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Abstract

Microbes have evolved sophisticated strategies to colonize biotic and abiotic surfaces. Forces play a central role in microbial cell adhesion processes, yet until recently these were not accessible to study at the molecular scale. Unlike traditional assays, atomic force microscopy (AFM) is capable to study forces in single cell surface molecules and appendages, in their biologically-relevant conformation and environment. Recent AFM investigations have demonstrated that bacterial pili exhibit a variety of mechanical responses upon contact with surfaces and that cell surface adhesion proteins behave as force-sensitive switches, two phenomena that play critical roles in cell adhesion and biofilm formation. AFM has also enabled to assess the efficiency of sugars, peptides and antibodies in blocking cell adhesion, opening up new avenues for the development of antiadhesion therapies against pathogens.

Introduction

Biofilms represent the major lifestyle of microorganisms [1,2]. These multicellular communities develop on a wide variety of biotic and abiotic surfaces, and are mechanically stabilized by self-produced extracellular polymeric substances (EPS) [3]. The EPS matrix provides protection to the cells within an heterogeneous physico-chemical 3D structure, that endows biofilm cells with remarkable emergent features such as quorum sensing, resistance to antimicrobials and cell differentiation [1,2,4-6], properties that clearly differ from those of free-living microorganisms. In nature, biofilms are ubiquitous: they can be beneficial when driving biogeochemical cycles or involved in biotechnological processes, but when associated with surface colonization (plants, animals, medical devices), they can lead to life-threatening infections [1,2,7]. In the medical context, biomaterials and host tissues are the target of chronic infections caused by multiple pathogens such as *Escherichia coli*, *Staphylococcus aureus, Staphylococcus epidermis* and *Pseudomonas aeruginosa* [8]. Biofilms show increased antibiotic resistance, likely due to the very slow diffusion of the drugs into these thick communities, and to the metabolic heterogeneity and cooperation of cells within the biofilm [9,10]. As biofilm formation starts with the initial adhesion of microbial cells to surfaces, many studies have focused on trying to understand the mechanisms, either specific or non-specific, driving this process [11]. What has become clear is that microbes have evolved a plethoric set of adhesive strategies during evolution, including non-specific surface forces, driven *e.g.* by surface charge and hydrophobicity, and specific interactions involving surface appendages like pili and flagella, and adhesive proteins (adhesins) expressed on the cell surface. Understanding these forces is crucial to fully understand cell adhesion and biofilm formation, and subsequently to design efficient antiadhesive therapies that are complementary to antibiotic treatments [12-15].

Unlike traditional ensemble techniques, atomic force microscopy (AFM) enables to quantify the forces engaged in microbial cell adhesion, at single cell and single molecule levels, and in physiological conditions [16-18]. AFM topographic images are obtained by scanning a sharp tip, mounted on a soft cantilever, over the sample in x and y directions. Due to interaction forces between the tip and the sample, the cantilever vertically bends and its deflection is detected via a laser beam focused at the free end of the cantilever and reflected into a photodiode. This deflection is proportional to force, which can therefore be measured with piconewton sensitivity. In single-molecule force spectroscopy (SMFS), the tip is approached towards - and retracted from - the sample, and the interaction force measured as a function of the separation distance. Modifying the tip with specific ligands provides information on the localization, binding strength, and mechanics of cell surface molecules. A variation of this is single-cell force spectroscopy (SCFS), where a single cell is attached to the cantilever to quantify single-cell adhesion forces. In this review, we discuss recent breakthroughs made in understanding the forces guiding microbial adhesion both at the cellular and molecular levels, and in assessing the antiadhesion potential of small molecules.

Bacterial pili as multifunctional adhesive structures

Many bacterial species produce cell surface protein filaments known as pili (or fimbriae) [19,20]. These appendages play multiple functional roles, among which promoting adhesion to other bacteria, host tissues, and abiotic surfaces. In Gram-positive pili, the attachment of the subunits (pilins) to each other is made through sortase-catalyzed covalent bonding, while Gram-negative pili are formed by noncovalent interactions between pilins. AFM and other single-molecule tools have demonstrated that these structural differences lead to very different mechanical responses when pili are subjected to force (Fig. 1).

