NANO LETTERS
Cite This: Nano Lett. 2018, 18, 5821−5826
Letter
pubs.acs.org/NanoLett

Bacterial Sexuality at the Nanoscale

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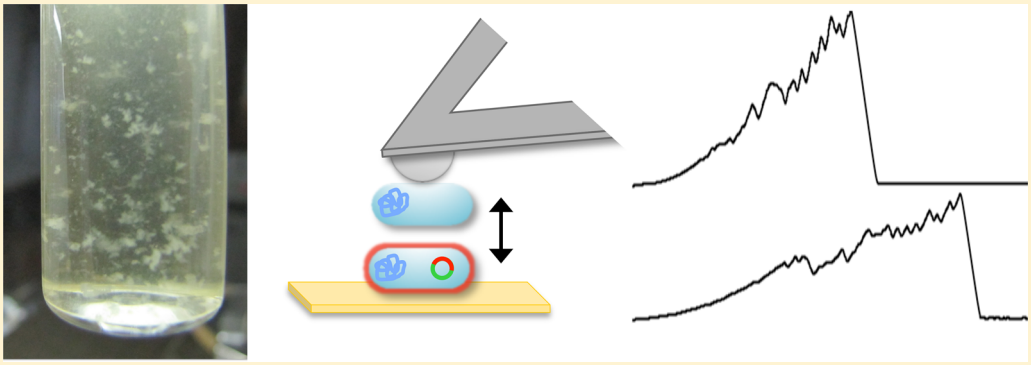
Supporting Information



ABSTRACT: Understanding the basic mechanisms of bacterial sexuality is an important topic in current microbiology and biotechnology. While classical methods used to study gene transfer provide information on whole cell populations, nanotechnologies offer new opportunities for analyzing the behavior of individual mating partners. We introduce an innovative atomic force microscopy (AFM) platform to study and mechanically control DNA transfer between single bacteria, focusing on the large conjugative pXO16 plasmid of the Gram-positive bacterium *Bacillus thuringiensis*. We demonstrate that the adhesion forces between single donor and recipient cells are very strong (∼2 nN). Using a mutant plasmid, we find that these high forces are mediated by a pXO16 aggregation locus that contains two large surface protein genes. Notably, we also show that AFM can be used to mechanically induce plasmid transfer between single partners, revealing that transfer is very fast (<15 min) and triggers major cell surface changes in transconjugant cells. We anticipate that the single-cell technology developed here will enable researchers to mechanically control gene transfer among a wide range of Gram-positive and Gram-negative bacterial species and to understand the molecular forces involved. Also, the method could be useful in nanomedicine for the design of antiadhesion compounds capable of preventing intimate cell−cell contacts, therefore providing a means to control the resistance and virulence of bacterial pathogens.

KEYWORDS: *Adhesion force, bacterial sexuality, conjugation, plasmid transfer, atomic force microscopy, single-cells*

Unlike eukaryotes, bacteria have the unique ability to physically transfer part of their DNA between cells. Bacterial sexuality, also known as horizontal gene transfer (HGT), is a major driver of genome evolution in most bacterial species, leading to improved fitness and adaptation under the variable selective pressure of the environment.[1,2] HGT is also responsible for the spread of many antibiotics resistance and virulence genes, which is of major concern in medicine.[3] Understanding the basic mechanisms leading to HGT could offer new opportunities for bacterial control in therapeutic approaches. Also, this knowledge could be exploited for bacterial domestication in the context of biotechnological applications such as the development of new genetic tools.

