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MICROREVIEW

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What makes bacterial pathogens so sticky?

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Abstract

Pathogenic bacteria use a variety of cell surface adhesins to promote binding to host tissues and protein-coated biomaterials, as well as cell–cell aggregation. These cellular interactions represent the first essential step that leads to host colonization and infection. Atomic force microscopy (AFM) has greatly contributed to increase our understanding of the specific interactions at play during microbial adhesion, down to the single-molecule level. A key asset of AFM is that adhesive interactions are studied under mechanical force, which is highly relevant as surface-attached pathogens are often exposed to physical stresses in the human body. These studies have identified sophisticated binding mechanisms in adhesins, which represent promising new targets for antiadhesion therapy.

1 | AFM TO STUDY MICROBIAL ADHESION AT THE SINGLE-MOLECULE LEVEL

Adhesion of microbial pathogens is often the first key step leading to successful infection. Bacteria and fungi have evolved a variety of cell surface proteins (adhesins) to specifically bind to host cells and protein-coated biomaterials. These interactions enable the cells to colonize their host and can trigger molecular signalling pathways in host cells. Whereas the biology of microbial adhesins has been widely investigated, little is currently known about their molecular interactions. Traditionally, biomolecular assays (e.g., surface plasmon resonance, SPR) are used to study the interactions (e.g., dissociation constant) of recombinant protein domains purified from *Escherichia coli*. These in vitro analyses may alter protein folding and functionality, and are performed at equilibrium, that is, in conditions that are often far from the real life.

Analysing fully functional adhesins directly on cell surfaces, in a biologically relevant conformation, is therefore very challenging. Atomic force microscopy (AFM) has proven to be uniquely suited to address this problem at the single-molecule level (Xiao & Dufrêne, 2016). The heart of the instrument is a very sharp tip that is brought in contact with the sample and scans the surface while probing the small forces between tip and sample. A piezoelectric scanner moves the tip with high accuracy, which is mounted on a soft cantilever, and deformation of this cantilever is measured with a laser beam, enabling precise determination of the force on the tip. This enables to image microbial structures to molecular resolution and in aqueous buffers, without the need for labelling or fixation. In single-molecule force spectroscopy the tip is moved towards—and retracted from—the sample, and the interaction force is measured as a function of separation distance. Modifying the tip with specific ligands provides information on the localization, binding strength and mechanics of cell surface molecules and appendages (pili) (Figure 1a). In single-cell force spectroscopy, a single cell is attached to the cantilever to quantify single-cell adhesion forces (Figure 1b). In these assays, binding mechanisms are probed under mechanical tension, *that is*, in nonequilibrium conditions, which is an important advantage as pathogens are exposed to physical stress (fluid flow, cell surface contacts or epithelial turnover) during colonization of host tissues and biomaterials (Persat, Nadell, et al., 2015). In this minireview, we discuss recent progress made in this fast moving area, focusing primarily on adhesins from bacterial pathogens. We emphasize that, when subjected to mechanical stress, adhesive proteins and appendages display a wealth of mechanical responses that play important roles in cell adhesion.

2 | FORCES IN HOMOPHILIC CELL–CELL ADHESION

Homophilic adhesion is a widespread phenomenon in the microbial world, in which specific adhesins on one cell bind to identical adhesins

