Atomic Force Microscopy in Microbiology: New Structural and Functional Insights into the Microbial Cell Surface

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ABSTRACT Microbial cells sense and respond to their environment using their surface constituents. Therefore, understanding the assembly and biophysical properties of cell surface molecules is an important research topic. With its ability to observe living microbial cells at nanometer resolution and to manipulate single-cell surface molecules, atomic force microscopy (AFM) has emerged as a powerful tool in microbiology. Here, we survey major breakthroughs made in cell surface microbiology using AFM techniques, emphasizing the most recent structural and functional insights.

The microbial cell is a highly dynamic system whose cell wall components constantly interact with their environment. The sophisticated functions of the cell surface are mediated by the complex and dynamic assembly of specific macromolecules, including proteins, polysaccharides, and lipids. Although much progress has been made in elucidating the composition and biosynthesis of cell surface constituents, we know little about the organization and interactions of the individual components on live cells.

Classical microbiological assays provide information on large populations of cells. In contrast, single-cell microbiology offers new opportunities for analyzing the behavior and heterogeneity of single cells, thereby enabling researchers to address cellular properties and interactions in a way that was not possible before (1, 2). Examples of single-cell technologies include fluorescence assays, flow cytometry techniques, microspectroscopic methods, mechanical, optical, and electrokinetic micromanipulations, microcapillary electrophoresis, biological microelectromechanical systems, and atomic force microscopy (AFM). During the past years, AFM-based techniques have been increasingly used for the multiparametric analysis of microbial cell surfaces, providing novel insight into their structure-function relationships. Compared to electron microscopy techniques, the main advantages of AFM for microbiologists are the possibility to image cellular structures at molecular resolution and under physiological conditions (i.e., in buffer solution), the ability to monitor *in situ* the structural dynamics of cell walls in response to stress and to drugs, and the capability to measure the localization, adhesion, and mechanics of single cell wall constituents.

Unlike other forms of microscopy, AFM operates by sensing the small forces acting between a sharp tip and the sample surface (Fig. 1) (3–8). A piezoelectric scanner allows high-resolution three-dimensional (3D) positioning of the tip. The latter is attached to a soft cantilever that deflects and quantifies the force. Cantilever deflection is detected by a laser beam reflected from the free end of the cantilever into a photodiode. In the imaging mode, the tip follows the contours of the cell in solution to generate a 3D image of the cell surface architecture with (near) molecular resolution (Fig. 1a). AFM imaging allows microbiologists to observe cell wall components directly on live cells, including polysaccharides (Fig. 2a) (9), peptidoglycan (Fig. 2b) (10), teichoic acids (Fig. 2c) (11), pili and flagella (Fig. 2d) (12), and crystalline protein layers like rodlets (Fig. 2e) (13) and S-layers (Fig. 2f) (14). Notably, correlated AFM-fluorescence imaging may be used to obtain a more complete view of cellular structures (Fig. 2g and h) (15).

In addition, AFM force spectroscopy can be used to quantify the forces between the tip and the sample. In single-molecule force spectroscopy (SMFS), the tip is brought into proximity of and retracted from the sample, and the cantilever deflection measures the interaction force as a function of the separation distance (Fig. 1b) (3–8). This yields a force-distance curve which provides key information on the localization, binding strength, and mechanics of cell surface molecules (Fig. 1b). In most SMFS experiments, the AFM tip is functionalized with specific biomolecules (Fig. 1b). The force sensitivity of AFM is on the order of only a few piconewtons (1 pN = 10^{-12} N). This allows researchers to probe single receptor-ligand bonds or to unfold single proteins, as such single-molecule measurements typically require forces that are in the 50 to 250 pN range (7, 8). Notably, spatially resolved SMFS enables researchers to quantitatively map cell surface structure, properties, and interactions. A variation of SMFS is single-cell force spectroscopy (SCFS), a method in which the tip is replaced by a living cell in order to probe single-cell adhesion forces (Fig. 1c). In this minireview, we survey recent discoveries made in probing the microbial cell surface using AFM, focusing on structural and functional insights.

STRUCTURAL INSIGHTS

AFM imaging enables microbiologists to visualize the organization and dynamics of microbial cell walls and appendages at (near) molecular resolution, thereby answering pertinent questions that could not be addressed before. A key benefit of AFM is that the specimen need not be stained, labeled, or fixed and can be imaged under physiological conditions. By revealing the ultrastructural details of the outermost cell surface, AFM complements fluorescence microscopy, which probes the entire cell wall at lower resolution.

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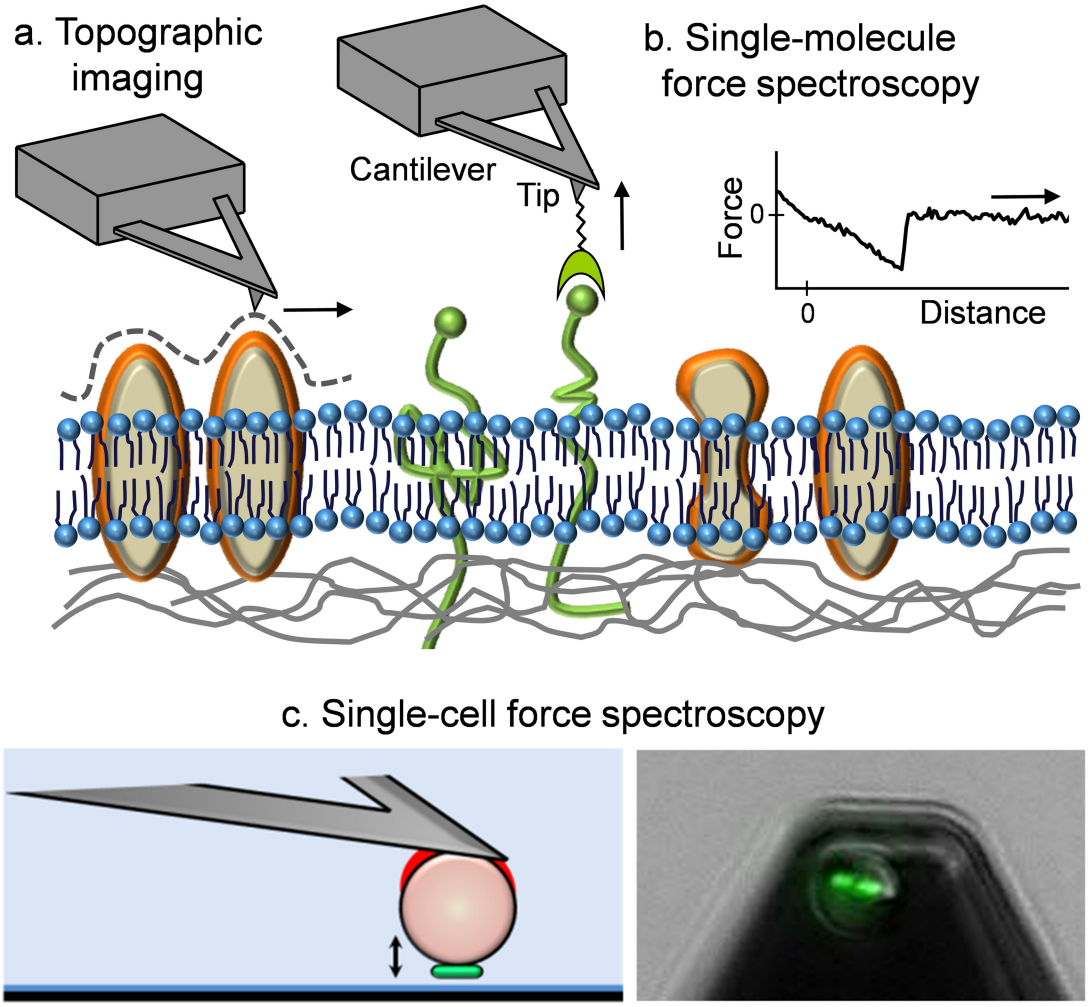

FIG 1 Atomic force microscopy: feeling the force. AFM works by sensing the tiny forces between a sharp tip and the sample surface. (a) In topographic imaging, the tip scans the cell surface in buffer with nanometer-scale resolution. (b) In single-molecule force spectroscopy, the small interaction force between the tip and cell surface molecules is measured while the distance between the tip and cell is varied, thereby yielding a force-versus-distance curve; as shown in the cartoon, the tip is generally labeled with a ligand to detect, localize, and manipulate individual receptors. (c) Single-cell force spectroscopy involves attaching a single live cell to the AFM cantilever, for instance using a colloidal probe coated with bioinspired polydopamine polymers, and recording the forces between the cell and a target surface (left). The viability of the cell can be checked using fluorescence stains (right [green color means that the membrane integrity is preserved]).