Bacterial sexuality essentially relies on two mechanisms, that is, competence for DNA transformation (capture and uptake of extracellular naked DNA)[4] and conjugation (transfer of DNA by cell-to-cell direct interactions).[5] These mechanisms are generally regulated by signaling molecules leading for social interactions and involve variable reprogramming of bacterial functions (e.g., cell division, DNA replication, recombination, cell envelope, and general metabolism), which for the extreme case of competence can be considered as a true developmental process.[4−8] In the conjugation process, a donor cell gets in

Received: June 18, 2018
Revised: August 27, 2018
Published: August 31, 2018

DOI: 10.1021/acs.nanolett.8b02463
Nano Lett. 2018, 18, 5821−5826

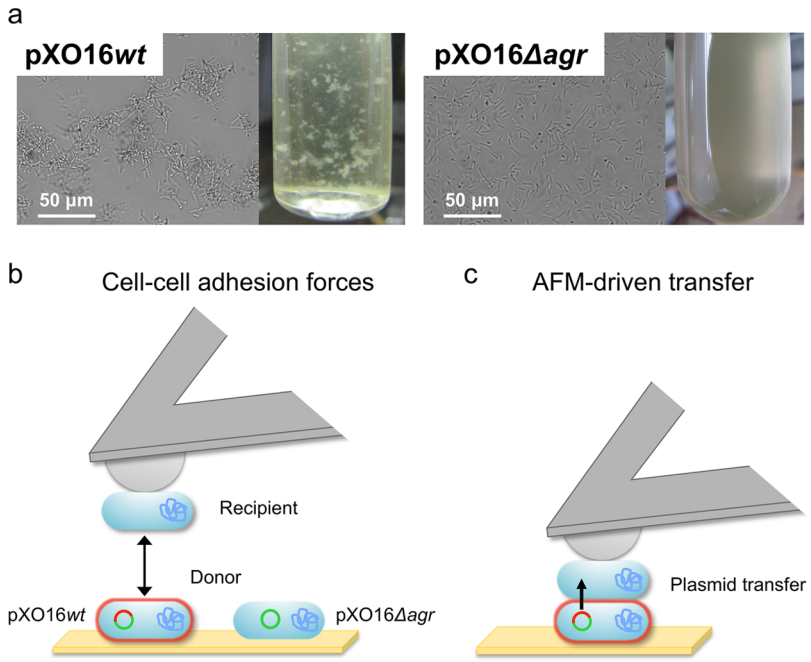


Figure 1. Probing and controlling bacterial sexuality at the nanoscale. (a) Optical microscopy images and stereomicrographs of bacterial suspensions of *B. thuringiensis* pXO16 recipient cells mixed with either pXO16*wt* donor cells harboring the full-length pXO16 plasmid, or pXO16Δ*agr* mutant cells lacking giant surface proteins. (b) We used AFM to measure adhesive forces between single pXO16*wt* or pXO16Δ*agr* donor cells and single recipient cells attached on the cantilever probe. The green circle inside the donor cell is the pXO16 plasmid, and the red outer layer the large surface proteins encoded by the *agr* region (shown also in red in the plasmid). (c) AFM also enabled us to induce plasmid transfer between mating partners and to probe the resulting changes in cell surface properties.

contact with a recipient cell to transfer part of its DNA, be it a plasmid or a chromosome. While conjugation mechanisms in Gram-positive bacteria are remarkably diverse, their molecular details are much less understood than those of Gram-negative model bacteria. In Gram-negative bacteria, sexual pili are produced by donor cells to mediate attachment to the target recipient cells.[9] Similar sexual appendages have not yet been discovered in Gram-positive systems. Instead, some encoded cell surface proteins are believed to be responsible for the aggregation of the mating partners.[5]

Strains from the Gram-positive bacterium *Bacillus thuringiensis* usually harbor large sets of plasmids, some of which carry the entomopathogenic δ-endotoxins.[10,11] Among these, the large conjugative plasmid pXO16 (350 kb) from *B. thuringiensis* sv. *israelensis* encodes an unusual conjugation mechanism that is accompanied by a macroscopic aggregation phenotype in liquid matings between two aggregation phenotypes, Agr+ and Agr−.[12] The Agr+ phenotype and its transfer capabilities were unambiguously correlated to the presence of pXO16. Correspondingly, strains cured of pXO16 displayed the Agr− instead of the Agr+ phenotype.[13] Addition of proteases in matings abolished the pXO16-encoded coaggregation, suggesting an aggregating substance of proteinaceous nature. Recently, the aggregation phenomenon was shown to promote pXO16 transfer while not being mandatory for transfer.[14] The fast transfer kinetics is another peculiar feature of pXO16, plasmid conjugation occurring within minutes and reaching 100% efficiency in just a few hours. The minimal time for a conjugative transfer is about 4 min.[15] A donor cell needs a recovery time of about 10 min before it can transfer the plasmid again. The maturation time needed for a transconjugant to become a donor is approximately 40 min. Clearly, the unique conjugation features of the giant conjugative plasmid pXO16 give us the opportunity to explore a novel realm of DNA exchange among Gram-positive bacteria.