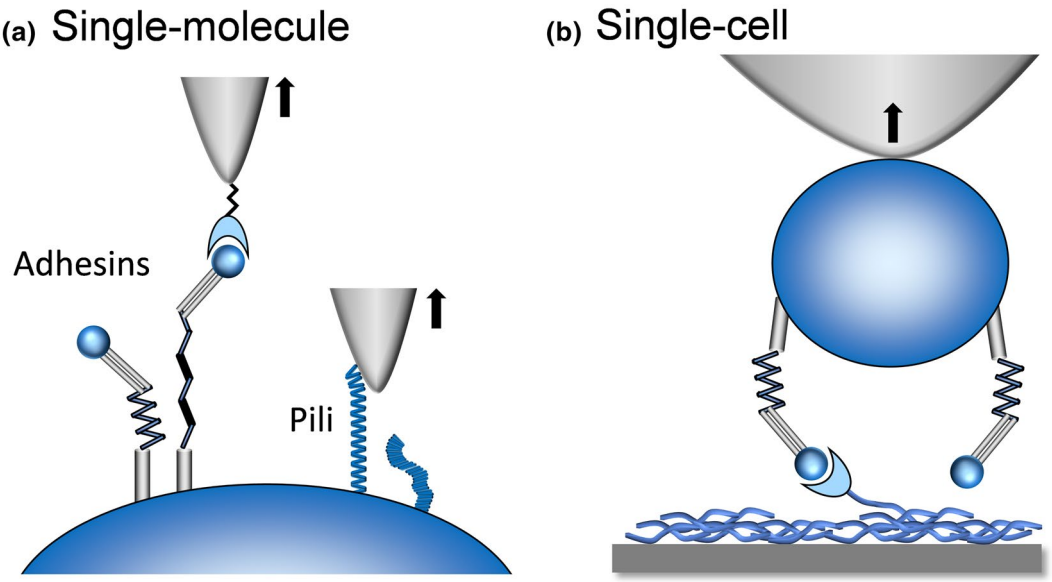

FIGURE 1 In single-molecule force spectroscopy (SMFS; a), AFM tips labelled (or not) with cognate ligands are used to force probe individual components like adhesins and pili, while in single-cell force spectroscopy (SCFS; b), the tip is replaced by a living cell in order to measure single-cell adhesion forces towards solid substrates, ligands or other cells. These methods provide information on the strength, specificity and dynamics of microbial cell surface interactions

on the other cell. AFM has been instrumental in understanding the forces guiding homophilic cell–cell adhesion. Prototypical examples are the fibronectin (Fn)-binding proteins (FnBPs) from *Staphylococcus aureus*, including clinically relevant methicillin-resistant strains. The strength of FnBPA-mediated cell–cell adhesion was found to originate from multiple low-affinity homophilic bonds, of ~150 pN force, between FnBPA A domains on neighbouring cells, and to depend on Zn^{2+} ions (Herman-Bausier, El-Kirat-Chatel, Foster, Geoghegan, & Dufrêne, 2015). Low-affinity binding might provide a means to the bacteria to detach and colonize new sites during biofilm formation. Along the same line, the *S. aureus* SasG adhesin favoured cell–cell adhesion *via* Zn^{2+}-dependent homophilic bonds (Formosa-Dague, Speziale, Foster, Geoghegan, & Dufrêne, 2016). The force required to unfold individual SasG domains was rather strong, up to ~500 pN, which resulted from tandemly arrayed mechanical clamps involving long stretches of hydrogen bonds and associated side chain packing interactions along the β-strands (Gruszka et al., 2015). This mechanical stability enables SasG to withstand physiological shear forces and maintain tight cell–cell contacts. Adsorption of zinc ions to cell wall components increased the cohesion of the cell surface, promoting the extension of SasG proteins and making them fully available for homophilic adhesion.

The molecular forces in yeast aggregation have also been measured. In the baker's yeast *Saccharomyces cerevisiae*, flocculin (Flo)-mediated co-adhesion involved well-known weak lectin-binding forces, but also unanticipated strong unfolding forces reflecting the force-induced extension of the hydrophobic tandem repeats of Flo proteins (El-Kirat-Chatel et al., 2015). In the pathogen *Candida albicans*, a novel form of homophilic cell–cell adhesion was identified, *that is*, β-sheet bonding of ~500 pN between amyloid sequences in Als (agglutinin-like sequence) proteins on opposing cells (Dehullu et al., 2019). Strong adhesion required Als expression on both interacting cells at high surface density, and were strongly inhibited by a short anti-amyloid peptide. The authors speculated that amyloid-based adhesion might be a widespread strategy among microbial pathogens to favour biofilm formation.

