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INVESTIGATION OF SERUM RESISTANCE IN HAEMOPHILUS PARAINFLUENZAE

Morgan J. McPartland
University of Rhode Island, mmcpartland_@uri.edu

INVESTIGATION OF SERUM RESISTANCE IN *HAEMOPHILUS PARAINFLUENZAE*

BY

MORGAN J. MCPARTLAND

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ABSTRACT

Around 50% of the United States adult population suffers from some form of periodontal disease. From gingivitis to severe periodontitis, periodontal disease is associated with a plethora of chronic and systemic diseases, like rheumatoid arthritis, cardiovascular disease, type-two diabetes, and Alzheimer's disease. Prevention of periodontitis relies on the establishment and maintenance of a resilient, eubiotic oral microbiome. A key member of the healthy oral microbiome is *Haemophilus parainfluenzae* (*Hp*). *Hp* is a highly prevalent and abundant oral generalist. As a health-associated commensal, understanding its relationships within the microbial community and its interactions with the host may shed light on what is required for a healthy, resilient oral microbiome to thrive. One way that *Hp* interacts with the host is through the host's immune responses. *Hp* is exposed to phagocytic immune cells like neutrophils and macrophages when living below the gumline as part of the subgingival plaque. As part of the subgingival and supragingival plaque, *Hp* comes into contact with gingival crevicular fluid (GCF), a serum-like exudate containing the complement system. Previous work in the M. Ramsey lab has shown that *Hp* is highly resistant to phagocytosis and complement-mediated killing. These high levels of immune evasion are inconsistent with the very low incidences of infection or invasive disease caused by *Hp*, and the observation that the abundance of *Hp* is negatively correlated with inflammation in the mouth. Additionally, the mechanism(s) of resistance to killing by the immune system is unknown. To address this knowledge gap, highly

saturated *mariner* transposon sequencing (TnSeq) libraries were created to identify conditionally essential genes in two strains of *Hp*, the type strain, ATCC33392, and a novel oral isolate, EL1, for survival in 50% normal human serum. Gene deletion mutants were created for conditionally essential genes in each strain that were identified as potentially conferring serum sensitivity. These mutants had their serum survival phenotypes and lipopolysaccharide structure characterized to identify mechanisms of complement resistance. Our results demonstrate that *Hp* does not rely solely on one mechanism to evade complement but has an assortment of tools to survive exposure to complement, from lipopolysaccharide to complement regulator-binding surface proteins.

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INTRODUCTION

Periodontal disease, known colloquially as “gum disease,” is an infection of the tissues surrounding and supporting the teeth. It is characterized by chronic inflammation of the gums, gum recession and bleeding, tooth loss, and, in severe cases, can lead to alveolar bone loss.[1–3] Periodontal disease can be triggered by the presence and abundance of keystone pathogens, like *Porphyromonas gingivalis*, and or by the shift of the oral microbiota from a “healthy” eubiotic community to a dysbiotic community.[4,5] This shift to dysbiosis can be due to many factors, such as a change in host diet or immune response, dental hygiene practices, or even salivary flow rate.[4,5] Regardless of what triggers the shift, a dysbiotic microbial community induces a chronic feedback loop of host inflammation and further microbial dysbiosis, eventually leading to a dysregulated immune response and progression in severity of periodontal disease (Figure 1).[3] Furthermore, periodontal disease is incredibly common, with 47.2% of US adults over the age of 30 having some form of periodontitis. Like many other diseases, the incidence of periodontal disease increases with age, illustrated by the fact that 70.1% of US adults over age 65 suffer from periodontitis.[2] Outside the United States, additional lack of access to dental healthcare, poor dental hygiene practices, and an increased prevalence of smoking have the World Health Organization estimating that 19% of the global adult population suffers from severe periodontal disease.[6] Periodontitis is not just an oral health concern. It has been linked to a plethora of other chronic illnesses, like cardiovascular disease,

metabolic disease, and Alzheimer's disease, and has even been shown to exacerbate certain already-existing illnesses, like inflammatory bowel disease (IBD) (Figure 2).[3,7]

Many chronic systemic diseases share the symptom of chronic low-grade systemic inflammation, and it is thought that periodontitis may play a role in stimulating that inflammation through the hematogenous spread of oral bacteria, their microbial-associated molecular patterns (MAMPs) or through spillover of local inflammatory mediators into the bloodstream.[7] Often, the worsening of chronic systemic inflammation is correlated to the worsening of disease symptoms or the development of other chronic systemic illnesses.[3,7–9] Importantly, though, the treatment of periodontal disease locally has been shown to decrease systemic inflammation.[7] For metabolic disease, a study showed periodontitis patients with type-two diabetes had a decrease in systemic inflammation and lower A_{1C} levels after treatment of their periodontal disease.[7] There have been numerous studies demonstrating a relationship between the oral microbiome, periodontal disease, and inflammatory bowel disease. IBD patients have an increased incidence of periodontal disease, and their periodontitis tends to be more severe.[7–9] Studies have also shown colonization of the IBD gut by oral microbes, which is not seen in healthy patients.[10] The presence of oral microbes in the IBD gut has also been correlated with the worsening of IBD symptomology and inflammation.[8,9] Periodontal disease has even been implicated as a possible causative agent and a risk factor for developing Alzheimer’s disease.[7,11] During autopsies, researchers

have been able to find *P. gingivalis* DNA in the brains of people with Alzheimer's disease. Additionally, the autopsies found a correlation between the abundance of gingipains, proteases secreted by *P. gingivalis*, and the abundance of tau protein and ubiquitin, both markers for Alzheimer's disease. *P. gingivalis* may not be the only link between periodontitis and Alzheimer's disease, though, as a mouse model study showed an increase in amyloid-beta and neuroinflammation in mice with periodontal disease in the absence of *P. gingivalis*. When periodontitis was treated, the amyloid-beta and neuroinflammation prevalence decreased.[7] Periodontal disease has also been linked to various autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus, and Sjögren syndrome.[7,11,12] Additionally, the presence and severity of periodontitis correlates with adverse pregnancy outcomes, like preterm birth and spontaneous abortion.[13,14]

Through the lens of the polymicrobial synergy and dysbiosis model of periodontal disease, which asserts that periodontal disease is characterized by a microbial community shift to dysbiosis leading to a feedback cycle of dysregulated immune response and proliferation of a dysbiotic oral microbiome, prevention of periodontitis relies on the formation and maintenance of a resilient, eubiotic oral microbiome.[4] While the Human Microbiome Project and many other studies have provided robust data sets illustrating “who” is present in the microbial community during health, we do not yet have an adequate appreciation of what inter-microbe and host-microbe relationships must be present for these communities to be

maintained and resist slipping into dysbiosis. Closing this knowledge gap requires studying oral microbes that are common amongst healthy adults and further exploring their microbial community and host relationships.

One member of the healthy oral microbiome is *Haemophilus parainfluenzae* (*Hp*). *Hp* is a Gram-negative, pleomorphic rod and a generalist of the oral cavity, meaning it does not favor any particular niche.[15] It is a highly prevalent and abundant member of supragingival plaque, the polymicrobial biofilm that forms on teeth above the gumline. According to data from the Human Microbiome Project, about a quarter of healthy adults have *Hp* making up 10% or more of their supragingival plaque.[16] *Hp* is consistently shown to be a health-associated oral microbe, meaning it is present during health with a reduced abundance during periodontal disease.[16–18] While as a member of the oral microbiome, *Hp* is associated with good health, *Hp* has also been known to cause extraoral infections and disease. *Hp* is a member of the HACEK group (*Haemophilus, Aggregatibacter*, *Cardiobacterium*, *Eikenella*, and *Kingella)*, a collection of bacterial genera known to colonize the oropharynx and can cause infective endocarditis.[19,20] It is theorized that these bacteria can enter the bloodstream through the mouth during teeth brushing, dental cleanings, or periodontal disease. Once in the bloodstream, they travel to the heart, where they settle, colonize, and cause disease. While infective endocarditis caused by a HACEK organism is rare, making up only 1.4% of total infective endocarditis cases, *Hp* is the most common HACEK organism to cause it.[19,20] Additionally, there have been case reports identifying *Hp* as the causative

agent of meningitis, septic arthritis, chronic osteomyelitis, septicemia, and a variety of other infectious diseases.[21–26]

Hp presents an intriguing dichotomy as it most often exists as a health-associated oral commensal, yet in some body sites and host conditions, it is an opportunistic pathogen. To be able to cause infective endocarditis and other infectious diseases, *Hp* must be highly resistant to killing by our immune system. A common observation of the microbiome during periodontitis is an explosion of "inflammo-philic" bacteria, bacteria that are immune resistant and thrive with the increased nutrients available during the immune response.[3,4,7] Theoretically, *Hp* should act "inflammo-philic." Yet, during periodontal disease, when there is inflammation and a dysregulated immune response, its abundance is reduced.[27–31] To better understand this phenomenon and *Hp*'s role in health and disease, we investigated how *Hp* can evade killing by one component of our immune response, the complement system. The complement system is made up of many proteins that act as a cascade and can lyse Gram-negative bacteria through the formation of the membrane attack complex (MAC).[32,33] Complement is present in our bloodstream, tissues, extracellular matrices, and in the mouth as a component of gingival crevicular fluid, a serum-like exudate from the periodontal tissues.[5,32,33] To investigate the mechanism of complement resistance used by *Hp*, we performed TnSeq, a high-throughput method to screen a library of bacterial mutants for fitness, in two *Hp* strains, the type strain, ATCC, and an oral isolate, EL1, identifying genes that were essential for survival in 50% normal human serum, a

cell-free component of blood that contains the complement system (Table 1). Deletion mutants were created for genes identified as essential for surviving serum complement in their respective parental strain. The gene deletion mutants and wildtype (WT) parental strains were tested for survival in 50% serum, SDS-PAGE was performed, and carbohydrates were stained to identify structural changes in their lipopolysaccharide (LPS). The results of this work will help identify how *Hp* is evading complement lysis and bring us one step closer to understanding its role as a health-associated commensal in the oral cavity and an extraoral opportunistic pathogen.

LITERATURE REVIEW

The Complement System

The complement system is part of our innate immune system and is thus one of the first lines of defense against invading microbes. It consists of around 40 soluble and cell-bound plasma proteins, which can be found in the bloodstream, the extracellular matrices of our tissues, and even within our cells. Under normal circumstances, the complement system helps maintain homeostasis by clearance of apoptotic host cells and monitoring for foreign antigens or compromised host cells. During infection, the complement system is critical in stimulating a pro-inflammatory response, "tagging" foreign surfaces, and assisting in phagocytosis. Additionally, the complement system can directly kill Gram-negative pathogens through the formation of the membrane attack complex (MAC). This information, and that summarized below, is from two reviews in *Frontiers in Immunology* titled, "Complement system part I – molecular mechanisms of activation and regulation" and "Complement system part II: role in immunity" by Nicolas S. Merle, *et. al*.[32,33]

The complement system acts as a cascade and can be activated through three different pathways: the classical pathway (CP), lectin pathway (LP), or the alternative pathway (AP) (Figure 3). The classical pathway, sometimes referred to as the antibody-mediated pathway, is activated when complement protein, C1q, interacts with a surface-bound target molecule (typically IgM, IgG, or C-reactive protein, but it can recognize over 100 different target molecules). The lectin

pathway has multiple pattern-recognition proteins that can trigger its activation, with mannose-binding lectin (MBL) being the best characterized. MBL and the other LP pattern recognition proteins activate the LP when they come into contact with microbe-associated carbohydrates. These are carbohydrates that are found on the surface of microbial cells and are not found on host cells. The alternative pathway is not activated by pattern recognition proteins but instead through spontaneous hydrolysis of complement protein, C3. This process is referred to as “tick-over” and works to maintain a low level of continuous complement activation to monitor against microbes and other foreign surfaces.

All three activation pathways functionally converge at the proteolytic cleavage of C3 into C3a and C3b. C3a is an anaphylatoxin and acts to stimulate a pro-inflammatory response. C3b is an opsonin and can covalently bind to the surface of a foreign invader, allowing for enhanced phagocytosis. Following C3 cleavage, each pathway forms its own “C3 convertase,” which rapidly cleaves C3, amplifying the signal for a proinflammatory response and continuing to deposit C3b on the pathogen's surface. The next major step in the cascade occurs when C3b binds a C3 convertase, forming the C5 convertase. The C5 convertase marks the beginning of the terminal pathway and cleaves complement protein C5 into C5a and C5b. C5a is also an anaphylatoxin that recruits innate immune cells to the site of infection. C5b recruits complement proteins C6, C7, C8, and C9, which together form the membrane attack complex. The MAC facilitates the direct killing of

pathogens by forming a pore in their cell membrane, causing the bacterial cell to lyse.

MAC assembles sequentially with single components of C5b, C6, and C7, forming a lipophilic complex that allows it to bind to the foreign cell membrane. Next, a single C8 component is added to the C5b-7 complex. That interaction prompts C8 to be the first component to penetrate the lipid bilayer. Lastly, C9 joins the complex and polymerizes to form the pore. The MAC pore is about 10nm in diameter and can contain between two and 18 C9 molecules. The MAC can kill Gram-negative bacteria and host cells but is unable to lyse Gram-positive bacteria due to their thick cell walls.

