriology. 2012;194(8):2062-73.
52. Heacock-Kang Y, Sun Z, Zarzycki-Siek J, McMillan IA, Norris MH, Bluhm AP, et al. Spatial transcriptomes within the *Pseudomonas aeruginosa* biofilm architecture. Molecular Microbiology. 2017;106(6):976-85.
53. Haussler S, Fuqua C. Biofilms 2012: new discoveries and significant wrinkles in a dynamic field. Journal of Bacteriology. 2013;195(13):2947-58.
54. Rumbaugh KP, Sauer K. Biofilm dispersion. Nat Rev Microbiol. 2020;18(10):571-86.
55. Petrova OE, Sauer K. Escaping the biofilm in more than one way: desorption, detachment or dispersion. Current Opinion in Microbiology. 2016;30:67-78.
56. Davies DG. Biofilm Dispersion. *In* Biofilm Highlights; Springer: Berlin. 2011:1-28.
57. Purevdorj-Gage B, Costerton WJ, Stoodley P. Phenotypic differentiation and seeding dispersal in non-mucoid and mucoid Pseudomonas aeruginosa biofilms. Microbiology. 2005;151(5):1569-76.
58. Yin W, Wang Y, Liu L, He J. Biofilms: The Microbial “Protective Clothing” in Extreme Environments. International Journal of Molecular Sciences. 2019;20(14):3423.
59. Mantzorou A, Ververidis F. Microalgal biofilms: A further step over current microalgal cultivation techniques. Science of The Total Environment. 2019;651:3187-201.
60. Prieto-Barajas CM, Valencia-Cantero E, Santoyo G. Microbial mat ecosystems: Structure types, functional diversity, and biotechnological application. Electronic Journal of Biotechnology. 2018;31:48-56.
61. Hao Y, Huang X, Zhou X, Li M, Ren B, Peng X, et al. Influence of Dental Prosthesis and Restorative Materials Interface on Oral Biofilms. International Journal of Molecular Sciences. 2018;19(10):3157.
62. Monds RD, O’Toole GA. The developmental model of microbial biofilms: ten years of a paradigm up for review. Trends Microbiol. 2009;17(2):73-87.
63. Dworkin M. Developmental biology of the bacteria: Benjamin/Cummings Pub. Co; 1985.
64. Brun YV, Shimkets LJ. Prokaryotic development: Asm Press Washington, DC; 2000.
65. Goodman AL, Kulasekara B, Rietsch A, Boyd D, Smith RS, Lory S. A Signaling Network Reciprocally Regulates Genes Associated with Acute Infection and Chronic Persistence in Pseudomonas aeruginosa. Dev Cell. 2004;7(5):745-54.
66. Merritt JH, Brothers KM, Kuchma SL, O'Toole GA. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. J Bacteriol. 2007;189(22):8154-64.

67. Chambers JR, Sauer K. Small RNAs and their role in biofilm formation. Trends Microbiol. 2013;21(1):39-49.
68. Petrova OE, Schurr JR, Schurr MJ, Sauer K. The novel *Pseudomonas aeruginosa* two-component regulator BfmR controls bacteriophage-mediated lysis and DNA release during biofilm development through PhdA. Molecular Microbiology. 2011;81(3):767-83.
69. Petrova OE, Sauer K. The novel two-component regulatory system BfiSR regulates biofilm development by controlling the small RNA *rsmZ* through CafA. Journal of Bacteriology. 2010;192:5275-88
70. Petrova OE, Sauer K. SagS contributes to the motile-sessile switch and acts in concert with BfiSR to enable *Pseudomonas aeruginosa* biofilm formation Journal of Bacteriology. 2011;193:6614-28.
71. Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jørgensen A, Molin S, et al. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Molecular Microbiology. 2003;48(6):1511-24.
72. Lee KWK, Periasamy S, Mukherjee M, Xie C, Kjelleberg S, Rice SA. Biofilm development and enhanced stress resistance of a model, mixed-species community biofilm. ISME J. 2014;8(4):894-907.
73. Barken KB, Pamp SJ, Yang L, Gjermansen M, Bertrand JJ, Klausen M, et al. Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. Environ Microbiol. 2008;10(9):2331-43.
74. Friedman L, Kolter R. Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. Molecular Microbiology. 2004;51(3):675-90.
75. Fabbri S, Li J, Howlin RP, Rmaile A, Gottenbos B, De Jager M, et al. Fluid-driven interfacial instabilities and turbulence in bacterial biofilms. Environ Microbiol. 2017;19(11):4417-31.