Pioneering laser tweezer experiments on the Gram-negative *Neisseria gonorrhoeae* type IV pili revealed that they retract and generate substantial force (100 pN) involved in twitching motility and host cell adhesion [21]. Use of a micropillar assay showed that pili form bundles made up of 10 filaments, that act as coordinated retractable units with forces in the nanonewton range that could be important for infection [22]. Upon detachment from hydrophobic surfaces, type IV pili from *P. aeruginosa* feature constant force plateaus resulting from force-induced conformational changes, and linear force peaks reflecting nanospring behaviors [23] (Fig. 1a). This enables individual pili to sustain forces up to 250 pN, thus helping the bacteria to withstand shear forces in physiological environments, such as mucus flow or bloodstream [24-26]. Adhesion of *P. aeruginosa* to host cells actually involves a complex mix of interactions: short-range cohesive interactions between the bacterial outer membrane and the host membrane, and long-range constant force interactions reflecting the extension of bacterial pili and the extraction of host tethers [23]. This highlights the role that type IV pili mechanics play in controlling bacterial attachment to biotic and abiotic surfaces. For *E. coli* fimbriae, the different Fim domains were found to be organized in a hierarchical mechanical architecture: domains close to the outer membrane present higher mechanical stability than domains exposed at the apex of the appendage [27].

Because of their covalent nature, Gram-positive pili show extreme stiffness and resistance to force. Under low loading force, pili from the probiotic *Lactobacillus rhamnosus* GG (LGG) shows a zipper-like adhesion involving multiple pilin subunits along the fiber, while at high loads, pili behave as inextensible nanosprings, meaning they resist force without unfolding [28,29] (Fig. 1b). These features contribute to favor bacterial attachment to intestinal host cells [30]. Along the same line, *Streptococcus pyogenes* pilins do not unfold even when subjected to high loads [31] as a result of stabilizing internal isopeptide bonds [32].

Echelman *et al.* demonstrated that CnaA loop domains present in pili of *Corynebacterium diphtheriae* and *Actinomyces oris* are highly stable, resist pulling forces of up to 500 pN thus helping bacteria to face shear forces [33]. On *Streptococcus pneumoniae*, it was shown that the RrgA adhesive domain of the pilus-1 contains two regions that bind simultaneously to host fibronectin, a glycoprotein present in the extracellular matrix. This favours an efficient gentle scan of the colonized surface for specific cell interaction and invasion [34]. Then, the backbone protein of this pilus, RrgB, binds to collagen I in a force-dependent manner to strongly anchor the bacteria to its host [35].

Microbial adhesins as force-sensitive switches in cell adhesion

Adhesins are proteins anchored into microbial cell walls that mediate the specific adhesion of the cells to protein-coated biomaterials and host tissues. Many adhesins are now well-characterized, yet their binding mechanisms remains often poorly understood. SMFS and SCFS have been instrumental in characterizing the strength and dynamics of adhesin interactions, particularly in *S. epidermidis* and *S. aureus* which represent a common source of nosocomial biofilm infections, including methicillin-resistant strains (MRSA). During infection, these microorganisms express different adhesins that bind to biomaterial and host surfaces [36-38].

An important finding of the past years is that staphylococcal adhesins, including SdrG, ClfA, and ClfB, bind to their host proteins with ultrastrong forces, similar to that of a covalent bond (2 nN), while showing moderate affinity [39-41]. For decades the archetypical streptavidin-biotin complex has been considered to be the strongest biomolecular bond in nature (0.2 nN). With these new discoveries we are now starting to appreciate that bacteria have evolved bonds that are ten times stronger, enabling them to firmly attach to their host

during colonization and infection. What is the molecular mechanism behind the extreme mechanical stability of these complexes? SdrG and related adhesins bind through the multi-step dock, lock, and latch (DLL) mechanism [42] involving dynamic conformational changes of the protein that result in a greatly stabilized adhesin-ligand complex [39,43]. Molecular dynamics simulations recently revealed that the target peptide is confined in a screw-like manner in the binding pocket of SdrG, and that the binding strength of the complex results from numerous hydrogen bonds between the peptide backbone and SdrG, independent of peptide side chains [41]. As a result, unbinding forces involve the simultaneous rupture of numerous hydrogen bonds in a cooperative shear geometry. These strong bonds are biologically important as they enable staphylococci to attach firmly, while resisting mechanical stresses associated with fluid flow, cell surface contacts or epithelial turnover. An important lesson from these single-molecule experiments is that the mechanostability of molecular bonds measured out of equilibrium may not correlate with binding constants studied at equilibrium with classical bioassays.

Another intriguing discovery is that some adhesins behave as force-sensitive molecular switches that activates bacterial adhesion under mechanical tension [15,44,45] (Fig. 2). AFM experiments revealed that binding between ClfA and the blood protein Fg is weak at low tensile loading, but is dramatically enhanced (~1,5 nN) at high tension, implying that the adhesive function of ClfA is tightly regulated by mechanical force. Similarly, the bond between ClfB and loricrin is weak at low stress, but extremely strong at high stress, consistent with a two-state model whereby adhesion is enhanced through force-induced conformational changes in the ClfB molecule, from a weakly binding folded state to a strongly binding extended state. Binding of *S. aureus* bacterial cell surface protein A (SpA) to the large plasma glycoprotein von Willebrand factor (vWF) was found to be extremely strong and activated by

mechanical tension, being able to resist forces which largely outperform the strength of typical receptor-ligand bonds (Fig. 2). These results suggest that force induces conformational changes in the vWF molecule, from a globular to an extended state, leading to the exposure of cryptic binding sites to which SpA strongly binds.

