Identifying Human Host Cell Protein Targets of the *Bartonella* Effector Protein (Bep) Fic Domains

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Abstract

The genus *Bartonellae* represents an increasing number of emerging bacterial pathogens that utilises an unusual infection strategy, parasitising the red blood cells of their mammalian host. The most common species to infect humans are *B. henselae* and *B. quintana*. *B. henselae* is transmitted between cats by the cat flea, although occasionally infects humans via cat scratches or bites, causing cat-scratch disease (CSD). CSD is characterised by enlarged tender lymph nodes and fever. *B. henselae* also infects the endothelial cells of both its hosts; likely a factor in disease progression. *B. quintana*, the cause of trench fever during WWI, is spread between people by the body louse. Trench fever is characterised by relapsing fever, headache, and bone pain. *B. quintana* is also able to infect human endothelial cells. These bacteria secrete a range of *Bartonella* effector proteins (Beps) via a Type IV secretion system, directly into endothelial cells, subverting host cell processes and resulting in internalisation of the bacteria.

Beps have a range of functions, many of which are not fully characterised. *B. henselae* secretes three Beps (BepA-C) that contain a filamentation induced by cAMP (Fic) domain and a *Bartonella* Intracellular Delivery (BID) domain, with BepA being the best studied. BepA's BID domain is responsible for intracellular delivery as well as inhibition of apoptosis by the host cell, however the exact function of the Fic domain remains unknown. Fic-containing bacterial toxins catalyse the transfer of an AMP moiety from ATP onto a host cell protein. This AMPylation frequently inactivates these proteins resulting in disrupted host cell processes and cytotoxicity. BepA has previously been shown to possess AMPylation activity, although the host target protein(s) are unknown. Evidence suggests that these proteins are novel targets.

The aim of this study was to create protein constructs containing these Fic domains, and to develop techniques to identify the host cell target proteins post AMPylation. To this end, both a fluorescent ATP analogue and a fluorescent click chemistry based approach were utilised. While no target protein was identified, a basic methodology was developed for protein production and target protein identification that could be further developed.

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Abbreviations

ACN – Acetonitrile

APS – Ammonium persulfate

ATCC – American Type Culture Collection

Bep – Bartonella Effector Protein

BID – Bartonella Intracellular Delivery

CTAB – cetyltrimethylammonium bromide

CSD – Cat-Scratch Disease

ddH₂O – Double distilled water

DMSO – Dimethyl sulfoxide

dNTP – Deoxynucleoside triphosphate

DTT – Dithioreitol

EDTA - Ethylenediaminetetraacetic acid

F1-ATP - N6-(6-amino)-hexyl-ATP-5-FAM

FAM – Fluorescein amidite

FCS – Fetal Calf Serum

Fic – Filamentation induced by cyclic AMP

FITC – Fluorescein isothiocyanate

GFP – Green fluorescent protein

HEPES – N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HUVEC – Human Umbilical Vein Endothelial Cell

IPTG – Isopropyl-1-thio-β-D-galactoside

LC-MS/MS – Liquid Chromatography-Mass Spectrometry/Mass Spectrometry

MES – 2-(N-morpholino)ethanesulfonic acid

N⁶pATP - N6-Propargyl-Adenosine-5'-triphosphate

NCBI – National Centre for Biotechnology Information

OD₆₀₀ – Optical Density at 600 nm

PAGE – PolyAcrylamide Gel Electrophoresis

Penstrep – Penicillin Streptomycin

PCR – Polymerase Chain Reaction

RIPA – RadioImmunoPrecipitation Assay

SDS – Sodium dodecyl sulfate

T4SS – Type IV Secretion System

TCA – Trichloroacetic acid

TBS – Tris-buffered saline

TBS-T – TBS-Tween

TE – Tris/EDTA

TEMED – N,N,N',N'-tetramethylethylenediamine

THPTA – Tris(3-hydroxypropyltriazolylmethyl)amine

Tris - Tris(hydroxymethyl)aminomethane

1 Introduction

1.1 *Bartonella* Species and Associated Disease

1.1.1 *Bartonella*

Newly emerging or re-emerging infectious diseases are a significant global health concern. A recent study found that, of pathogens that have been newly recognised since 1940, 60% are associated with zoonotic diseases, and 54% are of a bacterial or rickettsial origin¹. The *Bartonellae* represent an increasing number of emerging zoonotic bacterial pathogens. Prior to 1990, only two *Bartonella* species and their associated disease were known: *Rochalimae quintana*, the causative agent of Trench Fever during World War I and II²⁻⁴, and *B. bacilliformis*, the causative agent of Carrión's disease in Peru^{2,3,5,6}. *Bartonella* species have since reappeared as significant opportunistic infections during the AIDS epidemic, and have been subsequently identified and characterised. Through analysis of 16S ribosomal DNA sequences, the causative agent of cat-scratch disease (CSD) was found to be highly related to *R. quintana*⁷. This unknown organism was later identified as *R. henselae*⁸. Following this discovery, the genus *Rochalimaea* was unified with *Bartonellae*⁹ and has subsequently grown rapidly to 30 – 40 currently recognised species of *Bartonella*^{3,10}, at least seventeen of which have been reported as human pathogens¹¹.

The genus *Bartonellae* consists of fastidious, Gram-negative, rod-shaped bacteria¹²⁻¹⁴, that behave as facultative intracellular pathogens¹⁵. With the exception of *B. bacilliformis*, most *Bartonellae* are both microaerobic and capnophilic^{3,16}, requiring high CO₂ conditions for growth. *Bartonella* species utilise a relatively unique infection strategy whereby they alternate between mammalian and arthropod hosts, invading their mammalian host's erythrocytes and establishing a persistent infection^{10,12,15,17}. This ability to establish an intraerythrocytic infection defines the mammalian host as a reservoir host, and infection typically results in asymptomatic bacteraemia^{3,15}. Incidental hosts are those that are infected by a *Bartonellae* species from a different reservoir (e.g. CSD in humans). Intraerythrocytic infection does not occur in incidental hosts, and the result may be either a self-limiting or a chronic infection^{11,15,17,18}.

B. bacilliformis was the first described member of the genus and is spread through the bite of the sand fly, *Lutzomyia verrucarum*, found in Peru, Columbia, and Ecuador^{2,3,5,6,13,17}. The geographical restriction of the sand fly is believed to be the reason for the localised, endemic nature of *B. bacilliformis* infections². Infection can result in a life threatening haemolytic anaemia known as Oroya fever, which has a 40 – 90% mortality rate if left untreated, and occurs during the acute phase of the infection^{3,15}. In the chronic stages of the infection multiple vasoproliferative skin lesions, the verruga peruana, are formed^{2,6,15}. These lesions are characterised by lobular proliferations of atypical endothelial cells². This chronic phase of infection is associated with a much lower mortality than the acute phase¹⁵. Humans are the only known reservoir host^{2,15}.

Bartonellosis, a term originally used to describe this disease, now includes a range of human diseases associated with *Bartonella* infection; most of which have a much lower severity than Oroya fever^{2,3}. Bartonellosis conditions can involve multiple combinations of bacteraemia, low-grade fever, malaise, culture-negative endocarditis, vascular lesions of the skin, and or liver, as well as lymphadenopathy, although these conditions usually only occur in immunocompromised individuals³.

Similarly, *Bartonella* infections have been linked to endocarditis in both cats and dogs including cases reported from both New Zealand and Australia¹⁴. While infections are usually self-limiting, there have been more severe clinical manifestations associated with *Bartonella* infection including; encephalitis, neuroretinitis, anterior uveitis, haemolytic anaemia thrombocytopenia, glomerulonephritis, pneumonia, and osteomyelitis^{2,19}. Bacteraemia is also observed in many mammalian species infected with *Bartonella*, and as sensitivity of diagnostic methods improves, there is increasing evidence that many immunocompetent individuals are chronically infected with *Bartonella* as incidental hosts^{5,11,15,19-22}. People with occupational exposure to animals or biting arthropods, including veterinarians, avid hunters, or wildlife biologists, appear to be most at risk of chronic cryptic *Bartonella* infections. Currently, blood donors are not routinely screened for *Bartonella*, and studies have shown that the bacteria can survive the storage conditions for erythrocytes^{23,24}. The incidence of *Bartonella* infection acquired through blood transfusion is not known. The three most common pathogenic strains that infect humans are *B. bacilliformis*, *B. quintana*, and *B. henselae*^{2,15} with *B. henselae* being the most prevalent¹⁵.

