Measuring Attachment Force of B. bacteriovorus Over Short Time Scales

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ABSTRACT

The goal of this research is to examine the attachment of the predatory bacteria known as *Bdellovibrio bacteriovorus* onto its prey bacteria, *Escherichia coli*, and to increase the understanding of the *B. bacteriovorus* predatory process. *B. bacteriovorus* is being considered for predatory therapy, an alternative to antibiotics. Predatory therapy is the use of predatory bacteria to target pathogens in the body. The *B. bacteriovorus* has a hair-like protien filaments known as type IV pili that are believed to be the cause of the attachment. The pili extend and attach to the bacterium's prey, then pull the predatory bacterium into its prey where it transitions from its attack phase into its reproductive phase. We utilized optical tweezers to facilitate attachment of a trapped *B. bacteriovorus* to an immobilized *E. coli*. Upon attachment, we used optical tweezers to pull the two bacteria apart. The optical tweezers provided a way to measure the attachment force of the *B. bacteriovorus* associated with short attachment times ranging from 90 seconds to five minutes. For the short attachment times, we found the force to be a minimum of a few piconewtons. We hypothesize that the force will become greater over longer periods of time.

Keywords: Physics, Biophysics, Biology, Optical Tweezers, T4 Pili, *B. bacteriovorus*, *E. coli*, Attachment Force, Bacteria

1. INTRODUCTION

Predatory therapy is being examined as an alternative for antibiotics due to bacteria developing resistance to antibiotics.[1] Predatory therapy consists of injecting the body with a predatory bacteria to neutralize the pathogenic bacteria infecting the body. One bacteria that has been considered for this is *Bdellovibrio bacteriovorus*.[2] This gram-negative (has an inner and outer cell membrane with an space known as the periplasm in between them) periplasmic predatory bacteria is being studied as it could potentially be used as an option for predatory therapy because it has a wide variety of prey bacteria, including *E. coli*.[3] Another reason it is being considered for predatory therapy is it prefers to hunt gram-negative bacteria, not mammalian cells.[4] Some other bacteria *B. bacteriovorus* has been able to prey on are *P. aeruginosa* (collected from a patient with cystic fibrosis), and pathogens belonging tp the Proteus genus.[3] In addition to its large range of prey bacteria, it is believed that *B. bacteriovorus*' prey would not easily develop resistance to predation.[1,3]

This bacteria are easily trapped by an optical tweezer, providing a way for us to manipulate a single bacterium and learn more about the attachment of the predatory bacterium, specifically the forces involved. Each *B. bacteriovorus* is approximately 0.2 to 0.5μm $\times$ 0.5 to 2.5μm[5] and each *E. coli* is about 0.75μm $\times$ 2.0μm.[6,7] Due to the size and the rod shape of the *B. bacteriovorus* it is an optimal subject for trapping with our optical tweezers which utilizes a diode laser with a 660 nm wavelength. The reason for this belief is the wavelength and the bacteria are similar in magnitude which indicates it is a good size for our particular optical tweezer setup.[8] Being able trap the *B. bacteriovorus* with our optical tweezers system makes our attachment study possible.

2. BACKGROUND

2.1 *B. bacteriovorus*

The life cycle of *B. bacteriovorus*, illustrated in Fig. 1, consists of two phases, the attack phase and the reproductive phase.[9] The attack phase begins with the predatory bacterium propelling itself towards a prey bacterium using its flagellum, a "tail"-like appendage the bacteria use for movement.[10] The bacterium at this point is a small cell with a shape ranging from vibrioid to rod. This is followed by the attachment stage of a *B. bacteriovorus* to a prey bacterium, which typically occurs over approximately 10 to 20 minutes.[3] During this phase the *B. bacteriovorus* uses its type IV pili, extremely thin protein filaments that faciliate the attachment of the predatory bacteria, to attach to its prey.[11,12] A type IV pilus is made up of three helical strands that have twisted together.[12] After attachment has occurred the bacterium progresses to invading the periplasmic space of the prey bacterium's cell wall. After this stage the bacterium transitions from the attack phase to the reproductive phase. Once the *B. bacteriovorus* has fully penetrated into its prey bacterium, the prey cell becomes more round and is now referred to as a bdelloplast.[13] For the next stage, the *B. bacteriovorus* uses the nutrients of the prey bacterium to elongate in the bdelloplast. The following stage begins when the prey bacterium's nutrients are fully consumed by the predatory bacterium. In this stage, the elongated *B. bacteriovorus* divides into progeny cells.[3] In the final step of the reproductive phase, the bdelloplast lyses releasing progeny cells that swim away in search of new prey.[2,14] The cycle will repeats itself, as long as there is prey for the *B. bacteriovorus*.