The complement system is a powerful tool used by our immune system to monitor for foreign invaders, signal an immune response to the site of infection, and kill pathogens directly. Its potent downstream effects make regulating all steps of the complement cascade imperative for host health and dysregulation is present in some autoimmune disorders. Many complement regulators/inhibitors can be found as part of the extracellular matrix or on host cells. Factor I, a host serine protease, can inactivate C3b. C4 binding protein (C4BP) prevents formation of C3 and C5 convertases. Decay acceleration factor (DAF) accelerates the decay of the C3 and C5 convertases. Factor H inhibits formation of the C3 convertase, and acts as a cofactor for inactivation of C3b by Factor I. CD59, a protein expressed on many host cell types, prevents MAC insertion. Clusterin and vitronectin prevent

MAC insertion by making the complex water-soluble. There are additional mechanisms of complement “regulation” inherent in its biochemistry. The active components tend to have brief windows of reactivity, and the complexes formed tend to be short-lived and quick to dissociate if they are not bound by stabilizers. Additionally, the complement components and complexes have various binding affinities and reaction rates, which helps to keep them in check, not to mention the incredibly small spatial scale in which these reactions take place.

The complement system is essential for maintaining health. It plays a critical role in identifying and clearing foreign invaders, maintaining homeostasis, and bridging innate and adaptive immune responses. Those with deficits in the complement system are at increased risk of infection and invasive disease, while those with deficits in complement regulators reap the consequences of an overreactive inflammatory response, like an autoimmune disorder. Despite being one of the longest-known concepts in immunology, we are still learning and discovering new functions of the complement system. Continuing to study complement and its interactions with the host and foreign antigens will hopefully shed light on host-pathogen and host-commensal relationships, along with improving our ability to harness and modulate complement activity in clinical settings.

Gram-negative Bacteria: Lipopolysaccharide Biosynthesis and the Asymmetric Outer Membrane

Two characteristic and essential features of Gram-negative bacteria are the biosynthesis of lipopolysaccharides (LPS) and the maintenance of an asymmetric outer membrane. The cell envelope of Gram-negative bacteria consists of an outer membrane, a thin layer of peptidoglycan, and an inner, or cytoplasmic, membrane. The outer membrane is asymmetric in its distribution of lipids, having all LPS on the outer leaflet and only glycerophospholipids on the inner leaflet.[34,35] The asymmetry of the outer membrane is critical for maintaining the impermeability of the cell. Large, charged molecules cannot penetrate through the hydrophobic lipid bilayer, and hydrophobic molecules cannot pass the hydrophilic regions of LPS.[34]

Lipopolysaccharide (LPS) is a complex glycolipid unique to Gram-negative bacteria. Found in the outer leaflet of the outer membrane, LPS plays a critical role in protecting the bacterial cell from the outside environment and maintaining membrane stability. Additionally, it is often essential for virulence. LPS consists of lipid A, the core oligosaccharide, and O-antigen (Figure 4).[36] Lipid A anchors LPS to the outer membrane and is generally well-conserved between Gram-negatives. The core oligosaccharide, which can be further divided into inner and outer core oligosaccharides, consists of a chain of sugar moieties that attaches to the lipid A portion of LPS. The inner core oligosaccharide tends to be more conserved and typically includes 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and heptose residues. The outer core oligosaccharide is more varied and usually consists of

heptose and hexose sugars that the O-antigen is later attached to. The O-antigen is highly varied, even within the same species. For example, *Escherichia coli* has over 180 documented O-antigen structures.[37] The diverse assortment of O-antigens allows bacteria to evade recognition by the host immune system, which is critical in virulence.

The biosynthesis of LPS has been well characterized in the model organism, *E. coli*, with the synthesis of the conserved components remaining largely the same in other studied Gram-negative bacteria. LPS biosynthesis occurs at the cytoplasmic interface of the inner membrane and begins with the synthesis of lipid A through the Raetz pathway (Figure 5).[35] The starting substrates are UDP-N-acetylglucosamine (UDP-GlcNAc) and an acyl carrier protein-bound fatty acid. The first step of the pathway is catalyzed by LpxA, followed by the first committed step, which LpxC catalyzes. The following two steps are performed by LpxD and LpxH, forming 2,3-diacylglucosamine 1-phosphate, referred to as lipid X, as the product. Lipid X undergoes a series of reactions facilitated by LpxB, LpxK, WaaA, LpxL, and LpxM, completing the Raetz pathway and forming the lipid A-Kdo portion of LPS. The core oligosaccharide is then extended from the Kdo residue of lipid A-Kdo.[36] Upon the completion of core oligosaccharide synthesis, the lipooligosaccharide molecule can be transferred from the cytoplasmic side of the inner membrane to the periplasmic leaflet of the inner membrane. This transfer requires the help of a flippase, MsbA.

O-antigen biosynthesis occurs independently of the other LPS components at the cytoplasmic interface of the inner membrane. The O-antigen is made up of repeating polysaccharide units (RPUs) consisting of around five sugars. Given the broad diversity of O-antigens, there is no conserved pathway for RPU biosynthesis. However, the mechanisms for O-antigen assembly and transport are conserved. There are two dominant methods for O-antigen assembly and transport, the first being the Wzy-dependent pathway, and the second being the ABC transporter-dependent pathway.[37,38] The Wzy-dependent pathway synthesizes and flips, via the Wzx flippase, individual RPUs attached to undecaprenyl phosphate (C55-P) carriers across the inner membrane to the periplasmic leaflet of the inner membrane where they are then polymerized on the C55-P carrier by Wzy, the O-antigen polymerase, and Wzz, the O-antigen chain length regulator. The O-antigen is then ligated by WaaL to the core oligosaccharide portion of a lipooligosaccharide molecule, making it into a lipopolysaccharide molecule. The ABC transporter-dependent pathway polymerizes the entire O-antigen on one C55-P carrier on the cytoplasmic leaflet of the inner membrane. The entire O-antigen is then transferred across the inner membrane to the periplasmic leaflet where it is ligated to a molecule of lipooligosaccharide by WaaL. Once the LPS molecule is formed in the periplasm leaflet of the inner membrane, the Lpt (LPS transport) machinery transports it to the outer leaflet of the outer membrane.

LPS and the asymmetric outer membrane are iconic features of Gram-negative bacteria. LPS is critical for Gram-negative bacteria to evade and resist the host immune response and, sometimes, stimulate a pro-inflammatory response to assist in pathogen invasion. The asymmetric outer membrane is key to maintaining an impermeable cell envelope, helping to protect the bacteria from antimicrobials and environmental factors. Understanding the biosynthesis and maintenance of the Gram-negative cell envelope is imperative for antibiotic development, antimicrobial resistance research, and appreciating host-pathogen and host-commensal relationships involving Gram-negative bacteria.

Complement Evasion in *Haemophilus influenzae* and *Haemophilus parainfluenzae*

Little is known about the ability of *Haemophilus parainfluenzae* (*Hp*) to evade complement lysis; however, substantial effort has gone into investigating and characterizing the ability of *Haemophilus influenzae* (*Hi*), a close relative of *Hp*, to evade complement. As a frequent human pathogen, *Hi* is the major pathogen of the *Haemophilus* genus. *Hi* is a common causative agent of otitis media and can cause invasive diseases like bacteremia, pneumonia, cellulitis, and septic arthritis.[39] It is a Gram-negative, pleomorphic coccobacillus and can be encapsulated (typable) or unencapsulated (nontypeable). There are six serotypes of typable *Hi*, serotypes A through F. *Hi* serotype B (Hib) was the most common *Hi* type to cause invasive disease, but the introduction of the Hib vaccine, the only *Hi* vaccine, led to a significant decline in invasive disease caused by Hib. Nevertheless, 3 to 6% of invasive Hib cases in children are still fatal.[39] Nontypeable *Hi* (NTHi) has taken over, post-Hib vaccine, as the leading *Hi* type for causing invasive disease, with 55% of invasive *Hi* cases in children under 5 years old caused by NTHi.[40] Unlike many other Gram-negative pathogens, *Hi* does not produce O-antigen, meaning the outer leaflet of its out membrane consists of lipooligosaccharide (LOS) instead of lipopolysaccharide (LPS).[41–43] While many Gram-negative pathogens rely on the O-antigen for protection from immune mediated killing, *Hi* does not.

Hi has many mechanisms at its disposal that allow it to evade immune recognition and resist complement lysis. The typable strains have a capsule, which provides a buffer between the outer membrane of the cell and its environment. The capsule prevents MAC insertion through steric hindrance, protecting *Hi* from complement lysis. *Hi* also modifies its LOS with host-like molecules, like phosphorylcholine (ChoP) and sialic acid. These LOS modifications allow *Hi* to modulate host-immune response by evading immune recognition and, sometimes, provoking immune response to aid in further invasion of inflamed tissue. Sialylation of LOS is an especially powerful tool for evasion of complement.[41,44,45] Sialic acid inhibits complement activation via the classical pathway and can assist in the recruitment of complement regulators, like Factor H, to the bacterial cell surface. Additionally, these LOS modifications are often phase-variable.[42,44,46] Phase variation is a gene regulation phenomenon prevalent in some pathogenic bacteria where gene expression can be reversibly turned on and off. In *Hi*, this is achieved by regions of tetranucleotide repeats that have high frequencies of mutations (ex., a mutation that reduces the number of tetranucleotide repeats will turn gene expression off, and a mutation that adds to the number of tetranucleotide repeats turns gene expression back on). *Hi* also has a moderately variable LOS structure, which can help evade immune recognition.[41] Most of the LOS variation comes from LOS modification, especially the phase-variable modifications, but different strains have also shown some diversity in sugar moieties that make up the outer core oligosaccharide. Variation in the structure of LOS helps evade immune recognition,

especially by antibodies. The ability of Hi to bind complement regulators provides significant complement resistance. *Hi* expresses a diverse set of adhesions and complement regulator binding surface proteins, which allow it to bind C4b-binding protein (C4BP), Factor H (FH), Factor H-like protein 1 (FHL-1), vitronectin, and plasminogen, a proenzyme.[47–52] *Haemophilus* surface fibril (Hsf) binds vitronectin, protecting *Hi* from MAC insertion.[47] Protein E can bind plasminogen and vitronectin, potentially simultaneously.[50] The proenzyme plasminogen, when cleaved and in its active form of plasmin, degrades C3b. NTHi outer membrane protein 5 (P5) binds C4BP, which is the primary soluble inhibitor of the classical and lectin pathways.[48] These are just a few examples of complement regulator-binding proteins that *Hi* uses to evade complement lysis.

In contrast to the wealth of information available on the ability of *Hi* to evade complement, only a handful of studies have been published on the topic in *Hp*. From the literature available, we know that *Hp* has no signs of phase-variable LOS expression.[53] In a study of 18 *Hp* strains, only one contained a gene for capsule synthesis.[54] All 18 strains possessed an O-antigen synthesis cluster. The authors found that most of the *Hp* strains either synthesized 2-acetamido-4-amino-2,4,6-trideoxy- D-galactose (FucNAc4N) and used the Wzy-dependent O-antigen transport system or synthesized galactofuranose and used ABC transporters for O-antigen transport. 65% of the *Hp* isolates tested showed laddering indicative of O-antigen during SDS-PAGE analysis after silver staining. There is reason to believe that *Hp* should be able to bind vitronectin because Protein E of *Hi* is

conserved throughout the *Haemophilus* genus, including in *Hp*, but this has yet to be shown *in vitro*.[55] This is explored further in my own work below.

MATERIALS AND METHODS

Gene Deletion Plasmid Design and Construction

We made gene deletions in *Hp* via homologous recombination utilizing the counterselectable, suicide vector pEAKO, which contains *sacB* and a Spc^R cassette, as the backbone. 1kb regions of homology were chosen up and downstream of the gene to be deleted. Primers were designed using the NEBuilder Assembly Tool (New England Biolabs). pEAKO was digested with NotI-HF (New England Biolabs). Regions of homology and Kan^R cassette, when necessary, were PCR amplified using Q5 High-Fidelity 2X Master Mix (New England Biolabs). Digested plasmid and PCR product were run on an agarose gel, extracted, and purified using QIAquick Gel Extraction Kit (QIAGEN). Gibson assembly was performed using NEB HiFi DNA Assembly Master Mix (New England Biolabs) to assemble gene deletion vectors. All primers and vectors are included in Tables 3 and 4.

Complementation Plasmid Design and Construction

Complementation was done using pCRC1 or pMJM1, multicopy shuttle vectors. pCRC1 has a Kan^R cassette. pMJM1 has a Spc^R cassette. Primers were designed using the NEBuilder Assembly Tool (New England Biolabs). Native promotors of a gene were used when possible. If not possible, the *tac* promoter, which allows constitutive expression in *Hp*, on the vector backbone, was used. PCR of the gene was done using Q5 High-Fidelity 2X Master Mix (New England

Biolabs). Digested plasmid and PCR product were run on an agarose gel, extracted, and purified using QIAquick Gel Extraction Kit (QIAGEN). Gibson assembly was performed using NEB HiFi DNA Assembly Master Mix (New England Biolabs).

Gene Deletion Mutant Construction and Confirmation

Assembled plasmids were then transformed into NEB5α competent *E. coli* (New England Biolabs) and plated on appropriate selective media. Plasmids were extracted from overnight cultures of isolated colonies using the QIAprep Spin Miniprep Kit (QIAGEN). Plasmids were digested with NotI-HF (New England Biolabs) and run on a gel to verify proper assembly. Plasmid DNA was then transformed into MFDpir via heat shock/chemical transformation. MFDpir containing the plasmid was then plated for conjugation with the appropriate *Hp* strain. Primary integrants were then outgrown for 48h, passaging to warm, fresh media every 12h, and then plated on BHIYE + 10% sucrose, supplemented with hemin and NAD for counterselection. Isolated colonies were cross-picked, and colony PCR was performed to identify possible mutants. Prospective mutants had gDNA extracted, extragenic regions around the deleted gene were PCR amplified, and Sanger sequencing was performed on PCR products for verification of gene deletion through the INBRE Core Facility.