1.1.2 *Bartonella quintana*

B. quintana was first identified as a widespread pathogen among soldiers during World War I, causing a disease that became known as trench fever^{2,3,6,13,15}. Trench fever is estimated to have affected over one million Allied soldiers on the Western Front with an average weekly loss of 0.6% army strength in France, from evacuation due to sickness²⁵. However, *B. quintana* infections from long before this have been documented, as Drancourt et al. (2005) identified *B. quintana* DNA from a human tooth dating back at least 4000 years. Trench fever, also known as five-day fever⁴, is characterised by relapsing peaks of fever, headache, and bone pain¹⁵, particularly in the long bones of the legs^{2,3,13}. The incubation period for the disease is between 15 – 25 days and symptoms typically range from mild to severe, although some individuals may be asymptomatic^{3,4,13}. Infections are accompanied by consistent bacteraemia and, though most infections are usually self-limiting, 5-10% of sufferers may remain infectious for months to years after the initial infection^{2,13,15}. In immunocompromised individuals, *B. quintana* can also cause bacillary angiomatosis as well as endocarditis, an infection of the heart valves^{2,4,13,15}. Bacillary angiomatosis describes the outgrowth of multiple vasoproliferative tumours that can be found on the skin and on other organs of the body, and are similar to the verruga peruana lesions associated with *B. bacilliformis*^{13,15}. These skin lesions usually present as pink to deep red or purple papules or nodules^{2,13}. After reappearing during World War II, the incidence of trench fever decreased below detectable levels, before re-emerging with the AIDS epidemic as urban trench fever^{2,15}. This condition is associated with homelessness, alcoholism, and other conditions of poor hygiene^{2,15}. *Bartonella quintana* is mainly spread by the body louse, *Pediculus humanus corporis*, although other vectors have been reported^{2,15}. Individuals who wear unwashed clothing for long periods are susceptible to louse infestation and therefore infection by *Bartonella quintana*. Like *B. bacilliformis*, humans are the main reservoir^{4,6}.

1.1.3 *Bartonella henselae*

Cat scratch fever, later renamed cat scratch disease (CSD), was first described in 1950, but was thought to be caused by a viral agent, as no bacteria could be observed or cultured²⁷. *Bartonella henselae* was later discovered and isolated from immunocompromised patients in 1990, through the use of PCR⁷ and blood cultures²⁸. It wasn't identified however, until it was re-isolated from the blood of an HIV positive patient in 1992⁸. The reservoir host for *B. henselae* is the cat, either domestic or feral, and humans are an incidental host^{2,13,15}. Humans are typically infected by cat scratches or bites, resulting in a mild, self-limiting illness known as CSD. The infection is transmitted between cats via an arthropod vector, the cat flea (*Ctenocephalides felis*)^{2,13}. There is however, no conclusive evidence to suggest flea bites can transmit the bacteria to humans². Studies in New Zealand have shown that 11 – 17% of cats in a given region may be infected^{29,30}. Evidence has also shown that *B. henselae* infects feline erythrocytes, however there is controversy as to whether it naturally infects human erythrocytes like *B. bacilliformis* and *B. quintana*^{31,32}.

CSD is characterised mainly by lymphadenopathy, enlarged tender lymph nodes, and is usually accompanied by fever^{2,3,5,15}. In immunocompromised people, complications from *B. henselae* can include bacillary angiomatosis, endocarditis, and bacillary peliosis. Bacillary peliosis describes the formation of lesions on the liver or spleen and is very similar to bacillary angiomatosis. One study in New Zealand found 5% of blood donors tested were seropositive for *B. henselae*³³. There is however, increasing evidence that many bacteraemic individuals have negative serology results for *Bartonella* species^{5,11,21,34,35}. This has been discovered following the development of improved tests for *Bartonella*, such as PCR analysis, and BAPGM (*Bartonella* alpha Proteobacteria growth medium) to allow for enrichment culture of *Bartonella* from bacteraemic individuals^{11,21,34,36}. This challenges the notion that bacteraemia is rare in immunocompetent individuals, and suggests that the prevalence of *B. henselae* bacteraemia is much higher than expected, particularly in those working closely with animals^{5,11,19-22}. There is also increasing evidence that many of these individuals are co-infected chronically with multiple species of *Bartonella*^{5,11,21,34,36}. This has the potential to allow horizontal gene transfer between different *Bartonella* species to occur³⁷.

1.1.4 The *Bartonella* Genome

Bartonellae species share similarities between their genomes, such as low GC count (38%), and similar numbers of tRNA and rRNA molecules³⁸. There are also however, some major differences even between closely related species. Historically, the genus *Bartonellae* has been divided into 4 major lineages^{15,37,38}. Lineage 1 describes an ancestral lineage and is the most diverged from the remaining lineages. This lineage contains only 1 species, *B. bacilliformis*, which contains the smallest genome of all the currently sequenced *Bartonella* species (1.4 Mbp)³⁸. Conversely, Lineage 4 is very species rich and contains the most diverse group of species of any of the lineages³⁸. Recently, this lineage model has been challenged, as new sequence data and phylogeny analysis have found that *B. australis*, a species of *Bartonellae* that infects kangaroos, appears to have diverged earlier than *B. bacilliformis*, which clustered with the ruminant-infecting *Bartonella* that make up Lineage 2³⁷. It is proposed that, rather than being an ancestral strain, *B. bacilliformis* arose from reductive genomic evolution following a host switch. Based on these phylogenetic analyses, three *Bartonellae* groups were proposed: Group A, consisting of the Lineage 2 *Bartonellae*, Group B consisting of the Lineage 3 species, and Group C, consisting of Lineage 4 species³⁷. *B. australis* has not currently been assigned a lineage or group.

Both *B. henselae* and *B. quintana* are members of Group C and genomic studies have shown they are very closely related^{38,39}. In spite of this, the genomes of these two species differ in size. *B. henselae* has one of the largest *Bartonella* genomes currently sequenced (1.9 Mbp), while *B. quintana* has a much smaller genome (1.5 Mbp)^{38,39}. Interestingly, *B. quintana* has 67 pseudogenes as well as many gene remnants that are still expressed in *B. henselae*, whereas *B. quintana* has 6 functional homologues of *B. henselae* pseudogenes. *B. henselae* also contains a prophage region that encodes 62% of the genes not expressed in *B. quintana*, which only has gene remnants of this prophage, suggesting it was lost during *B. quintana* evolution. The differences in the genomes of the two species have led researchers to believe that the *B. quintana* genome is derived from a larger ancestral *B. henselae*-like genome by reductive genomic evolution³⁹.

1.1.5 *Bartonella* Lifecycle

Bartonella species typically alternate between a warm-blooded host and an arthropod vector. When an infected haematophagous arthropod takes a blood meal, *Bartonellae* are shed in the faeces and are inoculated when the host scratches the bacteria into the skin¹⁷. This results in bacterial infection of the dermal niche, with evidence suggesting dermal dendritic cells (DCs) are infected⁴⁰. Following dermal niche colonisation, it is thought that the bacteria induce infected DCs to migrate to the bloodstream, where *Bartonella* subsequently colonise the blood seeding, or primary, niche. This blood seeding niche is hypothesised to be endothelial cells or mononucleated cells of the reticulo-endothelial system, although this is still heavily debated^{15,17,40,41}. Five days after infection, the bacteria escape from the blood seeding niche and enter the bloodstream, where they invade the reservoir host's erythrocytes^{15,17}. The bacteria are repeatedly, synchronously, released from the blood seeding niche every few days giving rise to a recurring fever¹⁷. The infection of erythrocytes involves three main stages: adhesion, invasion, and intracellular persistence¹⁵. *Bartonellae* replicate inside erythrocytes until a critical population density is reached¹⁷. At this point, the number of bacteria remains static for the remainder of the erythrocyte's lifespan and these infected erythrocytes function essentially normally¹⁷. This infection of erythrocytes enables the bacteria to persist in an immunologically privileged site¹², and promotes transmission by the haematophagous arthropod vector¹⁷ (Figure 1.1). *B. bacilliformis* is an exception to this strategy, as it lyses erythrocytes after invasion, causing the potentially lethal haemolytic anaemia associated with Oroya fever^{2,15,17}.

