Measurements of the binding force between the *Helicobacter pylori* adhesin BabA and the Lewis b blood group antigen using optical tweezers

Oscar Björnham
Umeå University
Department of Applied Physics and Electronics
SE-901 87 Umeå
Sweden

Erik Fällman
Ove Axner
Umeå University
Department of Physics
SE-901 87 Umeå
Sweden

Jörgen Ohlsson
Ulf J. Nilsson
Lund University
Organic and Bioorganic Chemistry
P.O. Box 124
SE-221 00 Lund
Sweden

Thomas Borén
Umeå University
Department of Medical Biochemistry and Biophysics
SE-901 87 Umeå
Sweden

Staffan Schedin
Umeå University
Department of Applied Physics and Electronics
SE-901 87 Umeå
Sweden
E-mail: staffan.schedin@tfe.umu.se

Abstract. *Helicobacter pylori* is a world-wide spread bacterium that causes persistent infections and chronic inflammations that can develop into gastritis and peptic ulcer disease. It expresses several adhesin proteins on its surface that bind to specific receptors in the gastric epithelium. The most well-known adhesin is BabA, which has previously been shown to bind specifically to the fucosylated blood group antigen Lewis b (Leb). The adhesion forces between BabA and the Leb antigen are investigated in this work and assessed by means of optical tweezers. A model system for *in situ* measurements of the interaction forces between individual bacteria and beads coated with Leb is developed. It is found that the de-adhesion force in this model system, measured with a loading rate of ~100 pN/s, ranges from 20 to 200 pN. The de-adhesion force appears predominantly as multiples of an elementary force, which is determined to 25±1.5 pN and identified as the unbinding force of an individual BabA-Leb binding. It is concluded that adhesion in general is mediated by a small number of bindings (most often 1 to 4) despite that the contact surface between the bacterium and the bead encompassed significantly more binding sites.
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1 Introduction

Adhesion is a prerequisite for bacteria to persistently colonize epithelial surfaces. Bacteria express surface-associated adhesion molecules, i.e., adhesins, with often fine-tuned specificities for eucaryotic cell surface protein or carbohydrate structures. If bacteria are unable to adhere to the epithelial cells, they will be rapidly removed by the local unspecific host defense mechanisms, e.g., peristalsis, and turn-over of the epithelial cell populations and the mucus layer. Thus, bacteria need to efficiently adhere to withstand the forces they are exposed to. Persistent pathogens are well adapted to their host and can by intimate adherence utilize the target tissue cells as a nutrient source. The biological impact of the initial step of colonization and the notion that antiadhesive compounds (soluble receptor analogs) might be an alternative drug design for antimicrobial therapy have encouraged an increasing interest in the structures on bacterial and eucaryotic cell surfaces involved in these interactions.

H. pylori is a Gram-negative, microaerophilic, rod shaped (1.5 to 2.0×0.5 to 1.0 μm) bacterium with polar flagellae, which colonizes the human and primate gastric mucosa. It establishes persistent infections and chronic inflammations that can continue to gastritis and peptic ulcer disease. Sometimes, chronic gastritis can further develop into dysplasia through conditions of local atrophic gastritis, which is a risk factor for development of gastric cancer. *H. pylori* probably has several different adhesin molecules, most of which belong to the *H. pylori* outer membrane family of proteins, the HOPs, which are all unique for *H. pylori*. Among those proteins, the BabA adhesin is the best described. It has been shown to specifically bind the fucosylated blood group antigen Lewis b

Address all correspondence to Staffan Schedin, Department of Applied Physics and Electronics, Umeå University, SE-901 87 Umeå University, Sweden. Tel: +46–90–787487. Fax: +46–90–786469. E-mail: Staffan.Schedin@tfe.umu.se

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(Leb) and related ABO-antigens.[1,2] On red blood cells (erythrocytes), the H-1 and Leb antigens correspond to the blood group O phenotype in the ABO system, which is the blood group phenotype of people particularly prone to peptic ulcer disease.[2,3]

Interestingly, severe gastric disease is significantly associated with *H. pylori* strains that express BabA,[1,4] which suggests that BabA is a virulence factor. Experimental infections in mice that transgenically express the Leb (considered a human/primate specific antigen) suggested that *H. pylori* adherence enhances inflammatory responses in the gastric mucosa, which further argues for a role of *H. pylori* adherence in development of diseases.[5]

Scatchard analysis, which is based on affinity measurements during conditions of equilibrium, is a commonly used method to assess specific receptor bindings.[6] The Scatchard method can also, during conditions of soluble receptor saturation, provide an estimate of the full number of attachment molecules present in a certain volume of bacteria. However, to better understand the full impact of bacterial adhesion mediated by BabA as an attachment protein, e.g., the binding forces that result as a consequence of multivalency as well as cooperativity (possibly due to the organization of BabA adhesin in supramolecular complexes), it is of importance to directly measure the actual interaction forces acting on individual bacteria, e.g., with a force measuring technique. A distinct difference between the two approaches is that a Scatchard analysis provides the affinity for separate receptor-ligand complexes, whereas a direct force measurement provides information about the multivalent interactions that can take place *in vivo*, e.g., bacterial adherence to cell surfaces or to macromolecules (for example, the high molecular weight and densely glycosylated mucin molecules in the gastro-intestinal lining and secretions).

The adhesion behavior of individual *H. pylori* bacteria has, to our knowledge, not yet been investigated in terms of forces. The main reason is that such measurements require access to an *in situ* technique that is sensitive enough to detect the feeble forces (often in the low pN range) that are involved in the bacterial adhesion process at an individual bond level. Until recently, such techniques did not exist.

