Abstract

A Wzx-Wzy-like Polysaccharide Biosynthetic Pathway Contributes to Virulence in *Brucella abortus* 2308 through an Unknown Mechanism

by Dariel A. Hopersberger

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Director: R. Martin Roop II
Department of Microbiology and Immunology

Brucella abortus is an intracellular pathogen that causes spontaneous abortion in cattle and undulant fever in humans. As a member of the α-proteobacteria, *B. abortus* is closely related to the bacterial species *Caulobacter crescentus* and *Agrobacterium tumefaciens*, which have been shown to produce a holdfast and a unipolar polysaccharide (UPP), respectively, at one pole of the bacterial cell. Although little is currently known about exopolysaccharide production and function in *Brucella* species, recent evidence suggests that these bacteria are capable of producing exopolysaccharides, and with the exception of a flippase gene, *B. abortus* 2308 carries the genes for a complete UPP biosynthetic pathway.

In *A. tumefaciens*, UPP can be detected by crystal violet, Congo red, or lectin staining; however, UPP production was not detected in *B. abortus* 2308 using these assays. UPP production can also be increased in *A. tumefaciens* by overexpressing the diguanylate cyclase PleD, which regulates intracellular cyclic di-GMP levels, but we were unable to demonstrate that UPP is regulated by cyclic di-GMP in *B. abortus* 2308.

Nonetheless, *upp* homologs in *B. abortus* 2308 are able to restore biofilm production in *A. tumefaciens*. In *A. tumefaciens*, knockout mutants of the outer membrane transporter *uppC*

and the polyisoprenylphosphate hexose-1-phosphate transferase *uppE* result in strong biofilm phenotypes, wherein the ability of *A. tumefaciens* to produce a biofilm is nearly abrogated. *B. abortus* 2308 *uppC* and *uppE* homologs restore biofilm production in the respective *Agrobacterium upp* mutants, indicating that *uppC* and *uppE* from *B. abortus* 2308 produce functional proteins.

A Δ*uppCE* mutant in *B. abortus* 2308 also exhibits significant attenuation in mice and an altered intracellular replication profile in cultured murine macrophages. Accordingly, *uppCE* appears to be required for virulence in *B. abortus* 2308, although by a currently unknown mechanism. Additional studies will be needed to determine whether *B. abortus* 2308 produces an authentic UPP as well as define the mechanisms by which the putative *upp* genes contribute to virulence.

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by

Dariel A. Hopersberger

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By

Dariel A. Hopersberger

APPROVED BY:

DIRECTOR OF DISSERTATION:____

R. Martin Roop II, Ph.D.

COMMITTEE MEMBER:____

Jamie DeWitt, Ph.D.

COMMITTEE MEMBER:____

Md A. Motaleb, Ph.D.

COMMITTEE MEMBER:____

Everett C. Pesci, Ph.D.

COMMITTEE MEMBER:____

Michelle L. Ratliff, Ph.D.

CHAIR OF THE DEPARTMENT OF
MICROBIOLOGY AND IMMUNOLOGY:_____________________________________

Everett C. Pesci, Ph.D.

DEAN OF THE GRADUATE SCHOOL: ______________________________________

Kathleen Cox, Ph.D.

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Chapter One

Review of the Literature

Introduction to *Brucella* Species

Brucella species are facultative intracellular pathogens that are characterized as small, non-motile, Gram-negative coccobacilli belonging to the order of α-proteobacteria. *Brucella* species are as follows, with their natural host type in parentheses: *B. melitensis* (goats and sheep), *B. abortus* (cattle), *B. suis* (swine), *B. ovis* (sheep), *B. canis* (canines), *B. neotomae* (rats), *B. microti* (mice), *B. ceti* (cetaceans, such as dolphins and whales), *B. pinnipedialis* (seals), and *B. vulpis* (foxes) (Olsen & Palmer, 2014). Although *Brucella* strains exhibit host preference as previously outlined, they are not strictly limited to a single host type. Of particular importance to this study, human infections have been reported for the strains *B. melitensis*, *B. abortus*, *B. suis*, and *B. canis*. While the majority of *Brucella* species infect mammalian species, unnamed *Brucella* species, identified as belonging to the BO2 clade, infect amphibians (Tiller et al., 2010; Scholz et al., 2016; al Dahouk et al., 2017). In addition, the novel species *B. inopinata* has been isolated from a breast implant infection, although its natural host remains unknown (Scholz et al., 2010).

Brucella species belong to the *Rhizobiales* group in the α-proteobacteria class (Brown et al., 2012). Members of the *Rhizobiales* infect a wide range of hosts and include plant pathogens (*Agrobacterium tumefaciens*), plant symbionts (*Sinorhizobium meliloti*), mammalian pathogens with no intermediate vector (*B. abortus*), and mammalian pathogens transmitted by an arthropod vector (*Bartonella henselae*). Although members of this group infect a wide variety of hosts and inhabit diverse lifestyles, they exhibit a high degree of genetic similarity, indicating the ability of the various members of the *Rhizobiales* to adapt to the diverse range of lifestyles exhibited across the group (Batut et al., 2004a).

Phylogenetic analysis of the different members of the α-proteobacteria exposes a clear trend between free-living soil or symbiotic plant-associated members from their intracellular relatives. The former shows a trend towards genome expansion, and the latter demonstrates a tendency towards genome reduction (Batut et al., 2004). An explanation for the increases in genome size associated with these plant symbionts is that it allows these bacteria to adapt to highly variable soil environments, whereas the reduction in genome size observed in arthropod and mammalian pathogens indicates an evolutionary trend whereby only genes required for adaption to highly specific intracellular niches are maintained. The establishment of a symbiosis requires that the invasive species maintains a non-lethal relationship with the host. Similarly, to establish a chronic infection, intracellular pathogenic bacteria must also establish a non-lethal interaction with the host species. Therefore, it may come as no surprise that although the genomes of intracellular members of the α-proteobacteria are reduced, the genes required for pathogenesis are often homologous to those required to establish symbiosis in plant-associated members, indicating that both groups of the α-proteobacteria have adapted common ancestral genes to maintain long-term, non-lethal relationships with their hosts.

Global Economic Impact

Brucellosis is recognized as the world's leading zoonotic disease and is endemic in many parts of the world, typically in lower-income regions, including regions in Asia, Eastern Europe, Africa, the Middle East, and Central and Latin America, as well in some areas located along the Mediterranean Basin. Because of this, a large portion of the world's cattle are infected with brucellosis, with estimates as high as 300 million of the 1.4 billion worldwide cattle population carrying the disease (de Figueiredo et al., 2015). The people in these areas often depend on their livestock for basic sustenance as well as for economic gains, and because infection with *Brucella*

species causes reduced milk production, abortion, infertility, sterilization, morbidity, and mortality in these animals, this disease can have a major impact on the economies in these regions. For example, in some parts of Africa, it is estimated that infection with bovine brucellosis results in an income loss of 6-10% per affected animal (Mcdermott et al., 2013). The annual economic losses due to brucellosis in India, Argentina, Kyrgyzstan, Egypt, and Nigeria are estimated at $3.4 billion, $60 million, $10.6 million, $9.8 million, and $3.2 million per year, respectively (Mcdermott et al., 2013; Dadar et al., 2021).

As human cases of brucellosis in these regions often go underdiagnosed, the true human health care costs due to brucellosis in these same areas can be difficult to determine. One study estimates that patients infected with brucellosis pay $1000 more, on average, than patients of a randomly selected control group during the first year following diagnosis, further burdening these already economically disadvantaged regions (Vered et al., 2015).

Brucellosis in Animals

In their natural hosts, *Brucella* species target the reproductive system, leading to spontaneous abortion and reduced milk yield in females and sterility in males (Khan & Zahoor, 2018). *Brucella* infections can be transmitted vertically from mother to fetus or horizontally from one host to the next. Transmission can occur through sexual intercourse, contact with secretions (especially those associated with the reproductive tract), or contact with aborted fetuses (Olsen & Palmer, 2014). The latter often occurs due to a feature of maternal care often shared by mammalian species susceptible to *Brucella* that involves the mother and other members of the herd licking the newborn calf, even if the calf is still born (Kovács et al., 2021).

As previously discussed, *Brucella* remains endemic in certain low-income nations. *Brucella* remains prevalent in these areas due to the lack of funding for animal vaccines, surveillance, and other control programs that are typically found in countries that can afford such measures. Conversely, *Brucella* has largely been eradicated from countries such as the United States due to efforts to control the disease that began in the mid-20th century. In 1934, regulatory programs to control brucellosis in the United States were first established, and additional funding was approved by Congress in 1954 to facilitate the eradication of the disease via the efforts of the United States Department of Agriculture, which largely relied on surveillance, culling of infected animals, and animal vaccinations (Olsen, 2010). These control programs were remarkably successful, with all 50 states being declared free of bovine brucellosis in 2009 by the United States Department of Agriculture (Rhyan et al., 2013; US Code 9 CFR § 78, 2014)

Despite the low occurrence of brucellosis, the United States remains vigilant in its surveillance due to wild animal populations that serve as reservoirs for the disease. For example, wild elk and bison located in Yellowstone National Park and the surrounding areas pose a risk of transmission to domestic herds (Olsen, 2010). In addition, some feral swine populations in the eastern part of the United States harbor *Brucella suis*, posing a risk to hunters in those regions (Centers for Disease Control and Prevention, 2016).

Brucellosis in Humans

Brucellosis is the world's most widespread bacterial zoonotic disease, causing more than 500,000 human infections across the globe per year (Gwida et al., 2010). Species capable of causing disease in humans include *B. melitensis*, *B. abortus*, *B. suis*, and *B. canis*, although the latter has been implicated in human pathogenesis to a much lower degree than the other species. Also known as Malta fever, brucellosis is most commonly acquired through the ingestion of

unpasteurized, contaminated milk products. Other means of acquiring the disease include coming into direct contact with or inhaling infected materials, such as may happen during the butchering of an infected animal. Brucellosis is also one of the most frequent laboratory-acquired diseases in clinical and research facilities. The process of vaccinating livestock also puts veterinarians at risk of infection as the animal vaccines cause disease in humans (Boycott et all, 1964; Ashford et al., 2004; Traxler et al., 2013). Direct human-to-human transmission is rare, although it has been reported to occur (Franco et al., 2007).

Regions that experience endemic levels of brucellosis tend to be lower-income areas with inadequate health-care structures in place to manage cases of the disease. Cases of brucellosis are rare in the United States, and when cases are reported, they are typically attributed to travel outside of the country to regions in which the disease is endemic and proper measures are not taken to pasteurize local dairy products. Other means of acquiring the disease in the United States include accidental veterinary or laboratory infections and infections caused by hunting wild animals, such as feral swine.

Due to the zoonotic nature of the disease, control of brucellosis has largely been accomplished through widespread vaccination of livestock. Low occurrence of brucellosis in animal populations has been linked to low occurrence in the adjacent human population. As cases of brucellosis remain practically non-existent in most of the United States, very few regions still regularly vaccinate their animals, as the risk posed to veterinarians during the vaccination process and the cost to vaccinate outweigh the benefits of widespread vaccination; however, in regions where wild populations of elk and bison act as reservoirs for the disease, vaccination of cattle still occurs.

Brucella as an Agent of Biowarfare/Bioterrorism

It is estimated that an aerosolized infectious dose of *Brucella* could be as low as 10-100 microorganisms (Doganay & Doganay, 2013). Due to its high infectivity and the ease at which it may be aerosolized, *Brucella* species are a potential target for use in a weapon of bioterrorism, leading the US government to categorize *Brucella* as a class B Select Agent (Pappas et al., 2006). CDC defines Select Agents as "biological agents and toxins that have been determined to have the potential to pose a severe threat to public health and safety, to animal and plant health, or to animal or plant products" and treats such agents with additional regulatory scrutiny compared to other pathogens (United States Department of Health and Human Services, 2002). Rather than causing a high mortality rate, the use of *Brucella* as a biological weapon would mainly have an incapacitating effect, causing a high degree of morbidity and thus directing a significant amount of resources related to medical care towards those affected by the dissemination of such a weapon. A 1997 model predicted that an aerosolized attack using *Brucella* could cause 500 deaths and 125,000 cases of morbidity and would cost $477.7 million per 100,000 people exposed (Christopher et al., 1997; Lindeke, 2018).

The United States military has long recognized the potential for *Brucella* spp. to function as agents of biowarfare. In fact, in 1942, the United States began developing its own biological weapons and by 1954 had constructed its first agent of biowarfare, the M33 bomb, which contained *B. suis* as the infectious agent (Lindeke, 2018; Valderas & Roop, 2006). Fortunately, attitudes towards the use of biological warfare shifted, and in 1972, the United States signed the treaty at the Biological Weapons Convention, in which 140 nations pledged to discontinue production of such weapons and to dismantle and destroy any remaining stockpiles (Valderas & Roop, 2006). Nevertheless, the threat that some countries, either those who did not sign the

treaty or those who have not honored it, may still use biological agents such as *Brucella* spp. as biological weapons concerns the United States military, who remain interested in the development of a vaccine suitable for human use against this disease.

Pathogenesis

Brucella is a stealthy pathogen that largely relies on evading the immune system as a means to establish a chronic infection (Barquero-Calvo et al., 2007a). Instead of employing classical microbial virulence factors, such as pathogenicity islands, toxin release, or virulence plasmids, *Brucella* spp. rely on maintaining a low profile in the host and avoid provoking a strong pro-inflammatory response (Delrue et al., 2004). *Brucella* can infect a variety of professional phagocytes, including dendritic cells and neutrophils, but its ability to colonize macrophages establishes the initial acute phase of infection. *Brucella*-infected macrophages are carried through the lymphatic system, providing *Brucella* with both a niche within which to replicate and a means of transportation to allow the bacteria to invade other areas of the body, namely the bone marrow, lymph nodes, liver, and spleen. Infection of the spleen establishes the chronic stage of infection which can last for weeks to months. In the natural host, *Brucella* also travels to the reproductive tract and infects placental trophoblasts, leading to spontaneous abortion in cattle, although whether human fertility is affected remains controversial (Yousuf Khan et al., 2001, M. Z. Khan & Zahoor, 2018). Recent data indicates that brucellosis may indeed pose a risk to pregnant women, correlating with increased rates of spontaneous abortion or pre-term deliveries (Kurdoglu et al., 2015).

Aside from using phagocytic cells such as macrophages as “hide-outs” in which the bacteria can shield themselves from recognition by the immune system, *Brucella* spp. also employ other strategies to evade the host immune system. One of the main factors involved in

Brucella's ability to avoid detection by the host immune system is its unique lipopolysaccharide (LPS). LPS from enteric Gram-negative bacteria, such as *Escherichia coli*, tends to trigger a strong inflammatory response; however, *Brucella* LPS is only weakly endotoxic. The reason for this is largely attributed to the fact that the lipid A found in LPS from *Brucella* is characterized by very-long-chain fatty acids (VLCFAs) (Ferguson et al., 2004). The unusually long length of these VLCFAs prevents *Brucella* LPS from forming strong interactions with Toll-like receptor 4 (TLR4) found on the surface of immune cells such as macrophages. This limits the stimulation of anti-inflammatory cytokine production by these cells (Lapaque et al., 2006). *Brucella* LPS also plays a role in reducing *Brucella's* susceptibility to complement and interacts with lipid rafts on host cells which allows the bacteria to invade these cells via the endocytic pathway, a strategy that results in the ability of the *Brucella*-containing vacuole (BCV) to avoid fusion with lysosomes in the cell (Eisenschenk et al., 1999; Lapaque et al., 2006).

Brucella spp. also secrete a cyclic polymer known as cyclic β-1,2-glucan (CβG) into the periplasmic space. While this molecule appears to be involved in osmoregulation in other members of the α-protobacteria, CβG does not appear to play a major role as an osmoprotectant in *Brucella*. Instead, it appears that CβG contributes to virulence by interacting with lipid rafts on the hosts cells, which contributes to the ability of the BCV to circumvent fusion with lysosomes and therefore avoid degradation within the macrophage (Arellano-Reynoso et al., 2005).

Diagnosis and Treatment

Perhaps the most characteristic symptom of brucellosis is undulant fever, a fever that rises and falls in an unpredictable fashion (as opposed to a malaria-induced fever, for example,

which peaks and regresses at regular intervals). Symptoms due to infection with *Brucella* also include sweating, chills, arthralgia and myalgia, headache, fatigue, meningitis and endocarditis, and depression and mental status changes (Corbel et al., 2006). The standard means of detecting infections with *Brucella* is through culture of blood; however, *Brucella* may also be isolated from the bone marrow, cerebrospinal fluid, or joint tissue (di Bonaventura et al., 2021). Serology can also be used to detect infection with *Brucella*, but this method is not ideal as concurrent infections with other organisms can lead to false positive results (Yagupsky et al., 2020). Polymerase chain reaction (PCR) may also be used; however, because standards for sample preparation as a diagnostic tool have not been established and because success with this method varies between laboratories, PCR is not considered to be a means for a definitive diagnosis and is rather used to identify the specific species once a diagnosis has been confirmed through other means (Yagupsky et al., 2020). Due to the disadvantages of all these methods of diagnosis, one of the most important means by which a diagnosis is made is via patient history (i.e., recent travel to a region endemic with *Brucella*, known laboratory contact, hunting activities, etc.) along with culture or serology.

Brucellosis can be a debilitating disease as symptoms last 3 to 6 months, and the infection is difficult to clear; however, several antibiotic regimes are useful: doxycycline and rifampin (oral) for 6 weeks, doxycycline (oral) for 6 weeks in combination with streptomycin (intramuscularly) for 2-3 weeks, doxycycline (oral) for 6 weeks in combination with gentamicin (intramuscularly) for 7-10 days, or streptomycin and gentamicin (intramuscularly) (Alavi & Alavi, 2013). It should be noted, however, that even with antibiotic treatment, infections with *Brucella* species can often take weeks to months to clear.

Exopolysaccharides in Bacteria

Exopolysaccharides are polysaccharides consisting of repeating units of sugar molecules, such as glucose, galactose, mannose, etc., secreted by bacteria into the extracellular environment. Exopolysaccharide can function in facilitating cellular attachment to both biotic and abiotic surfaces, protecting against environmental factors such as desiccation or high salinity, or by providing resistance to complement and the antimicrobial activities of macrophages and neutrophils (Leid et al., 2005; Mishra et al., 2012; Muñoz et al., 2019). Common forms of bacterial exopolysaccharides include capsules and biofilms, the latter of which is sometimes referred to as a "slime layer." Some sources distinguish between biofilms and slime layers, characterizing the former as a structure that contains bacterial cells and the latter as containing all the matrix components except living cells.

A capsule is an extracellular polysaccharide layer that covers the outer surface of the cell and is closely associated with the cell surface. Capsules are generally characterized as well-organized structures that are not easily dissociated from the outer surface of the bacteria. Bacterial capsules function to protect against environmental stress factors, such as desiccation and low nutrient availability, as well as against antimicrobial compounds and host immune defenses such as complement activation (Singh et al., 2019; S. Sharma et al., 2020; Buffet et al., 2021). For example, unlike most facultative pathogens, *Klebsiella pneumoniae* lacks a diverse repertoire of virulence factors but does produce a bacterial capsule that is highly effective in protecting the cells from antimicrobial molecules, making *K. pneumoniae* infections particularly difficult to clear with antibiotics (Rendueles, 2020).

Biofilms are similar to bacterial capsules in that they form a protective matrix, but biofilms are generally characterized as being highly heterogenous in composition and may

encompass more than one cell. Biofilms also often contain a mixture of exopolysaccharide, extracellular DNA (eDNA), and protein that form only a loose association with the cell surface, in contrast to the tight association with the cell surface as seen with capsules. It should be noted, however, that while capsule formation is not required for biofilm production, it can be one of the initiating steps (H. Wang et al., 2015).

Biofilms provide protection against environmental stresses and aid in the colonization and persistence of a bacterial species by facilitating attachment and protecting the bacteria from phagocytosis, antimicrobial agents, and other host defenses (Ciofu & Tolker-Nielsen, 2019; Thi et al., 2020). In *Pseudomonas aeruginosa*, for example, the bacteria themselves comprise less than 10% of the biofilm mass, with the remainder of the biofilm structure being composed of exopolysaccharides, proteins, eDNA, and lipids which results in producing a physical barrier that blocks interactions with host immune cells, antibodies, and components of the complement system (Gunn et al., 2016; Maurice et al., 2018). Because of these protective attributes, biofilm formation is one of the leading causes of chronic infections, and such infections are difficult to treat with the use of traditional antibiotics, particularly in hospital settings (D. Sharma et al., 2019).

Exopolysaccharides in α-Proteobacteria

As previously discussed, bacteria across all taxa produce exopolysaccharides that serve a variety of functions, including biofilm formation, capsule production, adhesion and attachment, resistance to killing by complement or antimicrobial molecules, and aid in the evasion of the immune system (Smith et al., 2003; Nwodo et al., 2012; Jones & Wozniak, 2017; Le et al.,

2018). Numerous members of the α-proteobacteria produce exopolysaccharides that contribute to the specific lifestyles of each individual species, and several examples are discussed below.

Sinorhizobium meliloti EPSI and EPSII

Sinorhizobium meliloti produces two exopolysaccharides: succinoglycan (known as exopolysaccharide I) and galactoglucan (known as exopolysaccharide II) that allow the bacteria to invade the inner root cortex and establish a symbiotic relationship with the plant host (Jones, 2012). Both exopolysaccharides are found in two forms: high-molecular-weight (HMW) and low-molecular-weight (LMW). It was previously reported that the LMW form of succinoglycan is responsible for host invasion, but more recent evidence indicates that functionality of this exopolysaccharide relies not on molecular weight but rather on whether the molecule is succinylated (Bertram-Drogatz et al., 1998; Mendis et al., 2016).