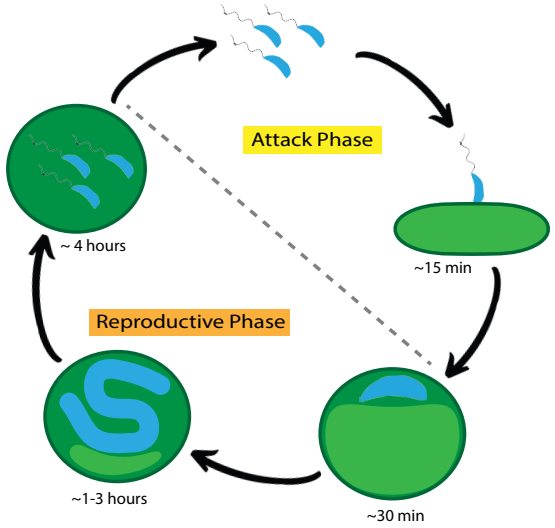


Figure 1: Life Cycle of *B. bacteriovorus* which consists of two phases: the attack and reproductive. Attachment occurs at approximately 15 minutes followed by penetration of the prey cell and the creation of a bdelloplast which occurs at approximately 30 minutes. Over the course of one to three hours the predatory bacteria feeds on the nutrients of the prey cell. After approximately four hours after attachment progeny cells are formed and released so the cycle can start again with the free swimming *B. bacteriovorus* hunting for prey.[15]

Pili play a part in multiple functions of the bacteria. Some of these functions are twitching motility (movement independent of the flagella[16]), host cell adherence and invasion of the prey cell, and the creation of fruiting bodies.[11,17] Out of these functions, the one we focused on was the host cell adherence and invasion of the prey cell during the attachment process of *B. bacteriovorus* to its prey. The pili stretch out and hold onto the prey bacteria, then are used by the predatory bacterium to pull itself towards its prey so it can penetrate its prey bacterium.[9] *B. bacteriovorus* bacteria have type IV pili.[18] Their pili are typically 5 to 8 nm wide and 1 to 2 μm long.[19] Type IV pili have been found to have retractile forces strong enough to pull the *B. bacteriovorus* to its prey and enable it to penetrate the prey cell's wall.[17]

2.2 Previous work

Multiple studies have been conducted over the past few years with the focus of investigating type IV pili. Many of these studies agree that the force these appendages can produce is greater than 100 pN.[12,17,20,21] A wide variety of techniques have been implemented to investigate the properties of type IV pili. Some of the methods utilitized were optical tweezers, other optical traps and atomic force microscopy (AFM).[22]

One study in 2015 used AFM to test for the rupture force of type IV pili using a mutant strain of *Pseudomonas aeruginosa* PAO1.[12] The experiment tested for the rupture force of the pili in four ways: a single pili with a single contact point to a surface, a single strand of pili with a segment of it adhered to a surface, a single pili with multiple contact points attached to a surface, and lastly, multiple pili attached to a surface.[12] The research supported a low-affinity, high-avidity model for the attachment of the type IV pili. These experiments were done on surfaces made of gold, polystrene and mica since type IV pili are known to attach to multiple surfaces.[12] In this research force plateaus occured which were suspected to be caused by the extension of the pili but were determined to be caused by the pili peeling from the surface. The maximum rupture force observed in this experiment was approximately 300 pN.[12]

AFM was utilized in a study conducted in 2014 to measure the forces relating to the attachment of *P. aeruginosa* to a variety of surfaces.[19] The reason behind investigating this property of the bacteria was to learn more about the biofilm produced by this bacteria. This was due to the association between biofilms and resistant bacteria. Before researching the pili, test were run to show that the pili were present on the *P. aeruginosa*. These tests resulted in force curves that displayed adhesion forces that ranged from 50 to 250 pN.[19] Therefore it could be presumed this bacteria is able to withstand forces up to 250 pN. After the pili had been proven to be present the forces involved in the attachment of the *P. aeruginosa* to pneumocytes were observed. The force curves showed adhesion forces peaks ranging from 50 to 500 pN at low pulling speeds which were often followed by force plateaus.[19]

