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Characterizing the mechanism of cell-cell fusion induced by the bacterium Burkholderia thailandensis

Author
Kostow, Nora

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Characterizing the mechanism of cell-cell fusion induced by the bacterium *Burkholderia thailandensis*

by

Nora Kostow

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Committee in charge:

Professor Matthew Welch, Chair
Professor David Drubin
Professor Daniel Fletcher
Professor Sarah Stanley

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Abstract

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Cell-cell fusion is important for biological processes including fertilization, development, immunity, and microbial pathogenesis. Bacteria in the pseudomallei group of *Burkholderia* species, including *B. thailandensis*, spread between host cells by inducing cell-cell fusion. Previous work showed that *B. thailandensis*-induced cell-cell fusion requires intracellular bacterial motility and a bacterial protein secretion apparatus called the type VI secretion system-5 (T6SS-5), including the T6SS-5 protein VgrG5. However, the cellular level mechanism and T6SS-5 proteins important for bacteria-induced cell-cell fusion remained incompletely described. Using live cell imaging, we found that bacteria used actin-based motility to push on the host cell plasma membrane to form plasma membrane protrusions that extended into neighboring cells. Then, membrane fusion occurred within these membrane protrusions, either proximal to the bacterium at the tip or elsewhere within a protrusion. Expression of VgrG5 by bacteria within membrane protrusions was required to promote cell-cell fusion. Furthermore, a second predicted T6SS-5 protein, TagD5, was also required for cell-cell fusion. In the absence of VgrG5 or TagD5, bacteria in plasma membrane protrusions were engulfed into neighboring cells. Our results suggest that the T6SS-5 effectors VgrG5 and TagD5 are secreted within membrane protrusions and act locally within membrane protrusions to promote membrane fusion. Continued investigation of this pathway will enhance our understanding of the cellular and molecular mechanisms of membrane fusion and cell-cell fusion.

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Abbreviations

T6SS - type VI secretion system
MNGC - multinucleated giant cell
PAAR - proline-alanine-alanine-arginine
Bt -Burkholderia thailandensis
T3SS –type III secretion system
T4SS –type IV secretion system

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Chapter 1

Introduction

The work in this thesis aims to understand how the bacterium *Burkholderia thailandensis* induces host cell-cell fusion to achieve direct cell-to-cell spread between host cells. I will first describe how cell-cell fusion occurs in other systems. I will then describe how other bacteria achieve cell-to-cell spread without cell-cell fusion. Because *B. thailandensis*-induced cell-cell fusion requires a bacterial protein secretion apparatus called the type VI secretion system (T6SS), I will review how the T6SS functions based on knowledge of other bacteria. Finally, I will review what was known previously about *B. thailandensis*-induced cell-cell fusion.

Cell-cell fusion is mediated by both cellular and molecular level mechanisms

Membrane fusion is the process by which two membranes become one. Examples of membrane fusion include: mitochondrial fusion, vesicle fusion, and viral envelop-host cell membrane fusion. A subcategory of membrane fusion is cell-cell fusion, which joins two plasma membranes of two different cells, resulting in a single, fused cell. Examples of cell-cell fusion include: gamete fusion during fertilization, myoblast fusion during muscle development, osteoclast fusion during bone resorption, and programmed cell-cell fusion during vulval development in *Caenorhabditis elegans* (Chen et al., 2007). Although membrane fusion and cell-cell fusion share overlapping features, cell-cell fusion presents unique mechanistic obstacles because the membranes make contact outside of the cell, where cells have less control over the components and environment. Additionally, plasma membranes can have unique features that prevent them from being closely apposed, such as cell surface proteins or the presence of a cell wall. Therefore, fusing cells have evolved mechanisms to overcome such challenges.

During cell-cell fusion, the plasma membranes of two cells are merged in a process that involves two key steps. In the first step, the two membranes, which are typically separated by extracellular components, are brought into close proximity (Figure 1.1A) (Chernomordik & Kozlov, 2003; Hernández & Podbilewicz, 2017). This often requires factors such as the cytoskeleton and cell adhesion molecules (Figure 1.1B) (Aguilar et al., 2013; Zito et al., 2016). In a second step, the remaining distance between the membranes is closed, the outer leaflets fuse to form a hemifusion intermediate, and the inner leaflets combine, resulting in a fusion pore without disrupting the plasma membrane integrity (Figure 1.1C) (Chernomordik & Kozlov, 2003; Hernández & Podbilewicz, 2017). This requires the activity of proteins called fusogens (Chernomordik & Kozlov, 2003; Hernández & Podbilewicz, 2017). Once membrane fusion occurs, the small fusion pore then expands to generate one continuous cell (Figure 1.1 A and C) (Hernández & Podbilewicz, 2017). Each step of the pathway including membrane apposition, outer leaflet fusion, inner leaflet fusion, and fusion pore expansion, pose energy barriers that must be overcome by cellular machinery and fusogens. Although the steps required for fusion are clear, the cellular and molecular mechanisms are poorly understood.

Certain cell-cell fusion pathways are well understood and offer insights into how cell-cell fusion occurs. Some illuminating examples of the first step of the cell-cell pathway come from *Drosophila* myoblast fusion, fusion-associated small transmembrane (FAST) fusion, and *Chlamydomonas reinhardtii* reproduction. During muscle cell development in *Drosophila*, two cell types are established, founder cells and myoblasts (Kim & Chen, 2019). Myoblasts form podosome-like structures that push up against founder cells and localize adhesion molecules while the founder cell exerts an actin-mediating resisting force (Sens et al., 2010; Kim et al., 2015). These invading and resisting forces are thought to bring the membranes into close contact so that fusion can occur in these structures (Sens et al., 2010; Kim et al., 2015; Kim & Chen,

2019). A group of nonenveloped, fusogenic reoviruses express cell-cell fusogens called FAST proteins (Duncan, 2019). FAST fusogens work by recruiting host factors that nucleate actin to their cytoplasmic tail and actin polymerization likely generates the force required to bring neighboring plasma membranes together so that the fusogenic extracellular domain can carry out its activity (Chan et al., 2020, 2021). In order to mate, *C. reinhardtii* cells undergo differentiation to form two mating types (Hernández & Podbilewicz, 2017). The mating cells dissolve their cell wall and undergo recognition via establishment of a mating junction (Hernández & Podbilewicz, 2017). This involves actin-mediated projections from both cells toward each other (Goodenough et al., 1982; Detmers et al., 1983; Hernández & Podbilewicz, 2017). At the junction between the two projections, the two cells adhere and membrane fusion can then take place (Hernández & Podbilewicz, 2017). These three examples highlight the importance of differentiation, recognition, actin, force generation, and membrane projections in the cellular pathways of diverse cell-cell fusion processes.

The cellular level mechanisms described above work together with the molecular components, which are called fusogens, to carry out membrane fusion. Fusogens are categorized by their sufficiency to induce membrane fusion, typically established using an in vitro membrane fusion assay or exogenous expression in tissue culture. Fusogens can work when they are present in one membrane (unilaterally) or need to be present on both membranes (bilaterally). They also typically do not require chemical energy to induce membrane fusion, for example from ATP hydrolysis, but instead use conformational changes and refolding. The fusogens for several membrane fusion processes are known but those for many intracellular membrane and cell-cell fusion processes have not yet been identified. For example, the fusogens responsible for mitochondrial fusion, vertebrate gamete fusion, *Drosophila* myoblast fusion, osteoclast fusion, macrophage fusion, and *B. thailandensis*-induced cell-cell fusion have not been identified. And for the cell-cell fusogens that have been identified, the mechanisms of fusogens that do not resemble viral fusogens are still poorly understood.

Most of our understanding of how fusogens achieve membrane fusion comes from a few examples of non-cell-cell fusogens. The best understood fusogens are SNARE proteins that carry out intracellular secretory vesicle fusion and Class I, II, or III viral fusogens used for virus envelope-host cell membrane fusion during viral entry into a host cell (Martens & McMahon, 2008; Hernández & Podbilewicz, 2017). SNAREs bind the donor and recipient vesicle membranes through transmembrane domains (Jahn & Scheller, 2006). Helical bundles from the SNAREs on both membranes bind together and refold, resulting in membrane fusion (Jahn & Scheller, 2006). For virus envelope-host cell membrane fusion, three classes of fusogens have been identified and a model for how they induce membrane fusion has been formed based on X-ray crystal structures of virus-membrane fusogens in pre-and post-fusion states (Podbilewicz, 2014). In this model, inactive fusogens are triggered (for example by a pH change) to undergo conformational changes that expose fusion peptides that insert into the target cell membrane (Podbilewicz, 2014). Another conformational change brings the transmembrane domains and fusion close together and provides the energy for membrane fusion (Hernández & Podbilewicz, 2017). The membrane proximity, transmembrane domains, and fusion peptide work together to fuse membranes via a hemifusion intermediate (Martens & McMahon, 2008; Podbilewicz, 2014).

Illuminating examples of the molecular level pathway leading to cell-cell fusion are vulval development in *Caenorhabditis elegans* and vertebrate myoblast fusion. *C. elegans* vulval development requires several cell-cell fusion steps which require the fusogens EFF-1 and AFF-1. Via X-ray crystallography, it was determined that this family of fusogens are similar to Class II viral fusion proteins and therefore act via a homologous mechanism (Pérez-Vargas et al., 2014; Podbilewicz, 2014). Vertebrate myoblast membrane fusion is mediated by two proteins: Myomaker (Millay et al., 2013) and Myomerger/Myomixer/Minion (Bi et al., 2017; Quinn et al., 2017; Zhang et al., 2017). Myomaker, a seven transmembrane domain protein, mediates outer leaflet fusion to form a hemifusion intermediate while Myomerger, an 84 amino acid protein with a transmembrane domain and a cytoplasmic tail, completes membrane fusion by promoting inner membrane leaflet fusion resulting in fusion pore formation (Brukman et al., 2019; Leikina et al., 2018). How these proteins carry out their individual roles and collaborate during cell-cell fusion remain unknown. These examples highlight the diversity of how fusogens achieve membrane fusion and highlight that the best understood cell-cell fusogens are homologous to viral fusogens.

Identification of both the cellular and molecular-level mechanisms of the cell-cell fusion pathway are key to achieving a holistic understanding of cell-cell fusion mechanisms. Membrane apposition and fusogen activity are tightly linked and coordinated. Evidence for this comes from a clever experiment in *Drosophila* S2 cells (Shilagardi et al., 2013). In this experiment, actin protrusions were induced by exogenous expression of the myoblast proteins Duf and Sns and a fusogen was provided by exogenous expression of EFF-1 (Shilagardi et al., 2013). When expressed alone, neither were sufficient to carry out cell-cell fusion (Shilagardi et al., 2013). But when expressed together, the system supported cell-cell fusion (Shilagardi et al., 2013). This concept is also exemplified by the FAST fusogens, which uniquely achieve both membrane apposition and fusogen activity within a single protein and the two functions cannot be separated (Chan et al., 2020, 2021).

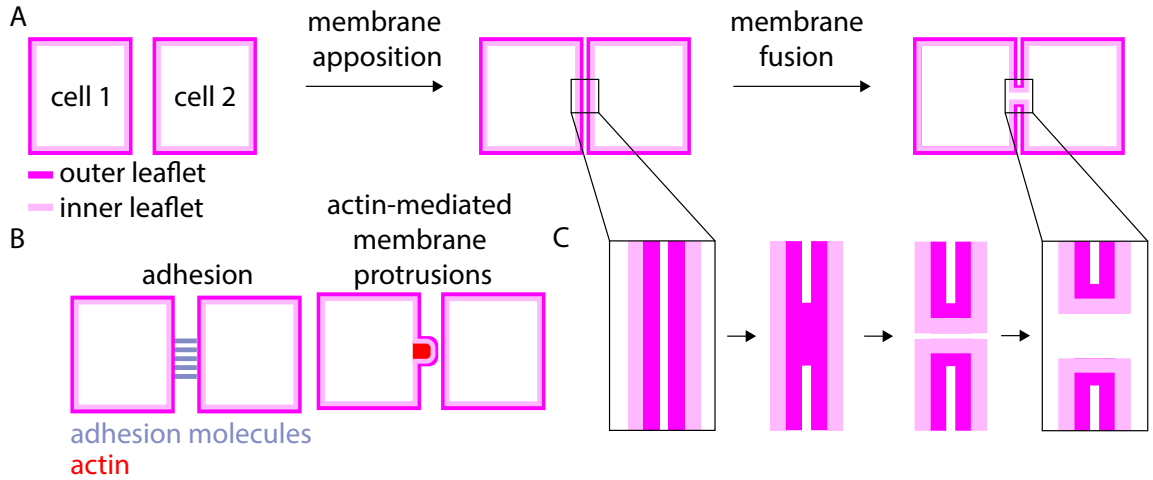

Figure 1.1 Models and examples of cellular and molecular level mechanisms of cell-cell fusion. (A) Cellular-level model of cell-cell fusion. (B) Membrane apposition is mediated by cellular factors. (C) Molecular-level model of membrane fusion during cell-cell fusion.

Cell-to-cell spread of bacteria

The *Burkholderia* species of the pseudomallei group undergo cell-to-cell spread by inducing cell-cell fusion, but this mechanism is unique among bacteria that undergo cell-to-cell spread. Bacterial pathogens that live in the host cell cytosol commonly spreading directly from cell-to-cell without accessing the extracellular environment to avoid extracellular dangers. The bacterial pathogens *Listeria monocytogenes*, *Shigella flexneri*, some *Rickettsia* species, pseudomallei group of *Burkholderia*, and *Mycobacterium marinum* all undergo direct cell-to-cell spread. *L. monocytogenes* and *S. flexneri* are two of the best studied species that undergo such cell-to-cell spread. Pseudomallei species of *Burkholderia* are the only bacterial species that spread from cell-to-cell by directly inducing cell-cell fusion but the pathway to achieving cell-cell fusion has not been established.

Initial insight into how cell-to-cell spread by engulfment occurs came from observations of the progression of *L. monocytogenes* infection in tissue culture by transmission electron microscopy (Tilney & Portnoy, 1989). Based on their findings, the authors proposed what is referred to here as the "engulfment" pathway of cell-to-cell spread (Tilney & Portnoy, 1989). In the engulfment pathway, bacteria form membrane protrusions from donor to recipient cells which are then engulfed into secondary double membrane vacuoles which the bacteria escape from to regain access to the cytosol (Tilney & Portnoy, 1989; Lamason & Welch, 2017; Dowd et al., 2021). The engulfment pathway was later confirmed by live-cell imaging (Robbins et al., 1999). Similar pathways have since been observed for *R. parkeri* (Lamason et al., 2016) and *S. flexneri* (Kuehl et al., 2014). Each species has evolved unique ways of achieving and controlling this pathway.

Studies of *L. monocytogenes* spread have revealed interesting insights into how bacteria manipulate their host to promote protrusion formation. *L. monocytogenes* form long plasma membrane protrusions formed by bacteria undergoing actin-based motility (Robbins et al., 1999; Lamason et al., 2016). Actin-based motility is induced by the bacterial protein ActA, which activates the Arp2/3 complex (Welch et al., 1998). *L. monocytogenes* protrusion initiation involves the secreted bacterial effector protein InlC (Rajabian et al., 2009). One of the functions of InlC is to block Tuba from carrying out its role in establishing membrane tension at the host cell cortex, thus resulting in lower cortical tension during *L. monocytogenes* infection and this lowered tension is hypothesized to reduce protrusion occurrence (Rajabian et al., 2009; Dowd et al., 2021). Proper protrusion formation also involves several host factors including Ezrin, Caveolin-1, Pacsin and Exo70 among others (Pust et al., 2005; Sanderlin et al., 2019; Dhanda et al., 2020; Dowd et al., 2021), although the mechanistic contributions of these factors is still under investigation. Based on these findings from *L. monocytogenes*, it is clear that both bacterial and host factors contribute to efficient protrusion formation.

Studies of *S. flexneri* have revealed insights into how bacteria might manipulate protrusion morphology. *S. flexneri* also forms long membrane protrusions (Bishai et al., 2013) using actin-based motility. This motility is mediated by the bacterial effector protein IscA which recruits N-WASP to activate the Arp2/3 complex (Makino et al., 1986; Bernardini et al., 1989). Cell-to-cell spread occurs more frequently at tricellular junctions, a feature that is unique among bacteria that undergo cell-to-cell spread, indicating that tricellular junctions may play a role in protrusion formation or spread (Fukumatsu et al., 2012). Protrusion formation also involves the activity of the secreted bacterial effector protein IpaC, which lowers cortical tension to promote

protrusion formation (Duncan-Lowey et al., 2020). Protrusion morphology of *S. flexneri* protrusions is unique in that the protrusion stalk collapses, forming a so-called vacuole like protrusion (VLP) (Dragoi & Agaisse, 2015). This protrusion morphology is dependent on the class II phosphatidylinositol 3-phosphate kinase PIK3C2A, a host protein which generates phosphatidylinositol- 3-phosphate (PI(3)P) which can be found along *S. flexneri* protrusions (Dragoi & Agaisse, 2015). These findings show that protrusion localization and morphology can be differentially controlled by bacteria.

R. parkeri also undergoes the engulfment cell-to-cell spread pathway, but this pathway looks very different from that of *L. monocytogenes* or *S. flexneri* (Lamason et al., 2016). *R. parkeri* does not undergo actin-based motility within membrane protrusions and forms very short protrusions (Lamason et al., 2016), although actin-based motility is important for spread (Reed et al., 2014). Interestingly, at the time of spread, *R. parkeri* undergoes formin-like actin nucleation leading to the formation of long, bundled actin filaments that lead to straight actin tails and paths of motility (Haglund et al., 2010; Kleba et al., 2010; Madasu et al., 2013) which differ greatly from the Arp2/3-mediated, curvy actin tails of *L. monocytogenes* or *S. flexneri*. This is evidence that the mechanism of protrusion formation can vary between bacteria and that actin-based motility does not always result in membrane protrusion formation. In addition, *R. parkeri* requires a secreted effector, Sca4, for efficient protrusion engulfment (Lamason et al., 2016), indicating that bacteria may actively mediate engulfment.

Although these examples highlight the importance of bacterial proteins during cell-to-cell spread, bacterial factors aside from actin nucleation promoting factors are not absolutely required. Monack and Theriot showed that *Eschericia coli*, which does not typically access the cytosol, could be engineered to access the cytosol and undergo actin-based motility which resulted in protrusion formation and engulfment into a recipient cell (Monack & Theriot, 2001). Notably, this engineered system did not recapitulate escape from the secondary vacuole, which is also an important step in the cell-to-cell pathway. This experiment showed that actin-based motility is sufficient for protrusion formation and engulfment (Monack & Theriot, 2001). Therefore, certain aspects of the cell-to-cell spread pathway likely rely on canonical host cell pathways or pathway manipulation rather than on bacteria developing spread mechanisms de novo.

Much is still unknown about how bacterial cell-to-cell spread occurs, especially for less well studied bacteria including *R. parkeri*, *Burkholderia* species, and *Mycobacterium marinum*. *Burkholderia* species are of particular interest because they induce cell-cell fusion, a pathway that is very different from the engulfment pathway. Understanding the pathways leading to cell-to-cell spread and how bacterial factors manipulate host biology will be key to understanding the mechanisms by which bacterial pathogens undergo cell-to-cell spread.

The type VI secretion system (T6SS) is a molecular syringe that secretes effector proteins, including VgrG and PAAR proteins

One feature that is known to be essential for *B. thailandensis*-induced cell-cell fusion is the bacterial protein secretion apparatus known as the type VI secretion system five (T6SS-5). Bacterial pathogens must deliver bacterial proteins into target cells, which is typically achieved through bacterial protein secretion systems termed the type III secretion system (T3SS), type IV secretion (T4SS), and the more recently identified type VI secretion system (T6SS). The T6SS

also functions during interbacterial communication and competition. The T6SS is composed of four main features: the baseplate, the sheath, the needle, and the tip. The baseplate anchors the system to the periplasm and bacterial membranes (Basler et al., 2012; Durand et al., 2015; Chang et al., 2017; Park et al., 2018; Nazarov et al., 2018). The sheath extends into the bacterial cytosol from the baseplate and surrounds the needle (Mougous et al., 2006; Basler et al., 2012; Chang et al., 2017; Wang et al., 2017). The tip sits inside of the baseplate and at the tip of the needle (Basler et al., 2012; Durand et al., 2015; Chang et al., 2017). Upon contraction of the sheath toward the baseplate, the needle/tip is shuttled across the baseplate, and outside of the cell (Pukatzki et al., 2007a; Leiman et al., 2009; Basler et al., 2012). This ejection force can puncture neighboring objects that are within rage, including a host cell, a vacuolar membrane, or a bacterium. Bacterial effector proteins can bind to T6SS needle and tip components, and these effector proteins are the only components of the T6SS that are released upon secretion (Jurėnas & Journet, 2021; Mougous et al., 2006; Pukatzki et al., 2006, 2007a). The tip components can contain C-terminal extensions that act as functional domains; I will term such proteins "extended" effectors (Jurėnas & Journet, 2021; Pukatzki et al., 2009). Proteins are secreted into the location that the T6SS is able to puncture. The T6SS components that shuttle effector proteins are: the needle building block Hcp (Mougous et al., 2006), the tip component VgrG (Pukatzki et al., 2007a) and the tip extending proline-alanine-alanine-arginine (PAAR) protein (Shneider, 2013).

VgrG and PAAR proteins are the tip components that are typically extended to carry effector functions. VgrG proteins trimerize to form a blunt cone-like structure (Spínola-Amilibia et al., 2016; Leiman et al., 2009). The N-terminus of the peptide is located at the base of the cone and the C-terminus is at the tip. For extended VgrG proteins, functional domains are translationally fused with the C-terminus, emanating from the cone tip (Pukatzki et al., 2007a). One example of an extended VgrG protein is VgrG1 of *Vibrio cholerae*, which is secreted into target host cells after bacterial internalization. VgrG1 is secreted across the vacuolar membrane into the host cytosol where it crosslinks host actin to impair the host's phagocytic function (A. T. Ma et al., 2009; Pukatzki et al., 2007b). Another example of an extended VgrG protein is VgrG1 of *Escherichia coli* which functions during interbacterial competition (Flaugnatti et al., 2016). VgrG1 binds to the phospholipase Tle1 through its C-terminal domain (Flaugnatti et al., 2016). Based on the Cryo-EM structure of the VgrG-Tle1 complex, three Tle1 molecules bind the VgrG1 cone structure and part of the VgrG1 C-terminal domain which emanates from the top of the VgrG cone (Flaugnatti et al., 2020). Determination of this structure established how TleI loads onto VgrG1 for secretion and how its phospholipase activity is blocked by its interaction with VgrG1 to prevent it from acting before secretion (Flaugnatti et al., 2020).

