Evidence of DNA Transfer through F-pilus Channels during *Escherichia coli* Conjugation

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The mechanism of DNA transfer from *Escherichia coli* (*E. coli*) Hfr donor strain AT2453 to recipient strain AB1157 during the conjugation process has been investigated by liquid atomic force microscopy (AFM). With the success of immobilizing both *E. coli* strains on gelatin-treated glass under aqueous solution, the F-pilus between an *E. coli* mating pair could be clearly imaged and dissected by an AFM probe. Another AFM probe functionalized with an anti-single-stranded DNA (ssDNA) antibody was then applied to detect transferring ssDNA. According to the AFM force spectrum, the transferring ssDNA could be detected only in the dissected area with a binding force of 109 ± 5 pN measured. Our results provide direct evidence indicating that the DNA was transferred through the F-pilus channel between an *E. coli* mating pair during their conjugation.

Introduction

Horizontal gene transfer is a fundamental mechanism for bacteria to adapt to environment changes that may lead to increased genetic variation by recombining DNA from different genetic sources. Conjugation is one of the major modes for horizontal gene transfer in the natural environment.[1] Bacterial conjugation between *Escherichia coli* cells has been a well-known phenomenon.[2] Based on the model established by previous reports,[3] the parental DNA (double-stranded DNA) from donor cells is unwound into single-stranded DNA (ssDNA), which is then transferred to recipient cells. The most visible feature of the F-plasmid-mediated conjugation system of *E. coli* is the F-pilus, a long and thin tubular structure,[4] extended originally from the bacterial cell surface. In the past decades, many elegant experiments about *E. coli* conjugation have been performed by using transmission electron microscopy (TEM) analyses,[5] and the results imply that the F-pilus plays an important role in the conjugation process.[6–9] Various theories have been brought up on the role that F-pilus plays in DNA transfer during *E. coli* conjugation. Gelvin[6] as well as Harrington and Rogerson[7] have reported that DNA transfer during *E. coli* conjugation might be via the F-pilus channel. On the other hand, Panicker and Minkley,[8] and Samuels and colleagues[9] described that the F-pilus interacts with the recipient cell and retracts by depolymerization into the donor cell, thereby allowing intimate wall-to-wall contact for DNA transfer in the conjugation process. Durrenberger et al. also reported DNA transfer between closely associated cells and suggested that the DNA goes through after the F-pilus retracts.[10] These proposed models were primarily derived from the results of TEM observations on nonliving cells and of biochemical studies. Direct evidence to demonstrate how DNA is transferred through the conjugation process remains lacking.

Deviated from previous works, liquid atomic force microscopy (AFM) is used in this work to investigate the DNA transfer mechanism during *E. coli* conjugation. Compared with other techniques such as scanning electron microscopy (SEM) and TEM, the most fascinating advantage of AFM is that the high-resolution microscope allows investigations and observations of biological specimens in physiological conditions.[11,12] In addition, AFM can also be used as an ultrasensitive force measurement apparatus as reported earlier.[13–15] By functionalizing an anti-ssDNA antibody onto AFM probes, we can utilize AFM force spectroscopy and the specific binding between ssDNAs and the anti-ssDNA antibody to detect the presence of ssDNA, so as to investigate the DNA transfer mechanism during *E. coli* conjugation in vivo.

In this study, both an *E. coli* conjugation donor and recipient were cultured and then immobilized on a glass coverslip. The F-pilus between the *E. coli* AT2453 and AB1157 mating pair was imaged by using an AFM probe and then dissected. Another AFM probe functionalized with anti-ssDNA antibody was applied to detect the ssDNA in the dissected area and measure the binding

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force between the anti-ssDNA antibody and ssDNA. As comparisons, force measurements were also conducted on undamaged F-pili or regions away from the dissected area. In addition, the measurements on pure ssDNA were carried out to further confirm the value of the binding force between the ssDNA and anti-ssDNA antibody.

Methods

Bacterial Growth and Mating Conditions. *E. coli* AT2453 (CGSC 4505: Hfr *thi1 lysA22 relA1 spoT1 e14*$^-$ λ^-), the mating donor,[16,17] was cultured at 37 °C on Luria−Bertani (LB) agar plates for 10 h until midexponential phase. *E. coli* AB1157 (ATCC 29055: F$^-$ *thr*$^-$ *leu*$^-$ *his*$^-$ *pro*$^-$ *arg*$^-$ *lac*$^-$ *gal*$^-$ *ara*$^-$ *xyl*$^-$ *mtl*$^-$ *T6*^{r} *rpsL*), the mating recipient,[18] was grown at 37 °C in LB broth supplemented with 100 μg/mL streptomycin overnight. Approximately 1×10^8 colony forming units of *E. coli* AB1157 from the overnight culture were inoculated into 4 mL of fresh LB broth and incubated at 37 °C with aeration until the culture reached midexponential phase.[19]