The structural changes of *Neisseria gonorrhoeae* type IV pili caused by external forces were examined in one study in 2010.[22] The study implemented the use of various methods to manipulate the type IV pili. The techniques used in this research include AFM, optical tweezers, magnetic tweezers, and molecular combing. These experiments showed that when the pili were subjected to high forces, around 100 pN, their structure was altered.[22] They stretched to approximately 3 times longer than their original length and the pili became 40% narrower as well. Once the pili was elongated unseen epitopes in the pili were uncovered. This study implied that it is probable that there is an elastic property of the type IV pili.[22] The structural change in the pili was found to be reversible.

Another instance where optical trapping was used to study the forces of the pili was preformed in 2004. The various physical properties possessed by a single pilus were investigated.[23] One of the properties focused on was the measurement of the force of the pilus. The system used in this experiment is similar to the general layout shown in Fig. 5. The system in this experiment implemented the use of a probe laser in addition to a trapping laser.[23] The trapping laser was a cw Nd:YAG laser and a HeNe laser was used as a probing laser which was used to alter the position of the trapped object. A bacterium was adhered to a large bead and a single pilus of that bacterium was attached to a smaller bead in the optical trap.[23] After which an automated procedured was run to measure multiple quantites, the trap stiffness and the automated force were just two of those quantites. The research ended up showing the three characteristic regions of an elongated pilus among other things. The first region consists of a pilus which due to expriencing a force is stretched to 10-15% its original length, in the second the pilus' structure begins to unfold, and in the third and final stage the pilus can be elongated to approximately 700% longer than its original length.[23]

2.3 Optical Tweezers

We implemented optical tweezers to trap a single *B. bacteriovorus*. An optical tweezer set-up includes a microscope objective with a high numerical aperture value (NA) to focus the laser beam. When a dielectric particle is introduced near the focus, a net force on the particle occurs and draws the particle towards the focus of the laser.[24] This net force is caused by the reflecting of incident photons which causes a force in the laser propagation direction and a bending of the photons transmitted through the particle which produces a force towards the

highest intensity region of the focus.[25] These two forces are called the scattering force and the gradient force, respectfully.[26] These forces, along with the force of gravity, are shown in Fig. 2 acting on a trapped particle.

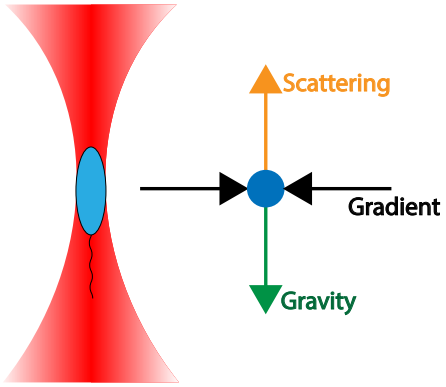


Figure 2: All forces acting on a particle trapped by the optical tweezers: Scattering force, $F_{opt,s}$, gradient force, $F_{opt,g}$, and the force of gravity, F_g