Exopolysaccharide I is composed of succinoglycan and is characterized by repeating octasaccharide units consisting of seven residues of D-glucose and one residue of D-galactose joined by β-1,3, β-1,4 and β-1,6 glycosidic linkages (Acosta-Jurado et al., 2021). Exopolysaccharide I plays a crucial role in the ability of *S. meliloti* to establish symbiosis with the host. Flavonoids produced by the plant host act as chemoattractants to draw *S. meliloti* to the plant roots. Also in response to plant flavonoids, *S. meliloti* expresses nodulation factors (NFs) that stimulate root hairs on the plant to curl around and engulf the bacteria that have congregated around the roots (Peleg-Grossman et al., 2007). The bacteria interfere with normal cell wall genesis, allowing a tube-like invagination, known as the infection thread, to form through which the bacteria invade the host (Gage, 2004). Although exopolysaccharide I has been shown to contribute to this process, the exact role that succinoglycan plays in infection thread development has yet to be determined (Jones et al., 2007; Jones, 2012; Maillet et al., 2020).

Exopolysaccharide II is composed of galactoglucan and is characterized by repeating disaccharide units of D-glucose and D-galactose in a molar ratio 1:1 linked by α-1,3 and β-1,3 bonds (Acosta-Jurado et al., 2021). *S. meliloti* must be able to withstand changing conditions characteristic of a soil environment. Exopolysaccharide II performs a protective role in hostile environments by increasing resistance to stress factors such as desiccation and high salinity (Primo et al., 2020). While the exact mechanism by which exopolysaccharide II protects against stress factors is unknown, it is speculated that it plays a role in biofilm formation and autoaggregation (Primo et al., 2020).

The regulation of exopolysaccharide production in *S. meliloti* is complex, but many of the proteins involved and their specific roles in this process have been identified. The two-component system ExoS/ChvI regulates the expression of exopolysaccharide genes responsible for the production of both succinoglycan and galactoglucan in *S. meliloti* and thus contributes to the establishment of symbiosis with the host, the transition between a mobile cell state to a planktonic state (known as the motile-to-sessile switch), and biofilm formation in this organism (Chen et al., 2008; Bélanger et al., 2009). While the transcription of exopolysaccharide genes is positively regulated by ExoS/ChvI, ExoR functions as a negative regulator of this process (Geiger et al., 2021). Disruptions to the domain in ExoR that interacts with ExoS results in increased production of exopolysaccharides in *S. meliloti* (Chen et al., 2008). Interestingly, under acidic conditions, ExoR is proteolyzed. Plant roots secrete organic acids and acid sugars regularly as metabolites or as a means to heal damaged tissues; thus, in the acidic environment that immediately surrounds plant roots, ExoR is mostly inactive, which allows the expression of exopolysaccharide genes that aid in the ability of *S. meliloti* to invade the plant host.

Caulobacter Holdfast

Another member of the α-proteobacteria, *C. crescentus* produces an adhesin, referred to as the holdfast, that allows for unipolar attachment to a surface and which is tightly controlled by cell-cycle regulators (Hardy et al., 2010; Berne et al., 2013; Fiebig et al., 2014; Hershey et al., 2019). *C. crescentus* has been used as a model organism to parse out the complicated regulation of cell cycle progression, to which approximately 20% of its genome is dedicated (Schrader et al., 2016). The lifecycle of *C. crescentus* depends on the organism's ability to moderate the transition from a mobile to a planktonic state (the motile-to-sessile switch).

Broadly speaking, *C. crescentus* exists in two forms: as motile swarmer cells and as stationary stalked cells. Swarmer cells possess a polar flagellum and pili and are fully motile but are unable to replicate. In order to replicate, *C. crescentus* must enter the sessile, stalked phase of the cell cycle, and this transition is dependent upon contact with a solid surface. Upon surface contact, motion of the flagellum is disrupted, and *C. crescentus* rapidly begins to generate a unipolar adhesin called the holdfast that allows the cell to attach to the detected surface (Snyder et al., 2020). This process has been shown to occur in a pilus-dependent manner, as when pilus retraction is blocked, *C. crescentus* is unable to initiate holdfast production (Berne et al., 2018).

The holdfast takes the form of a long protrusion from the cell surface with the adhesive exopolysaccharide located at the distal end of the structure. The stalk portion of the holdfast is a thin, cylindrical extension of the cell envelope (Sulkowski et al., 2019). The adhesive tip of the holdfast is comprised of an exopolysaccharide anchored to the stalk by a protein complex made up of the holdfast HfaABD proteins (Sulkowski et al., 2019). The exopolysaccharide portion of the holdfast has been reported to be a polysaccharide composed of glucose, 3-O-methylglucose, mannose, N-acetylglucosamine, and xylose residues (Hershey et al., 2019). Amazingly, the

adhesive properties of the holdfast surpass that of any known commercial or biological glue (Tsang et al., 2006; Berne et al., 2013b).

Upon synthesis of the holdfast, *C. crescentus* remains attached to the surface and enters the stalked phase of its cell cycle. Unlike swarmer cells, stalked cells are able to divide and replicate. Division occurs in a unipolar manner, whereby the daughter cell is generated at the pole opposite to where holdfast synthesis occurs on the original stalked cell. The pole of the cell at which division occurs is referred to as the "new pole," while the pole at which the holdfast is produced is referred to as the "old pole." The daughter cell generates a flagellum at the pole opposite to division before breaking off from the mother cell to become a non-divisional swarmer cell.

Regulation of the motile-to-sessile switch in *C. crescentus* is complicated and depends on a complex network of proteins that must be regulated in a spatial manner in order for unipolar holdfast synthesis and division to occur. This is partly accomplished through the production of cyclic di-GMP, a secondary messenger molecule that commonly plays a role in cell cycle progression, biofilm formation, and the motile-to-sessile switch in a variety of bacteria. Upon surface contact, PleD, a diguanylate cyclase that functions to synthesize cyclic di-GMP, is activated through phosphorylation by DivJ at the old pole of the cell (Paul et al., 2004; Abel et al., 2013; Lasker et al., 2016). In response to increased levels of cyclic di-GMP, the cell cycle histidine kinase CckA is activated, leading to a number of responses responsible for initiating cellular division and cell cycle progression, including holdfast production (Lori et al., 2015; Sprecher et al., 2017; Kaczmarczyk et al., 2020). The complete details of this process are complex and need not be expanded upon for the purposes of this review, but it is important to

note the role of cyclic di-GMP in this process as this molecule is commonly involved in exopolysaccharide synthesis in members of the α-proteobacteria.

***Agrobacterium* Exopolysaccharides**

The plant pathogen *A. tumefaciens*, a close relative of *Brucella* despite the discrepancies in lifestyles between plant and mammalian pathogens, produces several exopolysaccharides, including succinoglycan, cellulose, and a unipolar polysaccharide abbreviated as UPP. As is observed in *Sinorhizobium*, succinoglycan synthesis is negatively regulated by ExoR. The role of succinoglycan in the lifestyle of *A. tumefaciens* remains unclear as mutants unable to produce succinoglycan do not exhibit virulence defects; however, an *exoR* mutant results in over-production of succinoglycan, thereby inhibiting cellular motility (Tomlinson et al., 2010; Matthysse et al., 2014).

Cellulose and UPP both contribute to biofilm formation in *A. tumefaciens*. Cellulose plays a role in cellular attachment by forming a sticky matrix that allows both individual cells to bind tightly to the host cell as well as creates multicellular aggregates at the site of infection (Matthysse, 1983; Matthysse et al., 1995; Stredansky et al., 1998). As suggested by the name, UPP is responsible for unipolar attachment to the plant host cell, with the production of this exopolysaccharide being limited to only one pole of the cell. In the absence of cellulose, single cells of *A. tumefaciens* are able to bind to a surface, or a small number of cells may congregate into flower-shaped forms called rosettes bound together by the UPP produced by each cell, but the lack of cellulose production abrogates the formation of large cellular aggregates characteristic of biofilm formation (Xu et al., 2013).

Like in *C. crescentus*, cell growth in *A. tumefaciens* occurs in a polar fashion, with the pole at which adhesion occurs referred to as the old pole of the cell, and the opposite pole, where growth occurs, referred to as the new pole of the cell. UPP production only occurs at the old pole of the cell. Attachment with UPP is irreversible and is dependent upon surface contact; accordingly, UPP production is not observed in free-floating bacteria (Matthysse et al., 2014).

Once again mirroring *C. crescentus*, UPP production in *A. tumefaciens* is also regulated by cyclic di-GMP, although in *A. tumefaciens* this occurs through a pterin-dependent signaling pathway involving the pteridine reductase protein PruA (Feirer et al., 2015). PruA regulates UPP production by generating a pterin molecule that binds to a pterin-binding protein designated PruR. While the exact details of this interaction are currently unknown, PruR acts as a signaling intermediate between PruA and DcpA, a protein that can act as a diguanylate cyclase or a phosphodiesterase. In the presence of pterin-bound PruR, DcpA functions as a phosphodiesterase, which breaks down cyclic di-GMP, whereas in the absence of pterin-bound PruR, DcpA functions as a diguanylate cyclase, which generates cyclic di-GMP. UPP is produced when cyclic di-GMP levels are elevated.

Planktonic bacteria rarely produce UPP, which can make detecting UPP difficult under normal growth conditions (Matthysse et al., 2014). A Δ*pruA* mutant in *A. tumefaciens* is unable to produce the pterin required for PruR to interact with DcpA, therefore elevating cyclic di-GMP levels in the cell. Thus, a Δ*pruA* mutant can be used to artificially elevate UPP production in *A. tumefaciens*.

VisN and VisR, activators of flagellar motility in *A. tumefaciens*, also regulate UPP production by repressing the activities of various diguanylate cyclases, including DgcA, DgcB,

and DgcC (Xu et al., 2013). When these diguanylate cyclases are repressed, cyclic di-GMP levels are reduced, thereby suppressing UPP production; accordingly, increased biofilm production is observed in both a Δ*visN* or Δ*visR* mutant in *A. tumefaciens* (Xu et al., 2013).

MucR is a zinc-finger protein conserved across members of the α-proteobacteria that functions as a global transcriptional repressor implicated in regulating a variety of genes that contribute to establishing and maintaining the relationship between the bacteria and the eukaryotic host. More specifically, this protein has been linked to exopolysaccharide production in several members of the *Rhizobiales* (Fumeaux et al., 2014; Acosta-Jurado et al., 2021; Li et al., 2021; Jiao et al., 2022). MucR has been shown to regulate both succinoglycan and galactoglucan in *S. meliloti* in a quorum sensing-dependent manner (Bertram-Drogatz et al., 1998; Martín et al., 2000; Mueller & González, 2011). In *C. crescentus*, MucR mediates the cell cycle transition, which has been previously discussed as being involved in the generation of the holdfast (Fumeaux et al., 2014). The homolog for MucR in *A. tumefaciens* is known as Ros. Ros is required for the expression *exoY*, which encodes a glycosyltransferase gene involved in the initiating steps of exopolysaccharide synthesis (Mueller & González, 2011). Ros is elevated in an *exoR* mutant, which is characterized by mucoid colony formation due to the over-production of succinoglycan (Tomlinson et al., 2010).

Exopolysaccharides in *Rhizobium leguminosarum*

Rhizobium leguminosarum is a plant-associated member of the *Rhizobiales* responsible for nitrogen fixation in legumes. In *R. leguminosarum*, the process of cellular attachment relies on both cellular aggregation and unipolar attachment. Cellular aggregation allows a mass of bacterial cells to collect on the surface of the plant cell but does not involve binding to specific

receptors on the host cell. Unipolar attachment is achieved through specific interactions with the host cell. As in *A. tumefaciens*, cellulose contributes to cellular aggregation in *R. leguminosarum*. Individual cells that lack cellulose are still able to bind host root hairs but root hair "caps," clusters of *R. leguminosarum* that form on the tips of the root hair, are not produced (Williams et al., 2008).

Specific attachment to the host cell is due to the interactions of bacterial glucomannan with plant cell lectins, specifically pea (*P. sativum*) lectin (PSL) and vetch (*V. sativa*) lectin (VSL) (Laus et al., 2006). In a glucomannan mutant of *R. leguminosarum*, very few (<2%) of cells attach to root hairs on the host cell, indicating that glucomannan is responsible for attachment to the host (Williams et al., 2008). When *R. leguminosarum* cells are stained with either fluorescently labeled PSL or VSL, glucomannan can be visualized at a single pole of the cell, similar to what is observed when *A. tumefaciens* is incubated with fluorescently labeled WGA or DBA. The interaction between bacterial glucomannan and host cell lectins occurs under slightly acidic conditions (pH ~5.6); under alkaline conditions, plant lectins are soluble and are thus released from the root hairs, rendering bacterial attachment to these lectins unproductive under such conditions (Laus et al., 2006; Williams et al., 2008).

Exopolysaccharides in *Rhodopseudomonas palustris*

Genetic comparisons between *Rhodopseudomonas palustris*, another nitrogen-fixing bacterium belonging to the *Rhizobiales*, and *A. tumefaciens* reveal a high degree of genetic synteny between a putative cluster of genes in *R. palustris* predicted to synthesize an exopolysaccharide and the genes responsible for unipolar polysaccharide production in *A. tumefaciens* indicating that *R. palustris* may produce a similar unipolar adhesin (Fritts et al., 2017). In fact, when *R. palustris* is incubated with fluorescently labeled WGA, unipolar staining

is observed, and this staining is abrogated in mutants lacking key components of the putative UPP biosynthetic pathway (Fritts et al., 2017). Accordingly, it appears that both *A. tumefaciens* and *R. palustris* produce UPP that functions as an adhesin.

Exopolysaccharides in *Brucella*

Although information regarding exopolysaccharide production in many species of α-proteobacteria is abundant, relatively little is known about such production in *Brucella* species; nonetheless, there are some indications that *Brucella* species may produce an exopolysaccharide. As detailed earlier in this document, exopolysaccharides in bacteria commonly form protective structures known as a capsule (a layer of exopolysaccharide tightly bound to the outer surface of the cell) and biofilms (aggregates composed of bacteria as well as secreted materials such as polysaccharides, proteins, and eDNA). Exopolysaccharides may alternatively function as adhesins, which, unlike capsule or biofilm matrices, do not necessarily envelop the cell and can be confined to a particular region of the cell (for example, at a single pole). *Brucella* species are nonencapsulated bacteria that do not typically exhibit aggregation during *in vitro* cultivation; however, under specific conditions described below, cellular aggregation has been observed.

Experimental evidence suggests a negative correlation between quorum sensing and exopolysaccharide production in *B. melitensis*. VjbR is a quorum sensing-dependent transcriptional activator that regulates flagellar gene expression as well as the *virB* operon in response to *N*-acyl-homoserine lactones (AHLs). The *virB* operon encodes genes for the synthesis of a Type IV secretion system (T4SS) that secretes effector molecules to prevent fusion of the *Brucella*-containing vacuole with the phagolysosome in macrophages. When AHL is bound to VjbR, the *virB* operon is not activated. A *B. melitensis* mutant carrying a mutation to the quorum sensing regulator *vjbR* that renders VjbR unresponsive to AHL signaling displays a

clumping phenotype characterized by spontaneous aggregation in liquid culture, which is indicative of exopolysaccharide production (Uzureau et al., 2007). The presence of an exopolysaccharide was further confirmed in this mutant through staining with calcofluor white, a dye commonly used to detect exopolysaccharide production, and with fluorescently labeled concanavalin A (ConA) lectin, which indicates the presence of α-mannopyranosyl and α-glucopyranosyl residues (Uzureau et al., 2007). Production of this exopolysaccharide is not observed under normal *in-vitro* growth conditions, indicating that the exopolysaccharide genes are tightly regulated in *B. melitensis*. A clumping phenotype was not observed in an AHL-unresponsive *vjbR* mutant in *B. abortus*, but subsequent studies by another laboratory reported aggregation of a Δ*vjbR* mutant in *B. abortus* when grown under microaerophilic conditions, again suggesting that exopolysaccharide genes are only expressed under specific conditions not typically represented by normal *in-vitro* growth conditions (Almirón et al., 2013a). A clumping phenotype was also observed in *B. melitensis* when *aiiD* was overexpressed. AiiD is an enzyme that degrades AHL, further suggesting a link between exopolysaccharide production and the quorum sensing system in *B. melitensis* (Godefroid et al., 2010).

It should also be noted that the expression of flagellar genes, regulated by VjbR in *Brucella*, is also linked to exopolysaccharide production in other α-proteobacteria, including *A. tumefaciens* and *C. crescentus* (Heindl et al., 2014; Ellison et al., 2019). Interestingly, although *Brucella* species express genes required for flagellar protein synthesis, *Brucella* species are known to be non-motile as they do not possess the genes required for chemotaxis. Indeed, under specific nutrient conditions, a sheathed flagellar structure located at only one pole of the cells has been observed in *B. melitensis* using transmission electron microscopy (Fretin et al., 2005). The reason why *Brucella* expresses these flagellar genes but lacks the chemotaxis genes required for

mobility remains a mystery. As flagellar retraction is involved in both holdfast and UPP production in *S. meliloti* and *A. tumefaciens*, respectively, this raises an interesting possibility that the flagellar genes in *Brucella* may contribute to exopolysaccharide synthesis.

In *B. abortus*, a *wbkA* mutant also displays a clumping phenotype. WbkA is a glycosyltransferase previously implicated in O-polysaccharide synthesis (Mancilla et al., 2012). When subsequent researchers attempted to convert a rough strain of *Brucella* known as RB51 to a smooth phenotype by overexpressing *wbkA*, they expected that the overproduction of WbkA would result in increased O-polysaccharide synthesis to achieve this aim. Although overexpression of *wbkA* did not result in increased O-polysaccharide production, a clumping phenotype was observed in this strain (Dabral et al., 2015). Interestingly, in a separate study, proteomic and microarray analysis identified *wbkA* as a target of quorum sensing regulators, the only glycosyltransferase to be identified as such in the study, further linking the quorum sensing system to exopolysaccharide production in *Brucella species* (Uzureau et al., 2010). The exopolysaccharide observed in the *B. abortus wbkA* mutant was determined to be composed mainly of mannose but also contained galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine (Dabral et al., 2015). The fact that mannose is the main component of this exopolysaccharide is of interest as mannose residues on Gram-negative bacteria are known to bind to lectin molecules on their respective host cells, allowing for bacterial adherence (Eshdat et al., 1984; Dinadayala et al., 2006).

The expression of several genes predicted to function in polysaccharide biosynthesis and modification is increased in a *B. abortus* Δ*mucR* mutant, including three genes homologous to genes in *S. meliloti* that are known to contribute to succinoglycan production (Caswell et al., 2013). Congo red staining of the *B. melitensis* Δ*mucR* mutant results in increased uptake of the

Congo red dye. Increased Congo red staining has been observed in other members of the α-proteobacteria under conditions that result in the overproduction of exopolysaccharide, and thus the Congo red phenotype displayed by a *B. melitensis* Δ*mucR* mutant further suggests that *Brucella* strains have the genetic capacity to produce exopolysaccharides (Mirabella et al., 2013).

Brucella also encodes a two-component system known as BvrR/BvrS that is similar to the two component systems ExoS/ChvI in *S. meliloti* and ChvG/ChvI in *A. tumefaciens*, both of which regulate exopolysaccharide synthesis in their respective organisms (Sola-Landa et al., 1998; Wells et al., 2007). While no direct link between BvrR/BvrS and exopolysaccharide production in *Brucella* has been described in the literature thus far, the similarity of this two-component system to other two-component systems in closely related bacteria warrants further investigation. In fact, our laboratory has observed a Congo red phenotype in a *B. abortus* Δ*bvrRS* mutant (data unpublished), implicating a role for BvrR/BvrS in exopolysaccharide production. *B. abortus* also produces an ortholog for ExoR, which regulates BvrR/BvrS in *Brucella* and ExoS/ChvI and ChvG/ChvI in *S. meliloti* and *A. tumefaciens*, respectively. Although the ortholog for ExoR in *Brucella* has been reported to be non-essential for virulence, this does not rule out the possibility of its involvement in exopolysaccharide synthesis (Castillo-Zeledón et al., 2021).

As previously discussed, cyclic di-GMP contributes to UPP production in *A. tumefaciens* through the generation of a pterin molecule, synthesized by PruA, that binds to the protein PruR. VisN and VisR also influence cyclic di-GMP levels in *A. tumefaciens* by repressing the diguanylate cyclases DgcA, DgcB, and DgcC. Although there is a homolog for *pruA* in *B. abortus*, no homolog for *pruR* has been identified. While there is also no homologous protein for VisR in *B. abortus*, VisN shares 45% homology with the quorum sensing-dependent transcriptional activator VjbR, previously discussed in this section. Accordingly, based on

homology with *A. tumefaciens* alone, it is unclear whether the regulation of cyclic di-GMP would contribute to exopolysaccharide production in *B. abortus*. It should be noted, however, that in a knockout mutant of the phosphodiesterase *bpdA* (responsible for degrading cyclic di-GMP) in *B. melitensis* displays increased crystal violet staining indicative of biofilm formation, providing additional evidence that *Brucella* strains have the genetic capacity to produce exopolysaccharides (M. Khan et al., 2016).

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Chapter Two

A Wzx-Wzy-like Polysaccharide Biosynthetic Pathway Contributes to Virulence in *Brucella abortus* 2308 through an Unknown Mechanism

Biosynthetic Pathways for Exopolysaccharide Production

Bacteria employ one of four main strategies to produce exopolysaccharide, namely through the use of 1) extracellular enzymes, 2) synthase-dependent systems, 3) an ABC-transporter system, or 4) a Wzy-dependent assembly complex. As the name suggests, the first mechanism uses secreted enzymes that assemble the exopolysaccharide on the outer surface of the cell without the involvement of any membrane-bound proteins. The remaining three systems require proteins embedded in both the inner and outer membranes of the bacteria and are diagramed in Figure 1. In synthase-dependent pathways, synthesis and transport of the polysaccharide across the inner membrane occur at the same time (Pérez-Burgos et al., 2020). Products generated from this system tend to be involved in biofilm formation or protection of the bacteria against the host immune system (Whitfield et al., 2020).