Cell wall architecture. Peptidoglycan is the main constituent of bacterial cell walls. Despite the important functional roles of this polymer (mechanical strength, cell shape, and target for antibiotics), its three-dimensional organization has long been controversial (16). In the most widely accepted model, glycan strands run parallel to the plasma membrane, arranged perhaps as hoops or helices around the short axis of the cell, resulting in a woven fabric. In the past years, AFM imaging has complemented electron cryomicroscopy and tomography techniques in providing key structural details of peptidoglycan, such as strand orientation. Much of this work has been carried out on purified sacculi by the Foster research team (for a recent review, see Turner et al. [17]). In an initial study, they reported that the cell wall of the model rod-shaped bacterium *Bacillus subtilis* has glycan strands up to 5 μm, thus longer than the cell itself (18). The inner surface of the cell wall showed 50-nm-wide peptidoglycan cables running parallel to the short axis of the cell, together with cross striations with an average periodicity of 25 nm along each cable (Fig. 3a). The data favored an architectural model where glycan strands are polymerized and cross-linked to form a peptidoglycan rope, which is then coiled into a helix to form the inner surface cable structures. In another study, AFM was combined with optical microscopy with fluorescent vancomycin labeling to investigate the distribution of peptidoglycan in the spherical bacterium *Staphylococcus aureus* (19). Concentric rings and knobbly surface structures were observed and attributed to nascent and mature peptidoglycan, respectively (Fig. 3b). Peptidoglycan features were suggested to demark previous divisions and, in doing so, hold the necessary information to specify the next division plane. Peptidoglycan architecture and dynamics have also been investigated in bacteria with ovoid cell shape (ovococci), including a number of important pathogens (20). Here, AFM images showed a preferential orientation of the peptidoglycan network parallel to the short axis of the cells, while superresolution fluorescence microscopy unravelled the dynamics of peptidoglycan assembly. The results suggested that ovococci have a unique peptidoglycan architecture not

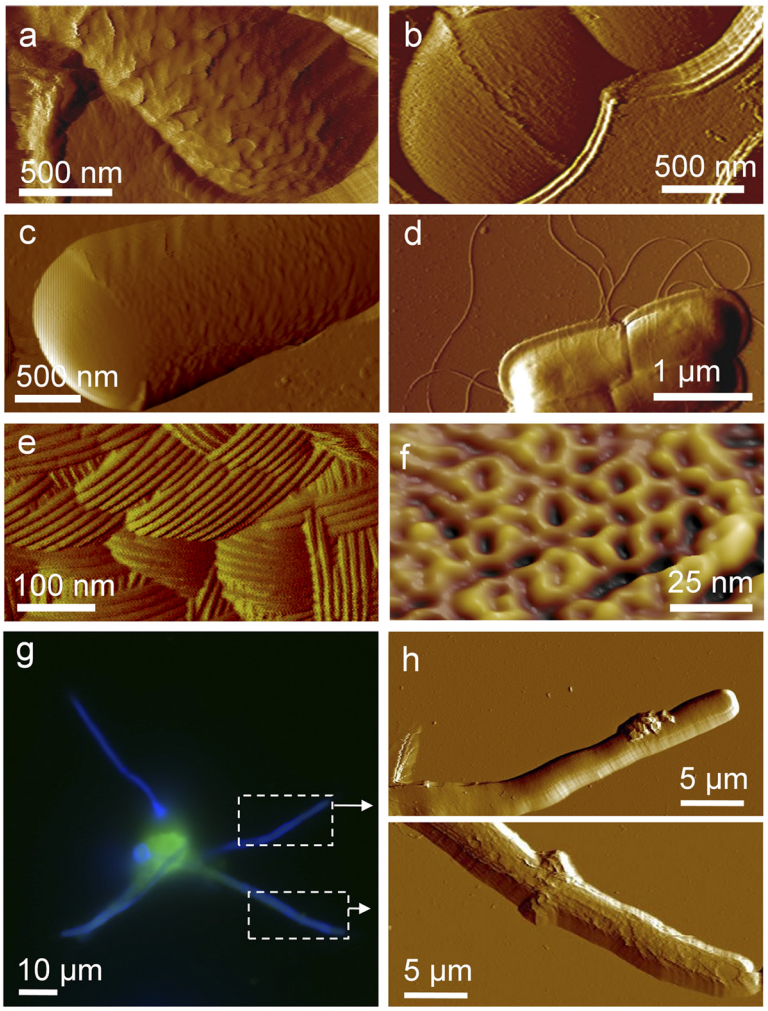

FIG 2 Seeing is believing: capturing the structural details of microbial cell surfaces. (a to f) High-resolution AFM images of individual cells from *Lactobacillus rhamnosus* GG (a), *Lactococcus lactis* (b), *Lactobacillus plantarum* (c), *Bacillus thuringiensis* (d), *Aspergillus fumigatus* (e), and *Corynebacterium glutamicum* (f). (g and h) Fluorescence image (g) and correlative AFM images (h) of a macrophage (in green) incubated for 3 h with cells from *Candida albicans* (in blue). The two images in panel h are enlarged views of the dashed areas shown in the fluorescence image. They reveal major structural differences between the two hyphae, one internalized (bottom), the other externalized (top). Adapted using data from references 9 to 15.

observed previously in other model organisms. Recently, the rod-shaped Gram-negative bacterium *Escherichia coli* was shown to feature peptidoglycan structures running parallel to the plane of the sacculus but in many directions relative to the long axis (21). The images also revealed bands of porosity running circumferentially around the sacculi (Fig. 3c). Superresolution fluorescence microscopy unravelled an unexpected discontinuous, patchy synthesis pattern. A model was suggested in which only the more porous regions of the peptidoglycan network are permissive for synthesis. Accordingly, these high-resolution studies have shown that bacterial species exhibit a variety of peptidoglycan architectures, thereby contributing to new structural models of peptidoglycan arrangement.

The organization of peptidoglycan has also been visualized in living cells. In early work, changes in *S. aureus* peptidoglycan architecture (nanoscale holes and concentric rings) were observed during growth (22). High-resolution images of *Bacillus atrophaeus* spores during germination revealed a porous network of peptidoglycan fibers, consistent with a honeycomb model structure for synthetic peptidoglycan oligomers (23). Interestingly, SMFS using functionalized tips provides a means to identify and localize single peptidoglycan chains in live cells (10, 24). Using vancomycin tips, D-Ala−D-Ala sites of peptidoglycan were shown to locate on the equatorial rings of *Lactococcus lactis*, suggesting that newly formed peptidoglycan was inserted in these regions (24). In the same vein, Andre et al. (10) used AFM tips modified with the lysine motif (LysM) to image peptidoglycan nanocables in *L. lactis*. Using topographic imaging, they found that wild-type cells display a featureless surface morphology, while mutant cells lacking cell wall exopolysaccharides featured 25-nm-wide periodic bands running parallel to the short axis of the cell (Fig. 2b). In addition, mapping wild-type cells with LysM tips confirmed that peptidoglycan was hidden by other cell wall constituents, while anisotropic peptidoglycan bands were detected on the mutant. Accordingly, high-resolution AFM images of sacculi and live cells have greatly contributed to refining our current perception of peptidoglycan architecture in a variety of bacterial species.

