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The Kinetic Signatures of Antibody Binding to M. Genitalium Adhesin Protein Fragments

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by

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Abstract

Mycoplasma genitalium is a sexually-transmitted bacterial pathogen that persists in patients by adherence to cells through matrix glycoproteins and evasion of host antibodies. The MgpB and MgpC adherence proteins consist of variable and conserved regions. Variable regions undergo antigenic variation to avoid specific antibodies. However, the C-terminus (MgpB-4a) does not vary, is highly immunogenic, and antibodies to this region inhibit attachment and promote bacterial killing in vitro. To better understand how *M. genitalium* avoids clearance by antibodies to MgpB-4a in vivo we used surface plasmon resonance (SPR) to measure kinetic values of binding events. Binding of polyclonal rabbit antibodies (3935 and 3936) raised against MgpB-4a was measured against recombinant protein fragments truncated at the N- and C-termini. The two antibody sera produced by two separate, identical inoculations exhibit different kinetics and thermodynamics of binding while binding tightly and specifically to fragments of the mycoplasma adhesin domain MgpB-4a. Antibodies from both rabbits bound tightly to MgpB-4a fragments with dissociation constants of 10 nM-1 pM. A higher temperature (37°C) reduced binding of fragments to both antibodies, affecting 3936 more than 3935. Each antibody exhibits different kinetic signatures, with 3935 binding more tightly to smaller protein fragments and 3936 binding more tightly to larger fragments. Both antibodies bind tightly and with similar dissociation rates too slow to measure, differing most in on-rates. The 3935 and 3936 antibodies are specific to the MgpB-4a domain, as other antibodies raised against MgpB-B bind to the same fragments at higher dissociation constants of 1 μM. Future experiments will compare these binding signatures to those of patient antibodies to explain how antibodies are avoided in vivo.

Introduction

Mycoplasma genitalium (Mg) is a sexually transmitted bacterial pathogen that causes a multitude of symptoms such as cervicitis in women and non-gonococcal urethritis in men (Wiesenfeld and Manhart). Mg infection can persist for months to years, and infection by Mg increases susceptibility to sexually transmitted infections such as HIV; Mg binding decreases the integrity of endocervical cells, increasing the passage of pathogens through epithelial barriers and causing infection (Vandepitte et al.) Mg infection affects between 1-3% of the US population and is primarily treated with Azithromycin and Doxycycline (Sliferski et al.; Bradshaw et al.). Alarmingly, multidrug resistance frequency is increasing, and previous treatment methods are becoming ineffective for patients leading to the necessity for the development of novel clinical treatments (Bradshaw et al.). These new therapies are aimed at replacing traditional antibiotics through identifying ways in which the bacteria persist in the body despite an immune response.

Mg is able to evade the immune response by adherence to matrix glycoproteins, and through antigenic variation of epitopes. Epitopes are regions of an antigen molecule where an antibody binds, while matrix glycoproteins are extracellular macromolecules to which Mg binds, allowing it to persist in the body. The antibodies developed in a typical immune response to infection are raised primarily against the MgpB and MgpC adhesin proteins (McGowin and Totten). MgpB and MgpC are two proteins that allow Mg to adhere to endothelial cells. Using sub-tomogram averaging, previous research shows

these two proteins form a dimer complex consisting of 4 lobes total in an extracellular domain* (Figure 1). These adhesin proteins are necessary for virulence, and sequencing of the MgpB and MgpC adhesin proteins reveal they consist of variable and conserved regions across bacterial samples. Specifically, a region within the C-terminus of MgpB (recombinant protein MgpB-4a) does not vary and is highly immunogenic, located near the transmembrane domain of the protein (Figure 2). Antibodies to this region inhibit attachment and promote bacterial killing in vitro (McGowen and Totten). Previous research on MgpB-4a has shown this region binds to fibronectin and plasminogen at different sites, suggesting this region consists of multiple binding sites for optimized cell adhesion (Mahlum et al.).