However, during the last years, novel techniques for *in situ* measurements of forces between biological objects have been developed. Two methods have emerged, based on atomic force microscopy (AFM) and optical tweezers (OT), respectively. Both these techniques have the potential to measure adhesive forces generated by various binding complexes on bacterial surfaces. However, the OT technique is close to one order of magnitude more sensitive than the AFM technique; it allows interaction measurements with a resolution down to parts of pN.

In short, OT consists of a microscope objective with a high numerical aperture into which a continuous laser beam is focused. A small object, with a refractive index larger than that of the surrounding medium, experiences a restoring force in the focal region, thus becoming trapped in the focus. If the small object is subjected to an external force, its position in the optical trap is shifted. The amount of shift is then a measure of the applied force. Quantitative results in terms of forces in the order of pN are obtained after a calibration procedure that converts the shift in position to a force.

Following the pioneering work by Ashkin et al.,[7,8] the OT technique was rapidly developed into a technique for force measurements in biological systems in general, and of adhesion forces in particular.[9–14] For example, Liang et al.[11] used OT to measure the forces that are involved in polyvalent adhesion of type-1 fimbriated *E. coli* bacterium to mannosylated structures. Another example of the use of OT for force measurements is the assessment of the detachment force between *S. epidermidis* and fibronectin-coated surfaces.[12] The OT technique has also been used to assess a variety of physical properties of the structure of *Escherichia coli* P pili that are associated with their ability to adhere and sustain forces from external cleaning actions.[13,14]

In this work, the OT technique has been applied to measurements of specific adhesion forces between individual *H. pylori* bacteria, which express the BabA adhesin, and its cognate receptor, the Leb. A model system has been constructed that consists of a closed sample chamber, in which a single bacterial cell is mounted on a spherical bead, while another bead serves as a force indicator in the OT. A *H. pylori BabA* mutant devoid of BabA is used to verify the existence of a specific binding in the system.[15] Moreover, as the OT technique allows studies of forces under controlled conditions, it also provides a possibility to evaluate elastic properties of the bacterium *H. pylori*.

2 Optical Tweezers System

2.1 *Instrumentation*

The optical tweezers system used is constructed around an inverted microscope (Olympus IX70) with a high numerical aperture oil-immersion objective (Zeiss CP-Achromat 100 $\times$NA=1.25). An argon ion laser-pumped continuous-wave titanium-sapphire laser, operating at a wavelength of 810 nm (which is a wavelength where the bacteria exhibit low absorption of light[16]), was employed for optical trapping. The laser beam was directed into the microscope by an optical arrangement consisting of a number of lenses and mirrors, and focused by a high numerical aperture objective. A stepper-motor controlled gimbal mounted mirror allows steering of the beam so that its focus can be moved in the lateral (x-y) direction in the focal plane without loss of trapping power. The focal point can also be moved along the depth (in the z direction) by changing the divergence of the beam by means of translation of one of the lenses that forms an afocal lens system. A complete description of the optical system for controlling the laser beam that forms the optical trap as well as the modifications made to the microscope to realize the OT system is found in Refs. 17 and 18.

2.2 *Force Measurement*

The displacement of a small object (in this case a spherical bead with a diameter of 3.2 μm) held in the optical trap constitutes a measure of the force that is exerted on the object. To monitor the displacement, a weak probe laser beam (HeNe) is conveyed into the microscope, using a similar optical arrangement as that of the trapping beam. The probe beam intensity was significantly lower than that of the trapping beam to minimize additional forces on the trapped bead. Furthermore, the probe beam is focused a short distance below the trapped bead, whereby the trapped bead acts as a lens and refracts the

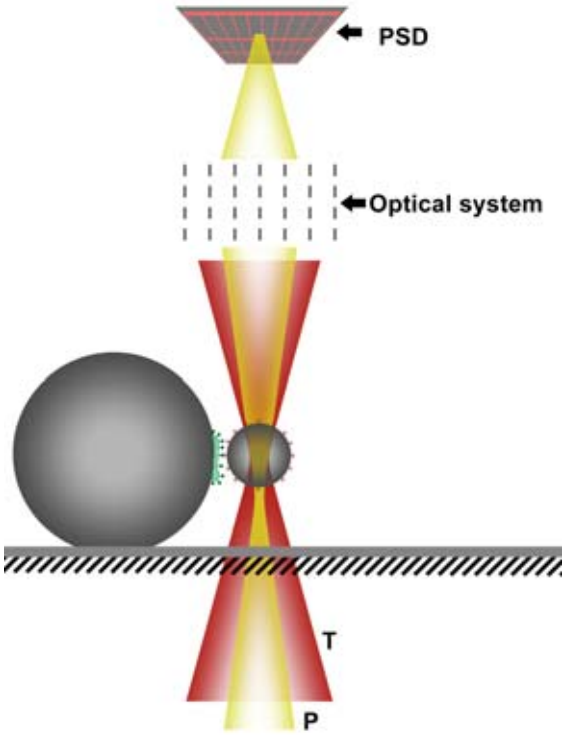

Fig. 1 An illustration of the measurement setup is shown. A bacterium is mounted on a large polystyrene bead. The trapping beam, marked T, has a small Leb-coated polystyrene bead in its focus. The thinner of the laser beams, marked P, is the probe laser, which eventually marks a spot on the position sensitive device (PSD) revealing the position of the trapped bead.

beam, together with the microscope condenser, in such a way that a distinct spot is produced in the far field. A position sensitive detector (PSD) is used to monitor the position of this spot, which is directly proportional to small lateral movements of the trapped bead. The position voltage signals are amplified, digitalized, and sampled by a data acquisition card in a computer. Figure 1 illustrates the optical trap and the position detection principle. Reference 18 presents a detailed description of the force measurement system.