The scattering force works on the trapped particle's axial movement, countering the force of gravity.[27] The force of gravity acting on the *B. bacteriovorus* is approximately -0.24 pN based on an estimated mass of 2.45×10^{-2} pg. As seen in Fig. 3, there is a change in momentum in the downward direction of the light as it is reflected off the trapped particle. Due to the conservation of momentum, the change in momentum of the particle will be in the opposite direction of the change in momentum of the light. Thus it will be be in the upwards direction.[24,28] The scattering force can be found with $F = \frac{\Delta p}{\Delta t} = \frac{2IA}{c}$. In these equations Δp is the change in momentum of the particle, Δt is the change in time, I is the intensity of the laser, A is the area of the beam, and c is the speed of light. Since the power of the laser is denoted by $P = IA$, we have $F_{opt,s} = \frac{2P_{reflected}}{c}$.[29] To find the reflected power, we solved for the Fresnel reflection coefficient, $r = \frac{n_{med}cos\theta_{med} - n_{bac}cos\theta_{bac}}{n_{med}cos\theta_{med} + n_{bac}cos\theta_{bac}}$. Assuming normal incidence both θ_{med} and θ_{bac} will be 0° resulting in the simplification of the equation to $r = \frac{n_{med} - n_{bac}}{n_{med} + n_{bac}}$.[24] Assigning 1.339 for the index of refraction of the HM (HEPES metal) buffer (n_{med})[30] and 1.384 for the index of refraction of the *B. bacteriovorus* (n_{bac}),[31] we found a reflection coefficient of 1.1623×10^{-2}. The fraction of power reflected is $R = 2.733e{-}4$. For example, a power of 50mW will result in a reflected power of $1.366 \times 10^{-}2$ mW which gives us a scattering force of about 0.1 pN. Therefore the scattering force and the force of gravity are on the same order of magnitude and thus will counteract each other as they are directed in opposite directions and hold the trapped particle at the focus of the beam.

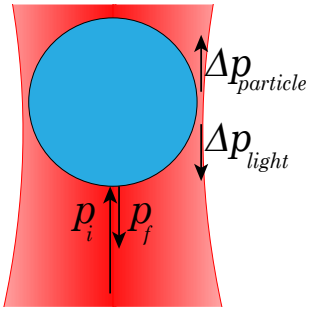


Figure 3: One instance of the change in momentum of a photon that is bouncing off the trapped particle causing change in the momentum of the particle to be in the upwards direction due to the conservation of momentum resulting in a force pushing the particle upwards in the same direction as the change in momentum of the particle.

The other force acting on the trapped particle is the gradient force.[29] This force affects both the transverse and axial movement. It keeps the particle centered in the beam.[25] As seen in the previous paragraph, the

intensity of the light affects the change in momentum of the particle and therefore the force acting on the the particle. The intensity of the light is greatest at the center of the beam and decreases further from the center. This means the change in momentum of a ray of light towards the center of the beam will be greater than that of a ray of light near the edge of the beam,[24] as seen in Fig. 4. The trapped particle will move towards the center of the beam due to the net force caused by the change in momentum of the particle.

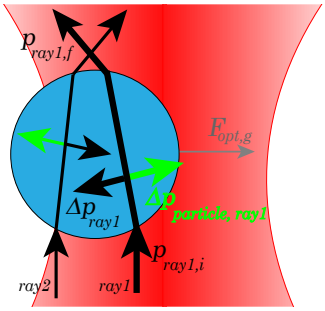


Figure 4: Illustration of the change in momentum of the two photons interacting with a trapped particle. One photon is at a higher intensity (ray 1) and the other is at a lower intensity (ray2). The change in momentum of the photon (black) and the resulting change in momentum of the particle (green) is shown for each photon. This change in momentum of the particle is greater at the higher intensity thus the overall change in momentum will point towards the center of the beam resulting in the gradient force (gray) pointing towards the center of the beam as well.

The gradient force can be treated as a spring force; therefore, $F_{opt,g} = -k\Delta x$, where k is the trap stiffness of the optical tweezer, and Δx is the displacement of the particle from the center of the beam.[24] The trap stiffness changes linearly with the power of the laser.[32] The axial trapping strength will be less than the transverse trapping strength. For an idea of the gradient force's magnitude an example of the beam set to a power of 50 mW with the bacteria having a displacement of 1 μm from the center of the beam will produce a gradient force of approximately 1.45 pN.

3. METHODS

Our optical tweezer setup shown in Fig. 5 was created using a 660 nm diode laser that has a power output of 5.0 mW to 100 mW. We utilized a 100X, 1.3 NA microscope objective which was followed by a sample region. A white light illumination lamp was implemented and shined through a condenser to light up the image on the camera. We used two dichroic mirrors; the first reflected the laser up into the microscope objective and allowed for lamp's light to pass through onto the imaging lens and camera and the second dichroic mirror reflected the beam on to a position sensitive detector called a quadrant photodiode.