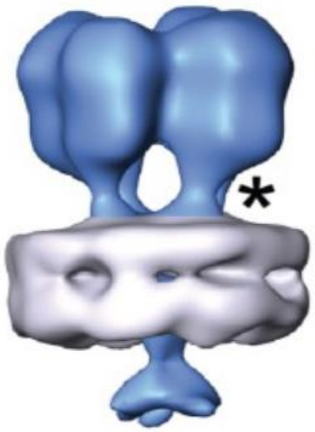

Figure 1: Sub-tomogram average of MgpB/C adhesin complex. (Scheffer et al)

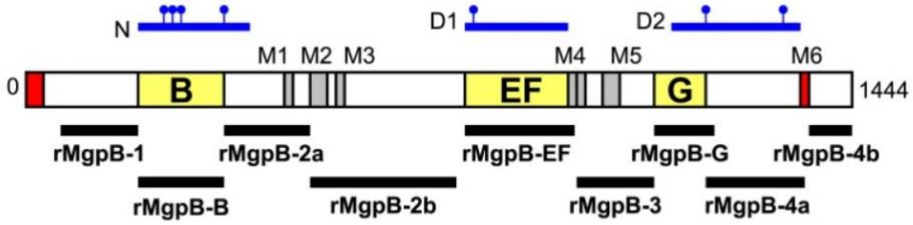

Figure 2: Representational graph of MgpB protein showing the location of the recombinant protein MgpB-4a relative to entire sequence. (Iverson-Cabral et al.)

To better understand how *M. genitalium* avoids clearance to MgpB-4a in vivo we used surface plasmon resonance (SPR) to measure kinetic and thermodynamic values of binding events to establish epitopes on the adhesin protein Mgp-4a. Knowledge of these binding sites can explain how antibodies are failing to bind sites that would result in clearance, and help us determine where they may be binding instead. Previous research

established SPR can be successfully used to measure binding affinities of antibodies to recombinant proteins (Mahlum et al.). SPR uses a light source aimed at a chip with bound protein, and this light is reflected off at a certain angle. When a protein is bound to a chip and an analyte in solution flows over, the analyte will then bind to the protein on the chip, and the total mass bound to the chip will increase. This results in a change in the refractive index (Figure 3). These changes in angles allow us to measure K_d, and on/off-rates of binding. A higher on-rate suggests little energy is required for binding, but a higher off-rate suggests the interaction formed is weak and dissociates quickly. The K_d relates these two values into a single constant, where $K_d = \frac{k_{on}}{k_{off}}$, so it is important to note equal K_d values can have differences in on and off rates, signifying different kinetics of binding despite a similar K_d. Overall, a lower K_d suggests a smaller concentration of antibody is necessary to bind antigen and the antibody has high affinity for the antigen, with the opposite true for a higher K_d. Using either the recombinant proteins or polyclonal antibodies as analytes or ligands, we are able to measure the binding signatures of their interactions in real time.

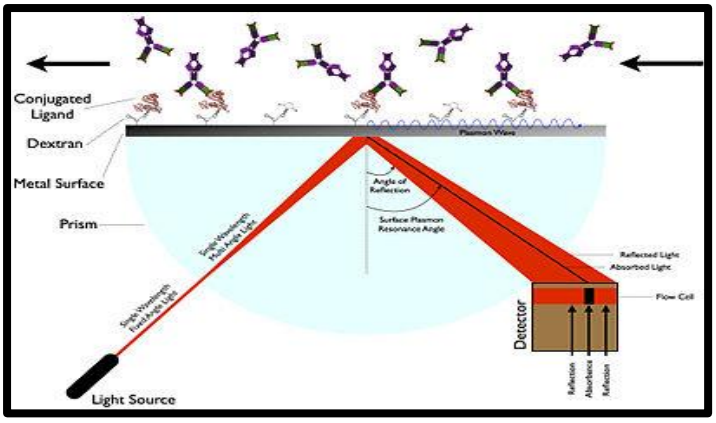

Figure 3: Surface Plasmon Resonance (SPR) SPR measures changes in resonance angle in real time. This change is directly related to the amount of protein near the surface of the chip through the change in refractive index. (Sabban, 2011)