Force-sensitive adhesion mechanisms have also been discovered in fungal biofilms [46]. In the pathogen *C. albicans*, cells adhere to biological and artificial surfaces, followed by cell aggregation. These interactions are chiefly mediated by cell surface Als (agglutinin-like sequence) adhesins [46,47]. Interestingly, Als proteins possess a short amyloid-forming core sequence that enables Als to form functional amyloids that are critical for cell adhesion and biofilm formation. A series of AFM and biological analyses have shown that under mechanical stress, structural changes in the adhesins lead to the exposure of hidden amyloid sequences, which favor cis β-sheet interactions between Als proteins laterally arranged on the cell surface [48-50]. This leads to the formation of nanodomains that promote cell aggregation [51]. In addition, Fluidic force microscopy (FluidFM), a recent force-controlled pipette technology [52], uncovered a novel mode of cell adhesion, that is, mediating trans β-sheet interactions between neighbouring cells [53,54]. In summary, force-activated amyloid β-sheet interactions play a dual role in cell-cell adhesion, that is, in formation of adhesin nanoclusters (*cis*-interactions) and in homophilic bonding between amyloid sequences on opposing cells (*trans*-interactions). Because amyloid-forming sequences are found in many microbial proteins, amyloid-based interactions could represent a general adhesion mechanism. Collectively, these studies demonstrate that mechanical force activates the binding of various adhesins, similar to what has been described for catch bonds between *E. coli* FimH and sugar residues on epithelial cells [55]. These catch bonds favor urinary tract infections by mediating strong

adhesion at a high flow rate. Owing to AFM and other force measuring techniques we are now appreciating that catch bond behaviors might be widespread among microbial pathogens.

Recently, force-sensitive behaviors have also been found in protein bridges between microbes and their host. Recent evidence indicates that Gram-positive bacteria such as *S. aureus* can invade host cells [56], enabling them to evade host immune defences and antibiotics. *S. aureus* attaches to endothelial cells *via* the binding of fibronectin (Fn)-binding proteins (FnBPA and FnBPB) to α5β1 integrins on the host cell surface. This interaction involves the extracellular matrix protein Fn, which acts as a bridging molecule between FnBPs and integrins. AFM demonstrated that FnBPA mediates bacterial adhesion to soluble Fn *via* strong forces (~1,500 pN), consistent with a high-affinity tandem β-zipper, and that the FnBPA-Fn complex further binds to immobilized α5β1 integrins with a much higher strength than the classical Fn-integrin bond (~100 pN). The high mechanical stability of the Fn bridge favors an invasion model in which Fn-binding by FnBPA leads to the exposure of cryptic integrin binding sites *via* allosteric activation, which in turn engage into a strong interaction with integrins. ClfA uses the blood plasma protein fibrinogen (Fg) to bind $\alpha_V\beta_3$ integrins by means of extremely strong forces, about ten times larger than those of classical integrin bonds [44]. This unexpected finding supports a model where the strong Fg bridge between ClfA and $\alpha_V\beta_3$ results from the force-induced exposure of strong, cryptic RGD binding sites in the Fg molecule. This stress-dependent interaction is believed to play a crucial role during sepsis, by causing endothelial cell apoptosis and loss of barrier integrity.

The use of force in antiadhesion therapy

Microbial pathogens are becoming more and more resistant to antibiotics. An appealing approach to complement antibiotics is the use of antiadhesive agents capable to

block the adhesion of pathogens to host tissues and biomaterials [12,13,15]. AFM is a valuable tool to assess the blocking activity of antiadhesion compounds, such as sugars, peptides and antibodies. The technique was used to assess the activity of sugars against clinical uropathogenic *E. coli* [57]. Compared to mannosidic monomers, fullerenes bearing multiple peripheral mannose residues showed greatly enhanced antiadhesion activity toward FimH, suggesting that the multivalent presentation of sugars is advantageous for antiadhesion therapy. Monoclonal antibodies raised against the *S. aureus* collagen-binding protein Cna, showed great efficiency in blocking bacterial adhesion to collagen surfaces [58]. Similarly, a peptide derived from β-neurexin was shown to inhibit homophilic cell-cell adhesion forces mediated by the surface adhesin SdrC [59].

Another branch of efforts is the design of antifouling coatings to prevent cell adhesion on implanted biomaterials. For instance, polyzwitterionic polymer brushes were found to reduce drastically the force needed to detach *Yersinia pseudotuberculosis* [60] and insertion of cationic nanoclusters in such brushes also enhanced the removal of *S. aureus* [61]. Similarly, the antiadhesive properties of negatively charged polymer brushes against *E. coli* were also demonstrated by AFM [62].