In addition to VgrG-mediated secretion, effector proteins can be secreted via extended PAAR proteins. One example of this is the *P. aeruginosa* Tse6, which contains a toxin domain that is delivered to the cytoplasm of neighboring bacteria during interbacterial competition (Quentin et al., 2018; Whitney et al., 2014, 2015). Tse6 forms a complex with VgrG1 that does not dissociate upon secretion (Quentin et al., 2018). Tse6 contains two transmembrane domains that are protected before secretion by chaperones that are required for stability and loading onto the T6SS (Hachani et al., 2016; Quentin et al., 2018; Whitney et al., 2014). When Tse6-VgrG1 is secreted into a neighboring bacterium's periplasm, the transmembrane domains self-insert to the inner membrane, and the toxin domain reaches the cytoplasm (Quentin et al., 2018). It was hypothesized that the transmembrane domains form a pore through which the toxin domain

could be threaded and then refolded, similar to how *Corynebacterium diphtheriae* diphtheria toxin is thought to be translocated (Murphy, 2011; Quentin et al., 2018).

The T6SS's function in targeting neighboring bacteria and transferring proteins across membrane barriers make it a unique bacterial secretion apparatus. There are many open questions regarding T6SS function during diverse processes including: How is T6SS firing controlled? How does T6SS regulate effector proteins localization? How do T6SS effector proteins function once they reach their destination? Understanding how the T6SS functions during processes aside from interbacterial competition will shed more light on the diverse functions of the T6SS.

The *Burkholderia* pseudomallei group of pathogens induce cell-cell fusion

The pseudomallei group of *Burkholderia* species are Gram-negative, intracellular bacterial species that directly induce cell-cell fusion (Kespichayawattana et al., 2000). The group consists of three species: *B. pseudomallei*, *B. mallei* and *B. thailandensis*. *B. pseudomallei* causes the human disease melioidosis, which is responsible for about 89,000 deaths worldwide per year and has a fatality rate of 10-50% (Wiersinga et al., 2018). *B. pseudomallei* is soil dwelling and infects humans through broken skin, inhalation, or ingestion (Wiersinga et al., 2018). *B. mallei* causes the equine disease glanders (Wilkinson, 1981) and cannot exist outside of a host but can be transmitted to humans and cause disease (Galyov et al., 2010). Both *B. mallei* and *B. pseudomallei* are accidental pathogens and cannot spread from human-to-human (Willcocks, 2016) but are nonetheless a major threat, especially because they are resistant to many antibiotics and no vaccines for them exist (Galyov et al., 2010). The third species, *B. thailandensis*, is not thought to be a human pathogen and is used as a model system for studying aspects of infection by its pathogenic relatives (Haraga et al., 2008; West et al., 2008).

Histopathology of human tissue samples from mellioidosis patients revealed giant cells that were presumably formed through cell-cell fusion (K. T. Wong et al., 1995). Giant cells were also observed in tissue culture for all three pseudomallei group species (Harley et al., 1998). That these species directly induce cell-cell fusion was ultimately shown by infecting a mixture of differentially labeled tissue culture cells with *B. pseudomallei* and observing overlapping labels within giant cells (Kespichayawattana et al., 2000). Although it is clear that *B. pseudomallei* induces cell-cell fusion in vitro and in a disease context, the reasons for inducing cell-cell fusion are not known.

B. thailandensis is used as a model system for studying cell-cell fusion of *Burkholderia* pseudomallei group species because it is non-pathogenic (Haraga et al., 2008; West et al., 2008), and therefore it will be the focus of the work presented in this thesis. During the *B. thailandensis* life cycle, bacteria invade mammalian host cells, escape the phagosome, live in the cytosol, and spread to neighboring cells by inducing cell-cell fusion (Harley et al., 1998; Kespichayawattana et al., 2000). After invasion, the type III secretion system (T3SS) mediates escape from the primary vacuole. French et al. elegantly showed that the T3SS is required only for vacuole escape when they inserted a *B. thailandensis* T3SS deletion mutant directly into the host cytosol using a nanoblade and observed that no features of the life cycle were altered, including actin-based motility and MNGC formation (French et al., 2011). Once in the cytosol, glutathione exposure triggers expression of factors required for spread, including those associated with actin-

based motility and the type VI secretion system five (T6SS-5) (J. Wong et al., 2015). *B. thailandensis* undergoes intracellular bacterial actin-based motility mediated by BimA as well as intracellular flagellar-based motility (French et al., 2011; Kespichayawattana et al., 2000; Schell et al., 2007; Sitthidet et al., 2010; J. M. Stevens et al., 2005; M. P. Stevens et al., 2005). The three species in the pseudomallei group of *Burkholderia* encode orthologs of BimA that mediate actin-based motility in unique ways. *B. thailandensis* BimA activates the Arp2/3 complex, forming short, curved tails (Sitthidet et al., 2010; Benanti et al., 2015). *B. mallei* and *B. pseudomallei* BimA orthologs use an Ena/VASP-like mechanism to form long tails consisting of bundled filaments (M. P. Stevens et al., 2005; Benanti et al., 2015). Although bacterial motility is important for efficient *B. thailandensis* induced cell-cell fusion, it is not absolutely required for cell-cell fusion as a mutant deficient in both modes of motility is still able to induce cell-cell fusion with substantially reduced efficiency (French et al., 2011). One consequence of intracellular bacterial actin-based motility is the formation of membrane protrusions which can be used for cell-to-cell spread (Lamason & Welch, 2017). *B. thailandensis* has been observed in plasma membrane protrusions (Kespichayawattana et al., 2000; J. M. Stevens et al., 2005; M. P. Stevens et al., 2005), but whether and how these protrusions contribute to cell-cell fusion is not known.

The final step in the *B. thailandensis* intracellular life cycle is inducing plasma membrane fusion. This requires a bacterial protein secretion apparatus called the T6SS-5 (Schell et al., 2007; Schwarz et al., 2010). *B. thailandensis* has five T6SS's, of which T6SS-5 is the only T6SS necessary for pathogenesis in a mouse model of infection (Burtnick et al., 2011; Hopf et al., 2014; Pilatz et al., 2006; Schell et al., 2007; Schwarz et al., 2010). Therefore, it is likely that a bacterial effector secreted by the T6SS-5 is responsible for inducing membrane fusion.

In order to identify bacterial effector proteins secreted by the T6SS-5, Schwarz et al. attempted to identify the *B. thailandensis* "secretome" (Schwarz et al., 2014). To do this, they grew *B. thailandensis* overexpressing the T6SS-5 master regulator VirAG in broth, identified proteins that were released into the broth via mass spectrometry, and compared this with a T6SS-5-deficient strain (Schwarz et al., 2014). They only identified VgrG5 (Schwarz et al., 2014) and demonstrated that it is required for cell-cell fusion (Schwarz et al., 2014; Toesca et al., 2014). However, this approach did not perfectly mimic the intracellular context for T6SS-5 function and could underrepresent the true T6SS-5 "secretome." VgrG5 contains a domain common to all VgrG proteins that trimerizes to form a blunt cone structure (Leiman et al., 2009; Spínola-Amilibia et al., 2016). The VgrG5 C-terminal domain (CTD) likely contains function since C-terminal extensions containing effector function are common features of VgrG proteins in other bacteria (Hachani et al., 2016; Jurėnas & Journet, 2021). Additionally, truncations that remove the entire CTD or portions of the CTD do not support cell-cell fusion (Schwarz et al., 2014; Toesca et al., 2014). The required regions of the CTD include a predicted transmembrane domain (TMD) and the C-terminal region. The TMD consists of two predicted transmembrane helices connected by a twelve amino acid linker. Although there is no structural data for VgrG5, its N-terminal domain is similar to the corresponding domain in other VgrG proteins, suggesting this domain likely mediates trimerization to form the standard cone-like structure of VgrG proteins. Emanating from this predicted cone is likely three predicted TMDs (containing a total of six predicted transmembrane helices), and additional functional domains, possibly at the C-termini of the three peptides. How VgrG5 contributes to cell-cell fusion remains unknown.

B. thailandensis also encodes a PAAR protein within the T6SS-5 gene cluster called TagD5 (Lennings, West, et al., 2019). TagD5 is small (119 amino acids) and contains a PAAR structural domain but does not contain a sequence extension present in some PAAR proteins that carry out effector functions (Shneider, 2013; Hachani et al., 2016) (Figure 1.2), so it is unclear how it could directly contribute to membrane fusion or have any effector function. However, whether TagD5 is required for cell-cell fusion and how it contributes to cell-cell fusion remain unknown.

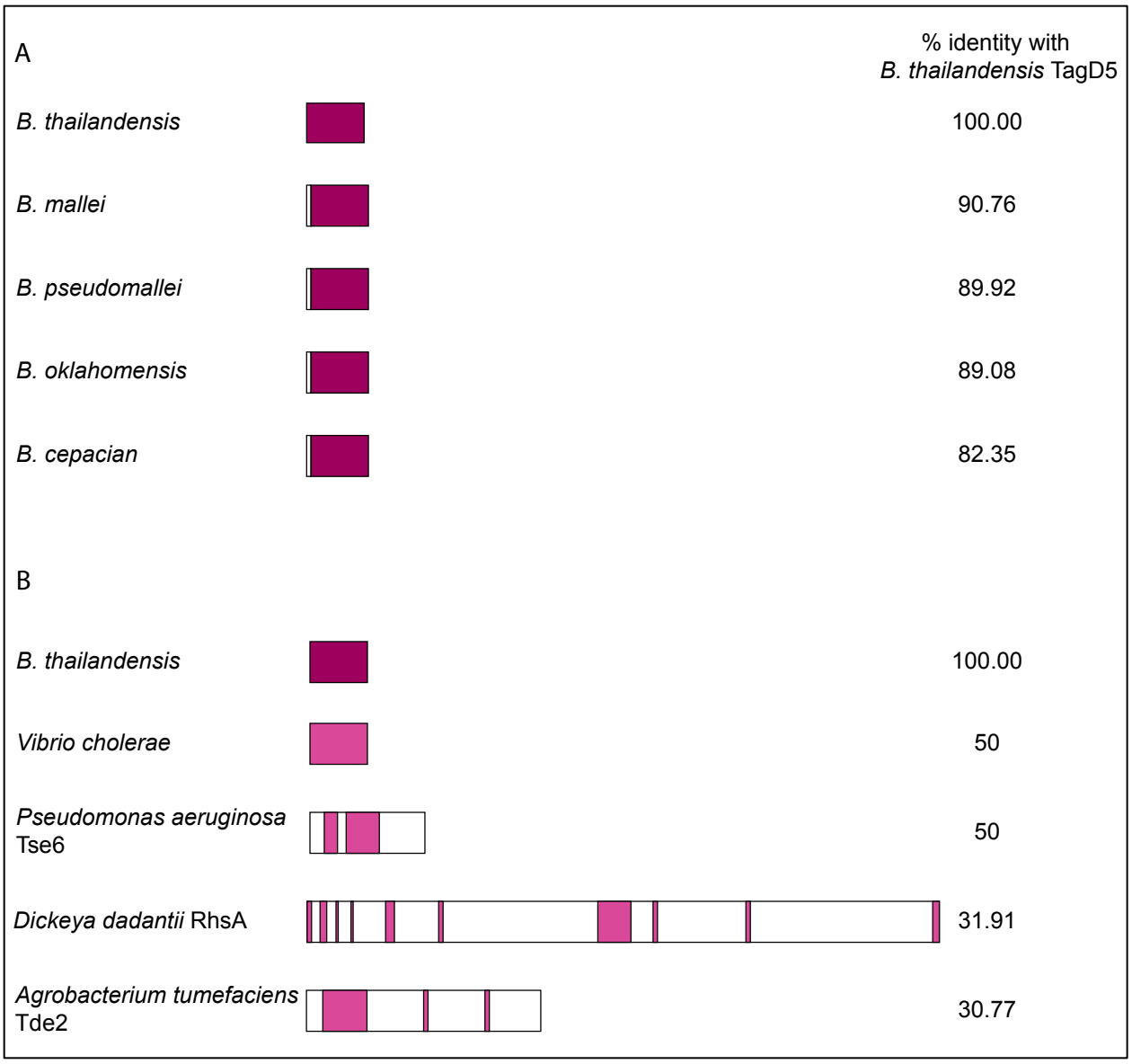

Figure 1.2: TagD5 homologs in *Burkholderia* and other species. (A) Amino acid identity of *B. thailandensis* TagD5 homologs compared with other *Burkholderia* species based on BLAST results. Sequence identity to *B. thailandensis* TagD5 are shown on the right. (B) Amino acid identity of *B. thailandensis* TagD5 compared with other PAAR domain containing proteins of other bacteria including *Vibrio cholerae* VCA0105 (Shneider, 2013), *Pseudomonas aeruginosa* Tse6 (Flaugnatti et al., 2016, 2020), *Dickeya dadantii* RhsA (L.-S. Ma et al., 2014), and Agrobacterium tumefaciens Tde2 (L.-S. Ma et al., 2014). Sequence identity to *B. thailandensis* TagD5 are shown on the right. Alignments illustrate that TagD5 does not contain additional sequences outside of the PAAR domain, similar to *V. cholerae* VCA0105. This is distinct from *P. aeruginosa* Tse6, *D. dadantii* RhsA, and A. tumefaciens Tde2 which do contain additional effector sequences. Maroon boxes represent regions with higher than 50% sequence identity to *B. thailandensis* VgrG5 and pink boxes represent regions that have below 50% sequence identity to *B. thailandensis* VgrG5.

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Chapter 2

Plasma membrane protrusions mediate host cell-cell fusion induced by *Burkholderia thailandensis*

Note: The information presented in this chapter was included in the publication Kostow N. and Welch M.D. Plasma membrane protrusions mediate host cell-cell fusion induced by *Burkholderia thailandensis*. bioRxiv 2022.02.24.481536

Abstract

Cell-cell fusion is important for biological processes including fertilization, development, immunity, and microbial pathogenesis. Bacteria in the pseudomallei group of *Burkholderia* species, including *B. thailandensis*, spread between host cells by inducing cell-cell fusion. Previous work showed that *B. thailandensis*-induced cell-cell fusion requires intracellular bacterial motility and a bacterial protein secretion apparatus called the type VI secretion system-5 (T6SS-5), including the T6SS-5 protein VgrG5. However, the cellular level mechanism and T6SS-5 proteins important for bacteria-induced cell-cell fusion remained incompletely described. Using live cell imaging, we found bacteria used actin-based motility to push on the host cell plasma membrane to form plasma membrane protrusions that extended into neighboring cells. Then, membrane fusion occurred within these membrane protrusions, either proximal to the bacterium at the tip or elsewhere within a protrusion. Expression of VgrG5 by bacteria within membrane protrusions was required to promote cell-cell fusion. Furthermore, a second predicted T6SS-5 protein, TagD5, was also required for cell-cell fusion. In the absence of VgrG5 or TagD5, bacteria in plasma membrane protrusions were engulfed into neighboring cells. Our results suggest that the T6SS-5 effectors VgrG5 and TagD5 are secreted within membrane protrusions and act locally to promote membrane fusion.

Introduction

Cell-cell fusion is important for biological processes including fertilization, development, and immunity (Chen et al., 2007). During cell-cell fusion, the plasma membranes of two cells are merged in a process that involves two key steps. In the first step, the two membranes, which are typically separated by extracellular components, are brought into close proximity (Chernomordik & Kozlov, 2003; Hernández & Podbilewicz, 2017). This often requires cellular factors such as the cytoskeleton and cell adhesion molecules (Zito et al., 2016; Hernández & Podbilewicz, 2017; Kim & Chen, 2019; Takito & Nakamura, 2020). In a second step, the remaining distance between the membranes is closed, the outer leaflets fuse to form a hemifusion intermediate, and the inner leaflets combine, resulting in the formation of a fusion pore without disrupting plasma membrane integrity (Chernomordik & Kozlov, 2003; Hernández & Podbilewicz, 2017). This requires the activity of proteins called fusogens (Chernomordik & Kozlov, 2003; Hernández & Podbilewicz, 2017). Once membrane fusion occurs, the small fusion pore then expands to generate one continuous cell (Hernández & Podbilewicz, 2017). Although the steps required for fusion are clear, the cellular and molecular level mechanisms are poorly understood. One approach to revealing cell-cell fusion mechanisms is to investigate microbe-induced cell-cell fusion processes.

The pseudomallei group of *Burkholderia* species are the only bacterial species known to directly induce cell-cell fusion (Kespichayawattana et al., 2000). This leads to the formation of multinucleated giant cells (MNGCs), both in cultured cells and in infected animals and human tissues (French et al., 2011; Harley et al., 1998; Kespichayawattana et al., 2000; West et al., 2008; K. T. Wong et al., 1995). Two species within this group, *B. pseudomallei* and *B. mallei*, cause the human disease melioidosis and equine disease glanders, respectively (Wiersinga et al., 2018; Wilkinson, 1981). A third species, *B. thailandensis*, is not thought to be a human pathogen and is used as a model system for studying aspects of infection with these pathogenic species (Haraga et al., 2008; West et al., 2008). *B. thailandensis* invades mammalian host cells, escapes the phagosome, and lives in the cytosol (Harley et al., 1998; Kespichayawattana et al., 2000). There, it undergoes intracellular bacterial actin-based (or flagellar) motility (French et al., 2011; Kespichayawattana et al., 2000; J. M. Stevens et al., 2005). Bacterial motility is important for efficient *B. thailandensis*-induced cell-cell fusion as a mutant deficient in both modes of motility induces cell-cell fusion with substantially reduced efficiency (French et al., 2011). Other bacterial pathogens, such as *Listeria monocytogenes* and *Rickettsia parkeri*, spread directly from cell-to-cell via a process that also involves actin-based motility (Lamason & Welch, 2017). Motility brings these bacteria to the plasma membrane where they enter into membrane protrusions that are engulfed into neighboring cells and are resolved into double membrane vesicles that they escape from to regain access to the cytosol (Tilney & Portnoy, 1989; Robbins et al., 1999; Monack & Theriot, 2001; Lamason et al., 2016; Lamason & Welch, 2017). *B. thailandensis* is also observed in plasma membrane protrusions (Kespichayawattana et al., 2000; J. M. Stevens et al., 2005; M. P. Stevens et al., 2005), but whether and how these protrusions contribute to cell-cell fusion is not known.

The second feature contributing to cell-cell fusion is a bacterial protein secretion apparatus called the type VI secretion system (T6SS) (Schell et al., 2007; Schwarz et al., 2010), a needle-like apparatus composed of a tube and a tip complex. To achieve secretion, bacterial

proteins with effector functions can be translationally fused with T6SS needle tip components or can bind to T6SS tip or tube components (Jurėnas & Journet, 2021; Mougous et al., 2006; Pukatzki et al., 2006, 2007a). The T6SS secretes proteins by ejecting the tube and tip of the needle from the bacterium, a process that can puncture into a neighboring bacterium or host cell, releasing effector proteins into the target cell. Secretion can also occur without puncturing nearby cells by ejecting proteins into the extracellular environment (Jurėnas & Journet, 2021). *B. thailandensis* has five T6SS's, of which T6SS-5 is the only T6SS necessary for pathogenesis in a mouse model of infection (Burtnick et al., 2011; Hopf et al., 2014; Pilatz et al., 2006; Schell et al., 2007; Schwarz et al., 2010). The T6SS-5 needle tip component VgrG5 is also required for cell-cell fusion (Schwarz et al., 2014; Toesca et al., 2014) and is the only protein known to be secreted by the T6SS-5 (Schwarz et al., 2014). VgrG5 contains a domain common to all VgrG proteins that trimerizes to form a blunt cone structure (Leiman et al., 2009; Spínola-Amilibia et al., 2016). Another component that is typically present at the T6SS tip is a PAAR (proline-alanine-alanine-arginine) family protein, which binds to the tip of a VgrG trimer resulting in an extended T6SS needle tip complex (Shneider, 2013). *B. thailandensis* encodes a PAAR protein within the T6SS-5 gene cluster called TagD5 (Lennings, West, et al., 2019). However, whether TagD5 is required for cell-cell fusion, and how the T6SS-5 tip components contribute to cell-cell fusion remain unknown.

To better understand the cellular pathway and bacterial factors leading to cell-cell fusion, we carried out live cell imaging of *B. thailandensis* as it induced cell-cell fusion. We found that cell-cell fusion occurred within host cell plasma membrane protrusions, with membrane fusion occurring both proximal to the bacterium at the protrusion tip or elsewhere in the protrusion. Expression of VgrG5 was required within membrane protrusions to promote cell-cell fusion. We also found that TagD5 was required for fusion. In the absence of VgrG5 or TagD5, bacterial protrusions were engulfed into neighboring cells. Our results suggest that the T6SS-5 effectors VgrG5 and TagD5 are secreted within membrane protrusions and act to promote cell-cell fusion.

Results

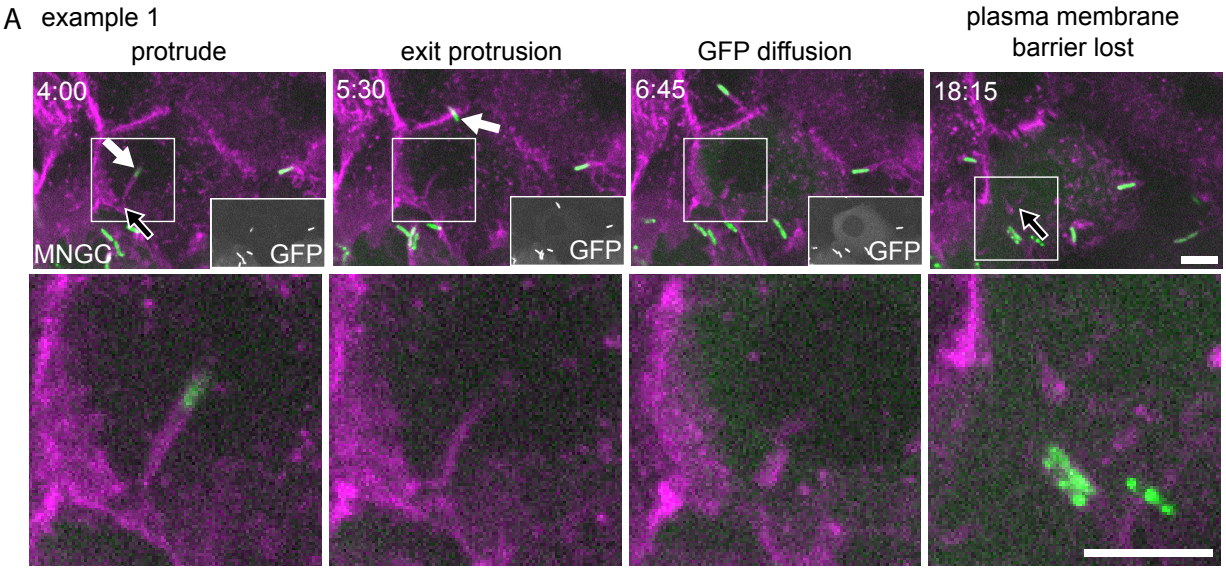
B. thailandensis induces cell-cell fusion at the tip or elsewhere within plasma membrane protrusions

To understand the cellular level mechanism by which *B. thailandensis* induces cell-cell fusion, we performed live cell imaging of cell-cell fusion events during *B. thailandensis* infection. We used a *B. thailandensis* strain deficient in flagellar motility (Δ*motA2*) but still competent for actin-based motility (hereafter called strain *Bt* WT) (French et al., 2011). For live cell imaging, we made a strain that also expressed GFP-tagged ClpV5 (ClpV5-GFP), a protein involved in disassembly of the T6SS in other bacteria (hereafter called strain *Bt*GFP WT) (Bonemann et al., 2009). ClpV5-GFP forms bright puncta in the bacterial cytosol of *B. thailandensis* (Lennings, Makhlouf, et al., 2019; Schwarz et al., 2014), allowing for clear visualization of the bacteria. Infections were carried out in monolayers of A549 human lung epithelial cells consisting of a 1:1 mixture of cells that stably expressed either an RFP plasma membrane marker (TagRFP-T-farnesyl) (Lamason et al., 2016) or stably expressed GFP in the cytosol. Upon infection and *B. thailandensis*-induced cell-cell fusion, MNGCs formed that expressed both the TagRFP-T-farnesyl plasma membrane marker and cytosolic GFP. For live cell imaging of cell-cell fusion, we observed bacteria that originated from an infected MNGC as they induced cell-cell fusion with a neighboring cell that expressed TagRFP-T-farnesyl plasma membrane marker but not cytosolic GFP. Therefore, as cell-cell fusion occurred, we observed the location of the bacterium relative to the RFP-labeled plasma membrane as well as the timing of cell-cell fusion as indicated by the diffusion of the cytosolic GFP from the MNGC into the cell that did not express GFP.