ABC-transporter pathways and Wzy-dependent pathways are similar. In both systems, polysaccharide synthesis is initiated by the transfer of a sugar-1-P from a UDP-sugar to an undecaprenyl phosphate (Und-P) molecule located on the cytoplasmic face of the inner membrane (Pérez-Burgos et al., 2020). The main difference is that synthesis of a polysaccharide in an ABC-transporter system is completed on the cytoplasmic side of the inner membrane, and the entire complete molecule is translocated across the inner membrane by the ABC transporter and then through the outer membrane exporter. In the Wzy-dependent pathway, only a portion of the polysaccharide is made on the cytoplasmic side of the inner membrane. A flippase transfers the polysaccharide fragment to the periplasm. These fragments are made available to the polymerase and co-polymerase proteins, which are imbedded in the inner membrane and which function to grow the polysaccharide using repeating units of the molecule transferred by the

flippase. As the polysaccharide grows, it is exported through a protein located in the outer membrane of the bacteria.

Wzx-Wzy Pathway

The biosynthetic pathway for UPP in *A. tumefaciens* is classified as a Wzy pathway, also known as the Wzx-Wzy pathway. The Wzx-Wzy pathway is comprised of the following components: 1) various glycosyltransferases and acetyltransferases located in the cytoplasm that modify the polysaccharide anchored to the PHPT protein, 2) a polyisoprenylphosphate hexose-1-phosphate transferase (PHPT) located in the inner membrane; this protein links undecaprenyl diphosphate (Und-PP) to hexose molecules that form the nascent polysaccharide, thereby acting as an anchor for this polysaccharide molecule, 3) a flippase protein that transfers the polysaccharide unit from the cytoplasmic surface of the inner membrane to the periplasmic surface of the inner membrane; this protein is designated as Wzx; 4) a polymerase molecule located in the outer membrane that grows the polysaccharide using units transferred to the periplasm by the flippase; this protein is designated as Wzy, 5) a co-polymerase that functions as a chain-length determinator for the growing polysaccharide; this protein is designated as Wzz, 6) and a transporter molecule that spans the outer membrane and is responsible for the transfer of the polysaccharide to the outer surface of the cell; this protein is designated as Wza. The genetic organization of the *upp* genes and a schematic for the organization and location of the UPP proteins in *A. tumefaciens* are shown in Figures 2 and 3, respectively.

Putative UPP Proteins in *Brucella abortus* 2308

Previous phylogenetic analysis indicated that several plant-associated species belonging to the group *Rhizobiales* are capable of producing UPP, but it was speculated that animal

pathogens in this group (including *Brucella* species) lacked the capacity to do so (Fritts et al., 2017). At the time this analysis was performed, only *uppA*, *uppB*, *uppC*, *uppD*, *uppE*, and *uppF* had been identified as potential *upp* biosynthetic genes in *Rhizobiales* species. The genes *uppA*, *uppD*, and *uppF* were determined to be absent from *Brucella* species, and *uppB* is only present in a truncated form, leading to the conclusion that *Brucella* species are incapable of producing UPP. Recently, additional UPP biosynthetic proteins have been identified in the closely related species *A. tumefaciens* (Onyeziri et al., 2022). Based on this additional knowledge of UPP production in *A. tumefaciens*, genetic analysis indicates that *B. abortus* 2308 does contain a complete set of genes that would fulfill the biosynthetic requirements for production of a similar exopolysaccharide, listed in Table 1 (Feirer et al., 2015; Onyeziri et al., 2022). Accordingly, we investigated whether these genes do indeed produce UPP and whether they affect the virulence of *B. abortus* 2308. The genetic organization of the putative *upp* genes and a schematic for the organization and location of the putative UPP proteins in *B. abortus* 2308 are shown in Figures 2 and 4, respectively.

UPP production begins with the conversion of glucose into glucose-6-phosphate (Glc-6-P), followed by conversion of Glc-6-P into to glucose-1-phosphate (Glc-1-P) by ExoC, a phosphoglucomutase that is involved in multiple polysaccharide biosynthetic pathways (Onyeziri et al., 2022). Glc-1-P is acetylated to produce *N*-acetyl glucosamine-1-phosphate (GlcN-1-P) by either UppA or UppQ. ExoN or GalU conjugate UDP to GlcN-1-P to form UDP-*N*-acetyl glucosamine (UDP-GlcNAc). It appears that *A. tumefaciens* produces two types of UPP: one composed of units derived from glucosamine and one composed of units derived from galactosamine. To generate galactosamine-derived UPP, GalE reversibly epimerizes UDP-GlcNAc to UDP-GalNAc. This process is shown in Figure 3. *B. abortus* 2308 encodes homologs

for *exoC* (BAB1_0055), *exoN*/*galU* (BAB2_0070), and *galE* (BAB1_1088), indicating that *B. abortus* 2308 has the genetic capacity to generate the precursor molecules for UPP production.

UDP-GlcNAc and UDP-GalNAc are further modified by glycosyltransferases, namely UppD, UppG, UppI, UppJ and UppL, and acetyltransferases, namely UppA, UppQ, and UppZ. Homologs for *uppJ*, *uppL*, and *uppQ* have been identified in *B. abortus* 2308 and are designated by the genes BAB2_0105, BAB2_0101, and BAB1_1948.

The polyisoprenylphosphate hexose-1-phosphate transferase (PHPT) (also known as a WbaP-like initiating glycosyltransferase) residing in the inner membrane of the cell is referred to as UppE in *A. tumefaciens*. UppE is responsible for adding the first monosaccharide unit to an undecaprenol pyrophosphate (Und-PP) lipid carrier on the cytoplasmic surface of the inner membrane (Onyeziri et al., 2022). Δ*uppE* and Δ*uppC* mutants of *A. tumefaciens* yielded the strongest biofilm phenotypes, indicating a reduction in exopolysaccharide production. The homolog for *uppE* in *B. abortus* 2308 is BAB1_0802 (Table 1). Interestingly, BAB1_0800, upstream of *uppE*, is homologous to *mlrA*. MlrA is a MerR-like transcriptional factor. In *E. coli*, MlrA interacts with cyclic di-GMP (known to regulate UPP production in *A. tumefaciens*) to activate genes required for the production of curli fibers, which contribute to adhesion and biofilm formation (Lindenberg et al., 2013).

Two Wzx-type polysaccharide flippases, designated as UppV and UppX, have been identified in *A. tumefaciens*. The Δ*uppX* mutant demonstrated a significant loss of biofilm formation, whereas the Δ*uppV* mutant indicated a moderate loss (Onyeziri et al., 2022). Wzx proteins are highly specific to their substrate and display the greatest genetic diversity of any protein in the Wzx-Wzy pathway between different bacterial species (Hong et al., 2015). No

homolog for the Wzx-type flippase has been identified in *Brucella*; however, due to the large degree of sequence diversity between proteins of this type, the fact that a flippase has not been identified based on homology does not necessarily indicate that *Brucella* species lack this protein.

Two Wzy-like polymerases have been identified in *A. tumefaciens*: UppW and UppY. UppW is responsible for generating UPP derived from glucosamine, and UppY is responsible for generating UPP derived from galactosamine. Only one homolog in *B. abortus* (BAB2_0106) has been identified, and whether this homolog produces a glucosamine or galactosamine-derived polysaccharide (or potentially a polysaccharide of a different composition) is yet to be determined (Table 1).

Two Wzz-like chain-length determinant co-polymerases have also been identified in *A. tumefaciens*, designated UppB and UppM. Unlike UppW and UppY, UppB and UppM do not appear to be specific to *N*-acetyl glucosamine or *N*-acetyl galactosamine-derived UPP and can be involved in either pathway. These proteins are located in proximity to the Wzy-like polymerase and function to control the length of the growing polysaccharide chain. One homolog, BAB2_0099, has been identified in *B. abortus* 2308 (Table 1).

The Wza-like outer membrane exporter protein (OPX) in *A. tumefaciens* is referred to as UppC. UppC is responsible for depositing UPP on the outer surface of the cell, and, like the Δ*uppE* mutant, the Δ*uppC* mutant displays a strong biofilm phenotype characterized by little to no detection of exopolysaccharide synthesis in a crystal violet staining assay. The Wza-like UppC protein is the only canonical member of the Wzy-pathway that appears to have one or more extracellular domains. The homolog for *uppC* in *B. abortus* 2308 is BAB1_0804 (Table 1).

As shown in Figure 2B, the gene BAB1_0803 lies between *uppE* and *uppC* in *B. abortus* 2308. In *A. tumefaciens*, the gene between *uppE* and *uppC* is identified as the glycosyltransferase *uppD*. Interestingly, a Δ*uppC* mutant in *A. tumefaciens* cannot be complemented with *uppC* alone (Onyeziri et al., 2022). In order to restore biofilm production in this mutant, both *uppC* and *uppD* must be expressed. The *B. abortus* 2308 gene BAB1_0803 lies in the same position as *uppD* in *A. tumefaciens* but is significantly truncated and lacks the glycosyl transferase catalytic motif present in *uppD* in *A. tumefaciens*; however, when a plasmid carrying *uppC* and BAB1_0803 from *B. abortus* 2308 is expressed on a plasmid and introduced into *A. tumefaciens* Δ*uppC*, the Δ*uppC* mutant is fully complemented (unpublished data from the laboratory of Clay Fuqua at Indiana University Bloomington). This suggests that BAB1_0803 from *B. abortus* 2308 may be homologous to *uppD* in *A. tumefaciens*, although how this gene could be functional is unclear since it lacks the glycosyl transferase domain. Alternatively, expression of *uppD* may not be required for the activity of *uppC* derived from *B. abortus* 2308.

At the early stages of UPP production, UppE is responsible for anchoring the nascent UPP molecule to the inner membrane, and UppC plays a pivotal role in this biosynthetic pathway by transporting UPP to the extracellular surface of the cell. In studies using *A. tumefaciens*, the starkest biofilm phenotypes were observed in the Δ*uppC* and Δ*uppE* mutants. Accordingly, we generated a Δ*uppCE* mutant in *B. abortus* 2308 for this study.

Although relatively little is known about exopolysaccharide production in *Brucella* species, recent studies indicate that *Brucella* species may be capable of producing such molecules (Uzureau et al., 2007; Godefroid et al., 2010; Almirón et al., 2013; Caswell et al., 2013). Genes predicted to contribute to UPP synthesis have been identified in several members of the *Rhizobiales* (Fritts et al., 2017). Recently, additional genes involved in UPP production

have been identified in *A. tumefaciens*, and this information has further contributed to our understanding of how this exopolysaccharide is made (Onyeziri et al., 2022). We aim to explore whether *B. abortus* 2308 produces UPP and how production of this exopolysaccharide may contribute to virulence in this organism.

Materials and Methods

Bacterial strains and culture conditions

The plasmids and strains used in this study are listed in Table 2. Liquid cultures of *B. abortus* 2308 and its derivative mutants were routinely grown in brucella broth and were incubated at 37°C with shaking at 250 rpm. Plated cultures of *B. abortus* 2308 and its derivative mutants were grown on Schaedler agar containing 5% bovine blood (SBA) and were incubated at 37°C under 5% CO_2. Kanamycin (45 µg/mL) was added to the medium, as necessary, for the selection of strains carrying a kanamycin-specific resistance marker. In assays requiring minimal medium, ATGN was used and prepared using the formula shown in Table 3 (Morton & Fuqua, 2012). ATGN is a minimal medium that provides an environment in which the bacteria receive limited nutrients but still allows for growth without inducing a stress response, therefore reducing the probability of auxotrophic mutant formation (Morton & Fuqua, 2012).

Construction of the *Brucella abortus* 2308 Δ*uppCE* mutant

An isogenic mutant containing an in-frame deletion of *B. abortus* 2308 BAB1_0802-BAB1_0805 was created using the unmarked gene excision strategy described by Caswell et al. (Caswell et al., 2012). A fragment approximately 1500 base pairs in size upstream of *uppE* (BAB1_0802) that included the first eight base pairs of this gene was amplified via PCR with the primers listed in Table 4. A BamHI restriction site was included at the 5' end of this fragment. Another fragment of approximately 1500 base pairs downstream of *uppB* (BAB1_0805) that included the last six base pairs of the gene was amplified via PCR using the primers listed in Table 4. A PstI restriction site was included at the 3' end of this fragment. The upstream and

downstream fragments were digested with the restriction enzymes BamHI and PstI, respectively, and a T4 polynucleotide kinase reaction in the presence of ATP was used to phosphorylate the blunts ends at the 3' end of the upstream gene fragment and the 5' end of the downstream fragment to allow for ligation. See Figure 5 for a schematic illustrating the deleted region.

The upstream and downstream fragments were ligated with T4 ligase, and the resulting fragment was ligated into the vector pNTPS138 that had been previously digested with BamHI and PstI. The plasmid pNTPS138 is a commonly used gene replacement vector, i.e., a vector used for allelic exchange, that carries a *sacB*-based gene that confers sucrose sensitivity for selection (West et al., 2002). The resulting plasmid was transformed into *B. abortus* 2308 using electroporation, and merodiploid transformants were selected on SBA containing kanamycin (45 μg/mL). Single kanamycin-resistant colonies were allowed to grow for 5-6 hours in brucella broth, and the resulting liquid culture was plated onto SBA plates containing 10% sucrose. Colonies that grew on SBA plates containing 10% sucrose were patch plated onto fresh SBA-kanamycin and SBA-sucrose plates, and colonies confirmed to be both sucrose-resistant and kanamycin-sensitive were grown overnight at 37°C with shaking at 250 rpm in brucella broth. Genomic DNA was isolated from these samples, and PCR was used to screen for the loss of the gene of interest. Putative mutants were finally confirmed by DNA sequencing.

Plasmid-borne complementation of *uppCE* locus

Nucleotide primers listed in Table 4 were used to amplify the region containing BAB1_0802 to BAB1_0805 from *B. abortus* 2308 genomic DNA using PCR, and this DNA fragment was cloned into pMR10, a low copy number plasmid, carrying kanamycin resistance. The resulting plasmid was then transformed into the Δ*uppCE* strain using electroporation, and

the strain carrying the plasmid was selected by screening on SBA plates containing kanamycin. This strain is referred to as DH05. See Figure 5 for a schematic illustrating the reconstructed region.

Genetic reconstruction of the *Brucella abortus* 2308 Δ*uppCE* mutated locus

Nucleotide primers listed in Table 4 were used to amplify the region containing BAB1_0802 to BAB1_0805 from *B. abortus* 2308 genomic DNA using PCR, and this DNA fragment was cloned into pNPTS138 carrying kanamycin resistance. The resulting plasmid was then transformed into JEP8 (the Δ*uppCE* strain) using electroporation, and selection of the desired strain was performed as described above to generate a strain carrying *uppCE* restored to the chromosome, referred to as DH06. See Figure 5 for a schematic illustrating the reconstructed region.

Construction of a *Brucella abortus* 2308 derivative that overproduces the diguanylate cyclase *pleD* (PleD+) from *Agrobacterium tumefaciens* upon IPTG induction

A plasmid carrying *pleD* from *A. tumefaciens* cloned into pSRKKm (pJW110) was provided by the laboratory of Clay Fuqua at Indiana University Bloomington (Table 2, Xu et al., 2013). This plasmid contains an *aphII* kanamycin resistance gene for selection and a *lacI* promoter site to render *pleD* expression inducible by IPTG (P_{lac}-*pleD*) (Khan et al., 2008). Plasmid pJW110 was transformed into *B. abortus* 2308, and this strain is designated as DH07 (Table 2). When used, DH07 was induced with 1mM IPTG and grown in medium containing kanamycin at a concentration of 45 μg/mL for selection.

Construction of the *Agrobacterium tumefaciens* strains carrying *uppC* and *uppE* from *Brucella abortus* 2308

Separately, *uppC* (BAB1_0804) and *uppE* (BAB1_0802) were PCR-amplified from *B. abortus* 2308 DNA. NdeI and SpeI restriction sites were encoded on the upstream and downstream ends of the fragments, respectively, to allow ligation into a plasmid. After restriction digestion, the resulting fragments were ligated into pSRKKm, which carries the *aphII* kanamycin resistance gene for selection and a *lacI* promoter site to render expression inducible by IPTG (Khan et al., 2008).

A. tumefacien C58 was grown on ATGN plates containing 1.5% agar for two days at 28°C. Electrocompetent cells were generated by scraping growth off these plates into 2-mL of LB medium, followed by incubation overnight at 28°C with shaking. A 50-μL aliquot of this overnight culture was added to 5-mL of LB broth and incubated at 28°C with shaking until the cultures reached an OD_{600} of 0.6-0.7. The cell cultures were centrifuged, and the resulting pellet was resuspended in 1-mL of ice-cold, sterile water. The process of re-suspension in water and pelleting was repeated an additional four times. The resulting solution was resuspended in 250-μL of ice-cold, sterile 30% glycerol in water, and the cells were stored at -80°C until use.

To transform the cells, 40-μL of electrocompetent cells were incubated with 2 to 10-μL of the plasmid, depending on the DNA concentration, on ice for 5 to 10 minutes. 60-μL of sterile water was added, and the mixture was transferred to an electroporation cuvette. The cells were electroporated at 25 volts/cm and were immediately added to 600-μL of cold LB medium in a sterile culture tube.

The cells were then incubated at 28°C with aeration for 90 minutes. Following incubation, the cells were centrifuged, the supernatant was removed, and the pellet was resuspended in 100-μL of ATGN. The resulting cell suspension was plated onto ATGN plates containing 1.5% agar and kanamycin at a concentration of 300-μg/mL for selection of colonies containing the pSRKKm plasmid, and the plates were incubated at 28°C for 2-4 days. Colonies were confirmed to contain the pSRKKm plasmid harboring the appropriate *Brucella*-derived genes using PCR.

Empty pSRKKm plasmid was also transformed into electrocompetent *A. tumefacien* C58, *A. tumefacien* Δ*uppC*, and *A. tumefacien* Δ*uppE* (prepared as described above) for use as controls in the crystal violet complementation experiments.

Complementation of *Agrobacterium tumefaciens* Δ*uppC* and Δ*uppE* with plasmid-borne *uppC* and *uppE*, respectively, from *Brucella abortus* 2308

Cell cultures were grown in ATGN overnight at 28°C with shaking and were adjusted the following day to an OD_{600} of 0.05 in ATGN medium containing kanamycin (300 μg/mL) and IPTG (400 μM). 22x22 mm PVC coverslips were inserted vertically into the wells of 12-well polystyrene plates previously sterilized by UV exposure for 20 minutes. The wells of these plates were filled with 3-mL of bacterial cell culture, and the plates were incubated at 28°C without shaking for 48 hours in the presence of an open bottle of saturated K_2SO_4 solution to reduce sample evaporation.

After 48 hours, the coverslips were removed and rinsed with water to wash off any residual cell culture. The coverslips were then stained with 0.1% crystal violet in water for 20 minutes at room temperature. While the coverslips were incubating in crystal violet, the cell

cultures remaining in the wells of the plate were pipetted up and down for thorough mixing, and a portion was removed to read the OD_{600} on a Biowave II spectrophotometer.

After the 20-minute incubation period, the coverslips were rinsed with water to remove any crystal violet not bound to the coverslip. The coverslips were inserted into the wells of a clean 12-well polystyrene plate containing 3-mL of 33% acetic acid in water, and this solution was pipetted over both sides of the coverslips to ensure that all crystal violet was dissolved. Upon removal of the coverslips, the remaining solution was again pipetted to thoroughly mix the dissolved crystal violet in the samples. The absorbance value (A_{600}) was read for each sample using a Biowave II spectrophotometer.

Crystal violet staining assay

When using the crystal violet staining assay as described above with *Brucella* strains, no crystal violet staining was detected on the coverslips for any strains analyzed, including the *B. abortus* 2308 Δ*mucR* mutant, which is predicted to exhibit increased polysaccharide production. We determined, however, that an alternative crystal violet staining assay wherein cultures were grown in a 96-well plate and crystal violet staining was based on the residue left in the wells yielded positive results when using *B. abortus* 2308 Δ*mucR* as the control. This assay is described below (O'Toole, 2010).

Strains were grown overnight in brucella broth and were adjusted to an OD of 1.65 (mid-exponential phase) the following day. 175-µL of each culture was added to four wells of a 96-well plate, and the plate was incubated at 37°C under 5% CO_2 for 48 hours. After incubation, the cultures were removed by overturning the plate and shaking out the remaining liquid. The plate was submerged under water, and the plate was once again overturned and shaken to remove the

liquid. This step was repeated a second time. 200-µL of 0.1% crystal violet dissolved in water was added to each well, and the plate was incubated at room temperature for 20 minutes. The plate was rinsed with water four times, as previously described, and the plate was turned upside down and tapped onto a dry paper towel to remove any remaining water. After drying, 225-µL of 30% acetic acid in water was added to each well, and the plate was incubated at room temperature for 20 minutes. The liquid in each well was pipetted up and down to fully dissolve the remaining crystal violet in the acetic acid solution. The plate was read on an ELx800™ Absorbance Microplate Reader at 550nm using 30% acetic acid in water as the blank.

Congo Red staining assay

Strains were grown overnight in brucella broth and were back-diluted to an OD of 0.1 or 1.0 the following day. 25-µL of each strain was deposited in triplicate onto tryptic soy agar plates containing 0.01% Congo red (made from a 1% stock solution dissolved in a 50/50 methanol/water solution). Plates were incubated at 37°C under 5% CO_2 for 8 days.

Microscopy with fluorescently labeled lectins

Cell cultures were grown to either mid-exponential or stationary phase. A 99-µL aliquot was transferred to a sterile Eppendorf tube, centrifuged, and re-suspended in ATGN. As a minimal medium, ATGN is ideal for use in lectin-staining microscopy as the monosaccharide content is limited to a low concentration of glucose (0.5%), thereby reducing interaction with the lectins used in staining. Although this medium is commonly used to culture *A. tumefaciens*, our lab has observed that *B. abortus* 2308 is also able to survive and grow in this medium.