2.3 *Force Calibration Procedures*

To convert the voltage signals received from the PSD to a force, an accurate calibration of the entire force measuring system has to be performed. The calibration was performed in two steps, in which the first step relates the PSD signal to the position of the trapped bead. The second step relates a given position in the trap to the force the optical trap exerts on the bead. Both calibration parameters are determined before each measurement to achieve as accurate assessments as possible.[18]

The first calibration step is done by moving the optical trap (focal point) in the lateral plane by means of the stepper-motor controlled mirror in the optical arrangement. A 3.2-μm bead is held by the optical trap during the movement, resulting in a shift of the position of the probe beam on the detector. The optical trap with the bead is moved in a stepwise manner a precise distance (± 0.40 μm) around the zero position to verify that the voltage-versus-position response is linear for bead displacements in this interval. Finally, the detector response factor V/nm) is determined by linear regression within the linear region of the voltage-versus-position curve.

To transform the position of the bead into the corresponding force, the second calibration step is needed. This calibration step is based on the Brownian motion (the random thermal movement) a small particle makes in a liquid. The bead is trapped by the OT, and the minute movements the bead makes when exposed to the combined effects of the forces of the trap and the thermal motion of the molecules in the liquid are recorded. This data, in combination with the well-known properties of Brownian motion in the absence of a trap, make it possible to deduce the required force constant of the trap in units of pN/nm through a power spectrum. The force constant of the optical trap in this work was ~ 0.2 pN/nm.

The Brownian motion calibration procedure was, in turn, validated by means of the Stoke's drag force technique.[19] The resulting force constants obtained by the two calibration techniques were found to agree within a few percent.

3 BabA-Leb Model System

The model system was compounded by the following components suspended in phosphate buffered saline (PBS): individual *H. pylori* bacteria, a large bead that served as an immobilization tool for the bacterium, and a small Leb-coated bead that served as a handle for the OT.

3.1 *Bacterium*

The *H. pylori* used in the measurements were the 17875Leb strain and the 17875babA1::kanbabA2::cam (denoted the BabA mutant), described in Ref. 15. Bacteria were grown for 40 to 45 h on Brucella agar medium supplemented with 10% bovine blood and 1% Iso Vitox (Becton Dickinson, Franklin Lakes, NJ) at 37 °C, under 10% CO_2 and 5% O_2.

3.2 *Large Beads*

The large functionalized polystyrene beads with carboxyl surface groups (Interfacial Dynamics Corporation, Portland, Oregon) had a diameter of 9.6 μm and were immobilized on the coverslip and served as a wall onto which a single *H. pylori* bacterium was mounted. The large beads were attached to the coverslip surface by means of a heating procedure. This procedure was accomplished by keeping the beads suspended in PBS in an oven at ~ 100 °C a few minutes until the liquid had evaporated and the beads were fixed to the glass. The large carboxyl beads were functionalized to allow attachment of bacteria. This was done by the use of two-step protocol.[20] In the first step, beads were washed once in 0.025-M MES buffer, pH 6.0 by centrifugation at 2500 rpm for 15 min and resuspended to 20-mg/ml solids. The second step was performed after the beads have been attached to the coverslip. A droplet (25 μl) of the activation buffer composed of 1 ml (0.1-M MES, 0.5-M NaCl, pH 5.0), 0.6-mg EDAC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] and 1.2-mg N-hydroxy-succinimide (NHS) was added to the coverslip and incubated at room temperature for 15 to 25 min. Next, the activation buffer was removed and 250-μl MES buffer was added to maintain the low pH and prevent the coverslip from drying. The bacteria were attached to the beads immediately before preparing the sample for measuring. The

measurements were performed in a PBS solution, which means that the pH was increased from 5 to 7.4 in the liquid surrounding the beads during preparation of the sample. The increased pH destabilized the reactive amine intermediate, and bacteria were allowed to bind covalently to the activated beads.

3.3 *Leb Beads*

The small carboxyl polystyrene beads (Interfacial Dynamics Corporation), with a diameter of 3.2 μm, were coated with Leb human serum albumin (HSA) conjugate (IsoSep AB, Tullinge, Sweden). Carboxy-functionalized polystyrene beads (1 mL, 40 mg/mL, prod. Nr. 7-3000, 3.2-μm, Interfacial Dynamics Corporation) were washed twice by suspending them in MES buffer (4 mL, 25 mM, pH 5.2), centrifuging, and decanting the mother liquor. The beads were then suspended in MES buffer (2 mL). Leb-HSA conjugate (0.25 mg in 94-μL MES buffer, prod. Nr. 61/08, Isosept AB) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (5 mg in 63-μL MES buffer) was added to a portion of the bead suspension (0.157 mL). The mixture was shook overnight, then washed four times by centrifugation, decanted of mother liquor, and resuspended in PBS buffer (pH 7.2). Finally, the beads were suspended in PBS (300 μL) and glycine (0.3 mg) and NaN_3 (0.3 mg) were added. The H-1 antigen is monofucosylated and defines the blood group O-phenotype. The difucosylated Lewis b antigen is formed by addition of a second fucose residue.