The purpose of this study is to evaluate the varying kinetic signatures of two polyclonal rabbit antibodies to explain how antibodies are avoided in vivo. Kinetic signatures involve on-rates, off-rates, and K_d binding constants: how quickly the

antibodies bind, how quickly they dissociate, and how much they bind overall. Using SPR, we are able to measure these different kinetic signatures and obtain 3 values for the binding events observed. Understanding the epitopes and kinetic signatures of these polyclonal antibodies will tell us how the immune system targets this bacterium and why it is failing to clear the infection. Future work focused on patient antibodies and how *M. genitalium* avoids clearance in vivo can help with the development of targeted therapies that can hinder bacterial adhesion or assist in a directed immune response.

Materials and Methods

Protein and Antibody Production and Purification (Aguila et. al.)

MgpB-4a truncations and mutations were cloned into a pET plasmid vector. Proteins were expressed in BL21 alpha E. coli competent cells and were purified using nickel affinity chromatography. Using a BCA assay, purified protein concentrations were quantified. Rabbits were immunized with recombinant protein 4a (Figure 2). Serum was subsequently collected, and antibodies were purified using affinity-based purification kits.

Surface Plasmon Resonance (SPR) Data Collection

All reactions were completed at 25 ^{o}C unless otherwise stated. BIACORE X machine collected data in real time using BIACORE X Control Software. HBSEP buffer was used as solvent in all solutions unless otherwise stated. HBSEP was taken up by inlet tube during all CM5 chip data acquisition. FC1 was set as the blank, with no protein or antibody bound while FC2 was used as protein-bound channel.

Determining Preconcentration

The pH of solution can change the overall charge on a protein to allow for a positive protein to bind to the negatively charged surface of the chip. Different pH values can create a charged protein, and the best pH value was determined before each coupling to ensure chip would bind protein properly. Run settings set as: Multichannel, >10μL/min rate with reference cell=1. A blank CM5 chip was docked in the machine, with different pH conditions injected into the loop to determine optimum pH for coupling. Different pH conditions of sodium acetate buffer (pH=4.05, 4.51, 5.08, and 5.49) with a 1:10 dilution of protein to pH were injected. Regeneration in-between samples was done with 50 mM

NaOH. Best pH conditions were selected looking for highest response difference in flow channel 2.

Collecting Preliminary Antibody/Protein Data

Highest concentration in data acquisition of antibodies/protein with a coupled chip was determined through the use of serial dilutions ranging from 1:10-1:3000 with HBSEP. A response difference of ~100 was optimal for the high concentrations, as this difference suggests a detectable amount of analyte bound to chip without saturating binding sites. This is then used in a two-fold dilution series down to 1/32 of the high concentration.

Regeneration of Surface Using NaOH

Different concentrations of NaOH (ranging from 10-50 mM) was used to regenerate surface and interrupt protein-antibody binding. Regeneration allows for chip to remove any analyte not removed during wash procedure and frees up binding sites on the bound protein without unfolding the protein irreparably.

Basic Data Acquisition

Data acquisition was done using multichannel, 40µl/min with reference cell=1. Analyte injections during data acquisition done by injecting liquid followed by air-bubbles to avoid diluting the analyte with buffer. A blank of HBSEP, no delay, was injected into the loop. This was then repeated with a 180 second wash delay blank injection. Analytes were then injected from 1/32 dilution to high concentration with a 180 second wash delay. Regeneration was used as needed and repeats were completed if baselines drifted during data acquisition or bubbles interrupted analysis of binding events. 3 separate runs of data acquisition were performed per analyte.

Surface Plasmon Resonance (SPR) Data Processing

Data processing of antibody-protein binding curves was completed on BIAevaluation software. Equilibrium data and kinetic data were analyzed per individual run. For kinetic data, blank was subtracted from all analyte concentrations. Kinetics were fit with association and dissociation 1:1 Langmuir binding model. Kinetics were fit either simultaneously or separately (during antibody on chip experiments). Using triplicate data sets, average k_{on} and k_{off} values were determined, with most averages less than 20%. Errors (SE) were determined by incorporating magnitude of error with the measured values in accordance to previous research (McFarland). Dissociation time was determined by the wash delay (180 seconds). For samples that did not dissociate detectably within the set time period, a minimum rate constant of 10^{-5} s^{-1} was set. Beyond this limit, off-rates are likely not biologically relevant.