The ATGN re-suspended pellet was then mixed with 1-µL of a stock solution (1 mg/mL) of wheat germ agglutinin (WGA) labeled with fluorescein. This solution was incubated at room

temperature under low-light conditions for 20 minutes. Cells were centrifuged, and the pellet was re-suspended in 100-µL of ATGN, and 5-µL of this cell suspension was pipetted onto an agarose pad (1.5% agarose). 5-µL of VECTASHIELD® Antifade Mounting Media (Vector Laboratories) was added on top of the cell suspension to help prevent bleaching of the fluorophore, and a coverslip was placed over the cell suspension and sealed with slide sealant. Samples were observed using fluorescence microscopy (Zeiss Axiocam 705) under a 100x oil immersion objective.

Cyclic di-GMP quantification

Cultures were grown to either mid-exponential or stationary phase. For strains carrying the P_{lac}-*pleD* plasmid, kanamycin was added at a concentration of 45 µg/mL and IPTG was added at a concentration of 1mM to the induced samples. 1-mL of cells was collected in a Eppendorf tube and centrifuged to achieve a pellet. The supernatant was removed, and the pellet was resuspended in 100-mL of extraction buffer (40% methanol, 40% acetonitrile, and 0.1 N formic acid). The samples were incubated at -20°C for 30 minutes and then centrifuged for 5 minutes to obtain a pellet. The supernatant was transferred to a clean Eppendorf tube, and after a 7-day process to confirm that the supernatants were sterile, the samples were sent to the laboratory of Christopher Waters at Michigan State University for UPLC-MS/MS analysis.

Virulence of *Brucella* strains in cultured murine macrophages

Assays to determine the ability of *B. abortus* 2308 strains to survive and replicate within bone-marrow derived macrophages obtained from 6- to 8-week old C57BL/6 mice were performed as per the referenced protocol (de Chastellier et al., 1993). Briefly, bone marrow from the femur, tibia, and fibula bones of 1-2 C57BL/6 mice were collected by flushing the bones with

DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (referred to as wash medium). The cells contained therein were differentiated into mature macrophages over the course of 6 days by culturing the cells in wash medium supplemented with 20% L929 supernatant at 37°C under 5% CO_2 (de Chastellier et al., 1993). L929 is a fibroblast cell line originally isolated from the normal subcutaneous areolar and adipose tissue of a cloned male C3H/An mouse(Cooper et al., 1984; Heap et al., 2021). L929 cells secrete macrophage colony-stimulating factor (M-CSF) which drives hematopoietic stem cells to differentiate into macrophages; thus, supernatant collected from cultured L929 cells can be used to differentiate the hematopoietic stem cell collected from murine bone marrow into macrophages for an infection assay (Heap et al., 2021).

The differentiated macrophages were collected and used to seed the wells of a 96-well plate at a concentration of $1x10^5$ cells/well in antibiotic-free DMEM supplemented with 10% (FBS). After a 24-hour time period to allow for the macrophages to attach to the wells, the wells were infected with the appropriate strain of *Brucella* at an MOI of 100. At 30 minutes post-infection, the medium was replaced with fresh medium containing gentamycin (50 μg/mL) to eliminate extracellular bacteria, and after the 2-hour timepoint, this medium was replaced with medium containing gentamycin at a reduced concentration of 10 μg/mL. At each 24-hour interval, the medium in the wells was removed and replaced with medium containing gentamycin (10 μg/mL).

At 2-, 24-, and 48-hours post-infection, the wells were washed with phosphate-buffered saline (PBS), and 0.1% sodium deoxycholate in PBS was added to lyse the macrophages. The resulting lysate was serially diluted and plated in triplicate at a volume of 25-μL on Schaedler

agar containing 5% bovine blood (SBA). Colonies were counted after 3-4 days of growth at 37°C under 5% CO_2.

Described in detail in Chapter 1 of this document, VjbR is quorum-sensing regulator that represses the *virB* operon, which is responsible for encoding the protein components of the T4SS that excretes effector molecules to prevent fusion of the BCV with the phagolysosome in macrophages; thus, a Δ*vjbR* mutant is highly attenuated in macrophages and was used as a control in the initial assay for comparison of the Δ*uppCE* mutant against a strain known to display a strong virulence defect.

Virulence of *Brucella* strains in infected mice

In-vivo virulence assays were conducted in mice to evaluate the ability of *B. abortus* 2308 strains to establish a chronic infection in the host. Briefly, 6- to 8-week old C57BL/6$^{Nramp+/+}$ mice were injected with 100-μL of a $1x10^5$ CFU/mL solution of the appropriate strain of *Brucella*. C57BL/6$^{Nramp+/+}$ mice have been consistently and reliably used by our lab and others to evaluate the capacity of *Brucella* and other intracellular bacterial species to establish and maintain a chronic infection in mice (Arpaia et al., 2011; Johnsrude et al., 2020). Mice were divided into groups of five mice per strain per time point. At 1-, 3-, and 5-weeks post-infection, the mice were euthanized, and the spleens were collected and homogenized in 10-mL of PBS. The spleen homogenate was serially diluted in PBS and plated in triplicate at a volume of 25-μL on Schaedler agar containing 5% bovine blood (SBA). Colonies were counted after 3-4 days of growth at 37°C under 5% CO_2.

Western Blot analysis using the α-UppC antibody

Whole, native UppC protein from *B. abortus* 2308 was purified as described below and sent to LabCorp. (Denver, PA, USA) for antibody production. Briefly, BAB1_0804 (*uppC*) that was PCR amplified from *Brucella abortus* 2308 genomic DNA was cloned into the anhydrotetracycline-inducible *E. coli* expression vector pASK-IBA2C (IBA-Lifesciences) via restriction cloning (BsaI). This created a recombinant UppC (rUppC) expression construct containing an N-terminal OmpA signal peptide to direct the expressed protein to the periplasmic space and a C-terminal Strep-tag® II for protein purification. The rUppC expression construct was then transformed into *E. coli* BL21(DE3) cells, 100 mL of which was grown to exponential-phase, and rUppC expression was induced overnight at 16°C. After pelleting the cells, the periplasmic fraction was isolated via osmotic shock as described in Ausubel & Albright, 1989. SDS-PAGE was used to confirm induction. rUppC was then isolated from the periplasmic fraction via Strep-Tactin affinity purification according to manufacturer's suggested protocols (IBA-Lifesciences).

Purified rUppC was sent to LabCorp for antisera production in New Zealand White rabbits. Briefly, the rabbits were initially injected with 250-µg of rUppC that was emulsified with Titermax Classic as an adjuvant. The rabbits received subcutaneous boosts (125-µg of rUppC emulsified with Titermax Classic) at 21, 42, and 63 days after the initial injection, and production bleeds were taken at 31, 52, and 73 days after the initial injection.

For the Western Blot analysis, *B. abortus* 2308 and Δ*uppCE* were grown on SBA plates for 3 days. Colony growth was scraped from the plates, suspended in 1X PBS, and adjusted to an approximate OD_{600} value of 1.0. The cell cultures were resuspended in an equal volume of 1:1

ethanol:acetone to ensure killing of the brucellae. The samples were then pelleted, resuspended in lysis buffer (0.3% SDS, 20mM DTT, 100mM NaCl, 25mM Tris, 10% glycerol, pH 8), and boiled 30 minutes. Samples were then separated on a 12.5% SDS gel and transferred to a 0.45 µm nitrocellulose membrane. The nitrocellulose membrane was blocked overnight at room temperature in blocking buffer (1X PBS, 0.1% TWEEN 20, 5% BSA, pH 7.4) and then incubated with α-UppC antisera (1:1000 in blocking buffer) overnight at room temperature.

The nitrocellulose membrane was washed three times with wash buffer (1X PBS, 0.1% TWEEN 20, pH 7.4) and then incubated with a secondary antibody (goat α-rabbit-HRP 1:2000 in 1X PBS, 0.1% TWEEN 20, pH 7.4) for five hours at room temperature. The membrane was washed three times using wash buffer (1X PBS, 0.1% TWEEN 20, pH 7.4).

The secondary antibody was detected via chemiluminescence using Thermo Scientific SuperSignal West Pico PLUS Chemiluminescent Substrate under the manufacturer's suggested guidelines. The resulting Western Blot was visualized in a Biorad ChemiDoc after 2 minutes of exposure.

Biosafety level 3 working conditions

All experiments using live *Brucella* strains were conducted in a biosafety level 3 (BSL3) laboratory certified by the Centers for Disease Control and Prevention's Division of Select Agents and Toxins.

Statistical analysis

All statistical analyses were performed using the Student's two-tailed t test. P values of less than 0.05 were considered significant.

Results

Brucella abortus 2308 carries the genes required for EPS synthesis via a Wzx-Wzy pathway.

Comparative genetic analysis of the *B. abortus* 2308 genome against *A. tumefaciens* shows that *B. abortus* 2308 carries the genes necessary for UPP production via a complete Wzx-Wzy pathway, with the exception of a flippase gene. One homolog for each of the Wza-, Wzy-, and Wzz-like proteins has been identified in *B. abortus* 2308, as well as homologs for three glycosyltransferases associated with UPP production in *A. tumefaciens* (Table 1). As noted, no flippase (Wzx) homolog has been identified; however, Wzx proteins are known to be highly diverse in sequence and structure, and thus identifying Wzx proteins through sequence homology alone can be a fruitless endeavor (Hong et al., 2018). Therefore, while a Wzx homolog has not currently been identified based on sequence comparisons, this does not exclude the possibility of the presence of a Wzx flippase molecule in *B. abortus* 2308. A schematic for the putative UPP biosynthetic pathway in *B. abortus* 2308 is shown in Figure 4.

A deletion of uppCE does not affect crystal violet or Congo red staining.

Two common methods used to detect the presence of exopolysaccharides are crystal violet staining (described in the previous section) and Congo red staining. In a Congo red staining assay, Congo red dye is added to solid medium on which the bacteria are grown, and exopolysaccharide production can be detected by observing changes to the amount of dye taken up by the colonies that grow on the plate. An observed increase in red coloration from the reference sample indicates an increase in exopolysaccharide production, and a decrease in red pigmentation indicates reduced exopolysaccharide production.

The global regulator MucR and its homologs are known to negatively regulate polysaccharide production in members of the α-proteobacteria; thus, a *B. abortus* 2308 *mucR* mutant was used in this assay as a control (Mueller & González, 2011; Caswell et al., 2013; Mirabella et al., 2013; Jiao et al., 2022). As expected, the *B. abortus* 2308 *mucR* mutant displayed increased crystal violet staining when compared to *B. abortus* 2308; however, no statistically significant difference was detected between the Δ*uppCE* mutant and *B. abortus* 2308 (Figure 6).

Although a *B. abortus* 2308 Δ*mucR* mutant is predicted to display increased Congo red staining, we have not observed this phenotype in our laboratory; however, a *B. melitensis* 16M Δ*mucR* does display the anticipated Congo red phenotype. Accordingly, *B. melitensis* 16M Δ*mucR* was used as the control in our Congo red assays. After 8 days of growth at 37°C/5% CO_2, increased Congo red staining was observed in the *B. melitensis* 16M Δ*mucR* mutant, but no difference was detected between the Δ*uppCE* mutant and *B. abortus* 2308 (Figure 7).

UPP production was not detected with lectin staining in Brucella abortus 2308.

UPP has been detected visually via microscopy in *A. tumefaciens* using fluorescently labeled lectins, specifically fluorescein-labeled wheat germ agglutinin (WGA) and fluorescein-labeled *Dolichos biflorus* agglutinin (DBA). In *A. tumefaciens*, WGA binds the *N*-acetyl glucosamine-derived portion of UPP generated from the UppW polymerase, and DBA binds the *N*-acetyl galactosamine-derived portion of UPP generated from the UppY polymerase (Onyeziri et al., 2022). Only one homolog for the UPP polymerase protein (BAB2_0106) has been identified in *B. abortus* 2308, but as it shares similar levels of homology to both UppW and UppY in *A. tumefaciens*, it is difficult to predict whether the polymerase in *B. abortus* 2308

would produce an *N*-acetyl glucosamine or an *N*-acetyl galactosamine-derived molecule. Accordingly, *B. abortus* 2308 and Δ*uppCE* were probed with both fluorescein-labeled WGA and DBA. Because many bacterial exopolysaccharides alternatively incorporate mannose as the primary monosaccharide subunit, *B. abortus* 2308 and Δ*uppCE* were also incubated with fluorescein-labeled Concanavalin A (ConA), which is specific for mannose residues(Dinadayala et al., 2006; Eshdat Y & Sharon N., 1984).

No fluorescence was detected when either strain was stained with fluorescein-labeled WGA, DBA, or ConA (data not shown). This may indicate the absence of an exopolysaccharide, or, because lectins bind specifically to sugar residues, that such an exopolysaccharide may be comprised of residues that do not interact with WGA, DBA, or ConA.

Increased intracellular levels of cyclic di-GMP do not affect the crystal violet or Congo red phenotype of B. abortus 2308

In both the crystal violet and Congo red assays, no difference was detected between the Δ*uppCE* mutant and *B. abortus* 2308; however, it should be noted that *B. abortus* 2308 displayed low levels of staining in both assays. Accordingly, decreased levels of staining with either dye, as expected in a Δ*uppCE* mutant, may be difficult to observe using these assays. The secondary messenger cyclic di-GMP is known to regulate UPP production in *A. tumefacien*s, with increased levels of exopolysaccharide being associated with increased levels of cyclic di-GMP. In order to artificially elevate exopolysaccharide production in *B. abortus* 2308, we increased intracellular levels of cyclic di-GMP by overexpressing the diguanylate cyclase PleD from *A. tumefaciens* on a plasmid under control of an IPTG-inducible promoter (P_{lac}-*pleD*). This strain is referred to as the PleD+ strain.

A statistically significant difference was not detected between *B. abortus* 2308 and the induced PleD+ strain in the crystal violet assay, nor was a difference in Congo red staining observed between the two strains. Likewise, no difference was detected in either assay when comparing the PleD+ strain under induced or non-induced conditions. Accordingly, we considered the possibility that our PleD+ strain was not overproducing cyclic di-GMP as we predicted. In order to test this, we compared intracellular levels of cyclic di-GMP between the *B. abortus* 2308, Δ*uppCE*, IPTG-induced PleD+, and uninduced PleD+ strains using ultraperformance liquid chromatography-mass spectrometry (UPLC-MS) in samples taken during both the exponential and stationary growth phases.

During exponential growth phase, cyclic di-GMP levels increased about 13-fold when PleD was induced with IPTG compared to *B. abortus* 2308 (Table 5). Discrepancies in cyclic di-GMP levels between the strains increased dramatically when samples were taken during stationary phase. Cyclic di-GMP levels increased nearly 26-fold when PleD was induced compared to the non-induced wild-type strain (Table 5). No significant difference was detected between *B. abortus* 2308 and the Δ*uppCE* mutant at either exponential or stationary growth phase. These results demonstrate that *pleD* from *A. tumefacien*s elevates cyclic di-GMP levels in *B. abortus* 2308 but this does not appear to impact the phenotype of this strain in the crystal violet or Congo Red assays.

Brucella abortus 2308 *uppC and uppE genes complement the respective mutants in Agrobacterium tumefaciens.*

The assays performed thus far (crystal violet staining, Congo red staining, and lectin staining) did not confirm production of UPP by *B. abortus* 2308. This led us to question whether

B. abortus 2308 produces functional UppC and UppE proteins. In order to test this, we performed a classical heterologous genetic complementation assay in which individual knockout mutants of *uppC* and *uppE* in *A. tumefacien*s were complemented with the homologous genes found in *B. abortus* 2308. Heterologous genetic complementation is a useful genetic tool to study the functionality of genes from bacteria in which genetic studies are difficult to perform. For example, *Mycobacterium tuberculosis* is notoriously difficult to grow *in vitro* due to specific nutrient requirements and the slow nature of its growth, and *Mycobacterium leprae* has never been successfully grown under laboratory conditions, so heterologous genetic complementation is performed in *E. coli* when homologous genes are present (de Mendonça et al., 2007; R. Wang et al., 2014). In this way, genes from one organism that may be difficult to culture or are only expressed under specific conditions can be assessed for their functionality in a different organism that is more amenable to grow under laboratory conditions.

Crystal violet assays are commonly used to detect biofilm production, and the resulting absorbance value is referred to as the "biofilm score." In a crystal violet staining assay, the biofilm score is nearly abrogated in the Δ*uppC* and Δ*uppE* deletions in *A. tumefacien*s. To determine whether *Brucella upp* homologs could restore the capability of the corresponding *Agrobacterium upp* mutants to produce a biofilm, we introduced plasmid-borne *uppC* and *uppE* from *B. abortus* 2308 into the respective mutants in *A. tumefacien*s.

B. abortus 2308 *uppC* carried on a pSRKKm plasmid was introduced into a Δ*uppC* mutant of *A. tumefacien*s, and *B. abortus* 2308 *uppE* carried on a pSRKKm plasmid was introduced into a Δ*uppE* mutant of *A. tumefacien*s, and expression of these genes was induced using IPTG. Wild-type *A. tumefaciens*, *A. tumefacien* Δ*uppC*, *A. tumefacien* Δ*uppE* harboring empty pSRKKm plasmid were also included in the assay for comparison. When *uppC* or *uppE*

from *B. abortus* 2308 was expressed in the Δ*uppC* and Δ*uppE* mutants of *A. tumefacien*s, respectively, the biofilm scores were restored to wild-type levels (Figure 8). This provides genetic evidence that *B. abortus* 2308 encodes *uppC* and *uppE* that can replace the homologous genes in *A. tumefacien*s.

Deletion of uppCE in Brucella abortus 2308 leads to attenuation in bone-marrow derived murine macrophages.

Although UPP was not detected in *B. abortus* 2308 using a crystal violet staining assay, Cong red staining assay, or microscopy using fluorescently labeled lectins, we determined that *B. abortus* 2308 *uppC* and *uppE* complement *A. tumefaciens* Δ*uppC* and Δ*uppE* mutants, respectively, indicating that the UppC and UppE proteins in *B. abortus* 2308 can replace the homologous proteins in *A. tumefaciens*. It is possible that UPP may be produced in *B. abortus* 2308 under conditions specific to an intracellular environment, which would not be detected using the aforementioned staining assays. As previously described, a pertinent step in the ability of *B. abortus* 2308 to establish an infection relies on its capacity to survive and replicate in macrophages. Accordingly, a standard macrophage cell culture infection assay is performed to evaluate the virulence of mutant *Brucella* strains.

In *A. tumefaciens*, UPP has been shown to be required for virulence, allowing *A. tumefaciens* to attach and subsequently invade the plant host cell (Onyeziri et al., 2022). To determine whether *uppC* and *uppE* are required by *B. abortus* 2308 to survive and replicate in mammalian macrophages, we infected bone-marrow derived murine macrophages with either wild-type *B. abortus* 2308 or the Δ*uppCE* mutant (Figure 9). Our data show that the *uppCE*

mutant was attenuated at both 24- and 48-hours post-infection in macrophages, indicating that this mutant has a reduced capability of surviving and replicating in macrophages.

The ΔuppCE mutant in Brucella abortus 2308 exhibits severe attenuation in the mouse model.

After analyzing the ability of *B. abortus* 2308 to survive and replicate within macrophages, we wanted to determine if *uppC* and *uppE* contribute to the maintenance of a chronic infection in the mouse model. In order to test this, C57BL/6$^{Nramp+/+}$ mice were infected intraperitoneally with either wild-type *B. abortus* 2308 or the Δ*uppCE* mutant. Both strains displayed the same profile at the one-week timepoint; however, after one week, the Δ*uppCE* mutant displayed significant attenuation compared to the wild-type strain, with the Δ*uppCE* mutant being attenuated by about 50-fold at the five-week timepoint (Figure 10). It should be noted that at the 5-week timepoint, no *Brucella* was recovered from one of the mice in the *uppCE* group. This mouse was determined to qualify as a statistical outlier and accordingly was omitted from the data set. The high attenuation in the Δ*uppCE* mutant demonstrates that the *uppCE* genes are required for virulence in *B. abortus* 2308.

We confirmed the link between the *uppC* and *uppE* genes and virulence using two different constructs to complement the Δ*uppCE* mutant strain: DH05 (complementation mutant with *uppCE* carried on pMR10) and DH06 (chromosomal restoration of *uppCE*). Reconstruction of the locus on the chromosome (DH06) prevents multiple copies of the gene from being expressed, which could affect the phenotype of the mutant. Complementation by expressing the genes on a pMR10 plasmid demonstrates that the phenotype observed in the original knockout mutant was due to the absence of only the targeted genes and that downstream genes were not unintentionally affected. Both complementation strains displayed virulence at wild-type levels,

whereas the Δ*uppCE* mutant strain was attenuated by about 10-fold at the 3.5-week timepoint (Figure 11).

UppC was not detected in a Western Blot using α-UppC antibody in the Δ*uppCE mutant.*

Although we did not detect an exopolysaccharide in *B. abortus* 2308 using a crystal violet staining assay, Congo red staining assay, or microscopy using fluorescently labeled lectins, a Δ*uppCE* mutant displays attenuation in both BMDMs and in the mouse model. Accordingly, we considered the possibility that *B. abortus* 2308 may not produce an exopolysaccharide as is observed in *A. tumefaciens*, but rather one or more of the putative Wzy/Wza proteins identified in *B. abortus* 2308 may instead directly interact with the host cell surface.

Brucella species are known to produce a family of highly conserved outer membrane proteins (OMPs) known as Omp 22, Omp25, Omp25b, Omp25c, Omp25d, Omp31, and Omb31b (Cloeckaert et al., 2002; Salhi et al., 2003). While these proteins often contribute to outer membrane integrity, members of this group of proteins have also been shown to directly interact with host cell-surface receptors to regulate virulence in *Brucella*. For example, Omp25 in *B. abortus* 2308 binds to SLAMF1 protein, found on the surface of dendritic cells, and impacts host cytokine production through this receptor.