H-1 Fucα1.2Galβ1.3GlcNAcβ1.3Galβ1.4Glc

Lewis b Fucα1.2Galβ1.3GlcNAcβ1.3Galβ1.4Glc
|
Fucα1.4

In addition to hosting the receptors, the Leb bead also served as a force indicator in the OT measurements.

3.4 *Sample Synthesis*

The sample mixture was prepared by applying a small portion of bacteria, suspended in a droplet of 25-μl PBS, onto a coverslip with attached large beads. Next, 5 μl of PBS solution containing Leb beads was added. All PBS solutions contained 1% bovine serum albumin (BSA, Sigma, Saint Louis, Missouri) to minimize the influence of unspecific bindings between the Leb bead and the bacterial surface. The sample mixture was placed in a chamber consisting of two glass coverslips fixed to each side of an aluminum slide holder. The chamber was completely sealed to prevent evaporation during the experiment and to allow for the use of an oil immersion condenser. A piezo stage, mounted on the microscope table, held the sample chamber and allowed precise translation of the sample at a controlled speed relative to the microscope objective.

4 Measurement Procedure

4.1 *Beads and Bacteria Mounting*

A free-floating bacterium was trapped and mounted, through covalent binding, onto the large bead using the OT. Low laser power (~100 mW measured at the entrance of the microscope objective) was used to minimize the effects of heating the bacterium by the light. A small Leb-coated polystyrene bead was trapped with increased laser power (~600 mW). The force calibration of the trap was then performed by the procedures described in Sec. 2.3.

4.2 *Finding a Start Position*

Before an actual force measurement could be performed, it was necessary to locate a good starting position for the Leb bead relative to the bacterium. First, the bead was moved to a starting position in close proximity to the bacterium, but still at such a distance from the bacterial surface that no binding could take place. The coverslip, with the attached large bead and bacterium, was then moved away from the Leb bead with a speed of 0.5 μm/s. At this first measurement cycle, there was no force detected, since no bacterial attachment had been established. When the coverslip had moved 5 μm it was returned to a new starting position located 0.1 μm closer to the Leb bead than the previous starting position, whereby a new measurement cycle was performed. This iterative procedure continued until contact occurred and an adhesion force appeared.

4.3 *Measurement Method*

A measurement cycle from a starting position that gave rise to bacterial adhesion is illustrated in Fig. 2. The Leb bead was in this case first gently pressed onto the bacterium, whereby the bead was displaced slightly from the center of the optical trap [Fig. 2(a)]. The contact between the adhesins and the receptors allowed specific bindings to take place. After 5 s, the bacterium was retracted from the Leb bead in the trap by a movement of the coverslip with the large bead and the bacterium. The binding force pulled the Leb bead away from the center of the trap [Fig. 2(b)]. The force between the bacterium and the bead increased until the binding suddenly ruptured [Fig. 2(c)]. The force at rupture is referred to as the de-adhesion force. Repeated measurements from the same starting position were performed to investigate the repeatability of the binding.

4.4 *Data Acquisition and Presentation*

The entire measurement procedure was computer controlled by a custom-made LabView® program. Position data of the trapped Leb bead were collected with a sample rate of 1000 sample/s while moving the coverslip. The position data was converted to forces by means of the calibration constants from the force calibration procedure. Although the movement of the coverslip was the entity that was externally controlled in the experiments, it is most often the distance between the bacterium and the Leb bead that is the entity of primary interest. These two entities differ by an amount given by the displacement of the Leb bead from the center of the trap. The software allows the output force data to be displayed either versus time or versus the bacterium-to-bead distance. For analysis purposes, the latter presentation form is more convenient, as the slope of the force curves in this form directly gives information of the elastic behavior of the bacterium.

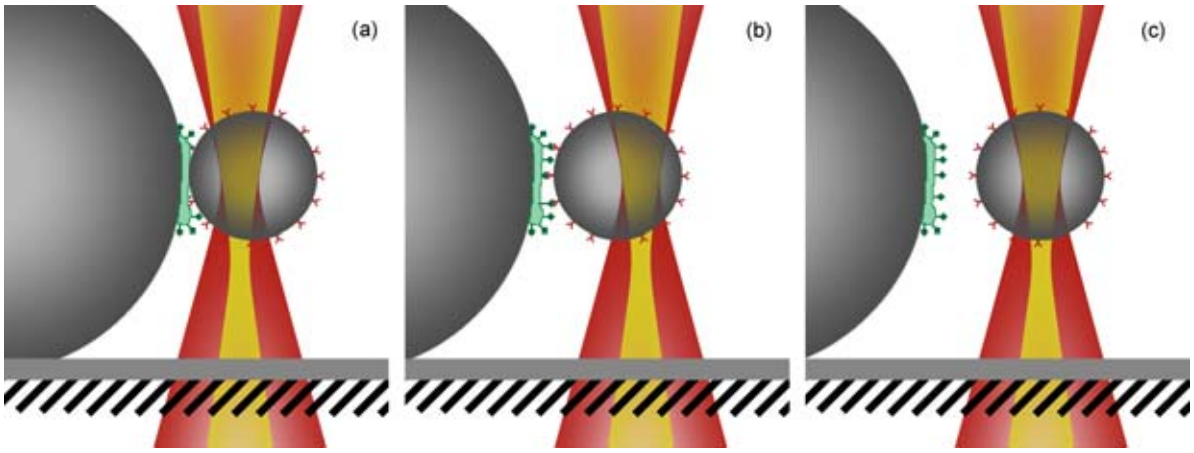

Fig. 2 (a), (b), and (c) illustrate the measurement procedure in three chronological steps. (a) The bead is pressed onto the bacterium and adhesion occurs. (b) The ligand is bound to the receptors and forces the bead from the focus of the trapping beam as the distance between the trap and the bacteria increases. (c) The bead returns to the center of the trap as the force from the laser trap exceeds the maximum adhesion force that the receptor-ligand binding can support.

