REVIEW ARTICLE

Emergent Heterogeneous Micro-environments in Biofilms: Substratum Surface Heterogeneity and Bacterial Adhesion Force-sensing

One sentence summary: Individual adhering bacteria can increase intrinsic, stochastically distributed substratum surface heterogeneities yielding different adhesion forces over a substratum surface that trigger emergence of heterogeneous micro-environments in biofilms.

Yijin Ren,[1] Can Wang,[1,2] Zhi Chen,[2] Elaine Allan,[3] Henny C. van der Mei[4*] and Henk J. Busscher[4]

[1] University of Groningen and University Medical Center Groningen, Department of Orthodontics, Hanzeplein 1, 9713 GZ Groningen, The Netherlands;

[2] Wuhan University, School & Hospital of Stomatology, Wuhan, 430079, Wuhan, China;

[3] UCL Eastman Dental Institute, University College London, 256 Gray's Inn Road, London WC1X 8LD, United Kingdom;

[4] University of Groningen and University Medical Center Groningen, Department of Biomedical Engineering, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands.

***Corresponding author:** Henny C. van der Mei, Department of Biomedical Engineering, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands.

Tel.: +31 503616096; E-mail: h.c.van.der.mei@umcg.nl

ABSTRACT

Phenotypically-heterogeneous micro-environments emerge as biofilms mature across different environments. Phenotypic-heterogeneity in biofilm sub-populations not obeying quorum sensing-dictated, collective group-behavior, may be considered as a strategy allowing non-conformists to survive hostile conditions. Heterogeneous phenotype development has been amply studied with respect to gene expression and genotypic changes, but “biofilm genes” responsible for pre-programmed development of heterogeneous micro-environments in biofilms

have never been discovered. Moreover, the question of what triggers the development of phenotypically-heterogeneous micro-environments has never been addressed. The definition of biofilms as "*surface-adhering and surface-adapted*" microbial communities contains the word "*surface*" twice. This leads us to hypothesize that phenotypically-heterogeneous micro-environments in biofilms develop as an adaptive response of initial colonizers to their adhering state, governed by the forces through which they adhere to a substratum surface. No surface is entirely homogeneous, while adhering bacteria can substantially contribute to stochastically occurring surface heterogeneity. Accordingly, bacterial adhesion forces sensed by initial colonizers differ across a substratum surface, leading to differential mechanical deformation of the cell wall and membrane, where many environmental sensors are located. Bacteria directly adhering to heterogeneous substratum domains therewith formulate their own local responses to their adhering state and command non-conformist behavior, leading to phenotypically-heterogeneous micro-environments in biofilms.

Keywords: quorum sensing, environmental sensing, swarming, antibiotic resistance, cooperativity, biosurfactants

TABLE of CONTENT

- How bacteria differentiate between different substratum surfaces
    - Adhesion forces between bacteria and substratum surfaces
    - Cell wall deformation and surface adaptation
- Heterogeneous surfaces and bacterial interactions
    - Surface heterogeneity due to protein adsorption
    - Surface charge heterogeneity
    - Heterogeneity in surface hydrophobicity and roughness
    - Nanoscopically heterogeneous substratum surfaces
- Substratum surface heterogeneities induced by adhering bacteria
    - Localized cooperative phenomena and biosurfactant release
    - Bacterially-induced changes in adsorbed protein conformation and positive cooperativity
    - Cooperativity through EPS production
- The commanding role of initial colonizers in biofilm formation
    - Adhesion force-sensing and biofilm composition
    - Adhesion force-sensing and EPS production
    - Adhesion force-sensing and quorum-sensing
- Summary

ABBREVIATIONS

AFM	atomic force microscopy
DDS	dichlorodimethylsilane
eDNA	extracellular DNA
EPS	extracellular polymeric substances
F_{adh}	adhesion force
HA	hydroxyapatite

PE	polyethylene
PEG	polyethylene glycol
PEO	poly(ethylene) oxide
PET	polyethylene terephthalate
PIA	polysaccharide intercellular adhesin
PDMS	polydimethylsiloxane
PMMA	polymethyl methacrylate
PS	polystyrene
QA	quaternary ammonium
SS	stainless steel
SR	silicone rubber
Ti-6Al-4V	titanium-aluminum-vanadium alloy
WCA	water contact angle

INTRODUCTION

Bacterial adhesion and biofilm formation

Bacteria adhere to surfaces in most industrial and natural environments, regardless of whether the surfaces are of synthetic or biological origin, and the latter includes the surfaces of prokaryotic and eukaryotic cells. Bacterial adhesion clearly marks the start of "*biofilm*" formation, but it still remains a challenge to define the end of biofilm formation. Biofilms are defined as surface-adhering and surface-adapted communities of microorganisms (Tolker-Nielsen 2015), that grow embedded in their self-produced matrix of extracellular polymeric substances (EPS: see Text Box 1) (Flemming and Wingender 2010). Note, that this definition includes cell-to-cell adhesion and therefore also encompasses planktonic aggregates (Vert *et al*. 2012).

Text Box 1. Extracellular polymeric substances

Polymers, such as polysaccharides, proteins, extracellular DNA (eDNA) or nucleic acids, secreted by bacteria and forming a 'glue' that holds a biofilm together, possibly serving other functions like nutrient trapping and protection against antimicrobial challenges (Flemming and Wingender 2010).

Emergent biofilm properties

The biofilm phenotype of bacteria is distinguished from the planktonic state by emergent properties ("*localized gradients, sorption and retention, cooperation and competition, tolerance and resistance*": see Text Box 2) (Flemming *et al*. 2016).

Text Box 2. Emergent biofilm properties

New properties that emerge in a biofilm that are not predictable from the properties of free-living bacterial cells (Flemming *et al*. 2016).

Biofilm phenotypes do not emerge homogeneously across a biofilm. Heterogeneous micro-environments with different microbial composition, pH, live-dead ratios of bacteria, EPS-production, including eDNA-rich or -poor domains, differential penetrability, density, water content and channelization have been observed in biofilms using fluorescent probes (Stewart and Franklin 2008) or optical coherence tomography (Wagner *et al*. 2010). Phenotypically heterogeneous micro-environments are present in biofilms of both Gram-negative and Gram-positive species in different environments (Figure 1), where non-conformists represent a bacterial sub-population that does not obey quorum-sensing commands (see Text Box 3), generally thought to coordinate a homogeneous response in an entire biofilm (Grote *et al*. 2015). Possession of heterogeneous micro-environments can be considered as a deliberate strategy of biofilm inhabitants, with the potential of offering multiple mechanisms to combat hostile conditions and therewith facilitate survival of non-conformists.

Text Box 3. Quorum-sensing

Intra- and interspecific bacterial communication by producing, releasing and detecting small, diffusible molecular auto-inducers. When auto-inducers reach a threshold concentration, it is commonly accepted that a whole population collectively obeys with homogeneous gene expression. Non-conformists represent a bacterial sub-population that does not obey quorum-sensing commands (Grote *et al*. 2015).

Phenotypically heterogeneous, emergent micro-environments

Heterogeneous gene expression or genotypic changes form the basis for the development of phenotypically heterogeneous micro-environments in biofilms. Gene expression is traditionally studied as an average behavioral property in a bacterial population. However, phenotypic heterogeneity occurs also already at the single-bacterium level (Dubnau and Losick 2006) and it could be argued that phenotypic heterogeneities at the single-bacterium level form the basis of heterogeneously-emerging properties in biofilms. The development of heterogeneous phenotypes at the level of biofilm communities, as well as at the level of single-bacteria, has been amply studied and reviewed with respect to gene expression and genotypic changes in planktonic bacterial aggregates and biofilms grown in well plates or on agar (Wolska *et al*. 2016). However, the question of what actually triggers the emergence of heterogeneous micro-environments in biofilms remains unanswered.

Hypothesis on the development of phenotypically heterogeneous, emergent micro-environments

Despite their frequent observation, heterogeneous micro-environments are usually taken for granted, without wondering why one only sees patches of EPS (Nuryastuti *et al.* 2011), polysaccharide intercellular adhesin (PIA) (Arciola *et al*. 2015) or other compounds (Dueholm and Nielsen 2016) appear in a microscopic image, why isolated regions of dead bacteria occur

(Muñoz-Egea *et al*. 2015), why pH varies across a biofilm (Hidalgo *et al*. 2009), why penetrability varies at different locations in a biofilm (Liu *et al*. 2016), or why some adhering bacteria develop motility while others remain non-motile (Prüss 2017)? Are these heterogeneous responses that emerge stochastically distributed by coincidence, are they a transient state in a kinetic process, are they a response to an environmental trigger or do they develop as a genetically preprogrammed, deterministic property in the transition from an adhering bacterium to a mature biofilm?

Since “biofilm genes” responsible for preprogrammed development of heterogeneous micro-environments in mature biofilms have consistently not been discovered (O’Toole *et al*. 2000), emergent phenotypic heterogeneity in biofilms is likely governed by environmental triggers (Vlamakis *et al*. 2008) and physical cues (O'Toole and Wong 2016; Chew and Yang 2017). However, the precise nature of the actual trigger or physical cue has not been addressed. The word “surface” occurs twice in the definition of biofilms by Tolker-Nielsen: “surface-adhering” and “surface-adapted” communities of microorganisms (Tolker-Nielsen 2015). This leads us to hypothesize that phenotypically heterogeneous, emergent micro-environments in biofilms develop as a response of bacteria to their adhering state and are governed by the local properties of the substratum surface.

Aim of this review

In this review, we summarize the events that stimulate different emergent phenotypes during biofilm formation on different non-biological materials with the aim of identifying substratum surface-associated triggers for the development of phenotypically heterogeneous, emergent micro-environments in a biofilm.

MICRO-ENVIRONMENTS IN BIOFILMS ON DIFFERENT SUBSTRATUM SURFACES

In Table 1 we summarize events stimulating emergent phenotypes across a wide variety of different bacterial strains and species and on different substrata. Data in the table are literature-derived without the intention of representing a complete overview of the literature. Instead, the table serves to identify substratum surface-associated triggers for emergent phenotypes, as discussed below. Opposite to the discussion below which is phenomenologically organized, the table is organized alphabetically for different strains.