76. James GA, Beaudette L, Costerton JW. Interspecies bacterial interactions in biofilms. Journal of industrial microbiology. 1995;15(4):257-62.
77. Murga R, Stewart PS, Daly D. Quantitative analysis of biofilm thickness variability. Biotechnol Bioeng. 1995;45(6):503-10.
78. Liu W, Russel J, Røder HL, Madsen JS, Burmølle M, Sørensen SJ. Low-abundant species facilitates specific spatial organization that promotes multispecies biofilm formation. Environ Microbiol. 2017;19(7):2893-905.
79. Sauer K, Steczko J, Ash SR. Effect of a solution containing citrate/methylene blue/parabens on *Staphylococcus aureus* bacteria and biofilm, and comparison with various heparin solutions. J Antimicrob Chemother. 2009;63(5):937-45.
80. Allegrucci M, Hu FZ, Shen K, Hayes J, Ehrlich GD, Post JC, et al. Phenotypic characterization of *Streptococcus pneumoniae* biofilm development. Journal of Bacteriology. 2006;188(7):2325-35.
81. Hall-Stoodley L, Hu FZ, Gieseke A, Nistico L, Nguyen D, Hayes J, et al. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. JAMA. 2006;296(2):202-11.
82. Hall-Stoodley L, Nistico L, Sambanthamoorthy K, Dice B, Nguyen D, Mershon WJ, et al. Characterization of biofilm matrix, degradation by DNase treatment and evidence of capsule downregulation in Streptococcus pneumoniae clinical isolates. BMC Microbiol. 2008;8:173.
83. Bakaletz LO. Bacterial biofilms in the upper airway - evidence for role in pathology and implications for treatment of otitis media. Paediatr Respir Rev. 2012;13(3):154-9.
84. Walker WT, Jackson CL, Allan RN, Collins SA, Kelso MJ, Rineh A, et al. Primary ciliary dyskinesia ciliated airway cells show increased susceptibility to Haemophilus influenzae biofilm formation. Eur Respir J. 2017;50(3).
85. Bjarnsholt T, Alhede M, Alhede M, Eickhardt-Sorensen SR, Moser C, Kuhl M, et al. The in vivo biofilm. Trends Microbiol. 2013;21(9):466-74.
86. Lebeaux D, Chauhan A, Rendueles O, Beloin C. From in vitro to in vivo models of bacterial biofilm-related infections. Pathogens. 2013(2):288-356.
87. Hall-Stoodley L, Stoodley P, Kathju S, Hoiby N, Moser C, Costerton JW, et al. Towards diagnostic guidelines for biofilm-associated infections. FEMS Immunol Med Microbiol. 2012;65(2):127-45.

88. Trego AC, Mills S, Collins G. Granular biofilms: Function, application, and new trends as model microbial communities. Critical Reviews in Environmental Science and Technology. 2021;51(15):1702-25.
89. Bjarnsholt T, Jensen PO, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, et al. Pseudomonas aeruginosa biofilms in the respiratory tract of cystic fibrosis patients. Pediatr Pulmonol. 2009;44(6):547-58.
90. Kirketerp-Moller K, Jensen PO, Fazli M, Madsen KG, Pedersen J, Moser C, et al. Distribution, organization, and ecology of bacteria in chronic wounds. J Clin Microbiol. 2008;46(8):2717-22.
91. Vasconcelos C, Warthmann R, McKenzie JA, Visscher PT, Bittermann AG, van Lith Y. Lithifying microbial mats in Lagoa Vermelha, Brazil: modern Precambrian relics? Sedimentary Geology. 2006;185(3-4):175-83.
92. Bjarnsholt T, Kirketerp-Moller K, Jensen PO, Madsen KG, Phipps R, Krogfelt K, et al. Why chronic wounds will not heal: a novel hypothesis. Wound Repair Regen. 2008;16(1):2-10.
93. Gloag ES, Wozniak DJ, Stoodley P, Hall-Stoodley L. Mycobacterium abscessus biofilms have viscoelastic properties which may contribute to their recalcitrance in chronic pulmonary infections. Sci Rep. 2021;11(1):5020.
94. Jensen LK, Koch J, Dich-Jorgensen K, Aalbaek B, Petersen A, Fuursted K, et al. Novel porcine model of implant-associated osteomyelitis: A comprehensive analysis of local, regional, and systemic response. J Orthop Res. 2017;35(10):2211-21.