Biological methods that are traditionally used to analyze HGT mechanisms provide information on whole cell populations. By contrast, single-cell technologies offer new opportunities for analyzing cellular properties, interactions, and heterogeneity in a way that was not possible before.[16−18] Single-cell methods include flow cytometry, micromanipulations, microcapillary electrophoresis, microfabrication and microfluidics[19,20] and nanoscopy techniques.[21,22] Until now, live-cell nanoscopy has never been used to study and control HGT in single bacteria. Here we use atomic force microscopy (AFM) to study the strength of cell−cell adhesion during conjugation of pXO16 (Figure 1b) and to mechanically control DNA transfer between single bacteria (Figure 1c). The results show that the forces between donor and recipient cells are very strong and mediated by specific surface proteins encoded in the *agr* region[14] of the plasmid. They correlate with the microscale cell aggregation behavior and with the occurrence of plasmid transfer, suggesting that they are critical to conjugation. AFM-induced mechanical contact between mating partners enables to achieve plasmid transfer within 15 min and to induce major changes in the surface properties of the transconjugant cells.

We first confirmed the aggregation phenotype of cells containing wild-type pXO16 (pXO16*wt*) using cell population assays (Figure 1a; see Methods in SI). When mixed together in liquid medium, recipient and pXO16*wt* donor cells formed aggregates of various sizes from ∼25 μm to millimeter-sized aggregates visible to the naked eye. In contrast, recipient cells and pXO16Δ*agr* donor cells,[14] that is, cells impaired in the expression of large surface proteins, did not lead to observable aggregates at the micro- or macroscopic scales. These