Phenotypic drug tolerance and resistance

Phenotypic heterogeneity with respect to drug tolerance and resistance has been observed frequently in bacterial bulk cultures. Correct mechanistic distinction between tolerance and resistance is difficult (see Text Box 4). Phenotypic resistance is thought to be mainly due to environmentally-triggered changes in bacterial cell wall permeability impeding drug access, activation of efflux pumps and release of drug-deactivating enzymes (Kester and Fortune 2014). Examples of environmentally triggered events are the reversible change in porin expression levels in enteric bacteria in response to high osmolarity or temperature (Dupont *et al*. 2007) or the reduced antibiotic sensitivity of *Enterobacter aerogenes* which results from reduced porin expression under antibiotic pressure (Bornet *et al*. 2000). Phenotypic tolerance on the other hand, involves an environmental trigger of bacterial dormancy, persistence, differentiation and biofilm formation, including EPS production (Kester and Fortune 2014; Kaldalu *et al.* 2016). Although the mechanisms of phenotypic heterogeneity with respect to tolerance and resistance likely unite in a biofilm, the role of the substratum surface and its specific properties as an environmental trigger for the development of biofilm heterogeneity has not been considered (Olsen *et al.* 2015; Brauner *et al.* 2016).

Text Box 4. Resistance and tolerance

Antibiotic resistance generally means an increase in the minimum inhibitory concentration (MIC) of an antibacterial agent due to a permanent change in the bacterium, e.g. by mutation or through horizontal gene transfer. Antibiotic tolerance is the ability of bacteria to survive the effect of an antibiotic due to a reversible phenotypic state. Two main forms of tolerance have been identified: 'tolerance by slow growth' (occurs at steady state) and 'tolerance by lag' (a transient state that is induced by starvation or stress) (Olsen *et al.* 2015; Brauner *et al.* 2016).

S. epidermidis and *S. aureus* biofilms grown on polycarbonate filters on agar possessed at least four distinct phenotypes: bacteria growing either aerobically or fermentatively, dead or dormant (Rani *et al*. 2007). Multiple strains of *S. epidermidis* containing the *ica* locus, which encodes for PIA, were found to produce biofilms on hydrophobic polyethylene surfaces (water contact angle, WCA of 84 degrees) which contained large patches of EPS. Alternatively, on more hydrophilic acrylic and stainless steel surfaces (WCA of 69 degrees and 33 degrees, respectively), heterogeneously occurring EPS production was less and concurrently, *ica*-gene expression was low in these biofilms as compared with biofilms on polyethylene (Nuryastuti *et al*. 2011). Similarly, EPS production in biofilms of *S. aureus* and *S. epidermidis* on hydrophobic silicone rubber surfaces (WCA of 110 degrees) was massive and yielded resistance to gentamicin, whereas on hydrophilic polyethylene-glycol (PEG), polymer-brush coated silicone rubber (WCA of around 40 degrees), EPS production was absent and bacteria remained susceptible to gentamicin. To a lesser extent, such differences were also observed in biofilms of the Gram-negative bacterium, *P. aeruginosa* (Roosjen *et al*. 2004; Muszanska *et al*. 2012). Expression of the membrane located sensor, NsaS and the NsaA two-component efflux pump in *S. aureus* SH1000, responsible for nisin resistance in the planktonic state, was enhanced when the organism was adhering to a substratum surface. Moreover, adhesion to a hydrophobic polyethylene surface triggered a greater expression of *nsaS* and *nsaA* than adhesion to a more hydrophilic stainless steel surface (Carniello *et al*. 2018). Despite the influence that the specific properties of the substratum surface have on emergent biofilm properties, most experiments are

reported in the literature without reference to the substratum material. In many cases, biofilm assays are performed in multi-well polystyrene plates and the type of polystyrene is not specified even though this will affect surface properties: for example, bacterial-grade polystyrene is more hydrophobic in the absence of surface treatment (WCA 78 degrees) than tissue culture-grade polystyrene after physical treatment (WCA 43 degrees), and these differences may severely impact on bacterial adaptive behavior. Moreover, often conclusions on surface adaptation are extrapolated from results obtained in biofilms grown on aqueous agar, which may not accurately reflect the conditions encountered on solid substratum surfaces.

Collectively, these examples demonstrate that the substratum surface, most notably its hydrophobicity or hydrophilicity (see Text Box 5), provides an environmental trigger for the development of antibiotic resistance and tolerance in biofilms. Importantly, in most of these examples, a uniform response of the entire biofilm has been inferred without evidence that the biofilm is homogeneous over its entire volume. However, where available, closer inspection of micrographs in the published literature (see Figure 1 for specific examples), clearly shows stochastically occurring non-conformists, providing clear evidence of heterogeneity.

Text Box 5. Surface hydrophobicity

"Surface hydrophobicity" and its opposite "surface hydrophilicity" literally indicate the "fear" or "love" of a surface for water. Surface hydrophobicity can be quantitated by placing a small water droplet on a surface and measuring its degree of spreading, full spreading being characterized by a zero degrees water contact angle (hydrophilic surface). On super-hydrophobic materials, like nanostructured hydrophobic surfaces, air can become entrapped and water has an almost 180 degrees WCA (Hizal *et al*. 2017), making it behave like a mercury droplet.

Swarming behavior

Swarming is another drug-resistance mechanism allowing bacteria to explore and subsequently escape an antibiotic-laden or otherwise hostile environment (Lai *et al*. 2009), and also enables bacteria to actively search for nutrients (Daniels *et al*. 2004). Swarming phenotypes are often characterized by being hyperflagellated, elongated, multinucleate (Toguchi *et al*. 2000) and antibiotic-resistant. In *Paenibacillus vortex* biofilms, antibiotic-refractory, swarming phenotypes function to explore the environment for antibiotic-laden regions that should be avoided by the 'builders' of the biofilm community (Roth *et al*. 2013).

Swarming bacteria either reside in 1) bulk suspension, where they are unlikely to experience any effects from a substratum surface, 2) surface-constrained, near the surface but still in suspension and experiencing hydrodynamic shear or 3) in direct interaction with the substratum surface (Tuson and Weibel 2013). Swarming in the surface-constrained regime requires reversible adhesion on the one hand, but in order to prevent detachment back into the bulk suspension, bacteria must have a means to rapidly transit between reversible and irreversible adhesion. Indeed studies on single cells of *C. crescentus* demonstrated that transitioning from reversible to irreversible adhesion is not a single event and most cells reversibly contact a surface multiple times before a final transition to irreversible adhesion takes place, with pili playing an important role in this transition (Hoffman *et al*. 2015).

Bacteria can sense the presence of a surface by obstruction of surface appendages such as flagella, pili or fimbriae (Friedlander *et al.* 2013; Ellison and Brun 2015) and subsequent activation of membrane located sensors (Belas 2014). In *C. crescentus,* arrest of flagellum rotation and concurrent stimulation of "just-in-time" polysaccharide adhesive occurs to maximize

adhesion and prevent untimely detachment back into suspension (Li *et al*. 2012). The presence of *P. aeruginosa* flagella and type IV pili increased bacterial adhesion to highly hydrophobic substratum surfaces (Bruzaud *et al*. 2015), suggesting a role for substratum surface properties on development of bacterial swarming phenotypes.

HOW BACTERIA DIFFERENTIATE BETWEEN DIFFERENT SUBSTRATUM SURFACES

Adhesion forces between bacteria and substratum surfaces

The observations that bacteria adapt differently to adhesion on different substratum surfaces, immediately raises the question of how bacteria sense that they are on a surface, and more importantly, how they tailor their adaptive response to the characteristic properties of the surface they adhere to. Adhesion, whether arising from specific, molecular ligand-receptor or non-specific interactions (Bos *et al*. 1999), is an interplay between ever present attractive Lifshitz-Van der Waals forces, attractive or repulsive acid-base interactions as a generalized form of hydrogen bonding, electrostatic forces with a magnitude depending on pH and ionic strength of the fluid environment and Brownian motion forces. The attractive Lifshitz-Van der Waals forces are the most long-ranged ones, acting over distances of up to 1 μm and becoming increasingly stronger when the interacting surfaces become closer. The sum total of these different forces determine the force by which a bacterium adheres to a substratum surface and this varies on different surfaces (Alam and Balani 2017), while at close approach Lifshitz-Van der Waals forces are usually able to overcome electrostatic barriers (Puddu and Perry 2012; Paula *et al*. 2014).

Text Box 6. Bacterial adhesion force measurement

Bacterial adhesion can be measured using atomic force microscopy (AFM). In bacterial probe AFM, a bacterium is attached to a highly flexible cantilever and brought into contact with a substratum surface, allowing contact between the bacterium and the surface for a defined time-period and applied loading-force. Upon retraction of the cantilever from the surface, the force required to break the bond between the bacterium and the substratum surface is recorded from the bending of the flexible cantilever. In this way, bacterial adhesion forces to biological and non-biological surfaces in the picoNewton (pN) to nanoNewton (nN) range have been measured (Dufrêne 2015).

Text Box 7. On the magnitude of bacterial adhesion forces to surfaces

Most forces by which bacteria adhere to surfaces are reportedly in the nN-range (Van der Mei *et al*. 2008; Beaussart *et al*. 2013; Sullan *et al.* 2014; Thewes *et al*. 2015), which is large compared to the gravity force experienced by bacteria. In air, the gravity force experienced by a bacterium is around 10^{-6} nN, while due to buoyancy, this force reduces in an aqueous suspension to around 10^{-8} nN. Assuming an adhesion force of around 1 nN, this implies that the forces by which bacteria adhere to a substratum surface are 10^{6} - 10^{8} fold higher than the gravity forces they experience.