5 Results

A total of 104 force measurements with bacteria were conducted in 17 series; the 17875Leb strain was used in eight series, whereas the BabA mutant, i.e., the negative control, was used in nine series. A series constitutes several measurements performed on a given bacterium. In addition, 41 measurements were conducted without any bacterium on the large bead for the purpose of investigating the surface-surface interaction of the two beads.

5.1 *Typical Force Measurement*

Figure 3 displays force data from a measurement on an individual bacterium of the 17875Leb strain. The graph can be seen as consisting of three characteristic regions. The first region, labeled (a) in the graph, consists of a negative force. This arises when the large bead, with the bacterium, is pressed onto the Leb bead, pushing the latter out of the center of the trap. The negative force data demonstrate that a close contact between the bacterium and Leb bead has been established. The second region, labeled (b) in the graph, consists of a linear force-versus-distance dependence, which demonstrates adhesion and originates from the stress response of the biological system under elongation. The force increases with elongation until the binding ruptures, whereby the Leb bead detaches from the bacterium and returns instantaneously to the center of the optical trap. The third part of the data, labeled (c), which constitutes of a zero force region, indicates that the binding has ruptured. The three regions observed in Fig. 3 correspond to the events described in Figs. 2(a)–2(c).

There are no data points between regions 2 and 3. This gap appears as a consequence of an immediate increase of the Leb bead-bacterium distance as the binding breaks. The maximum value of region (b) is considered to be an assessment of the de-adhesion force in the system under prevailing conditions. For the particular measurement shown in Fig. 3, the de-adhesion force amounted to 80 pN.

5.2 *Exclusion of Bead-to-Bead Interaction*

It is not trivial, because of diffraction effects in the microscope, to ensure that the bacterium is mounted at the same height as the Leb bead. If a measurement is performed with a slight difference in height between the two, direct contact may occur between the large mounting bead and the Leb bead, which might cause unspecific bead-to-bead interaction. Since this type of interaction does not provide any information about the specific receptor binding, it was necessary to find means to exclude them from the bacterium-to-bead interaction.

It was found that the force response from the two types of interaction was of similar magnitude, wherefore the exclusion could not be done based on the magnitude of the force. On the other hand, it was found that they could be distinguished from each other based on a difference in their elongation dependence. Since a bacterium to some extent is elastic, measurements involving a bacterium will give rise to force curves with lower slopes than measurements made on direct bead-bead interactions. An example of a force curve for a bacterium-to-bead interaction is given by curve A in Fig. 4. A corresponding measurement with a direct bead-to-bead interaction is shown in curve B in Fig. 4. As can be seen from the figure, the bead-to-bead interaction shows a response that has a significantly larger slope than the bacterium-to-bead interaction. Based on this type of analysis, all measurements with a slope indicating a bead-to-bead interaction were excluded from further analysis.

5.3 *Verification of Specific BabA-Leb Binding*

To verify the existence of a specific BabA-Leb binding, the maximum de-adhesion forces in each series for the 17875Leb and for the BabA mutant were compared. The reason for using the maximum forces as a mean for comparison is the observation that the binding force in a particular series of measurements tended to decrease with the number of measurement on a given bacterium, possibly indicating a destruction of the

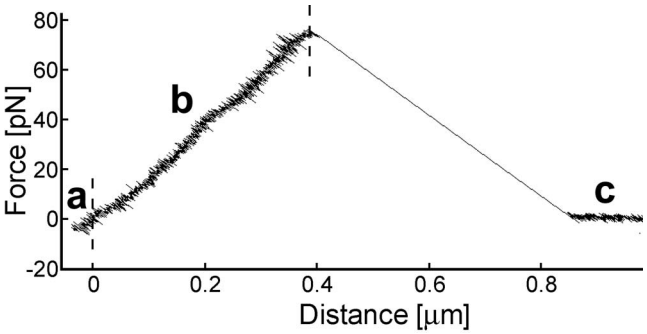

Fig. 3 A measurement curve starts at negative force when the bead is pressed onto the bacterium (a). The force increases linearly as the bead is pulled away (b) and then suddenly drops to zero as the binding breaks, whereby the bead immediately returns to the center of the laser trap (c).

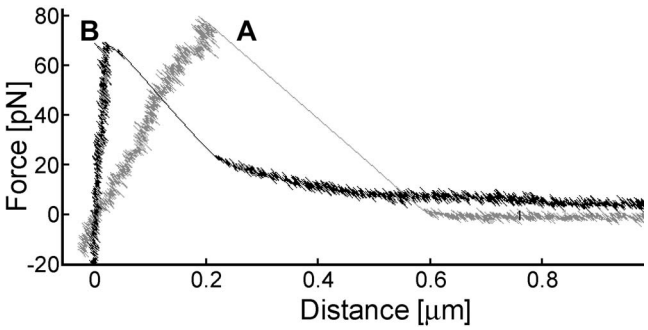

Fig. 4 Curve A shows a typical bacterium-bead interaction, whereas curve B is a result of a measurement performed without bacterium, thus only bead-bead interaction. The slope of the force curve reveals the source of the force.

adhesins after repeated interactions. To not be affected by any such possible destruction, the maximum de-adhesion force in each series was therefore identified. These maximum de-adhesion forces (one for each series, thus one for each bacterium) are presented in a force distribution diagram shown in Fig. 5 with the series with 17875Leb bacteria given by square markers and those of the BabA mutant represented by diamond-shaped markers. It was found that the average of the maximum forces for the 17875Leb strain was 97 pN, with a majority of the maximum forces in the 80 to 110-pN interval, whereas the average of the maximum forces for the BabA mutant was significantly smaller, 26 pN, with only one series (in fact, only one data point in one series) above 45 pN. This difference clearly indicates the existence of a specific BabA-Leb binding, which is in agreement with previous findings.[2] However, the data do not only verify the existence of a specific BabA-Leb binding, they also indicate that some minor unspecific bacterium-to-bead interactions are present in the system. These unspecific forces are, however, markedly weaker than the specific BabA-Leb binding.