95. Li C, Renz N, Trampuz A. Management of Periprosthetic Joint Infection. Hip Pelvis. 2018;30(3):138-46.
96. Dudareva M, Barrett L, Figtree M, Scarborough M, Watanabe M, Newnham R, et al. Sonication versus Tissue Sampling for Diagnosis of Prosthetic Joint and Other Orthopedic Device-Related Infections. J Clin Microbiol. 2018;56(12).
97. Fux CA, Wilson S, Stoodley P. Detachment characteristics and oxacillin resistance of Staphyloccocus aureus biofilm emboli in an in vitro catheter infection model. J Bacteriol. 2004;186(14):4486-91.
98. Bay L, Barnes CJ, Fritz BG, Thorsen J, Restrup MEM, Rasmussen L, et al. Universal Dermal Microbiome in Human Skin. mBio. 2020;11(1).
99. Burmolle M, Thomsen TR, Fazli M, Dige I, Christensen L, Homoe P, et al. Biofilms in chronic infections - a matter of opportunity - monospecies biofilms in multispecies infections. FEMS Immunol Med Microbiol. 2010;59(3):324-36.
100. Kim D, Barraza JP, Arthur RA, Hara A, Lewis K, Liu Y, et al. Spatial mapping of polymicrobial communities reveals a precise biogeography associated with human dental caries. Proc Natl Acad Sci U S A. 2020;117(22):12375-86.
101. Salta M, Wharton JA, Blache Y, Stokes KR, Briand J-F. Marine biofilms on artificial surfaces: structure and dynamics. Environmental Microbiology. 2013;15(11):2879-93.
102. Alhede M, Kragh KN, Qvortrup K, lesen-Holm M, van GM, Christensen LD, et al. Phenotypes of Non-Attached Pseudomonas aeruginosa Aggregates Resemble Surface Attached Biofilm. PLoS One. 2011;6(11):e27943.
103. Dastgheyb SS, Hammoud S, Ketonis C, Liu AY, Fitzgerald K, Parvizi J, et al. Staphylococcal persistence due to biofilm formation in synovial fluid containing prophylactic cefazolin. Antimicrob Agents Chemother. 2015;59(4):2122-8.
104. Pestrak MJ, Gupta TT, Dusane DH, Guzior DV, Staats A, Harro J, et al. Investigation of synovial fluid induced Staphylococcus aureus aggregate development and its impact on surface attachment and biofilm formation. PLoS One. 2020;15(4):e0231791.
105. Macias-Valcayo A, Staats A, Aguilera-Correa JJ, Brooks J, Gupta T, Dusane D, et al. Synovial Fluid Mediated Aggregation of Clinical Strains of Four Enterobacterial Species. Adv Exp Med Biol. 2020.

106. Bidossi A, Bottagisio M, Savadori P, De Vecchi E. Identification and Characterization of Planktonic Biofilm-Like Aggregates in Infected Synovial Fluids From Joint Infections. Front Microbiol. 2020;11:1368.
107. Kragh KN, Alhede M, Rybtke M, Stavnsberg C, Jensen PO, Tolker-Nielsen T, et al. The Inoculation Method Could Impact the Outcome of Microbiological Experiments. Appl Environ Microbiol. 2018;84(5).
108. Schleheck D, Barraud N, Klebensberger J, Webb JS, McDougald D, Rice SA, et al. Pseudomonas aeruginosa PAO1 preferentially grows as aggregates in liquid batch cultures and disperses upon starvation. PLoS One. 2009;4(5):e5513.
109. Haaber J, Cohn MT, Frees D, Andersen TJ, Ingmer H. Planktonic aggregates of Staphylococcus aureus protect against common antibiotics. PLoS One. 2012;7(7):e41075.
110. Kragh KN, Alhede M, Rybtke M, Stavnsberg C, Jensen PO, Tolker-Nielsen T, et al. Inoculation method could impact the outcome of microbiological experiments. Appl Environ Microbiol. 2017.
111. Hall-Stoodley L, Stoodley P. Biofilm formation and dispersal and the transmission of human pathogens. Trends Microbiol. 2005;13(1):7-10.
112. Secor PR, Michaels LA, Ratjen A, Jennings LK, Singh PK. Entropically driven aggregation of bacteria by host polymers promotes antibiotic tolerance in <em>Pseudomonas aeruginosa</em&gt. Proceedings of the National Academy of Sciences. 2018;115(42):10780.
113. Secor PR, Michaels LA, Ratjen A, Jennings LK, Singh PK. Entropically driven aggregation of bacteria by host polymers promotes antibiotic tolerance in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A. 2018;115(42):10780-5.