Results and Discussion

The Mgpb-4a domain is 168 amino acids long and consists of multiple charged amino acids thought to be involved with antibody binding. Using their previous experience with microbiology and immunology, our collaborators identified these amino acids of interest and truncations/point mutations were designed that could aid in identifying binding sites. SUMO-tagged MgB-4a recombinant proteins with domain truncations at the N- and C-termini or introduction of point mutations at key arginine residues (Figure 4) were bound to polyclonal anti-MgpB-4a antibodies produced in two rabbits. Antibodies from both rabbits bound tightly to these recombinant proteins with

Figure 4: MgpB-4a sequence, truncations, and mutations

Recombinant proteins 3-5, with truncated N, C, or both termini, shown above with respective length and size relative to fragment 1 (full 4a domain sequence without transmembrane region). N and C terminals highlighted in MgpB-4a sequence above truncation representation. 2a-c point mutations, Arginine to Alanine point mutations, are underlined/bolded in the sequence above. SUMO tags include one His tag at N-terminus.

dissociation constants in the nanomolar to picomolar range (Figure 5). Antibodies from rabbits immunized with a different domain of the MgpB adhesin protein (MgpB-B) bound MgpB-4a fragments with weak binding constants in the micromolar range or weaker, suggesting the two rabbit antibodies explored here exhibit specific binding to these SUMO MgB-4a constructs. SUMO tags were used for purification as previous data suggests using multiple His tags increases non-specific binding to antibody sera (Mahlum et al.).

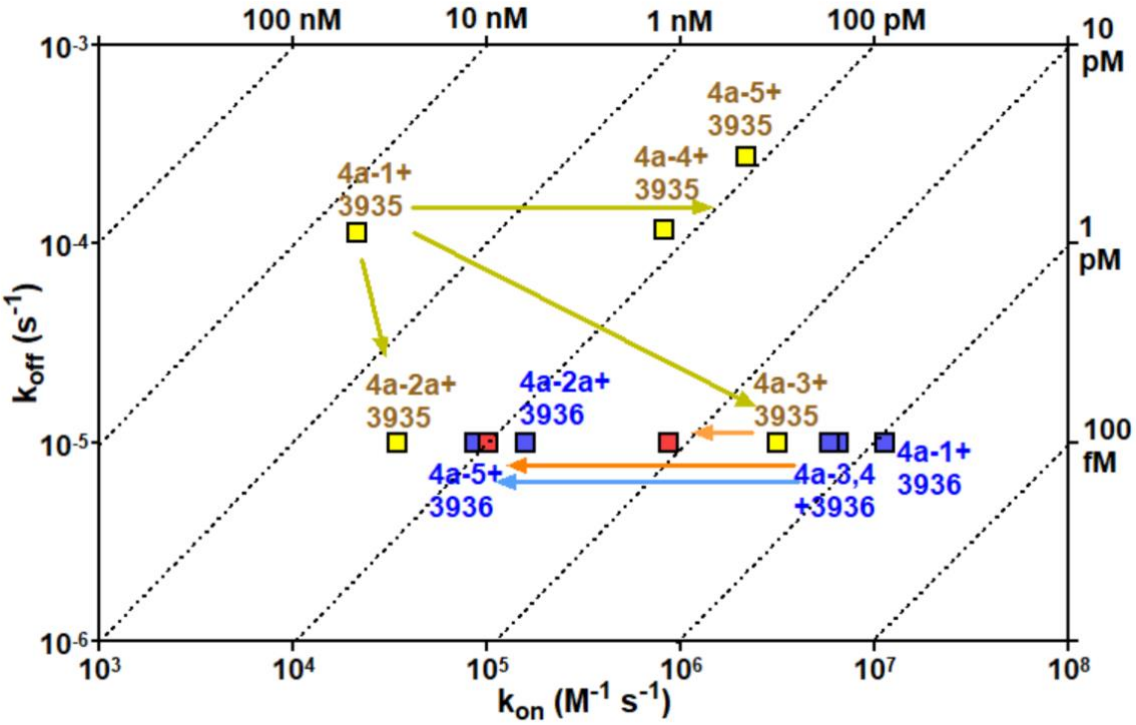

Figure 5: 3936 and 3935 antibodies bind tightly to antigen, but with different kinetic signatures