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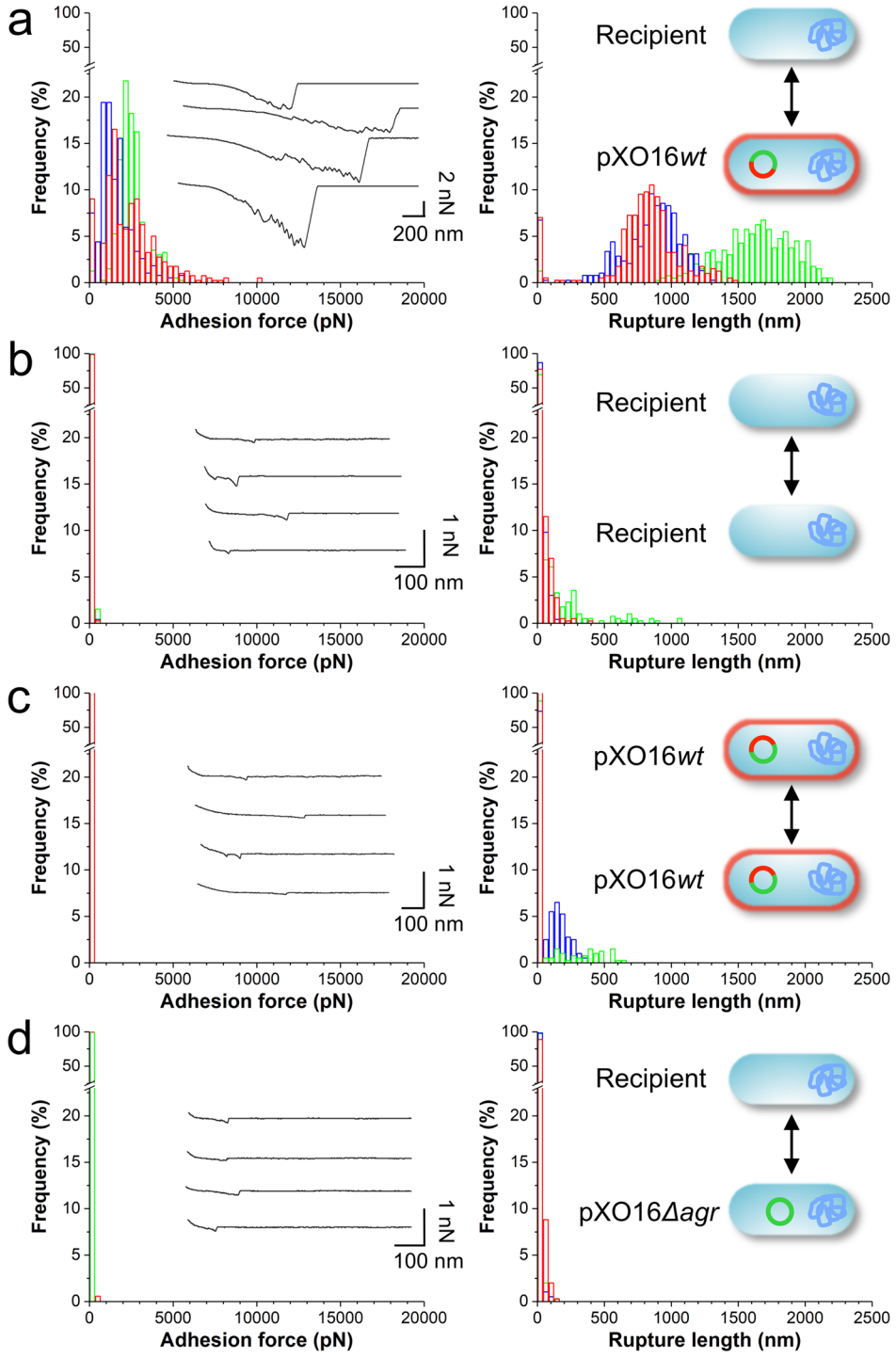

Figure 2. Adhesion forces between mating partners are very strong and involve large adhesion proteins encoded by plasmid pXO16. Adhesion force and rupture length histograms obtained in PBS between pXO16*wt* and recipient cells (a), recipient and recipient cells (b), pXO16*wt* and pXO16*wt* cells (c), and pXO16Δ*agr* and recipient cells (d). For each condition three independent cell pairs are presented (different colors). Shown in the insets are representative force signatures.

observations confirm the implication of pXO16 in the aggregation process, especially the 27-kb deleted part of the plasmid in the pXO16Δ*agr* mutant. This region codes for proteins with putative surface functions, notably two giant proteins of 3,526 and 2,558 residues believed to function as adhesins as they contain collagen-binding B-type domains and a cell-wall anchoring LPXTG domain. Suspensions of pXO16*wt* donor cells did not form aggregates either, suggesting that the large proteins exposed on the surface of pXO16*wt* cells specifically bind to ligands on recipient cells.

We sought to measure the forces involved in conjugative aggregation using single-cell force spectroscopy (SCFS; see Methods in SI).[23,24] In the standard experiment, a recipient cell is immobilized on the AFM cantilever, and the cell probe is then used to directly measure the interaction forces with a pXO16*wt* donor cell (Figure 1b). Figure 2a shows the