We observed that moving bacteria collided with the plasma membrane of the MNGC (termed the “donor” cell) and then moved into a membrane protrusion that extended into the neighboring cell (termed the “recipient” cell) (Figures 2.1 and 2.2). In the 144 cell-cell fusion events observed, we saw membrane protrusions in 141 events (in 3 events no plasma membrane protrusion was observed). Of these 141 events, 20 were selected for further analysis because the entire cell was visible, the process of protrusion formation and cell-cell fusion was captured from start to finish, and we could determine the location where cell-cell fusion was initiated. In 12/20 events, bacteria exited the protrusion at the protrusion tip and moved into the cytosol of the recipient cell (Figures 2.1A and B). Shortly thereafter, the cytosolic GFP diffused from the donor cell into the recipient cell (Figures 2.1A and B). This indicates that membrane fusion occurred at the tip of the membrane protrusion and formed a pore through which the bacteria moved (cartooned in Figure 2.1C). In the remaining 8/20 events, the bacteria remained at the plasma membrane protrusion tip, even as the GFP signal diffused from the donor cell into the recipient cell (Figures 2.2A and B). In one example, based on the TagRFP-T-farnesyl signal, the protrusion clearly appeared to be separated from the donor MNGC yet still contained the bacterium (Figure 2D). Therefore, in these examples, the membrane fusion occurred at a distance from the bacteria (cartooned in Figure 2.2C). In many events (n = 14), after the GFP diffused from the donor cell into the recipient cell, the now merged plasma membrane spread apart in the area where the protrusion had formed (Figures 2.1 and 2.2, Supplemental Figure 2.1). This indicates that membrane fusion occurred within the membrane protrusion and expanded, leading to a continuous cytoplasm between the donor and recipient cell and expanding the size of the MNGC. These observations indicate that cell-cell fusion occurs within membrane protrusions,

either at the protrusion tip or elsewhere in the protrusion.

To understand how protrusion morphology and timing might contribute to the cell-cell fusion pathway, we measured the maximum protrusion length and timing of cell-cell fusion events. The length of protrusions at their maximum was 8 +/- 2 μm (Figure 2.3A) (all experimentally determined values from this study are listed as mean +/- SD), shorter than the length previously observed for protrusions induced by *L. monocytogenes* (~17 μm on average) but longer than those induced by *R. parkeri* (~3 μm on average) (Lamason et al., 2016). There was no difference in maximum protrusion length during cell-cell fusion events that occurred at the protrusion tip versus elsewhere in the protrusion (Figure 2.3B), indicating that differences in the location of fusion are not related to protrusion length. To determine how long it takes for *B. thailandensis* to induce cell-cell fusion, we quantified the time from the start of protrusion formation to the time at which cytosolic GFP diffused into the recipient cell. This time was 8 +/- 3 min (Figure 2.3C; these data likely overrepresent shorter events due to the experimental difficulty of capturing long events) and there was no correlation between maximum protrusion length and the speed at which cell-cell fusion occurred (Figure 2.3D, R^2=0.09). When binned based on the location of membrane fusion, membrane fusion at the protrusion tip occurred slightly faster than membrane fusion that occurred elsewhere in the protrusion (Figure 2.3E). *B. thailandensis*-induced cell-cell fusion occurred with similar or faster timing compared with the protrusion uptake pathway of *R. parkeri* (~10 minutes) and *L. monocytogenes* (~20 minutes) (Lamason et al., 2016). Therefore, *B. thailandensis*-induced cell-cell fusion appears to occur quickly compared with the cell-to-cell spread processes of other bacteria.


A example 1
protrude
exit protrusion
GFP diffusion
plasma membrane barrier lost
4:00
5:30
6:45
18:15
MNGC
GFP
B example 2
1:30
7:30
17:30
22:30
MNGC
GFP
plasma membrane
host GFP
bacteria

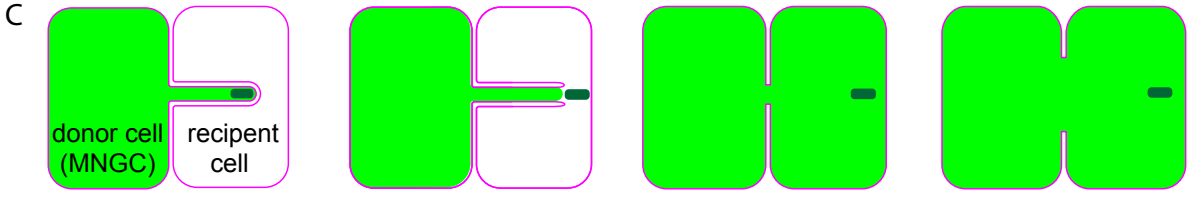

C
donor cell (MNGC)
recipent cell

Figure 2.1: In one observed pathway, *B. thailandensis* spreads by inducing cell-cell fusion at the protrusion tip. (A and B) Live-cell imaging stills of two examples of *Bt*GFP WT while inducing cell-cell fusion. A 1:1 mixture of A549 cells that expressed the plasma membrane marker TagRFP-T-farnesyl or cytoplasmic GFP were used. Times represent min:s post protrusion formation. Images taken at ~16 h post infection. Scale bars are 5 μm. White arrows highlight the bacterium forming the protrusion. Black arrows highlight the region of protrusion entry. Where GFP signal is difficult to see, insets with increased brightness are shown. (C) Model of cell-cell fusion occurring at the protrusion tip.

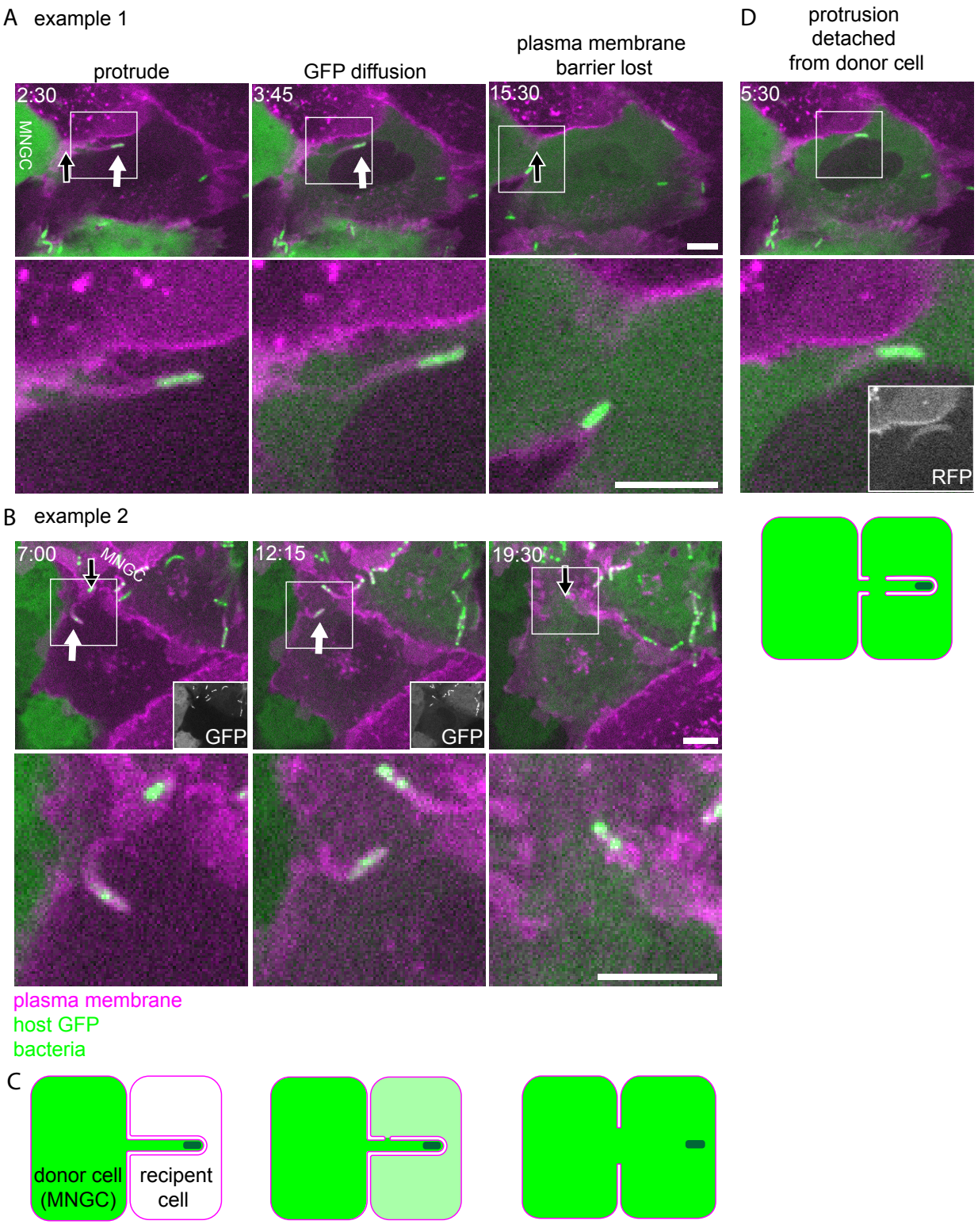

A example 1
protrude
GFP diffusion
plasma membrane barrier lost
2:30
3:45
15:30
MNGC
D
protrusion detached from donor cell
5:30
RFP
B example 2
7:00
12:15
19:30
MNGC
GFP
GFP
plasma membrane
host GFP
bacteria
C
donor cell (MNGC)
recipent cell

Figure 2.2: In another observed pathway, *B. thailandensis* spreads by inducing cell-cell fusion elsewhere within the protrusion. (A and B) Live-cell imaging stills of two examples of *Bt*GFP WT while inducing cell-cell fusion. A 1:1 mixture of A549 cells that expressed the plasma membrane marker TagRFP-T-farnesyl or cytoplasmic GFP were used. Times represent min:s post protrusion formation. Images taken at ~16 h post infection. Scale bars are 5 µm. White arrows highlight the bacterium forming the protrusion. Black arrows highlight the region of protrusion entry. Where GFP signal is difficult to see, insets with increased brightness are shown. (C) Model of cell-cell fusion occurring elsewhere within the protrusion. (D) Still showing visible detachment of the bacterium-containing protrusion from the donor cell.

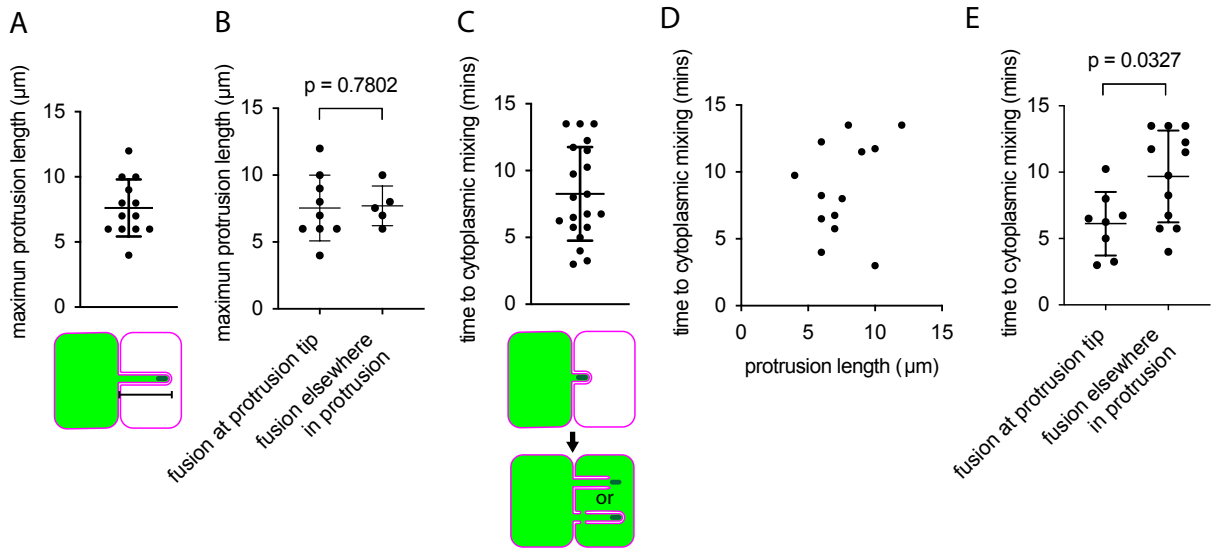


Figure 2.3: Quantification of *B. thailandensis* inducing cell-cell fusion live cell imaging dataset. (A) Graph of maximum protrusion length from videos where entire protrusion was visible (n=12). (B) Graph of maximum protrusion length for membrane fusion that occurred at the protrusion tip (n=9) versus elsewhere in the protrusion (n=4). (C) Graph of time to cytoplasmic mixing (n=20). (D) Graph of time to cytoplasmic mixing versus maximum protrusion length (n=12). $R^2 = 0.09197$, p = 0.3138. (E) Graph of time to cytoplasmic mixing for membrane fusion that occurred at the protrusion tip (n=8) versus elsewhere in the protrusion (n=12). For (A-D,E) P values were calculated by unpaired Mann-Whitney test, data are mean +/- SD.

VgrG5 acts at the membrane fusion step

The T6SS tip protein VgrG5 was previously found to be necessary for *B. thailandensis*-induced cell-cell fusion (Schwarz et al., 2014; Toesca et al., 2014). However, it was unknown at which stage of the cell-cell fusion pathway VgrG5 contributes. To investigate this, we generated identical a Δ*vgrG5* deletion mutants in both *Bt* (*Bt* Δ*vgrG5*) and *Bt*GFP (*Bt*GFP Δ*vgrG5*) strain backgrounds. We confirmed that *Bt*GFP Δ*vgrG5* did not express VgrG5 by western blotting using an anti-VgrG5 antibody we generated (Supplemental Figure 2.2). We then infected A549 cells that expressed TagRFP-T-farnesyl with a Δ*vgrG5* deletion mutant in the *Bt*GFP strain (*Bt*GFP Δ*vgrG5*) and performed live cell imaging. We found that *Bt*GFP Δ*vgrG5* formed membrane protrusions (Figure 2.4A) that appeared similar to protrusions formed by *Bt*GFP WT (Figures 2.1 and 2.2). Rather than inducing cell-cell fusion, *Bt*GFP Δ*vgrG5* bacteria in

protrusions were instead engulfed into the recipient cell (Figure 2.4A). Because this engulfment pathway is similar to the process that occurs during *R. parkeri* and *L. monocytogenese* cell-to-cell spread (Lamason & Welch, 2017), protrusions formed by *Bt*GFP Δ*vgrG5* are likely engulfed into double membrane vacuoles (cartooned in Figure 2.4B). These bacteria remained in these vacuoles for the duration of the imaging session. However, even though we did not observe such events, some bacteria still accessed the host cell cytosol after engulfment into recipient cells because some bacteria underwent actin-based motility as evidenced by their presence in plasma membrane protrusions of secondary cells (Supplemental Figure 2.3). These results indicate that VgrG5 is specifically involved in the membrane fusion step of the cell-cell fusion pathway.

To further compare the non-canonical cell-to-cell spread of *Bt*GFP Δ*vgrG5* with *Bt*GFP WT-induced cell-cell fusion, we measured the maximum protrusion lengths and timing of cell-cell fusion or engulfment for both strains. Maximum protrusion lengths were not significantly different between *Bt*GFP WT and *Bt*GFP Δ*vgrG5* (Figure 2.4C). This suggests that VgrG5 does not contribute to protrusion formation. Compared with the time it took for *Bt*GFP WT to induce cell-cell fusion, the engulfment of *Bt*GFP Δ*vgrG5* took significantly longer (Figure 2.4D). *Bt*GFP WT in membrane protrusions were also occasionally engulfed (Supplemental Figure 2.4). *Bt*GFP WT engulfment took significantly longer than *Bt*GFP WT-induced cell-cell fusion (Supplemental Figure 2.4B) and was not significantly different than engulfment of *B*tGFP Δ*vgrG5* (Supplemental Figure 2.4C). Our finding that *B*tGFP Δ*vgrG5* is engulfed into recipient cells, and that engulfment occurs more slowly than cell-cell fusion, suggests that inducing cell-cell fusion overrides a slower default engulfment pathway.

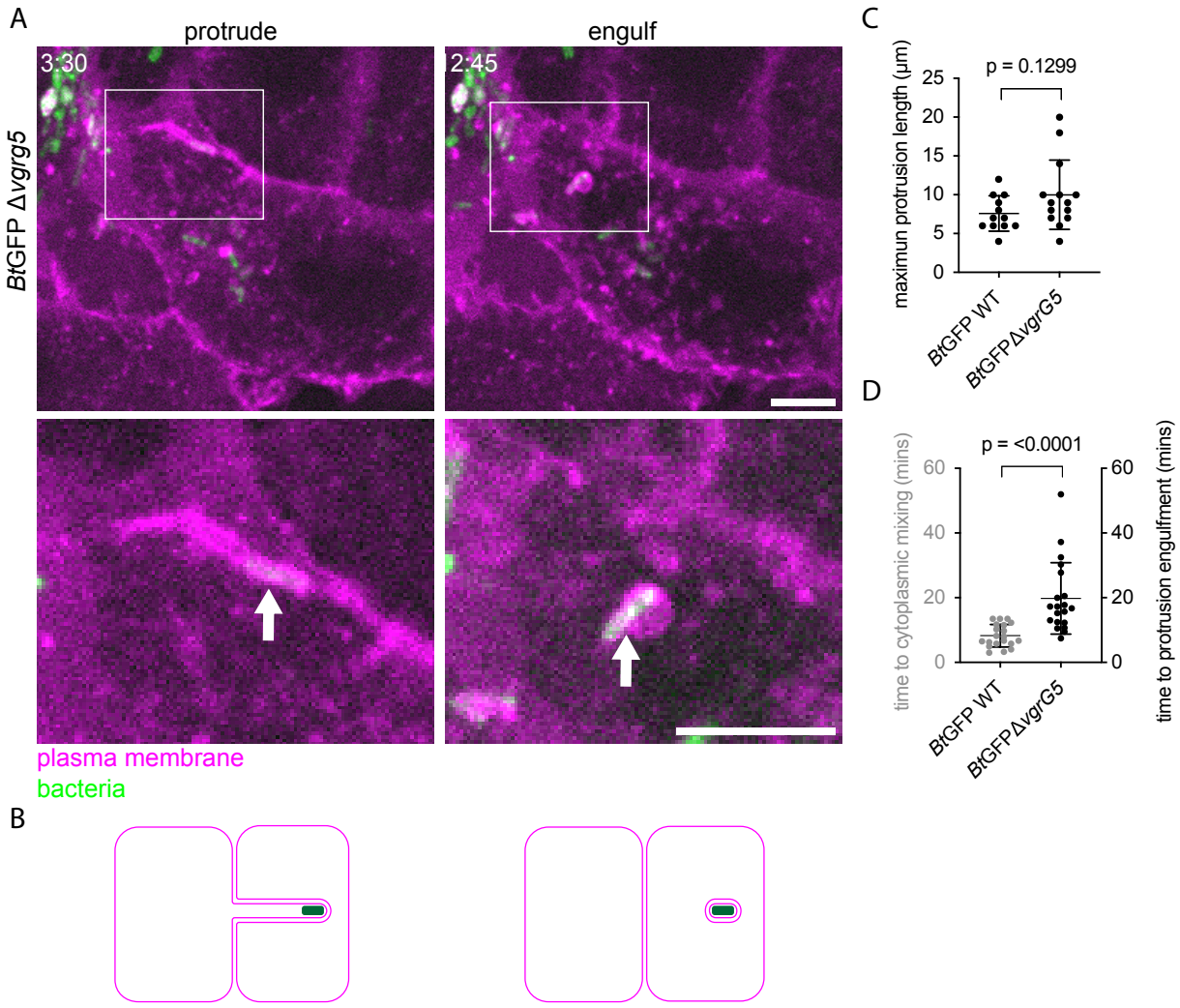


Figure 2.4: VgrG5 acts at the membrane fusion step. (A) Live cell imaging stills of *Bt*GFP Δ*vgrG5* during cell-to-cell spread. A549 cells that expressed TagRFP-T-farnesyl were used. Times represent min:s post protrusion formation. All images taken at ~24 h post infection. Scale bars are 5 µm. (B) Model of spread. (C) Graph of maximum protrusion length for *Bt*GFP WT (n=12) and *Bt*GFP Δ*vgrG5* (n=14). (D) Graph of time to cytoplasmic mixing (n=20) or protrusion engulfment (n=20). For (C-D), P values were calculated by unpaired Mann-Whitney tests, data are mean +/- SD.

***B. thailandensis* must express VgrG5 within a protrusion to induce cell-cell fusion**

Having determined that membrane fusion can occur at a distance from the bacterium and that VgrG5 functions at the membrane fusion step of the cell-cell fusion pathway, we wondered whether VgrG5 could be supplied by other bacteria elsewhere in an infected cell. To answer this question, we performed a co-infection experiment in monolayers of A549 cells that consisted of a mixture of cells that stably expressed either TagRFP-T-farnesyl or stably expressed GFP in the cytosol. We co-infected these monolayers with *Bt* WT that expressed BFP (*Bt*BFP WT) (Benanti et al., 2015) and *Bt*GFP Δ*vgrG5*. We then performed live cell imaging, with a focus on *Bt*GFP Δ*vgrG5* bacteria that formed protrusions from MNGC donor cells that extended into neighboring

recipient cells that expressed TagRFP-T-farnesyl (Figure 2.5A). If VgrG5 supplied by *Bt*BFP WT could rescue the ability of *Bt*GFP Δ*vgrG5* to induce cell-cell fusion, then we would observe diffusion of the GFP signal due to cell-cell fusion (Figure 2.5A, top). Alternatively, if VgrG5 supplied by *Bt*BFP WT could not rescue the ability of *Bt*GFP Δ*vgrG5* to induce cell-cell fusion, *Bt*GFP Δ*vgrG5* in membrane protrusions would be engulfed (Figure 2.5A, bottom). In all 10 instances observed, *Bt*GFP Δ*vgrG5* membrane protrusions were engulfed by the recipient cell and no cytosolic GFP diffused into the recipient cell during engulfment (Figure 2.5B). This observation suggests that VgrG5 must be expressed by bacteria within membrane protrusions to promote cell-cell fusion.