K_{on}/K_{off} plot for the 3936 and 3935 kinetic signatures measured via SPR and evaluated on BIAeval. Orange arrow shows differences of binding patterns at 37C for fragment 3. Blue arrows show differences in binding of 3936 between truncations, binding more tightly to larger recombinant proteins. 3936 binds most tightly to fragment 1 at close to 1pM. Yellow arrows shows difference in binding of 3935 between truncations, binding more tightly to the smaller protein fragments. 3935 binds most tightly to fragment 3 (N terminal deletion) at close to 5 pM.

The polyclonal rabbit antibodies responded differently to the truncated recombinant protein, the 2a, 2b, and 2c Arg-to-Ala mutation, and increased temperature (Figure 5). For both antibodies, a higher temperature reduced on-rates and off-rates were unaffected within detection limits.

For 3936, temperature and truncation decreased on-rates meaning there are multiple sites on the protein to which the polyclonal antibodies bind and truncating either end of the protein reduces the total binding sites available. Overall, this suggests 3936 possesses distributed epitopes across the C-terminal domain of MgpB. 3936 bound tightest to fragment 1, or the full 4a domain, with a K_d close to 1pM (Figure 4). Mutation R80A also decreased on-rates for 3936, destabilizing binding as might be expected for long-range electrostatic attraction.

Alternatively for 3935, smaller domains bound with increased on-rates which may indicate binding concentrated to the central region of the C-terminal domain. This region was conserved in all truncations, unlike flanking regions. 3935 preferentially bound to the C-terminal half of 4a-2a (fragment 3) with a K_d close to 5pM (Figure 5). Mutation R80A (R1236A relative to entire MgpB protein) decreased off-rates for 3935, stabilizing the interaction. We hypothesize that this arginine's positive charge (located in the center, near the N-terminal half of the domain) destabilizes the bound antibody-antigen complex due to positive charges in the antigen's epitope.

All experiments exploring effects of truncation and temperature on kinetic signatures of polyclonal antibodies were completed with the antigen bound to the chip. A set of experiments coupling the antibody to the chip with the antigen as analyte showed a

difference in binding signatures. We expected to see enhanced binding due to the avidity effect when the antigen is coupled to the chip relative to when the antibody is coupled.