5.4 *Identification and Assessment of Specific BabA-Leb Binding Forces*

The identification and assessment of specific BabA-Leb binding forces is based on the distribution of forces. The data from the eight series of measurements from the 17875Leb strain are displayed in terms of a histogram in panel A in Fig. 6, while the data from the nine series from the BabA mutant are shown in panel B. The intervals have been chosen as multiples of 12.5 pN, with a width of ±6.25 pN, for reasons discussed later. The data from the 17875Leb strain (panel A) shows a pronounced high-low staggering. The histogram shows that a significant fraction of all measurements (46 measurements, 75%) gave rise to forces that appear in the even intervals (marked with filled bars), whereas a substantially smaller fraction of all measurements (15 measurements, 25%) appeared in the odd intervals (unfilled bars). This distribution of forces suggest the existence of an elemental force in the system, F_{Leb}. The even intervals represent multiple bindings with a force of NF_{Leb}, with N being an integer, whereas the odd intervals correspond to intermediate force intervals, centered around $(N-0.5)F_{\mathrm{Leb}}$.

The elemental force used in Fig. 6 was found by maximizing the appearance of high-low staggering. Figure 7 shows the fraction of measurements that belongs to the even intervals (i.e., those consisting of multiples of an elemental force NF_{Leb}) as a function of elemental force F_{Leb}. As can be seen in the figure, a maximum of the high-low staggering appears for a F_{Leb} value of 25 pN. Taking the half-width-half-maximum (HWHM) of this curve as the uncertainty, it is possible to assess the elemental force in the BabA-Leb system to 25 ± 1.5 pN. The data from the BabA mutant (Fig. 6 panel B) do not show the same high-low-staggering feature, which demonstrates the absence of an elemental force in the control system.

5.5 *Bacterium Deformation*

A bacterium expands or contracts along the same direction at which the force is applied. The resistance to an elongation is often referred to as stiffness. The stiffness can be directly obtained from the data as the slope of a force-versus-distance curve. The experiments showed that the stiffness of the bacterium was uncorrelated to the de-adhesion force. The stiffness was also found to be the same for the 17875Leb as well as the BabA mutant. This suggests that it does not depend on the adhesion mechanism, but that it rather is related to a deformation of the bacterium. The mean value of the stiffness of all measurements (along the direction perpendicular to the length axis) was found to be ~ 280 pN/μm.

6 Discussion

6.1 *Verification of Origin of Forces*

Force measurements based on oligosaccharide-activated beads that interact with immobilized bacterial cells are challenged by unspecific bacterial binding as well as occasional direct contact between bead surfaces. In the force measurements reported here, the unspecific binding was reduced by efficient blocking of both beads and bacterial surfaces with albumin protein, which is a serum protein that is naturally nonglycosylated and will not take part in the BabA-Leb binding event. The pure bead-to-bead interaction could be readily identified and eliminated from the bacterium-to-bead interactions due to the lack of elasticity in the measurements (infinite slope of the force curve at the start). In addition, a BabA

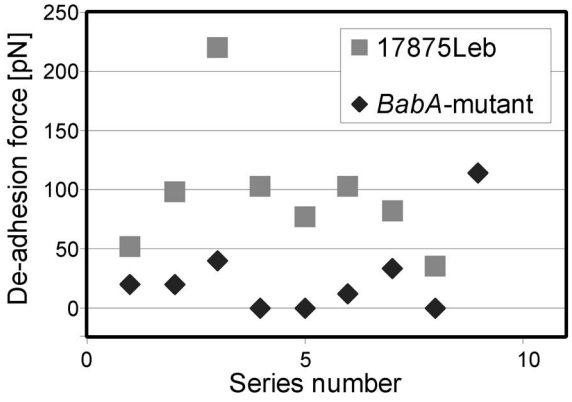

Fig. 5 Comparison between de-adhesion forces measured on 17875Leb (square markers) and the BabA mutant (diamonds). The maximum forces in each series are presented.

mutant, devoid of the BabA adhesin protein and thus lacking all Leb binding properties, was analyzed as a negative control. As was shown in Fig. 5, the maximum binding force of the BabA mutant (~26 pN) was distinctly different from and weaker than that of the 17875Leb bacteria (~97 pN). This indicates that the BabA protein-Leb antigen binding is both specific and, furthermore, involves multivalent interactions that collectively adds to the avidity of the tight bacterial-host cell interaction revealed by the OT-force measurements. The results are also in qualitative agreement with previous studies by means of Scatchard analyses, in which Leb has been identified as the most important receptor that mediates the binding to the BabA protein present on the bacterial surface.[2]