As only putative UPP protein predicted to contain an extracellular domain *B. abortus* 2308 is UppC, we generated an antibody to UppC in order to detect the UPP biosynthetic machinery (Figure 12). Western Blot analysis demonstrates that UppC (MW = ~21kDA) was detected in *B. abortus* 2308 but not in the Δ*uppCE* mutant, confirming that *B. abortus* 2308 expresses UppC at measurable levels when grown under laboratory conditions and that this

protein is not present in the *uppCE* mutant (Figure 12). Further studies will need to be conducted to confirm whether the UppC protein itself is responsible for interaction with host cells.

Figure 1. Comparison of bacterial exopolysaccharide transport pathways. This figure illustrates the three membrane-dependent systems: the synthase-dependent system, the ABC-transporter system, and the Wzy-dependent pathway.

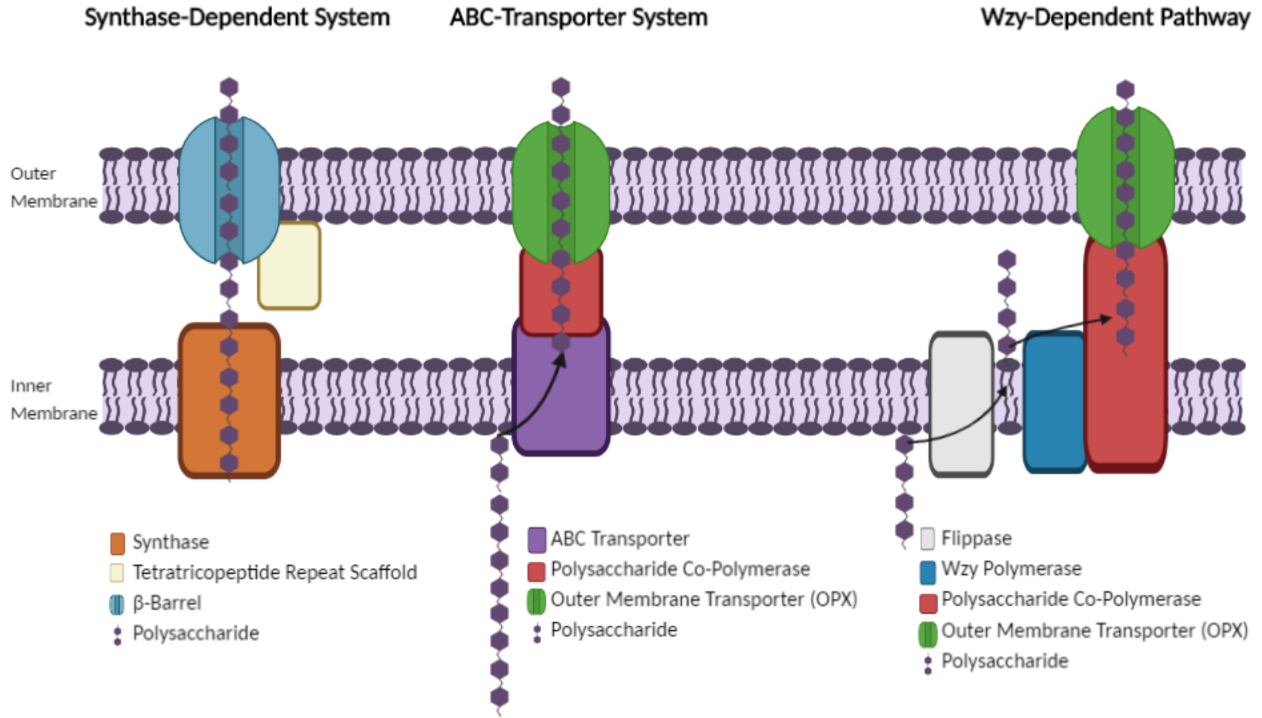
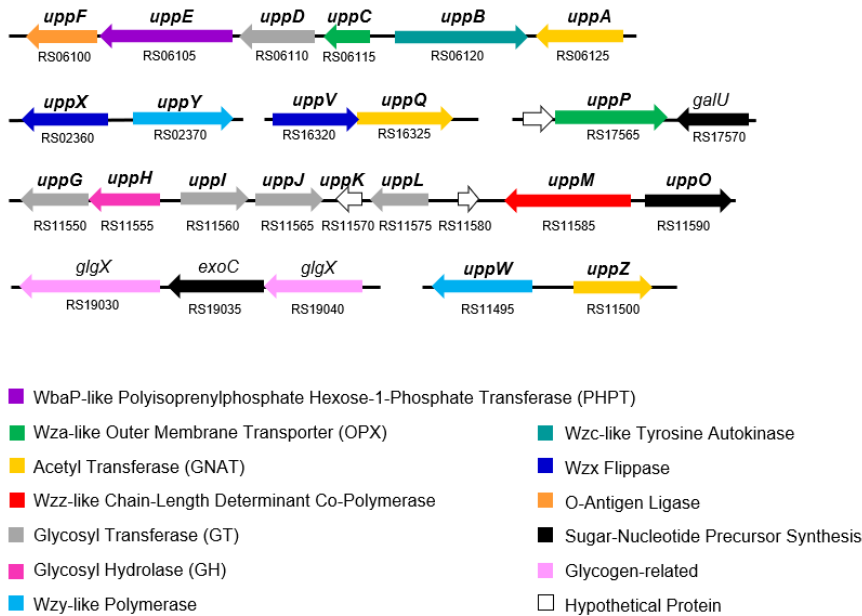


Figure 2. Comparison of the genetic organization of the *upp* genes in *Agrobacterium tumefaciens* (A) and the putative *upp* genes in *Brucella abortus* 2308 (B). Genes are color-coordinated with their predicted functions, shown below the schematics. An apostrophe (’) indicates that the gene is truncated. Figure A is modified from Onyeziri et al. (2022).

A) *Agrobacterium tumefaciens*



B) *Brucella abortus* 2308

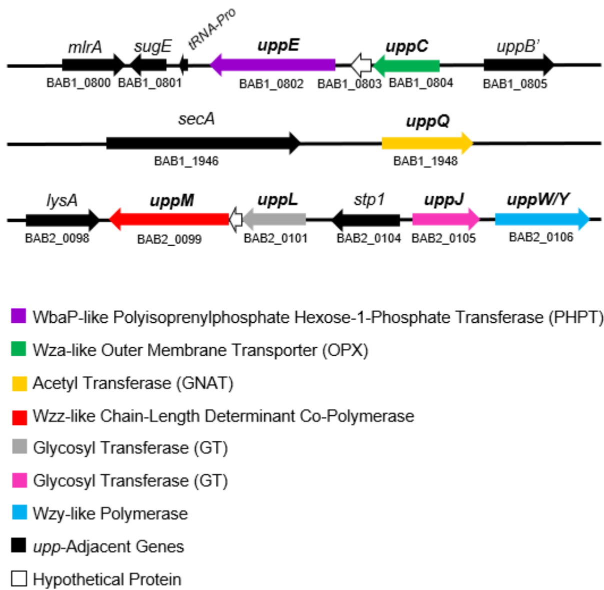


Figure 3. Schematic for the organization and location of the UPP proteins in *Agrobacterium tumefaciens*. Proteins are color-matched with their predicted functions, shown below the schematic. Black text represents proteins for which homologs have been identified in *B. abortus* 2308 and grey text represents proteins for which no homolog has yet been identified in B. *abortus* 2308. Modified from Onyeziri et al. (2022).

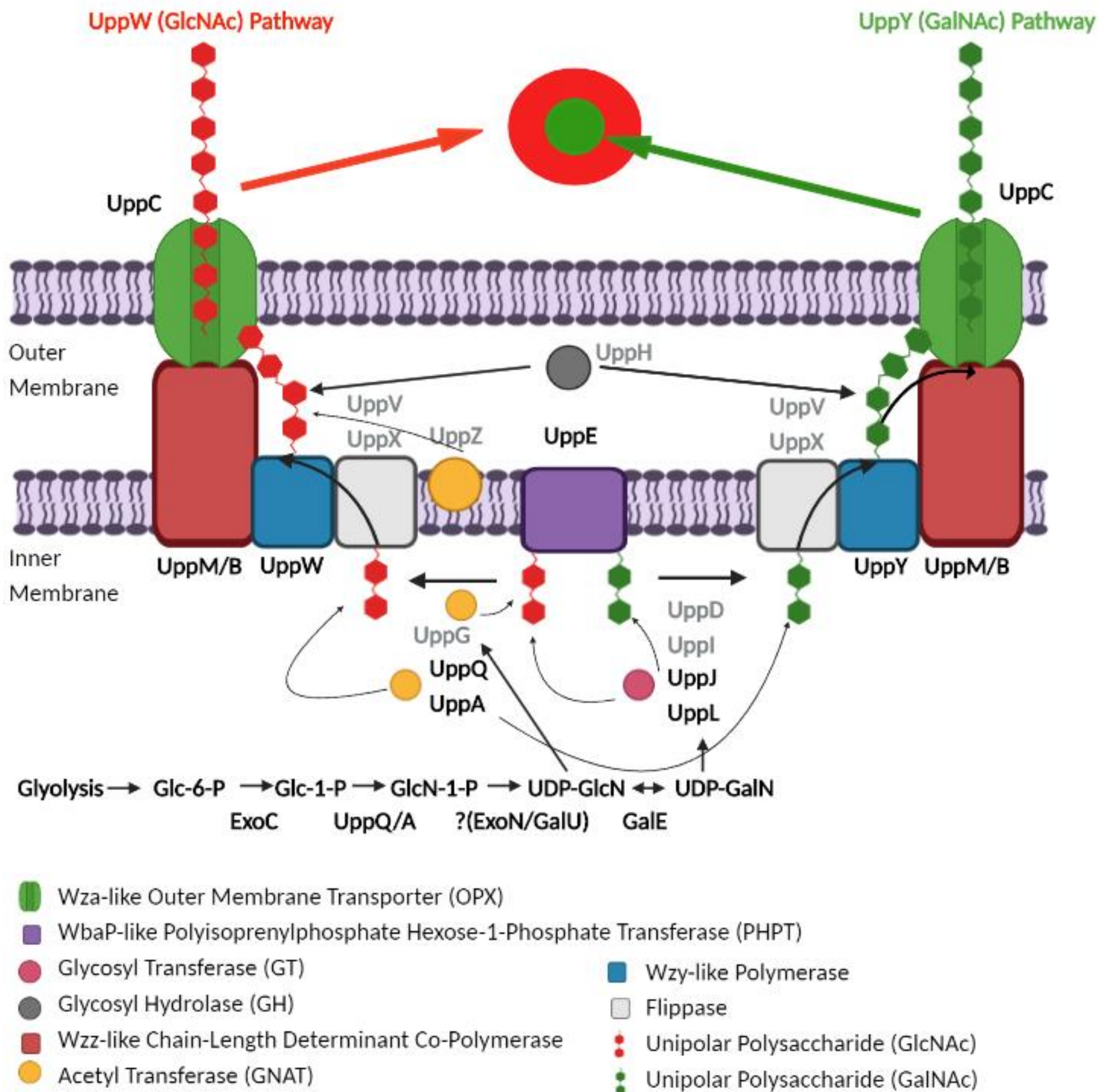


Table 1. Comparison of putative *upp* genes in *Brucella abortus* 2308 against confirmed *upp* genes in *Agrobacterium tumefaciens* C58.

B. abortus **2308 Gene Designation**	**Gene Name**	***A. tumefaciens*** **C58 Gene Designation**	**Homology between *Brucella* and *Agrobacterium***	**Protein Similarity**	**Polysaccharide Nomenclature**	**Localization**
BAB1_0804	*uppC*	ATU_RS06115	63% ID, 75% homology	OM Secretin	Wza (OPX)	Outer Membrane
BAB1_0802	*uppE*	ATU_RS06105	55% ID, 68% homology	PHPT	WbaP	Inner Membrane
BAB2_0105	*uppJ*	ATU_RS11565	35% ID, 51% homology	GT2	NA	Cytoplasm
BAB2_0101	*uppL*	ATU_RS11575	11% ID, 21% homology	GT26	NA	Cytoplasm
BAB2_0099	*uppM*	ATU_RS11585	32% ID, 50% homology	GumC	Wzz	Inner Membrane
BAB1_1948	*uppQ*	ATU_RS16325	43% ID, 66% homology	GNAT	BcsL	Cytoplasm
BAB2_0106	*uppW*	ATU_RS11495	23% ID, 40% homology	RfaL	Wzy	Inner Membrane
BAB2_0106	*uppY*	ATU_RS02370	23% ID, 42% homology	RfaL	Wzy	Inner Membrane

Figure 4. Schematic for the organization and location of the putative UPP proteins in *Brucella abortus* 2308. Proteins are color-matched with their predicted functions, shown below the schematic.

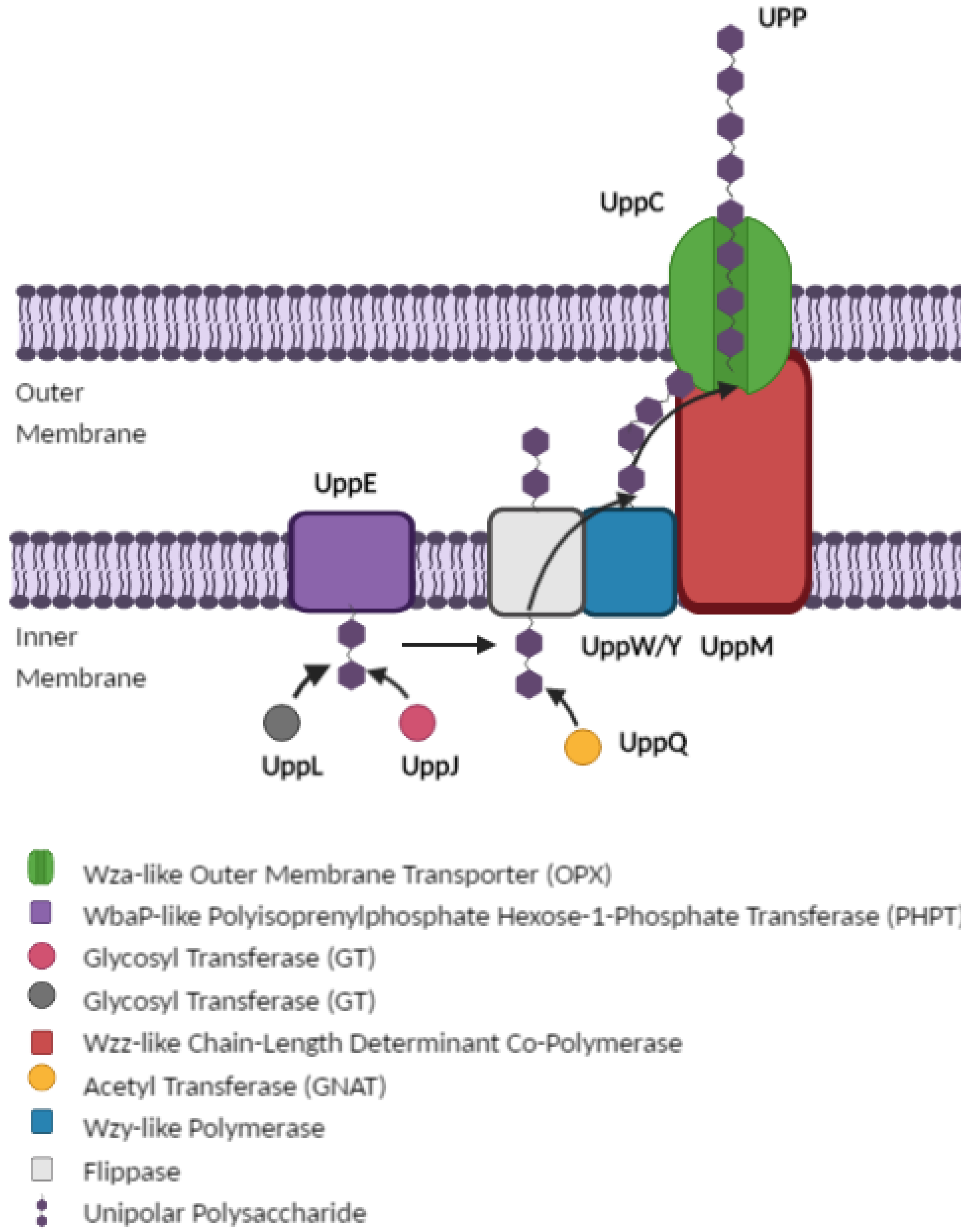


Table 2. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Genotype or Description	Reference/Source
Brucella abortus 2308		
2308	Virulent challenge strain	Jones et al., 1965
JEP8	2308 Δ*uppCE*	This study
DH05	JEP8 with *uppCE* complemented on pMR10	This study
DH06	JEP8 with chromosomally complemented *uppCE*	This study
DH07	2308 with plasmid-borne *pleD* (ATU_RS06385) from *A. tumefaciens* on pJW110	This study
CC092	2308 Δ*mucR*	Caswell et al., 2013
Brucella melitensis 16M		
16M	16M Δ*mucR*	Roop lab stock strain
Agrobacterium tumefaciens C58		
DH12	C58 with empty pSRKKm	This study
DH13	C58 Δ*uppC* with empty pSRKKm	This study
DH14	C58 Δ*uppC* with 2308 *uppC* on pSRKKm	This study
DH15	C58 Δ*uppE* with empty pSRKKm	This study
DH16	C58 Δ*uppE* with 2308 *uppE* on pSRKKm	This study
DH17	C58 Δ*uppC* with C58 *uppC* on pSRKGm	This study
DH18	C58 Δ*uppE* with C58 *uppE* on pSRKGm	This study
Escherichia coli		
DH5α	F- Φ80*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	Invitrogen
BL21(DE3)	F- *omp*T *hsd*S_B (r_B-, m_B-) *gal dcm* (DE3)	Invitrogen
Plasmids		
pNPTS138	*sacB*-based gene replacement vector; kanamycin resistance	West et al., 2002
pMR10	Cloning vector; broad-host range; low copy number (2 to 4 copies per cell); kanamycin resistance	Gee et al., 2005
pSRKKm	$LacI^Q$ encoding, IPTG inducible plasmid; kanamycin resistance	Khan et al., 2008
pSRKGm	$LacI^Q$ encoding, IPTG inducible plasmid; gentamycin resistance	Khan et al., 2008
pASK-IBA2C	Expression plasmid under transcriptional control of the *tet*-promoter/operator; C-terminal Strep-tag®II; chloramphenicol resistance	IBA-Lifesciences
pJW110	pSRKKm carrying P*lac* -*pleD*; kanamycin resistance	Xu et al., 2013

Table 3. Formula for ATGN medium.

20X AT Buffer

Substance	Grams added/L	Final conc. (1X)
KH_2PO_4 (pH to 7.0 w/ NaOH)	214	0.079 M

20X AT Salts

Substance	Grams added/L	Final conc. (1X)
$(NH_4)_2SO_4$	40	0.015 M
$MgSO_4 \cdot 7H_2O$	3.2	0.6 mM
$CaCl_2 \cdot 2H_2O$	0.2	0.06 mM
$MnSO_4 \cdot H_2O$	0.024	0.0071 mM

100 X Iron Stock

Substance	Grams added/L	Final conc. (1X)
$FeSO_4$	0.611	22 μM
HCl	0.61	10 mM

1X ATGN Medium

Substance	mL/L	Final conc. (1X)
20X Buffer	50	1X
20X Salts	50	1X
50% glucose	10	0.5% (13.9 mM)
100X Iron	10	1X
Agar*	15g	1.5%

*Added to make solid medium

Table 4. Oligonucleotide primers used for PCR amplification in this study.

Primer	Sequence (5'→ 3')
upp bab1_0802 up Bam F	GATGGATCCGAGCGCGGGAATTTGTTAATCCCGTTTCGATACGTT
upp bab1_0802 up R	CTTATTGAACATTATCGCCCTGTTAGAGCCGTTTCGATC
upp bab1_0805 down F	ATATAACCCGACAAATCAATGGGTTGAACGTTTTCC
upp bab1_0805 down Pst R	GATCTGCAGAATTGCACGGTCTTGGGATTCTCGAACA
upp compF PstI	GATCTGCAGCAAGATTATGATCGAAACGG
upp compR BamHI	GATGGATCCCTGATTCAAGGAAAACGTTC
*Agrobacterium uppC*del For	AAGCTACGTAATACGACTCACTAGTAGGATCTGCACCTGGCTGG
*Agrobacterium uppC*del Rev	GACTAGAGGGTCGACGCATGCGCAGGAAGAGCTGCCCCT
*Agrobacterium uppE*del For	CGCGCGGAAGCTATGCCCTATA
*Agrobacterium uppE*del Rev	AGACGTTGGGGTCCTGAAAGGC

Figure 5. Genetic regions deleted and cloned from *B. abortus* 2308 to create the Δ*uppCE* knockout and complemented mutants, respectively. An apostrophe (') indicates that the gene is truncated.

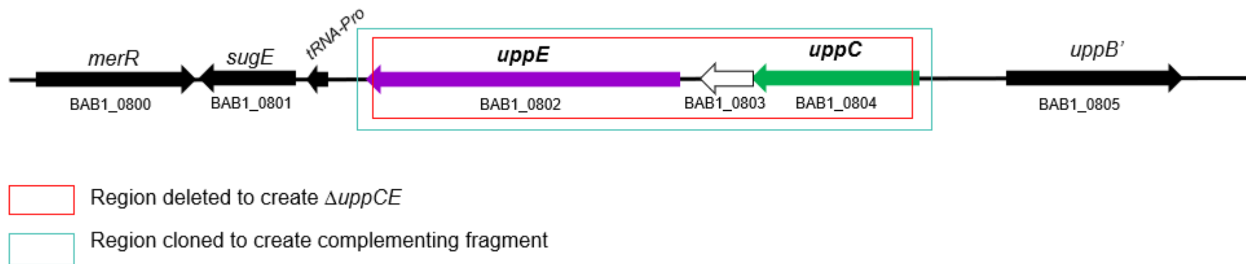


Figure 6. Crystal violet staining of *Brucella abortus* 2308 and Δ*uppCE*. To test whether exopolysaccharide production could be artificially increased to aid in its detection by crystal violet staining, plasmid-borne *pleD* was overexpressed by induction with IPTG. All absorbance values were read on a spectrophotometer at A_{550}, as per the method previously described. Statistical significance ($P \leq 0.05$) was determined using the Student's two-tailed t-test for comparison between *B. abortus* 2308 and the Δ*uppCE* mutant and is denoted by an asterisk (*). The results presented are from a single experiment that is representative of three experiments performed from which equivalent results were obtained. n = 4 for this experiment.