114. Dastgheyb S, Parvizi J, Shapiro IM, Hickok NJ, Otto M. Effect of biofilms on recalcitrance of staphylococcal joint infection to antibiotic treatment. J Infect Dis. 2015;211(4):641-50.
115. Knott S, Curry D, Zhao N, Metgud P, Dastgheyb SS, Purtill C, et al. Staphylococcus aureus Floating Biofilm Formation and Phenotype in Synovial Fluid Depends on Albumin, Fibrinogen, and Hyaluronic Acid. Front Microbiol. 2021;12:655873.
116. Gilbertie JM, Schnabel LV, Hickok NJ, Jacob ME, Conlon BP, Shapiro IM, et al. Equine or porcine synovial fluid as a novel ex vivo model for the study of bacterial free-floating biofilms that form in human joint infections. PLOS ONE. 2019;14(8):e0221012.
117. Dorken G, Ferguson GP, French CE, Poon WCK. Aggregation by depletion attraction in cultures of bacteria producing exopolysaccharide. Journal of The Royal Society Interface. 2012;9(77):3490-502.
118. Cornforth DM, Dees JL, Ibberson CB, Huse HK, Mathiesen IH, Kirketerp-Moller K, et al. Pseudomonas aeruginosa transcriptome during human infection. Proc Natl Acad Sci U S A. 2018;115(22):E5125-E34.
119. Rossi E, Falcone M, Molin S, Johansen HK. High-resolution in situ transcriptomics of Pseudomonas aeruginosa unveils genotype independent patho-phenotypes in cystic fibrosis lungs. Nat Commun. 2018;9(1):3459.
120. Koo H, Allan RN, Howlin RP, Stoodley P, Hall-Stoodley L. Targeting microbial biofilms: current and prospective therapeutic strategies. Nat Rev Microbiol. 2017;15(12):740-55.
121. Kragh KN, Hutchison JB, Melaugh G, Rodesney C, Roberts AE, Irie Y, et al. Role of Multicellular Aggregates in Biofilm Formation. MBio. 2016;7(2).
122. Hawes I, Sumner D, Jungblut AD. Complex Structure but Simple Function in Microbial Mats from Antarctic Lakes. In: Hurst CJ, editor. The Structure and Function of Aquatic Microbial Communities. Cham: Springer International Publishing; 2019. p. 91-120.
123. Franca RDG, Pinheiro HM, van Loosdrecht MCM, Lourenço ND. Stability of aerobic granules during long-term bioreactor operation. Biotechnology Advances. 2018;36(1):228-46.
124. Li Y, Xu D, Chen C, Li X, Jia R, Zhang D, et al. Anaerobic microbiologically influenced corrosion mechanisms interpreted using bioenergetics and bioelectrochemistry: A review. Journal of Materials Science & Technology. 2018;34(10):1713-8.

125. Bahrami A, Khouzani MK, Harchegani BB. Establishing the root cause of a failure in a firewater pipeline. Engineering Failure Analysis. 2021;127:105474.
126. Risse-Buhl U, Anlanger C, Kalla K, Neu TR, Noss C, Lorke A, et al. The role of hydrodynamics in shaping the composition and architecture of epilithic biofilms in fluvial ecosystems. Water Research. 2017;127:211-22.
127. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, et al. Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. J Clin Invest. 2002;109(3):317-25.
128. Kirketerp-Moller K, Stewart PS, Bjarnsholt T. The zone model: A conceptual model for understanding the microenvironment of chronic wound infection. Wound Repair Regen. 2020;28(5):593-9.
129. Sonderholm M, Kragh KN, Koren K, Jakobsen TH, Darch SE, Alhede M, et al. Pseudomonas aeruginosa Aggregate Formation in an Alginate Bead Model System Exhibits In Vivo-Like Characteristics. Appl Environ Microbiol. 2017;83(9).
130. Lee B, Haagensen JA, Ciofu O, Andersen JB, Høiby N, Molin S. Heterogeneity of biofilms formed by nonmucoid Pseudomonas aeruginosa isolates from patients with cystic fibrosis. J Clin Microbiol. 2005;43(10):5247-55.
131. . !!! INVALID CITATION !!! (95, 97).
132. Bay L, Kragh KN, Eickhardt SR, Poulsen SS, Gjerdrum LMR, Ghathian K, et al. Bacterial Aggregates Establish at the Edges of Acute Epidermal Wounds. Adv Wound Care (New Rochelle). 2018;7(4):105-13.