Glycopolymers represent another class of cell wall constituents

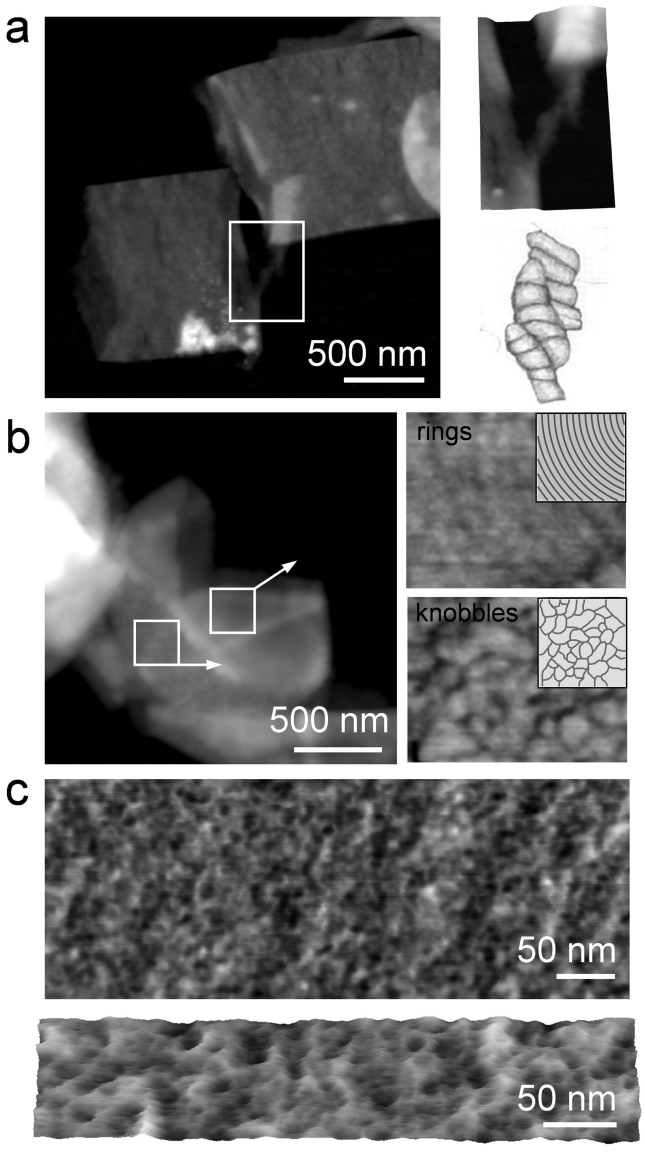

FIG 3 Unravelling peptidoglycan architecture. (a to c) AFM images of purified sacculi from *B. subtilis* (a), *S. aureus* (b), and *E. coli* (c), emphasizing key architectural details: nanocables running parallel to the short axis of the cell, with 25-nm-wide cross striations (a), concentric rings and knobbly surface structures (b), and bands of porous material running circumferentially around the sacculi (c). Adapted using data from references 18, 19, and 21.

which fulfill important functions, such as protecting the cell against unfavorable environmental conditions, mediating cellular recognition, and promoting biofilm formation. Stukalov et al. used AFM and transmission electron microscopy to study capsular polysaccharides of four different Gram-negative bacterial strains (25). While electron microscopy analysis revealed capsules for some but not all of the strains, AFM allowed the unambiguous identification of the presence of capsules on all strains. Moreover, AFM visualized bacterial cells within the capsules, indicating that the technique is capable of probing subsurface features. Francius et al. (9) probed the cell surface polysaccharides of the probiotic bacterium *Lactobacillus rhamnosus* GG (Fig. 2a). AFM images of the cells in buffer revealed a rough morphology decorated with nanoscale waves. These features reflected extracellular polysaccharides, as they were hardly seen in a mutant impaired in exopolysaccharide production. In addition, SMFS with tips functionalized with lectins was used to identify single polysaccharide chains, demonstrating the coexistence of polysaccharides of different nature on the cell surface. Although teichoic acids are known to play important roles during cell elongation and cell division (26), we know little about the relationships between the spatial localization of these components and their functional roles. To address this issue, AFM was combined with fluorescence microscopy to map the distribution of wall teichoic acids (WTAs) in *Lactobacillus plantarum* (11). Phenotype analysis of wild-type and mutant strains revealed that WTAs are required for proper cell elongation and cell division. Nanoscale imaging by AFM showed that strains expressing WTAs have a highly polarized surface morphology, the poles being much smoother than the side walls (Fig. 2c). SMFS and fluorescence imaging with specific lectin probes demonstrated that the polarized surface structure correlates with a heterogeneous distribution of WTAs, the latter being absent from the surface of the poles. These findings show that the polarized distribution of WTAs in *L. plantarum* plays a key role in controlling cell morphogenesis.

How about cell wall proteins? AFM has been intensively used to image proteins in purified membranes, at subnanometer resolution directly in aqueous solutions. These high-resolution studies are not covered here, as there are several reviews available on the subject (3, 27). An important challenge in membrane protein research is to increase the temporal resolution of AFM in order to monitor dynamic processes (28). In recent years, the Ando research group has made remarkable progress in developing new high-speed AFM instruments (28). While the time required to record a high-resolution image with conventional AFMs is about 60 s, high-speed technology makes it possible to obtain 10 images per second. This enabled them to observe dynamic molecular processes in photoactivated bacteriorhodopsin, showing that illumination of this light-driven proton pump induces major structural changes within 1 s (29). Also, high-speed AFM enabled the Scheuring team to track the motion of the outer membrane protein F (OmpF) from *E. coli* (30). High-resolution movies revealed that the proteins were widely distributed in the membrane as a result of diffusion-limited aggregation. Although the overall protein motion scaled with the local density of proteins in the membrane, individual protein molecules could also diffuse freely or become trapped by protein-protein interactions. From these data, they determined an interaction potential map and an interaction pathway for a membrane protein. Of note, the high-speed technology has also been applied to living bacteria, revealing the molecular dynamics of the cell surface (31). The bacterial outer membrane was covered with a net-like structure with slowly diffusing holes, presumably reflecting porin trimers. Collectively, the above studies have contributed to better understanding of the structural organization of microbial constituents, including peptidoglycan, glycopolymers, and membrane proteins.

Appendages. AFM has also been instrumental in studying the morphology and organization of bacterial pili and flagella, enabling direct quantification of their critical dimensions. Touhami and colleagues showed that AFM is a very sensitive tool for examining the general structure and elasticity of pili and flagella from *Pseudomonas aeruginosa* at high resolution (32). AFM imaging in buffer revealed the presence of pili surrounding cells from the

Gram-negative bacterium *Acinetobacter venetianus* RAG-1 (33). Pili were not seen with hydrophobic tips, suggesting that these appendages are hydrophobic in nature. Fälker et al. (34) analyzed the ultrastructural properties, including thickness and length, of pili from the Gram-positive pathogen *Streptococcus pneumoniae*. Pili were frequently tangled, or web-like, with two or three fibers wrapping around each other. High-magnification images revealed thin fibers with bulbous decorations both internal to the fiber and at the tip. High-resolution AFM images of *L. rhamnosus* GG cells revealed pili not only all around the cells but also in the form of star-like structures and elongated bundles assembled on the substrate (35). Some pili showed helical structures that may help the bacteria to withstand physiological shear forces encountered during colonization. AFM may be used for studying the expression of cell surface appendages in relation to function. In one such study, the amount of flagella expressed by six *Bacillus thuringiensis* strains was determined from AFM images and correlated with the microscopic swarming motility of the cells (12).