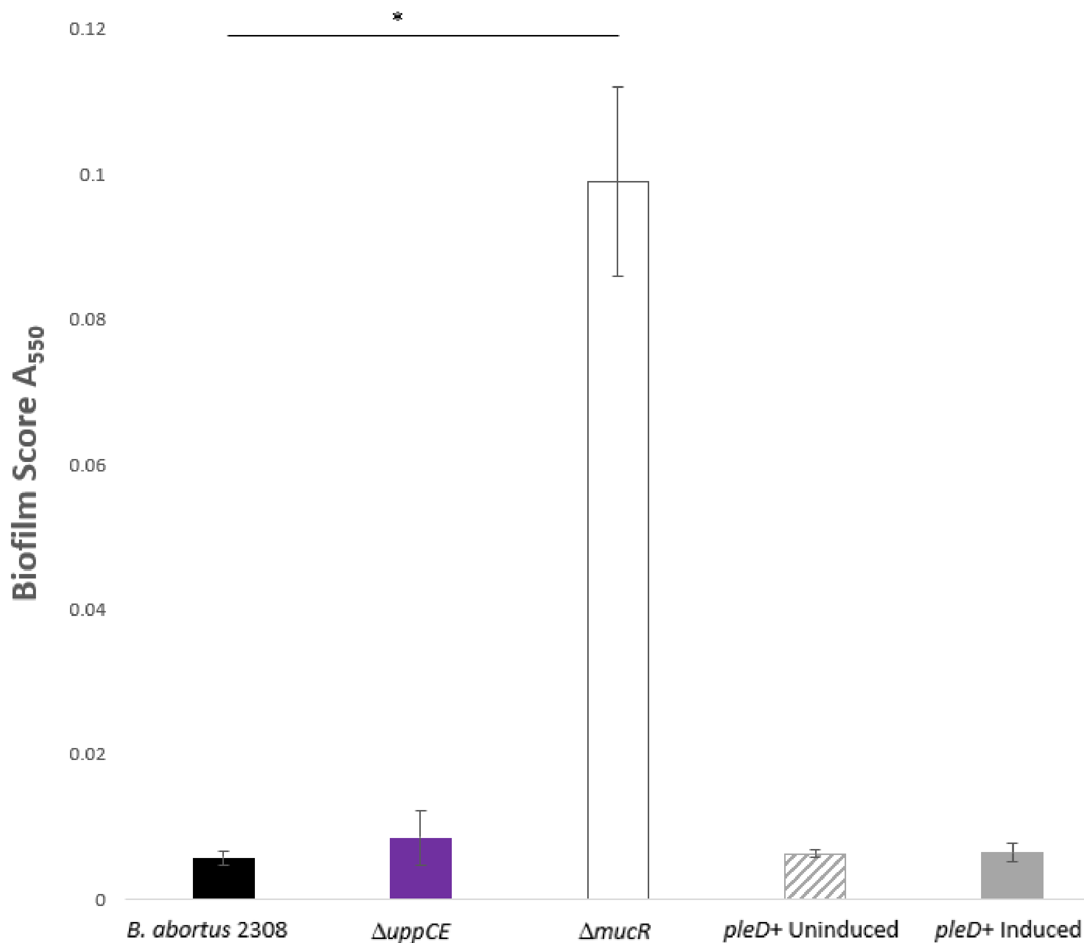


Figure 7. Comparison of Congo red staining between *Brucella abortus* 2308 and Δ*uppCE*. 25-µL of each strain was deposited in triplicate on tryptic soy agar plates supplemented with 0.01% Congo red. The plates were incubated for 8 days at 37°C under 5% CO_2. The results presented are from a single experiment that is representative of three experiments performed from which equivalent results were obtained. n = 3 for this experiment.

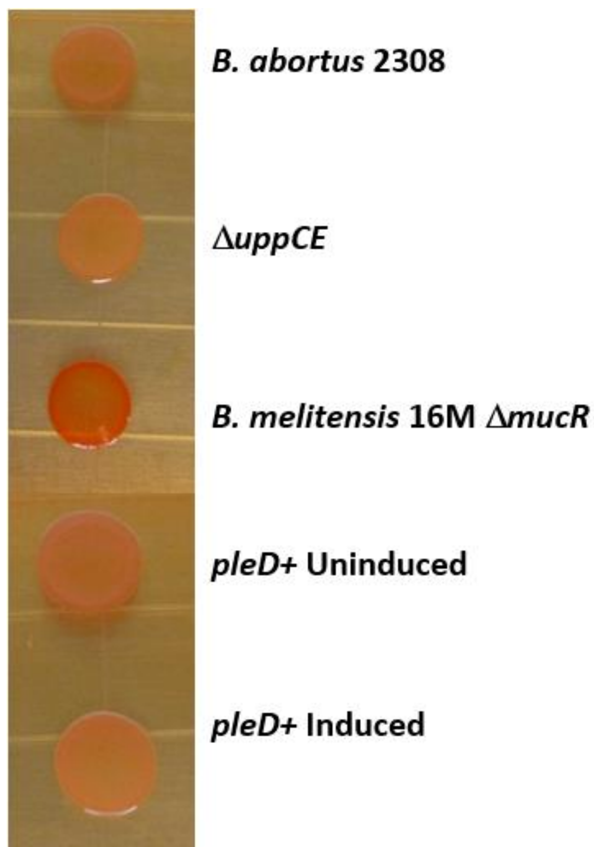


Table 5. Ectopic expression of *Agrobacterium tumefaciens pleD* elevates intracellular cyclic di-GMP levels in *Brucella abortus* 2308. Plasmid-borne *pleD* from *A. tumefaciens* was introduced into *B. abortus* 2308, and PleD expression was induced using 1mM IPTG. Cyclic di-GMP amounts were quantified by the laboratory of Christopher Waters at Michigan State University UPLC-MS/MS. A fold change of greater than or less than 2.0 was considered significant in this assay.

Exponential Growth Phase		
Strain	**Cyclic di-GMP (nM)**	**Fold Change**
B. abortus 2308	3.8	-
B. abortus 2308, P_{lac}-*pleD* Uninduced	7.5	2.0
B. abortus 2308, P_{lac}-*pleD* Induced	48.7	12.8
Stationary Growth Phase		
Strain	**Cyclic di-GMP (nM)**	**Fold Change**
B. abortus 2308	3.3	-
B. abortus 2308, P_{lac}-*pleD* Uninduced	4.8	1.3
B. abortus 2308, P_{lac}-*pleD* Induced	97.3	25.6

Figure 8. Biofilm phenotypes of *Agrobacterium tumefaciens* Δ*uppC* and Δ*uppE* complemented with *uppC* and *uppE* from *Brucella abortus* carried on pSRKKm. The OD_{600}, representing cell culture turbidity, and the absorbance of the acetic-acid solubilized crystal violet solution were recorded, and the ratio of A_{600}/OD_{600} was calculated. All samples were normalized to the *A. tumefaciens* C58 strain, whereby the biofilm scores are reported as the percentage of the A_{600}/OD_{600} ratio when the *A. tumefaciens* C58 value is set to 1.0. All strains were grown in the presence of kanamycin (300-µg/mL) and IPTG (400 µM). Statistical significance ($P \leq 0.05$) was determined using the Student's two-tailed t-test for comparisons of *A. tumefaciens* C58 to the single mutant strains and for comparisons of the *A. tumefaciens* single mutant strains versus the respective *Brucella*-derived complemented strains. Statistical significance ($P \leq 0.05$) is denoted by an asterisk (*). n = 3 for this experiment.

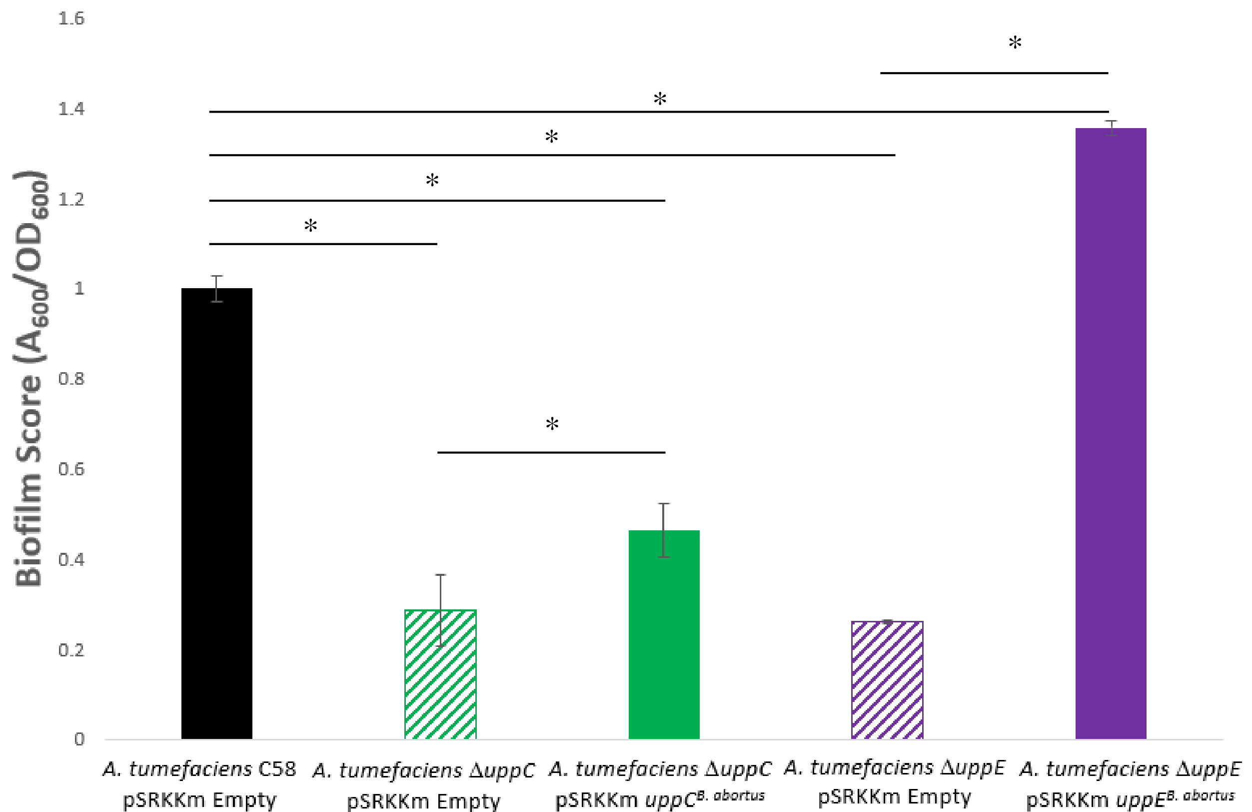


Figure 9. Intracellular survival and replication profile of *Brucella abortus* 2308 and isogenic *uppCE* and *vjbR* mutants in cultured bone marrow-derived macrophages obtained from C57BL/6 mice. Macrophages were infected with *Brucella* strains at an MOI of 100. Values shown on Y-axis are the number of intracellular brucellae cultured from lysed macrophages determined by serial dilution and plating. Statistical significance ($P \leq 0.05$) was determined using the Student's two-tailed t-test for comparison between *B. abortus* 2308 and the Δ*uppCE* mutant and is denoted by an asterisk (*). n = 3 for this experiment.

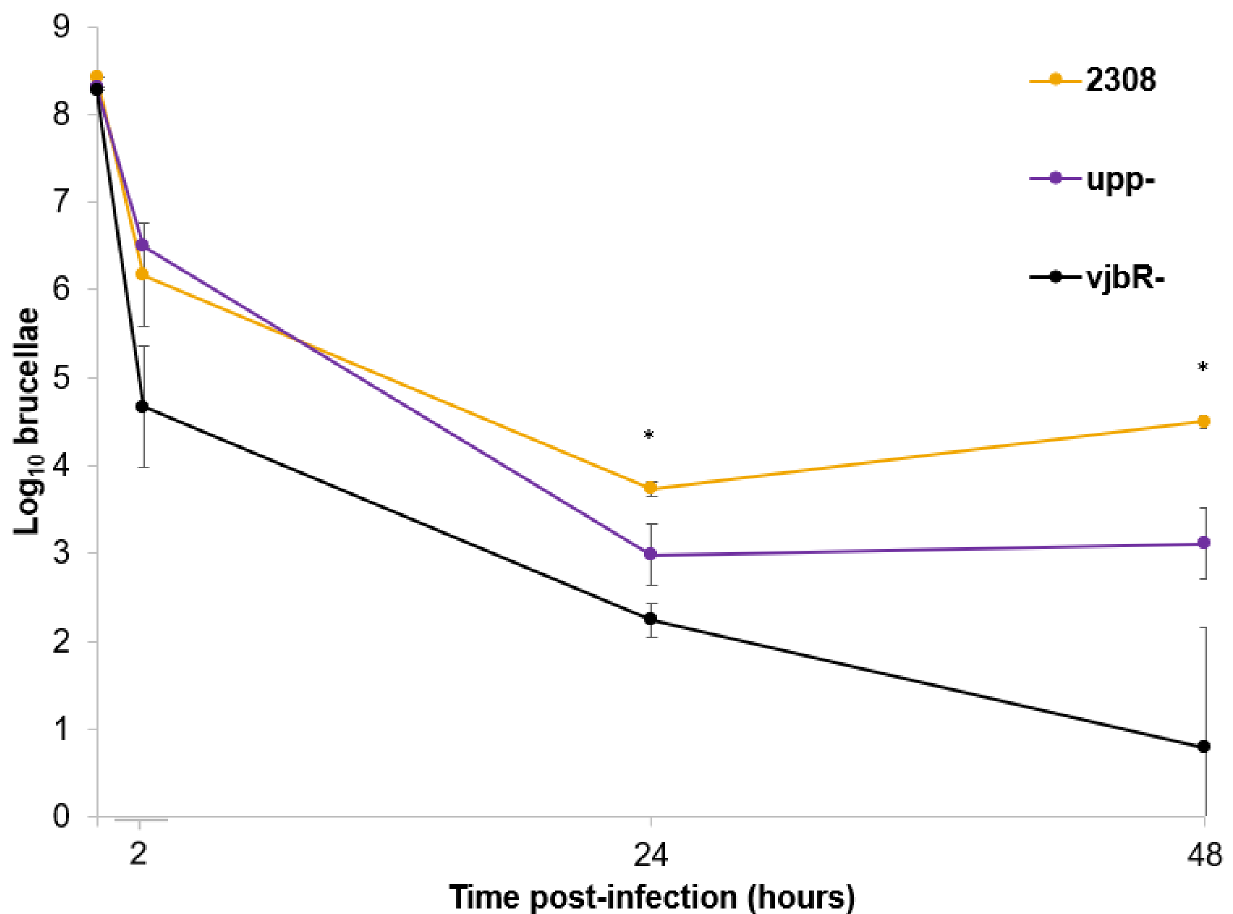


Figure 10. Spleen colonization profiles of *Brucella abortus* 2308 and an isogenic Δ*uppCE* mutant in C57BL/6$^{Nramp+/+}$ mice. Mice were infected with approximately 10^4 brucellae via the intraperitoneal route. The values shown on the Y-axis are the number of brucellae isolated from the spleens of mice at the experimental time points shown determined at necropsy by serial dilution and plating of spleen homogenates. Statistical significance ($P \leq 0.05$) was determined using the Student's two-tailed t-test for comparison between *B. abortus* 2308 and the Δ*uppCE* mutant and is denoted by an asterisk (*). n = 5 for this experiment.

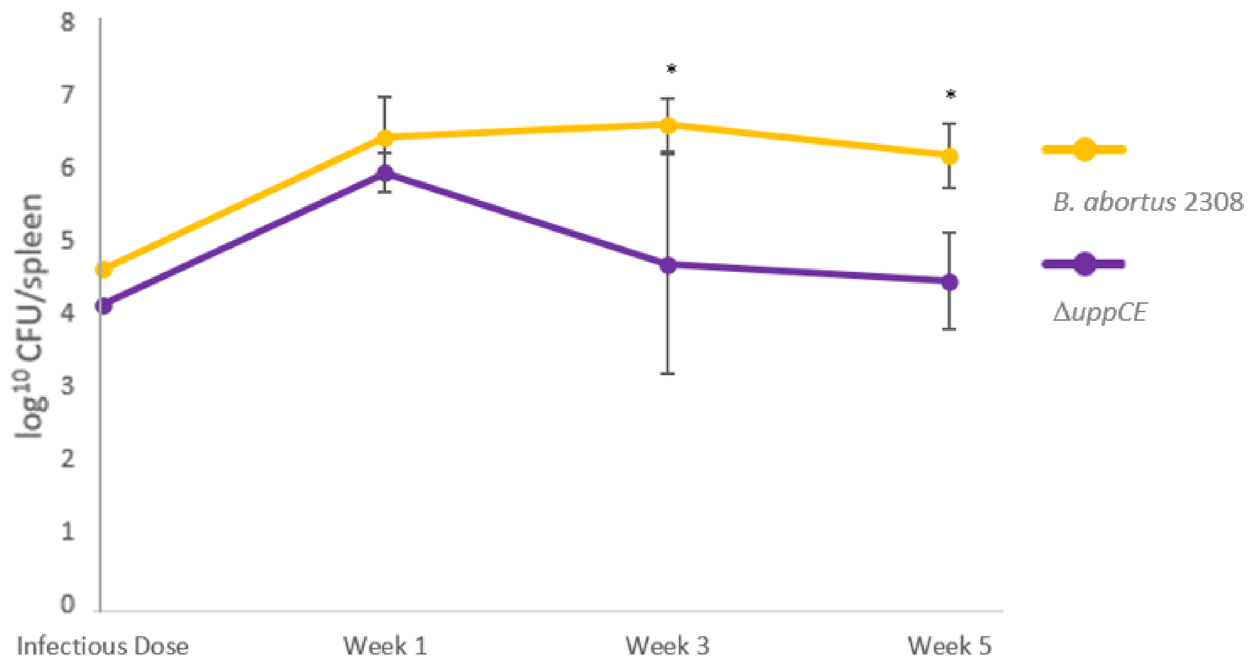


Figure 11. Spleen colonization profiles of *Brucella abortus* 2308, Δ*uppCE*, and *uppCE*+ complemented strains in C57BL/6$^{Nramp+/+}$ mice at 3.5 weeks post infection. Mice were infected with approximately 10^4 brucellae via the intraperitoneal route and the number of brucellae residing in the spleens of the mice were determined at necropsy by serial dilution and plating of the spleen homogenates. Statistical significance ($P \leq 0.05$) was determined using the Student's two-tailed t-test for comparison between strains and is denoted by an asterisk (*). n = 5 for this experiment.