133. Ring HC, Bay L, Nilsson M, Kallenbach K, Miller IM, Saunte DM, et al. Bacterial biofilm in chronic lesions of hidradenitis suppurativa. Br J Dermatol. 2017;176(4):993-1000.
134. Ring HC, Thorsen J, Saunte DM, Lilje B, Bay L, Riis PT, et al. The Follicular Skin Microbiome in Patients With Hidradenitis Suppurativa and Healthy Controls. JAMA Dermatol. 2017;153(9):897-905.
135. Qvist T, Eickhardt S, Kragh KN, Andersen CB, Iversen M, Hoiby N, et al. Chronic pulmonary disease with Mycobacterium abscessus complex is a biofilm infection. Eur Respir J. 2015;46(6):1823-6.
136. Folsom JP, Richards L, Pitts B, Roe F, Ehrlich GD, Parker A, et al. Physiology of Pseudomonas aeruginosa in biofilms as revealed by transcriptome analysis. BMC Microbiol. 2010;10:294.
137. Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T. Tolerance to the antimicrobial peptide colistin in Pseudomonas aeruginosa biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes. Mol Microbiol. 2008;68(1):223-40.
138. . !!! INVALID CITATION !!! (95, 99, 100).
139. Bjarnsholt T, Mastroianni E, Kirketerp-Moller K, Stewart PS, Mahr AM, Dominguez Cabanes A, et al. The impact of mental models on the treatment and research of chronic infections due to biofilms. APMIS. 2021.
140. Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science. 1998;280(5361):295-8.
141. O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol Microbiol. 1998;30(2):295-304.
142. O'Toole GA, Kolter R. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol. 1998;28(3):449-61.
143. Schembri MA, Kjaergaard K, Klemm P. Global gene expression in Escherichia coli biofilms. Mol Microbiol. 2003;48(1):253-67.
144. Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, et al. Gene expression in Pseudomonas aeruginosa biofilms. Nature. 2001;413(6858):860-4.
145. Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH. Microarray analysis of Pseudomonas aeruginosa quorum-sensing regulons: effects of growth phase and environment. J Bacteriol. 2003;185(7):2080-95.

924 146. Bagge N, Schuster M, Hentzer M, Ciofu O, Givskov M, Greenberg EP, et al. Pseudomonas
925 aeruginosa biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase
926 and alginate production. Antimicrob Agents Chemother. 2004;48(4):1175-87.
927 147. Heacock-Kang Y, Sun Z, Zarzycki-Siek J, McMillan IA, Norris MH, Bluhm AP, et al. Spatial
928 transcriptomes within the Pseudomonas aeruginosa biofilm architecture. Mol Microbiol. 2017;106(6):976-
929 85.
930 148. Liao J, Schurr MJ, Sauer K. The MerR-like regulator BrlR confers biofilm tolerance by
931 activating multidrug-efflux pumps in *Pseudomonas aeruginosa* biofilms. Journal of Bacteriology.
932 2013;195:3352-63.
933 149. Lenz AP, Williamson KS, Pitts B, Stewart PS, Franklin MJ. Localized gene expression in
934 Pseudomonas aeruginosa biofilms. Appl Environ Microbiol. 2008;74(14):4463-71.
935 150. Shrout JD, Chopp DL, Just CL, Hentzer M, Givskov M, Parsek MR. The impact of quorum
936 sensing and swarming motility on Pseudomonas aeruginosa biofilm formation is nutritionally conditional.
937 Mol Microbiol. 2006;62(5):1264-77.
938 151. Bielecki P, Puchalka J, Wos-Oxley ML, Loessner H, Glik J, Kawecki M, et al. In-vivo expression
939 profiling of Pseudomonas aeruginosa infections reveals niche-specific and strain-independent
940 transcriptional programs. PLoS One. 2011;6(9):e24235.
941 152. Potvin E, Lehoux DE, Kukavica-Ibrulj I, Richard KL, Sanschagrin F, Lau GW, et al. In vivo
942 functional genomics of Pseudomonas aeruginosa for high-throughput screening of new virulence factors
943 and antibacterial targets. Environ Microbiol. 2003;5(12):1294-308.

944 112. Knott S, Curry D, Zhao N, Metgud P, Dastgheyb SS, Purtill C, Harwood M, Chen AF, Schaer TP, Otto M,
945 Hickok NJ. 2021. Staphylococcus aureus Floating Biofilm Formation and Phenotype in Synovial Fluid
946 Depends on Albumin, Fibrinogen, and Hyaluronic Acid. Front Microbiol 12:655873.

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