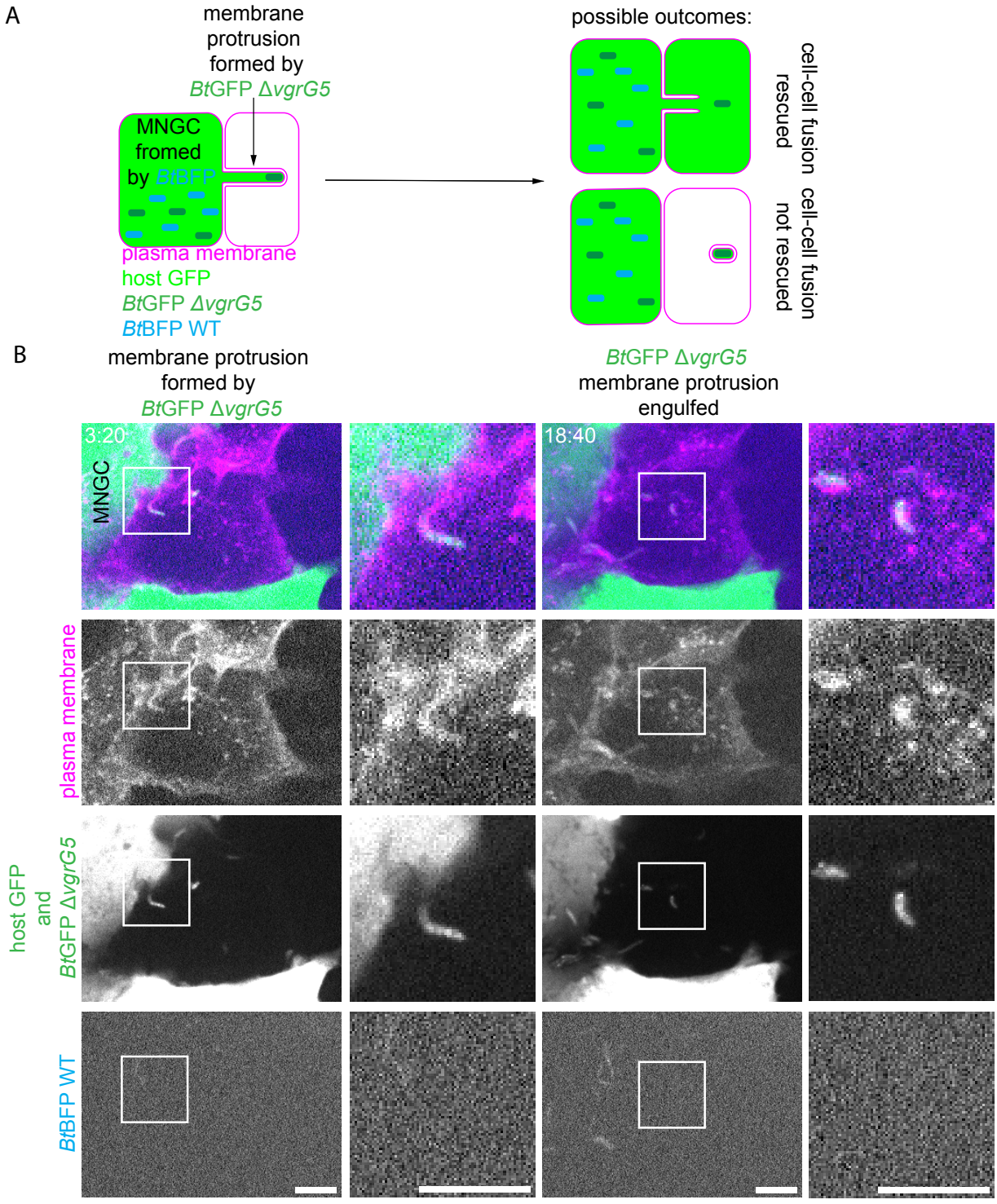


Figure 2.5: *B. thailandensis* must secrete VgrG5 within a protrusion to induce cell-cell fusion (A) Experimental design and possible outcomes. (B) Live cell imaging stills of *Bt*GFP Δ*vgrg5* spreading from an MNGC initially formed by cell-cell fusion induced by *Bt*BFP WT bacteria. Times represent min:s after the video began. Scale bars are 5 μm.

TagD5 is required for inducing cell-cell fusion and acts at the membrane fusion step

The PAAR protein TagD5 is encoded in the same T6SS-5 gene cluster as VgrG5 (Burtnick et al., 2011; Hopf et al., 2014; Lennings, West, et al., 2019; Pilatz et al., 2006; Schwarz et al., 2010), and based on the known interaction between VgrG and PAAR proteins (Shneider, 2013), we hypothesized that it functions with VgrG5 to induce membrane fusion. To test this, we generated identical Δ*tagD5* deletion mutants in both *Bt* (*Bt* Δ*tagD5*) and *Bt*GFP (*Bt*GFP Δ*tagD5*) strain backgrounds. We investigated whether *Bt*GFP Δ*tagD5* expressed VgrG5 by western blotting using our anti-VgrG5 antibody and found that *Bt* Δ*tagD5* exhibited reduced levels of VgrG5 protein (Supplemental Figure 2.2). Therefore, TagD5 influences VgrG5 expression or stability.

To test whether TagD5 is required for cell-cell fusion, we first employed a plaque size assay, which was previously used to determine the extent of cell-cell fusion (Benanti et al., 2015; French et al., 2011), in Vero cells. *Bt* Δ*tagD5* failed to form a plaque, as did *Bt* Δ*vgrG5* (Figure 2.6A). This is consistent with functions for both VgrG5 and TagD5 in cell-cell fusion.

To determine the step at which TagD5 acts in the cell-cell fusion pathway, we next performed live cell imaging of A549 cells that expressed TagRFP-T-farnesyl infected with *Bt* Δ*tagD5* made in the *Bt*GFP strain (*Bt*GFP Δ*tagD5*). The phenotypes exhibited by *Bt*GFP Δ*tagD5* were nearly identical to those of *Bt*GFP Δ*vgrG5*. *Bt*GFP Δ*tagD5* did not induce cell-cell fusion and instead formed membrane protrusions that were engulfed by the recipient cell (Figure 2.6B and C). Maximum protrusion lengths were not significantly different between *Bt*GFP WT and *Bt*GFP Δ*tagD5* (Figure 2.6D), similar to *Bt*GFP Δ*vgrG5* (Figure 2.4C). Furthermore, compared with the time it took for *Bt*GFP WT to induce cell-cell fusion, the engulfment of *Bt*GFP Δ*tagD5* took significantly longer (Figure 2.6E), similar to engulfment of *Bt*GFP Δ*vgrG5* (Figure 2.4D). There was no difference between the time to engulfment of *Bt*GFP Δ*vgrG5* and *Bt*GFP Δ*tagD5* (Supplemental Figure 2.4C). Therefore, both TagD5 and VgrG5 are required for the membrane fusion step of the cell-cell fusion pathway, consistent with them working together during this step.

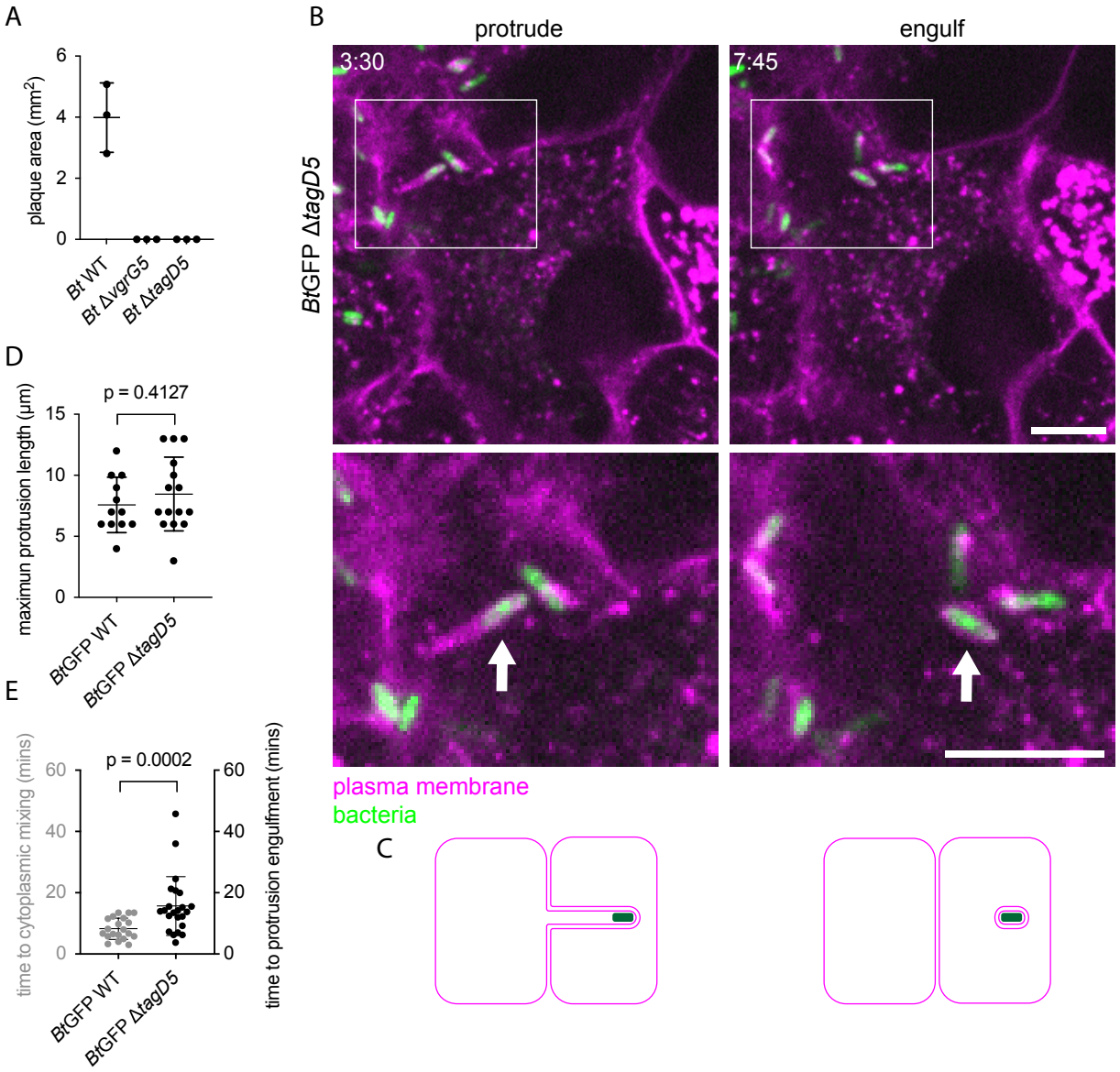


Figure 2.6: TagD5 is required for inducing cell-cell fusion and acts at the membrane fusion step. (A) Plaque areas of Vero cells infected with the indicated strains. N=3 experiments, 9-11 plaques per experiment. (B) Live-cell imaging stills of *Bt*GFP Δ*tagD5* during cell-to-cell spread. A549 cells that expressed TagRFP-T-farnesyl were used. Times represent min:s post protrusion formation. All images taken at ~24 h post infection. Scale bars are 5 μm. (C) Model of spread. (D) Graph of maximum protrusion length for *Bt*GFP WT (n=12) and *Bt*GFP Δ*tagD5* (n=15). (E) Graph of time to cytoplasmic mixing (n=20) or protrusion engulfment (n=23). For (A, D-E) P values were calculated by unpaired Mann-Whitney tests, data are mean +/- SD.

Discussion

Here we describe the cellular pathway leading to host cell-cell fusion induced by the bacterial pathogen *B. thailandensis*. We found that cell-cell fusion occurs within host cell plasma membrane protrusions formed by motile bacteria, with membrane fusion occurring either proximal to the bacterium at the protrusion tip or elsewhere in the protrusion. We also identified TagD5, a component of the T6SS-5 that likely interacts with the T6SS-5 protein VgrG5, as a factor critical for cell-cell fusion. We found that both TagD5 and VgrG5 function at the membrane fusion step of the cell-cell fusion pathway. We further showed that VgrG5 must be secreted within membrane protrusions to support cell-cell fusion. Our results suggest that the T6SS-5 components VgrG5 and TagD5 act within membrane protrusions to promote membrane fusion.

We demonstrated that the first step of the cell-cell fusion pathway is for bacteria undergoing actin-based motility to collide with the host cell plasma membrane and form membrane protrusions that extend from donor cells into recipient cells. Membrane protrusions containing *B. thailandensis* have been observed previously (Kespichayawattana et al., 2000; J. M. Stevens et al., 2005; M. P. Stevens et al., 2005). Our observations further indicate that cell-cell fusion occurs within these protrusions, suggesting that bacterially-induced plasma membrane protrusions function as mediators of cell-cell fusion. Protrusions might mediate key molecular steps leading to cell-cell fusion, such as membrane apposition, membrane fusion, or fusion pore expansion. However, a *B. thailandensis* strain deficient for motility can induce very limited cell-cell fusion (French et al., 2011), indicating that such protrusions, while important, are not absolutely required. Membrane protrusions formed by bacteria undergoing actin-based motility are reminiscent of actin-rich protrusions that promote cell-cell fusion in other contexts, including *Drosophila* myoblast fusion (Kim et al., 2015; Sens et al., 2010), osteoclast fusion (Oikawa et al., 2012), and macrophage fusion (Faust et al., 2019). Force from actin polymerization is also thought to promote virus induced cell-cell fusion by the fusion-associated small transmembrane (FAST) fusogens expressed by a group of nonenveloped, fusogenic reoviruses (Chan et al., 2020, 2021). Similar to these examples, bacterial actin-based motility within protrusions could provide the force necessary to bring neighboring plasma membranes close together, a key step in the cell-cell fusion process (Hernández & Podbilewicz, 2017).

Delineation of the *B. thailandensis*-induced cell-cell fusion pathway at the cellular level also enabled our subsequent analysis of the role of bacterial factors in this process. Because VgrG5 is the only protein known to be secreted by the T6SS-5 and because it is required for cell-cell fusion (Schwarz et al., 2014; Toesca et al., 2014), it is a candidate fusogen protein. Consistent with this idea, we found that VgrG5 must be expressed by a bacterium within a protrusion for cell-cell fusion to occur, placing VgrG5 in the location of the fusion event. Moreover, we found that VgrG5 is required for the membrane fusion step but not for earlier steps in the pathway. Our results are consistent with a direct role for VgrG5 in inducing plasma membrane fusion, although it is possible that VgrG5 does not directly mediate membrane fusion. Ultimately, to define the molecular level mechanism of cell-cell fusion, it will be necessary to demonstrate that the required proteins are sufficient to induce membrane fusion in a minimal system.

We found that an additional component of the T6SS-5, TagD5, is required for membrane fusion. TagD5 (Lennings, West, et al., 2019) is a member of the PAAR family proteins that interact with and are secreted along with VgrG proteins of other T6SS systems (Hachani et al., 2016; Shneider, 2013). This suggests that VgrG5 and TagD5 might form a complex and therefore function together. Consistent with this hypothesis, we showed that TagD5 is required for membrane fusion and that TagD5 contributes to VgrG5 stability or expression. TagD5 is small (119 amino acids) and contains a PAAR structural domain but does not contain a sequence extension present in some PAAR proteins that carries out effector functions (Shneider, 2013; Hachani et al., 2016), so it is unclear how it could directly contribute to membrane fusion. However, VgrG5 contains additional sequences beyond the VgrG structural features that are required for fusion, and therefore it likely carries out effector functions (Pukatzki et al., 2007a; Schwarz et al., 2014; Toesca et al., 2014). A TagD5-VgrG5 complex could act similarly to other PAAR protein-VgrG systems. One particularly relevant example is the *Pseudomonas aeruginosa* Tse6-VgrG1 PAAR protein-VgrG complex which delivers a toxin domain to the cytoplasm of neighboring bacteria during interbacterial competition with only Tse6 contributing effector activity (Quentin et al., 2018; Whitney et al., 2014, 2015). The *P. aeruginosa* Tse6-VgrG1 complex requires chaperones for stability and loading onto the T6SS (Hachani et al., 2016; Quentin et al., 2018; Whitney et al., 2014), suggesting a TagD5-VgrG5 complex may require other yet-to-be-identified bacterial proteins such as chaperones or even secreted effectors. A full understanding of the molecular mechanism of cell-cell fusion will require identification of all the required factors.

Because the T6SS is ejected from bacteria and can puncture neighboring cell membranes (Jurėnas & Journet, 2021), one hypothesis is that this process directly mediates fusion, for example, by disrupting membrane integrity. This hypothesis would predict that membrane fusion occurs proximal to a bacterium. However, we found that membrane fusion does not always occur in close proximity to a bacterium but frequently occurs elsewhere within a membrane protrusion, away from the bacterium. Therefore, our data support a canonical role for the T6SS-5 in secreting effector proteins (Jurėnas & Journet, 2021) rather than in inducing membrane fusion directly. Although our results are insufficient to determine the molecular-level mechanism of membrane fusion during *B. thailandensis* induced cell-cell fusion, they are consistent with membrane fusion involving a canonical fusogen-mediated hemifusion pathway (Hernández & Podbilewicz, 2017). Because we observed that VgrG5 must be expressed within membrane protrusions, we hypothesize that VgrG5 is secreted and released once a bacterium enters a protrusion and then acts within the protrusion to promote cell-cell fusion. This mechanism is consistent with the mechanism of other VgrG proteins that are released upon T6SS secretion (Hachani et al., 2016), including VgrG2b of *P. aeruginosa*, which targets host microtubules (Sana et al., 2015), and VgrG1 of *Vibrio cholerae*, which targets host actin (A. T. Ma et al., 2009; Pukatzki et al., 2007b). Therefore, our results are consistent with known functions of the T6SS in secreting bacterial proteins.

In the absence of fusion due to loss of TagD5 or VgrG5, bacteria in protrusions are engulfed by the recipient cell. This pathway is similar to the engulfment of protrusions containing other bacteria, such as *L. monocytogenes* and *R. parkeri*, into double-membrane vacuoles to achieve cell-to-cell spread (Lamason & Welch, 2017). We never observed an engulfed bacterium exiting its double membrane vacuole, and such a defect in accessing the

cytosol could explain prior observations that *Bt* Δ*vgrG5* has a growth defect in host cells (Bulterys et al., 2019). Our observations are also consistent with prior observations that protrusion formation through actin-based motility drives bacterial engulfment into the recipient cell (Monack & Theriot, 2001). In addition, we found that *Bt*GFP WT induce cell-cell fusion more quickly than the time it takes for cells to engulf *tagD5*- and *vgrG5*-deficient mutants, indicating that fusion must occur before engulfment occurs. Our work suggests that membrane fusion must be carried out quickly and efficiently to supersede a slower default double membrane protrusion engulfment pathway that is detrimental to the growth and spread of *B. thailandensis*.

Our findings define the cellular level pathway for *B. thailandensis*-induced cell-cell fusion resolving how bacterial motility, bacterial membrane protrusions, and T6SS-5 activity work together to induce cell-cell fusion. Although the T6SS components VgrG5 and TagD5 are directly implicated in membrane fusion, they do not resemble any known fusogens (Podbilewicz, 2014). Therefore, understanding how these proteins function during cell-cell fusion could reveal new insights into membrane fusion mechanisms. The conspicuous length of membrane protrusions formed by *B. thailandensis*, which lend themselves to imaging, makes this a powerful system for continuing to explore the conserved function of membrane protrusions during cell-cell fusion. Continued investigation of this pathway will enhance our understanding of the cellular and molecular mechanisms of membrane fusion and cell-cell fusion.

Materials and Methods

Bacterial and mammalian cell culture

Escherichia coli strains XL1-blue and BL21(DE3) were obtained from the UC Berkeley MacroLab and were used for plasmid construction and protein expression, respectively. *E. coli* was cultured in liquid or solid lysogeny broth (LB) with or without 100 μg/ml ampicillin or 50 μg/ml kanamycin, when appropriate. *E. coli* RHO3 (López et al., 2009) was grown in LB supplemented with diaminopimelic acid (DAP) (200 mg/ml). *B. thailandensis* E264 was cultured in liquid or solid LB.

Mammalian cell lines (Vero monkey kidney epithelial, RRID:CVCL_0059, HEK293T human embryonic kidney, RRID:CVCL_0045; A549 human lung epithelial, RRID:CVCL_0023; and U2OS human osteosarcoma, RRID:CVCL_0042) were obtained from the University of California, Berkeley Tissue Culture Facility, which authenticated these cell lines prior to freezing, and were not tested for mycoplasma contamination. Cells were grown at 37˚C in 5% CO_2. Vero cells were maintained in DMEM (Invitrogen, 11965-092v) containing 2% fetal bovine serum (FBS, GemCell Bio-Products, 100-500). HEK293T, A549, and U2OS cells were maintained in DMEM containing 10% FBS (Atlas Biologicals, FP-0500-A).

Plasmid construction

To visualize GFP (Wasabi) in A549 cells, the lentiviral expression vector Wasabi-pIPFCW2 was constructed. The gene encoding Wasabi was amplified by PCR from the plasmid F-tractin-Wasabi-pIPFCW2 (Benanti et al., 2015) with 5’ NheI and 3’ EcoRI cut-sites included in the primer overhangs for subcloning (forward primer 5’ GAACCGTCAGATCCGCTAGCATGGTGAGCAAGGGCG 3’, reverse primer 5’ GGGCGAATTCTTACTTGTACAGCTCGTCCATGC 3’). The F-tractin-Wasabi-pIPFCW2 and amplified *wasabi* gene and were cleaved with NheI and EcoRI and ligated together to produce Wasabi-pIPFCW2.

To make *B. thailandensis* mutants, we used plasmid pEXKm5 (López et al., 2009) for allelic exchange. We PCR-amplified DNA from *B. thailandensis* cells boiled in water. These DNA fragments contained ~500 bp 5’ and 3’ to the region of interest and were subcloned into pEXKm5. To generate the *clpV5-gfp* pEXKm5 plasmid, the 5’ and 3’ends were flanked by sequences in pEXKm5 surrounding the HindIII cut-site. We amplified *clpV5* (primers 5’CAACGCGCGCAGTAAAGGAGAAGAACTTTTCAC3’and GGGAACTCCTTTATTTGTATAGTTCATCCATGC3’), *gfp* (primers 5’CAACGCGCGCAGTAAAGGAGAAGAACTTTTCAC3’ and 5’GGGAACTCCTTTATTTGTATAGTTCATCCATGC3’), and ~500 bp 3’ to *clpV5* (primers 5’TATACAAATAAAGGAGTTCCCGATGTCTTCGTC3’ and 5’CTCGAGGCGGCCGGCTAGCATTGACGATATCGGGAATCG3’). pEXKm5 was digested with HindIII and the 4 fragments were assembled via Gibson cloning (New England Biolabs, E2611S).