Figure 1.1: Infection strategy commonly employed by the bartonellae. (A) When an infected haematophagous arthropod takes a blood meal it inoculates the host, resulting in colonisation of the dermal niche. (B) In the dermal niche, bacteria are taken up by dendritic cells. (C) *Bartonella* are transported by dendritic cells to the blood-seeding niche. (D) Bacteria escape from the blood-seeding niche and enter the bloodstream where they colonise the host's erythrocytes and re-infect the blood-seeding niche. (E) *Bartonella* persist and replicate inside the host erythrocytes until a critical population is reached (F). (G) Infected erythrocytes are taken up by a haematophagous arthropod for further transmission. Reproduced with permission from The American Society for Microbiology. Harms & Dehio (2012)¹⁵.

1.1.6 *Bartonella* and Endothelial Cells

Bartonella species are able to colonise endothelial cells resulting in vasoproliferative tumour growth, particularly in immunocompromised patients⁴¹. This is believed to be a crucial step in the formation of verruga peruana, bacillary angiomatosis, and bacillary peliosis syndromes. Experiments using both live bacteria and cell free extracts from the three *Bartonella* species detailed above stimulated the proliferation of human umbilical vein endothelial cells (HUVECs) *in vitro*⁴¹⁻⁴³. In addition, *B. quintana* and *B. henselae* stimulated migration of HUVECs through a porous membrane, while *B. bacilliformis* was angiogenic *in vivo* in a rat model^{41,42}.

Experiments with *B. bacilliformis* have shown that activation of the host Rho GTPase is involved in internalisation of the bacteria⁴⁴, and it is believed that both *B. quintana* and *B. henselae* utilise a similar mechanism⁴¹. This activation results in the formation of actin stress fibres, which are associated with altered cell morphology, impaired migration, and defects in cell morphogenesis in HUVEC primary cells^{41,45}. The invasion process is actin-dependent, occurs in a range of hours, and results in membrane bound clusters of bacteria⁴¹ reminiscent of bacterium-directed phagocytosis described in other bacterial species⁴⁶⁻⁵⁰.

In addition to this mechanism, *B. henselae* uses a second method for internalisation. The bacteria form aggregates on the endothelial cell surface that contain hundreds of bacteria. These aggregates are engulfed by plasma membrane-derived protrusions, eventually resulting in their internalisation in a host structure: the invasome^{41,51}. In *Bartonella henselae*, invasome formation is dependent on host cell proteins including integrin β 1 receptor on the cell surface and talin-1 activation inside the cell⁵². Talin-1 is required to bind integrin β -1 and to stabilise it in an open conformation allowing *B. henselae* to translocate effector proteins (discussed below) into the endothelial cells.

1.2 *Bartonella* Virulence Factors

Bartonella species possess a wide range of virulence factors. These include trimeric autotransporter adhesins, such as *Bartonella* adhesion A (BadA) in *B. henselae*, or the four variable outer membrane proteins (Vomps) in *B. quintana*, which mediate adhesion to the host cell extracellular matrix^{53,54}. BadA has been reported to be responsible for activation of hypoxia inducible factor 1 (HIF-1), and vascular endothelial growth factor (VEGF) secretion in macrophages and epithelial cells⁵⁵. *B. henselae* also expresses filamentous hemagglutinins which mediate adhesion in other gram-negative bacteria, but whose function is not known in *B. henselae*. Many *Bartonella* species also possess flagella which appears to have been lost in Group C^{15,37,56}.

B. quintana and *B. henselae* also encode two different type IV secretion systems (T4SSs). The first is the Trw T4SS, which forms surface pilus structures and has been shown to play a role in the colonisation of host erythrocytes^{15,56}. The Trw T4SS, as the bacterial factor that enables erythrocyte infection, also determines host specificity, as *Bartonella* are only able to invade erythrocytes from their reservoir host. Identification of Trw as a key component in host-specific adhesion for erythrocytes has enabled the identification of its target, band3, a major outer membrane glycoprotein of erythrocytes⁵⁷. Only *Bartonella* from Group C possess the Trw T4SS, which appears to be correlated with a loss of flagella in these *Bartonella* species^{15,37,53,56}. The other critical T4SS of *Bartonella* is the VirB/D4 system, which secretes a suite of *Bartonella* effector proteins (Beps) directly into the host cell¹⁵. This system is possessed by *Bartonella* species from both Groups B and C, with Group B containing multiple copies^{15,37}.

Experiments using the rodent-adapted *B. tribocorum* have demonstrated the importance of these T4SSs, with mutations in either the VirB4, VirD4, or Trw proteins resulting in complete loss of erythrocyte infection in a rat model^{58,59}.

1.2.1 VirB/D4 T4SS and Secreted Effector Proteins

T4SSs are macromolecular machineries that inject bacterial DNA or proteins directly into a host cell and are widely expressed in bacterial pathogens, having evolved from conjugation machinery¹⁵. They form a translocation channel that spans the inner and outer membranes of *Bartonella* and the host cell cytoplasmic membrane¹⁵. T4SSs typically exert their pathogenesis through the secretion of effector proteins directly into the host cell by a poorly understood mechanism.

The VirB/D4 T4SS is found in both Group B and Group C species of *Bartonella*, including *B. henselae* and *B. quintana*^{15,17,37}. It has been proposed that the VirB/D4 genes were originally acquired by two independent horizontal gene transfers, giving rise to the observed differences between Group B and Group C *Bartonella* species³⁷. However, gene transfers still frequently occur in this region between different *Bartonella* species, particularly during co-infection. Evidence for this is found in the *B. vinsonii* strain Winnie, isolated from a dog that had been co-infected with *B. henselae*. Phylogenetic analysis found that the VirB genes (*virB2* – *virB8*) in this strain of *B. vinsonii*, a dog-associated *Bartonella* species, were more closely related to *B. henselae* than *B. vinsonii*. Conversely, the VirB genes (*virB9-virD4*) were more closely related to those of *B. vinsonii* VirB/D4 genes³⁷. This provides strong evidence that during co-infection a gene transfer event occurred. In contrast, the Trw secretion system is believed to have been acquired through a single horizontal gene transfer³⁷.

The VirB/D4 T4SS contributes to virulence through its ability to translocate Beps directly into the host cell^{15,60}. The Houston-1 strain of *B. henselae*⁸ elicits many VirB/D4-dependent phenotypes in endothelial cells, suggesting that the VirB/D4 T4SS is an important factor in the manipulation of endothelial cells during infection¹⁵. These phenotypes include activation of NF- κ B and the pro-inflammatory response, inhibition of apoptosis, and capillary-like sprout formation of endothelial cell aggregates^{3,51,53,61-63}. This system is also important in mediating rearrangements in the host cell actin cytoskeleton, which results in internalisation of bacterial aggregates, formed on the cell surface, in the invasome^{15,63}.

Evidence has emerged that suggests this system is also able to translocate a *Bartonella* plasmid into a human cell, however the significance of this during infection is not known⁶⁴.

1.3 *Bartonella* VirB/D4 Secreted Effector Proteins

1.3.1 Bep Protein Structure

Both *B. henselae* and *B. quintana* contain a pathogenicity island that encodes the highly conserved VirB/D4 T4SS, as well as the secreted Bep proteins⁶⁵. These effector proteins display a modular structure with an N-terminal host interacting domain, and a C-terminal bipartite secretion signal. This bipartite signal consists of a roughly 142 amino acid Bep intercellular delivery (BID) domain followed by a non-conserved tail sequence with a net positive charge^{3,15,65}. Three of the *Bartonella henselae* Bep proteins contain more than one BID domain (BepE – G), although it is the most C-terminal BID domain that is involved in translocation^{51,62}. This suggests that the remaining BID domains may have functions other than translocation. The Bep proteins have a high degree of plasticity and are not completely conserved between the *Bartonella* species⁶⁵.

Several of the Bep proteins found in both *B. henselae* and *B. quintana* contain an N-terminal filamentation induced by cAMP (Fic) domain¹⁵. This domain is usually followed by a small oligonucleotide/oligosaccharide binding fold and finally the secretion signal. *B. henselae*'s pathogenicity island encodes 7 Bep proteins (BepA – G), three of which (BepA – C) are homologues and contain a Fic domain^{15,66}. In contrast, BepD – F contain multiple N-terminal motifs for tyrosine phosphorylation. These motifs are characterised by the amino acid sequence Glu-Pro-Ile-Tyr-Ala (EPIYA) and have been shown to undergo tyrosine phosphorylation by endogenous host cell kinases. EPIYA motifs are believed to interact with SH2 domain-containing host cell proteins which are specific for phosphorylated tyrosine⁶⁷. An example of this is BepF, which has been demonstrated to interact with the SH2 domain-containing signalling and adaptor proteins Grb2, RasGAP, and Crk^{51,65}. BepG differs from other Bep proteins, as it does not contain either of these N-terminal motifs, but instead consists of four BID domains with short repeat sequences interspersed⁶² (Figure 1.2).