Cell wall remodeling. Understanding how cell walls remodel in response to growth or to drugs and how such structural dynamics correlate with changes in biophysical properties are important topics in cellular microbiology. AFM imaging allows researchers to track dynamic structural changes, while force spectroscopy provides a means to correlate these changes with differences in cell wall rigidity. In their pioneering work, Plomp et al. used AFM to probe the high-resolution structural dynamics of single *Bacillus atrophaeus* spores germinating under native conditions (23). AFM images revealed previously unrecognized germination-induced alterations in spore coat architecture and topology as well as the disassembly of outer spore coat rodlet structures. Combined AFM-fluorescence imaging enabled us to visualize how the fungal pathogen *Candida albicans* takes advantage of the yeast- to hyphal-phase transition to facilitate piercing and escape from phagocytes (15). Besides growth, environmental stresses can also greatly alter the microbial cell wall. By way of example, the Dague team explored the effects of heat stress on the structural and mechanical properties of *Saccharomyces cerevisiae* (36). Heat stress induced the formation of circular rings on the cell surface and increased the cell wall stiffness with a concurrent increase in chitin content. Analysis of mutants suggested that the circular features reflect defective bud scars or bud emergence sites during temperature stress.

Many important antibiotics, including β-lactams (penicillin) and glycopeptides (vancomycin), target microbial cell walls. Owing to its ability to monitor drug-induced surface alterations in microbial pathogens, AFM has opened up new possibilities for understanding the mode of action of antibiotics and for screening new antimicrobial molecules capable to fight resistant strains (37–42). Using real-time imaging, Francius et al. (40) captured the structural dynamics of *S. aureus* cells exposed to lysostaphin, an enzyme that specifically cleaves the peptidoglycan cross-linking pentaglycine bridges and that represents an interesting potential alternative to antibiotics. The enzyme induced major changes in cell surface morphology (swelling, splitting of the septum, and nanoscale perforations) and cell wall mechanics, which were attributed to the digestion of peptidoglycan, leading eventually to the formation of osmotically fragile cells. Similarly, the *P. aeruginosa* cell wall was demonstrated to be structurally and biophysically affected at the nanoscale by two reference antibiotics, ticarcillin and tobramycin, with the cell wall stiffness decreasing dramatically after treatment (41). In a related study, the effect of a polycationic calixarene-based guanidinium compound, CX1, on an *P. aeruginosa* multidrug-resistant strain was investigated (42). CX1 caused substantial alteration of the cell wall morphology (increased roughness and perforations) and a major drop in the cell wall stiffness. Further analysis of artificial membranes suggested that CX1 destroys the outer membrane of the bacteria. Treatment of *C. albicans* with the antifungal agents flucytosine and amphotericin B led to perforation and deformation of the cell wall (43). Greater cell wall damages were observed when the drugs were combined with allicin, an organic compound from garlic (44). Caspofungin, a novel antifungal drug that targets the synthesis of cell wall β-1,3-D-glucans, caused major morphological and structural alterations of the *C. albicans* cell wall, which correlated with a change in the cell wall mechanical strength (45, 46). Moreover, the drug induced the massive exposure of the cell adhesion protein Als1 on the cell surface and led to increased cell surface hydrophobicity, two features that triggered cell aggregation (45).

The mode of action of antimicrobial peptides has also been examined (47–51). Among these peptides, colistin is being used in combination with other antibiotics to treat and control chronic lung infections in cystic fibrosis patients. The mechanism of action seems to involve electrostatic interactions between cationic peptides and the outer membranes of Gram-negative bacteria. Supporting this view, AFM showed that various bacterial species treated with colistin have disrupted cell surfaces, increased stiffness, and decreased adhesive properties (47–49). In contrast, treatment of *B. subtilis* with the peptide trichokonin VI induced collapse of the cell wall, increased roughness, and caused a progressive decrease in cell stiffness (50), suggesting that the leakage of intracellular materials is a possible mechanism of action. AFM was used to probe the interaction of chrysophsin-3 with *Bacillus anthracis* in sporulated, germinated, and vegetative states (51). Unlike sporulated and germinated cells, vegetative cells became stiffer after treatment, an effect attributed to loss of water content and cellular material from the cell due to disruption of the cell membrane.

These investigations indicate that AFM imaging has the potential to become an important tool in antimicrobial therapy and pharmacology. A key direction for future research is to improve the temporal resolution of the technique so that fast cell wall remodeling can be monitored (28). Using the high-speed technology, Fantner et al. (52) observed, in real time, the effect of the antimicrobial peptide CM15 on individual *E. coli* cells. The results suggested that bacterial killing is a two-stage process consisting of an incubation phase, followed by an execution phase in which most of the damage is completed in less than a minute. In the future, it is anticipated that AFM, and more specifically high-speed imaging, will allow us to better understand the action mode of antimicrobial agents, including antibiotics, antimicrobial peptides, and innovative compounds like nanoparticles.

FUNCTIONAL INSIGHTS

Besides providing structural insights into the microbial cell wall, a wealth of information on cell surface functions can also be gained. Recent findings have shown the power of AFM force spectroscopy to decipher the binding mechanisms of microbial adhesins, to unravel the mechanics of cell surface proteins and its role in cellular function, to understand how cell surface proteins assemble

into functional nanodomains, and to quantify the forces that drive single-cell adhesion.

Binding mechanisms of adhesins. The adhesion of microbes to each other as well as to host cells has major implications in microbiology, biotechnology, and medicine. Microbial infection is often initiated by the specific adhesion of pathogens to host tissues via cell surface adhesins. Although much is known about the structure and biosynthesis of microbial adhesins, the molecular details underlying their interaction with host receptors remain largely unknown. Such knowledge offers exciting perspectives for controlling pathogen-host interactions for therapy. SMFS has been used to unravel the binding mechanisms of adhesins, providing direct information on the adhesin binding strength, affinity, and specificity. This implies functionalizing the AFM tip with cognate ligands and measuring the specific receptor-ligand forces, either on model substrates or on live cells (6, 8). Several studies have concentrated on the binding properties of fibronectin-binding proteins (FnBPs) from the pathogen *S. aureus*. AFM tips bearing fibronectin were used to quantify the molecular strength of fibronectin-*S. aureus* interactions on living bacteria (53). Strong bonds were promoted by the transcription factor SigB and are likely to play a role in the mechanically resistant adhesion of *S. aureus* to host tissues. Multiple parallel bonds were measured between fibronectin and FnBPA or FnBPB and different *S. aureus* and *L. lactis* strains (54). FnBPA and FnBPB were necessary and sufficient for the binding of *S. aureus* to prosthetic devices that are coated with host fibronectin. When trying to understand the interaction forces of pathogens, the use of actual clinical isolates of bacteria rather than laboratory strains should be preferred. With this in mind, the Lower research team probed the FnBP binding strength on 46 bloodstream isolates of *S. aureus*, each from a patient with a cardiovascular implant (55). Binding events were consistent with a multivalent, cluster bond consisting of ~10 or ~80 proteins in parallel, and the bond lifetime was two times longer for bloodstream isolates from patients with an infected device. In a related study of 80 clinical isolates, bacteria from patients with an infected device exhibited a distinct binding force signature and had specific single amino acid polymorphisms in FnBPA (56). *In silico* molecular dynamics simulations demonstrated that, in these isolates, three residues in the adhesin form extra hydrogen bonds with fibronectin, complementing the higher binding force and energy measured by AFM.