Distinguishing three adhesion force regimes (Busscher and Van der Mei 2012), it was proposed that extremely weakly adhering bacteria (adhesion forces less than 1 nN) do not realize they are in an adhering state and therefore do not show any adaptive response to a substratum surface. Alternatively, when adhering very strongly (proposed adhesion forces above 10 nN) as on quaternary-ammonium coated surfaces (Muszanska *et al.* 2012), cell wall damage is inferred resulting in bacterial cell death (Tiller *et al.* 2001; Asri *et al.* 2014). The intermediate regime comprising adhesion forces between 1 and 10 - 15 nN as occurs on most common substratum surfaces across a wide variety of bacterial strains and species (Van der Mei *et al*. 2008; Beaussart *et al*. 2013; Thewes *et al.* 2015; Sullan *et al.* 2014), invokes bacterial adaptation with production of EPS according to the magnitude of the adhesion forces experienced (Harapanahalli *et al*. 2015).

The ability to measure bacterial adhesion forces using the AFM (see Text Box 6) creates an awareness of the enormous magnitude of bacterial adhesion forces as compared with the gravitational forces they experience (see Text Box 7). Thus, it is not surprising that a lethal regime exists in which bacteria die due to cell wall damage as result of experiencing adhesion forces that are 10^{6} – 10^{8} fold higher than the gravitational force they experience. It has been argued that bacterial cell walls are rigid to resist large internal pressures, but remarkably plastic in order to adapt to a wide range of external forces (Amir *et al.* 2014), including adhesion forces. In fact, it has been demonstrated using AFM (Chen *et al*. 2014) and surface enhanced fluorescence (see Text Box 8), that the bacterial cell wall deforms under the influence of the relatively large adhesion forces arising from a substratum surface (Figure 2), despite the rigidity provided to bacteria by their peptidoglycan layer. Also AFM imaging of *S. epidermidis* trapped in a filter has shown structural and mechanical deformation of the cell wall (Méndez-Vilas *et al*. 2007).

Text Box 8. Surface enhanced bacterial fluorescence

Surface enhanced fluorescence is the phenomenon that fluorophores within 20-30 nm from a metal surface show a stronger fluorescence intensity than expected for the same fluorophore in solution (Lee *et al*. 2011). Surface enhanced bacterial fluorescence of fluorescent bacteria adhering to metallic surfaces can be exploited to demonstrate bacterial cell wall deformation, because more of the fluorescent, intracellular content of a bacterium is brought into the close vicinity of the surface upon adhesion and subsequent cell wall deformation, and therewith subject to surface enhanced fluorescence (Li *et al.* 2014).

Cell wall deformation and surface adaptation

The role of cell wall deformation in triggering bacterial responses is difficult to demonstrate experimentally, as bacterial cell wall deformation is small due to the rigidity provided by the bacterial peptidoglycan layer surrounding the membrane. In mammalian cells however, lacking a rigid cell wall, the influence of substratum hydrophobicity is more obvious and many different types of tissue cells remained “cauliflower” shaped on hydrophobic substratum surfaces while deforming to a “pancake” shape on hydrophilic ones (Schakenraad *et al.* 1986). Also in mammalian cells, sensors located in the cell membrane-have been described which control the subsequent differentiation of stem cells in a substratum-dependent fashion (Engler *et al*. 2006).

Deliberate compression of bacteria between AFM cantilevers and substratum surfaces, has demonstrated that the bacterial cell wall deforms in a viscoelastic way (Vadillo-Rodriguez *et al*. 2008; Vadillo-Rodriguez and Dutcher 2009), although it should be noted that deformation under such conditions is not exactly the same as “spontaneous” deformation under the influence of adhesion forces arising from a substratum surface. *E. coli* and *B. subtilis* behaved like elastic rods when subjected to external forces, but deformed permanently in the plastic regime of viscoelastic deformation when cell wall synthesis occurred while the force was applied (Amir *et al*. 2014). Moreover, the offspring of plastically deformed bacteria always recovered their shape, but this required conditions allowing cell wall synthesis (Sliusarenko *et al*. 2010; Amir et al. 2014) over several generations (Si *et al*. 2015). Bacterial cell wall deformation changes the pressure profile across the lipid membrane (Perozo *et al.* 2002) which is laden with environmental sensors that can become activated by such changes (Kocer 2015) through gating of mechanosensitive channels (Haswell *et al*. 2011) or directly by conformational changes in membrane-located receptors (Otto and Silhavy 2002). Thus adhesion-force sensing and subsequent cell wall deformation provide an important mechanism for adhering bacteria to realize they are on a surface and begin the process of surface-adaptation. The role of rigid bacterial peptidoglycan layers in adhesion force-sensing and subsequent cell wall deformation is probably large, since a *S. aureus* Δ*pbp4* mutant, which lacks peptidoglycan cross-linking, seemed unable to adapt its response in line with the adhesion forces arising from a substratum surface (Harapanahalli *et al.* 2015).

HETEROGENEOUS SURFACES AND BACTERIAL INTERACTIONS

Surface heterogeneity due to protein adsorption

All naturally occurring and synthetic surfaces are heterogeneous, either on a micro- or nanoscopic scale and will exert different local adhesion forces on adhering bacteria to trigger different adaptive responses. Dental enamel is an excellent example of a naturally occurring heterogeneous surface with distinct crystalline hydroxyapatite structures comprised in an organic matrix, that in the oral cavity become covered within seconds with a conditioning film of adsorbed salivary proteins forming a network-structure over the enamel surface (Busscher *et al*. 1989; Simmons *et al*. 2011). Although the network-structure of adsorbed proteins is a heterogeneous surface structure in itself, saliva contains many different proteins (Marsh *et al*. 2016) that adsorb and displace each other in succession which further contributes to surface heterogeneity. In the oral cavity, formation and composition of salivary conditioning films varies on different surfaces (Aroonsang *et al.* 2014) and precedes adhesion of bacteria and subsequently influences bacterial adhesion forces and biofilm detachment (Song *et al.* 2015). A similar succession of protein adsorption and desorption occurs on cellular and synthetic graft surfaces exposed to blood (Vroman 2008). Note that, in the marine and other aqueous environments, conditioning films are often described as adsorbed films composed of dissolved organic carbon (Bakker *et al*. 2003). Since bacteria diffuse more slowly than proteins, bacteria mostly adhere to such heterogeneous, adsorbed conditioning films, regardless of whether in the oral cavity or in any other environment.

Surface charge heterogeneity

Strong electrostatic attraction between positively-charged quaternary ammonium-coated surfaces and negatively charged bacterial cell surfaces are reported to cause cell wall damage and subsequent cell death (Asri *et al.* 2014). Charge heterogeneity on glass surfaces, often thought to be homogeneous, became evident by repetitively allowing negatively-charged, 1 µm diameter polystyrene particles to adhere to the same glass surface. Under low ionic strength conditions, particles always adhered first to the same, previously occupied microscopic location through strong, local electrostatic attraction (Wit and Busscher 1998), demonstrating the existence of positively-charged heterogeneities on an overall negatively-charged glass surface.

Heterogeneity in surface hydrophobicity and roughness

Heterogeneity in surface hydrophobicity and roughness at the sub-micrometer scale are easily demonstrable by the measurement of water contact angle hysteresis on material surfaces (see Text Box 9). Large differences between advancing and receding contact angles on “smooth” surfaces with a roughness less than 0.1 µm indicate regions with a large difference in surface hydrophobicity. Roughened, hydrophobic surfaces may appear as “superhydrophobic”, while roughened, hydrophilic surfaces possess smaller water contact angles than expected based on the hydrophobicity, respectively the hydrophilicity of their smooth counterparts.

Text Box 9. Contact angle hysteresis

When a water droplet advances over a perfectly smooth surface, it can be stopped by a small, more hydrophobic heterogeneity or rugosity, which causes the contact angle to be higher than when the droplet is in an equilibrium state. Equally so, when receding over an already wetted surface, water tends to remain behind on a hydrophilic heterogeneity and the contact angle appears smaller than in an equilibrium state. The difference in advancing and receding contact

angles is called “contact angle hysteresis” (Timmons and Zisman 1966). Only perfectly smooth and chemically homogeneous surfaces have a zero degree contact angle hysteresis, which makes the measurement of contact angle hysteresis suitable for the measurement of surface heterogeneity in general at a sub-micrometer scale.

Bacteria themselves are in fact also ideal to demonstrate heterogeneity in substratum surface hydrophobicity due to differential interaction with hydrophobic and hydrophilic regions on a substratum surface. Micro-patterned substratum surfaces consisting of hydrophobic lines separated by wide hydrophilic spacings for instance, attracted equal numbers of streptococci over its entire surface, but when challenged with a detachment force, streptococci were retained only on the hydrophobic lines (Bos *et al*. 2000), suggesting that the strength of bacterial adhesion is higher to hydrophobic regions. Adhesion force measurement using AFM on a patterned substratum consisting of square arrays of non-adhesive PEG hydrogels comparable in size to a bacterial cell on a hydrophobic, silanized glass surface showed that *S. aureus* adhesion was decreased at the hydrogel spacings as these presumably impeded contact between the bacterial cell and the hydrophobic surface (Wang *et al*. 2011).

Nanoscopically heterogeneous substratum surfaces

Nanotechnological advances have enabled the production of nanoscopically heterogeneous surfaces, that are often bio-inspired (Tripathy *et al.* 2017) most notably by the so-called “lotus effect” (Huang *et al*. 2016). Such plant leaves, and also certain insect wings, remain free of bacteria through self-cleaning and antibacterial properties, thought to be mediated by nanopillared arrays (Hasan *et al*. 2013) that inherently represent a nanoscopically heterogeneous substratum surface. Electron micrographs have clearly demonstrated that the bacterial cell wall can locally severely deform under the influence of the adhesion forces arising from extruding random (Svensson *et al*. 2014) and periodic (Hizal *et al*. 2016) nanostructures to yield pressure-induced EPS production and even bacterial cell death in Gram-positive staphylococci. This is supported by observations that killing of *P. aeruginosa* and *S. aureus* on graphene nanosheets related with density of the edges of the graphene (Pham *et al*. 2015). Approximately 98% of *P. aeruginosa* cells and 97% of *S. aureus* cells were killed on superhydrophilic and superhydrophobic black silicon surfaces with well-defined surface geometries and wettability, smaller, more densely packed pillars exhibiting the greatest bactericidal activity (Linklater *et al*. 2017). It is speculated that the bactericidal activity is due to irreversible membrane bulging. In antibiotic-challenged *E. coli*, pores in the peptidoglycan network with a critical radius of around 20 nm, the typical distance between neighboring peptides and glycan strands, are required to cause bulging of the cytoplasmic membrane out through the pore. This bulging is irreversible and leading to loss of cell viability (Daly *et al*. 2011).