To construct the Δ*vgrG5* pEXKm5 plasmid, two fragments were PCR-amplified with 11 bp of homology to each other and this homologous region contained two in-frame stop codons at codon 108 of *vgrG5*. One fragment contained a 5’ XmaI cut-site and ~500 bp 5’ to codon 108 of

vgrG5 (primers 5’ CCCTGTTATCCCTACCCGGGACGCGCGACGCTTCAC 3’ and 5’ GCCTTCCTTCATCAATCGAGATGGCTCTCGTCGTACT 3’) and the other contained ~500 bp 3’ to codon 108 of *vgrG5* and a 3’ XmaI cut-site (5’ TCTCGATTGATGAAGGAAGGCCTCTACTACTACTTCGAGC 3’ and 5’ TCGACTTAAGCCGGCCCGGGGGTGCGCCTGCGAGC 3’). The two fragments were then stitched together via their 11 bp region of homology by overlap PCR.

To construct the Δ*tagD5* pEXKm5 plasmid, two fragments were PCR-amplified. The first fragment contained ~500 bp upstream of the *tagD5* start codon (primers 5’ATCCCTACCCGGGTCGTGCGCATCCGCATCCTCTT 3’ and 5’ GCTCATGCCCGCGCCGCACAGGCCGGAGGCGG 3’) and the second fragment contained ~500 bp downstream of the *tagD5* stop codon (primers 5’GCCTCCGGCCTGTGCGGCGCGGGCATGAGCGATC 3’ and 5’ AGCCGGCCCGGGGATTCGCAGCGGCACGTCGAA 3’). The two fragments were then stitched together via overlap PCR. The stitched fragments and pEXKm5 were digested with XmaI and ligated together.

To express 6xHis-MBP-VgrG5 CTD, we used a version of pETM1 expression vector containing a 6xHis tag, MBP tag, and TEV cleavage site downstream of the SspI cut site. A fragment of *vgrG5* encoding a C-terminal domain of *vgrg5* (aa718-1012, *vgrG5-ctd*) was amplified by PCR from *B. thailandensis* (primers 5’ ACCTGTACTTCCAATCCAATCGCACGCTGCTCTCGAAAATC 3’ and 5’ ATCCGTTATCCACTTCCAATGCCTAGCTGGATCAACTGTC 3’) and subcloned into the SspI site of the pETM1.

To express 6xHis-SUMO-VgrG5-CTD, *vgrG5-ctd* was amplified by PCR (primers 5’ACCTGTACTTCCAATCCAATCGCACGCTGCTCTCGAAAATC 3’ and 5’ ATTGGAAGTGGATAACGGATGCCTAGCTGGATCAACTGTC 3’) and subcloned into the SspI site of plasmid pSMTp3, 3’ to the portion encoding HIS-SUMO.

B. thailandensis strain construction

B. thailandensis strains were created by allelic exchange, as previously described (Benanti et al., 2015; López et al., 2009). Bi-parental matings between *B. thailandensis* strain E264 and *E. coli* RHO3 (López et al., 2009) harboring a pEXKm5 derivative were performed to introduce pEXkm5 into *B. thailandensis*, followed by selection on 50 μg/ml kanamycin-containing plates that lacked DAP to select against *E. coli* RHO3. Uptake of pEXKm5 was also confirmed by PCR detection of the *sacB* gene. The integrated vector backbone was removed by growth in non-selective YT media (5 g/l yeast extract (VWR, EM1.03753.0500), 5 g/l tryptone (Fisher Scientific, BP1421-500)) and screening for loss of b-glucuronidase activity via plating on YT plates containing 50 μg/ml X-Gluc (cyclohexlammonium salt, Gold Biotechnologies, G1281C1). Strains were confirmed by PCR amplification and DNA sequencing of the region of interest.

Transient transfections, transduction, and cell line production

For retroviral transduction to visualize GFP (Wasabi) in A549 cells, viral particles were packaged by transfecting HEK293s plated 24 h prior at $5x10^5$ cells/well (2 ml/well, 6-well plate),

via calcium phosphate transfection with 750 ng pMDL-RRE, 450 ng pCMV-VSVg, 300 ng RSV-Rev and 1500 ng Wasabi-pIPFCW2. Approximately 22 h after transfection, the media was replaced with 2 ml fresh media. After an additional 21 h, the supernatant, which contains viral particles, was collected from each well, and cell debris was cleared by filtration through a 0.45 μm syringe filter. The viral supernatant was added to A549 cells and polybrene (Santa Cruz Biosciences, sc-134220) was added to 10 μg/ml to enhance the infection efficiency. After transduction, fresh media was added at 24 h post infection (hpi), and at 48 hpi cells transduced with Wasabi-pIPFCW2 were selected with 3-4 mg/ml puromycin (Calbiochem, 540411) and sorted for mid-range expression of Wasabi.

Bacterial Infections of host cells

B. thailandensis strains (Δ*motA2;ClpV5-GFP* (*Bt*GFP WT) (this study), Δ*motA2;* Δ*vgrG5* (Δ*vgrG5*) (this study), *Bt*GFP Δ*vgrG5* (this study), Δ*motA2;* Δ*tagD5* (Δ*tagD5*) (this study), *Bt*GFP Δ*tagD5* (this study), Δ*motA2;BFP* (*Bt*BFP) (Benanti et al., 2015)) were streaked from frozen stocks onto LB agar plates. Bacteria were swabbed from plates to inoculate LB liquid media and were grown with shaking at 37˚C for 3-16 h. Prior to infections, the OD_{600} of cultures was measured in order to calculate the number of bacteria to infect with (OD_{600} of 1 =$5x10^8$ cfu/ml) to achieve the proper multiplicity of infection (MOI). Bacterial cultures were pelleted and resuspended in PBS (ThermoFisher, 10010049. Composition: Potassium Phosphate monobasic (KH2PO4), 1.0588236mM; Sodium Chloride (NaCl), 155.17241mM; Sodium Phosphate dibasic (Na2HPO4-7H2O), 2.966418mM). Mammalian cells were seeded at least 24 h before infection and immediately prior to infection were washed with PBS and provided with fresh DMEM with 10% FBS. Bacteria were added directly to media on cells, media was pipetted or rocked gently to mix, and bacteria were left to invade for 45 min to 1 h at 37˚C unless otherwise stated. Cells were rinsed once with PBS, then DMEM with 10% FBS and 0.5 mg/ml gentamicin (Fisher Scientific, MT30-005-cR) was added. For mixed-strain infections, infections lasted longer, as detailed below.

For live cell imaging of spread, confluent monolayers of A549 cells were infected. For infection with *Bt*GFP WT (Δ*motA2*;*clpV5-GFP*), a mix of A549 TagRFP-T-farnesyl (Lamason et al., 2016) and A549 GFP cells at a 1:1 ratio ($6x10^5$ cells/dish) were plated in 20 mm MatTek dishes (Mat Tek Corp., P35G-1.5-20-C). Cells were infected as described above at an MOI of 10-50 and imaged at 12-18 h. For live imaging of *Bt*GFP Δ*vgrG5* and *Bt*GFP Δ*tagD5*, A549 TagRFP-T-farnesyl were plated in 20 mm Mat Tek dishes ($6x10^5$ cells/dish) 24-48 h before infection. Cells were infected as described above at an MOI of 100 and imaged at 24-30 hpi.

For co-infections of *Bt*BFP WT and *Bt*GFP Δ*vgrG5*, a mix of A549 TagRFP-T-farnesyl and A549 GFP cells at a 1:1 ratio or 4:1 were plated in 20 mm Mat Tek dishes ($6x10^5$ cells/dish) at least 24 h before infection. Infections were done two ways. For two videos, *Bt*BFP WT were infected first at an MOI of 100, and allowed to invade for 2 h. Then at 5 h after the initial infection, *Bt*GFP Δ*vgrG5* were added at an MOI of 100 and allowed to invade for 2 h. Imaging was performed at 9-12 hpi. For the other eight videos, *Bt*BFP WT and *Bt*GFP Δ*vgrG5* were infected simultaneously, allowed to infect for 1.5 h, and imaging was performed at 14-19 hpi. For some of the experiments, each strain was used to infect at an MOI of 50 and in others, they were used to infect at an MOI of 20 (*Bt*BFP WT) and 80 (*Bt*GFP Δ*vgrG5*).

Live cell imaging

Before imaging, infected cells in 20 mm Mat Tek dishes were washed once with PBS before addition of 1.5 ml FluoroBrite DMEM Media (Invitrogen, A18967-01) supplemented with 10% FBS and 1XGlutaMAX (Gibco, 35050-061) and 0.5 mg/ml gentamycin.

Images were captured on a Nikon Ti Eclipse microscope with a Yokogawa CSU-XI spinning disc confocal, 60X (1.4 NA) Plan Apo objective, a Clara Interline CCD Camera (Andor Technology), and MetaMorph software (Molecular Devices). 3 image Z-stacks were captured at 15 s intervals for 30-90 m. For mixed infections, images were taken every 20 s. Images were processed using ImageJ (Version 2.1.0/1.53c) and assembled in Adobe Illustrator (version 25.3.1). Spread events were then observed and a dataset was collected of individual spread events in which we were able to identify which bacterium induced cell-cell fusion. The kinetics and membrane morphology for each spread event were recorded. Maximum protrusion was defined as the longest protrusion length observed before earliest sign of GFP diffusion into the recipient cell (for *Bt*GFP WT) or engulfment (for *Bt*GFP Δ*vgrG5* and *Bt*GFP Δ*tagD5*). Time of spread was defined as the time of protrusion entry to the earliest sign of GFP diffusion into the recipient cell (for *Bt*GFP WT) or engulfment (for *Bt*GFP Δ*vgrG5* and *Bt*GFP Δ*tagD5*).

Plaque assay

For plaque assays, Vero cells were plated in 6-well plates ($6x10^5$ cells/well), infected at an MOI of 2, and bacteria were allowed to invade for 45 min. Infected cell monolayers were washed once with PBS and overlayed with 3 ml of 0.7% agarose in DMEM with 5% FBS and 0.5 mg/ml gentamycin. At 31 hpi, 1 ml of 0.7% agarose in PBS containing neutral red (Sigma, N6264) at 1:20 dilution was overlayed onto wells (final concentration on cells was 1%). 14 h after addition of neutral red, plates were scanned and plaque area was measured using ImageJ (Version 2.1.0/1.53c).

Protein expression and purification

For expression of VgrG5 in broth culture (J. Wong et al., 2015), *B. thailandensis* strains were grown overnight and then diluted 1:10 in 3.5 ml LB. After 2 h, cultures were split into 2 tubes with 1.5 ml each and L-Glutathione reduced (GSH, Sigma-Aldrich, G4251) was added to 50 mM in one of them. Cultures were grown for 2 h followed by processing for western blotting as described below.

To generate the anti-VgrG5 antibody, 6xHis-MBP-TEV-VgrG5-CTD was expressed in *E. coli* BL21. Protein expression was induced with 1mM IPTG at 37˚C 1 h. Cells were pelleted at 4539.5 xg and resuspended in 50 mM Tris HCl pH 8.0, 200 mM KCl, 1 mM EDTA, and protease inhibitors (1 µg/ml each leupeptin (MilliporeSigma, L2884), pepstatin (MilliporeSigma, P5318), chymostatin (MilliporeSigma, E16), 1 mM phenylmethylsulfonyl fluoride (PMSF, 600 MilliporeSigma, 52332)) and stored at -80˚C. Cells were thawed, imidazole was added to 5 mM, and cells were incubated with 1 mg/ml lysozyme (Sigma, L4919-5G) for 15 min on ice and then sonicated at 4˚C (6x 12 s pulses, 50% power). The lysate was spun at 20198 xg, 4˚C, for 25 min. The supernatant was incubated for ~2 h rotating at 4˚C with Ni-NTA Resin (Qiagen, 1018244) that had been washed with wash buffer (20mM Tris HCl pH 8.0, 200 mM NaCl, 1mM DTT, 20mM imidazole). Cleared lysate was incubated with resin for ~2 h, rotating at 4˚C, and resin was washed with 3 ml wash buffer. Protein was eluted stepwise in 50 mM, 200 mM, and 500

mM imidazole. Elutions containing VgrG5-CTD were desalted using Amicon Ultra-4 Centrifugal Filter Units (Merck Millipore Ltd., UFC801096) into 10 mM imidazole, 50 mM Tris HCl pH 8.0, 200 mM NaCl, and incubated overnight at 4˚C with TEV protease at a VgrG5-CTD:TEV ratio of 1:100. MBP-VgrG5-CTD was run over an Ni-NTA column as described above but with 10 ml wash buffer containing 30 mM imidazole. The wash was collected in 1 ml fractions. The rest of the protein was eluted in elution buffer containing 200 mM imidazole. The washes and elution were pooled and then concentrated to 1 mg using a desalting column. This resulted in a mixed population of mostly uncleaved 6xHis-MBP-VgrG5-CTD and some VgrG5-CTD.

For antibody affinity purification, HIS-SUMO-VgrG5-CTD was expressed in bacteria as described above and purified using Ni NTA resin as described above but eluted with 200 mM imidazole. The protein was then further purified by concentrating and running over a gel filtration column (CYZ superdex 200 increase, Sigma) in 20 mM HEPES pH 8.0, 200 mM NaCl. Fractions containing HIS-SUMO-VgrG5-CTD were pooled and concentrated as described above to 1 mg/ml.

Antibody production, purification, and validation

To generate rabbit-anti VgrG5 antibodies, purified VgrG5-CTD protein was used to inoculate rabbits at Pocono Rabbit Farm and Laboratory (Canadensis, PA) where a 91-day custom antibody protocol was performed.

To purify the anti-VgrG5 antibody, purified HIS-SUMO-VgrG5-CTD was concentrated to 0.5 ml and was combined with 0.5 ml coupling buffer (200 mM $NaHCO_3$ pH 8.3, 500 mM NaCl). This was then coupled onto NHS-ester Sepharose 4 Fast Flow resin (GE Healthcare, 17-0906-01) for 4 h at 4˚C. 10 ml of serum was diluted 1:1 in binding buffer (20 mM Tris, pH 7.5), 0.2 µm filtered, and rotated for 1 h at room temperature with the resin. After washing with binding buffer, the antibody was eluted off of the resin with 100 mM glycine, pH 2.5, and 1 ml fractions were collected. Eluted fractions were immediately neutralized with 1 M Tris pH, 8.8 to 65.4 mM final concentration. Elutions that recognized VgrG5 via western blot (elutions 2 and 3) were pooled and dialyzed into 20 mM HEPES, pH 7.0, 100mM NaCl. Aliquots were flash-frozen and stored at -80˚C or supplemented with 35% glycerol and stored at -20˚C. To validate the VgrG5 antibody, western blots were performed as described below.

Western blotting

For detection of VgrG5 in glutathione-induced samples, 100 µl of broth culture was washed once with PBS and boiled in 1X SDS sample buffer three times for 10 min each. Samples were run on a 10% SDS-PAGE gel and the gel contents were transferred to a nitrocellulose membrane (ThermoFisher, 88018). Membrane was blocked for 30 min in TBS-T (20 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween 20 (Sigma, P9416) containing 5% milk (Genesee, 20-241), then incubated with 1:5000 anti-VgrG in 5% milk in TBS-T overnight at 4˚C. The membrane was then washed 3 x 5 min in TBS-T and incubated with 1:5000 goat anti-rabbit HRP secondary antibody (Santa Cruz Biotechnology, sc-2004) in 5% milk in TBS-T for 1 h followed by 3 x 5 min washes in TBS-T. To detect secondary antibodies, ECL HRP substrate kit (Advansta, K-12045) was added to the membrane for 1 min at room temperature and developed using HyBlot ES High Sensitivity Film (Thomas Scientific 1156P37).

Statistics and sample size

Statistical analyses were performed using GraphPad PRISM v.9. Statistical parameters and significance are reported in the Figure Legends. Comparisons were made using unpaired, two-tailed Mann-Whitney tests and differences were considered to be statistically significant when $P < 0.05$, as determined by an unpaired Mann-Whitney test. For comparing time to engulfment across different strains, a one-way ANOVA test with multiple comparisons was performed. Sample size of n = 20 independent events was selected for cell-cell fusion events and cell-cell engulfment spread events based on the experimental limitations of capturing such rare events. Protrusion lengths could not be measured during all cell-cell fusion events, resulting in a smaller sample size for those datasets. Engulfment of *Bt*GFP WT events were extremely rare, resulting in a smaller number of events observed and a smaller dataset. For live cell imaging experiments, each imaging session was a biological replicate without technical replicates. The sample size for plaque size assays was determined by the number of plaques present in two wells of a 6 well dish (9-11 plaques for *Bt* WT, 0 for *Bt* Δ*vgrG5* and *Bt* Δ*tagD5*). Each plaque measured was a technical replicate with n = 3 biological replicates performed. There was no randomization or blinding.

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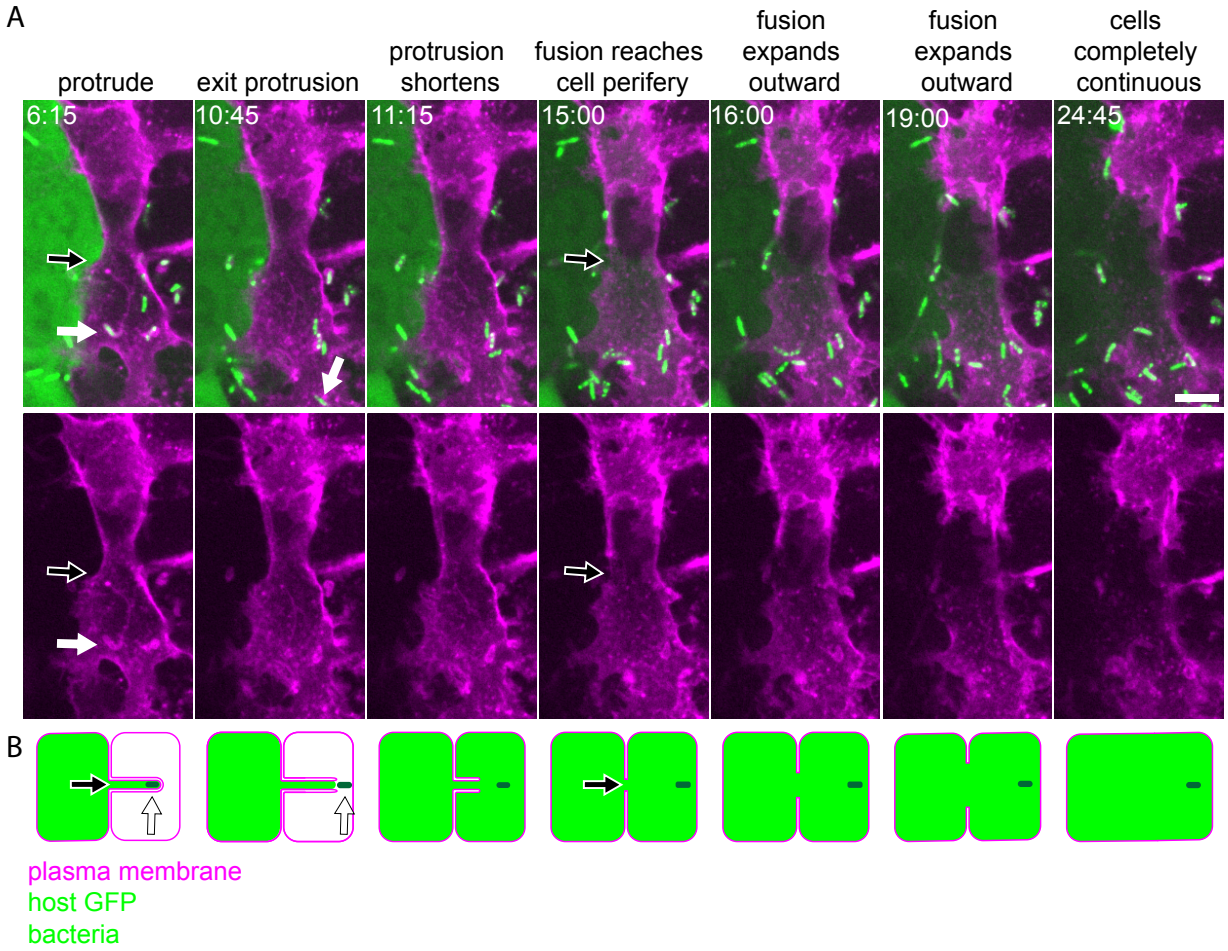
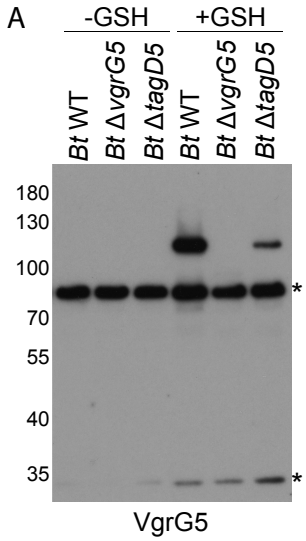
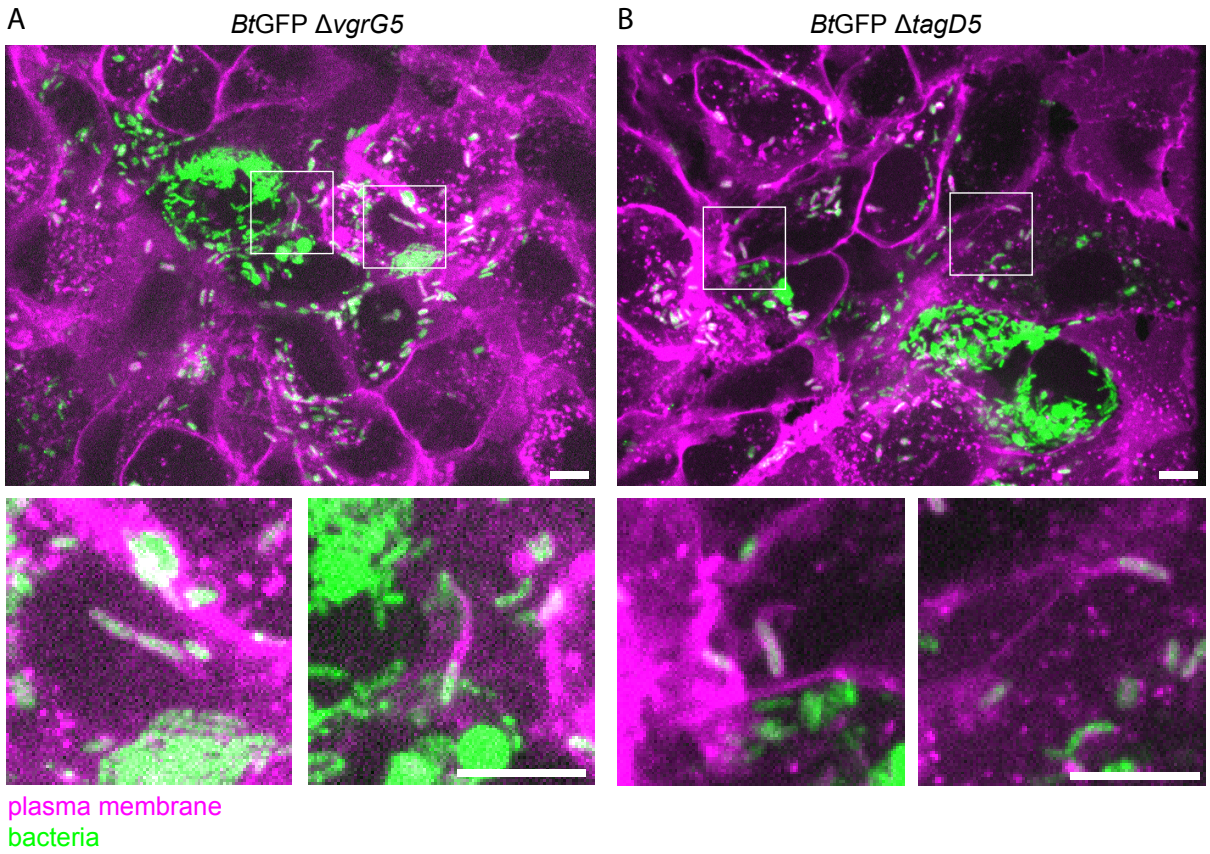
Supplemental Figures