Our sample region consisted of *E. coli* adhered to the surface of a slide and a pool of HM buffer containing free swimming *B. bacteriovorus* sealed with a glass cover slip. An example of the sample pool near the side of the slide at the location of the "*E. coli* carpet" is pictured in Fig. 7. The pool was a square shape with sides of approximately 3 mm and was roughly 100 μm deep as pictured in Fig. 6.

The *B. bacteriovorus* is prepared by placing a concentrated suspension of *E. coli* in HM buffer with a small amount of *B. bacteriovorus* in an orbital shaker at 30C over a course of 1 to 3 days. The solution containing the two bacteria initially has a cloudy appearance, indicating there is a higher concentration of *E. coli* than of *B. bacteriovorus*. Over the course of the 1 to 3 days the *B. bacteriovorus* prey on the *E. coli* thus the concentration of the *E. coli* decreases and the concentration of the *B. bacteriovorus* increases. While the solution still observed to be slightly to significantly cloudy, the shift in the two bacteria's concentrations causes the solution to become clearer than it previously was. After this change in appearance occurs to the solution, we take a 900 μL sample and centrifuge it for 15 minutes at 7500 rpm. The purpose of utilizing the centrifuge in this manner is to separate the heavier *E. coli* out from the rest of the solution providing us a sample consisting mostly of *B. bacteriovorus*.

Following the preparation of the *B. bacteriovorus* sample, we prepared the *E. coli*. This is done by inoculating an Ehrlenmeyer flask containing LB (Luria broth) media with a single colony of *E. coli*. The flask is then placed

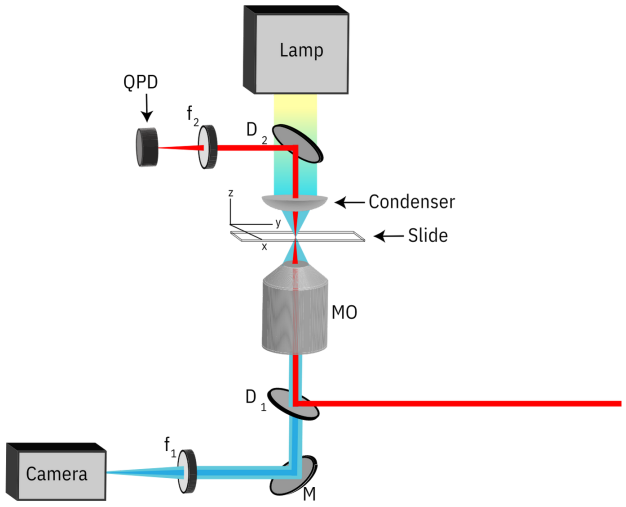


Figure 5: Beam was reflected off a dichroic mirror (D1) into a 100X, 1.3 NA microscope objective (MO) where it was focused in a sample pool attached to a glass slide. The beam then passed through a condenser and reflected off a second dichroic mirror (D2) on to a position sensitive detector known as a quadrant photodiode (QPD). A white light lamp was used to provide light for the camera and the light produced by the lamp passed through the same condenser. The slide was moved using a motorized translation stage (xyz axis).

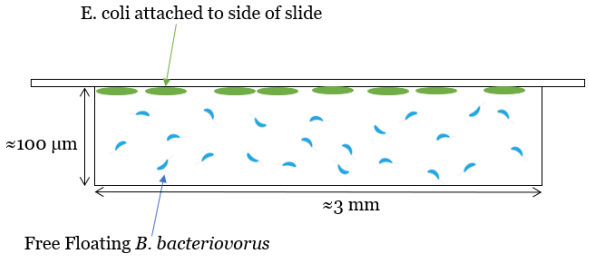


Figure 6: Side view illustration of the sample region where *E. coli* (green) was adhered to the slide and there were *B. bacteriovorus* (blue) swimming freely in the HM solution.

in the orbital shaker overnight. After this time, when the solution is cloudier, we collect a 900 μL sample of the *E. coli* suspension which is then centrifuge for four minutes at 9000 rpm. After the centrifuge has finished, we remove the supernatant and resuspend the *E. coli* in 200 μL of HM buffer using mechanical agitation. Next, we pipette 100 μL of this concentrated *E. coli* sample and drop it on a slide that has been coated with Cell-Tak. Cell-Tak is a cellular adhesive derived from mussels.[33] The slide is then left alone for approximately five minutes. The length of time the *E. coli* is allowed to sit on the coated slide affects the density of bacteria adhered to the slide.The sample becomes more dense with the more time it is allowed to sit before being rinsed. We found five minutes resulted in an ideal distribution and number of *E. coli* adhered to the slide. After waiting we rinse the slide with deionized water to remove excess *E. coli*. Once all excess *E. coli* has been washed off, we top off the slide with 100 μL of HM buffer.