SUBSTRATUM SURFACE HETEROGENEITIES INDUCED BY ADHERING BACTERIA

During adhesion, bacteria can create heterogeneities as a means of communication (Figure 3) to allow localized positive- or negative-cooperation in colonizing a substratum surface, that is, stimulate or discourage adhesion of other bacteria in their immediate surroundings (Sjollema *et*

al. 1990). In a broader sense, bacteria have been suggested to leave “footprints” when adhering to and detaching from a substratum surface (Neu 1992) that will contribute to substratum surface heterogeneity.

Localized cooperative phenomena and biosurfactant release

Biosurfactants (see Text Box 10), by their amphiphilic nature, are ideal molecules to be transported over large distances to reach remote areas of a substratum surface as a means to interact with other initial colonizers (Figure 3A). *S. mitis* strains excrete biosurfactants that modify their immediate surroundings to make it less attractive for their competitors to adhere (Loozen *et al*. 2014; Van Hoogmoed *et al*. 2000) and the spreading of oral biosurfactants excreted by initial colonizers such as *S. mitis* over dental enamel surfaces reduced the adhesion forces of other colonizers (Van Hoogmoed *et al.* 2006). Lactobacilli also claim substratum surface area by excretion of biosurfactants that discourage adhesion of enterococci and other uropathogens (Velraeds *et al*. 1996).

Quorum-sensing controlled expression of phenol-soluble modulin surfactants in *S. aureus* (Periasami *et al*. 2012) and rhamnolipids in *P. aeruginosa* (Davey *et al*. 2003) biofilms has been shown to mediate biofilm structuring and detachment. For *P. aeruginosa*, siderophores, eDNA and biosurfactants play multiple roles in the interaction between different sub-populations in a biofilm and influence its structural development, as related to biosurfactants concentration and composition (Pamp and Tolker-Nielsen 2007).

Text Box 10. Biosurfactants

Biosurfactants are amphiphilic compounds produced by living organisms, mostly microorganisms, and excreted extracellularly, that contain hydrophobic and hydrophilic moieties, accumulating at an interface and reducing interfacial tensions *versus* air, a liquid surrounding or another material (Sambanthamoorthy *et al*. 2014; Cochis *et al*. 2012).

Bacterially-induced changes in adsorbed protein conformation and positive cooperativity

Bacteria also have other means to modify their immediate surroundings on a substratum surface to exert positive cooperativity (Nesbitt *et al*. 1982; Van der Mei *et al*. 1993): several initial colonizers of protein-conditioned surfaces have the ability to induce conformational changes in the adsorbed protein film that surrounds them (Figure 3B), making the film more attractive for their peers to adhere. Initial colonizers of oral surfaces *in vivo* have slightly stronger adhesion forces with salivary conditioning films than later colonizers (Mei *et al*. 2009), that may be underlying their ability to induce conformational changes in the adsorbed proteins to which they adhere. Since clinically, the relative prevalence of initially colonizing strains on a surface depends on the forces by which specific bacterial strains are attracted to their substratum surface (Wessel *et al.* 2014), local induced changes in the conformation of adsorbed proteins may yield biofilm regions with a different bacterial composition.

Cooperativity through EPS production

EPS production can be considered as another cooperative phenomenon offering advantages in adhesion to neighboring bacteria by creating local surface heterogeneity around an adhering organism (see also Figure 3B) (Nadell *et al*. 2011) but, like for positive cooperativity in general, at the obvious expense of impairing dispersal of adhering bacteria to new locations. Psl for instance, is a cell wall anchored polysaccharide in *P. aeruginosa* (Ma *et al*. 2009) promoting aggregate formation between neighboring bacteria in micro-environments of a biofilm, that does not occur and subsequently yields less biofilm in strains lacking Psl (Wang *et al*. 2013). Mixed species oral biofilms on saliva-coated surfaces possess acidic niches in their EPS- matrix that selectively stimulate the localized growth of pathogenic *S. mutans* (Xiao *et al*. 2012; Koo and Yamada 2016).

THE COMMANDING ROLE OF INITIAL COLONIZERS IN BIOFILM FORMATION

Bacterial responses to prevailing environmental conditions is virtually always a survival strategy to maintain their adhering state in competition with others or under mechanical attack, while the production of EPS as an adaptive response embeds adhering bacteria in a matrix that also offers protection against chemical attacks (Carniello *et al.* 2016; de la Fuente-Núñez *et al.* 2013). Initially adhering bacteria have various ways to influence the development of micro-environments in the biofilm that grows on top of them, in which adhesion force-sensing plays a crucial role.

Adhesion force-sensing and biofilm composition

In the sequence of events that lead to a full grown biofilm with heterogeneously occurring micro-environments, the initially adhering bacteria firstly have various ways to induce local heterogeneities on a substratum surface to which they adhere. Newcomers can recognize these heterogeneities by the strength of the local adhesion forces they experience and interpret them as signs to “stay away” or “welcome, adhere here”. This in turn, will create micro-environments in a biofilm with different microbial composition. Therewith the basis of cooperation, and possible conflicts, in a mature biofilm (Xavier *et al*. 2007) is commanded by the initially adhering bacteria.

Adhesion force-sensing and EPS production

Emergent EPS production follows initial adhesion in the sequence of events leading to a mature biofilm, and is arguably one of the most important adaptive responses within a biofilm. Adhesion force-sensing constitutes an environmental trigger for EPS production. The production of the matrix molecule, poly-N-acetylglucosamine and the secretion of eDNA decreases with increasing adhesion force, suggesting that adhering staphylococci adjust their adaptive response to environmental need (Harapanahalli *et al*. 2015) to prevent unnecessary costs to their fitness (Brooks *et al*. 2014). Similarly, EPS production by bacteria adhering under fluid shear conditions is more extensive than under stagnant conditions, suggesting that its expression is induced only when required (Nivens *et al*.1993; Hou *et al*. 2017).

Since the effective range of adhesion forces is limited to maximally 1 µm, it is impossible for bacteria other than the initial colonizers to directly sense a substratum, while their immediate neighbors reside at distances between 1-3 µm and are embedded in an EPS matrix (Drescher *et al*. 2016). Accordingly, only initially adhering bacteria are able to sense and adapt to the adhesion forces exerted by a substratum surface and in fact, the majority of bacteria in a biofilm have never contacted the substratum surface (Zhao *et al*. 2013). Since the same will be true for the bacteria in emergent heterogeneous micro-environments, this leads to the conclusion that initially adhering bacteria command the development of emerging heterogeneous micro-environments by sensing and adapting to the substratum and communicating with neighboring bacteria information about that surface (see Figure 4). Stochastically occurring environmental triggers have been suggested before as being causative to phenotypic heterogeneity (Vega and Gore 2014), but have never been associated with triggers derived from stochastically occurring substratum surface heterogeneity.

Text Box 11. Surface adaptation

Bacterial surface adaptation comprises the particular response of a bacterium to the surface properties of the substratum to which it adheres.

The surface adaptation (Text Box 11) of initial colonizers in response to direct contact with a substratum surface likely do not disappear with the first generation of later colonizers, not in direct contact with the surface, but will most probably disappear only after a number of generations (Si *et al*. 2015) and the progeny returns to a more planktonic phenotype. Return to a planktonic phenotype does not necessarily imply bacterial return back into suspension, but may also occur in a biofilm, where bacteria are “suspended” or “free floating” in an EPS matrix at average distances of 1-3 µm from neighboring organisms (Drescher *et al*. 2016), i.e. more specifically formulated, outside the influence of adhesion forces exerted by their neighbors.

Adhesion force-sensing and quorum-sensing

Identifying initial colonizers that are in direct contact with a substratum surface as “commanding” bacteria, implies that there must be a communication means available within a biofilm to pass information derived from adhesion force-sensing to bacteria that are not in direct contact with the substratum enabling them to indirectly sense the surface. The initially adhering bacteria likely pass substratum information by producing and releasing auto-inducing molecules to which later biofilms colonizers respond. Since the distance over which auto-transducers can be transported and remain detectable is limited by diffusion (Vega and Gore 2014), quorum-sensing is eventually quenched which restricts the adaptive response to micro-environments in a biofilm, although “calling distances” between Gram-negative bacteria extending up to 78 µm have been reported (Elias and Banin 2012). However, most effective calling distances for producing and releasing, sensing and responding to auto-transducer gradients are suggested to be between 4 - 5 µm (Gantner et al. 2006; Elias and Banin 2012) and bacteria can optimize the use of auto-inducers by being in each other’s close vicinity. *Myxococcus xanthus*, *E. coli*, *B. subtilis* and lactobacilli for instance, use contact-dependent signaling for communication (Blango and Mulvey 2009). Direct physical contact between bacteria in a biofilm is generally absent,

unless co-adhering bacterial pairs are involved, that occur mostly in the oral cavity (Rickard *et al*. 2003).

SUMMARY

In summary, all surfaces are heterogeneous with respect to hydrophobicity, charge and/or the possession of micro- or nanoscopic structures. Such stochastically occurring heterogeneities exert different adhesion forces upon adhering bacteria. Bacteria sense these adhesion forces through cell wall deformation, which subsequently activates membrane located sensors to stimulate phenotypic responses in initially adhering bacteria in direct contact with the surface. The local adaptive response of initial colonizers is conveyed to other biofilm inhabitants through diffusion of auto-inducers produced by the initial colonizers and their first generations progeny. Later generation progeny will lose the surface-adapted phenotype of the initial colonizers, while diffusion of auto-inducers occurs only over limited distances. This puts initial colonizers in command of the development of localized, stochastically occurring heterogeneous domains in a biofilm.

The role of adhesion force-sensing in cell wall deformation as local triggers for the development of heterogeneous micro-environments in biofilms, puts a strong emphasis on the substratum surface on which biofilms are grown. Hitherto, in research on adaptive responses of bacteria to environmental triggers, conclusions are frequently extrapolated from agar-grown “biofilms” and biofilms on undefined well-plate materials to biofilms in general. Realization of the role of substratum properties in localized, adaptive responses of adhering bacteria and subsequent properties of a biofilm may accelerate development of much needed insight in the mechanisms of heterogeneous micro-environment development in biofilms.