Serum Survival Assay

Strains were tested in biological quadruplicate. The parental strain of the mutant and *E. coli* K12 were used as negative and positive controls for complement activity, respectively. Samples were grown to mid-log phase, and cultures were washed in BHIYE via centrifugation. Cultures were then normalized to an OD_{600} of 0.1. Each sample was inoculated into 50% active normal human serum and 50% heat-inactivated serum (Gemini Bio). Serum was heat-inactivated by incubating it for 30m at 56°C. The sample wells were 122.5µL of filter sterilized 1xPBS and 125µL of the respective active or inactive serum. Wells were inoculated with 2.5µL of normalized culture and incubated for 1h at 37°C with 5% CO_2. After an hour, samples were serially diluted and track-plated to calculate CFU mL^{-1}. Percent survival was calculated by dividing the CFU mL^{-1} after incubating in active serum for an hour by the CFU $mL^{-}1$ of samples incubated in inactive serum.

Lipopolysaccharide Characterization Gel

This procedure was adapted from Stead et al. (2010), cultures were grown to mid-log or stationary phase, and then 1mL of culture was normalized to an OD_{600} of 1. Samples were then washed once with 1xPBS, and cell pellets were resuspended in 100µL of 1×LDS sample buffer (Invitrogen) + 4% BME. Cell suspensions were boiled for 10m to lyse the cells and allowed to cool. Proteinase K (New England Biolabs) was added to a concentration of 125ng $µL^{-1}$, and the mixture was incubated at 55°C for 16h. The proteinase K was then heat-inactivated at 100°C for 5m. The proteinase K treated whole cell lysates were then separated

by SDS polyacrylamide gel electrophoresis using a 4–12% bis-tris gradient gel (Invitrogen). The CandyCane Glycoprotein Molecular Weight Standards (Invitrogen) was used as a ladder. The gels were stained with Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (Molecular Probes).

gDNA Extraction

gDNA was extracted from overnight cultures using DNeasy Blood and Tissue Kit (QIAGEN). Concentration and quality were determined via spectrophotometry using the BioTek Synergy HTX Multimode Reader (Agilent) and BioTek Take3 (Agilent). DNA was used for construction of gene deletion plasmids and complementation vectors.

RESULTS

TnSeq

TnSeq, transposon sequencing, libraries were created in two *Hp* strains, ATCC and EL1, by the Ramsey lab using a *mariner* transposon.[56] The *mariner* transposon inserts at TA sites, and *Hp* has an AT-rich genome (ATCC: 60.7% AT; EL1: 60.6% AT), which created saturated transposon mutant libraries (Figure 6). The TnSeq libraries were used to identify conditionally essential genes for survival in 50% serum for 1h. TnSeq identified 15 genes in ATCC and 7 genes in EL1 that were essential for survival in serum (Table 1). Most of these genes are involved with LPS and cell envelope biosynthesis. Both strains had a conserved gene of unknown function, containing a domain of unknown function number 218 (DUF218), identified as conditionally essential for surviving in 50% serum. Interestingly, the maintenance of lipid asymmetry, *mla*, genes were not identified as essential for survival in 50% serum. As described above, lipid asymmetry in the outer membrane of Gram-negative bacteria is critical for the function and stability of the cell envelope, especially when exposed to membrane stress like the MAC.

Gene Deletion Mutants

galE, *yhxB*, *mlaA*, *wzzB*, and DUF218 were deleted in the ATCC strain. *galE*, *yhxB*, *mlaA*, *wzzB*, DUF218, and *licD3* were deleted in the EL1 strain. Δ*galE* and Δ*mlaA* in the ATCC strain were created using allelic exchange with a kanamycin resistance cassette. Δ*yhxB* and Δ*mlaA* in the EL1 strain were created

using allelic exchange with a kanamycin resistance cassette. Gene deletion mutant strains that showed a significant change in their ability to survive in serum had their deleted gene complemented back. Complementation was performed using a multicopy shuttle vector. All complemented mutant strains had their serum survival phenotype restored to wildtype levels (Figure 10).

Serum Survival Assays

Wildtype ATCC had a 53.8% survival and wildtype EL1 had a 112% survival. In the ATCC parental strain, *galE*, *wzzB*, and DUF218 proved to be essential, as predicted by the TnSeq library (Figure 7). When exposed to 50% serum, the Δ*galE*, Δ*wzzB,* and ΔDUF218 mutants in the ATCC strain had a percent survival of 0%. In other words, they had a complete loss of their serum resistance phenotype. The essentiality of *yhxB* in ATCC was dependent on the growth phase. *yhxB* was essential for the ATCC strain to survive serum exposure in stationary phase but was not essential for survival in serum for one hour in mid-log growth phase (Figure 8). Δ*yhxB* ATCC in stationary phase had a 0% survival, and in mid-log it had a 73% survival after exposure to serum. In the EL1 parental strain, *licD3* proved to be essential, as predicted by the TnSeq library. The Δ*licD3* mutant had a 17.9% survival when exposed to serum; a 94.1% reduction in the ability to survive (Figure 9). *galE* was not predicted to be essential for survival in serum for one hour, but the loss of *galE* caused a 25.9% reduction in the ability to survive serum exposure. This reduced serum survivability does not necessarily indicate *galE* is essential for EL1 to survive in serum, as the Δ*galE* EL1 mutant still had 86.1%

survival, but it is a statistically significant reduction in the ability to survive in serum. The loss of *wzzB,* and DUF218 in the EL1 strain did not confer the TnSeq expected phenotype of serum sensitivity. Δ*wzzB* EL1 had a 104.7% survival in 50% serum for one hour. ΔDUF218 had 126.9% survival when exposed to serum. The Δ*wzzB* and ΔDUF218 EL1 gene deletion mutants are undergoing whole genome sequencing to identify potential suppressor mutations.

Complementation was performed for gene deletion mutant strains that demonstrated a statistically significant difference ($p < 0.05$) in the ability to survive in serum compared to their respective WT parental strains. These complemented mutants and the empty vector complemented mutants were tested in 50% serum to characterize their serum survival phenotypes (Figure 10). The addition of the *galE* gene back into the Δ*galE* ATCC mutant strain successfully restored its serum survival phenotype. The complemented Δ*galE* mutant showed 118.3% survival after exposure to serum. Δ*galE* ATCC, complemented with the empty shuttle vector, was unable to restore the serum survival phenotype. Complementation of the Δ*wzzB* ATCC and ΔDUF218 ATCC mutant strains also restored their serum survival phenotype. The addition of the *wzzB* and DUF218 genes restored their ability to survive serum exposure from 0% to 111.8% and 100.3%, respectively. Once again, complementation of the Δ*wzzB* ATCC and ΔDUF218 ATCC strains with the empty shuttle vector, pMJM1, was not able to restore their serum survival phenotype back to wildtype. In stationary phase, complementation of the Δ*yhxB* ATCC strain with the *yhxB* gene restored its ability to survive exposure to 50%

serum for one hour. The empty shuttle vector, pCRC1, was unable to restore the serum survival phenotype to stationary phase Δ*yhxB* ATCC. Complementation of the Δ*licD3* and Δ*galE* mutants in the EL1 strain are still in the process of being made.

Lipopolysaccharide Gels

Since many of the genes targeted for deletion are involved in lipopolysaccharide (LPS) biosynthesis, LPS gels were used to examine potential structural changes to the LPS. SDS-PAGE was performed on whole cell lysates that had been incubated with proteinase K for the previous 16 hours. The gel was stained with the Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit, which oxidizes the carbohydrates and labels them with a fluorescent dye for imaging. Uropathogenic *E. coli* strain CFT073 was run as a positive control for LPS sample preparation and O-antigen staining. CFT073 has O-antigen laddering between 42 and 82kDa. It has three segmented bands towards the bottom of the gel. The bottom-most band corresponds to incomplete lipooligosaccharide. The middle band is consistent with fully synthesized lipooligosaccharide, and the band above illustrates the addition of a very short O-antigen.

Wildtype ATCC showed a dark band towards the bottom of the LPS gel consistent with lipooligosaccharide (Figure 11). Above that band, there was a faint band indicating the addition of very short O-antigen. At around 180kDa, there is a smear that indicates the addition of long O-antigen with a discrete number of

RPUs. The Δ*galE* ATCC mutant has a significant reduction in the molecular weight of its lipooligosaccharide. This can indicate various changes to the LPS structure, including a reduced core or changes in the charge of lipid A. Additionally, the Δ*galE* ATCC mutant showed loss of the long O-antigen. The Δ*yhxB* ATCC mutant also has structural changes in its LPS, as illustrated by an even more drastic drop in the molecular weight of the lipooligosaccharide and the loss of long O-antigen. The Δ*mlaA* ATCC mutant appears identical to the wildtype ATCC, as expected. The Δ*wzzB* ATCC mutant shows normal lipooligosaccharide, the loss of long O-antigen, and gradient laddering above the lipooligosaccharide band, with the bottom of the gradient being darker than the top. This laddering is consistent with shortened O-antigen, and the increments correspond to the addition of one O-antigen RPU. The ΔDUF218 mutant has a robust lipooligosaccharide band with a thick band of short O-antigen hovering above it.

The LPS gel of the WT EL1 strain illustrated a lipooligosaccharide portion, with an O-antigen smear right above 82kDa (Figure 12). As seen in the Δ*galE* and Δ*yhxB* ATCC mutants, Δ*galE* and Δ*yhxB* EL1 mutants have reduced molecular weight lipooligosaccharide bands. Both Δ*galE* and Δ*yhxB* EL1 mutants have a very faint trace of O-antigen around 82kDa. The Δ*mlaA* EL1 LPS is identical to wildtype EL1. Δ*wzzB* EL1 has a normal lipooligosaccharide fragment and a dramatic gradient smear from around 82kDa to the core oligosaccharide. There is faint banding within the smear, but the poor resolution of the banding makes it difficult to tell if there is laddering. The Δ*wzzB* EL1 mutant does not have long O-antigen,

but the gradient smear indicates there is shortened O-antigen. The ΔDUF218 EL1 mutant has an identical LPS structure to wildtype EL1. The lipooligosaccharide of Δ*licD3* EL1 has a higher molecular weight than the wildtype. This may indicate an extended outer core oligosaccharide or a change in the charge of lipid A. The Δ*licD3* EL1 mutant is also missing long O-antigen.

Protein E

Protein E (PE) is an adhesin and complement regulator-binding protein in *Haemophilus influenzae*. PE is known to bind vitronectin at a very high affinity ([k_d] = $4x10^{-7}$ M), which prevents complement lysis by inhibiting the insertion of the MAC into the bacterial cell envelope.[57,58] The structure of PE of *Hi* serotype d has been solved at a resolution of 1.8 Å. PE is a 16kDa lipoprotein consisting of six beta sheets and one alpha helix.[55,59] The authors that solved the structure reported a vitronectin binding region (residues 84 to 108) and a region that can bind laminin and plasminogen (residues 41 to 68). Laminin and plasminogen are extracellular matrix proteins. Binding laminin helps with adhesion to host cells and tissue. Plasminogen is the inactive form of plasmin. When cleaved into plasmin, it helps in complement evasion by degrading C3b.[60]

PE is reportedly conserved amongst the *Haemophilus* genus.[55] When the amino acid sequence of the solved PE structure (WP_005694109.1) was put through pBLAST against the genome of the *Hp* ATCC strain, the protein sequence for the encoding gene (peg.289) showed 66% identity and 78% positivity, with an

E value of $1x10^{-74}$. The peg.260 protein sequence in EL1 showed 65% identity and 78% positivity, with an E value of $6x10^{-74}$, to the *Hi* PE amino acid sequence. The ATCC and EL1 PE's are nearly identical, with 99% identity and positivity. Both *Hp* strains had their PE structures modeled in Phyre^2. Phyre^2 matched both to the solved *Hi* structure of PE with 70% structural identity and 100% confidence. The Phyre^2 modeled structure of *Hp* PE was overlaid with the solved crystallized structure of *Hi* PE in PyMOL (Figure 13). The secondary structures were largely conserved between the solved and modeled PE structures. The *Hi* PE has six antiparallel beta sheets, one alpha helix, and eight loops, and the modeled *Hp* PE has five antiparallel beta sheets, two alpha helices, and seven loops (Figure 14). The vitronectin binding region and plasminogen/laminin binding regions were highlighted in PyMOL (Figure 15). The vitronectin binding region seems to be well conserved in the modeled *Hp* PE structure; thus, we are confident that this protein will also exhibit complement protection via vitronectin binding, which remains to be tested.

DISCUSSION

Haemophilus parainfluenzae (*Hp*) is highly resistant to complement lysis, but the mechanisms of its resistance have yet to be identified. To answer this unknown, we used two strains of *Hp*, the type strain ATCC, and an oral commensal isolate, EL1, to build TnSeq libraries and identify the essential genes in each strain for survival in 50% normal human serum, the cell-free component of blood containing the complement system. Genes identified as essential by TnSeq were deleted, and the subsequent isogenic mutants had their serum survival phenotype and lipopolysaccharide (LPS) structure characterized. Here, I discuss the role of our genes of interest in the cell and their impact on the ability of *Hp* to survive in serum, their impact on LPS structure, and the seemingly essential components of LPS for the survival of *Hp* in serum. Additionally, I address the potential role of complement regulator-binding proteins in the ability of *Hp* to survive in serum.