Single-molecule experiments have highlighted a fascinating trait of adhesion proteins, i.e., their multifunctional properties. A prototype of multifunctional adhesin is the mycobacterial heparin-binding hemagglutinin adhesin (HBHA). The force driving the specific recognition between HBHA and heparin receptors was quantified and found to depend on interaction time, suggesting that time-dependent conformational changes are needed for optimal binding (57). The adhesin was also capable of binding heparin sulfate proteoglycan receptors on living pneumocytes, leading upon detachment to the extraction of membrane tethers that could play a role in pathogen-host interactions (58). Further investigations revealed that the adhesin is engaged in homophilic interactions that may promote mycobacterial aggregation (59). In addition, HBHA was shown to specifically bind actin via both its N-terminal and C-terminal domains, strongly suggesting a role of the HBHA-actin interaction in the pathogenesis of mycobacterial diseases (60). As another example, the trimeric autotransporter adhesin from *Burkholderia cenocepacia* was shown to form homophilic *trans*-interactions engaged in bacterial aggregation and to bind collagen, a major extracellular component of host epithelia (61). Both homophilic and heterophilic interactions displayed low binding affinity, which could be important for epithelium colonization. Binding to living pneumocytes lead to the formation of membrane tethers that may play a role in promoting adhesion. Last, the peptidoglycan hydrolase Acm2 from the probiotic bacterium *L. plantarum* showed broad specificity. It was able to not only bind to structurally different peptidoglycans, with glucosamine as the minimal binding motif, but also to recognize mucin, the main extracellular component of the intestinal mucosal layer, thereby suggesting that this enzyme may also function as a cell adhesion molecule (62).

The mechanisms behind pathogen invasion have also been explored by means of force spectroscopy. The Lafont team uncovered the role of septins in the interaction between the *Listeria monocytogenes* invasion protein InlB and the Met receptor (63). Septins are unconventional cytoskeletal elements that regulate the entry of *L. monocytogenes* into host cells. The authors found that septin depletion significantly reduced the unbinding force of InlB-Met interaction on living cells and the viscosity of membrane tethers at locations where the InlB-Met interaction occurs. Consistent with a proposed role of septins in association with the actin cytoskeleton, cell elasticity was decreased upon septin or actin inactivation. 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.

Because polysaccharides on cell surfaces are also engaged in cell adhesion, studying their adhesion and conformational properties is important. SMFS with tips bearing lectins were used to probe polysaccharides on the surface of *L. rhamnosus* GG, revealing the coexistence of two polysaccharides of different natures (9). The measured polysaccharide properties—i.e., distribution, adhesion, and extension—of the wild-type bacterium were markedly different from those of a mutant strain with impaired biofilm formation and exopolysaccharide production, which suggests that these molecules play a role in bacterial adhesion and in promoting beneficial health effects. Force spectroscopy has contributed to our understanding of the role of lipopolysaccharides (LPS) in adhesion and virulence. The length and physicochemical properties of the LPS of eight *E. coli* strains were characterized, revealing a link between LPS length and adhesion for some strains (64). Force measurements of *P. aeruginosa* strains with LPS of various lengths showed that while adhesion forces were not correlated with LPS length, a relationship between adhesion force and bacterial pathogenicity was found in an acute pneumonia mouse model of infection (65). Adhesion forces were lower for strains with LPS mutations, suggesting that the wild-type strain is optimized for maximal adhesion.

In addition to providing new insights into the molecular mechanisms of adhesion, force spectroscopy experiments may be of biomedical interest for the design of molecules that promote (probiotics) or inhibit (pathogens) bacterial adhesion. The Camesano team has made interesting efforts toward this direction (66, 67). They showed that culturing P-fimbriated *E. coli* bacteria in the presence of cranberry juice cocktail lowers the adhesion toward AFM tips, probably by causing changes in bacterial fimbriae (66). They were further able to demonstrate that the antiadhesive components in the cocktail can reach the urinary tract and that these components are active in preventing nonspecific adhesion (67).

Protein mechanics. The mechanical properties of cell surface proteins play an essential role in defining cellular functions (7). While mechanosensors convert mechanical forces into biochemical signals, cell adhesion proteins mediate adhesion through force-induced conformational changes (68). To date, how such cellular proteins respond to mechanical stimuli to achieve function remains poorly understood. Owing to its ability to pull on single proteins, AFM has enabled researchers to tackle this problem. While protein mechanics has been widely investigated *in vitro* (7, 8, 27, 68), bringing these nanomechanical experiments into live cells has long been challenging (69). In a seminal study, SMFS was combined with the modern tools of molecular genetics to measure the elasticity of Wsc1 mechanosensors in *S. cerevisiae* (70–72). Mechanosensors were elongated and modified with a His tag in order to be specifically detected by a chemically modified AFM tip (70, 71). Pulling Wsc1 sensors on live cells revealed a fascinating behavior, i.e., they behave as nanosprings capable of resisting high mechanical force and responding to environmental stress (70). The technology was further developed as a tool for determining cell wall thickness *in vivo* in yeast cells (72).

In nature, microbial adhesins are often subjected to forces. Single-molecule experiments have demonstrated that, under an external force, adhesins exhibit striking mechanical responses that are important for cell adhesion (73–77). In biofilm research, pulling on the large adhesin protein LapA from *Pseudomonas fluorescens* yielded mechanical signatures made of a sequence of adhesion peaks with long extensions, reflecting the unfolding of the multiple repeats of the adhesin (73, 74). The modular nature of LapA could be important for its biological function in that it may strengthen bacterial adhesion by increasing the lifetime and energy of protein-substrate bonds. Hence, protein mechanics makes LapA ideally suited to function as a multipurpose bridging protein, enabling *P. fluorescens* to colonize various surfaces. Similarly, stretching the Ig terminal region of *C. albicans* Als adhesins revealed multiple adhesion peaks corresponding to the force-induced unfolding of hydrophobic tandem repeats engaged in cell adhesion (Fig. 4a) (75). Urea altered the shape of the unfolding peaks, reflecting a loss of mechanical stability of the domains due to hydrogen bond disruption. The unfolding probability increased with the number of repeats and was correlated with the level of cell-cell adhesion, pointing to a role of these modular domains in fungal adhesion, presumably via the force-induced exposure of hydrophobic residues. Like curli in *E. coli* (76), Als proteins have conserved amyloid-forming sequences and form amyloid fibers (77). Recently, SMFS demonstrated the role of Als amyloids in strengthening fungal adhesion (78). Pulling Als molecules through their amyloid sequence yielded force plateau signatures corresponding to the mechanical unzipping of amyloid β-sheet interactions formed between surface-arrayed Als proteins (Fig. 4b), thus demonstrating that amyloid interactions provide cohesive strength to the adhesins. We expect that AFM will have great value for understanding the role of functional amyloids in microbiology (76, 77).

Insights into the mechanics of bacterial pili have also been obtained, explaining how these structures are used to promote adhesion and resist mechanical stress. Gram-negative pili readily elongate under force as a result of the unfolding of their helical quaternary structure (32, 79, 80). Pili elongation is believed to help bacteria to redistribute external forces to multiple pili, thus enabling them to withstand shear forces. Moreover, type IV pili

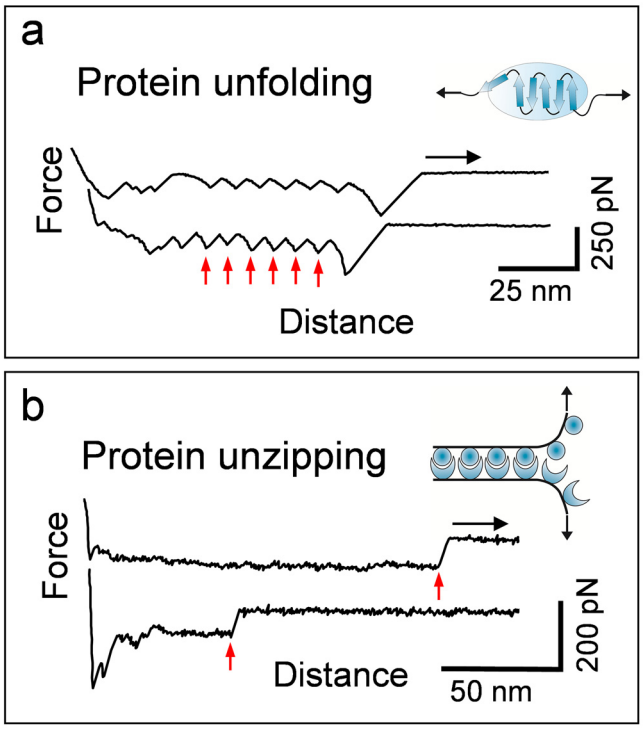

FIG 4 May the force be with you: force-induced unfolding and unzipping of adhesins. (a) Stretching single Als adhesins from *Candida albicans* through their terminal Ig region yields force-distance curves with periodic features reflecting the sequential unfolding of multiple tandem repeat domains. (b) In contrast, pulling Als proteins through their amyloid sequence leads to characteristic force plateaus corresponding to the mechanical unzipping of β-sheet interactions between surface-arrayed proteins. Both unfolding and unzipping behaviors are believed to strengthen adhesion of the pathogen. For both unfolding and unzipping experiments, two characteristic force curves are shown. The red arrows emphasize the characteristic force peaks in each case. Adapted using data from references 75 and 78.