3 | BACTERIAL ADHESINS ENGAGE IN ULTRASTRONG INTERACTIONS

There has been exciting progress in applying AFM to understand the strength, specificity and dynamics of heterophilic interactions between adhesins and their specific ligands. Again, the most widely investigated adhesins are the staphylococcal FnBPs. Several investigators (Bustanji et al., 2003; Casillas-Ituarte et al., 2017, 2019) suggested that the strength of single FnBP-Fn bonds is ~150 pN, in the range of that of typical receptor–ligand bonds (Müller, Helenius, Alsteens, & Dufrêne, 2009), and increases with mechanical stress according to the classical Bell–Evans model. *S. aureus* clinical isolates showed a distinct force signature and depending on specific single amino acid polymorphisms in FnBP, stronger and more resilient binding mechanisms can occur (Lower et al., 2011). Other studies have reported much larger adhesion forces for FnBP–Fn interactions (Buck et al., 2010; Casillas-Ituarte, Lower, Lamlertthon, Fowler, & Lower, 2012; Xu et al., 2008), meaning that the strength of single FnBP bonds has been a controversial issue for years.

Recently, advanced AFM experiments with controlled surface chemistry enabled to resolve this discrepancy. The force required to separate staphylococcal FnBPA and SpsD adhesins (Prystopiuk et al., 2018) from Fn was shown to be extremely strong (~1,500–2,000 pN), a unusual mechanostability which originates from the β-sheet

organization of a tandem β-zipper (Schwarz-Linek et al., 2003). Upon binding to FnI modules, the intrinsically disordered binding sequences of the adhesins shift into an ordered structure by forming additional β-strands along triple peptide β-sheets in the Fn molecule. For SpsD, this model was supported by dynamic force measurements showing that unbinding forces follow an unusual mechanism, as well as by SPR analysis revealing that SpsD binds Fn with high affinity.

Another breakthrough is that staphylococcal adhesins SdrG, ClfA, ClfB and Cna engaged in dock, lock and latch (DLL) interactions (Ponnuraj et al., 2003) (or variations of these) bind to host proteins through very high forces, in the ~1,000–2,000 pN range, similar to the strength of covalent bonds (Grandbois, Beyer, Rief, Clausen-Schaumann, & Gaub, 1999). AFM demonstrated that the DLL molecular recognition force between SdrG and fibrinogen (Fg) is ~2,000 pN, (Figure 2) (Herman et al., 2014). By means of steered molecular dynamics simulations, Milles, Unterauer, Nicolaus, and Gaub (2018a; Herman-Bausier & Dufrêne, 2018), unravelled the mechanics of the SdrG–Fg interaction with atomic resolution. They found 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. Under force, numerous hydrogen bonds rupture simultaneously in a cooperative shear geometry. Recently, it was demonstrated that SdrG B domains unfold at forces higher than 2 nN and that this high mechanostability relies on the calcium coordination sites of the adhesin (Milles, Unterauer, et al., 2018b). Herman-Bausier et al. (2016) studied the mechanical strength and inhibition of the collagen (Cn)-binding protein Cna, which plays important roles in bacterium–host adherence and in immune evasion. The strength of Cna–Cn bonds was very strong (~1,200 pN), consistent with the "collagen hug" mechanism. They discovered that the B region behaves as a nanospring capable of sustaining high forces. It therefore appears that the B region has a mechanical function that is important for strong ligand binding.

In a nutshell, tandem β-zippers and DLL (-like) complexes formed by staphylococcal adhesins have a mechanical stability that largely outperforms the strength of noncovalent biological interaction ever measured. This might partly explains the ability of the pathogens to withstand physiological shear forces during colonization of their host. These findings may find applications for the identification of inhibitory compounds to block staphylococcal adhesion and may help designing bioinspired adhesives.