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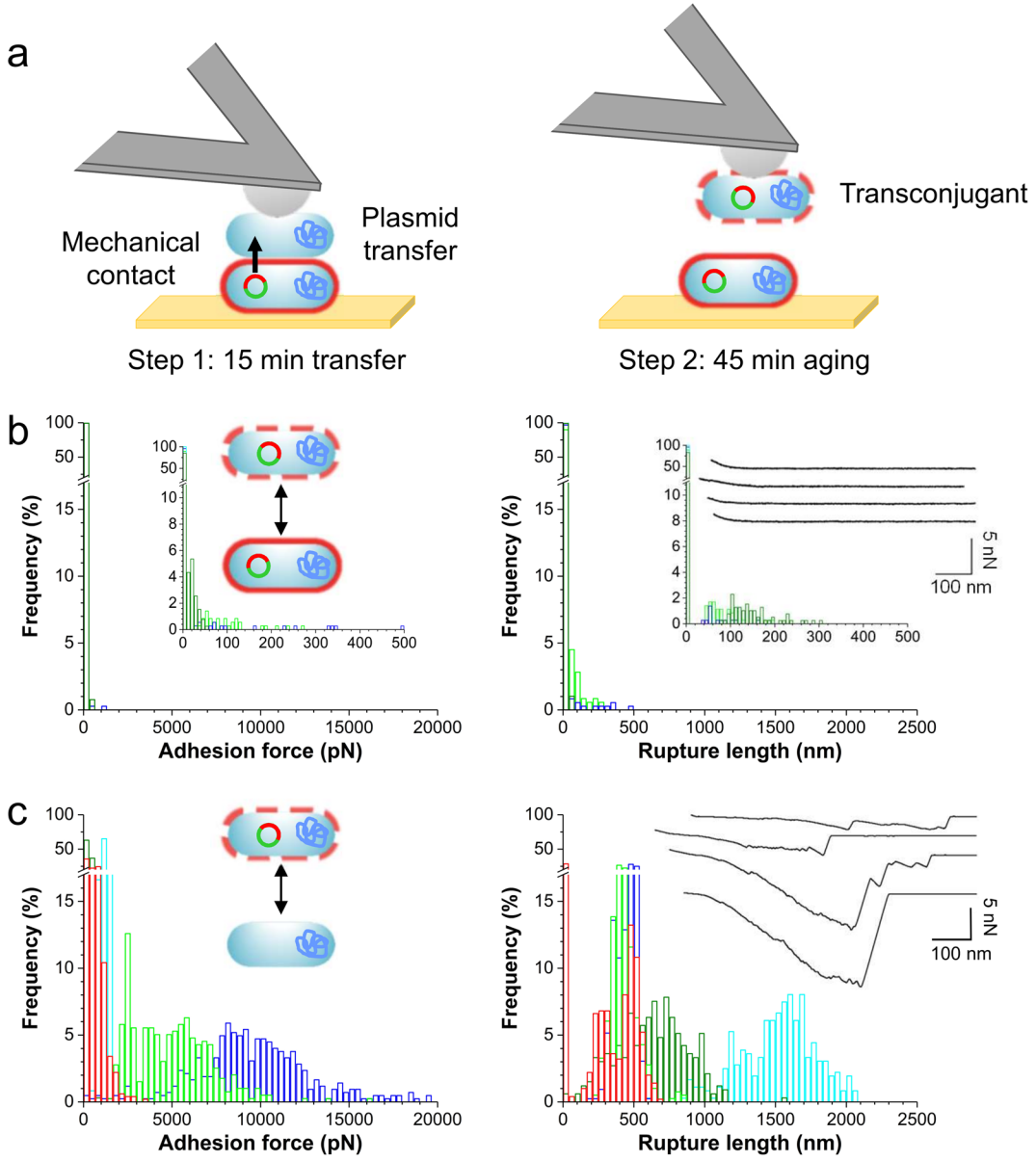

Figure 3. AFM as a tool to induce gene transfer between single cells. (a) AFM was used to mechanically set plasmid transfer in minimal medium during 15 min; after retraction of the cell probe and 45 min of maturation, cell−cell adhesion forces were measured to track changes in cell surface properties resulting from plasmid transfer. The thin surface coat on the transconjugant cell (in red) represents a heterogeneous layer of giant adhesion proteins coded by the plasmid. Force data obtained in minimal medium between transconjugant and pXO16*wt* cells (b), and between transconjugant and recipient cells (c). For each condition, five independent cell pairs are presented (different colors).