Conjugation was started by mixing the *E. coli* strains AT2453 and AB1157 in appropriate volume ratios to give final titers of about 2×10^8 donor cells and 4×10^9 recipient cells per milliliter in distilled water. The mating mixtures were gently agitated at room temperature for 30 min to form specific mating pairs and then diluted into distilled water. After they were applied onto coverslips for 15 min for *E. coli* immobilization, the coverslip was then rinsed gently with distilled water to remove unbound *E. coli*. Liquid AFM observation to find the *E. coli* pairs in conjugation was then conducted immediately after applying the distilled water.

Substrate Surface Treatment for *E. coli* Immobilization. A gelatin solution was prepared[20] by dissolving 0.5 g of gelatin (Sigma #G6144) and 10 mg of chromium ammonium sulfate in 100 mL of distilled water at 60 °C. After cooling to 40 °C, the solution was spin-coated on glass and air-dried overnight to provide a surface suitable for the immobilization and imaging of bacteria.

AFM Imaging/Dissection and Force Measurements. Following immobilization, appropriate *E. coli* mating pairs (with F-pilus in between) were chosen for imaging first and the AFM operation was then switched to manipulation mode for performing F-pilus dissection, back and forth for one time, both using silicon AFM probes without antibody functionalization. The dissected area was then imaged again to confirm the dissection of the F-pilus and record its location. The break in the pili after dissection could be enlarged and imaged. However, the ssDNA could not be imaged due to the size beyond the resolution limit of liquid AFM.[21] With precise alignment based on the recorded location, the AFM probe was then replaced by an anti-ssDNA antibody functionalized AFM probe to detect ssDNA signals and measure the force at the same location of the dissected area. All processes carried out under AFM were controlled at less than 90 min in our experiments to avoid the completion of conjugation (about 2.5 h after mixing).[22]

Two sets of AFM probes were used in this work: one set of unfunctionalized silicon probes for imaging and dissecting and the other set functionalized with an anti-ssDNA antibody for force measurement. It is critical that the position between these two AFM probes needs to be aligned precisely when the silicon probe for the imaging/dissecting is replaced by the anti-ssDNA antibody functionalized probe for force measurement at a particular point. This was carried out by roughly aligning the imaging/dissecting probe and force measurement probe via a charge coupled device (CCD) screen connected to the optical microscopy (OM) part of the AFM system. The force measurement probe was then used to image for precise alignment. The vertical force exerted on the surface during an AFM scan was about 0.1−0.3 nN, which may not impair the function of the antibody and the covalent binding between the probe and antibody.[14]

AFM images were recorded using a NanoWizard atomic force microscope (JPK Instruments AG Berlin, Germany). AFM imaging was carried out in liquid using contact-mode operation at a scan rate of 1 line/s and 256 pixels per line. The probes for imaging/dissecting and detecting ssDNA had the same type of silicon cantilevers (MikroMasch, Tallinn, Estonia) with a Cr (20 nm)/Au (20 nm) coating and a spring constant of 0.03 N/m determined by the thermonoise method.[23] The loading rate was 0.5 μm/s, and the contact time was 0 s, meaning the tip was retracted immediately after reaching the set point. In this work, all those parameters were well controlled, with the same loading rate and same contact time for all the measurements. The force we applied to rupture the F-pilus (in the midway) for force measurements was about 7.5 nN in order to make sure that the AFM tip had positively ruptured the F-pilus.

For the force measurements in this work, the sensitivity value S (V/nm) was also well calibrated. The optical lever sensitivity for each curve was different. The deviation of S is obvious between the AFM probes with and without functionalization, while it is slight between the probes of the same types. Therefore, the sensitivity was readjusted every time when the tip or the position of the laser was changed. Besides, the sensitivity was also calibrated to check if there is any contamination of the AFM tip.

F-pilus and Mating-Pairs Identifications. In this work, the F-pili were identified based on their diameter, which was different from that of the flagella. Typically, the diameter of the flagella is about 20 nm,[24] which is larger than that of the F-pili (8−10 nm).[25,26] Therefore, only the filamentous structures that showed a diameter of about 10 nm were considered as the F-pili for this study, otherwise they were regarded as flagella.