4 | MECHANICAL STRESS STRENGTHENS MICROBIAL CELL ADHESION

A striking property of adhesins is their ability to form catch bonds, that is, bonds that strengthen under tension (Sokurenko, Vogel, &

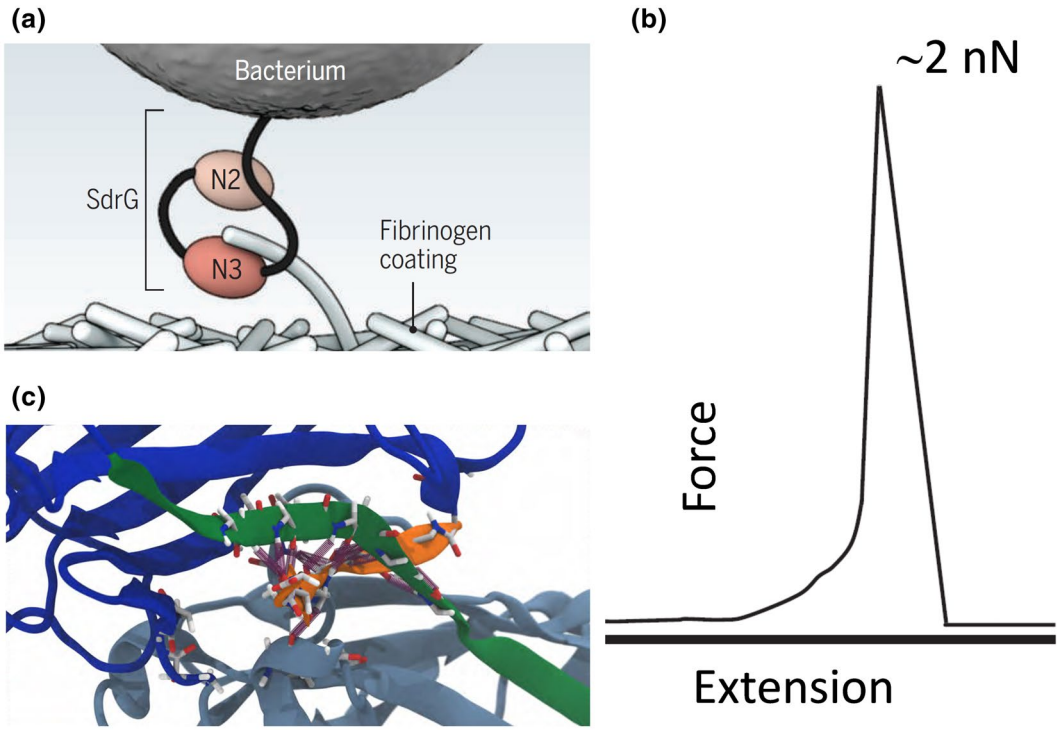

FIGURE 2 AFM captures the ultrastrong interaction of the staphylococcal adhesin SdrG. (a) The N2N3 subdomains of SdrG bind fibrinogen through the high-affinity dock, lock and latch (DLL) mechanism involving sequential conformational changes in the subdomains which stabilize the conformation of the complex. (b) AFM shows that the strength of interaction is equivalent to that of a covalent bond (~2 nN). (c) Simulations show that this extreme mechanostability results from an intricate hydrogen bond network (purple) between the ligand peptide backbone (orange) and the N2N3 subdomains (light and dark blue). Under load, the screw-like arrangement of hydrogen bonds maintains the peptide in a perfect shear geometry. Adapted with permission