adhesion forces, rupture lengths, and typical force signatures obtained for three representative donor-recipient cell pairs (for more cell pairs, see Figure S1 in the SI). Most force curves (~95%) showed large adhesion force peaks with a maximum adhesion force of 2,286 ± 573 pN (mean ± s.d., n = 1,127 adhesive curves from three cell pairs). There were substantial variations from one cell pair to another, which we attribute to differences in contact area between the two interacting rod-shaped bacteria, and to intrinsic cellular heterogeneity, that is, all bacteria in the cell population do not have the same surface properties. Rupture lengths varied from ~800 to ~1,600 nm (864 ± 13 nm, 1,623 ± 13 nm, and 855 ± 9 nm, for cells 1−3, respectively). Many adhesion signatures featured sawtooth patterns reflecting protein unfolding. These observations indicate that conjugation correlates with strong adhesion between mating partners that results from the binding and unfolding of surface proteins. Interestingly, these forces were never observed between two recipient or two donor cells (Figures 2b,c; mean adhesion frequency of 28 ± 4% and 12 ± 12%, respectively; mean adhesion forces of 94 ± 17 and 60 ± 26 pN; for more cell pairs, see Figure S1 in SI), supporting that donor−recipient cell adhesion forces involve specific interactions.

We postulated that these specific forces are mediated by large surface proteins encoded by the pXO16 plasmid. To test this hypothesis, we probed the interaction between recipient cells and pXO16Δ*agr* mutant cells (Figure 2d; for more cell pairs, see Figure S1 in SI). Both the adhesion frequency (9 ± 11%) and adhesion force (82 ± 9 pN) were strongly reduced, thereby enabling us to confirm the key role of surface proteins (aggregation substances) in cell−cell adhesion and to rule out the contribution of other cell wall components. Putative actors

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of aggregation are the two giant proteins identified in the pXO16 sequence.[14,25] Considering that each amino acid accounts for 0.36 nm of the contour length of an extended protein,[26] the 2,558 and 3,526 amino acid residues of the two proteins should give rise to molecular extensions of 921 and 1,269 nm, which is in good agreement with our rupture lengths in the 800−1,600 nm range. What is the molecular origin of the measured cell adhesion forces? Given the magnitude and shape of the adhesion events we believe that multiple giant proteins are involved. Variations in rupture lengths may originate from the extension of multiple loosely bound proteins attached to each other and making a bridge between the two cells. Further single-molecule experiments using purified giant proteins are needed to better understand their binding mechanism, particularly the strength of single bonds, the nature of the ligands involved, and the number of bonds between two cells. Taken together, these results show that high interaction forces are associated with donor−recipient mating and can be attributed to giant surface proteins coded for by pXO16. Such high forces can therefore be considered as a marker of the presence and expression of pXO16 by the cells.

We next demonstrated that AFM can be used to mechanically induce plasmid transfer between single partners. Expression of the pXO16 plasmid by the donor cells leads to the presence of giant surface proteins acting as aggregation substances. Hence, we expect that transconjugant cells should express these proteins following transfer. Therefore, we asked whether AFM could be used as a single-cell manipulation tool (i) to induce, through cell-to-cell contact, the transfer between donor and recipient cells, and (ii) to further probe pXO16-dependent surface remodelling in the new transconjugant cell (Figure 1c and Figure 3a; see Methods in SI). As the transfer of pXO16 occurs within 4 min,[15] we established a mechanical contact between donor and recipient cells for 15 min. To allow for surface modifications to take place post-transfer, we then retracted the transconjugant cell probe and let the cell rest for another 45 min, allowing the expression of the pXO16-encoded surface proteins in the transconjugants. We measured the forces between the matured transconjugant cell probe and a donor cell (Figure 3b). To ensure proper protein expression, plasmid transfer, cell rest and force measurements were performed in minimal medium. Compared to recipient-donor forces (Figure 2a) where very high adhesion frequencies (95%) and forces (2,286 pN) were observed, transconjugant-donor forces were much weaker (Figure 3b) with adhesion frequencies of 21 ± 2% (mean ± s.d. for five different cell pairs) and adhesion forces of 864 ± 205 pN (mean ± s.d. for five different cell pairs). The poor adhesion provides direct demonstration that the transconjugant cell surface has undergone major changes, thus that successful transfer of the plasmid has occurred. When the same experiment was performed in PBS (see Figure S2 in the SI), weaker adhesion was also noted but force profiles with unfolding patterns were still observed, suggesting that some protein-based interactions remained between the cells. A reasonable explanation for this is that protein expression is not favored in PBS, that is, in the absence of nutrients. Therefore, the cell surface is only partially covered with giant surface proteins, meaning putative ligands are still surface exposed.