Genes of Interest Identified by TnSeq, Their Role in the Cell, and Their Impact on Survival in Serum

The genes identified as essential by TnSeq in the *Hp* ATCC strain were *galE*, *yhxB*, *wzzB*, and DUF218 (Table 1). All but *yhxB* were confirmed to be essential for ATCC to survive in serum during mid-log phase (Figure 7). The Δ*yhxB* mutant did not have a statistically significant change in serum survival compared to wildtype ATCC, when tested in mid-log phase. The TnSeq assays were performed in stationary phase, and when exposed to serum in stationary phase,

the Δ*yhxB* mutant was no longer serum resistant (Figure 8). *yhxB* encodes a phosphoglucosamine mutase and turns D-glucosamine 1-phosphate into D-glucosamine 6-phosphate. It is involved in the biosynthesis of peptidoglycan and lipopolysaccharide and is essential for the production of UDP-N-acetylglucosamine (UDP-GlcNAc).

galE has been well documented as essential for serum survival in *Haemophilus influenzae* (*Hi*) and other Gram-negative bacteria.[37,43,61] *galE*, UDP-glucose 4-epimerase, catalyzes the reversable reaction of UDP-glucose (UDP-Glc) to UDP-galactose (UDP-Gal). *galE* impacts the availability of UDP-Gal for core oligosaccharide biosynthesis and can affect O-antigen biosynthesis if UDP-Gal is part of the organism's O-antigen. Shortening of the core oligosaccharide and/or loss of the O-antigen would likely impact serum resistance by decreasing membrane stability and decreasing steric hindrance, allowing for easier insertion of the membrane attack complex in the bacterial outer membrane.

WzzB is the O-antigen chain length-determining protein and is part of the O-antigen synthesis cluster in ATCC. WzzB regulates the length of the O-antigen during polymerization by Wzy. The length of the O-antigen chain is dependent on how many repeating polysaccharide units (RPUs) are polymerized. Typically, the size of the O-antigen chains follows a modal distribution, creating a laddering pattern on an LPS gel where each interval is the addition or loss of an RPU. Work in *Shigella flexneri* has demonstrated that Δ*wzzB* strains lose the modal O-antigen

size distribution and have a larger proportion of short O-antigen compared to WT.[62] Having most of the O-antigen lengths long and within a certain range provides the bacterial cell with a buffer between its outer membrane and its environment. Shortened and irregular O-antigen chain lengths may compromise the proposed buffer and could create opportunities for MAC insertion.

The ORF belonging to DUF218 is annotated as an "Uncharacterized DUF218 membrane protein PM0506," and its role in the ability of ATCC to survive in serum is unclear. The closest pBLAST match is annotated as a "YdcF family protein." Phyre[2], which works similarly to BLAST but instead of aligning nucleotide or amino acid sequences, aligns a modeled protein structure with known protein structures, showed *E. coli* YdcF as the closest structural match to DUF218. The function of YdcF in *E. coli* is unknown, but the protein can bind S-adenosyl-L-methionine, potentially allowing it to function as a methyltransferase.[63] The "PM0506" in its annotation refers to a gene of that name in *Pasteurella multocida*, whose function is also unknown.[64]

Δ*mlaA* was the last gene deletion mutant made in ATCC and tested for serum survival. *mlaA* was identified as not essential for ATCC to survive in serum and was deleted to further validate the TnSeq library. *mlaA* encodes an outer membrane lipoprotein, one of the maintenance of lipid asymmetry (Mla) proteins.[35,65,66] As the name suggests, MlaA and the other Mla proteins (MlaB-F) are essential for maintaining the asymmetry of the outer membrane. MlaA removes

mislocalized phospholipids from the outer leaflet of the outer membrane. The mislocalized phospholipids impair the tight packing of LPS required for membrane stability and can congregate to form patches of phospholipid bilayer in the outer membrane, which MAC can more easily insert into. In *Hi*, the deletion of *mlaA* (notated as *vacJ* in *Hi*) significantly reduces its ability to survive in serum.[43,52,61] The ATCC Δ*mlaA* mutant strain did not have a statistically significant difference in percent survival in serum compared to wildtype ATCC. This finding may be due to a handful of explanations: 1) The mechanism used by ATCC and the Δ*mlaA* mutant strain to survive in serum is independent of membrane stability, or 2) ATCC has another, redundant mechanism for removing mislocalized phospholipids. In *E. coli*, PldA, an outer membrane phospholipase, can remove mislocalized phospholipids, but pBLAST of PldA (CAD6023050.1) against ATCC showed no matches.[67] In *Pseudomonas aeruginosa*, a novel mechanism of phospholipid removal by MlaYZ has been reported. pBLAST of MlaZ (NP_251929.1) showed a partial hit to MlaA in ATCC. pBLAST of MlaY (AAG06626.1) returned no matches.[68] Further work is required to investigate the mechanism(s) used by *Hp* to deal with mislocalized phospholipids.

For EL1, the commensal oral isolate, the genes identified by TnSeq as essential for survival in serum were *licD3*, *wzzB*, and DUF218 (Table 1). The Δ*licD3* mutant strain validated that *licD3* is essential for EL1 to survive in serum (Figure 9). *wzzB* and DUF218, however, are not essential for EL1 to survive in serum. Δ*wzzB* and ΔDUF218 in EL1 both did not show a statistically significant

difference in their percent survival in serum when compared to wildtype EL1. The Δ*wzzB* and ΔDUF218 EL1 strains were also tested in stationary phase for serum survival to ensure their essentiality was not growth phase-dependent like *yhxB* in ATCC. There was no significant difference in their ability to survive in serum during stationary phase (data not shown). It is reasonable to conclude that *wzzB* may have been determined as essential for serum survival by TnSeq due to polar effects. *wzzB* is the first gene in the O-antigen synthesis cluster in EL1. It is very likely that interruption of that gene by the TnSeq transposon may have led to more detrimental downstream effects for the rest of the operon. This explanation, however, is unfitting for the ΔDUF218 mutant, as there was only one gene downstream in its operon, and it was determined to be not essential for serum survival by TnSeq. Whole genome sequencing will be performed in hopes of identifying potential secondary mutations that could account for the apparent discrepancy. Gene deletion mutants of *galE*, *yhxB*, and *mlaA* were also made in our EL1 strain to explore strain-strain differences and further validate the TnSeq library. TnSeq identified each of those genes as not essential for EL1 to survive in serum, and the mutants validated that.

licD3, a lipopolysaccharide choline phosphotransferase, is part of EL1's O-antigen biosynthesis cluster along with *wzzB*. In *Hi*, the *licD* alleles are part of the *lic1* locus, which can modify the outer core oligosaccharide with phosphorylcholine (ChoP).[46] ChoP expression and modifications require the entirety of the *lic1* locus (*licA*, *licB*, *licC*, and *licD*). EL1 does not possess the other components of the *lic1*

locus, making it unlikely that *licD3* modifies the outer core oligosaccharide of *Hp* EL1 with ChoP and its role in serum survival unclear.

Genes of Interest's Impact on Lipopolysaccharide (LPS) Structure

To further illuminate the mechanisms *Hp* uses to evade complement lysis, we characterized the structure of the wildtype and mutant strain's lipopolysaccharide (LPS) and looked for structural changes that would explain differences in serum survival. The LPS gels showed structural changes associated with the loss of *galE*, *yhxB*, and *wzzB* for both *Hp* strains (Figure 11&12). The Δ*galE* and Δ*yhxB* mutants in both *Hp* strains showed lipooligosaccharide of a lower molecular weight than the wildtype. This could be indicative of a reduced core oligosaccharide or changes in the charge of lipid A. Loss of *wzzB* did not affect the lipooligosaccharide portion of LPS. However, the Δ*wzzB* mutants had a gradient effect with some O-antigen laddering (laddering is apparent in the ATCC mutant strain but not well resolved in the EL1 mutant strain). The loss of *galE*, *yhxB*, and *wzzB* evidently also conferred the loss, or near-complete loss, of long O-antigen in both *Hp* strains. The loss of DUF218 conferred a structural change in LPS for the ATCC strain but not for EL1. The ΔDUF218 ATCC mutant had normal lipooligosaccharide, with a thick band above indicating the addition of short O-antigens. Notably, the ΔDUF218 ATCC mutant was missing long O-antigen. Deletion of *licD3* in the EL1 strain also changed the structure of LPS. The Δ*licD3* EL1 mutant does not have long O-antigen and has a slightly higher molecular

weight lipooligosaccharide. The Δ*mlaA* mutant strains for both ATCC and EL1 did not show any structural change in their LPS.

The results of the LPS gels largely support the proposed role each gene was thought to perform in LPS biosynthesis. GalE and YhxB were proposed to affect sugar availability for the biosynthesis of O-antigen and the core oligosaccharide, and the LPS of those mutants did, in fact, have reduced molecular weight lipooligosaccharide and reduction or complete loss of the long O-antigen. The WzzB mutants could not regulate their O-antigen size, demonstrated by their shortened O-antigen without modal distribution. The MlaA mutants had no structural change to the LPS because MlaA is involved with maintaining lipid asymmetry, not the structure of LPS.

The Impact of Lipopolysaccharide (LPS) Structure on Ability to Survive in Serum

While the LPS gels provided some insight into the mechanism used by the *Hp* strains to evade complement lysis, they did not allow us to identify a universal trait that conferred serum resistance in either strain background. In the ATCC strain, the loss of long O-antigen conferred serum sensitivity. However, this does not seem to be the only factor to influence the ability of the ATCC strain to survive in serum, as illustrated by the Δ*yhxB* ATCC mutant. It is difficult to identify an explanation for the growth-phase-dependent essentiality of *yhxB* for ATCC to survive in serum. In the EL1 strain, complete loss of long O-antigen confers serum sensitivity. Yet, again, long O-antigen does not appear to be the only factor at play.

The Δ*galE* and Δ*yhxB* EL1 mutants had greatly reduced abundance of long O-antigen, and they survived in serum. The lack of an obvious or straightforward mechanism being identified as an explanation for the ability of the two *Hp* strains to survive in serum tells us that the ability to survive in serum is multifactorial. Long O-antigen confers serum resistance via steric hindrance, and it is highly likely that *Hp* gains serum resistance by other mechanisms, like molecular mimicry, too.

Potential Role of Complement Regulator-Binding Proteins in the Ability of *Hp* to Survive in Serum

Although TnSeq did not identify any complement regulator-binding proteins as essential for survival in serum, this is likely not because the *Hp* strains don't have any complement regulator-binding proteins. They should express at least one, Protein E (PE). PE is conserved across the *Haemophilus* genus and is known to bind vitronectin and plasminogen, which both protect against complement through molecular mimicry.[55] I used the solved structure of the *Hi* PE to identify if the *Hp* PE would be homologous and to investigate if the known vitronectin and plasminogen binding regions were conserved. I did this by modeling the structures of the ATCC and EL1 PE in Phyre^2. The ATCC and EL1 PE structures were identical and shared 70% structural homology (at 100% confidence) with the solved *Hi* PE. I overlaid the EL1 PE with the solved *Hi* PE structure in PyMOL and highlighted the respective binding regions. The vitronectin binding region is well conserved, and the lysine and arginine (Lys85 and Arg86), thought to be involved in binding vitronectin in *Hi*, are functionally conserved (the residues are both

arginine in the *Hp* strains). It is entirely feasible that the *Hp* strains possess a functional complement regulator-binding protein, but it most likely wasn't identified by TnSeq as essential for serum survival because it is only one tool in the toolbox to survive the complement cascade. It is likely that the loss of one complement regulator-binding protein most likely does not confer enough serum sensitivity on its own to be considered essential.

Conclusion

Survival in serum and evasion of complement lysis can be achieved in various ways. The three most common mechanisms are steric hindrance, molecular mimicry, and protease secretion.[45,52] Steric hindrance can be accomplished by the production of capsule and or LPS, especially with long O-antigens, which provide the bacterial cell a buffer between its outer membrane and the environment. Molecular mimicry, evading immune recognition, occurs through the expression of host-like carbohydrates or proteins on the bacterial cell surface or by the bacteria expressing surface proteins that steal host immune regulators, and protease secretion enables the bacteria to degrade host immune factors. True immune evasive and resistant bacteria do not rely solely on one resistance mechanism. They have an arsenal of tools at their disposal and use a multifaceted approach to evade killing by our immune system. Our data show that *Haemophilus parainfluenzae* uses multiple mechanisms of immune evasion and resistance to survive in normal human serum. We have shown that major structural changes to LPS alone don't necessarily confer serum sensitivity. Loss of long O-antigen also

does not always impede the ability of our *Hp* strains to survive complement. Even the loss of a complement regulator-binding protein doesn't rid the *Hp* strains of their serum resistance. Complement resistance is a multifaceted ability in *Haemophilus parainfluenzae*.

TABLES

Table 1: TnSeq identified conditionally essential genes in ATCC and EL1 for serum survival. All mutants (in grey) were significantly underrepresented compared to the remaining mutants, indicating a loss of fitness when grown in active serum vs. inactive serum.