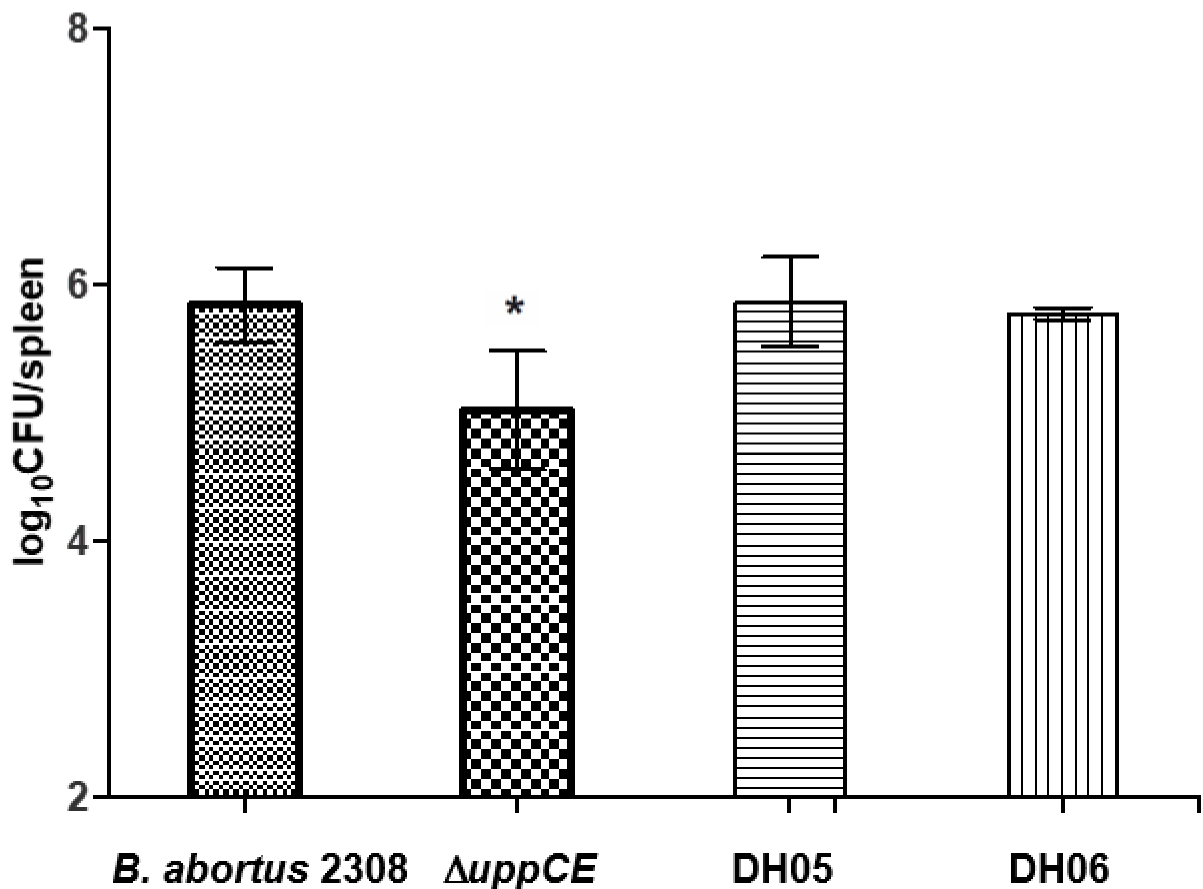
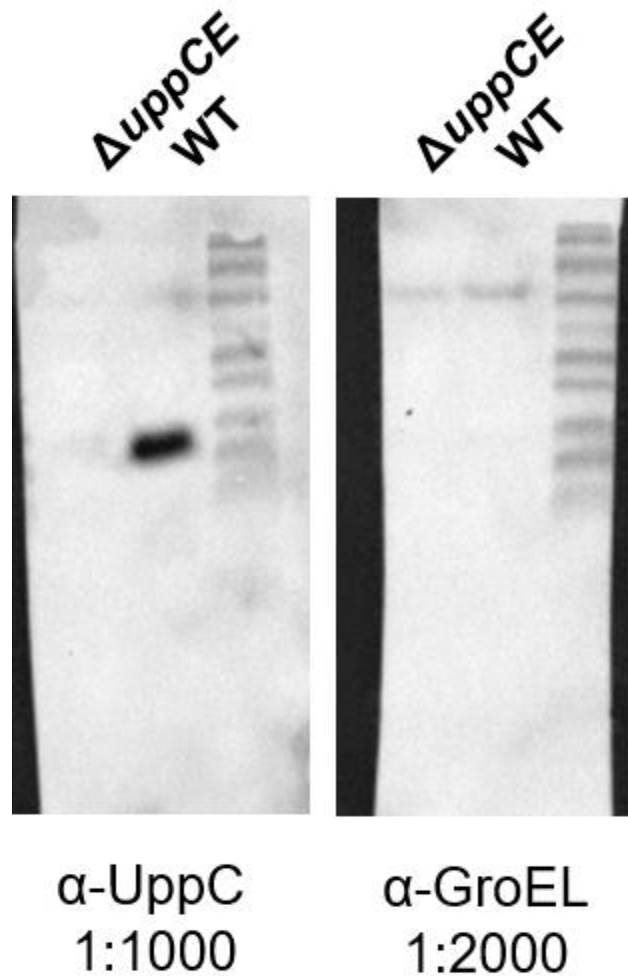


Figure 12. UppC is detected with an α-UppC antibody in *Brucella abortus* 2308 but not in the Δ*uppCE* strain. Using an α-UppC antibody generated in rabbits, the presence of UppC was confirmed by Western Blot analysis in *B. abortus* 2308, but this protein is absent from the Δ*uppCE* mutant. The predicted molecular weight of *B. abortus* 2308 UppC is approximately 21kDa. α-GroEL antibody (1:2000) was used as a control to ensure that the assay was successfully performed, and BenchMark™ Pre-Stained Protein Ladder was used to show the protein sizes.



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Chapter Three

Discussion and Future Directions

Bacterial exopolysaccharide production can contribute to pathogen virulence in a variety of ways, including facilitating interactions with hosts cells, allowing for attachment to surfaces (both biotic and abiotic), providing protection from the antimicrobial activities of complement and other host immune defenses, aiding in the formation of biofilms, and providing resistance to desiccation (Nwodo et al., 2012; Jones & Wozniak, 2017; Singh et al., 2019; Sharma et al., 2020; Buffet et al., 2021). Bacteria belonging to the classification of α-protobacteria are known to produce exopolysaccharides to facilitate their lifestyles in several different ways. For example, *Caulobacter crescentus* produces a holdfast to attach to abiotic surfaces, *Sinorhizobium meliloti* generates succinoglycan and galactoglucan to invade the root system of legumes, and *Agrobacterium tumefaciens* makes cellulose and unipolar polysaccharide to adhere to and aggregate around plant host cells (Matthysse et al., 1995, Jones, 2012; Hershey et al., 2019).

Stemming from a common ancestor, members of the class α-protobacteria have adapted to a highly diverse range of environments and host species. Members of the *Rhizobiales*, a subgroup of the class α-protobacteria, can infect a wide range of hosts, including plants, mammals, and arthropods, and yet these species share a close phylogenetic relationship. Interestingly, in species adapted to an intracellular niche, genes that contribute to virulence in mammalian and arthropod hosts are often homologous to genes that function to establish symbiosis with the plant host in plant-associated bacteria (Batut et al., 2004b). Accordingly, new insights into one member of the *Rhizobiales* can deepen our understanding of other members of this group, despite their seemingly different lifestyles.

As previously discussed, *A. tumefaciens* has recently been shown to produce an exopolysaccharide known as unipolar polysaccharide (UPP), which facilitates attachment of this bacterium to plant cell surfaces so that it may transfer its DNA to the host. UPP production in

Agrobacterium occurs through a Wzx-Wzy pathway, which is a common biosynthetic pathway utilized by bacteria for exopolysaccharide production (Islam & Lam, 2014). Although *A. tumefaciens* and *B. abortus* inhabit two very different niches, with *Agrobacterium* infecting plant cells and *Brucella* invading mammalian cells, genetic comparisons between *A. tumefaciens* and *B. abortus* demonstrate that the latter possess the genetic capability of producing a UPP molecule. With the exception of a gene encoding a flippase, the genome of *B. abortus* 2308 contains all of the required proteins for a complete Wzx-Wzy pathway, suggesting that *B. abortus* 2308 may also employ the use of an exopolysaccharide in its lifecycle. In this study, we aimed to determine whether *B. abortus* 2308 does indeed produce UPP, and, if so, whether this exopolysaccharide contributes to the virulence of this bacterium.

Detection of UPP in B. abortus 2308

Bacterial exopolysaccharide production may be detected using a variety of assays, usually involving a dye known to bind polysaccharides or by using a specific molecule (such as a lectin) known to bind monosaccharide residues in the polymer. For example, both Congo red and crystal violet dyes are commonly used to detect exopolysaccharide production as these dyes readily bind to polysaccharide molecules. Mutants lacking individual *upp* genes in *A. tumefaciens* exhibit reduction in both Congo red and crystal violet staining, indicating that exopolysaccharide production in these mutants is disrupted. The lectins WGA and DBA bind specifically to *N*-acetyl glucosamine or *N*-acetyl galactosamine residues, respectively, and UPP has been detected in *A. tumefaciens* using fluorescein-labeled WGA and DBA via microscopy.

Accordingly, we aimed to employ these same techniques to detect UPP production in *B. abortus* 2308; however, in the case of each assay, we did not detect a change in exopolysaccharide production. While this may suggest that UPP is not produced by *B. abortus*

2308, alternatively, the conditions under which these experiments were performed may not be conducive to UPP production. In the studies involving *A. tumefaciens*, for example, the bacteria were often genetically manipulated in order to increase UPP production so that UPP was more easily and readily detected. UPP is not strongly or uniformly expressed during in vitro cultivation of *A. tumefaciens*, and UPP production is subject to complex genetic regulation in response to a variety of environmental stimuli. While some of the regulators in this multifaceted system have been identified, many remain to be characterized. The complexity involved in UPP regulation may contribute to the difficulty we experienced in detecting it in *B. abortus* 2308. In addition, as a facultative intracellular pathogen of mammalians species, *B. abortus* 2308 are exposed to different environmental stimuli than soil-dwelling *A. tumefaciens*, so insights into environmental triggers of UPP production in *A. tumefaciens* may provide little information on similar stimuli for *B. abortus* 2308. Finally, while *B. abortus* 2308 carries all the necessary genes for a complete Wzx-Wzy pathway (except the flippase), *B. abortus* 2308 does not carry homologous genes for some of the known regulators of UPP production in *A. tumefaciens*. For example, as discussed in Chapter 1, PruA generates a pterin molecule that binds to PruR in *A. tumefaciens*, and pterin-bound PruR interacts with the phosphodiesterase DcpA to reduce cyclic di-GMP levels in *A. tumefaciens*. A Δ*pruA* mutant lacks the ability to generate the pterin molecule, and thus unbound PruR does not interact with DcpA, allowing cyclic di-GMP to accumulate in the cell. As high levels of cyclic di-GMP are associated with UPP production, a Δ*pruA* mutant in *A. tumefaciens* functions as a UPP-overproducing strain. Although *B. abortus* 2308 contains a homolog for *pruA*, no homolog exists for *pruR*, and previous studies with a Δ*pruA* mutant in mice did not exhibit at attenuated phenotype. Accordingly, the *pruA*-*pruR* system cannot be manipulated in *B. abortus* 2308 to increase UPP production.

It should be noted, however, that *B. abortus* 2308 encodes a MerR-like transcriptional factor homologous to *mlrA* upstream of *uppC* and *uppE*. MlrA contributes to adhesion and biofilm formation in *E. coli* through cyclic di-GMP binding, which is also known to regulate UPP production in *A. tumefaciens*. Accordingly, future studies with a knockout mutant of *mlrA* in *B. abortus* 2308 could potentially identify this gene as a regulator of UPP production in *Brucella*.

If *mlrA* does acts as a regulator of UPP production in *B. abortus* 2308, this suggests that cyclic di-GMP may play a role in this process as it does in *A. tumefaciens*. In fact, one way that UPP production can be manipulated in *A. tumefaciens* is by increasing cyclic di-GMP (Feirer et al., 2015). By overexpressing the diguanylate cyclase PleD, cyclic di-GMP levels can be increased in *A. tumefaciens*, resulting in a stark Congo red phenotype. Although relatively little is known about cyclic di-GMP signaling in *Brucella* species, 11 cyclic di-GMP metabolizing genes have been identified in *B. melitensis*, including the phosphodiesterase *bpdA* (M. Khan et al., 2016). In *B. melitensis*, a Δ*bpdA* mutant exhibits significant attenuation in macrophages and the mouse model and has been linked to the transcription of the flagellar genes *fliF*, *flgE*, and *flgB* (Petersen et al., 2011; M. Khan et al., 2016). It therefore appears plausible that cyclic di-GMP may contribute to virulence in *Brucella* species.

Accordingly, we expressed *pleD* from *A. tumefaciens* on an IPTG-inducible plasmid so cyclic di-GMP levels could be controlled in *B. abortus* 2308 and confirmed that our *pleD*+ strain does in fact elevate cyclic di-GMP levels; however, when *pleD* was overexpressed in wild-type *B. abortus* 2308, we still did not observe a Congo red or crystal violet staining phenotype. It should be noted, however, that spatial organization of the UPP machinery in *A. tumefaciens* is highly regulated and PleD is known to localize to the old pole of the cell (i.e., the pole at which

UPP is produced) (Heindl et al., 2019). It is unclear whether *A. tumefaciens*-derived PleD localizes adjacent to the UPP machinery in *B. abortus* 2308, which would be pertinent in detecting UPP if it is made in *Brucella*. Recent studies do suggest that in various bacteria, clustering of cyclic di-GMP biosynthetic machinery occurs so that processes affected by this molecule can be spatially confined (Kunz & Graumann, 2020). If PleD from *A. tumefaciens* does not co-localize with the UPP machinery in *B. abortus* 2308, this may explain why cyclic di-GMP levels appear to have no effect on exopolysaccharide production in *Brucella*. Accordingly, additional studies utilizing native *pleD* from *B. abortus* 2308 should be performed.

Therefore, although we were unable to detect exopolysaccharide production in *B. abortus* 2308 using Congo red, crystal violet, or lectin staining, even upon increasing cyclic di-GMP levels, this does not necessarily indicate that *B. abortus* 2308 cannot or does not produce UPP or a similar polysaccharide. Rather, UPP production may occur only under specific environmental conditions that are not represented by the conditions under which these assays were performed. Although very little work has been performed regarding the role of exopolysaccharides in *Brucella* species, the notion that exopolysaccharides may only be produced under very specific conditions is supported by what data we do have. The role of *vjbR* as a quorum sensing-dependent transcriptional activator is discussed in greater detail in Chapter 1 of this document, but, briefly, a *vjbR* mutant in which the quorum sensing domain was rendered insensitive to AHL has also been linked to a clumping phenotype in *B. melitensis*, but a clumping phenotype was not observed in *B. abortus* 2308 using the equivalent mutant. Interestingly, when a mutant in which *vjbR* was knocked out in its entirety was grown under microaerobic conditions, aggregation was observed in *B. abortus* 2308 (Almirón et al., 2013b). The fact that cellular aggregation has only

been observed under limited circumstances in *B. abortus* 2308 highlights the challenges in creating conditions under which UPP may be produced in these bacteria.

One striking difference between the lifestyles of *Agrobacterium* and *Brucella* species is that *Agrobacterium* survives extracellularly in the soil environment and produces UPP upon contact with the host plant cell. Conversely, *B. abortus* 2308 is a facultative intracellular pathogen that is unable to live freely in the environment for significant periods of time (Roop et al., 2009; Vila et al., 2016; Gopalsamy et al., 2021). Accordingly, the possibility exists that *B. abortus* 2308 may only produce UPP under conditions that mimic the intracellular environment. *B. abortus* 2308 colonizes macrophages and must be able to survive and replicate within these cells in order to maintain an infection. As part of the frontline defense against microbial invaders, macrophages phagocytize pathogens and present antigens to T-cells. In order to kill these pathogens and break down their structures to present antigen to host immune cells, the intracellular environment found within macrophages is intentionally hostile and damaging to microbes that have been phagocytized. Pathogens taken up by macrophages are exposed to reactive oxygen species produced by oxidative burst as well as an acidic environment (≤ pH 5) to kill and disassemble the intracellular microbes (Westman & Grinstein, 2021). The *virB* operon, which encodes the protein components of a Type IV secretion system (T4SS) for the release of various effector molecules that allow the *Brucella*-containing vacuole to evade fusion with the lysosome in macrophage, is transcribed when the pH is low (~4.5), indicating that these genes are activated in response to the acidic environment found within the macrophage (Rouot et al., 2003). Interestingly, many plant associated bacteria respond to the acidic conditions found around plant roots. Plants secrete organic acids and secondary metabolites that lower the pH of soil, and, when damaged, plant cells also secrete a mixture of compounds, including acidic

sugars, that help heal the wound (Subramoni et al., 2014). Members of the *Rhizobiales* capitalize on the low pH environment that surrounds plant roots and release virulence factors in response to these acidic conditions. For example, the exopolysaccharide glucomannan in the plant symbiont *Rhizobium leguminosarum* binds to host cell lectins when the pH of the surrounding environment is acidic (pH ~5.6) (Laus et al., 2006; Williams et al., 2008). In addition, the ChvG/ChvI two-component system, which regulates biofilm formation and motility in *A. tumefaciens*, is also activated under acidic conditions (Subramoni et al., 2014). This provides an intriguing link between the life cycles of plant-associated members of the *Rhizobiales* and *Brucella* species: the expression of virulence factors in both types of bacteria occurs in response to low pH.

Accordingly, additional studies should be performed under conditions that mimic the intracellular environment of macrophages to test whether UPP might be produced by *B. abortus* 2308 under these conditions. Previous work with *B. melitensis* and *B. suis* has employed the use of pH-adjusted E medium, first described by Vogel and Bonner, for this purpose (Vogel & Bonner, 1956; Kulakov et al., 1997). *B. melitensis* and *B. suis* may be grown in low-pH E medium for limited periods of time (3-4 hours) to simulate the acidic environment of the *Brucella*-containing vacuole; however, the use of E medium has heretofore been unsuccessful in sustaining cultures of *B. abortus* 2308. Accordingly, additional modifications to this medium could be explored to generate a medium that could sustain *B. abortus* 2308 while mimicking the intracellular environment of macrophages to determine whether UPP is produced under these conditions. In lieu of developing such a medium, macrophages could be infected with *B. abortus* 2308 and intracellularly stained with fluorescently labeled lectins, eliminating the need to develop a medium that imitates the intracellular environment of macrophages.

Although sequence analysis demonstrates that the genome of *B. abortus* 2308 contains the genes required for a complete Wzx-Wzy pathway (apart from the flippase gene, which is known to have a high degree of sequential diversity), it should be noted that the component proteins of a Wzx-Wzy pathway are highly substrate specific, and therefore the composition of exopolysaccharides between different bacterial species can vary greatly despite the use of similar biosynthetic machinery (Hong et al., 2015). Therefore, while insights into the composition of UPP in *A. tumefaciens* may help direct efforts to characterize UPP made by *B. abortus* 2308, it is possible that the composition of UPP in *B. abortus* 2308 could deviate significantly from that of UPP in *A. tumefaciens.* We stained *B. abortus* 2308 with fluorescein-labeled wheat germ agglutinin (WGA) and fluorescein-labeled *Dolichos biflorus* agglutinin (DBA) based on previous studies in *A. tumefaciens* that demonstrated that these lectins bind to the *N*-acetyl glucosamine and *N*-acetyl galactosamine residues in UPP, respectively. We additionally probed our samples with fluorescein-labeled concanavalin A (ConA) as this lectin is specific to mannose residues, which are commonly found in exopolysaccharides produced by bacteria. Although we did not detect UPP production in *B. abortus* 2308 using these lectins, it is possible that *B. abortus* 2308 may produce UPP that is uniquely tailored in its composition to allow interactions specific to an intracellular life cycle within mammalian cells. Thus, additional lectins could be probed to identify the residues that comprise UPP in *Brucella*.

Efforts to isolate UPP from *A. tumefaciens* are currently being undertaken by the laboratory of Clay Fuqua at Indiana University Bloomington. Once a successful method has been established, the composition of UPP can be analyzed by mass spectrometry to identify the individual components. This would alleviate the need to probe samples with various fluorescently labeled lectins and would rather allow the use of targeted lectins known to bind the

specific saccharide components identified by mass spectrometry. This would allow UPP to be detected by fluorescent microscopy, which would provide information regarding the location of UPP on individual cells as well as characterize aggregation patterns. Alternatively, if only analyzing for the presence or absence of UPP, antibodies specific to the known polysaccharide components could be generated and Western Blot analysis performed.

In both Gram-negative and Gram-positive bacteria, the Wzx-Wzy pathway is responsible for producing the majority of cell-surface polysaccharides, including O-antigen for LPS synthesis, capsule, spore coat, and other exopolysaccharides (Islam & Lam, 2014). Putative components of a Wzx-Wzy pathway are often identified by sequence homology and predicted membrane topology, but their specific activities are determined by analyzing the phenotypes of individual mutations to the genes predicted to compromise a Wzx-Wzy pathway (Cuthbertson et al., 2009). Accordingly, the end product of a Wzx-Wzy pathway, whether it be LPS, capsule, or UPP, can only be accurately determined through experimental analysis.

This raises the question as to whether the putative *upp* genes identified in *B. abortus* 2308 contribute to an exopolysaccharide other than UPP, such as LPS. We have several reasons to believe that the putative *upp* genes identified in *B. abortus* 2308 are not involved in LPS production. The genes for LPS production in *Brucella* species have been explored in great detail, and none of the putative *upp* genes identified in these studies have been previously implicated in LPS synthesis in any of these studies (Iriarte et al., 2004; Tumurkhuu et al., 2006; Gil-Ramírez et al., 2014; Fontana et al., 2016; Salvador-Bescós et al., 2018). Furthermore, we performed a crystal violet stain upon creating the Δ*uppCE* mutant and verified that it is a smooth strain, indicating that the LPS has been left intact. Not to be confused with a crystal violet stain used to detect biofilm formation, this crystal violet stain is used to determine whether a strain displays a

smooth or rough phenotype. Upon crystal violet staining, smooth phenotypes are characterized by a glossy sheen on colonies that appear white, whereas rough strains take up the dye more easily and thus appear dark purple in comparison to smooth strains (Salvador-Bescós et al., 2018). Smooth strains of *Brucella*, which include all species except *B. ovis* and *B. canis*, express complete LPS composed of the O-chain, core, and lipid A, whereas rough strains lack the O-chain. Rough mutants of *B. abortus* 2308 are highly attenuated in murine macrophages; they are both engulfed at an increased rate (~10-20 fold) when compared to wild-type smooth strains and are highly susceptible to intracellular killing once inside the macrophage (Pei & Ficht, 2004). In the mouse model, our Δ*uppCE* mutant behaved like *B. abortus* 2308 through the 1-week timepoint, displaying attenuation only at the 3- and 5-week timepoints. *Brucella* mutants lacking O-chain generally display significant attenuation early in the infection (i.e., 1 week or earlier) as such mutants are more sensitive to elimination by host immune defenses. The delay in attenuation observed in the Δ*uppCE* mutant suggests that the O-chain is not disrupted as we would expect a rough mutant to be attenuated early in the infection.

It should be noted, however, that some LPS modification proteins in *B. abortus* 2308 do display delayed attenuation in mice. For example, *wadC* is a glycosyltransferase gene involved in the synthesis of LPS core in *B. abortus* 2308. A Δ*wadC* mutant displays attenuation in mice but only after the 3-week timepoint (Conde-Álvarez et al., 2012). As previously mentioned, LPS synthesis in *Brucella* has been described in detail, and although none of the putative *upp* genes have been identified in these studies, this does not nullify the possibility that these genes do contribute to LPS synthesis in some way. Accordingly, silver stain analysis, commonly used to compare LPS profiles, could be performed to determine if there are any differences in LPS production between *B. abortus* 2308 and the Δ*uppCE* mutant. To further determine whether the

putative *upp* genes are involved in LPS synthesis, Western Blot analysis using monoclonal antibodies specific for the O-chain or the LPS core could be performed (Caswell et al., 2013). Differences in a Western Blot between wild-type *B. abortus* 2308 and the Δ*uppCE* mutant could indicate that LPS synthesis is disrupted in the Δ*uppCE* mutant. Alternatively, mass spectrometry could be used to compare the LPS structures of wild-type *B. abortus* 2308 and the Δ*uppCE* mutant.