ACKNOWLEDGEMENT

This study was supported by the University Medical Center Groningen-University of Groningen, Groningen both in The Netherlands. HJB is also director-owner of SASA BV. Opinions and assertions contained herein are those of the authors and are not meant to be construed as the representing views of the organizations to which the authors are affiliated.

REFERENCES

Alam F, Balani K. Adhesion force of *Staphylococcus aureus* on various biomaterial surfaces. *J Mech Behav Biomed Mater* 2017;**65**:872-80.

Amir A, Babaeipour F, McIntosh DB *et al.* Bending forces plastically deform growing bacterial cell walls. *Proc Natl Acad Sci U S A* 2014;**111**:5778-83.

Arciola CR, Campoccia D, Ravaioli S *et al.* Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects. *Front Cell Infect Microbiol* 2015;**5**:7.

Aroonsang W, Sotres J, El-Schich Z *et al.* Influence of substratum hydrophobicity on salivary pellicles: organization or composition? *Biofouling* 2014;**30**:1123-32.

Asri LA, Crismaru M, Roest S *et al.* A Shape-adaptive, antibacterial-coating of immobilized quaternary-ammonium compounds tethered on hyperbranched polyurea and its mechanism of action. *Adv Funct Mater* 2014;**24**:346-55.

Bakker DP, Klijnstra JW, Busscher HJ *et al*. The effect of dissolved organic carbon on bacterial adhesion to conditioning films adsorbed on glass from natural seawater collected during different seasons. Biofouling 2003;**19**:391-7.

Beaussart A, El-Kirat-Chatel S, Herman P *et al.* Single-cell force spectroscopy of probiotic bacteria. *Biophys J* 2013;**104**:1886-92.

Belas R. Biofilms, flagella, and mechanosensing of surfaces by bacteria. *Trends Microbiol* 2014;**22**:517-27.

Blango MG, Mulvey MA. Bacterial landlines: contact-dependent signaling in bacterial populations. *Curr Opin Microbiol* 2009;**12**:177-81.

Bornet C, Davin-Regli A, Bosi C *et al.* Imipenem resistance of *Enterobacter aerogenes* mediated by outer membrane permeability. *J Clin Microbiol* 2000;**38**:1048-52.

Bos R, Van der Mei HC, Busscher HJ. Physico-chemistry of initial microbial adhesive interactions–its mechanisms and methods for study. *FEMS Microbiol Rev* 1999;**23**:179-230.

Bos R, Van der Mei H, Gold J *et al.* Retention of bacteria on a substratum surface with micro-patterned hydrophobicity. *FEMS Microbiol Lett* 2000;**189**:311-5.

Brauner A, Fridman O, Gefen O *et al.* Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat Rev Microbiol* 2016;**14**:320-30.

Brooks JL, Jefferson KK. Phase variation of poly-N-acetylglucosamine expression in *Staphylococcus aureus*. *PLoS Pathog* 2014;**10**:e1004292.

Bruzaud J, Tarrade J, Coudreuse A *et al.* Flagella but not type IV pili are involved in the initial adhesion of *Pseudomonas aeruginosa* PAO1 to hydrophobic or superhydrophobic surfaces. *Colloids Surf B: Biointerfaces* 2015;**131**:59-66.

Busscher HJ, Uyen HMW, Stokroos I *et al.* A transmission electron microscopy study of the adsorption patterns of early developing artificial pellicles on human enamel. *Arch Oral Biol* 1989;**34**:803-10.

Busscher HJ, Van der Mei HC. How do bacteria know they are on a surface and regulate their response to an adhering state? *PLoS Pathog* 2012;**8**:e1002440.

Carniello V, Hou J, Van der Mei HC *et al.* The transition from bacterial adhesion to the production of EPS and biofilm formation. In: Flemming *et al*. (eds.). *The Perfect Slime—Microbial Extracellular Substances(EPS)*. London: IWA publishing, 2016;61-78.

Carniello V, Harapanahalli AK, Busscher HJ *et al.* Adhesion force sensing and activation of a membrane-bound sensor to activate nisin efflux pumps in *Staphylococcus aureus* under mechanical and chemical stresses. *J Colloid Interface Sci* 2018;**512**:14-20.

Chen Y, Harapanahalli AK, Busscher HJ *et al.* Nanoscale cell wall deformation impacts long-range bacterial adhesion forces on surfaces. *Appl Environ Microbiol* 2014;**80**:637-43.

Chew SC, Yang L. Biofilms: microbial cities wherein flow shapes competition. *Trends Microbiol* 2017;**25**:331-2.

Cochis A, Fracchia L, Martinotti MG *et al*. Biosurfactants prevent *in vitro Candida albicans* biofilm formation on resins and silicone materials for prosthetic devices. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2012;**113**:755-61.

Daly KE, Huang KC, Wingreen NS *et al.* Mechanics of membrane bulging during cell-wall disruption in Gram-negative bacteria. *Physical Review E* 2011;**83**:041922.

Daniels R, Vanderleyden J, Michiels J. Quorum sensing and swarming migration in bacteria. *FEMS Microbiol Rev* 2004;**28**:261-89.

Davey ME, Caiazza NC, O'Toole GA. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 2003;**185**:1027-36.

de la Fuente-Núñez C, Reffuveille F, Fernández L *et al.* Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Curr Opin Microbiol* 2013;**16**:580-9.

Drescher K, Dunkel J, Nadell CD *et al.* Architectural transitions in *Vibrio cholerae* biofilms at single-cell resolution. *Proc Natl Acad Sci U S A* 2016;**113**:E2066-E72.

Dubnau D, Losick R. Bistability in bacteria. *Mol Microbiol* 2006;**61**:564-72.

Dueholm MS, Nielsen PH. Amyloids-a neglected child of the slime. In: Flemming *et al*. (eds.). *The Perfect Slime—Microbial Extracellular Substances(EPS)*. London: IWA publishing, 2016, 113-133.

Dufrêne YF. Sticky microbes: forces in microbial cell adhesion. *Trends Microbiol* 2015;**23**:376-82.

Dupont M, James CE, Chevalier J *et al.* An early response to environmental stress involves regulation of OmpX and OmpF, two enterobacterial outer membrane pore-forming proteins. *Antimicrob Agents Chemother* 2007;**51**:3190-8.

Elias S,Banin E. Multi-species biofilms: living with friendly neighbors. *FEMS Microbiol Rev* 2012;**36**:990-1004.

Ellison C, Brun YV. Mechanosensing: a regulation sensation. *Curr Biol* 2015;**25**:R113-R15.

Engler AJ, Sen S, Sweeney HL *et al.* Matrix elasticity directs stem cell lineage specification. *Cell* 2006;**126**:677-89.

Flemming H-C, Wingender J. The biofilm matrix. *Nat Rev Microbiol* 2010;**8**:623.

Flemming H-C, Wingender J, Szewzyk U *et al.* Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol* 2016;**14**:563-75.

Friedlander RS, Vlamakis H, Kim P *et al.* Bacterial flagella explore microscale hummocks and hollows to increase adhesion. *Proc Natl Acad Sci U S A* 2013;**110**:5624-9.

Gantner S, Schmid M, Dürr C *et al. In situ* quantitation of the spatial scale of calling distances and population density-independent N-acylhomoserine lactone-mediated communication by rhizobacteria colonized on plant roots. *FEMS Microbiol Ecol* 2006;**56**:188-94.

Gao L, Liu Y, Kim D *et al.* Nanocatalysts promote *Streptococcus mutans* biofilm matrix degradation and enhance bacterial killing to suppress dental caries *in vivo*. *Biomaterials* 2016;**101**:272-84.

Ge Y, Ren B, Zhou X *et al*. Novel dental adhesive with biofilm-regulating and remineralization capabilities. *Materials* 2017;**10**:26.

Grote J, Krysciak D, Streit WR. Phenotypic heterogeneity, a phenomenon that may explain why quorum sensing does not always result in truly homogeneous cell behavior. *Appl Environ Microbiol* 2015;**81**:5280-9.

Harapanahalli AK, Chen Y, Li J *et al.* Influence of adhesion force on *icaA* and *cidA* gene expression and production of matrix components in *Staphylococcus aureus* biofilms. *Appl Environ Microbiol* 2015;**81**:3369-78.

Hasan J, Webb HK, Truong VK *et al*. Selective bactericidal activity of nanopatterned superhydrophobic cicada *Psaltoda claripennis* wing surfaces. *Appl Microbiol Biotechnol* 2013;**97**:9257-62.

Haswell ES, Phillips R, Rees DC. Mechanosensitive channels: what can they do and how do they do it? *Structure* 2011;**19**:1356-69.

Hidalgo G, Burns A, Herz E *et al.* Functional tomographic fluorescence imaging of pH microenvironments in microbial biofilms by use of silica nanoparticle sensors. *Appl Environ Microbiol* 2009;**75**:7426-35.

Hizal F, Choi C-H, Busscher HJ *et al.* Staphylococcal adhesion, detachment and transmission on nanopillared Si surfaces. *ACS Appl Mater Interfaces* 2016;**8**:30430-9.

Hizal F, Rungraeng N, Lee J *et al.* Nanoengineered superhydrophobic surfaces of aluminum with extremely low bacterial adhesivity. *ACS Appl Mater Interfaces* 2017;**9**:12118-29.

Hoffman MD, Zucker LI, Brown PJ *et al.* Timescales and frequencies of reversible and irreversible adhesion events of single bacterial cells. *Anal Chem* 2015;**87**:12032-39.

Hou J, Veeregowda DH, Van de Belt-Gritter B *et al.* Extracellular polymeric matrix production and relaxation under fluid shear and mechanical pressure in *Staphylococcus aureus* biofilms. *Appl Environ Microbiol* 2017;doi:10.1128/AEM.01516-17.

Huang YF, Yi SP, Lv ZS *et al*. Facile fabrication of superhydrophobic coatings based on two silica sols. *Colloid Polymer Sci* 2016;**294**:1503-9.