Once we finish with the bacterial colletion, we move on to creating the sample pool on the glass slide. A small square that was approximately the same size as the area the Cell-Tak was applied to is cut into a piece of double sided tape. The tape is then carefully placed on the slide to insure a secure seal with the hole located over the Cell-Tak area. A sample of the *B. bacteriovorus* diluted with HM buffer is added to the pool via a pipette. We cover the pool with a glass cover slip and finish making the sample slide by applying a drop of immersion oil to the top of the cover slip directly above the pool.

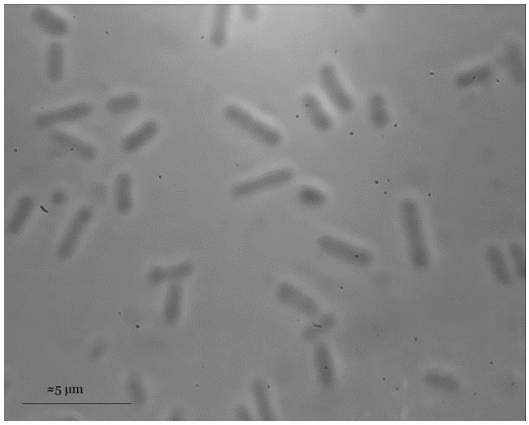


Figure 7: Image of sample region containing the *E. coli* adhered to the slide and free swimming *B. bacteriovorus* with the trap off.

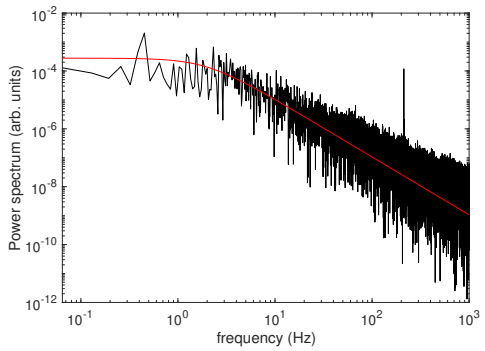
4. RESULTS AND DISCUSSION

In order to find the trap stiffness of the optical tweezers with a trapped *B. bacteriovorus*, we trapped a single bacterium and observed its vibrational motion. The movement of the trapped bacterium was then recorded by the quadrant photodiode. We then used a MATLAB program to produce a power spectrum which we applied a Lorenzian fit to as seen in Fig. 8a. With this, we were able to determine the corner frequency of the bacterium which we used to calculate the trap stiffness. The linear relationship between the trap stiffness and the power of the laser can be seen in Fig. 8b.

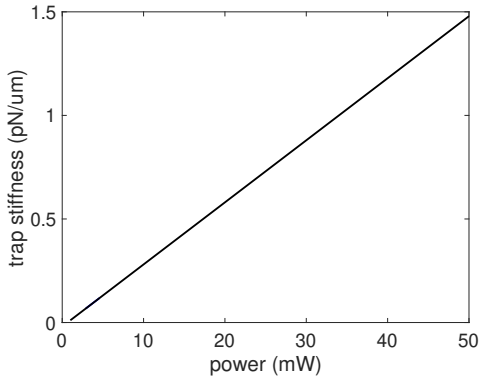
After preparing the *E. coli* coated slide and the *B. bacteriovorus*, we transitioned to measuring the attachment force. In order to measure the attachment force of the *B. bacteriovorus*, a single predatory bacterium was trapped by the optical tweezers. The motorized stage was used to position the bacterium near an immobilized *E. coli* on the side of the slide. After waiting for approximately 30 seconds to four minutes which allowed initial attachment to occur, we would untrap the *B. bacteriovorus* bacterium to see if attachment had occurred. If attachment was observed, the bacterium was retrapped by the optical tweezers and the motorized stage was used to horizontally move the *E. coli* away as illustrated in Fig. 10. If detachment occurred that indicated the force of the optical tweezers was greater than the attachment force of the *B. bacteriovorus* bacterium. Alternatively, if the bacterium remained attached to its prey then the optical force was less than the attachment force of the predatory bacterium. An example of a trial where there was no detachment upon translation of the slide is shown in Fig. 10 This experiment was repeated at varying powers. Our goal with this experiment was to determine the minimum force where the optical force was powerful enough to detach the predatory bacterium from its prey as this force would be equivalent to the attachment force of the *B. bacteriovorus* bacteria.