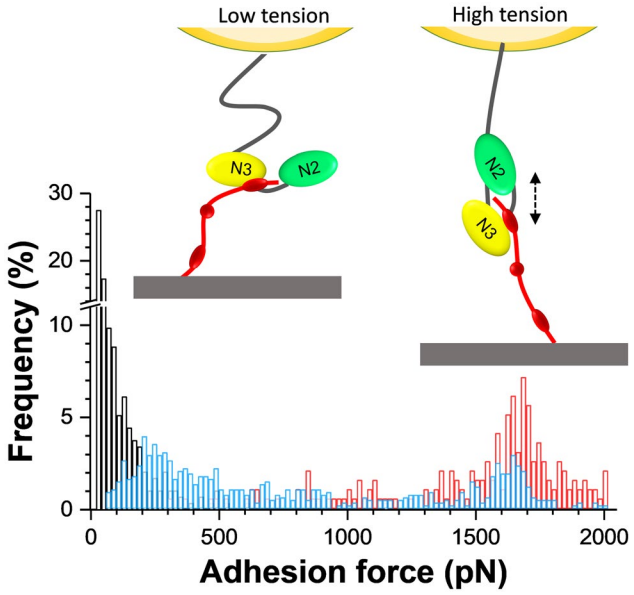

FIGURE 3 Force-activated adhesion of ClfA. Under low mechanical tension, fibrinogen weakly binds to ClfA, whereas under high tension, extension and conformational changes of the ClfA N2N3 subdomains enable the formation of a fully secured DLL-like interaction. Adapted with permission

Thomas, 2008). The prototypical example of a catch bond is found in *E. coli*, in which FimH binds to mannose residues on epithelial cells. Binding of FimH to mannose is weak and short lived at low flow, whereas this bond is strengthened at high flow. The underlying mechanism involves a model in which tensile mechanical force induces an allosteric switch from a low- to a high-affinity conformation (Yakovenko et al., 2008). These catch bonds favour urinary tract infections by mediating strong adhesion at a high flow rate.

Recent AFM studies have provided compelling evidence that catch bonds occur in *S. aureus* adhesins ClfA, ClfB and Spa, which is not that surprising as staphylococci are often subjected to physical stresses, including hydrodynamic flow and cell surface contacts. The strength of these stress-activated interactions outperform the binding force of classical receptor–ligand bonds by an order of magnitude. The bond between ClfB and loricrin was shown to be dramatically strengthened by mechanical tension, with strong DLL bonds (~1,500 pN) being favoured at high stress (Vitry et al., 2017). The authors suggested that bacterial adhesion to loricrin is enhanced through force-induced conformational changes in the ClfB molecule, from a weakly binding folded state to a strongly binding extended state. Similarly, binding of ClfA to Fg was weak at low tensile force, but enhanced (~1,500 pN) by mechanical tension (Figure 3) (Herman-Bausier et al., 2018). Strong bonds were inhibited by a peptide mimicking the C-terminal segment of Fg. These results led to the conclusion that ClfA interacts with Fg via two distinct binding sites, whose adhesive function is regulated by mechanical tension. Lastly, mechanical tension also activated the adhesive function of SpA (Viela, Prystopiuk, et al., 2019). Under tensile loading, the adhesin binds to the large plasma glycoprotein von Willebrand factor with a force of ~2,000 pN. The proposed mechanism was 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.

In summary, the force sensitivity of *S. aureus* adhesins demonstrated in these studies provides pathogen with a means to finely tune its adhesion to host tissues and implanted biomaterials, helping cells to attach firmly under high shear stress and to detach and spread under low stress. The loading rates (*LR*) in AFM experiments, *that is*, the rates at which force is applied to the complex, are consistent with physiological stresses occurring within specific host niches. Depending on their location, pathogens are subjected to a variety of shear conditions in the body, from low-shear conditions on implanted biomaterials to high-shear conditions in circulatory systems. Bacteria in biological flowing fluids are exposed to *LRs* that can exceed 10^5 pN/s (Yakovenko et al., 2008), meaning that values of 10^3 pN/s typically used in AFM studies are sufficient to favour strong adhesion in vivo in a number of situations.