It is difficult to differentiate between AT2453 and AB1157 after they were mixed, as they all appear rod shaped. As two *E. coli* cells with an F-pilus in between were observed under AFM (approximately 5% of immobilized *E. coli* pairs with this feature), the F-pilus was then dissected for force curve measurement. Two groups were obtained (about 50% for each group), which include one with specific binding (17 binding events for about 30 measurements) and the other with the forces similar to that obtained on the dissected F-pilus of AT2453. Since the latter was very likely due to "two cells are near one another", only those cases with specific binding measured were considered as the mating pairs.

Functionalizing AFM Probes with Antibodies. The AFM probes used for force measurement were commercial silicon probes (MikroMasch, Tallinn, Estonia) coated with 20 nm of chromium and 20 nm of gold in sequence. The probes were first thiolated by immersion in 0.15 M cysteamine for 4 h at room temperature in darkness. The resulting monolayer-modified probes were gently rinsed with phosphate buffer saline (PBS) to remove physically adsorbed cysteamine.[27] After the probes were soaked in 2.5% glutaraldehyde (in PBS) for 3 h at room temperature in darkness, they were washed gently with PBS to remove unbound glutaraldehyde. The treated probes were then functionalized with monoclonal anti-ssDNA antibody MAB3034 (Chemicon International, Temecula, CA) for 3 h at room temperature. The time elapsed between modification of the AFM tip and the subsequent force spectroscopy was about 10−20 min.

The reason for using the antibody MAB3034 to functionalize AFM tips is that the transferring DNA during conjugation is ssDNA,

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a single strand of DNA.[28] The antibody MAB3034 with great specificity to ssDNA, 90% reactivity to ssDNA, and 12% reactivity to double-stranded DNA (dsDNA)[29,30] is therefore used as the anti-ssDNA antibody to functionalize AFM tips for detection of the transferring ssDNA.

Verification of Anti-ssDNA Antibody Functionalized onto AFM Probes. Rhodamine-labeled goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc. PA) was used to verify whether the anti-ssDNA antibody had bound onto the Au-coated silicon AFM probes. The AFM probes were functionalized with the anti-ssDNA antibody first and then treated with 50 μL (1:100 dilution) of anti-IgG antibody sequentially for fluorescent labeling, followed by gentle shaking for 1 h at room temperature in the darkness. The AFM tips were then rinsed with fresh buffer to remove any unbound antibody and observed under the fluorescence microscope. A controlled experiment was conducted on similar Au-coated silicon AFM probes prefunctionalized with bovine serum albumin (BSA), using the same process and the same fluorescence dye-labeled anti-IgG antibody for fluorescent labeling.

Measurement of Specific Binding between ssDNA and Anti-ssDNA Antibody. A controlled experiment was conducted to measure the specific binding between ssDNA purified from bacteriophage M13 (immobilized on thiolated gold substrate) following a standard method[31] and anti-ssDNA antibody (MAB3034, functionalized on AFM probe). The immobilization protocol of ssDNA to thiolated gold substrate (deposited by an E-gun system) was similar to the AFM modification protocol described above, except that the anti-ssDNA antibody was replaced by 8 ng/L ssDNA extracted from bacteriophage M13 (in distilled and deionized water). As the concentration of ssDNA is sufficiently low allowing one to image an individual ssDNA string on a mica surface as reported,[32] it could also ensure that the force measured here is mostly from a single binding between the AFM tip and ssDNA.

In this work, the conjugation type of the strain is the Hfr strain that does not contain the plasmid form F factor. The use of the Hfr strain is to prolong the conjugation process (DNA transferring) for the convenience of experiments. The source of ssDNA used in the controlled experiment was purified from ssDNA bacteriophage M13. The specific binding of anti-ssDNA (MAB3034) with M13 ssDNA should be same as that with the ssDNA in F-pili.

Application of the Poisson Statistical Method. The Poisson statistical method[33–35] was used to determine the forces required to separate a single pair of ssDNA and its antibody. The advantage of this method is that it provides accurate rupture forces of individual molecule bonds in the presence of moderate-to-large amounts of different types of variations or noises. Based on the assumptions, the measured total adhesive force is contributed by a finite number of binding forces resulting from interacting molecular pairs (ssDNA and its antibody in this case). For a fixed contact area, the number of interacting molecular pairs will follow the Poisson distribution. Therefore, the measured total adhesive force also follows a similar distribution, which can be derived as follows:

$$\sigma_m^2 = \mu_m F_i - F_i F_0$$

where the terms F_i and F_0 represent individual molecule binding forces and long-range interactions (background force that may be present), respectively.[33,34] F_i can be derived from the slope and intercept of the linear regression curve, that is, the variance (σ_m^2) versus the mean (μ_m) of the pull-off force.