are able to exert retractile forces involved in twitching motility and host cell adhesion, presumably through filament disassembly into the inner membrane (81). In contrast, Gram-positive pili exhibit striking mechanical responses not observed in Gram-negative pili, consistent with the notion that they are formed by covalent polymerization and stabilized by internal isopeptide bonds. At low force, the *Lactobacillus rhamnosus* GG (LGG) pilus was shown to mediate zipper-like interactions involving multiple adhesins distributed along the pilus (zipper-mode rupture), while at high force, the pilus behaved as a nanospring capable of withstanding large mechanical loads (shear-mode rupture) (82). Zipper-like interactions and spring-like properties are believed to be critical for strengthening bacterium-host and bacterium-bacterium interactions in the intestinal environment. In summary, nanomechanical experiments have enabled new light to be shed into how cell surface sensors, adhesins, and pili respond to mechanical stimuli in relation to function. Note that besides protein mechanics, cell mechanics can also be addressed by AFM force spectroscopy, enabling us for instance to assess the impact of antibiotics on cell stiffness (83, 84).

Protein clustering. Knowledge of the distribution and dynamics of cell surface receptors is critical to our understanding of the cell surface functions. A hot topic is to understand how surface-

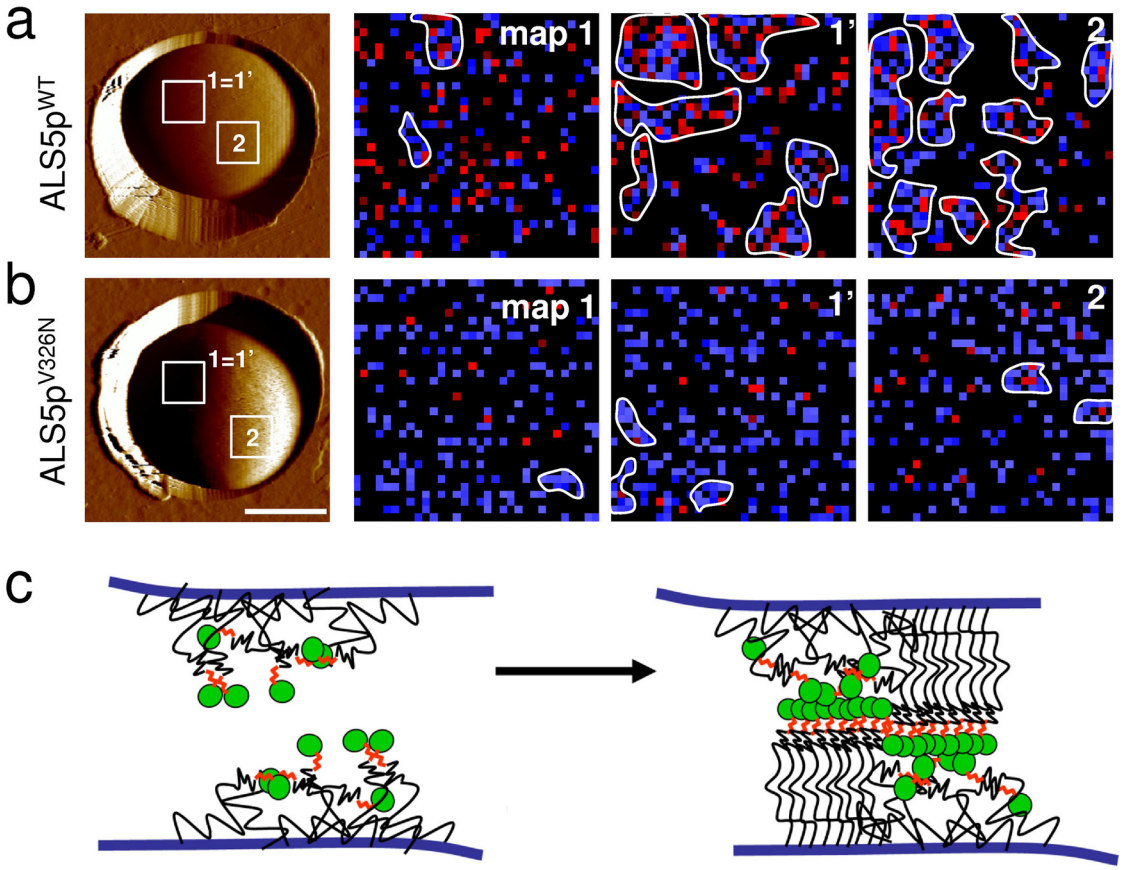

FIG 5 Together we are stronger: functional amyloids create adhesion nanodomains on living cells. (a) Single-molecule imaging of yeast cells expressing V5-tagged Als5pWT proteins. (Left) AFM topographic image (bar, 2 μm) of a single cell. Map 1 is an adhesion force map (1 μm by 1 μm) recorded with an anti-V5 tip on a given target area of the native cell that was never subjected to force (recorded on the square shown in the topographic image). Blue and red pixels correspond to Als5p recognition and unfolding, respectively. Map 1′ is a second adhesion force map recorded on the same target area documenting the formation of nanoscale clusters (outlined in white). Map 2 is an adhesion force map recorded on a remote area localized several hundred nanometers away from the first map. (b) Same sequence of data as in panel a obtained on cells expressing the single site mutation Als5pV326N. (c) Proposed model. Force-induced amyloid-dependent clustering of Als5p strengthens cell-cell adhesion. Adapted using data from reference 89.

associated proteins assemble to form micro- and nanodomains (68). Because AFM can map the distribution of single proteins on living cells (85, 86), including microbial cells (57, 87), it is well-suited to address this challenge. Single-protein imaging generally involves scanning the cell surface by means of spatially resolved SMFS with tips bearing specific ligands or antibodies (6, 8). This method has enabled key discoveries in microbiology, such as the stress-induced formation of protein clusters that activate cell signaling and cell adhesion. Individual Wsc1 mechanosensors were localized on yeast cells and found to form clusters of 200-nm size (88). Analyses of mutants indicated that the cysteine-rich domain of Wsc1 has a crucial, unanticipated function in sensor clustering and signaling. Protein clustering was strongly enhanced in deionized water or at elevated temperature, suggesting its relevance in proper stress response. In the cell adhesion context, a key finding was the clustering of microbial adhesins in response to mechanical stress. Pulling single Als adhesins from *C. albicans* with AFM tips terminated with specific antibodies triggered the formation and propagation of adhesion nanodomains on the cell surface (Fig. 5a) (89). Single-site mutation in the conserved amyloid-forming sequence of the protein revealed that amyloid interactions represent the driving force underlying Als clustering (Fig. 5b). Hence, the strength of cell-cell adhesion results from the force-activated amyloid-like clustering of hundreds of proteins on the cell surface to form arrays of ordered multimeric binding sites (Fig. 5c). These results, together with the zipper binding mechanism described above (Fig. 4b) (78), highlight the role that amyloids can play in microbial cell adhesion, both in clustering the adhesins to increase binding avidity, and in the formation of stable amyloid interactions between cells. Further investigations have demonstrated the impact of cellular morphogenesis, i.e., the yeast- to hyphal-phase transition, on the distribution and adhesion of Als adhesins and their associated mannans (90), which are critical for microbe-host interactions.