6.2 *Verification of an Elementary Binding Force*

The distribution of forces shown in Fig. 6(a) is strong evidence for an elemental force in the BabA-Leb system. If there was not an elemental force in the system, the number of measurements would be expected to distribute evenly in odd and even intervals, since their widths are all the same. However, it was found that 75% of all measurements ended up in even intervals, whereas 25% appeared in the odd. The large difference in population of the two groups of intervals can be investigated using standard binomial distribution theory. Assuming that each measurement has the same probability (50%) to appear in an odd and even interval results in a symmetric distribution function. Using this function, it is possible to assess the probability to measure such an uneven distribution of forces as 75% versus 25% to less than 0.5%. Due to this regularity in the distribution of forces and the low number of force measurements at 12.5 pN, it is possible to conclude, with a high certainty, that there exists an elemental force of ~25 pN in the BabA-Leb system. Since there is no similar high-low staggering in the control system, it is furthermore reasonable to assume that this force corresponds to an individual BabA-Leb binding. Unspecific forces in combination with natural variations in the BabA-Leb binding are assumed to be responsible for the spread of forces into odd intervals.

Although the previous discussion is not an irrefutable proof, the distribution of forces displayed in Fig. 6 supports the assumption that there is a minimum specific elemental force of 25±1.5 pN in the BabA-Leb system under the pertinent conditions (at a loading rate of ~100 pN/s). The absence of a high-low staggering in the control system provides evidence that the elementary force corresponds to the de-adhesion force associated with an individual BabA-Leb binding site.

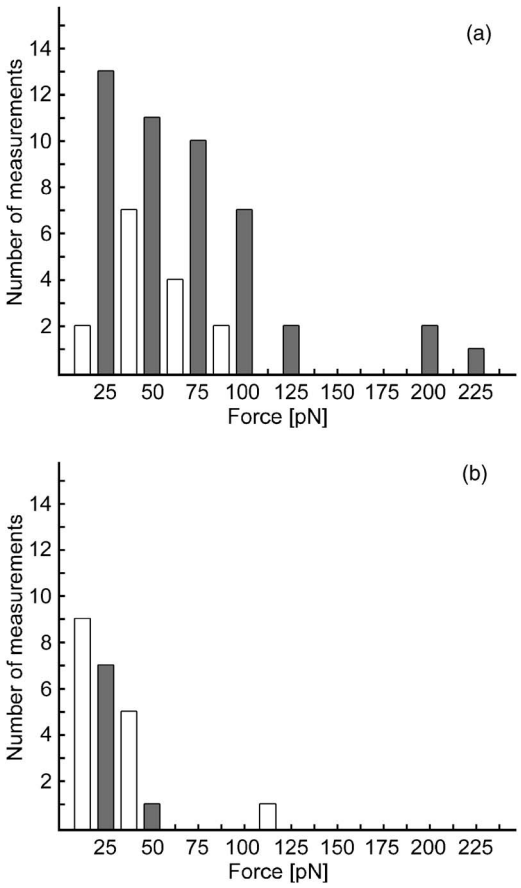

Fig. 6 Histograms of the distribution of de-adhesion forces. The intervals are centered at multiples of 12.5 pN with a width of ±6.25 pN. (a) and (b) correspond to the 17875Leb and the BabA-mutant strains, respectively.

6.3 *Assessment of the Number of Binding Sites*

From the data shown in Fig. 6(a), and under the assumption that an individual BabA-Leb binding corresponds to ~25 pN, it can further be concluded that BabA-Leb adhesion was, in a majority of the cases, mediated by a small number of bonds, in general, a few (1 to 4), and occasionally slightly more. These numbers seem at first sight to be unexpectedly low, since the number of binding sites in the contact area can be estimated to be significantly larger than this. It has previously been shown by Scatchard analysis that 17875Leb has an average of about 500 adhesins per bacterium.[2] It can further be estimated that about 15% of the total bacterium surface was in contact with the Leb bead under the pertinent experimental conditions (with a 20-pN application force). From a pure geometrical consideration, it is possible to derive an expression for the fraction of the total area of the bacterium that is in contact with the Leb bead, A_F, that reads

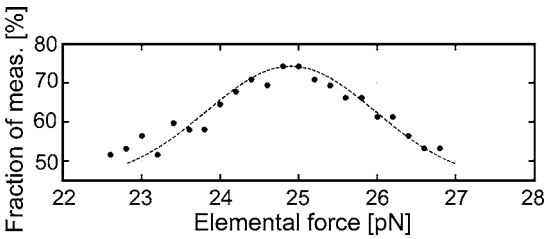

Fig. 7 The fraction of measurements on the 17875Leb strain that belongs to the even intervals of a histogram representation of the measurements data as a function of elemental force F_{Leb}. A maximum is observed at 25 pN.

$$A_F \approx (FR/2k_H B_L^2)^{1/2},$$

where F is the force by which the Leb bead is pressed onto the bacterium (the maximum negative force in region a), R is the radius of the Leb bead, k_H is the stiffness of the bacterium perpendicular to the length axis, and B_L is the length of the bacterium. Inserting typical geometrical values for the bacterium and using a stiffness of the bacterium of 280 pN/μm (assessed by experiments) show that the contact area is about 15% of the total bacterium surface. Assuming an even distribution of the 500 adhesins over the bacterial surface implies that the average number of binding sites in contact with the Leb bead can be estimated to ~75. There are, however, reasons to expect that the number of bonds that mediate the binding at any particular time should be significantly less than the number of binding sites in the contact area. The main reason is that the bonds are in general very short, which implies that only a limited number of them can mediate the force under elongation at any given time. After the bead has been docked with the rather flexible bacterium (where the bead has deformed the bacterium) and the separation of the two has started, it is, at any given time, mainly the bonds at the periphery of the contact area between the bead and the bacterium that mediate the force (the amount of deformation of the bacterium body is largest at the periphery of the contact surface), whereas the bonds that reside in the interior of the contact area do not mediate any substantial amount of force. Another reason is that some of these binding sites might be inactive and therefore not able to support force. This implies that one should expect that not more than a few bonds mediate the force at any given time. The experimental findings presented earlier are in qualitative agreement with these general expectations. Moreover, the fact that the measured adhesion force decreases (although not strictly monotonically) within a series indicates that the bond is being permanently destroyed after rupture.