Force-induced adhesion has also been demonstrated in yeasts. Under mechanical force, conformational changes in *S. cerevisiae* flocculins (Chan et al., 2016) and *C. albicans* Als adhesins (Alsteens, Garcia, Lipke, & Dufrêne, 2010; Garcia et al., 2011) lead to the exposure of hidden amyloid sequences, which favour β-sheet interactions between Als proteins laterally arranged on the cell surface. This leads to the formation of nanodomains that strengthen cell aggregation. So lectin-like (Flo) and immunoglobulin-like (Als) fungal adhesins may share common adhesive properties as a result of their structural similarities. Accordingly, owing to single-molecule experiments, we are now starting to appreciate that catch bond mechanisms might be widespread among microbial pathogens.

5 | NANOMECHANICS OF BACTERIAL PILI

The surface of many pathogenic bacteria is decorated with protein filaments known as pili or fimbriae which promote bacteria–bacteria interaction, bacterial adhesion to host tissues or abiotic surfaces. These appendages are formed by pilins (fibrous protein subunits) that assemble by noncovalent or covalent interactions in Gram-negative and Gram-positive bacteria respectively. An interesting finding from AFM and other biophysical techniques like optical tweezers is that pili feature various mechanical responses when subjected to force, and that pili mechanics is important for cell adhesion, a primary step leading to infection.

The most widely investigated pili are type IV pili from Gram-negative bacterial pathogens like *Neisseria gonorrhoeae* and *P. aeruginosa*, and the model bacterium *Myxococcus xanthus*. These pili can extend and retract enabling bacteria to walk or crawl on surfaces and undergo slingshot movements through coordinated pulling and release by several pili allowing efficient movement during biofilm formation. In 2000, Merz et al. discovered that type IV pili retract and generate substantial force (100 pN) involved in twitching motility and host cell adhesion (Merz, So, & Sheetz, 2000). The retraction of these pili involves the ATP- and

force-dependent action of the PilT retraction motor in *N. gonorrhoeae* (Maier, Koomey, & Sheetz, 2004) and *M. xanthus* (Clausen, Jakovljevic, Søgaard-Andersen, & Maier, 2009). Moreover, these pili can form bundles made up of 10 filaments, that act as coordinated retractable units with forces in the nN range that could be important for infection (Biais, Ladoux, Higashi, So, & Sheetz, 2008). Beaussart et al. (2014) quantified the nanoscale forces guiding the adhesion of *P. aeruginosa* type IV pili to biotic and abiotic surfaces. Pili strongly bound to hydrophobic surfaces, in a time-dependent manner, while they weakly bound to hydrophilic surfaces. Single pili could sustain forces of up to 250 pN, explaining how they resist mechanical stress. These findings were independently corroborated in a *P. aeruginosa* PilT$^-$ mutant incapable of retracting its pili (Lu et al., 2015).

Several studies have shown that type IV pili, P-pili and type I pili from Gram-negative bacteria (Forero, Yakovenko, Sokurenko, Thomas, & Vogel, 2006; Lugmaier, Schedin, Kühner, & Benoit, 2008; Miller, Garcia, Hultgren, & Oberhauser, 2006) readily elongate under force, a behaviour resulting from the uncoiling (unfolding) of their helical quaternary structure. As type IV pili do not have a helical conformation, it has been suggested that their elongation forces may originate from desorption of stretches of pili adhered to a surface (Lu et al., 2015). Different pilin domains may also show different force profiles. *E. coli* type 1 pilus domains closer to the tips of these pili appear to be mechanically less stable than domains closer to the pilus rod that can bear greater forces (Alonso-Caballero et al., 2018). Using a combination of AFM and optical and magnetic tweezers, Biais, Higashi, Brujić, So, and Sheetz (2010) showed that under force *N. gonorrhoeae* type IV pili undergo a molecular reorganization revealing hidden epitopes and leading to narrower and longer appendages. These molecular and morphological rearrangements provide means for the bacteria to maintain attachment to the host while withstanding intermittent forces. Accordingly, pili respond to force by transitioning into an extended quaternary structure that may expose hidden residues promoting adhesion. The mechanical response of pili enables the bacteria to firmly attach to surfaces, and thus to maintain attachment when subjected to high shear forces.