Despite the great potential of SMFS-based imaging for cell surface analysis, the technique has long been limited by its poor spatiotemporal resolution. However, new multiparametric imaging modalities now allow researchers to image the structure and physical properties (elasticity and adhesion) of cellular samples simultaneously at increased speed and lateral resolution (91). In microbiology, multiparametric imaging has already been applied to the purple membrane from *Halobacterium salinarum* (92, 93) and to the outer membrane protein F (OmpF) (94). Chopinet et al. (95) imaged the structure, elasticity, and adhesion of *E. coli*, *C. albicans*,

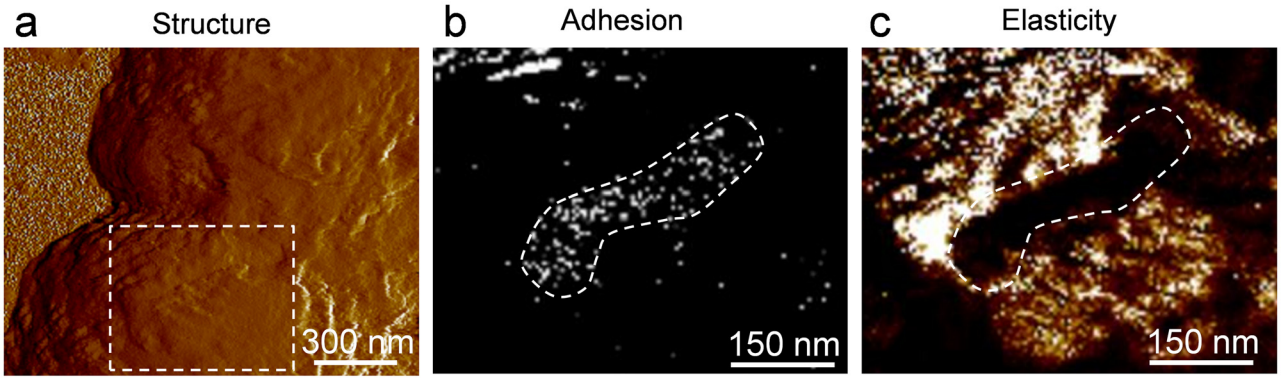

FIG 6 Multiparametric imaging of viruses on living bacteria. (a to c) High-resolution AFM structural image (error signal) (a) and directly correlated adhesion map (b) (z-scale shows adhesion values ranging from 0 to 350 pN) and elasticity map (c) (z-scale shows Young's modulus values ranging from 0 to 20 MPa) of *E. coli* cells infected by Fwt phage particles recorded in phosphate-buffered saline (PBS) with Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) AFM tips. Images b and c are higher-magnification views of the area outlined by a white dashed line in panel a. The dashed lines emphasize the organization of the bacteriophages into soft nanodomains surrounded by stiff material. Adapted using data from reference 97.

and *Aspergillus fumigatus* cells. Alsteens et al. (96) developed a multiparametric imaging method with chemically modified tips in order to map hydrophobic forces on *A. fumigatus* and to detect and manipulate single sensor proteins on yeast cells at near molecular resolution. Using this approach, single filamentous bacteriophages extruding from living bacteria were observed, revealing that the sites of assembly and extrusion localize at the bacterial septum in the form of soft nanodomains surrounded by stiff cell wall material (Fig. 6) (97). Because the assembly machinery of the phages is localized where peptidoglycan synthesis takes place, these nanodomains may interfere with peptidoglycan assembly, leading to the accumulation of newly formed cell wall material around these sites. These breakthrough experiments demonstrate that advanced multiparametric imaging techniques, combined with biochemically sensitive tips, represent a powerful platform for the simultaneous structural and functional analysis of microbial cells.

Cell surface interactions. Another fast-moving area is the use of force spectroscopy to understand the fundamental forces guiding cell-cell and cell-substrate interactions. The idea is to immobilize microbial cells on an AFM cantilever and to measure the interaction forces between the cellular probe and target surfaces (for two pioneering studies, see references 98 and 99). Cell probe experiments complement traditional methods used to investigate microbial adhesion, i.e., electron and optical microscopy examination, flow chamber experiments, surface chemical analysis, as well as surface charge and hydrophobicity measurements. They also offer a means to correlate single-molecule and single-cell data, thus providing an integrated view of cell adhesion mechanisms.

In most cell probe studies, chemical treatments were used to prepare the cell probes, thus leading to cell surface denaturation or even cell death. Also, multiple cells were generally attached to the cantilever, meaning the number of interacting cells was not controlled. To obtain biologically relevant information, there is therefore an urgent need to develop SCFS assays capable of true single-cell analysis, as is routinely achieved with animal cells (100). A noninvasive approach for SCFS is to simply attach the cells on cantilevers using specific receptor-ligand interactions (101). However, in most cases, the microbe-cantilever bond is too weak, leading to cell detachment. An elegant alternative is FluidFM, a new technology that uses hollow cantilevers for local liquid dispensing and manipulation of single living cells (102, 103). As opposed to other SCFS methods, FluidFM allows us to probe numerous cells in a short period of time; in this way, statistically relevant data can be recorded. However, it requires more sophisticated instrumentation than classical commercial instruments. A simpler protocol is to combine the use of colloidal probe cantilevers and of a bioinspired polydopamine wet adhesive. Living bacteria are picked up with a polydopamine-coated colloidal probe, enabling researchers to quantify the adhesion forces between single bacteria and target surfaces (104, 105). This procedure provides excellent control of cell positioning, thus ensuring reliable single-cell analysis.

In the past, cell probe assays have been increasingly used to understand the cellular interactions of medically important organisms, i.e., probiotics and pathogens. The nonspecific and specific forces engaged in the adhesion of *L. lactis* to mucin were measured, revealing the important role of mucin oligosaccharides (106, 107). Variations in the loading rate and contact time enabled researchers to assess the kinetic dissociation and association constants of the bonds. Using SCFS, Sullan et al. (108) investigated the forces guiding pili-mediated adhesion in probiotic *L. rhamnosus* GG (LGG) bacteria. On a hydrophobic substrate, bacterial pili strengthened adhesion through nanospring properties, thus corroborating earlier single-molecule experiments (82). On mucin, nanosprings were more frequent and adhesion forces were larger, reflecting the influence of specific pili-mucin bonds. On human intestinal Caco-2 cells, constant force plateaus were observed instead of nanosprings. These plateaus were suggested to originate from the extraction of membrane tethers and to substantially increase the lifetime of the interaction. Hence, the adhesion mechanisms of LGG bacteria strongly depend on the nature of the target substrate and involve different mechanical responses. For the large adhesin protein LapA from *P. fluorescens*, expression of the adhesin on the cell surface via biofilm-inducing conditions or deletion of the gene encoding the LapG protease increased the cell adhesion strength toward hydrophobic and hydrophilic substrates, explaining the adherent phenotypes observed under these conditions (73). In agreement with single-molecule experiments,

individual LapA repeats unfolded when single bacteria were subjected to force. Collectively, single-molecule and single-cell data unravelled the mechanical properties of LapA, providing a molecular basis for its "multipurpose" adhesion function (73, 74). Remarkably, combined SMFS and SCFS analyses demonstrated that the bond between the staphylococcal adhesin SdrG and the blood plasma protein fibrinogen is very strong and stable, equivalent to the strength of a covalent bond (109). This binding mechanism provides a molecular foundation for the ability of *Staphylococcus epidermidis* to colonize implanted biomaterials and to withstand physiological shear forces.