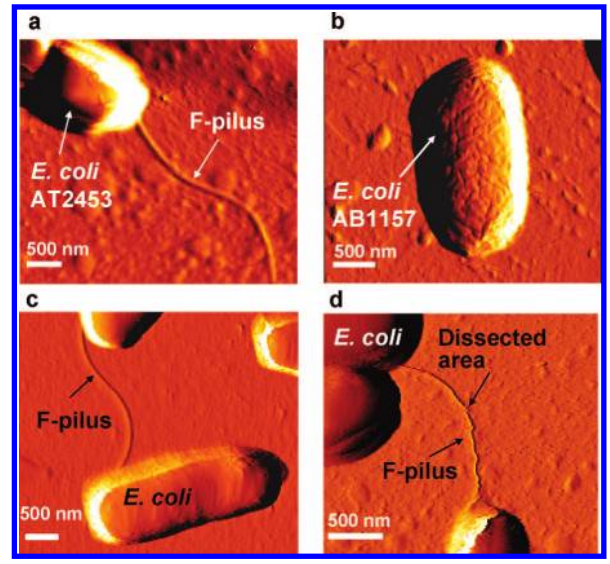

Figure 1. AFM images under aqueous solution of (a) *E. coli* AT2453, (b) *E. coli* AB1157, (c) *E. coli* AT2453 and AB1157 mating pairs, and (d) dissected F-pilus between *E. coli* AT2453 and AB1157.

Results and Discussion

Immobilization of *E. coli* and F-pili. The major issue of observing biological structures in their living state is that the samples must be firmly attached to a flat and solid support to resist the lateral forces applied by an AFM probe. Several strategies for immobilizing bacteria in liquid have been established for AFM studies.[20,36–42] Depending on the cell surface characteristics, the optimal conditions for immobilization of different microbial strains may vary significantly and thus need to be determined empirically.

After various trials of immobilization processes, gelatin-treated coverslips were found to be able to immobilize *E. coli* successfully so that both *E. coli* and the F-pili can be clearly imaged in liquid AFM. Figure 1 shows the success of imaging *E. coli* AT2453 carrying an F-pilus, *E. coli* AB1157, an F-pilus between an *E. coli* mating pair, and a dissected F-pilus between the *E. coli* mating pair in liquid with the use of this immobilization process and contact-mode AFM imaging. Other immobilization strategies using agar, glutaraldehyde, poly-L-lysine, and 3-aminopropyltriethoxysilane (APTES) failed to immobilize *E. coli* properly or to produce even surfaces for AFM imaging.

The percentage of cells undergoing conjugation at any time during AFM imaging and measurement was very low as measured by the auxotrophic markers His^+ and Thr^+. According to the observation under liquid AFM, approximately 5% of the immobilized *E. coli* pairs were found to have an F-pilus in between, which were likely in conjugation. The reasons remained

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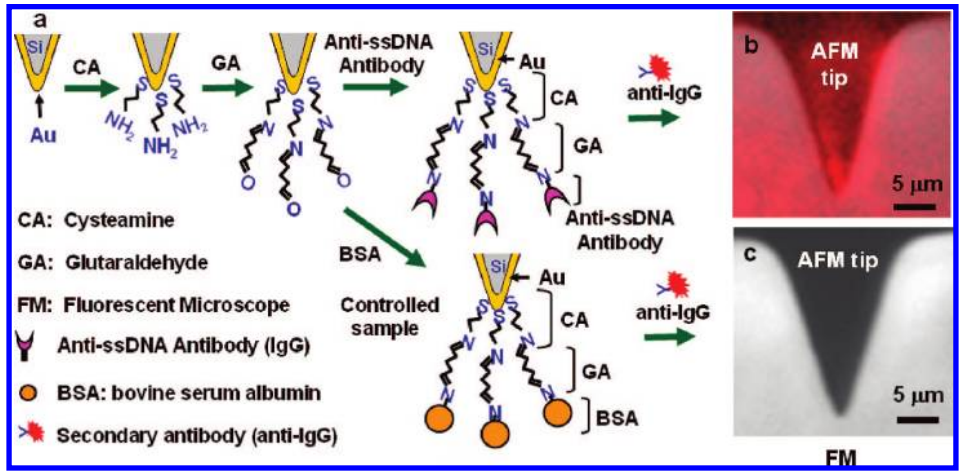

Figure 2. Verification of anti-ssDNA antibody (IgG) functionalized onto AFM probes. (a) Schematic of the AFM probe functionalized with anti-ssDNA antibody (IgG) or BSA (controlled sample). Fluorescent microscopy images after secondary antibody labeling of the AFM probes modified by the same process as in (a) for the probe functionalized with (b) anti-ssDNA antibody and (c) BSA.

unknown, possibly due to some of F-pili being removed during sample preparation or floating in liquid AFM, which were hard to observe.