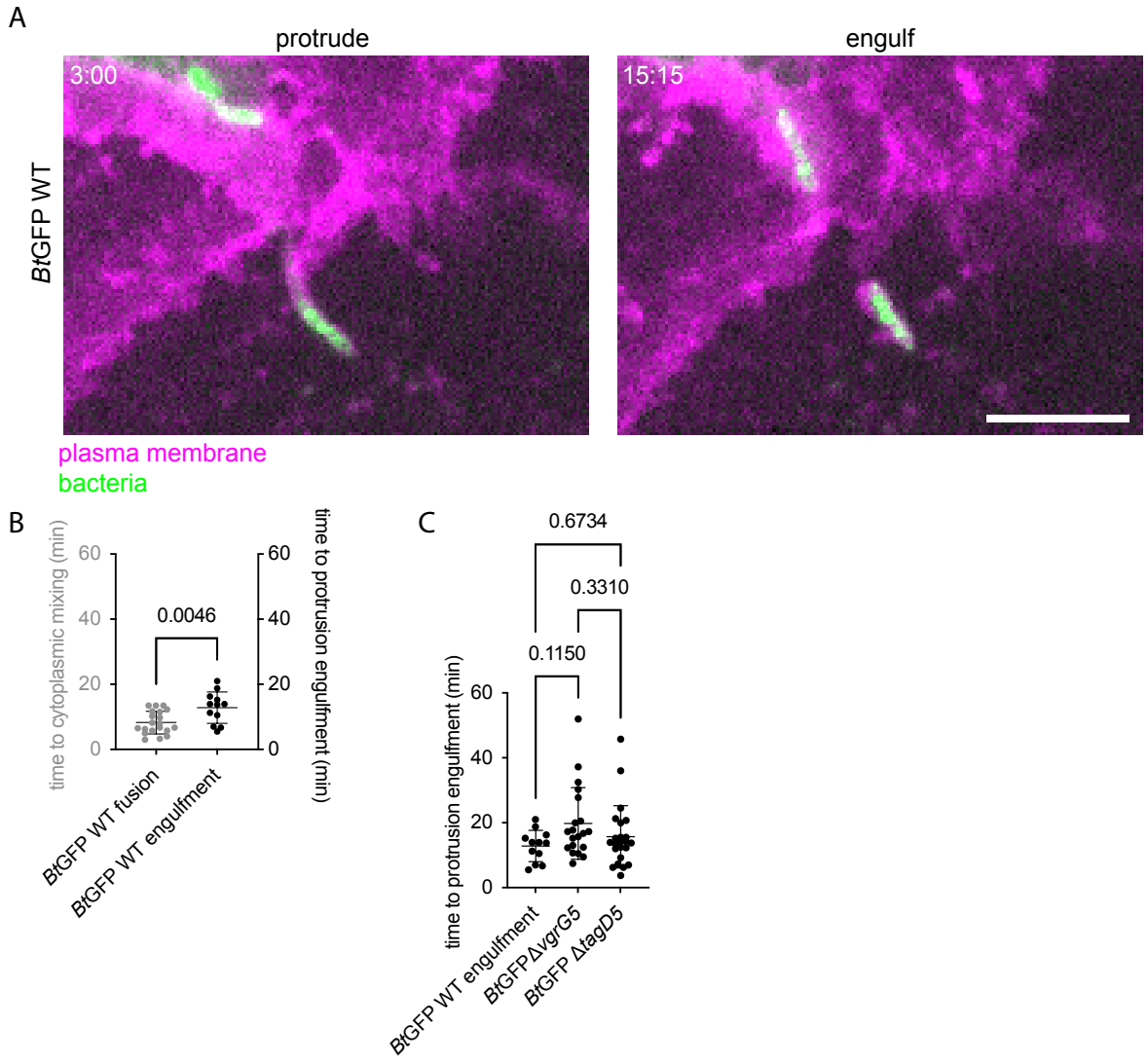
Supplemental Figure 2.1: Details of *B. thailandensis* inducing cell-cell fusion expanding from a membrane protrusion. (A) Live-cell imaging stills of two examples of *Bt*GFP WT while inducing cell-cell fusion. A 1:1 mixture of A549 cells that expressed the plasma membrane marker TagRFP-T-farnesyl or cytoplasmic GFP were used. Times represent min:s post protrusion formation. Images taken at ~16 h post infection. Scale bars are 5 µm. White arrows highlight the bacterium forming the protrusion. Black arrows highlight the region of protrusion entry. (B) Model of cell-cell fusion occurring in this example. Note that this movie is not part of the quantification dataset because the protrusion formed before the movie began.



Supplemental Figure 2.2: VgrG5 expression in glutathione-induced *Bt* WT, *Bt* Δ*vgrG5* and *Bt* Δ*tagD5*. (A) Western blot of cell lysates from the indicated strains grown in liquid cultures induced or uninduced with glutathione (GSH). Blot was probed with anti-VgrG5 antibodies. The asterisks indicate non-specific bands.



Supplemental Figure 2.3: *Bt*GFP Δ*vgrG5* and *Bt*GFP Δ*tagD5* can form protrusions from secondary cells. (A and B) Live imaging stills showing protrusions formed by motile bacteria in secondary cells after spread. Host cells are A549 cells expressing TagRFP-T-farnesyl. Images taken at ~24 h post infection with *Bt*GFP Δ*vgrG5* or *Bt*GFP Δ*tagD5*. Scale bars are 5 µm.



Supplemental Figure 2.4: Quantification of engulfment of *Bt*GFP WT, *Bt*GFP Δ*vgrG5* and *Bt*GFP Δ*tagD5.* (A) Live cell imaging stills of *Bt*GFP WT during engulfment into recipient cells. A549 cells that expressed TagRFP-T-farnesyl were used. Times represent min:s post protrusion formation. Images taken at ~16 h post infection. Scale bar is 5 µm. (B) Time to cytoplasmic mixing (n = 20) or protrusion engulfment for *Bt*GFP WT (n = 14). P value was calculated by an unpaired Mann-Whitney test. (C) Time to engulfment of *Bt*GFP WT (n = 14), *Bt*GFP Δ*vgrG5* (n = 20), or *Bt*GFP Δ*tagD5* (n=23). P = 0.1172, calculated by one-way ANOVA with Tukey's multiple comparisons test. Data are mean +/- SD.

Chapter 3

Characterizing the localization of *Burkholderia thailandensis* T6SS-5 proteins VgrG5 and TagD5

Introduction

Burkholderia thailandensis is a bacterium that spreads between host cells by inducing cell-cell fusion tissues (French et al., 2011; Harley et al., 1998; Kespichayawattana et al., 2000; West et al., 2008; K. T. Wong et al., 1995). The bacterial secretion apparatus T6SS-5 is required for this process (Schell et al., 2007; Schwarz et al., 2010), leading to the hypothesis that the T6SS-5 secretes bacterial effector proteins that mediate cell-cell fusion. In Chapter 2, we defined the cellular-level pathway that *B. thailandensis* uses to induce cell-cell fusion which involves bacterial motility, plasma membrane protrusion formation, and membrane fusion within these membrane protrusions (Chapter 2). We also found that two predicted T6SS-5 secreted effectors, VgrG5 and TagD5 (Schwarz et al., 2014; Toesca et al., 2014, Chapter 2), are required for cell-cell fusion and act at the membrane fusion step of the cell-cell fusion pathway and that VgrG5 must be expressed by bacteria within membrane protrusions in order to promote cell-cell fusion (Chapter 2). However, how these proteins contribute to cell-cell fusion on a molecular mechanistic level remains unknown.

VgrG5 is currently the only candidate fusogen for *B. thailandensis* induced cell-cell fusion. VgrG5 contains a domain common to all VgrG proteins that trimerizes to form a blunt cone structure (Leiman et al., 2009; Spínola-Amilibia et al., 2016). The VgrG5 C-terminal domain (CTD) likely contains effector function since C-terminal extensions containing effector function are common in VgrG proteins in other bacteria (Hachani et al., 2016; Jurėnas & Journet, 2021). Additionally, truncations that remove the entire CTD or portions of the CTD do not support cell-cell fusion (Schwarz et al., 2014; Toesca et al., 2014). The required regions of the CTD include a predicted transmembrane domain (TMD) and the C-terminal region. The TMD consists of two predicted transmembrane helices connected by a twelve amino acid linker, suggesting that VgrG5 may at some point be an integral membrane protein. Transmembrane helices are a common feature of fusogens and the present of predicted transmembrane helices is consistent with the potential for VgrG5 to participate in inducing membrane fusion. The VgrG5 C-terminus is sensitive to deletion of the final ten amino acids or an HA tag, indicating that this is an important region for VgrG5 function (Toesca et al., 2014). How these regions contribute to cell-cell fusion remains unknown.

Even less is known about TagD5, which was only recently shown to be required for *B. thailandensis*-induced cell-cell fusion. TagD5 is small (119 amino acids) and contains a PAAR structural domain but does not contain a sequence extension that is present in PAAR proteins that carry out effector functions (Shneider, 2013; Hachani et al., 2016). Thus, it is unclear how TagD5 could directly contribute to membrane fusion or have any effector function. VgrG and PAAR proteins typically bind to one another and function together (Jurėnas & Journet, 2021). Therefore, it is likely that TagD5 and VgrG5 form a complex and it is possible that such a complex functions during membrane fusion.

To better understand how VgrG5 and TagD5 function during *B. thailandensis*-induced cell-cell fusion, we characterized relevant aspects of these proteins. We found that exogenous overexpression within host cells of VgrG5, TagD5 or VgrG5 and TagD5 together did not result in cell-cell fusion. We found that VgrG5 and TagD5 exhibited similar localization patterns with no recognizable subcellular localization. We found regions of VgrG5 that are insensitive to epitope tagging for future studies. Finally, we found that the linker region of the TMD was

insensitive to amino acid substitution and lengthening but sensitive to shortening. Our results suggest that exogenous overexpression in host cells and genetic perturbation in *B. thailandensis* of VrgG5 and TagD5 are challenging, which suggests that T6SS-5-mediated translocation is important for the secretion, localization, and function of these proteins.

Results and Discussion

VgrG5 was not detected in infected host cells by live cell imaging or immunofluorescence microscopy

To understand how VgrG5 functions during cell-cell fusion, we sought to determine its localization in host cells. We first generated bacterial strains in which the *B. thailandensis vgrG5* gene was tagged with GFP in three locations: at the VgrG domain-CTD junction, at an internal site in the CTD identified as dispensable to cell-cell fusion (Toesca et al., 2014), and at the very C-terminus (Figure 3.1). For all three strains, GFP signal could be seen within bacteria, indicating that the proteins were expressed (data not shown). However, none of these strains induced cell-cell fusion, indicating the proteins were non-functional. We next endogenously tagged VgrG5 with a smaller FLAG epitope tag with the hope that it would not disrupt the function of VgrG5. The strain expressing VgrG5 with an N-terminal FLAG tag did not induce cell-cell fusion (Figure 3.1), indicating this protein was also non-functional. However, a strain expressing VgrG5 with a FLAG in the middle of the TMD linker region or at the CTD internal site was able to induce cell-cell fusion but with reduced efficiency (Figure 3.1). Interestingly, VgrG5 expression was lower when VgrG5 was tagged with FLAG at the CTD internal site, possibly explaining the reduction in plaque size but when VgrG5 was tagged with FLAG in the middle of the TMD linker region, there was no reduction in VgrG5 expression but a reduction in plaque size (Figure 3.1). Therefore, VgrG5 was functional when tagged with FLAG in two different locations.

To detect FLAG-tagged VgrG5 in *B. thailandensis*-infected cells, we used immunofluorescence microscopy with anti-FLAG antibodies. However, these experiments did not yield any signal above background (data not shown). In addition, tagged VgrG5 overexpressed in bacteria was not detectable by western blotting using anti-FLAG antibodies (data not shown), even though western blotting with a polyclonal anti-VgrG5 antibody was able to detect VgrG5. VgrG5 was also not detected above background in infected host cells by immunofluorescence using the anti-VgrG5 polyclonal antibody (data not shown). These results suggest that, if secreted by *B. thailandensis*, VgrG5 is present in low abundance and will be very difficult to detect by immunofluorescence using anti-FLAG or anti-VgrG5 antibodies.

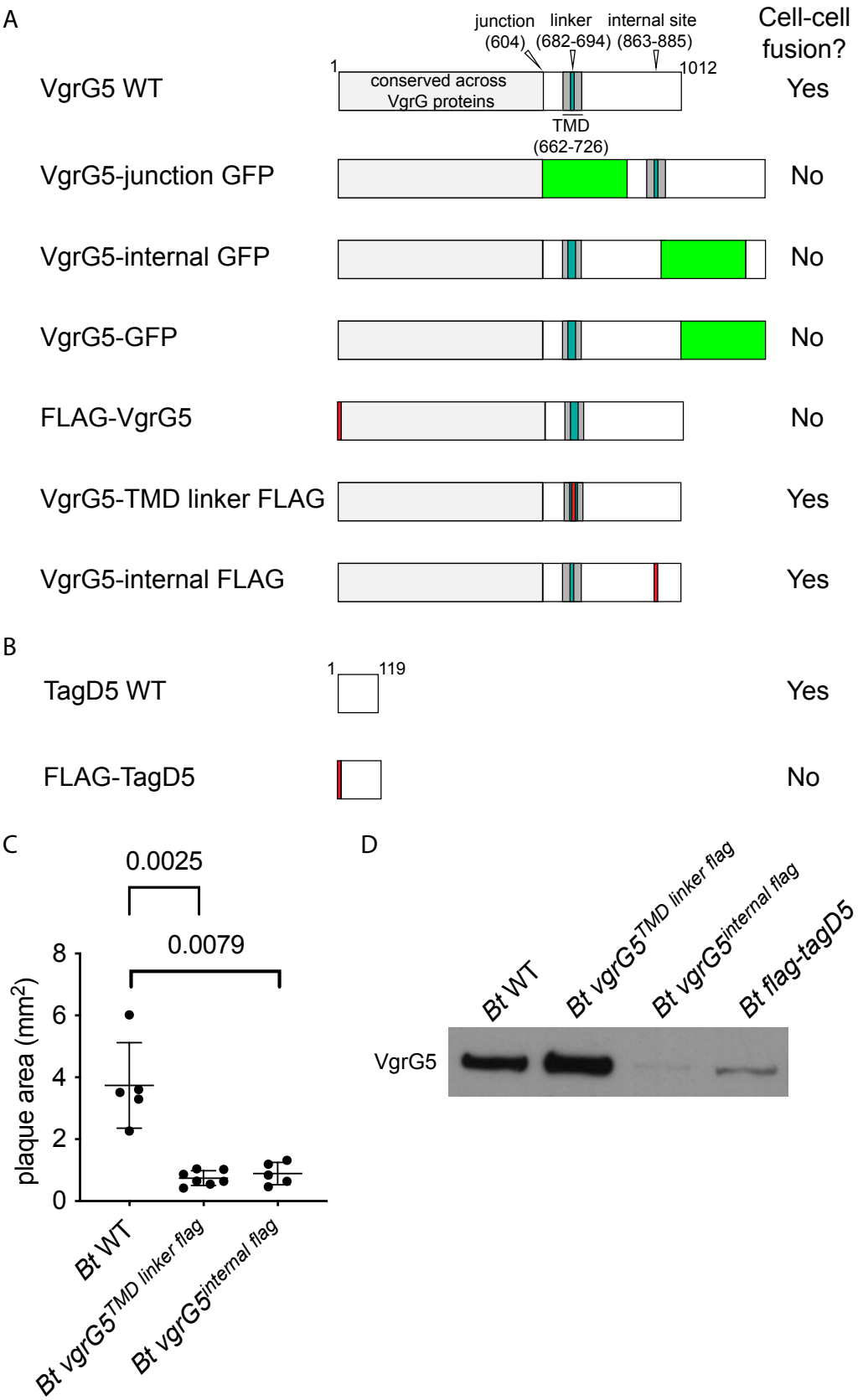

A
junction (604)
linker (682-694)
internal site (863-885)
Cell-cell fusion?
1
1012
conserved across VgrG proteins
TMD (662-726)
VgrG5 WT
Yes
VgrG5-junction GFP
No
VgrG5-internal GFP
No
VgrG5-GFP
No
FLAG-VgrG5
No
VgrG5-TMD linker FLAG
Yes
VgrG5-internal FLAG
Yes
B
1
119
TagD5 WT
Yes
FLAG-TagD5
No
C
0.0025
0.0079
plaque area (mm2)
8
6
4
2
0
Bt WT
Bt vgrG5TMD linker flag
Bt vgrG5internal flag
D
Bt WT
Bt vgrG5TMD linker flag
Bt vgrG5internal flag
Bt flag-tagD5
VgrG5

Figure 3.1: VgrG5 and TagD5 epitope tagging. (A) *B. thailandensis* VgrG5 and derivatives illustrating epitope tag locations. The abilities of strains containing these epitope tags to induce cell-cell fusion are shown on the right. (B) *B. thailandensis* TagD5 and derivative illustrating epitope tag location. The abilities of strains to induce cell-cell fusion are shown on the right. Graphics are not to scale. Light grey, VgrG domain; red, FLAG; green, GFP, dark grey, predicted transmembrane helices; teal, linker region. (C) Plaque areas of Vero cells infected with the indicated strains. n=1 experiment, 5-7 plaques per strain. P values were calculated by unpaired Mann-Whitney test, data are mean +/- SD. (D) Western blot analysis of cell lysates from the indicated strains grown in liquid cultures induced with glutathione (GSH). Blot was probed with anti-VgrG5 antibodies.

Exogenous expression of VgrG5 within host cells does not result in cell-cell fusion or reveal subcellular localization

We next tried to characterize VgrG5 localization and function following overexpression within host transfected U2OS host cells. Following transfection, cells were probed for VgrG5 using the anti-VgrG5 antibody. Although the VgrG5 signal was clear, VgrG5 did not exhibit any obvious subcellular localization, including membrane localization (Figure 3.2). VgrG5 overexpression also did not result in cell-cell fusion of the host cells as determined by the inability to observe multinucleated cells (Figure 3.2).

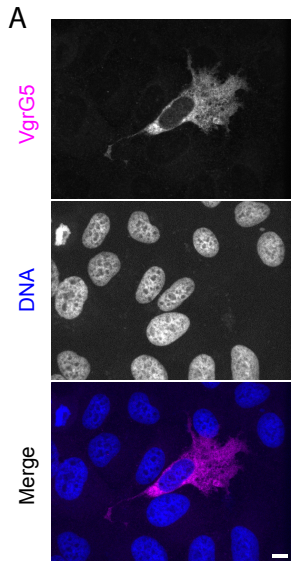


Figure 3.2: VgrG5 localization during overexpression in host cells. (A) U2OS cells transfected to express VgrG5. Immunofluorescence of VgrG5 (magenta) using the anti-VgrG5 antibody and DNA (blue) using Hoechst. Average intensity projection is shown. Scale bar is 5 μm.

Exogenous expression of TagD5 within host cells does not result in cell-cell fusion or reveal subcellular localization

To further understand the function of TagD5 during cell-cell fusion, we sought to localize TagD5. We generated a *B. thailandensis* strain expressing TagD5 tagged at its N-terminus with a FLAG tag from the endogenous site. However, this strain did not support cell-cell fusion (Figure 3.1), indicating that the FLAG tag might interfere with TagD5 function. Then, to assess localization, we transfected U2OS cells to overexpress FLAG-TagD5 and probed for TagD5 using an anti-FLAG antibody. Similar to VgrG5, FLAG-TagD5 showed no obvious subcellular localization patter. Moreover, overexpression of FLAG-TagD5 did not result in host cell-cell fusion as determined by the inability to observe multinucleated cells (Figure 3.3).

We further hypothesized that VgrG5 and TagD5 might form a complex and therefore we needed to overexpression both VgrG5 and TagD5 to determine their subcellular distribution or ability to induce membrane fusion. When co-expressed, VgrG5 and FLAG-TagD5 showed similar diffuse localization to the singly expressed proteins (Figure 3.3). Furthermore, co-expression of these proteins did not result in cell-cell fusion as determined by the inability to observe multinucleated cells (Figure 3.3).

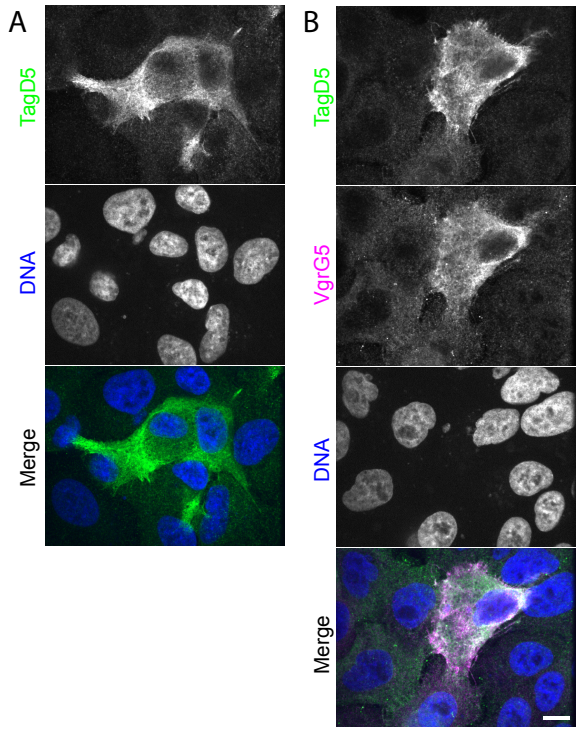


Figure 3.3: TagD5 and VgrG5 localization during overexpression in host cells. (A) U2OS cells transfected to express FLAG-TagD5. Immunofluorescence staining of FLAG-TagD5 (green) using anti-FLAG antibody and DNA (blue) using Hoechst. **(**B) U2OS cells transfected to express both FLAG-TagD5 and VgrG5. Immunofluorescence staining of FLAG-TagD5 (green) using anti-FLAG antibody, VgrG5 (magenta) using the anti-VgrG5 antibody, and DNA (blue) using Hoechst. Average intensity projections are shown. Scale bar is 5 μm.