6.4 *Single Rupture*

Almost no multiple peaks are found in the force curves. A multiple peak structure would be expected if the bonds that act in parallel would rupture one after another in succession. However, again because of the short length of the bonds, it is expected that when one or a few of several bonds have ruptured, the remaining bonds will directly be further elongated and exposed to such a high force (both from the increase in force due to the sudden increased bead-to-bacterium distance, but also from the dynamics of the movement) that they would immediately rupture. This can be seen as an immediate breakdown sequence appearing on a timescale below that of the measurements. Hence, it is not expected that multiple peak structures should be frequent in these types of experiments. This is also in agreement with the experimental findings.

6.5 *Elasticity*

It is also of interest to investigate the origin of the measured stiffness $k_H \sim 280$ pN/μm of the bacterium. In general, the force exerted on the bacterium results in a deformation that can have two origins: an elastic stretching of the cell wall (like the elongation of a mechanical spring) or an alteration of the geometrical shape of the bacterium. In both cases, a restoring force is produced.

In the work reported in Ref. 21, it is suggested that the stability of the cell wall of Gram-negative bacteria is associated with the peptidoglycan layer, which has a thickness of ~3 nm. The stretching of the cell wall can be expressed in terms of Young's modulus. This entity, denoted by Y_H, can, for a 1-D material, be estimated from the relation

$$Y_H = k_H \frac{L}{A}, \tag{1}$$

where k_H is the stiffness of the material, and L and A are the length and the cross section of the elastic part of the body, respectively. The authors of Ref. 21 report a Young's modulus of $\sim 10^7$ Pa based on a theoretical calculation of pure elastic stretching of the peptidoglycan layer. Our measurements indicate, however, a Young's modulus that is several orders of magnitudes lower than this [inserting reasonable values for the entities in Eq. (1) (a spring constant k_H of 280 pN/μm, a cross section A of 10^{-14} m^2, and a length L of 0.75 μm, given by the width of the bacterium) results in a Young's modulus of $\sim 10^3$ Pa]. Thus we can conclude that the measured elasticity cannot be explained by elastic stretching of the cell wall.

A bacterium also acts as a pressure chamber that will strive to maintain its original shape when exposed to deformations. The turgor pressure is the osmotic pressure difference between the inside of the bacterium and the surrounding medium. This pressure thus determines the resistance of the bacterium to an alteration of its geometrical shape. The turgor pressure is normally found to be in the range from 1 to 5 atmospheres.[21] A pressure of ~3 atmospheres is enough to produce restoring forces in the order of 100 pN if the bacterium is deformed an amount of ~0.3 μm, which is in agreement with the stiffness measured in these experiments ($k_H \sim 280$ pN/μm). The conclusion is therefore that the turgor pressure gives rise to the restoring forces of the bacterium body as it is deformed from applied forces.

6.6 *Comparison with Other Specific Bacterial Adhesion Forces*

Only a few results of specific bacterial adhesion forces seem to be reported in the literature. For example, in Ref. 11 the specific force required to break a single interaction between an *E. coli* pilus and its receptor (a mannose group) is estimated to 1.7 pN, a value that seems surprisingly low with respect to the thermal energy in the system. The thermal en-

ergy, given by the product of the Boltzmann's constant and the absolute temperature k_BT, is in the order of 4 pN·nm, which for a bond length in the interval of 0.2 to 0.5 nm corresponds to a thermal force in the range from 8 to 20 pN. Reference 12 reports on a more reasonable value of 18 pN for the force required to rupture a single, specific *S. epidermidis* bond with fibronectin. Based on general considerations regarding the thermal energy and bond lengths, it is reasonable to assume that specific bacterial adhesion should be mediated by a force that is larger than the thermal force. This is consistent with our BabA-Leb measurements that indicate a binding force of 25 pN.

6.7 *Future Work*

A noncovalent bond between a ligand and a receptor can generally be described as a process driven by Brownian-thermal fluctuations, which can be characterized by an energy landscape. The energy landscape describing the BabA-Leb bond is so far unknown. Exposing the bond to an external force lowers the energy barriers and increases the likelihood of unbinding. Theory predicts that the de-adhesion force has a logarithmic dependence on the rate at which the force is applied.[22] In this study we have only used one loading rate, ~100 pN/s, and the forces measured are thus statistical reflections of the energy landscape for this rate. A spectrum of the average unbinding force as a function of loading rate would provide a means for characterization of the energy landscape of the BabA-Leb bond in some detail. The experimental setup and the biological assay used here provide a possibility to investigate a large number of features and entities in this biological system. This work covers only a first study in which specific adhesion forces and some elastic properties of *H. pylori* have been investigated. Further investigations are planned to cover issues such as the impetus of the contact time, loading rate, and pH of the solvent, which all are assumed to be important factors for a general characterization of the adhesion properties of *H. pylori*, but whose influence is yet unknown. Also different variations of the Leb conjugate will be investigated.

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