In this work, the F-pili were identified based on their diameter, which is different from that of flagella. Typically, the diameter of flagella is about 20 nm (ref 24), which is larger than that of F-pili (8−10 nm) (refs 25 and 26). As the resolution of AFM in the *Z* direction is normally much better than that in the *X-Y* direction,[43] the diameter of 8 nm for F-pili was measured by using AFM height imaging, which could provide the information of the vertical distance between the highest point of the pili and the substrate (in the *Z* direction) that can be considered as the F-pili diameter. As the resolution of liquid AFM is about 1 nm in the *Z* direction,[43] the F-pili of 8 nm in height (i.e., diameter) can be imaged. By using the error signal (i.e., deflection image) to enhance the edge further,[44] the F-pili can be imaged clearly, as is observed in Figure 1a and c. However, as the resolution in the *X-Y* plane is limited by the size of the AFM cantilever tip (about 20 nm used in this work) due to the tip shape effect,[45] the F-pilus with a diameter of 8 nm will display an image with a width of 28 nm (20 + 8 nm) in the *X-Y* plane.

Verifying Functionalization of Anti-ssDNA Antibody onto AFM Probes. To detect the presence of ssDNA, an AFM probe was functionalized with an anti-ssDNA antibody using the immobilization protocol described in the Methods section. Besides, a fluorescence dye-labeled anti-IgG antibody was applied onto an anti-ssDNA antibody functionalized AFM probe to verify whether the anti-ssDNA antibody had been successfully bound onto the Au-coated silicon AFM probes, following the scheme shown in Figure 2a. A controlled experiment was also conducted on a BSA functionalized AFM probe using the same process as shown in Figure 2a. The result in Figure 2b shows that an anti-ssDNA antibody functionalized AFM glows red under the fluorescent microscope after it was applied with fluorescence dye-labeled anti-IgG antibody sequentially. On the other hand, Figure 2c shows that no red fluorescence was observed under fluorescent microscope for the BSA functionalized AFM probe applied with the same fluorescence dye-labeled anti-IgG antibody. Comparing Figure 2b with Figure 2c, it can be observed that the red fluorescence of the anti-IgG antibody only appears on the AFM probe prefunctionalized with the anti-ssDNA antibody (Figure 2b) but not on that prefunctionalized with BSA, as no binding occurs between the BSA and anti-IgG antibody. The result in Figure 2b indicates that the AFM probe used in this work for the force curve measurement has been successfully functionalized with anti-ssDNA antibody.

Though the tips were rinsed with fresh buffer to remove any unbound antibody, a high background signal in Figure 2b was observed, mostly due to the fluorescence from the backside of the AFM probe. As the AFM probe used in this experiment was overall coated with gold and its anti-ssDNA antibody, modification was based on the reaction between gold and cysteamine; not only the tip but also the other part of the AFM probe will glow with red fluorescence.

Detecting ssDNA Signals by Anti-ssDNA Antibody Functionalized AFM Probe. Before detecting ssDNAs via measuring the binding force with anti-ssDNA antibody functionalized AFM probes, it is crucial to calibrate the magnitude of the specific binding force first. This was conducted by the force measurement on bacteriophage M13 ssDNA, which was fixed onto a thiolated gold substrate with glutaraldehyde using an anti-ssDNA antibody functionalized AFM probe. The upper figure of Figure 3a shows the surface morphology of gold deposited by using an E-gun system, with granular Au features observed. The lower part of Figure 3a shows the surface morphology which has been changed after the ssDNA is bound, featured by the speckles as observed.

The Poisson statistical method, developed by Beebe and coworkers,[33–35] has been used to determine the forces required to separate a single pair of ssDNA and its antibody. Several sets of measurements ($n = 56$ for each) for ssDNA and its antibody interactions were taken at different substrate locations. The mean of the binding force (μ_m) and the relative standard deviation (σ_m) were then calculated and summarized (see Table 1). According to Figure 3b, there appears to be a good linear relationship between the force variance (σ_m^2) and the mean force (μ_m) plotted from the experimental data. The binding force of a single ssDNA molecule and anti-ssDNA antibody binding pair was calculated to be 98 ± 8 pN.