Figure 1.2: *Bartonella henselae* Bep protein structure. Fic domains are indicated in blue, BID domains are indicated in brown. Yellow boxes represent tyrosine-containing sequence motifs (indicated by Y). Based off figure used by Schulein et al. (2005).

While *B. henselae* Bep proteins are relatively well characterised, little is known about the function of the *B. quintana* Bep homologues. Interestingly, *B. quintana* also contains an gene similar to *yopJ* in *Yersinia pestis* (*yopP* in *Y. enterocolitica*) that is not found in *B. henselae*³⁹. *Yersinia* YopJ is a Type III secreted effector protein that catalyses acetylation reactions on conserved regions in the activation loop of MAP kinase signalling proteins. This prevents activation of these signalling proteins by phosphorylation and ultimately induces macrophage cell death^{68,69}. This suggests that YopJ interferes with the innate immune response, as it has also been shown to inhibit NF- κ B signalling^{68,69}. However, no function has been described for the *B. quintana* homologue.

1.3.2 Bep Protein Functions

The effector proteins BepA – G are all secreted by the VirB/D4 T4SS which together are responsible for all of the VirB/D4-dependent cellular phenotypes of *B. henselae* infected endothelial cells described above. While not all the functions of the Bep proteins are known, there have been some characterised effects. For example, either a combination of BepC and BepF, or BepG independently, are responsible for invasome formation^{3,51}. It is believed that these two protein systems represent a form of functional redundancy, although they have been shown to act via different pathways⁵¹. These protein systems are also responsible for inhibiting endocytic uptake of bacteria to enable the invasome process, as this is the slower mechanism for bacterial internalisation.

It is hypothesised that BepC and BepF may act at an intermediate level of the signalling cascade that leads to invasome formation⁵¹, while BepG is believed to interact further down the cascade. Interestingly, BepG also appears to have strong anti-angiogenic effects, while BepD has relatively moderate pro-angiogenic effects⁶¹. Whether this is a direct or indirect effect is not known. Recently BepE has been shown to inhibit the deleterious effects of BepC, which can cause host cell fragmentation in the absence of BepE⁴⁰. BepE exerts this effect by interacting with the host cell Rho signalling pathway via its second BID domain, resulting in the stabilisation of stress fibres. Loss of BepE in *Bartonella tribocorum* results in a strain unable to establish bloodstream infection in a rat model using intradermal inoculation⁴⁰. BepE also appears to be important in establishing colonisation of the dermal niche. The function of BepB currently remains unknown.

1.3.3 BepA

BepA from *B. henselae* is one of the best studied Beps and contains both a Fic domain and a BID domain^{60,66}. The BID domain of BepA has previously been shown to inhibit apoptosis by directly binding host adenylyl cyclase in the presence of the G α subunit to increase cAMP levels^{60,70}. The BID domain of BepA from *B. quintana* also demonstrated this effect. The *bepA* gene in *B. quintana* was originally annotated as a pseudogene^{39,60}. However, it was noted that the gene encoded an internal stop and start codon in frame splitting the gene into two open reading frames, BepA1 and BepA2. The first open reading frame encodes the Fic domain and the second encodes the BID domain and C-terminal tail⁶⁰. It has not been determined whether the Fic domain of *B. quintana* BepA is also functional. Interestingly, the BID domain of *Bartonella tribocorum*'s BepA did not demonstrate this anti-apoptotic effect. BepA from *Bartonella henselae* has also been shown to have a strong pro-angiogenic effect⁶¹; however, the exact function of the BepA Fic domain remains unknown.

1.4 The Fic Domain

Bioinformatic analyses reveal that over 2000 species from all forms of life encode a Fic domain. The catalytic function of this domain was discovered by Yarbrough et al. (2009), who described its ability to covalently modify protein side chains with an AMP moiety, typically on a serine, threonine or tyrosine residue. This reaction is known as adenylation or AMPylation. AMPylation was first described in 1970 as an enzymatic reaction carried out by an adenylyl transferase enzyme in *E. coli* to regulate the activity of an endogenous enzyme, glutamine synthetase^{72,73}.

This post translational modification is reversible, as indicated by deadenylation of glutamine synthetase by another *E. coli* enzyme. Only recently has the role of AMPylation in bacterial toxicity and pathogenicity been discovered^{66,71,74-76}.

1.4.1 *Vibrio parahaemolyticus* and VopS

One of the first proteins where Fic domain activity in pathogenicity was analysed is VopS, a protein delivered into the host cell by the *Vibrio parahaemolyticus* Type III secretion system^{71,74}. When expressed in HeLa cells, this protein induced cell rounding, an indication of cytotoxicity. Yarbrough et al. (2009) demonstrated that when a constitutively activated Rac protein was co-expressed with VopS, the Rac protein mass increased by 329 daltons, consistent with addition of an AMP moiety. Further analysis revealed that an AMP moiety was added to Thr35, a highly conserved residue located in the effector loop of the switch I region of the Rac protein. VopS specificity was demonstrated by substituting Thr35 with an Ala residue, which abrogated the AMPylation reaction. Further experiments showed that VopS catalysed AMPylation reactions on other members of the Rho-GTPase family, namely Rho and Cdc42⁷¹. The transfer of AMP results in steric hindrance and prevents activated Rho GTPase proteins from binding downstream signalling partners, interrupting signalling and leading to collapse of the actin cytoskeleton and cell rounding⁷¹. VopS has also been demonstrated to have *in vitro* auto-AMPylation activity⁷⁷. Other Fic domain-containing proteins, such as the fruit fly protein CG9523 from *Drosophila melanogaster*, also have *in vitro* auto-AMPylation activity^{66,77}.

1.4.2 *Histophilus somni* and IbpA

Another key bacterial cytotoxin, IbpA, contains two Fic domains, and is secreted by the cattle pathogen *Histophilus somni*⁷⁵. Like VopS, this protein also catalyses an AMPylation reaction on the Rho family of GTPases, preventing binding to their downstream signalling partners^{71,75}. When expressed in HeLa cells, IbpA caused plasma membrane retraction and actin cytoskeleton collapse, and the Fic2 domain was shown to be sufficient for this effect. The Fic2 domain of IbpA, like the Fic domain of VopS, catalyses the transfer of an AMP to the switch I region of the GTPases. However, in the case of IbpA, the AMP is transferred to a conserved Tyr residue rather than the Thr residue found in Rac^{71,75}. The AMPylation reaction was specific for the active form of the GTPases as no activity was seen with dominant negative mutants of the GTPases.

1.4.3 Fic Domain Motif

For both VopS and IbpA, the function of the Fic domain is dependent on a conserved histidine residue in the Fic domain's core motif, HPFxxGNR^{71,74,75}. Mutants constructed with an Ala substitution for the His residue resulted in a complete loss of function as all mutants failed to induce AMPylation on their targets^{66,71,74,75}. This core motif is conserved in *B. henselae* BepA⁶⁶ as well as BepC from both *B. henselae* and *B. quintana*. In the case of *B. henselae* BepB, the last two amino acids differ from the motif (GR → EH); in the case of *B. quintana* BepA1, the last glycine is substituted with a glutamate. However, in all instances the invariant histidine remains. This core motif is also found in death on curing (Doc) domain proteins, and it is believed that the Doc domain evolved from Fic domains⁷⁷. For this reason, Fic and Doc proteins belong to the same protein superfamily, the Fido superfamily⁷⁷. Interestingly, the Fic motif of IbpA showed a high sequence similarity to a human protein HYPE, which also weakly AMPylates Rho GTPases *in vitro*⁷⁵. Recently, it was revealed that HYPE localises to the ER lumen where it plays a critical role in the unfolded protein response⁷⁸. HYPE acts to AMPylate the molecular chaperone BiP at Ser365 and Thr366 in the ATPase binding domain. This results in increased BiP activity for protein refolding⁷⁸, and demonstrates a novel function for Fic domain-containing proteins.