Gene:	Sig ATCC:	Sig EL1:
UDP-glucose 4-epimerase *galE*	3.9E-60	2.5E-1
O-antigen ligase *waaL*	1.9E-112	3.5E-36
Nucleoside-diphosphate sugar epimerase	4.7E-147	9.1E-107
UDP-N-acetylgalactosaminyltransferase	1.1E-38	8.3E-26
UDP-glucose 4-epimerase	2.0E-105	-
UDP-N-acetylgalactosaminyltransferase	5.1E-58	-
Glycosyltransferase	1.4E-91	-
Alpha-1-2C4-N-acetylgalactosamine transferase *pglH*	5.5E-91	-
O-antigen chain length determining protein *wzzB*	1.1E-116	2.8E-57
ADP-heptose—LOS heptosylfransferase II *waaF*	1.9E-34	8.2E-10
ADP-L-glycero-D-manno-heptose-6-epimerase *rfaD*	4.9E-64	7.2E-04
Phosphoglucosamine mutase *yhxB*	2.6E-131	4.5E-01
D-sedoheptulose 7-phosphate isomerase	5.8E-35	4.1E-09
Uncharacterized DUF218 membrane protein	4.6E-49	1.1E-29
D-glycero-β-D-manno-heptose 1-P adenylyltransferase	7.8E-53	9.8E-43
2-C-methyl-D-erythritol 4-P cytidylyltransferase	8.8E-36	9.3E-37
Lipopolysaccharide cholinephosphotransferase *licD3*	-	3.8E-50
dTDP-glucose 4-2C6-dehydratase	-	2.4E-56

Table 2: Strains Used

Strain:	Strain Number:	Source:
Haemophilus parainfluenzae ATCC33392	MR469	American Type Culture Collection
Haemophilus parainfluenzae EL1	MR479	Matthew Ramsey Lab
Δ*galE* ATCC	MR536	This study
Δ*galE* ATCC + *galE* ATCC	MR552	This study
Δ*galE* ATCC + pMJM1	MR555	This study
Δ*yhxB* ATCC	MR528	This study
Δ*yhxB* ATCC + *yhxB* ATCC	MR533	This study
Δ*yhxB* ATCC + pCRC1	MR531	This study
Δ*mlaA* ATCC	MR539	This study
Δ*wzzB* ATCC	MR550	This study
Δ*wzzB* ATCC + *wzzB* ATCC	MR553	This study
Δ*wzzB* ATCC + pMJM1	MR556	This study
ΔDUF218 ATCC	MR548	This study
ΔDUF218 ATCC + DUF218 ATCC	MR554	This study
ΔDUF218 ATCC + pMJM1	MR557	This study
Δ*galE* EL1	MR529	This study
Δ*yhxB* EL1	MR537	This study
Δ*mlaA* EL1	MR540	This study
Δ*wzzB* EL1	MR549	This study
ΔDUF218 EL1	N/A	This study
Δ*licD3* EL1	MR547	This study
Haemophilus influenzae ATCC33533	MR530	American Type Culture Collection
Escherichia coli K12	MR189	Jodi Camberg Lab
NEB5α	N/A	New England Biolabs
MFDpir	MR142	Marvin Whiteley Lab

Table 3: Plasmids Used

Plasmid name:	MR strain number:	Plasmid function:
pEAKO	MR254	Suicide/Knockout
pCRC1	MR496	Shuttle/Complementation
pMJM1	MR551	Shuttle/Complementation

Table 4: Primers Used

	Mutant:	Primer number:	Sequence (5' to 3'):
Knockout Vector	Δ*galE*	oMR699	ggacgagctgtacaagtagcggccgcatacccataatgcgcgattaatc
		oMR700	aagtgaggtccttactcttgttgggcgg
		oMR701	caagagtaaggacctcactttggtgtgatc
		oMR702	tcacgaacgaaaatcgatgcggccgctctcaccactggtgacaaattg
	Δ*yhxB*	oMR693	ggacgagctgtacaagtagcggccgcccaattccatgcatgtg
		oMR694	agtctatccaacgctaggtttgatgcctag
		oMR695	aacctagcgttggatagactaacggaaaatg
		oMR696	tcacgaacgaaaatcgatgcggccgcgaagttcaagctgcaaaag
	Δ*mlaA*	oMR703	ggacgagctgtacaagtagcggccgcttgactaactcatcaaagtc
		oMR704	acgagtattcattataaccaacttgaaagataac
		oMR705	tggttataatgaatactcgtataagcgataaagtc
		oMR706	tcacgaacgaaaatcgatgcggccgcaaccgcacgacgtaataaag
	Δ*wzzB* EL1	oMR795	ggacgagctgtacaagtagc*ggccgc*tatcagaatttaaaatccttttgag
		oMR796	ggaattgatgttataagggtaaaaaatgaataaactag
		oMR797	acccttataacatcaattcctttaagtacttc
		oMR798	tcacgaacgaaaatcgatgc*ggccgc*ttagtcatcgaagctcatc
	ΔDUF218	oMR837	ggacgagctgtacaagtagc*ggccgc*cacagcagaaaaaaatttcttg
		oMR838	ttgaccgcacggctgtaatcagttttttc
		oMR839	gattacagccgtgcggtcaaaagatcac
		oMR840	tcacgaacgaaaatcgatgc*ggccgc*tagccacgtttgccatgtc
	Δ*licD3* EL1	oMR811	ggacgagctgtacaagtagc*ggccgc*gtagtaatgttataaaaaaaacaagtg
		oMR812	ggtgtgtaatgaaggggaaaaataatgaac
		oMR813	ttccccttcattacacaccttcttcaac
		oMR814	tcacgaacgaaaatcgatgc*ggccgc*gaactaaaagaatcaacggtaatg
	Δ*galE* ATCC	oMR730	attgttttagcttactcttgttgggcgg
		oMR731	caagagtaagctaaaacaattcatccagtaaaatataatattttattttc
		oMR732	aagtgaggtctgaggtgataggtaagattatac
		oMR733	tatcacctcagacctcactttggtgtgatc
	Δ*yhxB* EL1	oMR740	ggacgagctgtacaagtagcggccgcttaggggcgatttttccaaag
		oMR741	attgttttagttaagcaaactattaaatgataaaaaacg
		oMR742	gtttgcttaactaaaacaattcatccagtaaaatataatattttattttc
		oMR743	agaaaacatctgaggtgataggtaagattatac
		oMR744	tatcacctcagatgttttctccttgtaaaaag
		oMR745	tcacgaacgaaaatcgatgcggccgcatttgtccaattgaagaagtag

	Δ*mlaA*	oMR746	*ggacgagctgtacaagtagcggccgcgttagaaataaccgcatttttattc*
		oMR747	attgttttagtccactttcctatcacaaaaaattg
		oMR748	ggaaagtggactaaaacaattcatccagtaaaatataatattttattttc
		oMR749	acgagtattctgaggtgataggtaagattatac
		oMR750	tatcacctcagaatactcgtataagcgataaag
		oMR751	*tcacgaacgaaaatcgatgcggccgctgtaagcattcgaaatcag*
	Δ*wzzB* ATCC	oMR833	ggacgagctgtacaagtagc*ggccgc*agatatatcagaagctaagctatag
		oMR834	ccctgtagaacctattcacaaaagctaaataag
		oMR835	tgtgaataggttctacagggcaccactattc
		oMR836	tcacgaacgaaaatcgatgc*ggccgc*gtgcggtagctccaattg
Checking Primers	Δ*galE*	oMR713	tattcacccgcaatcacg
		oMR714	atactacggttaccggctg
	Δ*yhxB*	oMR697	cttggttggcctatcgtcgt
		oMR698	ggcaacgtgtgctcaatgat
	Δ*mlaA*	oMR775	gatgcgatggctgttgaga
		oMR776	tgcacctgggtgttcgtta
	Δ*wzzB* EL1	oMR807	acacaccttcttcaacgag
		oMR808	cggtggcaccaattgat
	ΔDUF218	oMR843	atcagataccacccgcagcta
		oMR844	atgtagtggcgccat
	Δ*licD3* EL1	oMR841	gcatggcattcattg
		oMR842	gagtatgccagattatcctg
	Δ*wzzB* ATCC	oMR845	ctaaccactgagtatgcc
		oMR846	cgaaggcggtaacaatgc
Complementation	Δ*galE*	oMR723	gctcggtacccggggatcctctagataattaaggagtaactcatgg
		oMR724	tgcatgcctgcaggtcgactctagattaatctcggtatcctttcg
	Δ*yhxB*	oMR719	gctcggtacccggggatcctctagacttttacaaggagaaaacatc
		oMR720	tgcatgcctgcaggtcgactctagattagcaatcttgtttaccg
	Δ*mlaA*	oMR725	gctcggtacccggggatcctctagacaatttttgtgataggaaag
		oMR726	tgcatgcctgcaggtcgactctagattaatcaatctcttttaatacatcatc
	ΔDUF218	oMR855	cctgcaggtcgactctagag*gatcc*ggaacagcgattttcgaatac
		oMR856	attcgagctcggtacccggg*gatcc*ggtgttcttagcgaatgac
	Δ*licD3* EL1	oMR851	cctgcaggtcgactctagag*gatcc*ggttccgcctgcaaaaattatc
		oMR852	attcgagctcggtacccggg*gatcc*gcagtctatgaaagaacttgtg
	Δ*wzzB* ATCC	oMR853	cctgcaggtcgactctagag*gatcc*gactatgaattcttaacgcaagg
		oMR854	attcgagctcggtacccggg*gatcc*gcttctggcgttagcaaac
	Δ*galE*	oMR847	cctgcaggtcgactctagag*gatcc*ggataacgcatgccatctttac

		oMR848	attcgagctcggtacccggg*gat*ccaactacgacgacgacc

FIGURES

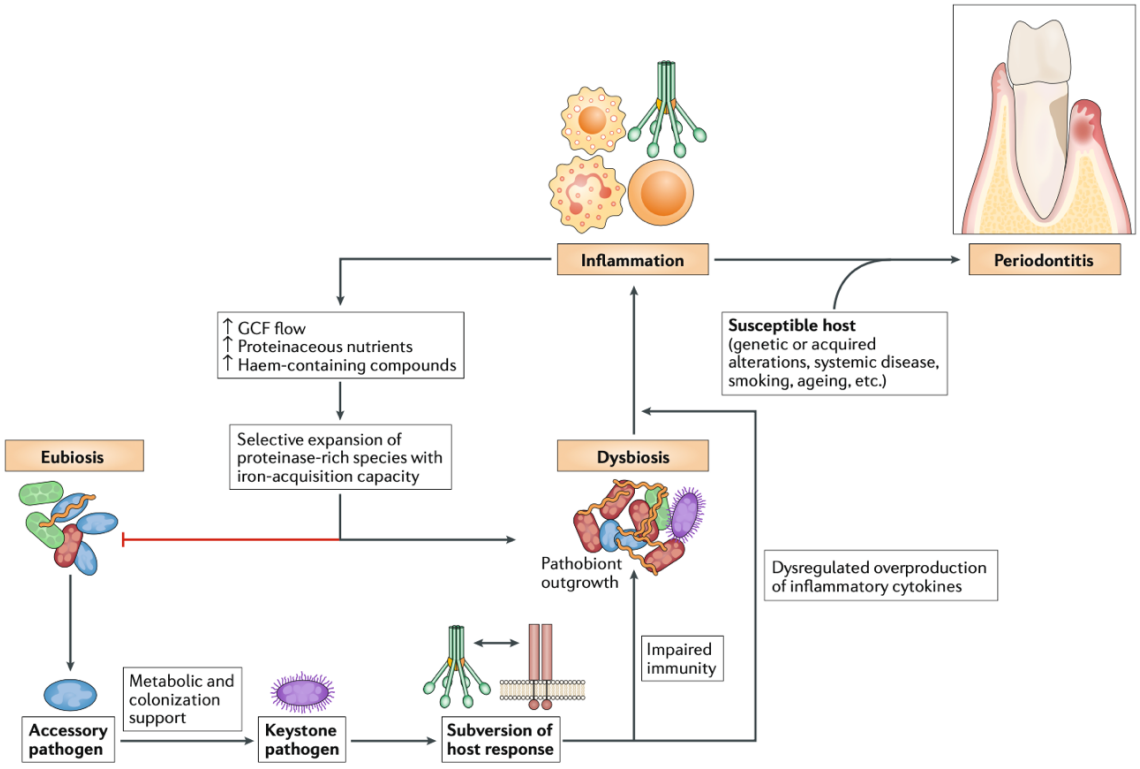

Figure 1: The oral microbiome and localized host immune response in periodontal disease. Eubiotic oral microbial communities can contain accessory pathogens, who, on their own, do not cause a community shift to dysbiosis. However, the accessory pathogens can provide metabolic and colonization support to keystone pathogens, like *Porphyromonas gingivalis*. These keystone pathogens can manipulate and trigger host immune responses, which kill off immune susceptible commensal microbes. The influx of nutrients from the host immune response can cause the outgrowth of pathobionts in the oral microbial community, which, under healthy conditions, make up a much smaller proportion of the oral microbiome. This now dysbiotic oral microbial community triggers the host immune response, entering a dysfunctional feedback cycle of inflammation and dysbiosis, which manifests as worsening, chronic periodontitis.

Original figure source: Lamont, R.J., Koo, H. & Hajishengallis, G. The oral microbiota: dynamic communities and host interactions. *Nat Rev Microbiol* **16**, 745–759 (2018). https://doi.org/10.1038/s41579-018-0089-x

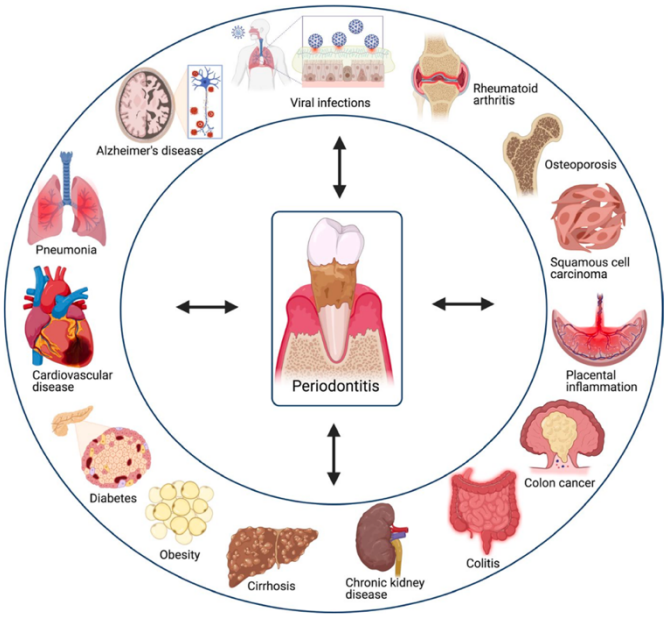

Figure 2: The interconnection of periodontitis and other systemic, chronic diseases. The presence and severity of periodontal disease have been associated with a plethora of systemic, chronic diseases. Many, if not all, of these diseases share the symptom of chronic low-grade inflammation.