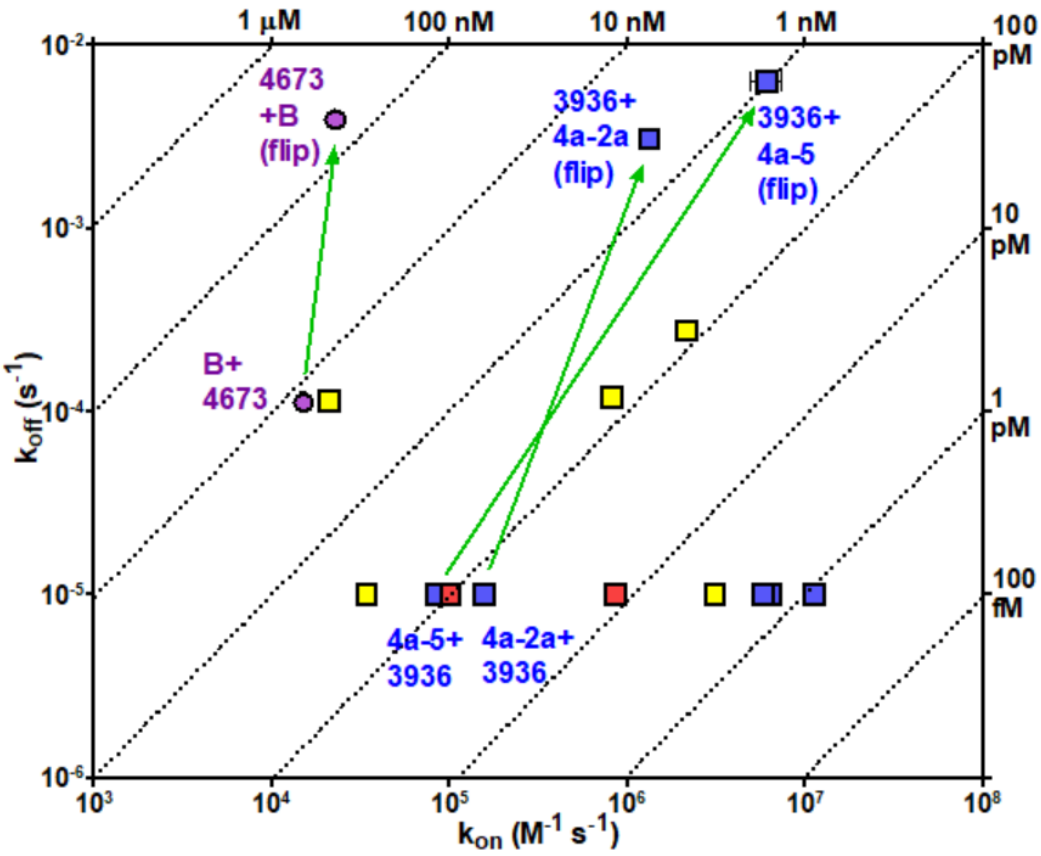

Figure 6: Attaching the antibody rather than the antigen to the chip results in binding changes that are consistent with the avidity effect

K_{on}/K_{off} plot for the 3936 and 4673 (anti-MgpB-B) kinetic signatures measured via SPR and evaluated on BIAeval. Green arrow shows differencing of binding patterns between recombinant protein on chip and antibody on chip ("flipped" chip).

Once the antibody was coupled to the chip (creating a "flipped chip") and a recombinant protein was used as an analyte, on-rates increased slightly, and off-rates increased significantly compared to previous rates with the antigen coupled to the chip. For all 3 flipped chips, K_d decreased by ~1-2 orders of magnitude with an antibody chip, consistent with increased avidity (Figure 6). When an antigen is attached to a surface, we expect the avidity effect to enhance binding of the antibody analyte flowing over the

surface as it is bivalent, resulting in a lower Kd relative to when the antibody is instead coupled to the chip.