After conducting approximately 50 trials, we produced 15 cases of attachment. There was a low attachment rate of 30% that we observed. Fig. 11a shows the number of times a bacterium remained attached (green) or was removed by the trap (blue) as a function of the time when detachment was attempted. Out of these 15 cases, there were only three cases where detachment occurred. We attempted detaching at three minutes the most frequently, thus there is a greater number of occurrences at that time. Fig. 11b shows the number of occurrences as a for each of the estimated trapping force. The lowest force at which we could detach a bacterium was on the order of 0.10 pN and the greatest force we attempted was 1.5 pN. Using this information we made can estimate the minimum attachment force to be approximately 1.5 pN.

Since this research is being conducted with living organisms they are not always predicatable so there is a multitude of possible reasons for attachment to not occur that may vary for each attempted attachment. Some of these reasons include the bacteria being too far from each other, and the flagellum pole of the *B. bacteriovorus* [instead of the pole possessing the bacterium's pili which play a role in attachment] being the one held closer to the *E. coli*. Other causes for attachment to not occur could be another *B. bacteriovorus* has attached or invaded the prey cell or the predatory bacterium could have damaged pili preventing the attachment to occur.

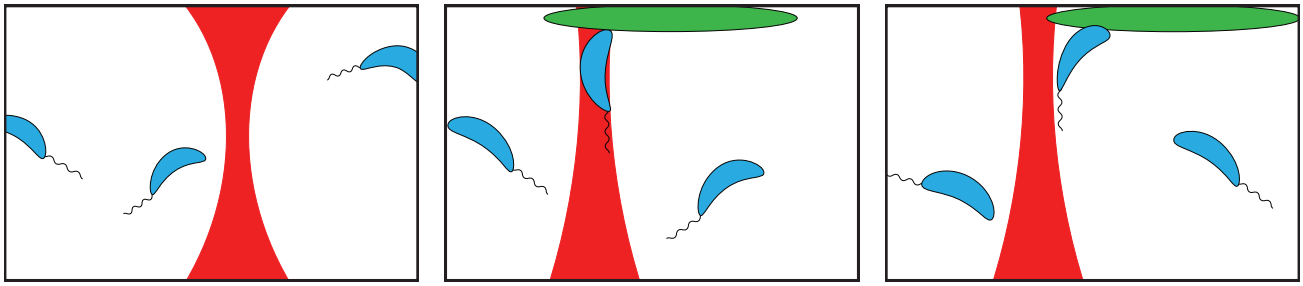


(a) Lorentzian fit on a power spectrum.



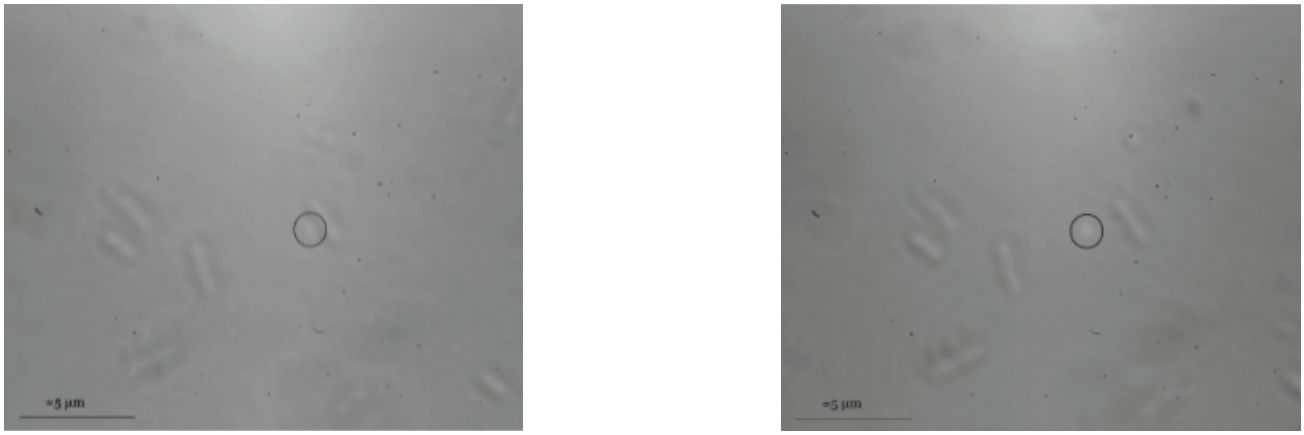
(b) Linear relationship between trap stiffness and power of the diode laser used in the experiment.