Kaldalu N, Hauryliuk V, Tenson T. Persisters—as elusive as ever. *Appl Microbiol Biotechnol* 2016;**100**:6545-53.

Kester JC, Fortune SM. Persisters nad beyond: Mechanisms of phenotypic drug resistance and drug tolerance in bacteria. *Crit Rev Biochem Molec Biol* 2014;**49**:91-101.

Kocer A. Mechanisms of mechanosensing—mechanosensitive channels, function and re-engineering. *Curr Opin Chem Biol* 2015;**29**:120-7.

Koo H, Yamada KM. Dynamic cell-matrix interactions modulate microbial biofilm and tissue 3D microenvironments. *Curr Opin Cell Biol* 2016;**42**:102-12.

Lai S, Tremblay J, Déziel E. Swarming motility: a multicellular behaviour conferring antimicrobial resistance. *Environ Microbiol* 2009;**11**:126-36.

Lee K, Hahn LD, Yuen WW *et al.* Metal-enhanced fluorescence to quantify bacterial adhesion. *Adv Mater* 2011;**23**:H101-H104.

Li G, Brown PJ, Tang JX *et al.* Surface contact stimulates the just-in-time deployment of bacterial adhesins. *Mol Microbiol* 2012;**83**:41-51.

Li J, Busscher HJ, Swartjes JJ *et al.* Residence-time dependent cell wall deformation of different *Staphylococcus aureus* strains on gold measured using surface-enhanced-fluorescence. *Soft Matter* 2014;**10**:7638-46.

Linklater DP, Juodkazis S, Rubanov S *et al*. Influence of superhydrophobicity on the bactericidal efficiency of black silicon surfaces. *ACS Appl Mater Interf* 2017;doi: 10.1021/acsami.7b05707

Liu Y, Busscher HJ, Zhao B *et al.* Surface-adaptive, antimicrobially loaded, micellar nanocarriers with enhanced penetration and killing efficiency in staphylococcal biofilms. *ACS Nano* 2016;**10**:4779-89.

Loozen G, Ozcelik O, Boon N *et al.* Inter-bacterial correlations in subgingival biofilms: a large-scale survey. *J Clin Periodontol* 2014;**41**:1-10.

Ma L, Conover M, Lu H *et al.* Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathog* 2009;**5**:e1000354.

Marsh PD, Do T, Beighton D *et al.* Influence of saliva on the oral microbiota. *Periodontol 2000* 2016;**70**:80-92.

Mei L, Busscher HJ, Van Der Mei HC *et al.* Oral bacterial adhesion forces to biomaterial surfaces constituting the bracket–adhesive–enamel junction in orthodontic treatment. *Eur J Oral Sci* 2009;**117**:419-26.

Méndez-Vilas A, Gallardo-Moreno AM, González-Martín ML. Atomic force microscopy of mechanically trapped bacterial cells. *Microsc Microanal* 2007;**13**:55-64.

Muñoz-Egea M-C, García-Pedrazuela M, Mahillo I *et al.* Effect of ciprofloxacin in the ultrastructure and development of biofilms formed by rapidly growing mycobacteria. *BMC Microbiol* 2015;**15**:18.

Muszanska AK, Nejadnik MR, Chen Y *et al.* Bacterial adhesion forces with substratum surfaces and the susceptibility of biofilms to antibiotics. *Antimicrob Agents Chemother* 2012;**56**:4961-4.

Nadell CD, Bassler BL. A fitness trade-off between local competition and dispersal in *Vibrio cholerae* biofilms. *Proc Natl Acad Sci U S A* 2011;108:14181-5.

Nesbitt WE, Doyle RJ, Taylor KG *et al.* Positive cooperativity in the binding of *Streptococcus sanguis* to hydroxylapatite. *Infect Immun* 1982;**35**:157-65.

Neu TR. Microbial footprints and the general ability of microorganisms to label interfaces. *Can J Microbiol* 1992;**38**:1005-8.

Nivens DE, Chambers JQ, Anderson TR *et al.* Long-term, on-line monitoring of microbial biofilms using a quartz crystal microbalance. *Anal Chem* 1993;**65**:65-9.

Nuryastuti T, Krom BP, Aman AT *et al.* Ica-expression and gentamicin susceptibility of *Staphylococcus epidermidis* biofilm on orthopedic implant biomaterials. *J Biomed Mater Res Part A* 2011;**96**:365-71.

Olsen I. Biofilm-specific antibiotic tolerance and resistance. *Eur J Clin Microbiol Infect Dis* 2015;**34**:877-86.

O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. *Ann Rev Microbiol* 2000;**54**:49-79.

O'Toole GA, Wong GC. Sensational biofilms: surface sensing in bacteria. *Curr Opin Microbiol* 2016;**30**:139-46.

Otto K, Silhavy TJ. Surface sensing and adhesion of *Escherichia coli* controlled by the Cpx-signaling pathway. *Proc Natl Acad Sci U S A* 2002;**99**:2287-92.

Pamp SJ, Tolker-Nielsen T. Multiple roles of biosurfactants in structural biofilm development by *Pseudomonas aeruginosa. J Bacteriol* 2007;**189**:2531-39.

Paula AJ, Silveira CP, Martinez DSfT *et al.* Topography-driven bionano-interactions on colloidal silica nanoparticles. *ACS Appl Mater Interfaces* 2014;**6**:3437-47.

Periasamy S, Joo H-S, Duong AC *et al.* How *Staphylococcus aureus* biofilms develop their characteristic structure. *Proc Natl Acad Sci U S A* 2012;**109**:1281-6.

Perozo E, Kloda A, Cortes DM *et al.* Physical principles underlying the transduction of bilayer deformation forces during mechanosensitive channel gating. *Nat Struct Mol Biol* 2002;**9**:696.

Pham VT, Truong VK, Quinn MD *et al*. Graphene induces formation of pores that kill spherical and rod-shaped bacteria. *ACS Nano*. 2015;**9**:8458-67.

Poltak SR, Cooper VS. Ecological succession in long-term experimentally evolved biofilms produces synergistic communities. *ISME J* 2011;**5**:369.

Prüss BM. Involvement of two component signaling in bacterial motility and biofilm development. *J Bacteriol* 2017;**199**:10.1128/JB.00259-17.

Puddu V, Perry CC. Peptide adsorption on silica nanoparticles: evidence of hydrophobic interactions. *ACS Nano* 2012;**6**:6356-63.

Rani SA, Pitts B, Beyenal H *et al.* Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. *J Bacteriol* 2007;**189**:4223-33.

Rickard AH, Gilbert P, High NJ *et al.* Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends Microbiol* 2003;**11**:94-100.

Roosjen A, Van der Mei HC, Busscher HJ *et al.* Microbial adhesion to poly(ethylene oxide) brushes: influence of polymer chain length and temperature. *Langmuir* 2004;**20**:10949-55.

Roth D, Finkelshtein A, Ingham C *et al.* Identification and characterization of a highly motile and antibiotic refractory subpopulation involved in the expansion of swarming colonies of *Paenibacillus vortex*. *Environ Microbiol* 2013;**15**:2532-44.

Sambanthamoorthy K, Feng XR, Patel R *et al*. Antimicrobial and antibiofilm potential of biosurfactants isolated from lactobacilli against multi-drug-resistant pathogens. *BMC Microbiol* 2014;**14**:197.

Schakenraad J, Busscher H, Wildevuur CR *et al.* The influence of substratum surface free energy on growth and spreading of human fibroblasts in the presence and absence of serum proteins. *J Biomed Mater Res Part A* 1986;**20**:773-84.

Si F, Li B, Margolin W *et al.* Bacterial growth and form under mechanical compression. *Sci Reports* 2015;**5**:11367.

Simmons LM, Al-Jawad M, Kilcoyne SH *et al.* Distribution of enamel crystallite orientation through an entire tooth crown studied using synchrotron X-ray diffraction. *Eur J Oral Sci* 2011;**119**:19-24.

Sjollema J, Van der Mei HC, Uyen HM *et al*. Direct observation of cooperative effects in oral streptococcal adhesion to glass by analysis of the spatial arrangement of adhering bacteria. *FEMS Microbiol Lett* 1990;**69**:263-70.

Sliusarenko O, Cabeen MT, Wolgemuth CW *et al.* Processivity of peptidoglycan synthesis provides a built-in mechanism for the robustness of straight-rod cell morphology. *Proc Natl Acad Sci U S A* 2010;**107**:10086-91.

Song F, Koo H, Ren D. Effects of material properties on bacterial adhesion and biofilm formation. *J Dent Res* 2015;**94**:1027-34.

Song F, Brasch ME, Wang H *et al.* How bacteria respond to material stiffness during attachment: a role of *Escherichia coli* flagellar motility. *ACS Appl Mater Interfaces* 2017;**9**:22176-84.

Stewart PS, Franklin MJ. Physiological heterogeneity in biofilms. *Nat Rev Microbiol* 2008;**6**:199.

Stoodley P, Wefel J, Gieseke A *et al.* Biofilm plaque and hydrodynamic effects on mass transfer, fluoride delivery and caries. *J Amer Dent Assoc* 2008;**139**:1182-90.

Sullan RMA, Beaussart A, Tripathi P *et al.* Single-cell force spectroscopy of pili-mediated adhesion. *Nanoscale* 2014;**6**:1134-43.

Svensson S, Forsberg M, Hulander M *et al.* Role of nanostructured gold surfaces on monocyte activation and *Staphylococcus epidermidis* biofilm formation. *Int J Nanomedicine* 2014;**9**:775.

Thewes N, Thewes A, Loskill P *et al.* Stochastic binding of *Staphylococcus aureus* to hydrophobic surfaces. *Soft Matter* 2015;**11**:8913-9.

Tiller JC, Liao C-J, Lewis K *et al.* Designing surfaces that kill bacteria on contact. *Proc Natl Acad Sci U S A* 2001;**98**:5981-5.

Timmons C, Zisman W. The effect of liquid structure on contact angle hysteresis. *J Colloid Interface Sci* 1966;**22**:165-71.