Changes in pilus structure after adhesion and upon stretching could also act as a mechanical signal (mechanosensing) leading, through mechanotransduction, to cellular responses. Persat, Inclan, Engel, Stone, and Gitai (2015) showed *P. aeruginosa* use type IV pili to sense initial contact with surfaces. This leads to a signalling cascade that results in the expression of hundreds of genes associated with pathogenicity and surface-specific twitching motility. Thus, bacteria use pili not only to attach and move, but also to sense mechanical features of their environment and regulate cellular processes of surface-associated lifestyles. Other mechanoresponses include upregulation of virulence factors, initiation of biofilm development and twitching motility in *P. aeruginosa* (Rodesney et al., 2017; Talà, Fineberg, Kukura, & Persat, 2019) and hold fast synthesis in *Caulobacter crescentus* (Ellison et al., 2017). Interestingly, it has also been found that *N. gonorrhoeae* pilus retraction upon adhesion to host epithelial cell membranes increased infection-related gene expression in the host cells (Howie, Glogauer, & So, 2005). These findings help to explain how pili play a critical role in colonization of the host.

Gram-positive pili are more rigid and capable to sustain higher mechanical force than Gram-negative pili, previously described. Isopeptide bonds between *Streptococcus pyogenes* pilins impede their mechanical extension even at pulling forces as large as 800 pN (Alegre-Cebollada, Badilla, & Fernández, 2010; Wang, Xiao, Edwards, & Gräter, 2013). The pili of *Corynebacterium diphtheriae* and *Actinomyces oris* could withstand similar high pulling forces (500 pN) through unfolding of polypeptide loops within their CnaA domains (Echelman et al., 2016). *Streptococcus pneumoniae* employs its pili in a host invasion mechanism whereby it binds with moderate forces of around 50 pN to host extracellular matrix fibronectin using its pilus-1 tip protein, RrgA, allowing initial contact (Becke et al., 2018); while under shear the pilus-1 backbone protein, RrgB, binds collagen with forces of up to 1.5 nN, strongly anchoring the bacteria (Becke et al., 2019). In a similar fashion, *Lactobacillus rhamnosus* uses the SpaC adhesion proteins of its pili to bind specifically to the epithelial extracellular matrix proteins, mucin and collagen, in a molecular zipper fashion at low pulling forces, allowing initial contact, while under high forces their pili behave like nanosprings favouring attachment to host cells even under shear stress (Lebeer et al., 2012; Sullan et al., 2014; Tripathi et al., 2013).

6 | FORCES IN CELLULAR INVASION

AFM has also provided new insights into the complex molecular mechanisms driving cellular invasion. In one such study, the role of septins in the interaction between the *Listeria monocytogenes* invasion protein InlB and the Met receptor was investigated (Mostowy et al., 2011). The results highlighted a function for septins in regulating the dynamics of the Met receptor at the cell surface, and possibly its linkage to the underlying cytoskeleton. A striking finding is that *S. aureus* cellular invasion involves protein bridges between bacterial adhesins and integrins on host cells that are extremely strong. Force spectroscopy experiments showed that FnBPA bind to Fn via a mechanically strong β-zipper structure (~1,500 pN), which further binds to α5β1 integrins with a strength much higher than that of the classical Fn–integrin bond (~100 pN) (Prystopiuk et al., 2018). This suggests an activation mechanism in which Fn binding by FnBPA leads to the exposure of cryptic integrin-binding sites via allosteric activation, which in turn engage in a strong interaction with integrins.