The VgrG5 linker region between the two predicted transmembrane domains is not responsible for inducing cell-cell fusion

VgrG5 contains a predicted transmembrane domain (TMD) region that is required for cell-cell fusion (Toesca et al., 2014). All fusogens contain at least one transmembrane helix and many contain a TMD (Hernández & Podbilewicz, 2017), which is why the TMD is a region of interest for understanding the mechanism by which VgrG5 contributes to cell-cell fusion. The TMD consists of two predicted transmembrane helices connected by 12 amino acids (referred to here as the "linker") (Figure 3.4) (Toesca et al., 2014). It is not known which portion of the TMD is important for fusion. We hypothesized that the linker might function as a fusion peptide similar to FAST fusogens (Duncan, 2019). To examine the function of the linker, we generated *B. thailandensis* strains in which we mutated all the linker amino acids to glycines and alanines (*Bt vgrG5*$^{GA\ linker}$), cut the GA-linker in half (*Bt vgrG5*$^{1/2\ linker}$), and doubled the length of the linker sequence using the canonical amino acid sequence (*Bt vgrG5*$^{2x\ linker}$) (Figure 3.4). We then measured MNGC formation by plaque size assay, which was previously used to determine the extent of cell-cell fusion (Benanti et al., 2015; French et al., 2011), in Vero cells. We found that *Bt vgrG5*$^{1/2\ linker}$ was unable to induce cell-cell fusion whereas *Bt vgrG5*$^{GA\ linker}$ and *Bt vgrG5*$^{2x\ linker}$ were able to induce cell-cell fusion (Figure 3.4). Therefore, the precise sequence of the linker is unimportant, but a minimum length of the linker is important for cell-cell fusion. Because this region of the TMD is flexible with regards to length and amino acid sequence, it is likely not the most important aspect of the TMD.

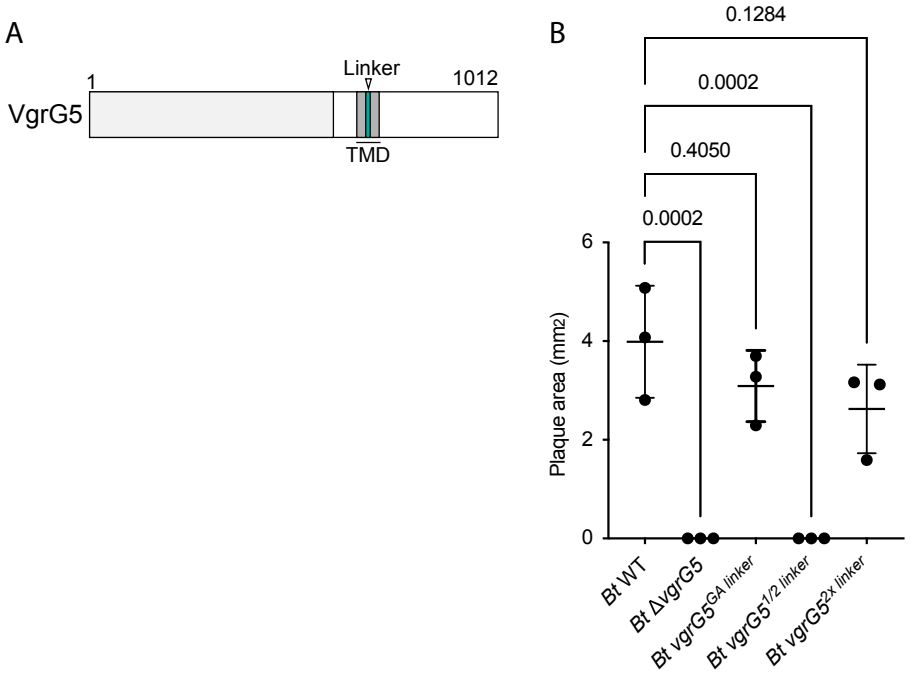


Figure 3.4: Dissection of the VgrG5 predicted TMD linker region. (A) Schematic of VgrG5 protein showing the TMD, including the predicted transmembrane helices (dark grey) and linker region (green). (B) Plaque areas of Vero cells infected with the indicated strains. N=3 experiments, 9-11 plaques per experiment. P = 0.0001, calculated by one-way ANOVA. P values shown were calculated with Tukey's multiple comparisons test. Data are mean +/- SD.

Conclusions

Here we characterized two proteins essential for *B. thailandensis* induced cell-cell fusion, VgrG5 and TagD5. We identified regions within VgrG5 that could tolerate the introduction of a FLAG tag and still support protein function in cell-cell fusion. We further found that exogenously expressed VgrG5 and TagD5, either alone or together, did not assume a defined subcellular localization patter and did not cause cell-cell fusion. We also characterized the linker sequence within the predicted TMD of VgrG5 and found that the amino acid identity and length of this linker were flexible, suggesting that it does not directly mediate membrane fusion.

We identified two regions of VgrG5 that could tolerate the insertion of a FLAG tag and resulted in a biologically functional protein. However, attempts to visualize VgrG5 by immunofluorescence microscopy using the anti-VgrG5 antibody were not fruitful. These results suggest that, if secreted by *B. thailandensis*, VgrG5 is present in low abundance and is very difficult to detect by immunofluorescence using anti-FLAG or anti-VgrG5 antibodies. This is also supported by our finding in Chapter 2 that VgrG5 must be expressed by bacteria within protrusions for the bacterium to induce cell-cell fusion. VgrG5 is likely secreted within membrane protrusions at very low amounts that might not be easily detected by immunofluorescence microscopy. That VgrG5 is difficult to localize by immunofluorescence is consistent with many bacterial effector proteins and fusogens, for example, InlC in *L. monocytogenes* (Rajabian et al., 2009) and Myomerger/Myomixer/Minion (Bi et al., 2017; Quinn et al., 2017; Zhang et al., 2017) respectively. Identification of which regions can be functionally tagged is important for future research. For example, a 3x FLAG tag could be engineered to boost the signal for western blotting or a Strep tag could be inserted in the same regions for affinity chromatography experiments to identify interacting proteins.

Exogenous overexpression of VgrG5, TagD5 or VgrG5 and TagD5 together did not result in cell-cell fusion. Similarly, this experiment did not reveal a particular subcellular localization pattern for VgrG5 or TagD5 and therefore did not suggest any additional hypotheses about where these proteins might function during infection. These results are not surprising because overexpression systems have many drawbacks and caveats. For example, because VgrG5 and TagD5 are likely delivered by the bacterial T6SS-5 within bacterial plasma membrane protrusions (see Chapter 2), they might not be folded, trafficked, or localized properly when overexpressed in host cells. Additional limitations to this approach include the absence of possible bacterial protein binding partners and membrane protrusions, which are key mediators of cell-cell fusion. Therefore, these results do not rule out a role for VgrG5 or TagD5 in membrane fusion and suggest that the localization or function of VgrG5 cannot be determined through an overexpression approach.

We also found that the VgrG5 linker sequence within the TMD is flexible with regards to its amino acid identity and that it can be lengthened but not shortened. This finding points to other regions of VgrG5 as being functional domains, such as the predicted transmembrane helices themselves or the C-terminal region, which is sensitive to deletion or tagging (Toesca et al., 2014). One possibility is that the linker region functions to hold the TMD together while the predicted transmembrane helices function during cell-cell fusion. Such functions could include membrane disruption, membrane channel formation, or protein-protein interaction, among others. The C-terminus of VgrG5 might also contain a functional domain as it was previously

shown that the 10 C-terminal-most residues are essential (Toesca et al., 2014). This sequence (KLDGQLIQLG) is not similar to sequences in other proteins as determined by BLAST and does not have any particular characteristics that suggest a function, for example, it is not enriched for hydrophobic residues. Therefore, it is possible that this sequence is required because it is part of a larger functional domain.

While the experiments shown in this work did not reveal the function or localization of VgrG5 or TagD5, they are useful because they reveal that standard cell biological and genetic approaches including tagging, exogenous expression, and truncation analyses may not be sufficient to understand how these proteins contribute to cell-cell fusion. Future work should focus on other approaches such as biochemical and structural studies, as discussed in Chapter 4.

Materials and Methods

Bacterial and mammalian cell culture

Escherichia coli strains XL1-blue and BL21(DE3) were obtained from the University of California, Berkeley MacroLab and were used for plasmid construction and protein expression, respectively. *E. coli* was cultured in liquid or solid lysogeny broth (LB) with or without 100 μg/ml ampicillin or 50 μg/ml kanamycin, when appropriate. *E. coli* RHO3 (López et al., 2009) was grown in LB supplemented with diaminopimelic acid (DAP) (200 mg/ml). *B. thailandensis* E264 was cultured in liquid or solid LB.

Mammalian cell lines (Vero monkey kidney epithelial, RRID:CVCL_0059, A549 human lung epithelial, RRID:CVCL_0023; and U2OS human osteosarcoma, RRID:CVCL_0042) were obtained from the University of California, Berkeley Tissue Culture Facility, which authenticated these cell lines prior to freezing, and were not tested for mycoplasma contamination. Cells were grown at 37˚C in 5% CO_2. Vero cells were maintained in DMEM (Invitrogen, 11965-092v) containing 2% fetal bovine serum (FBS, GemCell Bio-Products, 100-500). A549 and U2OS cells were maintained in DMEM containing 10% FBS (Atlas Biologicals, FP-0500-A).

Plasmid construction

To make *B. thailandensis* mutants, we used plasmid pEXKm5 (López et al., 2009) for allelic exchange. We PCR-amplified DNA from *B. thailandensis* cells boiled in water. These DNA fragments contained ~500 bp 5' and 3' to the region of interest and were subcloned into pEXKm5. To generate these fragments, upstream (US) and downstream (DS) fragments were designed with 11-36 base pairs of overlap between them for stitching by overlap PCR. The fragments were amplified with primers containing XmaI cut sites that would flank the full-length fragment once it was stitched. The primers used for amplifying US and DS (as well as middle fragments for GFP-containing plasmids) are listed in the table below.

Plasmid		Upstream fragment	Middle fragment	Downstream fragment
pEXKm5 VgrG5 junction GFP	Forward	ATCCCTACCCG GGCAGTACAAA GTGCGCTTTCCG TT	CTGCAGGGCAA CAGTAAAGGAG AAGAACTTTTC ACTGGAGTTG	TGAACTATACAAA CTGCGCTGGATGC TGCC
	Reverse	TTCTCCTTTACT GTTGCCCTGCA GCGTCGT	CATCCAGCGCA GTTTGTATAGTT CATCCATGCCAT GTGTAA	CCGGCCCGGGCCC TTGCCGACGCTCA T
pEXKm5 VgrG5 internal GFP	Forward	ATTACCCTGTTA TCCCTACCCGG GACGGTGCGCA AGTTCATGAT	GTCGGCGCGCT CGTGCCCACGC CGAGTAAAGGA GAAGAACTTTT CACTGGAGTTG	CATGGCATGGATG AACTATACAAAAA GGAAGCGAAGCT CGTCGAG
	Reverse	AGTGAAAAGTT CTTCTCCTTTAC	CAGCTCGACGA GCTTCGCTTCCT TTTTGTATAGTT	CTTAAGCCGGCCC GGGAGCCGGTCG ATTCCTTCGAA

		TCGGCGTGGGC ACGAG	CATCCATGCCAT GTGTAA	
pEXKm5 Flag-VgrG5	Forward	CCCTGTTATCCC TACCCGGGTCG CGCGAATGACC GAGAT	ACAAGGACGAC GACGACAAGTC TTCGTCCCATCG ACACTACG	
	Reverse	ACTTGTCGTCGT CGTCCTTGTAGT CCATCGGGAAC TCCTGGGCA	TTGTCGACTTAA GCCGGCCCGGG TAGGTAGCGCA GCGCGAGATC	
pEXKm5 VgrG5 linker Flag	Forward	ATCCCTACCCG GGCGAGCACAA CCAGCTCTACAT GAA		CTTGTCGTCGTCG TCCTTGTAGTCGC CCCCGATGAGCCC CGCAGCG
	Reverse	CGACGACAAGG GCGGCGGCTTC GGCGTGTCCG		AGCCGGCCCGGGC TTCGCTTCCTTCG CCTTGAT
pEXKm5 VgrG5 internal Flag	Forward	ATTACCCTGTTA TCCCTACCCGG GACGGTGCGCA AGTTCATGAT		GACTACAAGGAC GACGACGACAAG AAGGAAGCGAAG CTCGTCG
	Reverse	CTTGTCGTCGTC GTCCTTGTAGTC CGGCGTGGGCA CGAGCGC		GACTACAAGGAC GACGACGACAAG AAGGAAGCGAAG CTCGTCG
pEXKm5 Flag-TagD5	Forward	ATCCCTACCCG GGTCGTGCGCA TCCGCATCCTCT T		GACTACAAGGAC GACGACGACAAG AGTCCCGCCGACG TCTGCA
	Reverse	CTTGTCGTCGTC GTCCTTGTAGTC CATGCACAGGC CGGAGGCGG		AGCCGGCCCGGG GATTCGCAGCGGC ACGTCGAA
pEXKm5 VgrG5 GA linker	Forward	ATCCCTACCCG GGCGAGCACAA CCAGCTCTACAT GAA		GGCGCAGGTGGC GCTGGCGGCGCAG GTGGCGCTGGCGG CTTCGGCGTGTCC G
	Reverse	GCCAGCGCCAC CTGCGCCGCCA GCGCCACCTGC GCCGATGAGCC CCGCAGCG		AGCCGGCCCGGGC TTCGCTTCCTTCG CCTTGAT
pEXKm5 VgrG5	Forward	ATCCCTACCCG GGCGAGCACAA		GGCGCAGGTGGC GCTGGCGGCGCAG GTGGCGCTGGCGG

1/2GA linker		CCAGCTCTACATGAA		CTTCGGCGTGTCCG
	Reverse	AAGCCGCCAGCGCCACCTGCGCCGATGAGCCCCGCAGCGGTG		ATCGGCGCAGGTGGCGCTGGCGGCTTCGGCGTGTCCGCC
pEXKm5 VgrG5 2x linker	Forward (PCR 1)	ATCCCTACCCGGGCGAGCACAACCAGCTCTACATGAA		CCGTGGGCCAAGGGGGGCGGCGCCCTCGCCGATCTGCCGTGGGCG
	Forward (PCR 2)			CGGCGGCGCGCTCGCCGACCTGCCGTGGGCCAAGGGGGGC
	Reverse	GGCGAGCGCGCCGCCG		ATCGGCGCAGGTGGCGCTGGCGGCTTCGGCGTGTCCGCC

For VgrG5 and TagD5 transfections, expression vectors for transfection were generated in the pCDNA3 backbone. The gene encoding VgrG5 was amplified by PCR from boiled *B. thailandensis* with 5' 17 bp and 3' 17 bp overhangs homologous to the backbone (forward primer 5' GTACCGAGCTCGGATCCATGTCTTCGTCCCATCGACACTACG 3', reverse primer 5' GAATAGGGCCCTCTAGATtAGCCTAGCTGGATCAACTGTCCG 3'). The pCDNA backbone was amplified (forward primer 5' GACAGTTGATCCAGCTAGGCTaATCTAGAGGGCCCTATTCTATAG 3', reverse primer 5' TGTCGATGGGACGAAGACATGGATCCGAGCTCGGTAC 3'). The *vgrG5* fragment and linearized backbone were assembled using Gibson Assembly (New England Biolabs, E2611S).

The gene encoding TagD5 was amplified by PCR from boiled *B. thailandensis* with 5' *flag* sequence (forward primer 5' GACTACAAGGACGATGATGACAGTCCCGCCGACGTCTG 3', reverse primer 5' ACTCATCACCATCACTTTCTGCTGG 3'). The pCDNA backbone was amplified with 3' *flag* sequence and 5' 20 bp homology to the 3' end of *tagD5* (forward primer 5' AGAAAGTGATGGTGATGAGTTAATCTAGAGGGCCCTATTCTATAG 3', reverse primer 5' GTCATCATCGTCCTTGTAGTCCATGGATCCGAGCTCGGTAC 3'). The *tagD5* fragment and linearized backbone were assembled using Gibson Assembly (New England Biolabs, E2611S).

B. thailandensis **strain construction**

B. thailandensis strains were created by allelic exchange, as previously described (Benanti et al., 2015; López et al., 2009). Bi-parental matings between *B. thailandensis* strain E264 and *E. coli* RHO3 (López et al., 2009) harboring a pEXKm5 derivative were performed to introduce pEXkm5 into *B. thailandensis*, followed by selection on 50 μg/ml kanamycin-containing plates that lacked DAP to select against *E. coli* RHO3. Uptake of pEXKm5 was also confirmed by PCR detection of the *sacB* gene. The integrated plasmid backbone was removed by growth in non-selective YT media (5 g/l yeast extract (VWR, EM1.03753.0500), 5 g/l tryptone (Fisher Scientific, BP1421-500)) and screening for loss of b-glucuronidase activity via plating on YT plates containing 50 μg/ml X-Gluc (cyclohexlammonium salt, Gold Biotechnologies,

G1281C1). Strains were confirmed by PCR amplification and DNA sequencing of the region of interest.

Bacterial Infections of host cells

B. thailandensis strains were streaked from frozen stocks onto LB agar plates. Bacteria were swabbed from plates to inoculate LB liquid media and were grown with shaking at 37˚C for 3-16 h. Prior to infections, the OD_{600} of cultures was measured (OD_{600} of 1 =$5x10^8$ cfu/ml) for calculating the number of bacteria to infect with to achieve the proper multiplicity of infection (MOI). Bacterial cultures were pelleted and resuspended in PBS (ThermoFisher, 10010049. Composition: Potassium Phosphate monobasic (KH2PO4), 1.0588236mM; Sodium Chloride (NaCl), 155.17241 mM; Sodium Phosphate dibasic (Na2HPO4-7H2O), 2.966418 mM). Mammalian cells were seeded at least 24 h before infection and immediately prior to infection were washed with PBS and provided with fresh DMEM with 10% FBS. Bacteria were added directly to media on cells, media was pipetted or rocked gently to mix, and bacteria were left to invade for 45 min to 1 h at 37˚C unless otherwise stated. Cells were rinsed once with PBS then DMEM with 10% FBS and 0.5 mg/ml gentamicin (Fisher Scientific, MT30-005-cR) was added. For mixed-strain infections, infections lasted longer, as detailed below.

Transfection

For VgrG5 transfection, U2OS cells were plated in a 24-well plate (250,000 cells/well) with coverslips 24 hours prior to transfection. Plasmids were diluted to 66.6 µg/µl and mixed 1:1 with Opti-mem (Invitrogen, 31985-070). Lipofectamine (LFA) 2000 (ThermoFisher, 11-668-027) was added to Opti-mem at 2.5 µl LFA 2000 per 50 µl and mixed by flicking. The DNA mix was added to LFA 2000 at a 1:1 ratio and incubated for 5 minutes. Media on cells was replaced and 50 µl of the LFA 2000/DNA mix was added to the media on cells and mixed by shaking the plate back and forth. After ~14 hours, media was changed to fresh media containing 2% FBS. Cells were fixed for immunofluorescence staining 48 hours post transfection.

For transfections of FLAG-TagD5 and VgrG5 together and FLAG-TagD5 alone, U2OS cells were plated in a 24-well plate (250,000 cells/well) with coverslips 24 hours prior to transfection. Plasmids were diluted to 40 µg/µl and mixed 1:1 with Opti-mem (Invitrogen, 31985-070). Lipofectamine (LFA) 2000 (ThermoFisher, 11-668-027) was added to Opti-mem at 2 µl LFA 2000 per 50 µl and mixed by flicking. The DNA mix was added to LFA 2000 at a 1:1 ratio and incubated for 15 minutes. Media on cells was replaced. 50 µl of the LFA 2000/DNA mix was added to the media on cells and mixed by shaking the plate back and forth.

Immunofluorescence microscopy

Cells were immediately fixed at room temperature with cold 4% paraformaldehyde (ThermoFisher, O4042-500) (PFA) in PBS. Cells were washed 3x with PBS and then blocked and permeabilized in blocking buffer (5% bovine serum albumin (BSA) (Sigma-Aldrich, A3803-10g) in PBS with 0.3% Triton X-100 (ThermoFisher, BP151-100) and 0.02% NaAzide) for 30 minutes. Coverslips were incubated with 50 µl primary antibody diluted in blocking buffer for 30 mins -1 hour. For immune-staining of VgrG5, rabbit anti-VgrG5 (1:250) was used, and for immune-staining of FLAG, mouse anti-FLAG (1:500) was used. Coverslips were washed 3x with PBS and then incubated with Alexa 488 and/or 568-conjugated secondary antibody at 1:500 dilution and Hoechst (Fisher Scientific, H3570) at 1:2000 dilution. Coverslips were washed 3x in PBS, liquid was blotted away, and coverslips were mounted on slides using 3 µl ProLong Gold

Antifade Mountant (Fisher Scientific, P36930) and stored short term at room temperature or for long term at 4˚C.

Plaque assay

For plaque assays to investigate the VgrG5 TMD, Vero cells were plated in 6-well plates ($6x10^5$ cells/well), infected at an MOI of 2, and bacteria were allowed to invade for 45 min. Infected cell monolayers were washed once with PBS and overlayed with 3 ml of 0.7% agarose in DMEM with 5% FBS and 0.5 mg/ml gentamycin. At 31 hpi, 1 ml of 0.7% agarose in PBS containing neutral red (Sigma, N6264) at 1:20 dilution was overlayed onto wells (final concentration on cells was 1%). 14 h after addition of neutral red, plates were scanned, and plaque area was measured using ImageJ (Version 2.1.0/1.53c).

For plaque assays to investigate FLAG-tagged strains, experiments were carried out with the method above but with the following changes: an MOI of 20 was used, the neutral red overlay was added at 41 hpi, and plaques were imaged at 7 h after addition of neutral red.

References

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Chapter 4

Future Directions

The research described in this thesis establishes the cellular level mechanism of cell-cell fusion induced by *B. thailandensis* and inspires hypotheses about the mechanism that *B. thailandensis* uses to induce cell-cell fusion. The work also identifies two proteins, VgrG5 and TagD5, that function at the membrane fusion step of the cell-cell fusion pathway. Future work should focus on understanding how these proteins function during cell-cell fusion and how cell-cell fusion occurs mechanistically at a molecular level. Such insights will expand our knowledge of cell-cell fusion and membrane fusion processes more generally. In this chapter, I outline five key outstanding questions that should be addressed in future studies.

Do VgrG5 and TagD5 form a complex?