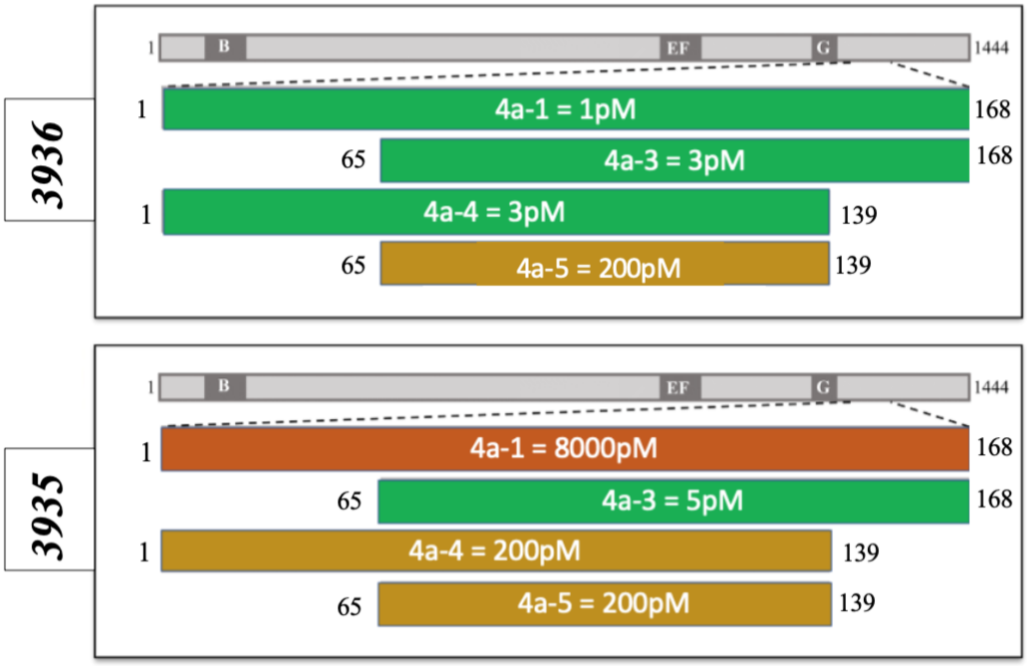

Figure 7: MgpB-4a antibody Kd affinity

Recombinant proteins 4a-3,4, and 5, with truncated N, C, or both termini shown above with length and size relative to fragment 4a-1. Fragment 4a-1 refers to full sequence + transmembrane region. His tags on beginning and end of fragments. 3935, on bottom, is more specific and binds best to C-terminal recombinant protein. 3936, on top, is less specific and binds best to longer proteins. 4a-2a has a point mutation from Arg-Ala at R80A (R1263A), not shown above. Smaller K_d values in green, increasing K_d in yellow, and largest K_d in orange. Decreasing K_d is indicative of tighter binding and greater affinity.

Overall, both antibodies bind tightly, but with distinct kinetic signatures and distinct specificities to truncated domains despite similar experimental origins (Figure 7). The 3936 antibody sample binds more peptides, consistent with a broader interaction in which epitopes are distributed throughout the 4a domain. The 3935 antibody sample preferentially binds truncated peptides containing C-terminal regions rather than N-terminal regions, indicating epitopes in the C-terminal half of the domain.

Conclusion

Mapping the kinetic signatures of these rabbit polyclonal antibodies establishes groundwork for understanding how *Mycoplasma genitalium* avoids the immune response in vivo. Our findings suggest the two polyclonal antibody sera have differing targets on the adhesin protein and range in their distribution of epitopes. Comparing binding signatures to disease severity and longevity can help map what antibody epitopes and distributions are effective in clearing infection and which are not. Future work involving mapping human antibody epitopes to MgpB-4a or other immunogenic regions of the MgpB adhesin protein can aid in the process of assisting the natural immune response and creating effective strategies that can target the bacteria in vivo. We also hope to repeat this research similarly using primate and human sera or other recombinant proteins designed from the adhesin protein MgpC.

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Appendix on Faith and Science

As a Christian scholar, the intersection between my faith and passion for science is dynamic and closely intertwined. As Francis Collins gracefully explains, "the God of the Bible is also the God of the genome. He can be worshipped in the cathedral or in the laboratory" (Collins 78). My own worship takes place intellectually; as a person focused on the accumulation of knowledge and reasoning, my appreciation for God's creation is found within the natural laws that He has given to us to explore. This understanding of my own personal strengths and my strong background within the Nazarene church have helped me view a Christian scholar as one who views God as the truth behind all things, even science.

Growing up in a Nazarene church in Olathe, Kansas provided me with many wonderful benefits to understanding Christianity, but also introduced obstacles for engaging in outside cultures. The Nazarene church places heavy emphasis on the value of "reason, experience, sanctification, and freedom" (Hughes 346). While the community is an important aspect of these tenants of holiness, individual journeys are stressed, and each walk of faith is seen as a personal experience. To understand fully the culture within the Nazarene church, recognition of its past is important. After WWI, an increase in a counter-culture movement spread throughout all Nazarene churches as isolation from the temptation of the world was equivalent to moral integrity. Legalism, intolerance, and quarantine were promoted throughout the church across the nation. A "Christ against culture" community was fostered, prompting many to attempt to protect themselves from the temptations of people not like them (Hughes 351). College Church of the Nazarene

in Olathe has echoes of these legalistic sentiments still found in the congregation, specifically among older generations who were raised within its boundaries. While the church is full of loving, God-fearing people, these intense rules of self-restraint can feel restrictive to many and the church has recently been declining in numbers. Modernization is re-shaping the church in many ways, but a balance of understanding and growing with the world without the danger of secularism is still being molded.