Figure 8: Computer generated graphs used to determine the trap stiffness and its relationship to power.



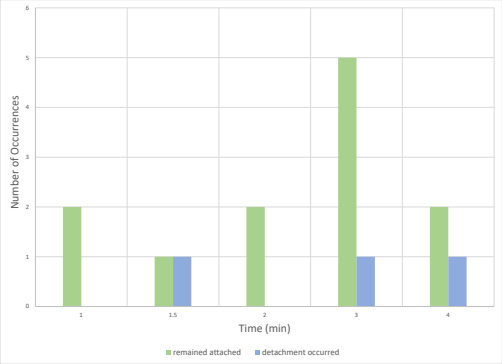
(a) (b) (c)

Figure 9: Method for measuring trap stiffness a) in a pool of free swimming *B. bacteriovorus* a single bacterium is trapped by the beam b) the trapped *B. bacteriovorus* is brought into range of the *E. coli* adhered to the slides surface for attachment to take place c) after attachment has occurred the slide translated pulling the *E. coli* away, resulting in either detachment of the *B. bacteriovorus* or attachment of the bacteria remaining.

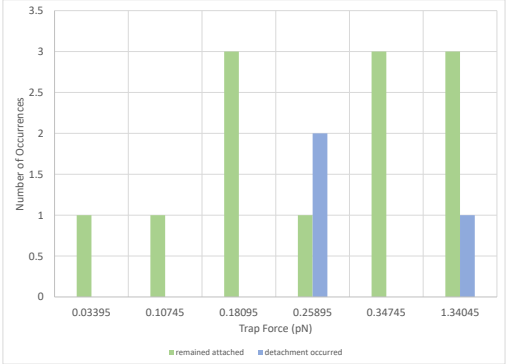


(a) (b)

Figure 10: (a) *B. bacteriovorus* attached to *E. coli* (b) *B. bacteriovorus* still attached to the *E. coli* after the slide has been translated away using a motorized transition stage. The circle indicated the location of the optical tweezers.



(a) Attachment time vs. the number of times the trapped *B. bacteriovorus* was detached from the *E. coli* (blue) and the number of times the trapped *B. bacteriovorus* remained attached to the *E. coli*(green).



(b) The trap force for each occurrence of attachment. The greatest attachment force measured was approximately 1.5 pN. Instances where the bacteria remained attached are in green and the blue shows the instances when detachment occured.

Figure 11: Graphical representation and analysis of the 15 occurences of attachment out of the 50 trials.

5. CONCLUSION

The intent of this research was to determine what the attachment force of *B. bacteriovorus* was on its prey *E. coli* with the use of optical tweezers. We developed a technique for establishing attachment and for measuring the force required to detach the predatory bacterium from its prey in order to determine the attachment force of *B. bacteriovorus*. We concluded from our results that the minimum trap force able to detach the bacteria was 1.5 pN. Based on comparisons with results from atomic force microscopy, this is a low estimate for an attachment strength which we expected as we were focusing on the initial attachment times in short time frames. We believe that the attachment force will increase over longer periods of time for attachment. We anticipate that a more extensive set of data will demonstrate this trend. Moving forward with this research, we will be exploring the mechanism of pili attachment by disabling the extension of pili using a cranberry juice solution that deactivates the pili. This will allow us to test whether the *B. bacteriovorus* can still attach to their prey while their pili is disabled.

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