Binding of ClfA to endothelial cell integrin $\alpha_V\beta_3$ via the blood plasma protein Fg plays a role during sepsis. Viela, Speziale, et al. (2019) showed that the ClfA–Fg–$\alpha_V\beta_3$ complex is extremely stable, being able to sustain forces of ~800 pN, again much stronger than the classical bond between the RGD sequence and integrins. Adhesion was strongly inhibited by an anti-integrin antibody, the RGD peptide and the cyclic RGD cilengitide, showing that formation of the complex involves RGD-dependent binding sites. A force-dependent binding mechanism was proposed where, in the absence of mechanical stress, RGD sequences in the Fg molecule mediate weak

binding to $\alpha_V\beta_3$, whereas under high stress, exposure of cryptic RGD sequences leads to extremely strong binding.

The nanoscale interactions between pathogens and host immune cells have also been measured (El-Kirat-Chatel & Dufrêne, 2016). The adhesion forces between *C. albicans* and macrophages were found to involve multiple specific molecular bonds between lectin receptors on the macrophage membrane and mannan carbohydrates on the fungal cell surface. Adhesion strengthens with time, suggesting that the macrophage membrane engulfs the pathogen quickly after initial contact, leading to its internalization. Riet et al. also found that specific receptors of dendritic cells recognizing mannan patterns in *C. albicans* cell wall strengthen the pathogen–host interaction and trigger the invasion through a clathrin-dependent mechanism (te Riet et al., 2014; te Riet, Joosten, Reinieren-Beeren, Figdor, & Cambi, 2017).

7 | PERSPECTIVES

In the past years, AFM has enabled to unravel the binding mechanisms of adhesins from a variety of microbial pathogens. Major examples include unravelling homophilic cell–cell adhesion forces mediated by specific adhesins, uncovering ultrastrong interactions between staphylococcal adhesins and their ligand and their activation by tensile loading, understanding the mechanical behaviour of pili, and unveiling the complex molecular mechanisms driving pathogen cellular invasion. Owing to these novel experiments, it has become clear that microbes have evolved a variety of adhesion strategies, with specific single-molecule binding forces ranging from ~100 pN (e.g., homophilic binding), similar to that needed to unfold single protein domains, to 2,000 pN (e.g., DLL ligand binding), equivalent to the strength of covalent bonds. Back to our title *What makes bacterial pathogens so sticky*, we believe that one of the key answers is that they have evolved adhesins that function as force-sensitive switches, capable of featuring ultrastrong binding strengths and making the cells capable to sustain high shear stresses encountered during colonization and infection.

In the future, AFM might become an important tool to design antiadhesion compounds to block cell adhesion. In early work, the Camesano group showed that culturing fimbriated *E. coli* bacteria in the presence of cranberry juice lowers their adhesion to abiotic surfaces (Pinzón-Arango, Liu, & Camesano, 2009). Measuring the adhesion forces between the bacteria and human uroepithelial cells exposed to cranberry juice provided insight into the mechanisms by which these compounds inhibit bacterial adhesion (Liu, Pinzón-Arango, Gallardo-Moreno, & Camesano, 2010). Fullerenes bearing multiple peripheral mannose residues showed greatly enhanced antiadhesion activity towards *E. coli* FimH, suggesting that the multivalent presentation of sugars is advantageous for antiadhesion therapy (Beaussart, Abellán-Flos, El-Kirat-Chatel, Vincent, & Dufrêne, 2016). Antibodies against the *S. aureus* Cna adhesin strongly blocked bacterial adhesion to collagen surfaces (Herman-Bausier et al., 2016). A peptide derived from β-neurexin was shown to inhibit homophilic cell–cell adhesion forces mediated by the surface adhesin SdrC. Rivas-Pardo, Badilla, Tapia-Rojo, Alonso-Caballero, and Fernández (2018) recently reported on a short peptide that blocks isopeptide bond formation between pilin subunits of the Gram-positive human pathogen *Streptococcus pyogenes*, resulting in unfolding of the pili at very low forces. All these studies show the potential suitability of AFM for the study of antiadhesive properties of small inhibitors targeting adhesins and pili.

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