1.4.4 Catalytic Mechanisms

Orth et al. (2010) have obtained a crystal structure of VopS and from this have proposed a catalytic mechanism. The Fic motif residues His348, Phe350, Asp352, Asn354, and Arg356, as well as the distal Arg299, are critical for full catalytic activity. In particular, the mutations H348A and R356A had the most diminished function of any of the mutations in these critical amino acids⁷⁹. As shown in Figure 1.3 below, the histidine residue acts as a base in the reaction to activate the threonine residue of the Cdc42 protein. This is supported by the fact that the VopS enzyme is less active at a pH below 6.0, the pKa of histidine⁷⁹. Kinetic results have shown that both Asn354 and Arg356 are involved in binding the ATP substrate. Asp352 binds ATP indirectly via its bound Mg^{2+} ion. Finally, Arg299 stabilises the binding of the target protein, Cdc42 (not shown).

Figure 1.3: Proposed mechanism of VopS catalysed AMPylation of Cdc42. The diagram shows the stabilisation of the phosphate groups by the VopS active site amino acids. The Cdc42 threonine is activated by the critical histidine in the VopS active site resulting in nucleophilic attack on the primary phosphate group. Diagram based off catalytic mechanism illustrated by Orth et al. (2010)

Similarly, Dixon et al. (2010) have published the crystal structure for the IbpA Fic2 domain bound to Cdc42. They propose a similar catalytic mechanism to that illustrated in Figure 1.3. The invariant histidine residue is thought to act as a base and interact with the proton of the Cdc42 tyrosine hydroxyl group⁸⁰. This interaction increases the nucleophilicity of the tyrosine hydroxyl group, which attacks the α -phosphate of the bound ATP (Figure 1.4).

Figure 1.4: Proposed mechanism of IbpA-Fic2 catalysed AMPylation of Cdc42. The diagram shows the activation of the Cdc42 tyrosine by the critical histidine in the IbpA-Fic2 activation site. This results in nucleophilic attack on the α-phosphate by the activated tyrosine. Diagram based off catalytic mechanism illustrated by Dixon et al. (2010)

1.4.5 Toxin/Antitoxin Modules

Fic domain-containing proteins have also been identified in toxin/anti-toxin modules, which play a role in maintenance of bacterial plasmids⁸¹. Toxin/anti-toxin systems include a stable protein (toxin), which exerts a toxic effect on the plasmid-containing cell, while another, less stable molecule (protein or non-coding RNA) inhibits the activity of the toxin^{81,82}. In many cases, toxin/antitoxin modules are found on plasmids and effectively kill bacteria that lose the plasmid (losing the less stable anti-toxin, but retaining the stable toxin). However, these modules are also found on bacterial chromosomes⁸². *Bartonella schoenbuchensis*, a Group A species, expresses VbhT, a protein with both a Fic domain and a BID domain, like several *Bartonella* effectors^{15,23}. While it is unknown if VbhT has a mammalian target cell or function, it arrests growth in *E. coli* when expressed²³. Like the other Fic domain proteins described above, it contains the conserved motif and invariant histidine residue and catalyses an AMPylation reaction on an unknown 80 kDa *E. coli* protein²³.

Interestingly, co-expression with VbhA, a protein encoded upstream of VbhT, completely represses VbhT toxicity²³. The VbhA protein was shown to bind to the ATP binding site of VbhT and block the AMPylation reaction²³. These two proteins represent an example of a Fic toxin-antitoxin module²³.

1.4.6 Other Fic domain activities

It should be noted that not all Fic proteins AMPylate their target substrates. A study of the *Legionella pneumophila* Fic protein Ank showed that it transferred a phosphocholine moiety to its target proteins, in this case the Rab GTPases⁷⁶. Intriguingly, the *L. pneumophila* Type IV secreted protein Drr lacks a Fic domain, yet AMPylates RabB1-GTPase⁷⁶.

Recently, the activity of a related *Bartonella* Fic-containing protein was described. Using a mass spectrometry approach, the Fic domain of Bep2 from *B. rochalimae*, a recently discovered Group B *Bartonella*⁸³, was found to AMPylate vimentin, a type III intermediate filament of mesenchymal derived cells^{84,85}. This illustrates the range of diverse activities, even among proteins with conserved domains.

1.4.7 Role of *Bartonella henselae* BepA Fic Domain

B. henselae BepA is the best studied Bep protein and is responsible for inhibiting apoptosis through its BID domain. BepA also contains a Fic domain which Palanivelu et al. (2011) have heterologously expressed and proteolytically cleaved to yield a fragment containing this domain. This fragment displayed auto-AMPylation activity, and also AMPylated two unknown HeLa cell proteins of around 40 and 50 kDa⁶⁶. These masses do not correspond with any of the Rho-GTPase family and likely represent two uncharacterised AMPylation targets⁶⁶. The AMPylation reaction was also dependent on the conserved Fic domain histidine residue. Interestingly, Palanivelu et al. (2011) found that the reaction was not strictly dependent on ATP, as GMP and CMP moieties were incorporated with almost equal efficiency when incubated with GTP and CTP respectively⁶⁶.

1.5 Research Aims

The aim of this research was to identify the eukaryotic host cell target protein(s) of the Fic domains of BepA, BepB, and BepC, from *B. henselae*, and BepA1 and BepC, from *B. quintana*. To do this, portions of the *bep* genes, containing the Fic domain but excluding the BID domain, were cloned into an expression plasmid and the Fic-containing protein purified using nickel affinity chromatography. Whole cell extracts from human endothelial and epithelioid cells were incubated with one of two labelled ATP analogues and the purified Fic domain peptide. The resulting reactions were run on SDS-PAGE gels, with the aim of identifying target proteins by fluorescence. The goal of this work was to detect and identify the host cell protein(s) modified by the transfer of the ATP analogue by the *Bartonella* Fic domains. Candidate protein(s) would then be excised from the gel and analysed by mass spectrometry to identify the targeted proteins, modified by the transfer of the ATP analogues by the recombinant Bep proteins.

Recently vimentin was identified as a host cell target protein of Bep2 from *B. rochalimae*, a related species to *B. henselae* and *B. quintana*. Vimentin is a cytoskeletal protein found in endothelial cells, and could represent a potential target of *B. henselae* and *B. quintana* Beps. To this end, vimentin polymerisation assays were also performed along with mass spectrometry analysis to determine if the recombinant Bep proteins were able to modify vimentin, and if this modification caused interference in the polymerisation of vimentin monomers.

2 General Methods

2.1 Media Recipes

All media were autoclaved at 121°C for at least 15 minutes, and cooled until supplements could be added.

2.1.1 Liquid Media

Lysogeny Broth (LB)

- 1% Tryptone
- 0.5% Yeast Extract
- 0.5% NaCl

2X YT Media

- 1.6% Tryptone
- 1.0% Yeast Extract
- 0.5% NaCl

SOC Media

- 2% Tryptone
- 0.5% Yeast Extract
- 0.05% NaCl
- 1% 250 mM KCl

Autoclaved then 0.5% 2 M sterile MgCl_2 and 2% 1 M sterile glucose added.

2.1.2 Solid Media

Chocolate Agar

Solutions were boiled before being autoclaved separately and then combined

- 500 mL 2% BBL Freeze-dried Bovine Haemoglobin solution (Fort Richard Labs)
- 500 mL 7.2% GC agar (Thermo Fisher Scientific) solution
- 1% IsoVitalEx Enrichment Medium (Becton Dickinson) added once autoclaved solution had cooled

LB agar

LB agar was made up by adding 1.5% agar to the LB solution described above.

2.1.3 Supplement Solutions

<u>Solution</u>	<u>Final Concentration</u>
Kanamycin Sulfate	50 µg/mL
Ampicillin	100 µg/mL
Isopropyl-D-1-thiogalactopyranoside (IPTG)	1 mM
Glucose	20 mM

Table 2.1: Supplements added to media.

2.2 Bacterial Strains and Growth Conditions:

2.2.1 Bacterial Strains

<u>Strain</u>	<u>Characteristics</u>	<u>Source</u>
<i>Bartonella henselae</i> Houston-1 Strain	Isolated from the blood of an HIV-positive male	ESR (ATCC 49882) ⁸
<i>Bartonella quintana</i> JK-31 Strain	Isolated from a cutaneous bacillary angiomatosis lesion of a human patient	Dr Jane Koehler ⁵⁴
<i>E. coli</i> DH5α	recA1 and endA1 mutations, blue/white screening	Life Technologies
<i>E. coli</i> BL21	Deficient in Lon and OmpT Proteases	Lab Stock
<i>E. coli</i> TOP10	endA1, and recA1 mutations, hsdR, mcrA, lacZΔM15 for blue/white screening	Thermo Scientific

Table 2.2: Bacterial strains used in this study.