Original figure source: Hajishengallis G. Interconnection of periodontal disease and comorbidities: Evidence, mechanisms, and implications. Periodontol 2000. 2022 Jun;89(1):9-18. doi: 10.1111/prd.12430. Epub 2022 Mar 4. PMID: 35244969; PMCID: PMC9018559.

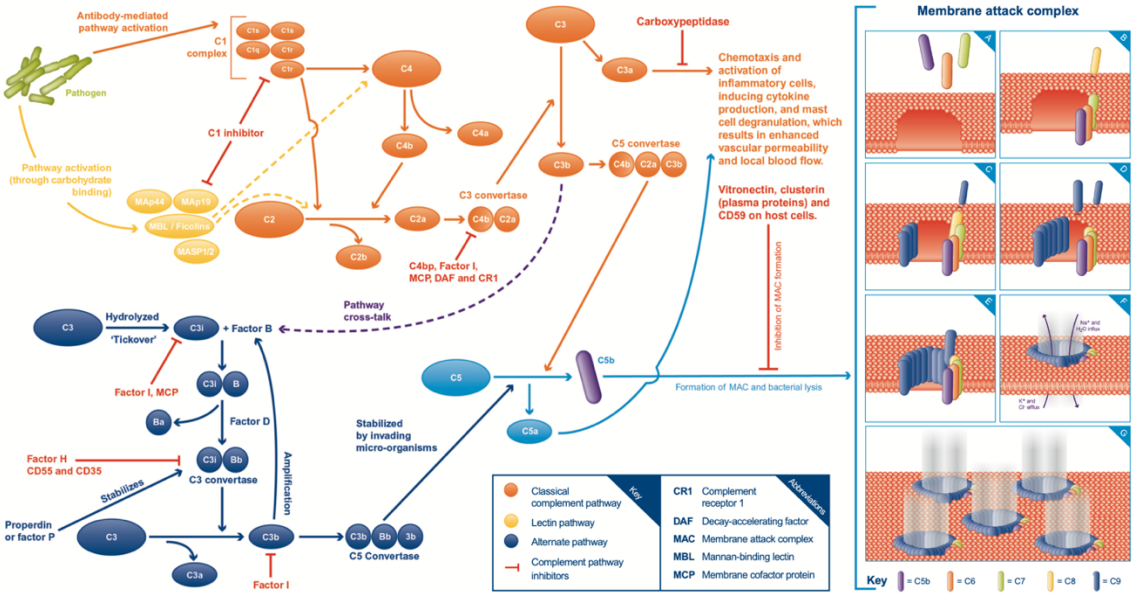

Figure 3: A simplified complement cascade and its regulators. The complement cascade can be activated through three different pathways: the classical pathway (sometimes referred to as the antibody-mediated pathway), the lectin pathway, and the alternative pathway. All three pathways converge on the terminal pathway, with the formation of the membrane attack complex (MAC). MAC functions like a pore-forming toxin, forming holes in the outer membrane of Gram-negative bacteria. This causes the bacteria to lyse and die.

Original figure source: Abcam – Complement cascade and its inhibitors

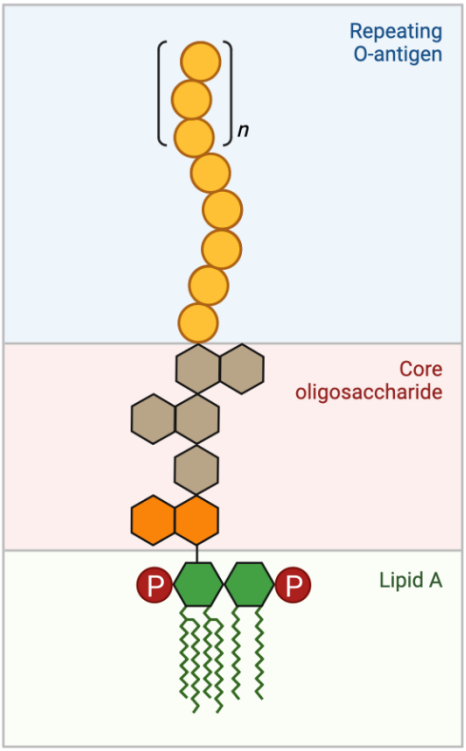

Figure 4: The structure of lipopolysaccharide (LPS). Lipopolysaccharide consists of the conserved lipid A, the core oligosaccharide, and the highly variable O-antigen. Some Gram-negative bacteria have lipooligosaccharide (LOS) instead of LPS. LOS is the lipid A and core oligosaccharide. LPS and LOS are characteristic of Gram-negative bacteria and play important roles in protecting the bacterial cell from the outside environment.

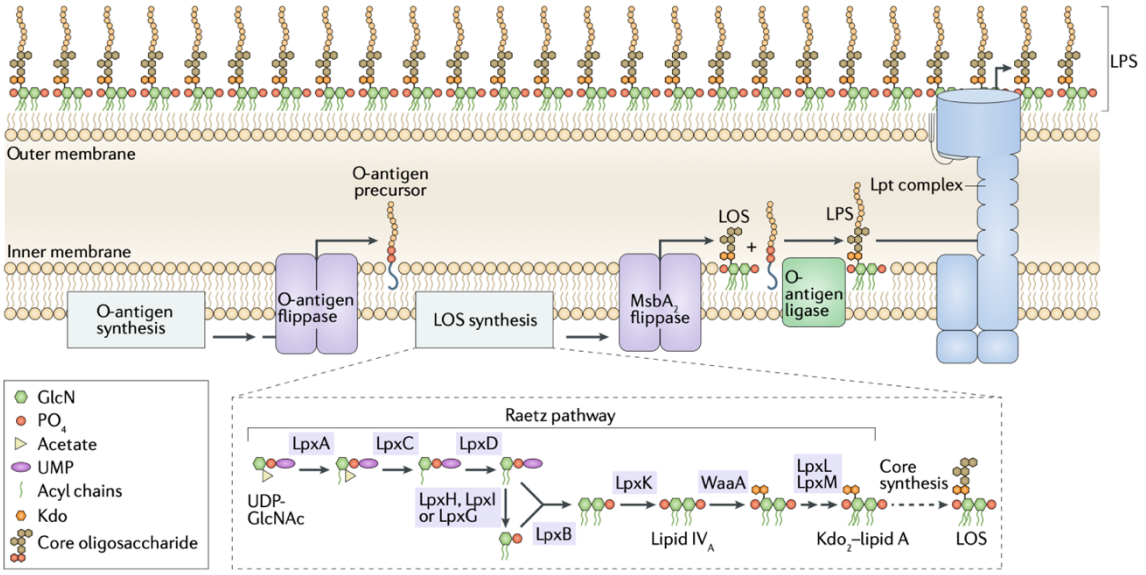

Figure 5: Lipopolysaccharide (LPS) biosynthesis and export. LPS biosynthesis begins with the synthesis of lipid A-Kdo through the Raetz pathway. Since lipid A is well conserved across Gram-negative bacteria, the Raetz pathway tends to be well conserved too. After lipid A-Kdo is formed, the core oligosaccharide is extended off of it. The inner core oligosaccharide is more conserved than the outer core oligosaccharide. Lipooligosaccharide (LOS) is formed when the core oligosaccharide extension has been completed. If the organism produces O-antigen, it is synthesized independently of the LOS and then ligated onto the LOS on the periplasmic leaflet of the inner membrane. Once the O-antigen has been liagated on, the LOS is LPS. The LPS molecule is then transferred to the outer leaflet of the outer membrane by the LPS transport machinery, which makes up the Lpt complex.

Original figure source: Simpson, B.W., Trent, M.S. Pushing the envelope: LPS modifications and their consequences. *Nat Rev Microbiol* **17**, 403–416 (2019). https://doi.org/10.1038/s41579-019-0201-x

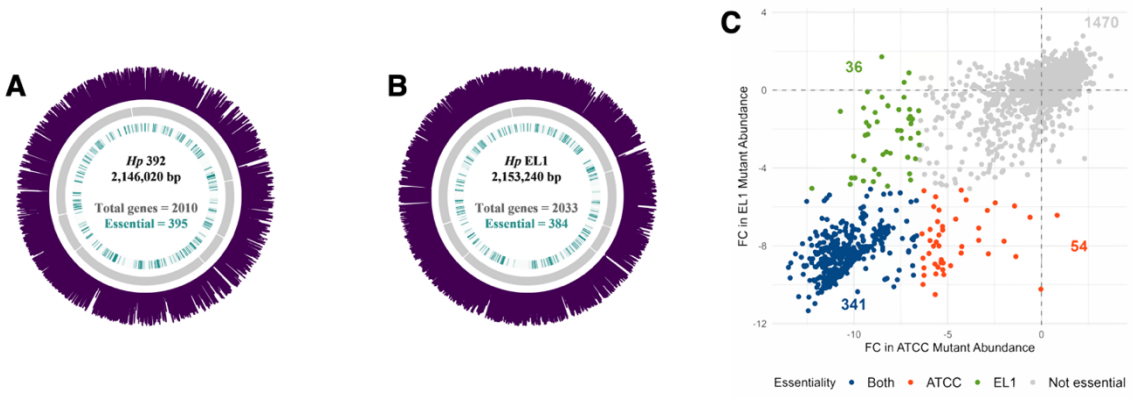

Figure 6: TnSeq libraries of *Hp* ATCC and EL1 and their absolutely essential genes. Saturated mariner transposon libraries of **A)** ATCC and **2)** EL1 *Hp* strains. The absolutely essential genes are highlighted in teal on the inner circle. **C)** Volcano plot comparison of conserved ATCC and EL1 genes and the fold-change in abundance vs. their expected abundance.

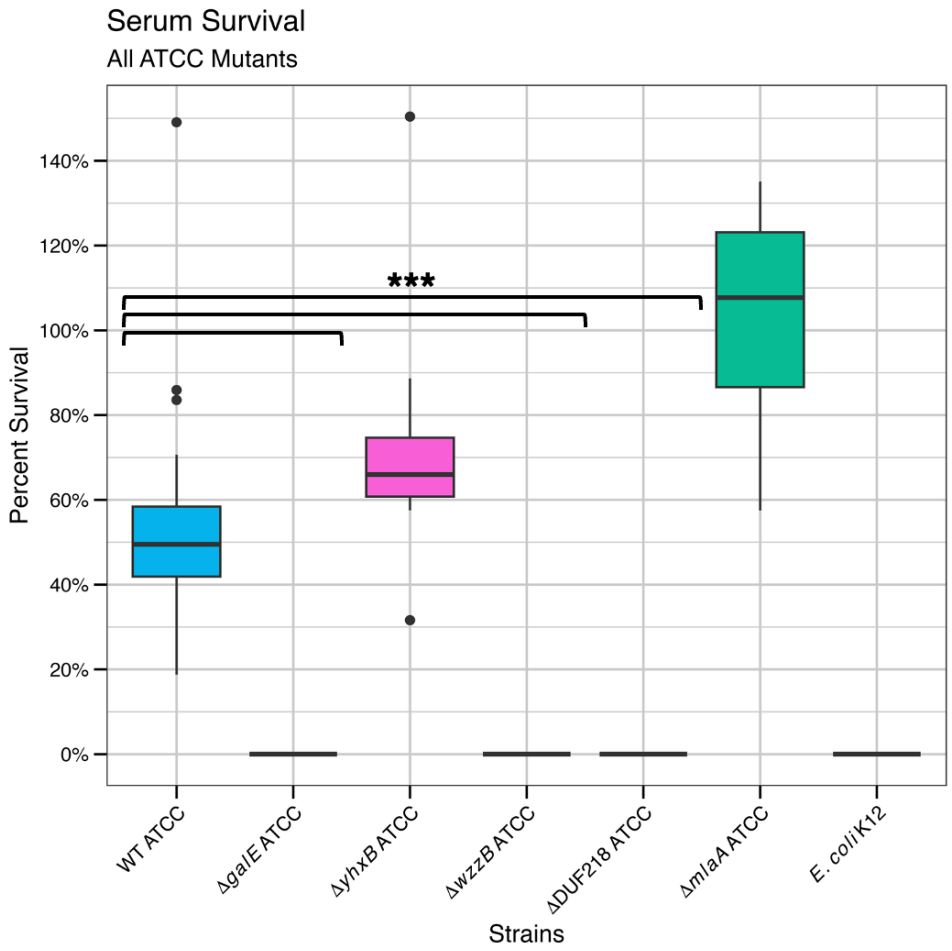

Figure 7: Percent survival of WT ATCC and the ATCC gene deletion mutants after one hour in 50% serum. Percent survival of the parental strain, *Haemophilus parainfluenzae* ATCC33392 (WT ATCC), and the gene deletion mutants after one hour in 50% normal human serum. All strains were tested in mid-log growth phase. Δ*galE*, Δ*wzzB*, and ΔDUF218 completely lost their serum survival phenotypes. Δ*yhxB* and Δ*mlaA* remained resistant to killing in serum. Box plots were graphed in R. Welch Two Sample t-test was also performed in R. *** $p = 3.46 \times 10^{-11}$