Conclusion

The studies discussed here emphasize that force and function are intimately connected in microbial cell adhesion. AFM is uniquely suited to tackle this problem at the single cell and single molecule levels. Major breakthroughs from the past years include unveiling the adhesive and mechanical responses of bacterial pili during cell adhesion, demonstrating that molecular complexes formed by adhesins show extreme mechanostability, explaining how pathogens can resist mechanical stresses during surface colonization and infection, and identifying new antiadhesion agents capable to potentially block microbial infection.

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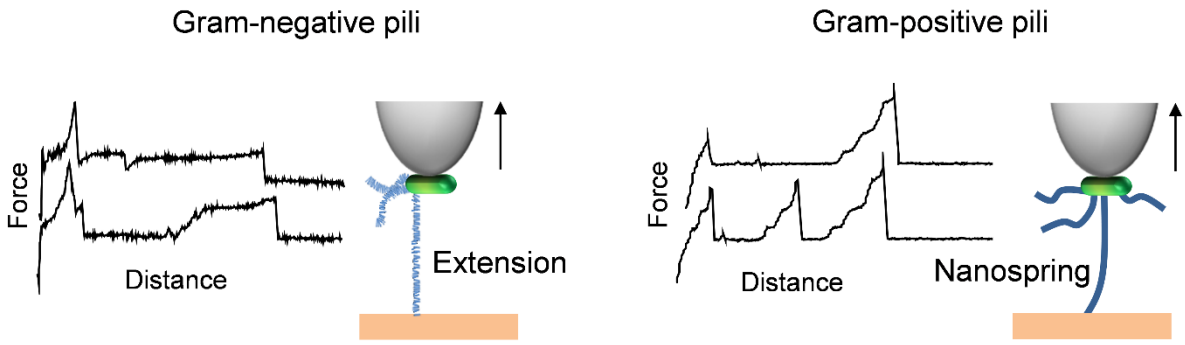

Figure 1. Bacterial pili feature a variety of mechanical and adhesive properties. (Left) Elongation of Gram-negative pili. (Right) Spring behaviour of Gram-positive pili.

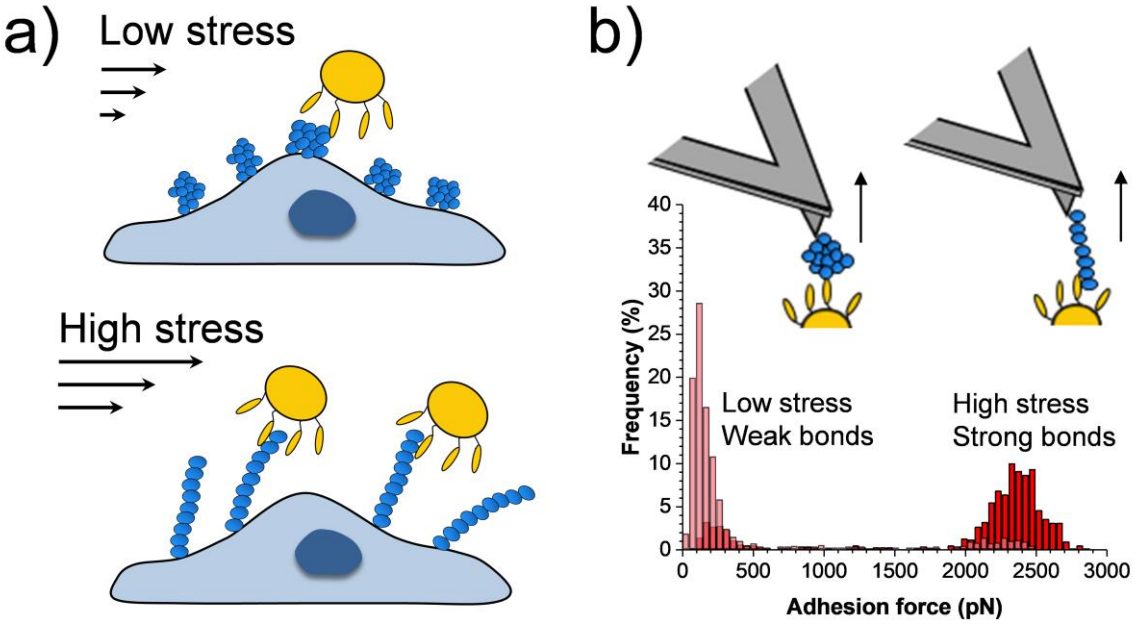

Figure 2. Force-induced activation of the SpA-vWF bond. (a) *S. aureus* bacteria adhere in larger amounts to vWF surfaces when the shear rate is increased. (b) AFM identifies an extremely strong SpA-vWF bond, capable of withstanding forces > 2 nN.