To detect the presence of ssDNA transferred between *E. coli* cells, the interacting force between the anti-ssDNA antibody functionalized AFM probe and different regions around the F-pilus

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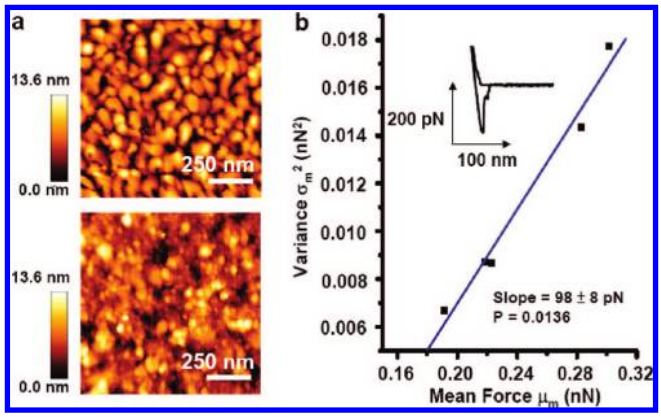

Figure 3. (a) AFM images of the granular Au surface deposited by using an E-gun system (upper), and after binding of ssDNA on a thiolated gold substrate with glutaraldehyde, featured by the speckles (lower). (b) Plots of measured binding force variance (σ_m^2) versus mean binding force (μ_m) obtained from the measurement of ssDNA on a thiolated gold substrate with glutaraldehyde using an anti-ssDNA antibody functionalized AFM probe. The top left-hand inset of plot (b) is an actual force−distance curve indicating the binding force. More than 20 AFM probes were used for measurements.

was measured. To ensure the force measured was contributed by the interaction between the anti-ssDNA antibody and ssDNA, controlled experiments were also conducted. These were carried out by comparing the results of the force measurements on the dissected area of the F-pilus between an *E. coli* mating pair (experimental sample A, results in Figure 4a) with those on gelatin-treated coverslips (controlled sample B, results in Figure 4b), the undissected region of the same F-pilus as that of the dissected one between an *E. coli* mating pair (controlled sample C, results in Figure 4c), and the dissected area of the F-pilus not in conjugation (controlled sample D, results in Figure 4d). Besides, the AFM probe without anti-ssDNA antibody decoration was also applied for the force measurement on the dissected area of the F-pilus between an *E. coli* mating pair (controlled sample E, results in Figure 4e).

Figure 4a shows a typical force measurement on the dissected area of the F-pilus between an *E. coli* mating pair using an anti-ssDNA antibody functionalized probe, revealing a sharp adhesion point observed in the retract phase of the measurement. It can observed that a much larger force of 109 ± 5 pN (n = 17) was measured from the dissected F-pilus (Figure 4a) than those measured from the controlled samples of the gelatin-treated coverslip surface (45 ± 9 pN, n = 51) and undissected region of the same F-pilus as the dissected one between *E. coli* mating pairs (11 ± 1 pN, n = 12), as shown in Figure 4b and c, respectively. Besides, a much smaller adhesive force 6 ± 2 pN (n = 16) was detected from the dissected area of the F-pilus extended from the *E. coli* AT2453 not in conjugation (Figure 4d). Furthermore, the adhesive force on the dissected area of the F-pili between *E. coli* mating pairs measured by the AFM probe without anti-ssDNA antibody decoration was only about 18 ± 7 pN (n = 23), as shown in Figure 4e.

It can be observed that the insets of Figure 4b−e do not look like specific binding events as in Figure 4a. The force values calculated in Figure 4b−e were mainly based on the definition extracted from the force curve for quantification. However, it can be regarded as an indication that the specific binding event does not occur to the samples of Figure 4b−e. Only the force curve for the F-pilus between two *E. coli* cells in conjugation exhibits this specific binding event, that is, the binding between the ssDNA and anti-ssDNA antibody.

Figure 4f summarizes the forces measured in Figure 4a−e (samples A−E, respectively) to further compare the results of experimental samples (A) with control samples (B−E); it can be clearly observed that the binding force of 109 ± 5 pN measured on the dissected area of the F-pilus between an *E. coli* mating pair is well above those of the control samples. This indicates that the nonspecific adhesive force, because of the absence of either ssDNA (Figures 4b-4d) or anti-ssDNA antibody (Figure 4e), is much smaller than the specific binding force between anti-ssDNA antibodies and ssDNA (Figure 4a). As for the relatively higher value of the force measured on the gelatin-treated coverslip (45 ± 9 pN, n = 51) compared to other controlled samples, it is suspected to be caused by the increased contact area between the AFM tip and gelatin surface due to the surface roughness of the gelatin coat as observed from Figure 1. Because the average adhesive force (109 ± 5 pN) between the anti-ssDNA antibody functionalized AFM probe and the F-pilus at the dissected point is very close to that measured on pure ssDNA (98 ± 8 pN, Figure 3b), this strongly suggests the presence of ssDNA in the breakage point of the dissected F-pilus between *E. coli* mating pairs. As the specific binding force was only detected in the dissected area of the F-pilus between an *E. coli* mating pair, it provides direct evidence that DNA was transferred through an F-pilus channel during *E. coli* conjugation.