2.2.2 *Bartonella* Growth Conditions

Bartonella henselae Houston-1 strain⁸ was cultured on chocolate agar plates under an enriched CO₂ atmosphere, using GenBags (Biomérieux) to establish capnophilic conditions. The bacteria were incubated at 37°C for 5 – 7 days.

2.2.3 *E. coli* Growth and Storage Conditions

All *E. coli* were grown overnight at 37°C, either in 5 mL LB liquid media, shaking at 200 rpm, or by plating bacterial suspension (100 µL) on LB agar plates. Where necessary, the media was supplemented with antibiotic. Glycerol stocks were preserved with 30% glycerol in cryovials and stored at -80°C.

2.3 Plasmid Transformation of Competent Cells

2.3.1 Preparing Electrocompetent Cells

DH5α or BL21 *E. coli* were cultured overnight in LB media. Overnight cultures were inoculated into two autoclaved, pre-warmed conical flasks containing 250 mL LB at a starting OD₆₀₀ of 0.1. Flasks were incubated at 37°C with shaking; until the OD₆₀₀ reached 0.3 – 0.5. Cultures were cooled in an ice cold water bath and swirled occasionally for 30 minutes, then transferred to ice cold centrifuge bottles and spun for 15 minutes at 1000 x *g* at 4 – 10°C. The supernatant was discarded and the pellets were washed three more times with the following, once each:

1. Ice cold sterile ddH₂O (250 mL)
2. Ice cold glycerol (10%, 125 mL)
3. Ice cold glycerol (10%, 5 mL)

Each wash was spun for 20 minutes at 1000 x *g* at 4–10°C. Cells were re-suspended in 1 mL of 10% ice cold glycerol and distributed in 40 µL aliquots to cryovials which were frozen at -80°C until needed.

2.3.2 Electroporation

Electrocompetent *E. coli* cells were defrosted on ice before addition of a purified ligation mixture of plasmid (5 µL), then incubated on ice for 30 seconds. The suspension was transferred to a dry, ice cold 0.2 cm electroporation cuvette. The bacterial suspension was electroporated (Biorad Gene Pulser Xcell) using standard settings for *E. coli* (25 µF, 2.5 kV, 200 Ω). The cells were immediately placed in 1 mL of warm 2X YT or SOC media and incubated for 1 hour at 37°C and 200 rpm. After recovery, the transformed cells were plated onto selective media at normal and 1:10 dilutions (100 µL), with the remainder being pelleted and re-suspended in 100 µL LB before plating. Resulting cultures were incubated overnight at 37°C.

2.3.3 Transforming Chemically Competent Cells

TA cloning solution (2 μ L) was added to a vial of TOP10 chemically competent cells (Thermo Scientific) and mixed by flicking the tube. Cells were then incubated on ice for 10 minutes before being heat shocked at 42°C for 30 seconds. The cells were transferred back to ice and SOC medium (250 μ L) was added. The cells were incubated at 37°C and 200 rpm for 1 hour.

Cells were then plated at varying concentrations on LB plates containing 100 μ g/mL ampicillin. Plates were incubated overnight at 37°C.

2.4 Standard Molecular Biology Techniques

2.4.1 Primers

All primers used in this study were synthesised by Integrated DNA Technologies (IDT, Singapore) and supplied lyophilised. For long term storage, primers were suspended in sterile ddH₂O to a final concentration of 100 μ M. For working stocks, aliquots were suspended in sterile ddH₂O to 10 μ M final concentration. All primers were stored at -20°C.

Forward primers were designed so that the ATG start site for each gene was included in the NdeI restriction site, enabling an in-frame insertion of the open reading frame gene downstream of an N-terminal His₆-tag in the pET28a vector. *B. quintana* BepC was the exception, as this gene contained an internal NdeI restriction site. In this case an NheI site was engineered in the primer before the start of the gene. The reverse primers were designed to contain an XhoI restriction site immediately following the gene. These primers also contained an engineered stop codon to prevent addition of a C-terminal His-tag.

2.4.1.1 Primers for Bacterial Expression

<u>Primer Names</u>	<u>Primer Sequence</u>
BepFicNdeI_For	5'- GCG CAT ATG CCA AAG GCA AAA GCA AAA ACG -3'
BepFicXhoI_Rev2	5'- GCG CTC GAG <u>TCA</u> GGC TTT TGG AGC TGT AAA GGT -3'
BhBepBFicNdeI_For	5'- GCG CAT ATG CCA AAA GCA AAA GCA -3'
BhBepBFicXhoI_Rev	5'- GCG CTC GAG <u>TCA</u> GGC GAT AAA GGT AAT TGT -3'
BhBepCFicNdeI_For2	5'- GCG CAT ATG TTA GAG CAT AAT TAT CTG TAT AAA AAC AGC -3'
BhBepCFicXhoI_Rev2	5'- GCG CTC GAG <u>TCA</u> GAC TAT GCG ATC ACC GTT CTG -3'
BqBepAFic_NdeIF	5'- GCG CAT ATG CCA AAA GCA AAG TCA AAA GAA ACG -3'
BqBepAFic_XhoIR	5'- GCG CTC GAG TCA AAC AGA TGT TTC ATA GGC TCT AGA TTG -3'
BqBepCFic_NheIF	5'- GCG GCT AGC ATG TTA GAG CAT AAT TAT TTT TAT AAA AAC AGC GCA -3'
BqBepCFic_XhoIR	5'- GCG CTC GAG <u>TCA</u> CTG AAA GGA GAT GAA ATC ACC ATT CTG -3'
BhBepA_H159A_F	5'- CAA CCA ACT CGC CCC ATT CAG AGA AGG GAA TGG ACG -3'
BhBepA_H159A_R	5'- TTC TCT GAA TGG GGC GAG TTG GTT GAG AGA ATT AAA C -3'
T7 Promoter	5'- TAA TAC GAC TCA CTA TAG GG -3'
T7 Terminator	5'- GCT AGT TAT TGC TCA GCG G -3'

Table 2.3: Primers used for cloning into E. coli. Bold shows the restriction site, underline shows an engineered stop codon.

2.4.1.2 Primers for Expression in Mammalian Cells

<u>Primer Names</u>	<u>Primer Sequence</u>
mCT-GFP-BhBepA_F	5' – ACC ATG GGG ATG CCA AAG GCA AAA GCA AAA ACG – 3'
mCT-GFP- BhBepA_R	5' - G GGC TTT TGG AGC TGT AAA GGT AAT TTT ATC ACC – 3'
GFP Reverse	5'- TAG AAG GCA CAG TCG AGC -3'

Table 2.4: Primers used for cloning mammalian cell expression plasmids. Bold indicates the engineered Kozak Sequence.

2.4.2 Plasmids

2.4.2.1 Bacterial Plasmids

<u>Strain</u>	<u>Properties</u>	<u>Primers</u>	<u>Source</u>
pET28a (Novagen)	Bacterial Expression vector, His-Tag	N/A	Merck Millipore
pBhBepAFic_37	Derivative of pET28a encoding <i>Bh</i> BepA fragment (aa 1 – 303)	BepFicNdeI_For BepFicXhoI_Rev2	This Study
pBhBepBFic_19	Derivative of pET28a encoding <i>Bh</i> BepB fragment (aa 1 – 298)	BhBepBFicNdeI_For BhBepBFicXhoI_Rev	This Study
pBhBepCFic_10	Derivative of pET28a encoding <i>Bh</i> BepC fragment (aa 1 – 285)	BhBepCFicNdeI_For BhBepCFicXhoI_Rev	This Study
pBqBepA1_1	Derivative of pET28a encoding <i>Bq</i> BepA1	BqBepAFic_NdeIF BqBepAFic_XhoIR	This Study
pBqBepCFic_2	Derivative of pET28a encoding <i>Bq</i> BepC fragment (aa 1 – 289)	BqBepCFic_NheIF BqBepCFic_XhoIR	This Study
pBhBepAFicH159A_40	Derivative of pET28a encoding a mutated (H -> A) <i>Bh</i> BepA (aa 1 – 303)	BepFicNdeI_For BepFicXhoI_Rev2 BhBepA_H159A_F BhBepA_H159A_R	This Study
pBrBep2Fic_5	Derivative of pET28a encoding <i>Br</i> Bep2 fragment (aa 1 – 360)	VopS_pET28a (gBlock)	This Study
pVpVopS_10	Derivative of pET28a encoding <i>Vp</i> Serotype O4:K12 VopS	Bep2_pET28a (gBlock)	This Study

Table 2.5: Bacterial plasmids generated in this study.