Attachment via UPP in B. abortus 2308

The attachment assays we performed involved abiotic surfaces (plastic wells for the crystal violet stain) or other surfaces that do not mimic the intracellular environment of a cell (blood agar plates for the Congo red stain), and thus the necessary conditions under which UPP may aid in the attachment of *B. abortus* 2308 may not be fulfilled in these assays. In the case of intracellular pathogens such as *Brucella*, initial adhesion to host cells often plays a vital role in the successful establishment of an infection, and such bacteria often bind to components of the extracellular matrix (ECM) to attach to host cells (Bialer et al., 2021). We have previously discussed the role of macrophages in the establishment of infection by *B. abortus* 2308, but it should be noted that placental trophoblasts are the targeted cells in later stages of the infection. It is the infection of these placental epithelial cells that leads to the hallmark symptom of infection in the natural host (cattle): spontaneous abortion. Several protein adhesins have been identified in *Brucella*, including SP29 and SP41 (sialic acid-binding proteins), BigA and BigB (proteins containing an Ig-like domain), BmaA, BmaB, and BmaC (monomeric autotransporters), and BtaE, BtaF, and Bp26 (trimeric autotransporters) (Bialer et al., 2020). These adhesins mainly interact with epithelial cells, and some also aid in attachment to erythrocytes, synoviocytes, osteoblasts, and/or trophoblasts (Bialer et al., 2020). Attachment by these adhesins is often

achieved through interactions with common components of the ECM, such as fibronectin, collagen, hyaluronic acid, and vitronectin (Castañeda-Roldán et al., 2004; Posadas et al., 2012; Nica Ruiz-Ranwez et al., 2013; Ruiz-Ranwez et al., 2013, Eltahir et al., 2019). Accordingly, additional studies should be performed to determine whether UPP in *B. abortus* 2308 may bind to components of the ECM as this would be relevant to the placental stage of infection.

Unipolar adhesion appears to be a shared characteristic between members of the α-proteobacteria. Holdfast in *C. crescentus*, UPP in *A. tumefaciens*, and exopolysaccharide in *R. palustris* are all expressed on a single pole of the cell. Interestingly, two of the adhesins listed above, BtaE and BmaC, are known to be expressed on *B. suis* at one pole of the cell (Ruiz-Ranwez et al., 2013). These proteins, which bind hyaluronic acid and fibronectin, respectively, on epithelial cells, have been shown to function as adhesins in *B. suis*, allowing the bacteria to bind to HeLa cells in a unipolar fashion. Conversely to UPP on *A. tumefaciens*, which is produced at the old pole of the cell, both BtaE and BmaC are expressed on the new pole of *B. suis*. This diversion from what is seen in *A. tumefaciens* could reflect a response of *Brucella* species to an evolutionary pressure resulting from the adaptation to an intracellular lifestyle, or this could indicate that different adhesins are required during different stages of infection, and thus some adhesins are produced at the old pole of the cell while others are produced at the new pole of the cell.

As previously discussed, invasion of placental trophoblast cells is essential for the progression of an infection with *B. abortus* 2308 in the natural host, and thus *B. abortus* 2308 must employ methods to invade these cells. HeLa cells are often substituted for other epithelial cells for cellular attachment assays as these cells are easier to culture and provide equivalent results when compared to other epithelial cell lines. In order to determine whether UPP is

involved in attachment to epithelial cells such as trophoblasts, additional studies should explore whether differences in attachment to HeLa cells occur between wild-type *B. abortus* 2308 and the Δ*uppCE* mutant strain.

As human infections with *Brucella* species often occur after the ingestions of infected dairy products, one may assume that *Brucella* infection occurs through absorption in the stomach or intestines; however, *Brucella* is sensitive to the extreme pH of gastric juices and thus does not appear to penetrate beyond the oropharynx (Gorvel et al., 2009). As high levels of *Brucella* have been recovered from the tonsils, it is currently believed that *Brucella* invades through this organ. In our mouse model, the mice are infected through the intraperitoneal (IP) route, which bypasses any initial interactions with epithelial cells found in the membranes of the mouth and oropharynx (as would occur in a natural infection). In this way, IP infection represents an "artificial" route of infection that does not occur outside of the laboratory environment. Intranasal or intraoral infections of mice could shed light on whether UPP may play a role in interacting with epithelial cells at early sites of entry as these methods of infection better mimic a natural route of infection.

Considering the important role that macrophages play in the ability of *B. abortus* 2308 to establish an initial infection, the possibility that UPP from *B. abortus* 2308 may directly interact with macrophage cells should be explored. This would not be the only known occurrence of a polysaccharide binding directly to macrophages. When the O-chain of LPS on smooth *Brucella* strains interacts with lipid rafts on the surface of mammalian cells, including macrophages, *Brucella* enters the cells via the endocytic pathway, allowing the *Brucella*-containing vacuole to avoid fusion with lysosomes (Watarai et al., 2002; Porte et al., 2003). Accordingly, one mechanism by which UPP could contribute to virulence in *B. abortus* 2308 is through an initial

interaction with a surface molecule on macrophages that allows the bacteria to be taken up and trafficked through a non-lethal route in the macrophage.

Innate immune cells, which include macrophages, dendritic cells (DCs), and neutrophils (PMN), detect pathogen-associated molecular patterns (PAMPs) using a variety of pattern recognition receptors (PRRs). One type of PRR is C-type lectin receptors (CLRs). Interactions between CLRs and bacterial glycans induce localized cytokine production that attracts other immune cells to the site of infection (Mnich et al., 2020). Mannose residues, which are commonly found in bacterial exopolysaccharides, are detected by the C-type lectin mannose receptors (MRs), which are expressed on both macrophages, dendritic cells, and lymphatic or liver endothelial cells (Azad et al., 2014). Mannose receptors can trigger phagocytosis of a pathogen or endocytosis of antigens. Interestingly, antigens endocytosed via mannose receptors are targeted to non-degradative early endosomes to prevent fusion with lysosomes, allowing the antigens to be processed for antigen presentation on MHC I molecules instead (van der Zande et al., 2021). Although CLRs are mainly expressed on myeloid cells, some epithelial cells also express CLRs. For example, the C-type lectin Dectin-1 promotes inflammatory cytokine production and phagocytosis and is found on most mucosal epithelial cells (Günther & Seyfert, 2018). While lectins found on immune cells can interact with polysaccharides found on bacterial surfaces, lectins found on pathogens can also interact with immune cells. Some lectins of microbial origin have been found to interact with the *N*-glycans of toll-like receptors (TLRs), leading to an increase in production of pro-inflammatory cytokines (Ricci-Azevedo et al., 2017).

One intriguing category of lectins expressed on macrophages and dendritic cells includes sialic acid-binding immunoglobulin (Ig)-like lectins, known as siglecs. The ability of the immune system to differentiate between self and non-self is essential for the survival of an organism.

Sialic acid-capped polysaccharides are characteristic of self-associated molecular patterns (SAMPs) that allow the immune system to make this distinction (Lübbers et al., 2018). These SAMPs interact with siglecs to promote immune tolerance. A variety of bacteria, viruses, and eukaryotic parasites have taken advantage of this fact and either synthesize their own sialic acid-containing molecules or acquire them from the host to drive an anti-inflammatory response to subvert the hosts immune system (Khatua et al., 2013; Higuchi et al., 2016). The possibility that UPP could interact with lectins on the surface of immune cells such as macrophages provides an intriguing explanation for the observed attenuation seen in both the macrophage and mouse models of infection with the Δ*uppCE* mutant.

Mechanism by which UPP contributes to virulence in B. abortus 2308

As previously stated, we did not detect UPP in *B. abortus* 2308 using Congo red, crystal violet, or lectin staining. Although this may not conclusively determine that *B. abortus* 2308 does not produce a UPP, for the reasons formerly described, the possibility exists that such an exopolysaccharide is not produced. As our Δ*uppCE* mutant strain does exhibit significant attenuation in the mouse model, *uppCE* must contribute to virulence by some mechanism. One explanation for this could be that rather than producing an exopolysaccharide, a protein component of the putative UPP biosynthetic pathway may directly interact with the host cell.

Of the putative UPP proteins identified in *B. abortus* 2308, only one is predicted to be exposed to the outer surface of the bacterial cell: the Wza-like outer membrane exporter protein UppC. *Brucella* species express several highly conserved outer membrane proteins (OMPs) that interact directly with receptors on the host cell surface to modulate the host response (Cloeckaert et al., 2002; Salhi et al., 2003). In *B. suis*, tumor necrosis factor alpha (TNF-α) production is regulated through the interaction of Omp25 with host macrophage cells, and in *B. abortus* 2308,

Omp25 regulates nuclear factor-κB (NF-κB) to limit acute inflammation by directly interacting with the receptor SLAMF1 on dendritic cells (Jubier-Maurin et al., 2001; Degos et al., 2020). If *B. abortus* 2308 does not in fact produce UPP, perhaps UppC instead functions similarly to other OMPs via direct interaction with the surface of immune cells.

To test whether the UppC protein contributes to the ability of *B. abortus* 2308 to invade macrophages, macrophages could be infected with wild-type *B. abortus* 2308, *B. abortus* 2308 pre-treated with our UppC antibody (α-UppC) (to block any active sites that may interact with host cells), and the Δ*uppCE* mutant. If the α-UppC pre-treated cells display a phenotype similar to that seen in the Δ*uppCE* mutant, this would indicate that the UppC protein may itself interact with host cells rather than an exopolysaccharide. In addition, cellular attachment assays using both HeLa cells and macrophages, as previously described, could be performed using α-UppC pre-treated cells. If the extracellular number of α-UppC pre-treated cells is reduced to a significant degree when compared to *B. abortus* 2308, this could indicate that UppC is responsible for direct interaction with these cells.

Individual mutants of *uppC* and *uppE* could also be used to evaluate whether UppC interacts with the host cell surface. If an attenuated phenotype is observed in the Δ*uppC* mutant but not in the Δ*uppE* mutant in macrophage and mouse assays, this could indicate that UppC alone contributes to the virulence phenotype seen in the Δ*uppCE* mutant. To further confirm this, additional single mutants of the remaining putative *upp* genes (*uppJ*, *uppL*, *uppM*, *uppQ*, and *uppW/Y*) in *B. abortus* 2308 should be created and tested for a virulence phenotype. If none of the additional mutants display an attenuated phenotype, this would suggest that only UppC is responsible for virulence, further implicating it as having a role in direct interaction with the host cell.

As previously discussed, interactions between Omp25 and receptors on macrophages and dendritic cells regulate the production of TNF-α and NF-κB, respectively, effectively reducing the host inflammatory response (Jubier-Maurin et al., 2001; Degos et al., 2020). The Δ*uppCE* mutant exhibits strong attenuation in the mouse model compared to wild-type *B. abortus* 2308; however, attenuation is not detected at the initial stages of infection. For at least the first week post-infection, *B. abortus* 2308 and the Δ*uppCE* mutant display similar replication phenotypes, but at the 3-week timepoint, the Δ*uppCE* mutant no longer maintains wild-type levels of replication and fails to establish a chronic infection. One possible explanation for this observation could be that the Δ*uppCE* mutant is unable to effectively regulate cytokine production in the macrophages, thus disrupting the "stealthy" nature of *Brucella* in evading early detection by the host immune system.

In order to determine whether this strong virulence phenotype is a result of a disruption of cytokine regulation, whether due to exopolysaccharide production or direct interaction with the UppC protein, cytokine production by the host could be assayed using an enzyme-linked immunosorbent assay (ELISA) using supernatants collected from infected macrophages. Once again using *wadC*, a glycosyltransferase involved in synthesis of the core section of *Brucella* LPS, as an example, a Δ*wadC* mutant of *B. abortus* 2308 is attenuated in bone marrow-derived dendritic cells (BMDCs) (Conde-Álvarez et al., 2012). This appears to be due to higher levels of pro-inflammatory cytokines such as IL-12 and TNF-α, which were detected in the supernatants of BMDCs. Interestingly, attenuation was not observed in bone marrow-derived macrophages (BMDMs). Although this document has focused largely on the role of macrophages in infection with *Brucella*, it should be noted that recent studies have shown that *Brucella* species invade and proliferate in human dendritic cells (Papadopoulos et al., 2016). *Brucella*-infected neutrophils are

engulfed by both macrophages and dendritic cells, and many of the same mechanisms used by *Brucella* to restrict stimulation of a pro-inflammatory immune response by macrophages have the same effect in dendritic cells. For example, as seen in macrophages, lipid A from *Brucella* species interacts only weakly with TLR4 receptors on dendritic cells to limit pro-inflammatory cytokine production. Accordingly, studies measuring cytokine production in the Δ*uppCE* mutant compared to wild-type *B. abortus* 2308 should be performed using the supernatants of both BMDMs and BMDCs. In addition, it should be noted that some cytokines relevant to an infection with *B. abortus* 2308 are not typically produced by macrophages. For example, interferon gamma (IFN-γ) is required for successful clearance of *Brucella*, but this cytokine is typically produced by T-cells and natural killer (NK) cells and is detected at later stages of a *Brucella* infection (Murphy et al., 2001). Accordingly, mouse serum should also be analyzed for cytokine production when comparing the Δ*uppCE* mutant to *B. abortus* 2308. Although ELISA is frequently used in such assays to quantify cytokine production, qPCR could alternatively be used as a method to analyze cytokine production by measuring the levels of expression of the cytokine genes.

Studies to characterize the cytokine profile of *Brucella*-infected cells should assay a combination of the pro-inflammatory and anti-inflammatory cytokines relevant to a *Brucella* infection, including interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-12 (IL-12), interleukin-4 (IL-4), and interleukin-10 (IL-10). The pro-inflammatory cytokines IFN-γ, TNF-α, and IL-12 are crucial for host clearance of *B. abortus* 2308 (Pasquali et al., 2001; Barquero-Calvo et al., 2007b; Vitry et al., 2012). Conversely, IL-4 and IL-10 are indicative of an anti-inflammatory response associated with post-infection recovery (Pasquali et al., 2001; Barquero-Calvo et al., 2007b; Grillá et al., 2012; Vitry et al., 2012). Anti-inflammatory cytokines

such as IL-4 and IL-10 promote a host "healing" response, as opposed to the "killing" response triggered by pro-inflammatory cytokines. It would be important to determine whether the Δ*uppCE* mutant drives an anti-inflammatory response as this mechanism could contribute to the stealthy nature of *Brucella* and aid in its ability to avoid triggering a strong host immune response. In addition, IL-4 and IL-10 are associated with alternatively activated macrophages, also known as M2 macrophages, which are the favored host cells during the chronic stages of infection (Xavier et al., 2013).

Smooth *Brucella* strains are characterized by strong resistance to complement-mediated killing (González et al., 2008). This has led to an assumption that complement plays little to no role in a *Brucella* infection; however, recent studies have shown that *B. abortus* is more readily cleared in complement depleted mice (Hoffmann & Houle, 1995; González-Espinoza et al., 2018). As complement depletion typically enhances bacterial growth, these results seem contradictory to our understanding of how this part of the innate immune system functions, indicating that the role of complement in a *Brucella* infection may be intertwined with other components of the immune system by a currently unknown mechanism. Complement can be activated by three different pathways: the classical pathway, the lectin pathway, or the alternative pathway. The classical pathway relies on the binding of antigen-antibody complexes to complement proteins, whereas the lectin and alternative pathway are independent of antibody and instead rely on interaction between mannose binding lectin with microbial exopolysaccharides or direct binding of complement proteins to a microbial surface, respectively. As previously described, bacterial exopolysaccharides commonly incorporate mannose residues, which are detected in the lectin pathway of complement activation. Deletion of the O-chain glycosyltransferase *wboA* increases the deposition of mannose-binding lectin to

the surface of *B. abortus* 2308, indicating that surface polysaccharides can contribute to resistance to complement activated by this pathway (Fernandez-Prada et al., 2001). Accordingly, future studies of UPP in *Brucella* species should investigate whether this exopolysaccharide may confer complement resistance.

Neutrophils have also been shown to contribute to the progression of an infection with *Brucella* species. *B. abortus* 2308 induces neutrophils to present phosphatidylserine on their cell surface, which functions as a signal for ingestion by macrophages. Crucially, *Brucella*-infected neutrophils do not undergo chromatin condensation or other signs of necrosis, allowing macrophage uptake of the dying neutrophils in a manner that does not induce inflammation. In this way, the neutrophils act as a "Trojan Horse," allowing delivery of the bacteria to the macrophages in a manner that does not alert the immune system (Gutiérrez-Jiménez et al., 2019). In fact, macrophages that have phagocytosed *Brucella*-infected neutrophils express high levels of the anti-inflammatory cytokine IL-10 and low levels of the pro-inflammatory cytokine TNF-α, indicating that the initial interaction between neutrophils and *Brucella* drives an immunomodulatory effect that aids in the evasion of the innate immune system by these bacteria. The mechanism whereby neutrophils act as a delivery system of *Brucella* to macrophages in a way that does not elicit a strong immune response also holds true for the delivery of *Brucella*-infected neutrophils to dendritic cells. In addition, the interactions of Omp25 on *Brucella* with SLAMF1 on the surface of dendritic cells drives an anti-inflammatory response (Degos et al., 2020). Cyclic β-1,2-glucan, discussed in chapter 1, also affects cytokine production by both neutrophils and dendritic cells, implicating a role for this exopolysaccharide in modulating the innate immune response in a *Brucella* infection (Martirosyan et al., 2012; Degos et al., 2015). As surface molecules, including the exopolysaccharide cyclic β-1,2-glucan, are known to interact

with neutrophils and dendritic cells to regulate the immune response, future studies should explore whether UPP may also interact with these cells.

Another possibility for how *uppCE* contributes to virulence may have to do with an effect on trafficking through the macrophage. The capacity of *Brucella* species to survive and replicate within these cells relies on the ability of the *Brucella*-containing vacuole to avoid degradation within the macrophage. VjbR, previously discussed in detail, regulates the expression of the *virB* operon. The *virB* operon encodes a set of proteins that form a Type IV secretion system (T4SS) for the transport of various effector molecules that prevent fusion of the *Brucella*-containing vacuole with lysosomes in the macrophage (Xiong et al., 2021). One of the putative *upp* genes in *B. abortus* 2308, BAB1_1948 (identified as *uppQ* in this document) has been previously described as an effector molecule secreted by the VirB Type IV secretion system. In this study, the protein encoded by BAB1_1948 is referred to as BspF. BspF has been shown to be essential for successful replication of *Brucella* within the replicative *Brucella*-containing vacuole (rBCV). By inhibiting the transport of recycling endosomes within the *trans*-Golgi network (TGN), BspF recruits TGN-derived vesicles to the rBCV (Borghesan et al., 2021). This remodeling of the vesicular traffic supports replication-permissive conditions that enhances bacterial growth within the rBCV (Borghesan et al., 2021). Our sequence analysis has identified BAB1_1948 as an *N*-acetyltransferase. It is currently unclear whether BAB1_1948 functions as an *N*-acetyltransferase that can acetylate molecules in the UPP biosynthetic pathway as well as function as an effector molecule secreted by the VirB Type IV secretion system or whether these two processes are intertwined. The description of BAB1_1948 as an effector molecule that interferes with trafficking by the *trans*-Golgi network raises the question as to whether UPP production may play a role in this process.

Summary

It had been previously speculated that UPP production was limited only to plant associated *Rhizobiales* species and that mammalian pathogens in this group lacked the genetic capacity to make UPP (Fritts et al., 2017). This information was, however, founded on a limited knowledge of the *upp* genes involved in a complete biosynthetic pathway in members of the *Rhizobiales*. Additional *upp* genes have been identified and characterized in the closely related species *A. tumefaciens*, and we have shown that based on sequence homology, *B. abortus* 2308 does carry the genes required for a Wzx-Wzy pathway for UPP production (except for the flippase, a protein type known to exhibit large degree of sequence diversity).

If *B. abortus* 2308 does in fact produce a unipolar polysaccharide that contributes to the virulence of this organism, this would be first instance that such an exopolysaccharide has been described in a mammalian pathogen belonging to the group *Rhizobiales.*

Although we were unable to detect production of an exopolysaccharide in *B. abortus* 2308 using crystal violet, Congo red, or lectin staining, we have shown that both *uppC* and *uppE* from *B. abortus* 2308 complement the respective mutants in *A. tumefaciens* and restore biofilm production in that species. We have also shown that a Δ*uppCE* mutant is attenuated in both BMDMs and in the mouse model, indicating that these *upp* genes are required for virulence in *B. abortus* 2308, although we have not yet elucidated the mechanism by which these genes have this effect. Accordingly, further studies should 1) explore how *uppC* and *uppE* contribute to virulence, 2) determine whether a genuine exopolysaccharide is made by the putative *upp* genes in in *B. abortus* 2308, and 3) if an exopolysaccharide is not produced, investigate whether UppC, the only protein in the biosynthetic machinery predicted to contain an extracellular domain, functions to directly interact with the host cell. As our Δ*uppCE* mutant displays such strong

attenuation, particularly in the mouse model, further characterization of these genes could shed light on important insights into the virulence of this organism.

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Appendix: Animal Use Protocol

Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27834 – 4354
252-744-2436 office | 252-744-2355 fax

February 22, 2022

Marty Roop, Ph.D.
Department of Microbiology and Immunology, ECU

Subject: Protocol K167b, original approval date 08/20/2020

Dear Dr. Roop:

The amendment#9 to your Animal Use Protocol entitled, "Virulence of defined *Brucella* mutants in the mouse model of chronic infection" (AUP#K167b) was reviewed by this institution's Animal Care and Use Committee on 02/22/2022. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle prior to any hazard use

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.

Sincerely yours,

SBMcRae

Sue McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/GD

enclosure