The possible number of binding pairs between the ssDNA and its antibody on the AFM probe was theoretically calculated as follows. The tip radius of the AFM probe is less than 20 nm as confirmed by SEM analysis, and the size of IgG is about 19 nm in chord and 10 nm in radii of gyration.[46] The ssDNA diameter is about 0.24 nm (ref 47), which is relatively smaller than the size of the AFM probe radius. It is very likely that one to four anti-ssDNA antibodies on the functionalized AFM probe have a chance to bind with the ssDNA exposed in the dissected area of the F-pilus between the *E. coli* mating pair. The force measured on the dissected area of an F-pilus by a functionalized AFM probe is 109 ± 5 pN, which was obtained from the measurements on 17 different F-pili (n = 17) after dissection with a mean value of 109 pN and a small standard deviation of ±5 pN, indicating that the all of the 17 measured forces mostly fell in a confined range consistently and repeatedly. As the sulfur−gold bond that links the AFM tip surface and anti-ssDNA is reported to be 1.4 ± 0.3 nN (ref 48), it is much larger than the force measured here (109 ± 5 pN) so that its contribution can be excluded. Besides, as the binding force between a single ssDNA and anti-ssDNA antibody is about 98 ± 8 pN (Figure 3b), calculated by applying statistical methods,[18–20] it implies that the force measured on the dissected area of the F-pilus is mostly from a single ssDNA/anti-ssDNA antibody binding pair.

A controlled experiment was also carried out by using the anti-ssDNA antibody functionalized AFM probe to measure the samples with ssDNA (on thiolated gold) and those with anti-6x His antibody (on thiolated gold) or gold-only as controls, similar to the experiments shown in Figure 3. Again, an average specific binding force of about 107 pZ for each binding between anti-ssDNA and ssDNA was measured (based on the largest common denominator). However, the average force measured is about 21 pZ for the anti-6x His antibody and 19 pZ for the gold-only samples, indicating no specific binding events. The above results provide evidence again to confirm that the binding force measured

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Table 1. Results of the Binding Forces between ssDNA and the Anti-ssDNA Antibody Functionalized AFM Probe in Distilled and Deionized Water[a]

data set	probe modification	mean binding force μ_m (nN)	relative standard deviation σ_m (nN)	force variance σ_m^2 (nN^2)	size of set (n)
A	anti-ssDNA antibody	0.283	0.12	0.014	64
B	anti-ssDNA antibody	0.302	0.133	0.018	64
C	anti-ssDNA antibody	0.223	0.093	0.009	64
D	anti-ssDNA antibody	0.191	0.082	0.007	64
E	anti-ssDNA antibody	0.218	0.093	0.009	64

[a] More than 20 AFM probes were used for measurements.