New movements of "sanctification as a journey" and the revitalization of the community are spreading within the Nazarene church, aligning to the time I was growing up in CCN (Hughes 347). I still have recognized many of the legalistic tendencies that prevail within my own background and are continuously growing and shaping this area of my life. As "Christian faith constantly becomes enmeshes with cultural forces", my own church's culture shaped my faith into many great things and many things that do not reflect the glory of God (Marsden 100). As a science-driven person, the strict adherence to rules was an easier way for me to live a life of faith and I was comfortable living inside the Nazarene church "bubble". However, moving to Seattle has shown me that a faithful life also takes passion and deep love for others, not just an understanding of how to live with self-restraint. Jesus was full of intensity for loving all people and a life without sin – not the following of a set of rules. I appreciate the value my church has given me in following the bible, but I have had to develop ways to interact with the world outside the church on my own and learn that this adventuring out of the church's walls is inherent to a strong Christian faith.

The Nazarene church still continues to hold reason in high regard, specifically promoting the ideas of a higher education after high school. As shown, this can result in authoritarian-like regulations, but also results in the fostering of intellect within the community. Science, in general, is not feared by the Nazarene church but misunderstood; as many in older generations have told me, they do not "believe" in evolution simply because they do not understand it fully but do not think it is anti-Christian. Because of this appraisal of science within my community, my enjoyment of investigation and exploration in my science classes was always met with positivity. As a woman, many encouraged me even further to enter the STEM fields as they believed it would be great for gender equality; the Nazarene church, while still needing more gender reform, was one of the first denominations to promote the ideas of women pastors. At one point in high school, I attended a sermon interviewing leading scientists on new interpretations of the creation story in tandem with evolution alongside those with a young earth creationism background. This promoted my individual desire to be a scientist, while also showing to me that the church and science belong in conversation and debate together; one does not negate the necessity for the other. I think I owe much of my appreciation for science to CCN and am forever grateful for the fostering of intellectual growth that took place there.

Growing up as a quiet, intellectually-driven girl I have always tried to understand how the gifts of scientific investigation could be used to help others, as a part of me thought I could never do anything for the glory of God like the missionaries did. While my church never disregarded my scientific desires, I always felt slightly guilty about my desire to work in science rather than going out to preach the word of God. After learning

more about my strengths throughout high school and realizing that God can use them to help others medically, I have always felt confident in my decision to pursue a higher education and research in the war against cancer. As Paul Farmer puts it, “I feel most alive… when I'm helping people” (Kidder 57). Paul Farmer is a perfect example of using the strengths God has given to bring light back into the world. While I may never be a doctor who travels around the world helping others or a missionary gifted with eloquent speech, I can do my own part to make God’s will a reality. None of these testaments to God’s will are more holy than the other; I have learned my work is just as important.

Within cancer research, I’ve always had a strong personal connection to my work outside of my love for scientific exploration. After losing my mother to lung cancer at the age of 13, I have always desired to rid the world of the disease completely. I have always strongly argued against the ideas of “everything happens because God wills it to be”, instead choosing to believe that God uses us within the circumstances of the fallen world to bring light back. God can work despite the suffering of the world, but he does not will it so. Because of this, I have always believed my calling was to work within cancer research because God was able to spark a fire for my passion despite a terrible tragedy. My honors project participates in the understanding of a disease linked to an increase in susceptibility to cancer, reflecting my long-term desires to work in this field to do my own part of healing this broken world.

As a person of faith, I am able to use the gifts God has given me to improve the world around me. These gifts, to many, seem antithetical to faith but are actually complex and intertwined. My journey growing up within the Nazarene church and suffering

through grief has provided me with the opportunity to be a Christian scholar within cancer research. My academic work is a way in which I can worship God as He is the truth behind all things, including scientific investigations. A relationship with God, to me, involves an analysis of how one works best to bring God's glory into the world and allowing this understanding of self to be a way in which God can be truly worshiped.

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