We discovered that TagD5, a PAAR family protein, is required for cell-cell fusion (Chapter 2). TagD5 is small (119 amino acids), containing only a PAAR structural domain but no sequence extension that is present in some PAAR proteins that carry out effector functions (Shneider, 2013; Hachani et al., 2016). Therefore, it is unclear how TagD5 could have effector functions, such as directly contributing to membrane fusion. Basic characterization of TagD5 will help elucidate how this protein or such a complex might function. This will require development of an anti-TagD5 antibody for use in western blotting, immunofluorescence microscopy, and immunoprecipitation assays to identify expression, investigate localization, and facilitate biochemical assays.

VgrG and PAAR proteins typically bind to one another and function together (Jurėnas & Journet, 2021). Therefore, it is likely that TagD5 and VgrG5 form a complex. We found that VgrG5 protein levels are diminished in the absence of TagD5, which suggests that TagD5 is required for VgrG5 stability or expression. We also found that the phenotypes of *vgrG5* and *tagD5* mutants are identical, indicating that they act at the same step of the cell-cell fusion pathway. These data are consistent with VgrG5 and TagD5 forming a complex. Further investigation into whether these proteins interact will require co-immunoprecipitation of VgrG5 and TagD5 from *B. thailandensis* and co-immunoprecipitation of purified VgrG5 and TagD5.

Other bacterial proteins might be part of a VgrG5-TagD5 complex and understanding VgrG5-TagD5 function will require identification of these interacting proteins. To achieve this, an immunoprecipitation and mass spectrometry approach could be used, either from infected host cells or broth-grown *B. thailandensis* in the presence of glutathione to induce T6SS-5 component expression (J. Wong et al., 2015). Additional proteins could be involved in chaperoning the complex, in protecting the predicted transmembrane domain, in inducing membrane fusion directly, or in other functions. Identifying interacting proteins will help reveal the complete molecular-level mechanism through which these proteins function in membrane fusion.

How does VgrG5 function?

VgrG5 is important for cell-cell fusion, but how it contributes remains unknown. The CTD likely contains effector activity since it is required for cell-cell fusion and C-terminal extensions of other extended VgrG proteins typically contain effector activity (Hachani et al., 2016; Jurėnas & Journet, 2021). The VgrG5 CTD does not have any predicted functions or regions except for the transmembrane domain (TMD), which is required for cell-cell fusion

(Toesca et al., 2014). Because the C-terminus is highly sensitive to mutation (Toesca et al., 2014), truncation experiments are not interpretable. To understand which domains of VgrG5 function during cell-cell fusion, alanine scanning (Weiss et al., 2000) might be a more suitable approach because this method allows for identification of required regions while retaining other functional regions of the protein.

Although mutational studies to identify which regions of VgrG5 are important for cell-cell fusion will help reveal required features of the protein, this approach will not provide enough information to suggest meaningful hypotheses about the mechanism of action of VgrG5. Therefore, a structural analysis of VgrG5 or a VgrG5-containing complex will be needed. Such experiments should at the minimum include TagD5, as it likely binds to VgrG5. Although high resolution structure would ideally be determined by x-ray crystallography or cryo-electron microscopy, other methods such as negative staining and electron microscopy would give a general sense of the organization and overall shape of VgrG5 or a VgrG5-containing complex. Because VgrG5 contains two predicted transmembrane helices in its TMD, it might be necessary to purify the complex in a lipid environment, possibly in a lipid nanodisc as in structural studies of the *Pseudomonas aeruginosa* PAAR protein Tse6 (Quentin et al., 2018). In addition, obtaining the structures of both a pre-secretion state and a post-secretion state of VgrG5 will help elucidate how the protein is transferred from the bacterium to the host cell and therefore how it functions within the host cell. Once the structure has been determined, apparent functional domains will need to be characterized by structure-function mutational studies to glean meaningful mechanistic insights.

Where does VgrG5 function during the cell-cell fusion pathway?

One important and outstanding question is where VgrG5 acts during cell-cell fusion. Our finding that bacteria in plasma membrane protrusions must express VgrG5 in order to induce cell-cell fusion suggests that VgrG5 localizes and acts within protrusions (Chapter 2). We hypothesize that once the bacterium enters into the membrane protrusion, VgrG5 localizes to the plasma membrane where it carries out its function. Localization of VgrG5 to the plasma membrane and membrane protrusions would support such a hypothesis. Alternatively, localization of VgrG5 elsewhere would suggest new hypotheses about how VgrG5 might function during cell-cell fusion.

My preliminary studies suggest that VgrG5 is associated with host cell membranes (Figure 4.1). In a membrane floatation, lysates from infected host cells underwent centrifugation in a sucrose gradient to float membranes. This experiment revealed that a population of VgrG5 co-migrates with the host plasma membrane protein transferrin receptor (TFR) (Figure 4.1A). This finding indicates that VgrG5 might associate with, bind to, or integrate into host membranes, possibly the plasma membrane. Interestingly, migration of a small population of VgrG5 with TFR also occurred when the membrane floatation experiment was performed using a mixture of *B. thailandensis* grown in liquid media with glutathione to induce expression of T6SS-5 components, including VgrG5, (J. Wong et al., 2015) together with host cell lysates (Figure 4.1B and C). Higher amounts of VgrG5 migrated the samples were dounced together (Figure 4.1B) and less when host cells were dounced separately and samples were subsequently mixed together (Figure 4.1C). Importantly, VrgG5 did not migrate to this fraction without host cells present (data not shown), indicating that the migration was not due to bacterial or bacterial

membrane floatation. That VgrG5 associates with host membranes following mixing of bacteria with cell lysates as well as following infection presents a complication to interpreting our findings. It is possible that VgrG5 does not associate with host membranes during infection, but rather that the douncing process, which is required for performing the floatation experiment on an infected sample, leads to the association. The findings from the membrane floatation assays raise the intriguing possibility that VgrG5 becomes integrated or associated with host membranes simply by proximity to or contact with membranes.

Additional preliminary studies suggest that VgrG5 is exposed on the extracellular surface during infection. The studies involved surface biotinylation using a membrane impermeable probe followed by pulldown of biotin using streptavidin beads or StrepTactin resin, an approach previously used to identify surface-exposed proteins (Sirkis et al., 2017; Chan et al., 2021). Using this approach on infected host cells, VgrG5 was identified in the elution from resin (data not shown). Therefore, a population of VgrG5 may be surface exposed during infection. When broth-grown bacteria were induced with glutathione and biotinylated, VgrG5 was again found in the elution from resin (data not shown). This finding suggests that VgrG5 might be exposed on the surface of bacteria. One hypothesis to explain this result is that rather than detaching from the T6SS-5 complex upon secretion, VgrG5 decorates the surface of the bacterium. Another hypothesis is that VgrG5 is partially exposed and accessible to the biotin reagent in its pre-secretion state. Either way, our preliminary results suggest that bacterially-associated VgrG5 is detectable by this biotinylation assay which is a major complication to interpreting the results of the biotinylation assay done on infected host cells. To establish that there is a population of VgrG5 exposed on the host cell surface during infection, it must be ruled out that bacterially-associated VgrG5 is being biotinylated upon biotinylation of infected host cells. To investigate this, one approach is biotinylation followed by pulldown and mass spectrometry to identify biotinylated proteins, including bacterial surface proteins. If we find that no bacterial surface proteins are biotinylated by approach, this experiment would also determine the biotinylated residues on VgrG5 which could give mechanistic insight into which regions of VgrG5 are potentially surface exposed and likely involved in membrane fusion.

Taken together, the biotinylation and membrane floatation results point to VgrG5 localization at the bacterial surface as well as in the host cell plasma membrane during infection. This suggests the hypothesis that, in the context of a membrane protrusion, the *B. thailandensis* T6SS-5 could localize VgrG5 simply by bringing VgrG5 into contact with the host cell plasma membrane where it could then self-integrate into the membrane and promote cell-cell fusion. Further experiments and controls to confirm these findings and to test such a hypothesis are needed before concrete conclusions can be drawn.

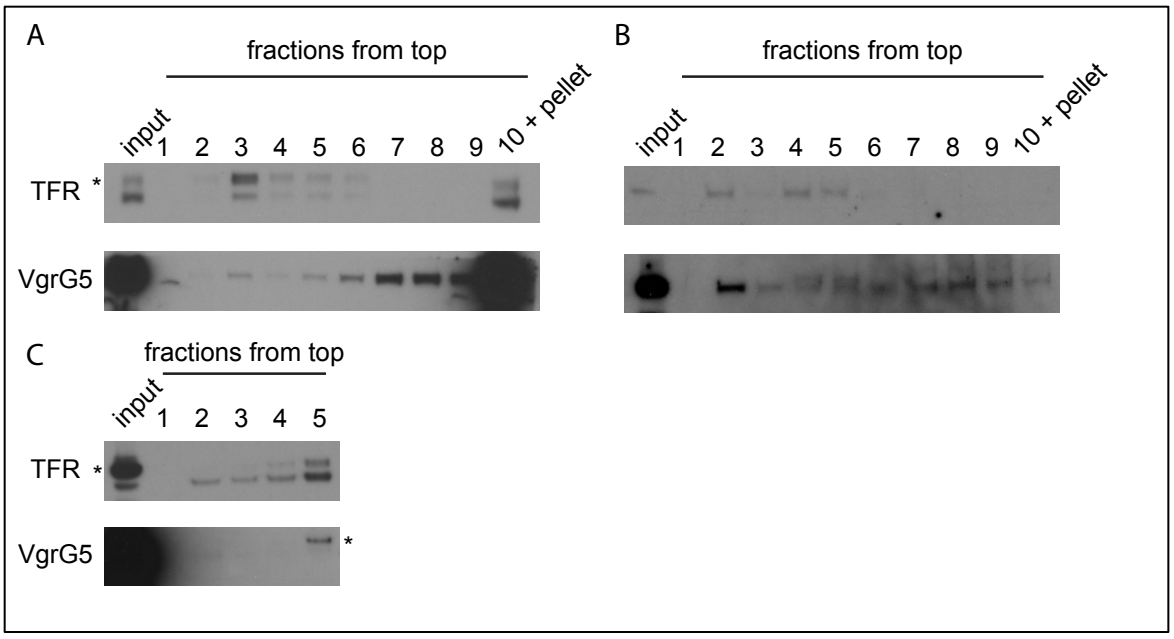

Figure 4.1: VgrG5 comigrates with transferrin receptor in membrane floatation assay. (A) Western blot analysis of floatation assay. Infected cells were lysed by hypotonic shock and douncing, mixed with 70% sucrose to 42.7% sucrose. 750 µl was overlayed with 750 µl 37.6% sucrose and then 250 µl 8.5% sucrose. This was centrifuged for 2-4 hours at 120k xg. 175 µl fractions were collected from the top of the gradient. (B) The same experiment was performed with uninfected host cells mixed with GSH-induced broth-grown *B. thailandensis* before the hypotonic shock. (C) The same experiment was performed with uninfected host cells mixed with GSH-induced broth-grown *B. thailandensis* after the host cells were douced.

Does VgrG5 or a VgrG5-TagD5 complex directly induce cell-cell fusion?

VgrG5 is currently the only candidate fusogen protein for *B. thailandensis* induced cell-cell fusion. In order to establish that a protein or protein complex functions as a fusogen, it is necessary to demonstrate the ability for it to induce membrane fusion or cell-cell fusion in a minimal system (Hernández & Podbilewicz, 2017). As presented in Chapter 3, attempts at overexpressing VgrG5 in host cells by transfection did not result in host cell-cell fusion. Overexpression in host cells by retroviral transduction might be necessary to observe VgrG5-mediate cell-cell fusion, as is necessary for observing cell-cell fusion by overexpression of the myoblast fusogens in tissue culture (Millay et al., 2013; Bi et al., 2017; Quinn et al., 2017; Zhang et al., 2017). However, given the results in Chapter 3 and what we know about how T6SS proteins are secreted, it is unlikely that exogenous expression in host cells will yield cell-cell fusion, even if VgrG5 is the fusogen.

An alternative approach is to test this hypothesis in vitro. This would require developing a membrane fusion assay, possibly using liposomes or a supported lipid bilayer. This would also require purifying VgrG5 or a VgrG5-containing complex from *B. thailandensis* or purified components from *E. coli*. Although I have never attempted to purify full-length VgrG5, it may be difficult as VgrG5 contains predicted transmembrane helices and likely exists as a trimer. Such difficulties should be considered in designing a purification strategy. Developing a membrane fusion assay to test VgrG5's activity as a membrane fusogen would likely require the use of in vitro membrane fusion techniques such as a liposome fusion assay, which determined the minimal machinery for SNARE-mediated membrane fusion (Weber et al., 1998). Although an in vitro approach will be difficult, it is the only way to establish what the fusogen for *B. thailandensis* is, which is key to understanding *B. thailandensis* induced cell-cell fusion.

How does motility contribute to *B. thailandensis* induce cell-cell fusion?

The work presented in this thesis suggests that bacterial motility is important for the pathway leading to *B. thailandensis* induced cell-cell fusion (Chapter 2). We hypothesize that bacterial motility promotes cell-cell fusion through the formation of membrane protrusions that might contribute to key steps in the cell-cell fusion pathway including T6SS-5 secretion activity, VgrG5 localization, membrane apposition, and fusion pore expansion. Interestingly, *B. thailandensis* can induce cell-cell fusion in the absence of intracellular bacterial motility, albeit with a delay and much lower efficiency (French et al., 2011). Therefore, *B. thailandensis* that is deficient in both forms of intracellular motility can be a useful tool to understand this pathway.

To understand the contribution of bacterial motility to the cell-cell fusion pathway, it is important to investigate the pathway leading to cell-cell fusion without bacterial motility. Therefore, we predict that without bacterial motility, membrane protrusions will not form and cell-cell fusion will occur through a different, undescribed pathway. Understanding such a pathway could also reveal new insights into the actin-based motility-mediated cell-cell fusion pathway. Preliminary observations show that, as expected, a mutant that is completely deficient in both flagellar and actin-based motility, *Bt*GFP Δ*bimA*Δ*motA2I*, did not form plasma membrane protrusions (data not shown). When cell-cell fusion did occur, it was difficult to identify the cellular-level mechanism. Sometimes fusion occurred in an area crowded with bacteria and other times it occurred without a bacterium obviously in the vicinity of the initial

membrane fusion (data not shown). In one example, a fusion pore seemed to form and expand from a single bacterium that was near the host cell cortex with no detectable membrane protrusion (Figure 4.2A). Therefore, cell-cell fusion appears to occur through a different pathway in this context, possibly involving the formation of much smaller membrane protrusions or another membrane apposition mechanism that is difficult to observe using live cell imaging by spinning disc confocal microscopy.

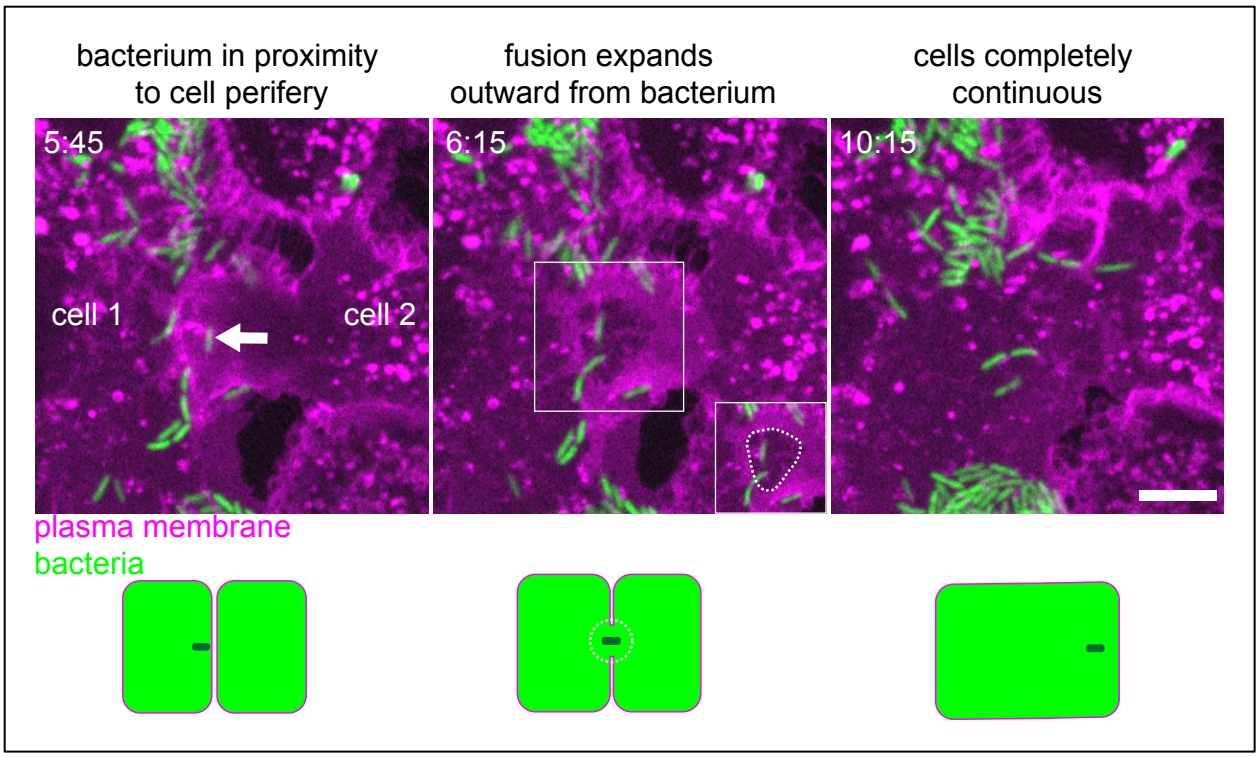

Figure 4.2: Live cell imaging of cell-cell fusion induced by non-motile *B. thailandensis*. Live-cell imaging stills of *Bt*GFP Δ*bimA*Δ*motA2* while inducing cell-cell fusion. A549 cells that expressed the plasma membrane marker TagRFP-T-farnesyl were used. Times represent min:s post start of movie. Images taken at ~24 h post infection. Scale bar is 5 µm. White arrows highlight the bacterium near the site of cell-cell fusion. Inset shows the outline of the fusion pore expanding around the central bacterium. Cartoons show models of how cell-cell fusion occurs in this video and illustrates the region that is highlighted in the inset.

Because *B. thailandensis* can induce cell-cell fusion in the absence of intracellular bacterial motility (French et al., 2011), we know that the fusogen can be localized and functional without bacterial motility, albeit to a severely reduced degree. This presents an opportunity to investigate the contribution of membrane protrusions to this process. We hypothesize that membrane protrusions function to help locally deliver and concentrate VgrG5 in the host plasma membrane while also mediating host plasma membrane apposition. To test whether membrane protrusions function, at least in part, to mediate membrane apposition, we can perform the mixed host cell cell-cell fusion live cell imaging assay with *Bt*GFP Δ*bimA*Δ*motA2*-formed MNGCs coinfected with *Bt*BFP Δ*motA2*Δ*vgrG5*, which can undergo actin-based motility but cannot induce cell-cell fusion. Timing of the infections and imaging would need to be determined because *Bt*GFP Δ*bimA*Δ*motA2* has significantly delayed MNGC formation (French et al., 2011) and it takes several hours for *Bt*BFP Δ*motA2*Δ*vgrG5* to begin undergoing actin-based motility. The *Bt*GFP Δ*bimA*Δ*motA2* infection would likely have to be done first, with the *Bt*BFP Δ*motA2*Δ*vgrG5* done later and then imaged 8-12 h later. This experiment differs from the mixed infection assay presented in Chapter 2 (in which *Bt*BFP WT and *Bt*GFP Δ*vgrG5* were coinfected). In that assay, the *Bt*BFP Δ*motA2*-formed MNGCs result from extensive cell-cell fusion that could dilute the VgrG5 present on the plasma membrane. Therefore, VgrG5 would need to be secreted within the protrusion by the T6SS-5 in order to function. In contrast, MNGCs formed by *Bt*GFP Δ*bimA*Δ*motA2* are small and could increase the likelihood that VgrG5 will be present at the plasma membrane and therefore locate to protrusions formed by *Bt*BFP Δ*motA2*Δ*vgrG5*. We predict that membrane protrusions formed by *Bt*BFP Δ*motA2*Δ*vgrG5* will increase cell-cell fusion in *Bt*GFP Δ*bimA*Δ*motA2*-induced MNGC's. This experiment would help expand upon and possibly reveal new insights into the cellular level understanding of cell-cell fusion by determining whether VgrG5 and bacterial motility can act in trans and whether bacterial motility contributes to cell-cell fusion partially by promoting membrane apposition.

Another approach to understanding how motility contributes to *B. thailandensis*-induced cell-cell fusion would be to investigate the cell-cell fusion pathway of *B. thailandensis* strains that undergo different modes of intracellular motility. The work in Chapter 2 focused on intracellular bacterial motility mediated by the *B. thailandensis* protein BimA which activates the Arp2/3 complex and forms short, curved actin tails (Sitthidet et al., 2010; Benanti et al., 2015). In contrast, *B. mallei* and *B. pseudomallei* BimA orthologs use an Ena/VASP-like mechanism to form longer tails consisting of bundled filaments (M. P. Stevens et al., 2005; Benanti et al., 2015). Because *B. mallei* and *B. pseudomallei* form actin tails with such different morphology compared with *B. thailandensis*, *B. thailandensis* strains engineered to express *B. mallei* or *B. pseudomallei* BimA might undergo different pathways leading to cell-cell fusion. Interestingly, other than *B. mallei* and *B. pseudomallei*, *R. parkeri* is the only other bacterial pathogen that does not undergo Arp2/3 mediated actin-based motility during spread (Lamason & Welch, 2017; Dowd et al., 2021) and also does not form long membrane protrusions during spread (Lamason et al., 2016). Therefore, *B. thailandensis* strains engineered to express *B. mallei* or *B. pseudomallei* BimA might form protrusions with different morphology from *B. thailandensis* BimA-mediated actin tails. Understanding the pathway that these strains use to induce cell-cell fusion could reveal how actin tail shape or protrusion morphology contribute to the cell-cell fusion pathway.

While the work in this thesis focused on bacteria only capable of undergoing actin-based motility, *B. thailandensis* also undergoes flagellar-based motility (French et al., 2011).

Interestingly, *B. thailandensis* deficient in actin-based motility but capable of undergoing intracellular flagellar-based motility cause cell-cell fusion but do not form membrane protrusions (French et al., 2011). Understanding the pathway that the Δ*bimA* strain uses to induce cell-cell fusion could reveal how cell-cell fusion can occur efficiently without membrane protrusions.

Long-term impact

Membrane fusion is a fundamental element of life in all organisms. Understanding the molecular-level mechanism of how membranes fuse is challenging and there are several membrane fusion processes that remain poorly understood. Investigating how pathogens manipulate this process is an important approach that will yield unique insights into these mechanisms. In particular, very little is known about *B. thailandensis*-induced cell-cell fusion, a process that is not described for other bacteria and that does not appear to use proteins homologous to known fusogens. The future directions presented in this thesis are important next steps in pursuit of understanding the molecular mechanism of cell-cell fusion and membrane fusion.

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