Toguchi A, Siano M, Burkart M *et al.* Genetics of swarming motility in *Salmonella enterica serovar Typhimurium*: critical role for lipopolysaccharide. *J Bacteriol* 2000;**182**:6308-21.

Tolker-Nielsen T. Biofilm development. *Microbiol Spectrum* 2015;**3**:MB-0001-2014.

Tripathy A, Sen P, Su B *et al*. Natural and bioinspired nanostructured bactericidal surfaces. *Adv Colloid Interface Sci* 2017;**248**:85-104.

Tuson HH, Weibel DB. Bacteria–surface interactions. *Soft Matter* 2013;**9**:4368-80.

Vadillo-Rodriguez V, Beveridge TJ, Dutcher JR. Surface viscoelasticity of individual Gram-negative bacterial cells measured using atomic force microscopy. *J Bacteriol* 2008;**190**:4225-32.

Vadillo-Rodriguez V, Dutcher JR. Dynamic viscoelastic behavior of individual Gram-negative bacterial cells. *Soft Matter* 2009;**5**:5012-9.

Van der Mei HC, Cox SD, Geertsema-Doornbusch GI *et al.* A critical appraisal of positive cooperativity in oral streptococcal adhesion: Scatchard analyses of adhesion data versus analyses of the spatial arrangement of adhering bacteria. *Microbiology* 1993;**139**:937-48.

Van der Mei HC, Rustema-Abbing M, De Vries J *et al.* Bond strengthening in oral bacterial adhesion to salivary conditioning films. *Appl Environ Microbiol* 2008;**74**:5511-5.

Van Hoogmoed C, Van der Kuijl-Booij M, Van der Mei HC *et al.* Inhibition of *Streptococcus mutans* NS adhesion to glass with and without a salivary conditioning film by biosurfactant-releasing *Streptococcus mitis* strains. *Appl Environ Microbiol* 2000;**66**:659-63.

Van Hoogmoed C, Dijkstra R, Van der Mei HC *et al.* Influence of biosurfactant on interactive forces between mutans streptococci and enamel measured by atomic force microscopy. *J Dent Res* 2006;**85**:54-8.

Vega NM, Gore J. Collective antibiotic resistance: mechanisms and implications. *Curr Opin Microbiol* 2014;**21**:28-34.

Velraeds M, Van der Mei HC, Reid G *et al.* Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from Lactobacillus isolates. *Appl Environ Microbiol* 1996;**62**:1958-63.

Vert M, Doi Y, Hellwich K-H *et al.* Terminology for biorelated polymers and applications (IUPAC Recommendations 2012). *Pure Appl Chem* 2012;**84**:377-410.

Vlamakis H, Aguilar C, Losick R *et al.* Control of cell fate by the formation of an architecturally complex bacterial community. *Genes Dev* 2008;**22**:945-53.

Vroman L. Finding seconds count after contact with blood (and that is all I did). *Colloids Surf B: Biointerfaces* 2008;**62**:1-4.

Wagner M, Taherzadeh D, Haisch C *et al.* Investigation of the mesoscale structure and volumetric features of biofilms using optical coherence tomography. *Biotechnol Bioeng* 2010;**107**:844-53.

Wang S, Parsek MR, Wozniak DJ *et al.* A spider web strategy of type IV pili‐mediated migration to build a fibre‐like Psl polysaccharide matrix in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol* 2013;**15**:2238-53.

Wang Y, Subbiahdoss G, Swartjes J *et al.* Length-scale mediated differential adhesion of mammalian cells and microbes. *Adv Funct Mater* 2011;**21**:3916-23.

Wessel SW, Chen Y, Maitra A *et al.* Adhesion forces and composition of planktonic and adhering oral microbiomes. *J Dent Res* 2014;**93**:84-8.

Wit PJ, Busscher HJ. Site selectivity in the deposition and redeposition of polystyrene particles to glass. *J Colloid Interface Sci* 1998;**208**:351-2.

Wolska KI, Grudniak AM, Rudnicka Z *et al.* Genetic control of bacterial biofilms. *J Appl Genet* 2016;**57**:225-38.

Xavier JB, Foster KR. Cooperation and conflict in microbial biofilms. *Proc Natl Acad Sci U S A* 2007;**104**:876-81.

Xiao J, Klein MI, Falsetta ML *et al.* The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. *PLoS Pathog* 2012;**8**:e1002623.

Zhao K, Tseng BS, Beckerman B *et al.* Psl trails guide exploration and microcolony formation in early *P. aeruginosa* biofilms. *Nature* 2013;**497**:388.

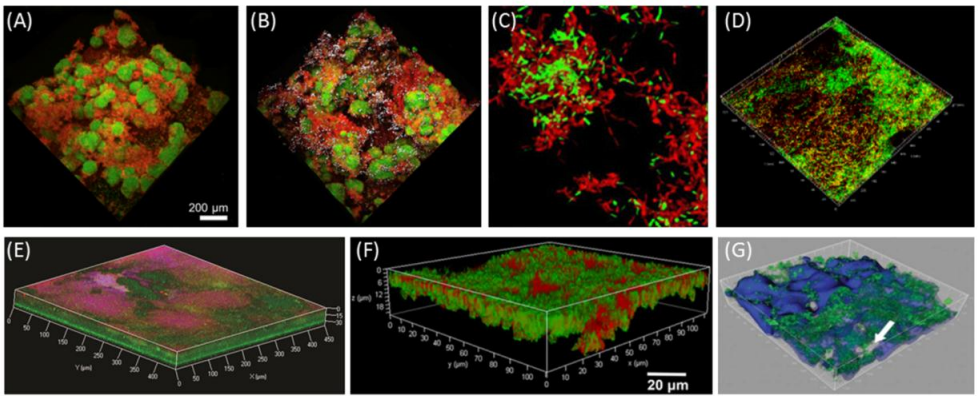

Figure 1. Examples of heterogeneously developing micro-environments in biofilms.

(A) Red-fluorescent patches of EPS in a *Streptococcus mutans* (green-fluorescent) biofilm on saliva-coated hydroxyapatite. (Gao *et al*. 2016, reprinted with permission from Elsevier Ltd.).

(B) Scattered red-fluorescent patches corresponding to EPS in 24 h *Staphylococcus epidermidis* (green-fluorescent) biofilm grown on saliva-coated hydroxyapatite discs with orthogonal distribution of catalytic nano particles (white) (Gao *et al*. 2016, reprinted with permission from Elsevier Ltd.).

(C) Live (green-fluorescent) and dead (red-fluorescent) *Mycobacterium smegmatis* scattered through a biofilm on a hydrophobic polystyrene surface after 72 h exposure to ciprofloxacin (Muñoz-Egea *et al*. 2015, reprinted with permission from BioMed Central), indicating differential susceptibility to ciprofloxacin and presumably reflecting a variation in physiological state.

(D) Distribution of bacteria and EPS after live-dead staining in a multispecies oral biofilm with *S. mutans, Streptococcus sanguinis,* and *Streptococcus gordonii*, formed on a dental adhesive surface (Ge *et al*. 2017, reprinted with permission from MDPI).

(E) Evolution of spatially-segregated communities in *Burkholderia cenocepacia* biofilms on polystyrene, with different colony morphotypes showing differently colored fluorescence (Poltak and Cooper 2011, reprinted with permission from the Nature Publishing group). Three distinct colony morphotypes reproducibly emerged within biofilms inoculated with a single ancestor.

(F) Uneven pattern of penetration and accumulation of Nile-red loaded micelles into a staphylococcal biofilm grown on glass (Liu *et al*. 2016 reprinted with permission from American Chemical Society). The micelle carriers have a poly(ethylene)glycol shell and are biologically invisible allowing them to enter a biofilm, where they acquire a cationic charge at low pH to

interact electrostatically with the bacterial cell surface. Thus the observed distribution of Nile-red likely demonstrates heterogeneity with respect to channelization and possibly low pH micro-environments within the biofilm.

(G) *In vitro* grown *S. mutans* biofilm on hydroxyapatite, with green-fluorescent bacteria and blue-fluorescent EPS patches occurring unevenly across the biofilm (Stoodley *et al*. 2008**,** reprinted with permission from Elsevier Ltd.).

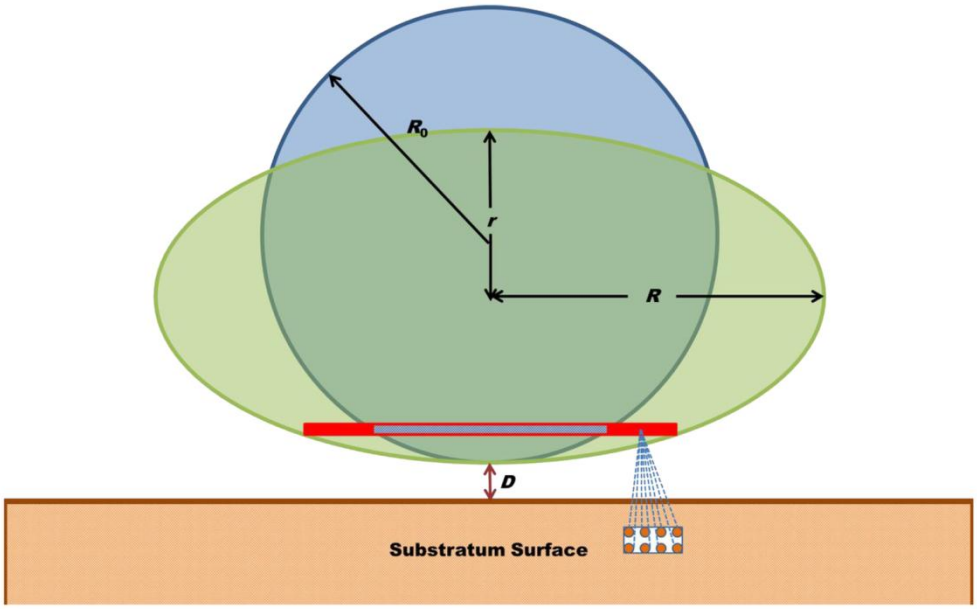

Figure 2. Bacterial cell wall deformation under the influence of adhesion forces arising from a substratum surface. (Chen *et al*. 2014, reprinted with permission from American Society for Microbiology). An undeformed bacterium with a radius R approaching a substratum surface comes under the influence of the adhesion forces arising from the substratum. It gradually deforms, which brings more molecules (solid red region) under the influence of the adhesion forces, stimulating further adhesion until opposing forces arising from the rigid bacterial cell wall and increased intracellular pressure fully counteract the adhesion force.