Recent findings have also deciphered the forces driving cell-cell adhesion, which are critical to host colonization and biofilm formation. Younes et al. (110) showed that lactobacilli display strong adhesion forces toward virulent *S. aureus* strains, thus explaining how coaggregation could eliminate these pathogens. Ovchinnikova et al. (111) investigated the physical interactions between *P. aeruginosa* and different forms of *C. albicans*. Adhesion of *P. aeruginosa* to hyphae was always accompanied by strong adhesion forces. A bacterial mutant unable to produce quorum-sensing molecules was less adherent, emphasizing the role of quorum sensing in establishing polymicrobial communities. Along the same line, Beaussart et al. (112) quantified the forces driving the coadhesion between *S. epidermidis* and *C. albicans*, revealing that bacteria bind strongly to *C. albicans* germ tubes but poorly to yeast cells. Analysis of fungal mutant strains altered in cell wall composition showed that coadhesion primarily involves Als proteins and O-mannosylations, which presumably recognize Als ligands and lectins on the bacterial surface. Alsteens et al. (113) uncovered the forces engaged in *C. albicans* yeast-hypha adhesion, which is of prime importance for biofilm formation. Using mutant strains, they found that Als3 proteins, primarily expressed on the germ tube, play a key role in establishing strong cohesive adhesion. The work favors a model in which cohesive adhesion during biofilm formation originates from tight hydrophobic interactions between Als tandem repeat domains on adjacent cells. Liu et al. (114) showed that cell probe assays also offer exciting prospects for antiadhesion therapy. By probing the adhesion forces between P-fimbriated *E. coli* and human uroepithelial cells exposed to cranberry juice, they shed new light into the mechanisms by which these compounds inhibit bacterial adhesion. Accordingly, cell probe experiments, and more specifically SCFS, nicely complement SMFS analyses to enhance our understanding and control of the molecular mechanisms by which microbes colonize surfaces.

NANOMECHANICAL SENSORS

In addition to providing structural and functional insights into the microbial cell surface, AFM cantilevers may also be used as biosensors, enabling the ultrasensitive detection of bioanalytes and cells without the need for labeling (115). Cantilevers are coated with receptor molecules such as specific antibodies. Upon interaction with ligands or cells, specific binding is monitored by measuring the bending or resonance frequency shift of the cantilever. AFM biosensors were used to detect *Aspergillus niger* and *B. anthracis* spores at low concentrations (116–118). Remarkably, recent investigations have demonstrated the power of AFM cantilevers for expanding our understanding of the biophysical mode of action of antibiotics. Nanomechanical detection was applied to the vancomycin-cell wall interaction, suggesting that surface stress causes mechanical disruption of the bacterial cell wall (119). The combination of nanomechanical cantilevers together with equilibrium theory made it possible to describe quantitatively the mechanical response of surface receptors to vancomycin and oritavancin in the presence of competing ligands (120). There were variations among strong and weak competing ligands, such as proteins in human serum, that determine dosages in drug therapies. The Kasas research team showed that the fluctuations of AFM cantilevers can detect low concentrations of bacteria, characterize their metabolism, and quantitatively screen, within minutes, their response to antibiotics (121). The method enabled studying the dynamic effects of ampicillin on *Escherichia coli* and *Staphylococcus aureus* species, establishing quantitative antibiograms within a matter of just a few minutes instead of days or weeks. These reports show that AFM-based biosensors hold great promise for understanding the binding mechanisms of antibiotics.

CONCLUSIONS AND FUTURE CHALLENGES

The use of AFM techniques in microbiology is a fast moving area which has greatly contributed to enhancing our understanding of the structures and functions of the microbial cell surface. These single-molecule and single-cell analyses will have an important impact not only on microbiology but also on medicine for elucidating the molecular mechanisms behind pathogen-host and pathogen-drug interactions and for developing new antimicrobial strategies.

AFM imaging has provided novel insight into the architecture and assembly of the major components of the cell wall, including peptidoglycan, polysaccharides, teichoic acids, membrane proteins, pili, and flagella, and has contributed to elucidate their roles in cellular processes like growth, division, morphogenesis, motility, and adhesion. In addition, the technique has allowed us to understand how cell walls remodel in response to growth and to drugs, shedding new light into the action mode of antimicrobial agents.

A variety of functional insights have been gained from SMFS and SCFS experiments. SMFS has been used to unravel the binding mechanisms of adhesins, like FnBPs and HBHA, and of polysaccharides, providing direct information on their binding strength, affinity, specificity, and multifunctionality. These experiments are of interest for the design of molecules that promote or inhibit bacterial adhesion. Researchers have also deciphered how mechanosensors (Wsc1), adhesins (Als and LapA), and pili respond to mechanical stress (protein unfolding, zipper-like interactions, and spring-like properties), and how this response is used to modulate cellular functions (mechanosensing and adhesion). The results have demonstrated the fascinating role of functional amyloids in cell adhesion and biofilm formation. SMFS-based imaging with tips bearing specific bioligands has enabled us to map the distribution of cell surface receptors, showing that, in response to stress, they form functional nanodomains that activate cell signaling and cell adhesion. Multiparametric imaging has emerged as a new AFM modality to understand the structure and physical properties of such cell surface nanodomains. SCFS complements SMFS by enabling a direct quantification of the fundamental forces guiding cell-cell and cell-substrate interactions that are critical to biofilm formation and host colonization. Finally, AFM-based mechanical sensors enable the ultrasensitive, label-free detection of bioanalytes and cells, which should find exciting applications for studying the binding mechanisms of antibiotics,

for the rapid detection of bacterial resistance to antibiotics, and for functional drug discovery.

Despite the great potential of AFM, its widespread use in microbiology has been limited by technical bottlenecks, including the low temporal resolution of the technique and the invasiveness of the analyses. However, the recent development of advanced AFM modalities has contributed to solving these problems. Currently, most AFM instruments are coupled to high-quality inverted optical microscopes, enabling targeted AFM measurements on single cells, and their correlation with fluorescence imaging. In future research, combining AFM with superresolution light microscopy techniques, such as photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), structured illumination microscopy (SIM), and stimulated emission-depletion (STED) microscopy, should provide a powerful imaging platform to structurally and functionally analyze the microbial cell surface.

Compared to light microscopy, AFM imaging is limited by its poor temporal resolution, i.e., the time required to record an image (60 s) is much longer than the time scale of many biological processes. Recently, there have been rapid advances in developing high-speed AFM instruments, capable of tracking molecular and cellular dynamics with millisecond time resolution (28). This technology has enabled researchers to observe the bacteriolysis of *Bacillus subtilis* subjected to lysozyme and the surface change of *E. coli* upon interaction with antimicrobial peptides (28). We expect that this newer modality will allow us to address a wealth of dynamic processes in microbial cells.

An important issue in cell surface biology is to simultaneously characterize the structure, physical properties, and interactions of cell surfaces under physiological conditions. While spatially resolved SMFS enables researchers to correlate structural images of cells with quantitative maps of their biophysical properties, the low speed and poor spatial resolution of this method have limited its use in microbiology (6, 8). The past years have seen major breakthroughs in developing new quantitative multiparametric imaging techniques, such as quantitative imaging and peak force tapping. Combined with the use of biochemically modified tips, multiparametric imaging now makes it possible to simultaneously image the structure, physical properties, and interactions of microbial cells at increased speed and resolution. Recent examples of such analyses include the quantitative mapping of hydrophobic properties on microbial pathogens (96), the fast localization and mechanical analysis of cell surface mechanosensors (96), and the imaging of single bacteriophages extruding from living bacteria (97).

Finally, the use of SCFS to quantify the forces guiding microbial cell adhesion has long been hampered by the lack of appropriate protocols for the controlled attachment of single microbial cells on AFM cantilevers. Until recently, cells were attached to cantilevers by means of electrostatic interactions, hydrophobic interactions, glue, or chemical fixation. These methods show several problems. The cell-cantilever bond is too weak, leading to cell detachment. The use of chemicals or drying leads to cell surface denaturation and/or cell death. Multiple cells are often attached and probed together, meaning reliable single-cell analysis is not accessible. Two approaches have recently been introduced for reliable SCFS in microbiology, i.e., FluidFM (102, 103) and colloidal probes combined with bioinspired polydopamine adhesives (104, 105). We anticipate that these assays will be increasingly used to gain insight into the molecular mechanisms driving cell adhesion and biofilm formation.

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