2.4.2.2 Plasmids for Mammalian Expression

<u>Strain</u>	<u>Properties</u>	<u>Primers</u>	<u>Source</u>
pBhBepAFicGFP_14	Derivative of pcDNA3.1-CT-GFP-TOPO (source) containing BhBepAFic	mCT-GFP-BhBepA_F mCT-GFP- BhBepA_R	This Study
pBhBepAFicH159AGFP_10	Derivative of pcDNA3.1-CT-GFP-TOPO (source) containing a mutated BhBepAFic	mCT-GFP-BhBepA_F mCT-GFP- BhBepA_R BhBepA_H159A_F BhBepA_H159A_R	This Study

Table 2.6: Eukaryotic plasmids generated in this study.

2.4.2.3 Isolation of Plasmid DNA

DNA minipreps were performed using the Presto Plasmid Miniprep kit (Geneaid) as per the manufacturer's instructions. Plasmid DNA was eluted in 50 μ L of elution buffer (10 mM Tris-HCl, pH 8.5).

2.4.3 Isolation of Genomic DNA

Genomic DNA was isolated using a modified version of the CTAB extraction method⁸⁶. Briefly *B. henselae* from 5 – 7 day old chocolate agar plates was scraped off and re-suspended in 567 μ L of TE buffer (10 mM Tris, 1 mM EDTA). Re-suspended bacteria were lysed with 30 μ L of 10% SDS and proteins removed with 3 μ L of 20 mg/mL proteinase K, followed by a 1 hour incubation at 37°C. To precipitate protein and lipid breakdown products, the solution was treated with 100 μ L of 5 M NaCl followed by 80 μ L of CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) and incubated at 65°C for 10 minutes. Genomic DNA was purified with a chloroform: isoamyl alcohol (24:1) extraction, followed by a phenol: chloroform: isoamyl alcohol (25:24:1) extraction. DNA was precipitated with 0.6 volumes of isopropanol and washed with 70% ethanol solution. Finally, DNA was re-dissolved in 100 μ L TE buffer.

B. quintana strain JK-31 genomic DNA was a gift from Dr Jane Koehler.

2.4.4 Polymerase Chain Reactions

2.4.4.1 Cloning PCR

PCR cloning reactions were performed using the Advantage polymerase (Clontech) with the following reaction components:

<u>Reagents</u>	<u>Stock Concentration</u>	<u>Final Concentration</u>
Advantage 2 PCR Buffer	10X	1X
DNA Template	100 ng/ μ L	2 ng/ μ L
Forward primer	10 μ M	0.2 μ M
Reverse primer	10 μ M	0.2 μ M
dNTP mix (Biolabs)	10 mM each	200 μ M each
Advantage 2 Polymerase Mix	50X	1X

Table 2.7: Reaction components for PCR with Advantage Polymerase.

Standard PCR reaction conditions are outlined below:

Pre-Cycle	95°C	1 minute	
Denaturing Step	95°C	30 seconds	30 Cycles
Annealing Step	60°C	30 seconds	
Extension Step	68°C	1 minute	
Post-Cycle	68°C	1 minute	

Table 2.8: Reaction conditions used for Advantage PCR with Bep-Fic primers.

For TA cloning into the pcDNA3.1 mammalian expression vector, the post-cycle extension step was lengthened to 10 minutes. Interestingly, for both *bepC* genes, the Advantage kit did not result in product, so the Phusion Hi-Fidelity PCR kit (Thermo Scientific) was used with the following reaction conditions:

<u>Reagents</u>	<u>Stock Concentration</u>	<u>Final Concentration</u>
5X GC Buffer PCR Buffer	5X	1X
DNA Template	100 ng/μL	2 ng/μL
Forward primer	10 μM	0.2 μM
Reverse primer	10 μM	0.2 μM
dNTP mix (Biolabs)	10 mM each	200 μM each
Phusion Polymerase Mix	50X	1X

Table 2.9: Reaction components for PCR with Phusion Polymerase.

Pre-Cycle	98°C	30 seconds	
Denaturing Step	98°C	10 seconds	30 Cycles
Annealing Step	60°C	30 seconds	
Extension Step	72°C	30 seconds	
Post-Cycle	72°C	5 minutes	

Table 2.10: Reaction conditions used for Phusion PCR for BepC-Fic constructs.

Control reactions with no added template DNA were routinely run.

2.4.4.2 BepA_H159A mutant

In order to construct the BhBepA_H159A mutant, a PCR fusion site-directed mutagenesis strategy was employed. Overlapping primers were engineered to contain two base changes converting the histidine in the active site into an alanine. A two-part PCR reaction was carried out; in the first step two overlapping fragments with the engineered mutation were amplified by PCR (Reactions 1 and 2). These PCR products were purified and checked, then used as a template in Reaction 3, which fused and amplified these two products, incorporating the mutation. The primers used for Reactions 1-3 are listed in Table 2.11.

	<u>Reaction 1</u>	<u>Reaction 2</u>	<u>Reaction 3</u>
Forward Primer	BhBepA_H159A_F	BepFicNdeI_For	BepFicNdeI_For
Reverse Primer	BepFicXhoI_Rev2	BhBepA_H159A_R	BepFicXhoI_Rev2

Table 2.11 Site directed mutagenesis for BhBepA_H159A-Fic construction. The table indicates the primers used for each PCR reaction. For Reactions 1 and 2, *B. henselae* genomic DNA was used as the template. For Reaction 3, the PCR products from Reactions 1 and 2 were combined and used as a template. Primer sequences are described in Table 2.3.

All reactions were performed using the Advantage PCR conditions described in Table 2.7, however the extension length was increased to 3 minutes. For the pcDNA3.1 mammalian expression vector, the mCT-GFP-BhBepA primers were used in place of the BepFic primers (Table 2.4)

2.4.4.3 Colony PCR

To screen colonies for plasmids with the correct insertion, colonies on solid media were added to premixed PCR reagents with a sterile inoculating loop. Colony PCR was carried out using Biomix Red Polymerase Mastermix (Bioline) with the following reaction components in a 10 μ L total volume:

<u>Reagents</u>	<u>Stock Concentration</u>	<u>Final Concentration</u>
2X Biomix Red	2X	1X
Forward primer	10 μ M	0.5 μ M
Reverse primer	10 μ M	0.5 μ M

Table 2.12: Reaction components for colony PCR.

A positive control using 1 μ L plasmid DNA, and a negative control with no added DNA, were also run. The PCR reactions were carried out under the following conditions:

Pre-Cycle	95°C	10 minutes	
Denaturing Step	95°C	30 seconds	30 Cycles
Annealing Step	45°C	30 seconds	
Extension Step	72°C	45 seconds	
Post-Cycle	72°C	5 minutes	

Table 2.13: Reaction conditions for colony PCR using T7-primers.

2.4.5 gBlocks

Positive control proteins, *V. parahaemolyticus* (serotype O4:K12) VopS (GenBank: KEE64915.1⁸⁷) and *B. rochalimae* Bep2 (NCBI WP_035006139) were synthesised by IDT. In the case of VopS, NheI and XhoI restriction sites were included, while for Bep2, BamHI and XhoI restriction sites were included. To resuspend the gBlocks, tubes were briefly centrifuged, then TE buffer was added to reach a final concentration of 10 ng/ μ L. The gBlocks were subsequently vortexed and incubated at 55°C for 20 minutes.

Before cloning reactions were performed, gBlock DNA (35 μ L) was cleaned and concentrated on a Zymo column (Zymo Research), before eluting in 10 μ L ddH₂O, to remove any potentially interfering EDTA.