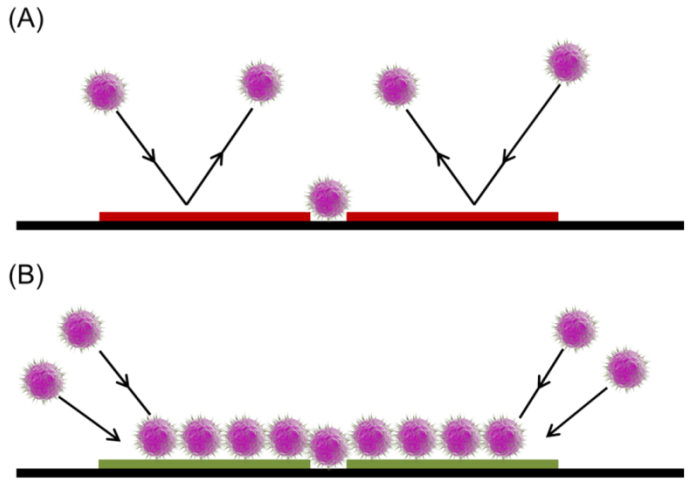

Figure 3. Bacterially-induced substratum surface heterogeneities as a means of communication and interaction between initially adhering bacteria.

(A) Certain strains of bacteria excrete biosurfactants that spread over the substratum surface, modifying the immediate surrounding surface so that it is less favorable (red colored) for adherence by other bacteria.

(B) Positive cooperativity is the mechanism by which an adhering bacterium changes the conformation of adsorbed proteins in its immediate surroundings or produces adhesive EPS, generating a more favorable surface (green colored) for adherence by other bacteria.

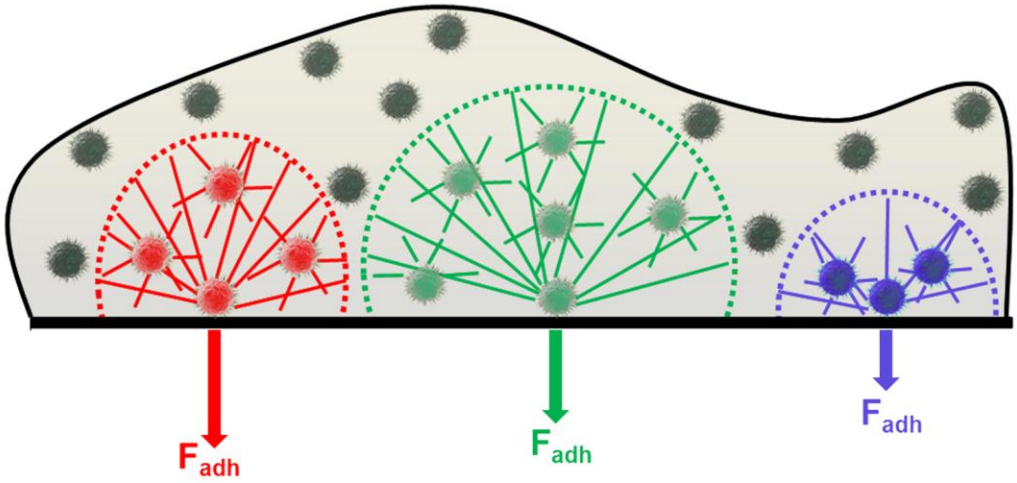

Figure 4. The commanding role in adaptive responses of initial colonizers in a biofilm. Initially adhering bacteria sense different local adhesion forces which triggers different adaptive responses that spread through the biofilm by diffusion of quorum-sensing molecules until their concentration is below a detectable threshold and the commands given are lost, limiting heterogeneous micro-environment development in space and time. Micro-environments, including the adhesion forces that trigger differential responses, the commanding organisms and obeying inhabitants of the micro-environment are indicated by different colors.

Table 1. Summary of observations involving the emergence of different phenotypes across a wide variety of different bacterial strains and species and on different substrata. Relevant experimental details are included, when available in the references used.

STRAIN	SUBSTRATA	OBSERVATIONS	RELEVANT DETAILS	REFERENCES
SINGLE SPECIES STUDIES				
Caulobacter crescentus	glass	bacteria made multiple surface contact before transitioning from reversible to irreversible adhesion.	WCA < 30 degrees; microfluidic flow conditions	Hoffman *et al.* 2015
Escherichia coli	micron-scale patterned	surface appendages enable bacteria to overcome unfavorable	static conditions	Friedlander *et al.* 2013

	PDMS	surface patterns		
E. coli	PS well plates	pH heterogeneity within biofilms	type of polystyrene and WCA not reported; shaking conditions (30 rpm)	Hidalgo *et al.* 2009
E. coli	hydrophobic glass beads	Cpx pathway regulates adhesion-induced gene expression		Otto *et al.* 2002
Lactobacillus plantarum	lectin monolayer and hydrophobic coatings	time-dependent binding to lectin layers; fast, time-independent binding to hydrophobic coatings		Beaussart *et al.* 2013
Mycobacteria	hydrophobic slides	biofilm viability and structure affected by antibiotic presence	30 min initial adhesion; orbital shaking (80 rpm)	Muñoz-Egea *et al.* 2015
Pseudomonas aeruginosa	glass, SS, PET, hydrophobic SS, hydrophilic PET	flagella increase adhesion on hydrophobic surfaces; straight and long flagella on PET and SS; curved and short flagella on glass	WCA and surface roughness provided for all surfaces	Bruzaud *et al.* 2015
Staphylococcus aureus	PE, SS	adhesion force and nisin efflux pump efficacy was highest on hydrophobic PE surfaces	WCA for PE 85 and for SS 35 degrees; static conditions	Carniello *et al.* 2018
S. aureus	PE, SS, Ti–6Al–4V alloy, HA	adhesion forces, bacterial retention and viability are substratum related	WCA for SS 49, for PE 82, for Ti–6Al–4V 69 and for HA 95 degrees	Alam *et al.* 2017
S. aureus	PE, SS, PMMA	matrix production and *ica*A gene expression is inversely related with adhesion forces	WCA for SS 33, for PMMA 69 and for PE 84 degrees; submicron roughness	Harapanahalli *et al.* 2015
S. aureus	glass	cell wall deformation and long-range adhesion forces are related		Chen *et al*. 2014
S. aureus	glass	heterogeneous pattern of penetration and accumulation of Nile-red loaded micelles into		Liu *et al.* 2016

		biofilms		
Staphylococcus epidermidis	QA-coatings	strong adhesion forces cause bacterial death	surfaces carry a positive charge	Asri *et al.* 2014
S. epidermidis	steel (SS) SS, PMMA, PE	substratum dependent EPS production and gentamicin susceptibility		Nuryastuti *et al.* 2011
Streptococcus sobrinus	DDS coatings	substratum hydrophobicity determines bacterial retention, with less impact on adhesion	WCA for DDS coatings 90 and glass 20 degrees	Bos *et al.* 2000
MULTIPLE SPECIES STUDIES				
S. aureus *E. coli*	nanoporous or nanopillared, hydrophobized aluminum oxide	adhesion to hydrophobic, nanopillared surfaces smaller than to hydrophilic or nanoporous surfaces	WCA varies from 0 - 162 degrees; static and flow conditions	Hizal *et al*. 2017
S. aureus *P. aeruginosa*	plasma etched black silicon	smaller, more densely packed pillars exhibited the greatest bactericidal activity	WCA varies from 8 - 160 degrees; pillar heights of 212, 475 to 610 nm	Linklater *et al.* 2017
S. aureus *S. epidermidis*	nanopillared-Si wafers	nanopatterning stimulates EPS-production and yields bacterial killing	regular patterning with sharply pointed pillars; flow conditions	Hizal *et al.* 2016
P. aeruginosa *S. aureus*	graphene nanosheets	graphene nanosheets creates pores in bacterial cell walls, causing bacterial death.	roughness of the graphene sheets varies between 19 - 44 nm.	Pham *et al*. 2015
Branhamella catarrhalis *Bacillus*	Cicada wing, nanopatterned surfaces	nanopatterning kills only Gram-negative bacteria		Hasan *et al*. 2013

subtilis *E. coli* *P. aeruginosa* *Pseudomonas fluorescens Pseudomonas. maritimus* *S. aureus*				
Asticcacaulis biprosthecum *Agrobacterium tumefaciens* *C. crescentus*	glass	reversible attachment of bacterial cells is mediated by motile cells bearing pili triggering adhesin production.		Li *et al*. 2012
S. aureus *S. epidermidis* *P. aeruginosa*	SR; SR with Pluronic brush	adhesion forces dictated the transition from a planktonic to a biofilm mode of growth	flow conditions; WCA for SR 110 degrees	Muszanska *et al.* 2012
Actinomyces naeslundii *Lactobacillus acidophilus* *Streptococcus mitis* *Streptococcus mutans* *Streptococcus oralis* *Streptococcus sanguinis* *S. sobrinus*	SS, bovine enamel	salivary conditioning films reduce adhesion forces	salivary films reduced WCA of SS to 23 and of enamel to 26 degrees; sub-micron roughness	Mei *et al*. 2009
S. aureus *S.*	various substrata	staphylococcal biofilms show four distinct states, growing aerobically,	different reactor systems	Rani *et al*. 2007

epidermidis		growing fermentatively, dead, and dormant, contributing to their tolerance to antimicrobials		
P. aeruginosa *S. epidermidis*	PEO-coatings	PEO-brush coating reduced adhesion of all strains and species	flow conditions	Roosjen *et al*. 2004
Marinobacter hydrocarbonoclasticus *Psychrobacter sp.* *Halomonas pacifica*	glass	dissolved organic carbon alters surface properties with an impact on adhesion	flow conditions; surfaces conditioned with natural seawater	Bakker *et al*. 2003