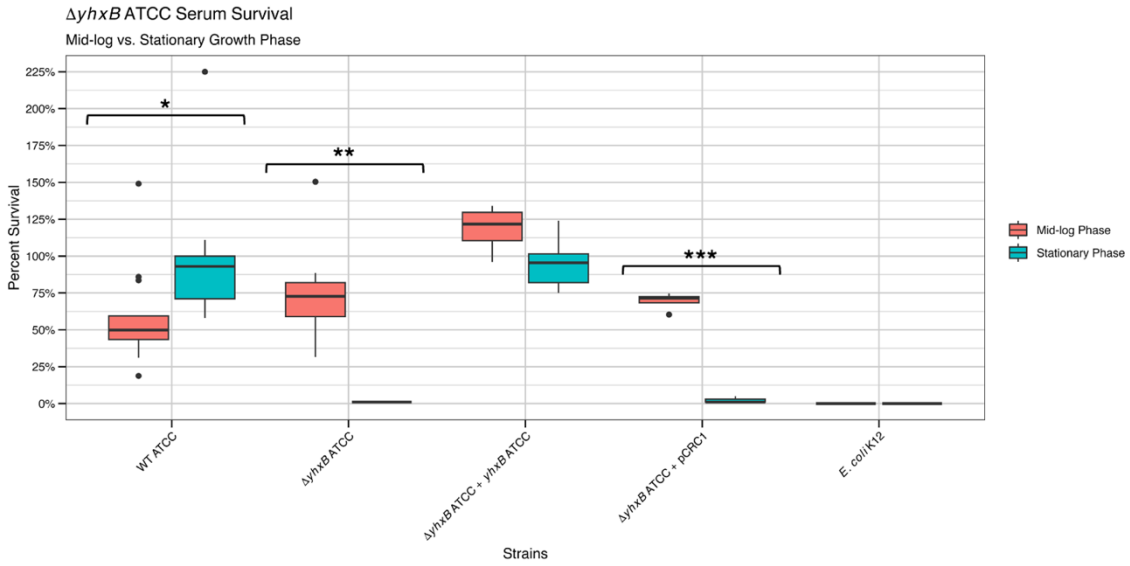

Figure 8: Δ*yhxB* ATCC serum survival phenotype is dependent on growth phase. Percent survival of WT ATCC, Δ*yhxB* ATCC, complemented Δ*yhxB* (Δ*yhxB* + *yhxB*), empty vector complemented Δ*yhxB* (Δ*yhxB* + pCRC1), and *E. coli* K12 in stationary or mid-log growth phase after one hour in 50% normal human serum. Δ*yhxB* ATCC is resistant to complement lysis in mid-log phase and sensitive to it in stationary phase. WT ATCC has a baseline higher percent survival after serum exposure in stationary phase. Typically, bacterial cells have less permeable membranes during stationary phase. *pvalue < 0.05, **pvalue < 0.005, ***pvalue = 4.0×10^{-05}

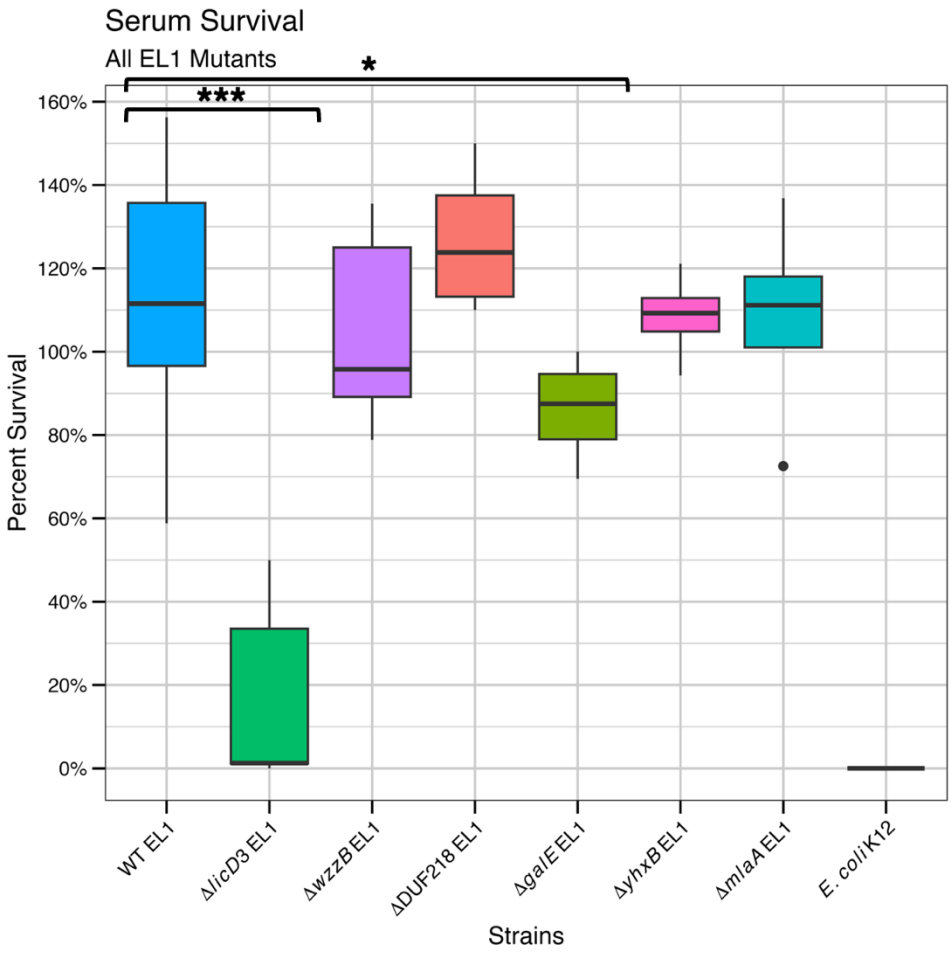

Figure 9: Percent survival of WT EL1 and the EL1 gene deletion mutants after one hour in 50% serum. Percent survival of the parental strain, *Haemophilus parainfluenzae* EL1 (WT EL1), and the gene deletions mutants after one hour in 50% normal human serum. Δ*licD3* and Δ*galE* were sensitive to complement lysis. Δ*wzzB*, ΔDUF218, Δ*yhxB*, and Δ*mlaA* EL1 mutants remained serum resistant. All strains were tested in mid-log growth phase. Box plots were graphed in R. Welch Two Sample t-test was also performed in R. *p< 0.05, *** $p = 3.34 \times 10^{-09}$

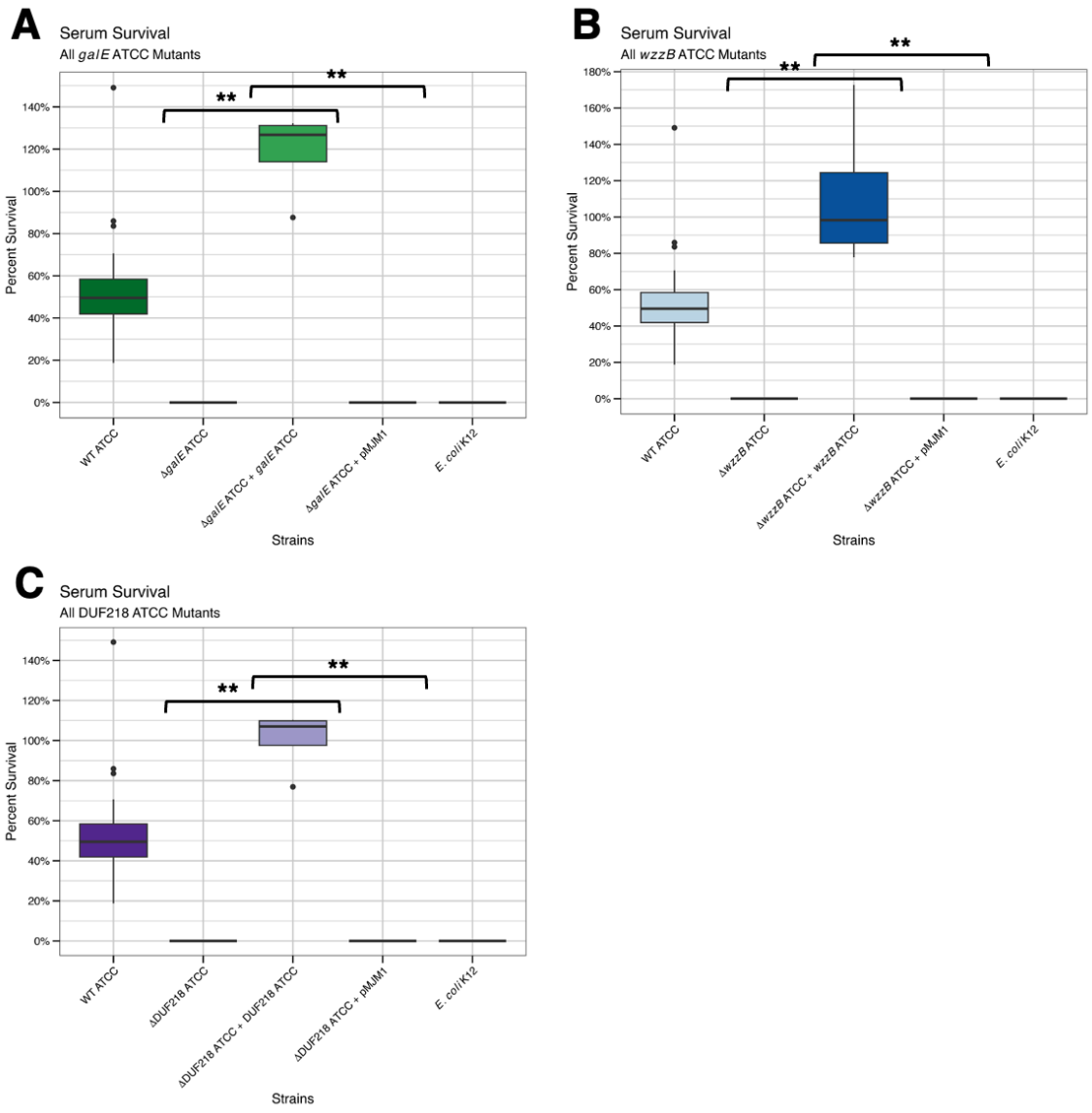

Figure 10: Complemented Δ*galE* ATCC, Δ*wzzB* ATCC, and ΔDUF218 ATCC have their serum survival phenotypes restored. Percent survival after one hour in 50% serum. **A)** WT ATCC and the *galE* knockout strain (Δ*galE* ATCC) graphed with complemented Δ*galE* (Δ*galE* ATCC + *galE* ATCC) and Δ*galE* with the empty shuttle vector used for complementation (Δ*galE* ATCC + pMJM1). **B)** WT ATCC and the *wzzB* knockout strain (Δ*wzzB* ATCC) graphed with complemented Δ*wzzB* (Δ*wzzB* ATCC + *wzzB* ATCC) and Δ*wzzB* with the empty shuttle vector used for complementation (Δ*wzzB* ATCC + pMJM1). **C)** WT ATCC and the DUF218 knockout strain (ΔDUF218 ATCC) graphed with complemented ΔDUF218 (ΔDUF218 ATCC + DUF218 ATCC) and ΔDUF218 with the empty shuttle vector used for complementation (ΔDUF218 ATCC + pMJM1). All strains were tested in mid-log growth phase. Box plots were graphed in R. Welch Two Sample t-test was also performed in R. ** $p < 0.0015$

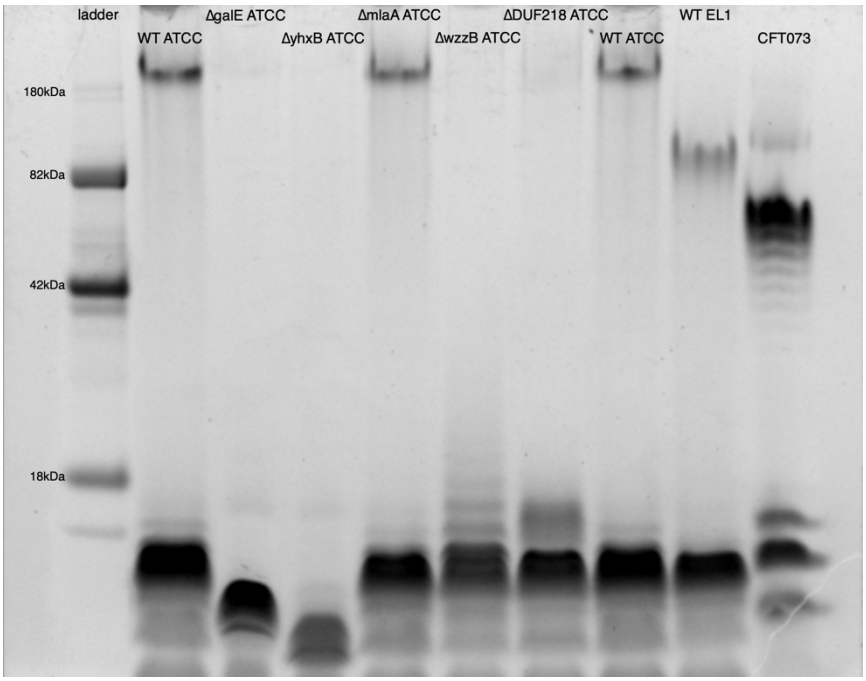

Figure 11: Lipopolysaccharide gel of WT ATCC and the ATCC gene deletion mutants. LPS gel of whole cell lysates treated with proteinase K and stained with the Pro-Q Emerald 300 Lipopolysaccharide staining kit. Compared to WT ATCC, the Δ*galE* ATCC, Δ*yhxB* ATCC, Δ*wzzB* ATCC, and ΔDUF218 ATCC mutants showed structural changes in their LPS. Δ*mlaA* ATCC has identical LPS structure to WT ATCC. From left to right: Candycane glycoprotein molecular weight standards, WT ATCC, Δ*galE* ATCC, Δ*yhxB* ATCC, Δ*mlaA* ATCC, Δ*wzzB* ATCC, ΔDUF218 ATCC, WT ATCC, WT EL1, and uropathogenic *E. coli* strain CTF073.