To further confirm that transfer and protein expression occurred in these single-cell experiments, the forces between transconjugant cells and recipient cells were also analyzed (Figure 3c). Compared to recipient−recipient pairs (Figure 2b) where weak adhesion frequencies and forces were found, here the adhesion was dramatically enhanced (94 ± 2%; 3,367 ± 789 pN; mean ± s.d. for five different cell pairs). While cell adhesion forces varied from one cell to another, the force characteristics were quite similar to those observed for recipient−donor cells, suggesting they involved giant surface proteins. High forces were not observed in PBS, confirming again that proper plasmid expression requires nutrient sources. Collectively, these findings demonstrate that a 15 min long, mechanically induced contact between mating partners was sufficient to achieve transfer, and that another 45 min of maturation dramatically strengthened the adhesive properties of the transconjugants. From a nanotechnology perspective, our results show the power of AFM to mechanically control and activate bacterial sexuality (HGT) between individual partner cells and to further track subsequent changes in cell surface properties. An important challenge will be to increase the throughput of the method so that larger number of cells can be readily analyzed. Using our experimental setup, a number of cells were found to detach from the cantilever owing to the very strong cell−cell adhesion forces, thus limiting the throughput of the analyses (Figure S3 in SI). In the future, we anticipate that the fluidic force microscope (FluidFM) technology should solve this problem.

In summary, we have shown that AFM represents a powerful platform for controlling gene transfer between single mating partners, and for probing the nanoscale forces involved in the process. Our main findings are that (i) the transfer mechanism of the conjugative plasmid pXO16 involves strong adhesion forces between mating partners, (ii) these forces reflect specific interactions mediated by two large surface proteins encoded in the *agr* region of the plasmid, (iii) single-cell adhesion correlates with microscale aggregation and with plasmid transfer, supporting a model where conjugation is promoted by cell−cell adhesion, (iv) AFM can be used to mechanically induce plasmid transfer between single partners, (v) this method reveals that pXO16 plasmid transfer is very fast (<15 min) and induces major changes in cell surface properties of the transconjugant cell.

Our nanoscale experiments offer exciting prospects for studying the mechanisms of conjugative DNA transfer at the single-cell level. We expect that AFM will contribute to understand the binding mechanisms of conjugative pili in Gram-negative bacteria and surface adhesins in Gram-positive bacteria. As HGT is responsible for the spread of many antibiotics resistance and virulence genes,[3] understanding the basic mechanisms involved in this process may offer opportunities for the development of antibacterial therapies. In particular, the design of antiadhesion compounds capable of preventing intimate cell−cell contacts could contribute to control the resistance and virulence of bacterial pathogens.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.8b02463.

Supplementary Figures and additional information about the methods (PDF)

DOI: 10.1021/acs.nanolett.8b02463
Nano Lett. 2018, 18, 5821−5826

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Work at the Université catholique de Louvain was supported by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (Grant Agreement 693630), the FNRS-WELBIO (Grant WELBIO-CR-2015A-05), the National Fund for Scientific Research (FNRS, Grants to A.G. and J.M.), and the Research Department of the Communauté française de Belgique (Concerted Research Action, Grants ARC 12/17-046 and ARC 17/22-084). Y.F.D. is Research Director at the FNRS.

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DOI: 10.1021/acs.nanolett.8b02463
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