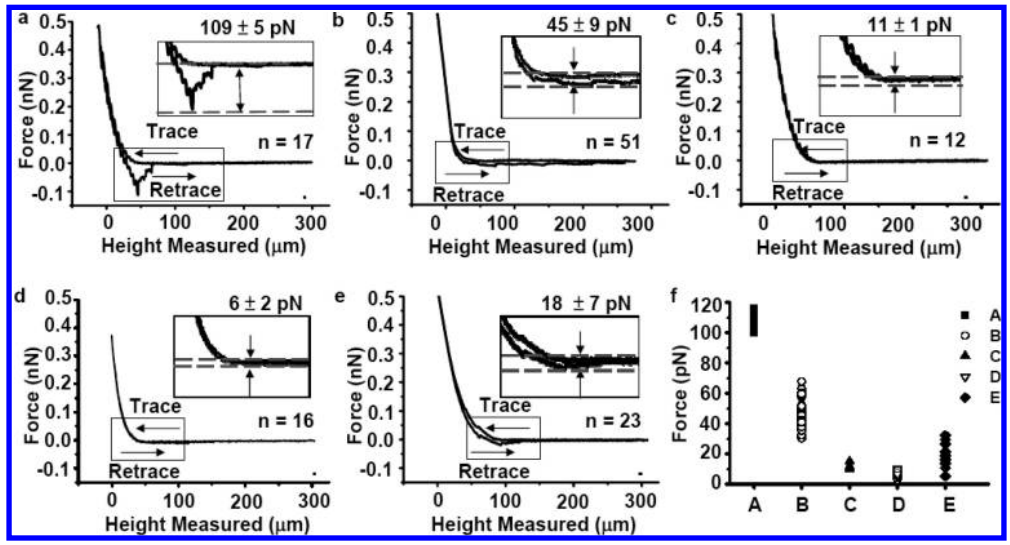

Figure 4. Typical force−distance curves for the measurements of adhesive forces between the anti-ssDNA antibody functionalized AFM probe and (a) the dissected area of the F-pilus between an *E. coli* AT2453 and AB1157 mating pair (n = 17, 17 specific binding events, 17 different samples, 17 different probes), (b) gelatin-treated glass (n = 51, 51 force curves measured from more than 20 samples and more than 20 probes), (c) the same F-pilus between an *E. coli* AT2453 and AB1157 mating pair as in (a) while away from the dissected area (n = 12, 12 samples, 2 probes), and (d) the dissected area of the F-pilus carried by an *E. coli* AT2453 not in conjugation (n = 16, 16 dissected areas, 10 samples, 2 probes). (e) Adhesive force measured from the dissected area of the F-pilus between an *E. coli* AT2453 and AB1157 mating pair measured by a unfunctionalized AFM probe (n = 23, 23 dissected areas, 10 samples, 2 probes). (f) Plot to summarize the forces measured in (a)−(e), as shown in A−E, respectively. Data are mean ± SD, n = 5. The AFM tip was washed after each measurement.

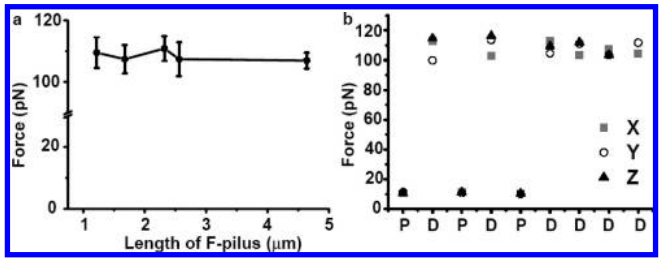

Figure 5. (a) Binding forces measured on the dissected are of the F-pili between *E. coli* AT2453 and AB1157 mating pairs, with a length of 1−5 μm. Data are mean ± SD, n = 6. Data were measured from more than 17 curves, 17 samples, and 17 AFM probes. (b) Use of a functionalized AFM probe to repeatedly probe dissected (D) and undissected areas (P) of the F-pili between *E. coli* AT2453 and AB1157 mating pairs in turns for several times. Data were measured from three samples using three AFM probes.

on the dissected F-pili between *E. coli* mating pairs is similar to that between the anti-ssDNA antibody and ssDNA and that the DNA is through the F-pili channel.

Another interesting finding on the binding force between the dissected area of the F-pilus and the anti-ssDNA antibody functionalized on AFM probes is that its magnitude is nearly independent of the length of the F-pilus (1−5 μm), as shown in Figure 5a. To ensure the validity of the force measurement without being influenced by the probe degradation,[49] possibly resulting from the antibody block by ssDNA on a dissected area or the gelatin on an undissected area during repeated probing, a reliability test for the functionalized AFM probe was designed and conducted via the force measurements of three individual AFM probes (X, Y, and Z in Figure 5b) functionalized with the anti-ssDNA antibody on three different samples. Each probe was applied on the dissected (D) and undissected (P) areas of the same F-pilus between an *E. coli* mating pair in turns for force measurements. After repeating several times, two groups of force values could be observed between those from dissected and undissected areas of the F-pilus with an obvious deviation of about 90 pN in between; it did not matter whether measurements started from the dissected (D) or undissected (P) area first for this reliability test. It indicates that each anti-ssDNA antibody functionalized AFM probe can be used without degradation, at least for nine consecutive force measurements as shown in Figure 5b.

In summary, the ssDNA transfer mechanism of *E. coli* conjugation has been studied by utilizing an anti-ssDNA antibody functionalized AFM technique in liquid. With the success of immobilizing both *E. coli* strains AT2453 and AB1157 on gelatin-treated glass under aqueous solution, the F-pilus between an *E. coli* mating pair could be clearly imaged and dissected by using an AFM probe. The anti-ssDNA antibodies were also successfully functionalized onto AFM probes, which were verified by florescent labeling, to detect ssDNA signals. Comparing the force measured on the dissected area of an F-pilus between the *E. coli* mating pair with those on a gelatin-treated glass coverslip, the same F-pilus between the *E. coli* mating pair away from the dissected area, the

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F-pilus carried by the *E. coli* AT2453 not in conjugation, and pure ssDNAs, it can be confirmed that the force of about 109 ± 5 pN is mainly contributed by the specific binding between the ssDNA and its antibody, which is independent of the length of the F-pilus. As the specific binding force was only measured in the dissected area of the F-pilus between an *E. coli* mating pair, it provides direct evidence that the DNA was transferred through the F-pilus channel during *E. coli* conjugation.

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