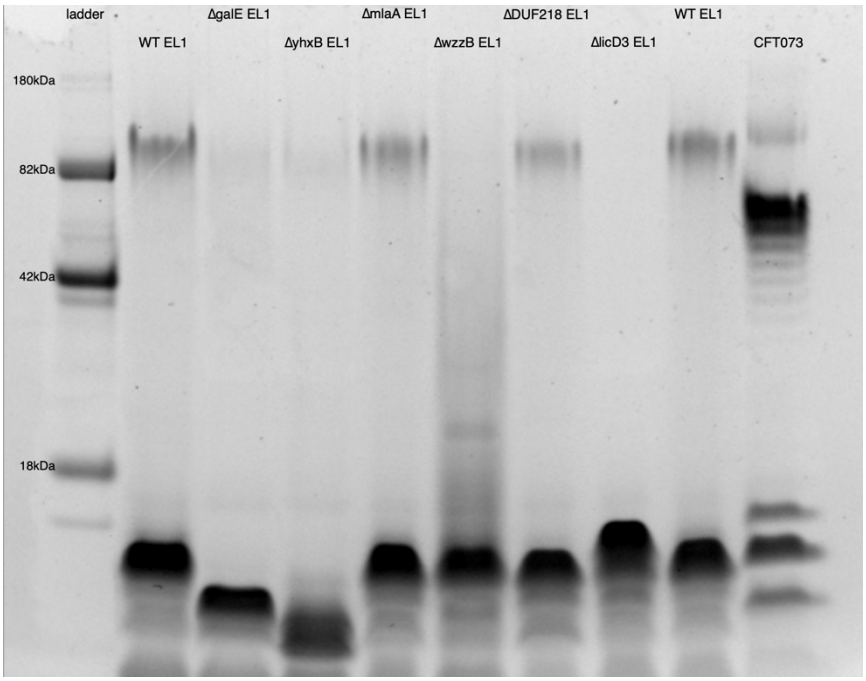

Figure 12: Lipopolysaccharide gel of WT EL1 and the EL1 gene deletion mutants. LPS gel of whole cell lysates treated with proteinase K and stained with the Pro-Q Emerald 300 Lipopolysaccharide staining kit. Δ*galE* EL1, Δ*yhxB* EL1, Δ*wzzB* EL1, and Δ*licD3* EL1 had structural changes in their LPS when compared to WT EL1. Δ*mlaA* EL1 and ΔDUF218 EL1 had identical LPS structure to WT EL1. From left to right: Candycane glycoprotein molecular weight standards, WT EL1, Δ*galE* EL1, Δ*yhxB* EL1, Δ*mlaA* EL1, Δ*wzzB* EL1, ΔDUF218 EL1, Δ*licD3* EL1, WT EL1, and uropathogenic *E. coli* strain CTF073.

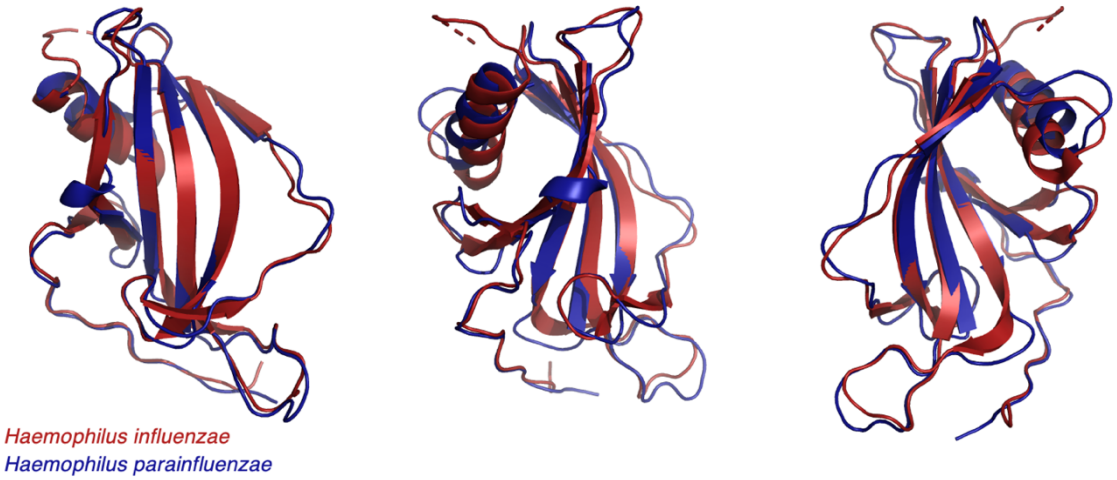

Figure 13: Solved *H. influenzae* Protein E structure overlaid with the modeled *H. parainfluenzae* Protein E. The solved structure of *Hi* PE, shown in red, was overlaid with the modeled *Hp* PE structure, shown in blue. The solved structure and modeled structure have 70% structural identity with 100% confidence.

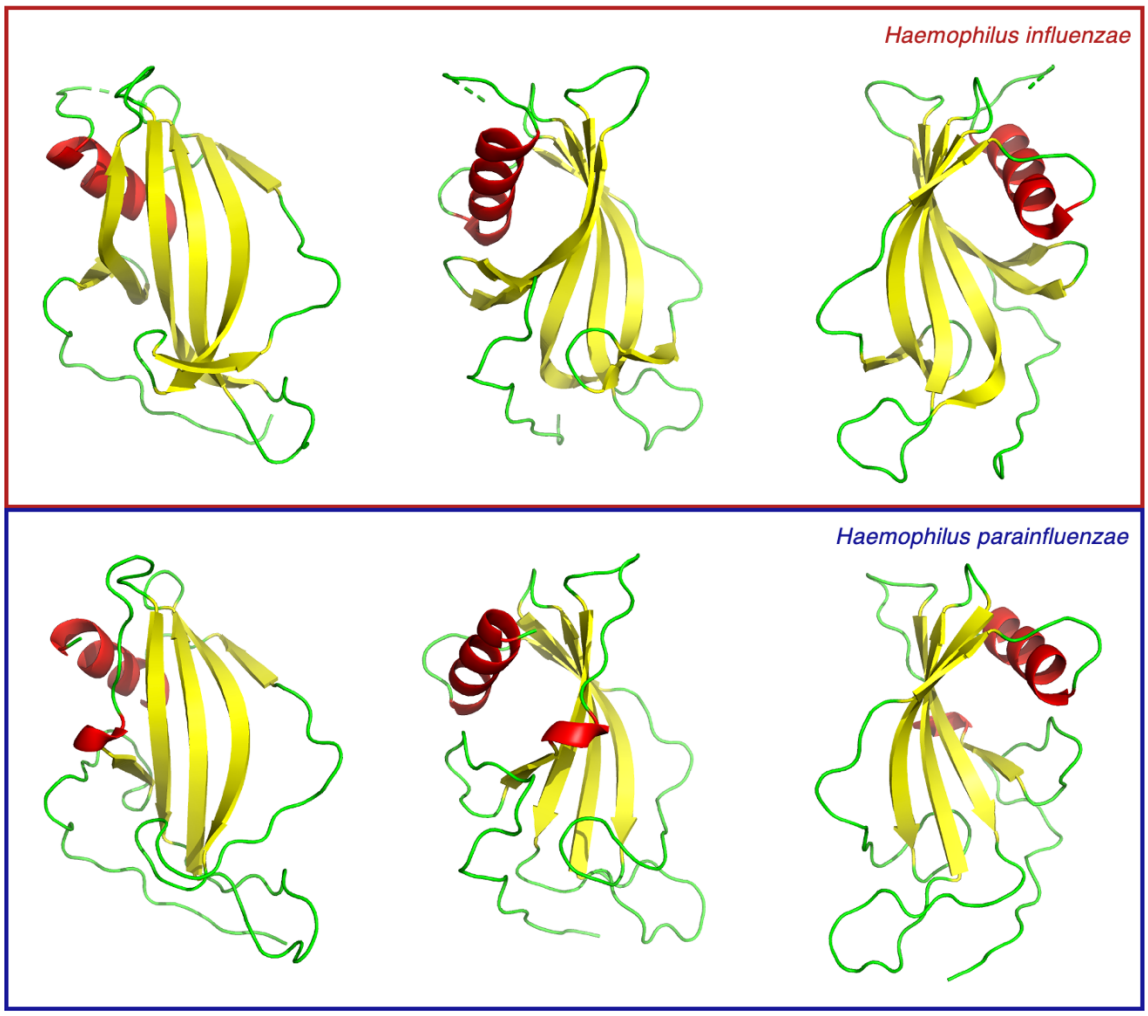

Figure 14: Secondary structure comparison of the solved *H. influenzae* Protein E and the modeled *H. parainfluenzae* Protein E. *Hi* PE has six antiparallel beta sheets, one alpha helix, and eight loops. The modeled *Hp* PE has five antiparallel beta sheets, two alpha helices, and seven loops. Beta sheets are yellow, alpha helices are red, and loops are green. The top panel, in the red box, shows the *Hi* PE. The bottom panel, in the blue box, shows the modeled *Hp* PE.

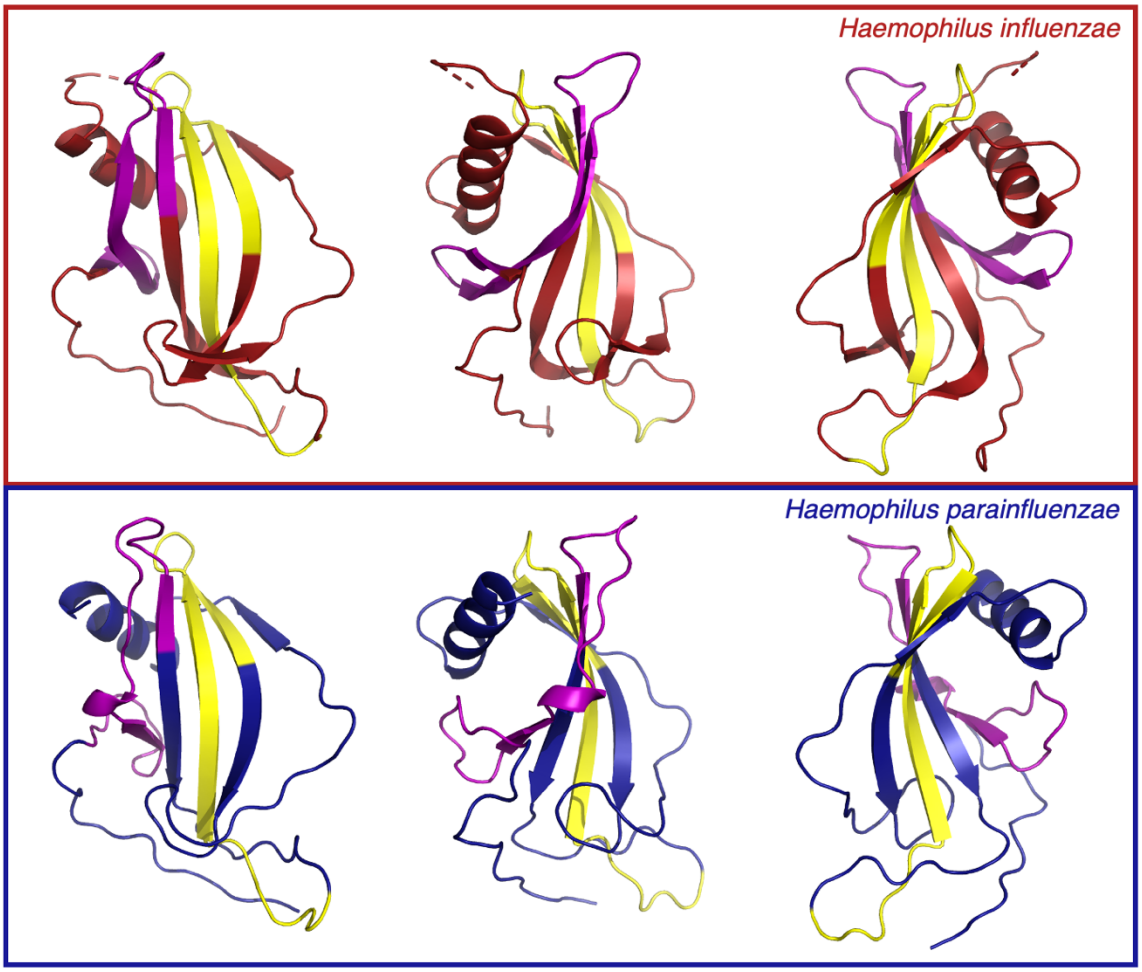

Figure 15: *H. influenzae* Protein E and modeled *H. parainfluenzae* Protein E structures with the vitronectin binding regions and plasminogen/laminin binding regions highlighted. The top panel, in the red box, shows the *Hi* PE. The bottom panel, in the blue box, shows the modeled *Hp* PE. The vitronectin binding region is highlighted in yellow. The plasminogen/laminin binding region is highlighted in purple. The vitronectin binding region seems to be well conserved between the solved and modeled structures. The plasminogen/laminin binding region is not as well conserved between the structures. The modeled *Hp* PE has different secondary structures in the plasminogen/laminin binding region compared to the solved *Hi* structure. Differing secondary structures make it less likely that the binding region could perform the same function in the different structures.

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