2.4.6 DNA Modification Reactions

2.4.6.1 Restriction Enzyme Reactions

Purified PCR products and plasmids were digested with Nde-I and Xho-I (20 U; New England Biolabs) at 37°C for 1 hour in 1X CutSmart Buffer (20 μ L final volume), followed by heat inactivation of the enzymes at 65°C for 20 minutes. For *B. quintana* *bepC*, the PCR product and pET28a plasmid were first digested with Xho-I as above, before purifying on a Zymo column (Zymo Research). Purified PCR product and plasmid was subsequently digested with Nhe-I (10 U; Fermentas) at 37°C for 1 hour in 1X Tango Buffer, followed by heat inactivation of the enzyme at 65°C for 20 minutes. For *bep2*, the gBlock DNA and the pET28a plasmid were digested with BamH-I and Xho-I (10 U; Thermo Scientific) in 1X Fast Digest Buffer for 1 hour at 37°C, followed by heat inactivation at 65°C for 20 minutes.

2.4.6.2 Ligation Reactions

Ligations were performed overnight at 16°C on all purified restriction digest samples (8 µL) using T4 DNA ligase (5 U) in 1X Ligation Buffer (New England Biolabs), in a final volume of 10 µL. Ligation reactions were dialysed by drop dialysis with ddH₂O for an hour on 0.025 µm membrane filters (Millipore). Resulting ligations (5 µL) were used for electroporation.

2.4.6.3 TA Cloning

To clone PCR products into the pcDNA3.1/CT-GFP-TOPO[®] mammalian expression vector (Life Technologies), the following reaction was set up:

<u>Reagents</u>	<u>Volume Used</u>
PCR Product	4 µL
Salt Solution (1.2 M NaCl, 0.06 M MgCl ₂)	1 µL
pcDNA3.1 vector	1 µL

Table 2.14: Reaction components for pcDNA3.1 TA cloning.

The reaction was gently mixed then incubated at ambient temperature for 30 minutes. Resulting solutions (2 µL) were used to transform TOP10 chemically competent cells.

2.4.7 DNA Purification

PCR products and restriction digests were purified using the GeneHlow Gel/PCR purification kit (Geneaid). The products were eluted in 20 – 50 µL Elution Buffer (10 mM Tris-HCl, pH 8.5) or heated ddH₂O, as required.

2.4.8 DNA Quantification

The quantity and purity of DNA samples was determined using a NanoDrop ND-1000[™] spectrophotometer (Thermo Scientific).

2.4.9 TAE Agarose Gels (1%)

DNA samples were analysed on agarose gels (1% w/v agarose in 1X TAE buffer [40 mM Tris-acetate, 1 mM EDTA]), supplemented with 1X GelRed[™] (Biotium), covered with 1X TAE at 100 V for 60 minutes. DNA was visualised under UV light. DNA loading dye (Thermo Scientific) was added to samples, which were run with 2-log DNA ladder (New England Biolabs) or Hyperladder (Bioline) as DNA size standards.

2.4.10 Sequencing

All sequencing reactions were performed by Macrogen Inc (Seoul, South Korea). Sequence data was confirmed using the Geneious 8.1.7 Multiple Sequence Alignment function.

2.5 Protein Manipulation Protocols

2.5.1 Inducing cellular protein

2.5.1.1 Preliminary Induction

Overnight cultures of plasmid-containing BL21 cells were grown at 37°C, 200 rpm, and used to inoculate 5 mL of fresh kanamycin supplemented LB at a starting OD₆₀₀ of 0.1 the following morning. Cells were returned to the incubator and after 2 hours of growth, 1 mL of the culture was removed pelleted by centrifugation at 16,100 x *g* for 1 minute and set aside (un-induced control). Recombinant protein production was induced for 4 hours at 37°C by the addition of IPTG. After the induction, 1 mL of culture was removed to a fresh tube and pelleted by centrifugation, with the pellet being retained. Pellets were re-suspended in 100 µL PBS. The samples were heated at 95°C for 10 minutes before being stored at -20°C until they could be analysed on a 12.5% SDS-PAGE gel.

2.5.1.2 Full Scale Induction

For protein purification, BL21 expressing the correct plasmid were cultured overnight. The resulting culture was used to inoculate 250 mL of kanamycin supplemented LB at a starting OD₆₀₀ of 0.1. Once an OD₆₀₀ of 0.3–0.6 was reached, a 1 mL un-induced sample was removed and centrifuged at 16,100 x *g* for 1 minute to pellet, before setting aside. Protein expression was induced with IPTG for 4 hours at 37°C. After induction the OD₆₀₀ was re-measured, and 1 mL induced sample was collected for the induced sample, before the remaining culture was centrifuged at 5,000 x *g* for 10 minutes. The resulting cell pellets from the induced samples were lysed with 4 mL B-Per (Thermo Scientific) per gram of pellet, according to the manufacturer's instructions. Both un-induced and induced pellets were re-suspended in PBS as previously described.

2.5.2 SDS-PAGE

2.5.2.1 Recipes

5X SDS Loading Dye

- 250 mM Tris-HCl pH 6.8
- 100 g/L SDS (10% w/v)
- Glycerol (30% v/v)
- Bromophenol blue (0.02% v/v)

SDS 10X Running Buffer

- 30 g/L Tris base
- 144 g/L glycine
- 10 g/L SDS

2.5.2.2 Protocol

SDS-PAGE gels (12.5%) were made using the TGX FastCast™ Acrylamide Kit (Bio-Rad) as per the manufacturer's instructions summarised here.

Resolving solutions were made up by mixing 8 mL of Resolver A and Resolver B solutions together, followed by the addition of 10% APS (80 µL) and TEMED. (8 µL) Stacking solutions were made up by mixing 2 mL of Stacker A and Stacker B solutions together, followed by 10% APS (20 µL) and TEMED(4 µL).

Alternatively, samples were run on pre-cast 8 – 16% Novex Tris-Glycine SDS-PAGE gels (Life Technologies). SDS-PAGE samples were made up by adding β-mercaptoethanol (0.7 M final concentration) and 5X SDS loading dye to protein samples at a 1:4:15 ratio. Samples were heated at 95°C for 10 minutes before loading onto the gel. Gels were run at 125 V for 45 – 90 minutes. Following the run, gels were washed with ddH₂O 3 times before staining with Bio-Safe pre-mixed Coomassie stain (Bio-Rad) for an hour. This was followed by de-staining in ddH₂O for 30 minutes.

2.5.3 Protein Extraction and Purification

The bacterial lysates containing His-tagged Bep proteins were applied to equilibrated Pierce HisPur-Ni-NTA 1 mL spin columns (Thermo Scientific), as per the manufacturer's instructions with some modifications, summarised below.

All steps were kept at 4°C where possible. Protein extracts were equilibrated by adding an equal volume of equilibration buffer (PBS with 10 mM imidazole). Equilibration buffer (2 mL) was used to wash the 1 mL resin columns before adding the prepared protein extracts. The columns were mixed on an orbital shaker for 30 minutes at 4°C, before centrifuging to collect the flow through. The resin was washed three times with 2 mL wash buffer (PBS and 20 mM imidazole). The purified proteins were then eluted three times with 1 mL elution buffer (PBS and 200 mM imidazole). All fractions, including the purified lysate and wash buffer flow-through, as well as both un-induced and induced samples, were run on 12.5% SDS-PAGE gels to check for correct protein isolation, as well as successful purification. To regenerate the resin, the manufacturer's instructions were followed whereby the resin was washed with MES buffer (10 mL), followed by ultrapure H₂O (10 mL). The resin was stored as a 50% slurry in 20% ethanol.

Protein eluates were desalted using Microcon-10kDa centrifugal filter columns (Millipore). Each elution sample (500 µL) was run on an individual column and spun at 14,000 x *g* for 10 minutes. This was repeated until all elution samples had been loaded onto the column. To each column, 500 µL of HEPES buffer (20 mM, pH 7.5) was added and the column re-spun at 14,000 x *g* for 10 minutes to dialyse the buffer. This was repeated to ensure effective buffer transfer. The protein was eluted by inverting the column into a new collection tube and spinning at 1,000 x *g* for 2 minutes. Resulting protein solutions were stored at 4°C for up to a week before use.

2.5.4 Protein Quantification

Protein quantification was performed using the Pierce BCA Protein Assay kit (Thermo Scientific) as per the manufacturer's instructions for microplate samples.

Unknown samples were diluted 1:10 and 1:100 in ddH₂O or HEPES buffer and compared with diluted HEPES for background. Each standard or unknown sample replicate (25 µL) was pipetted into a microplate well and 200 µL of the BCA working reagent (WR) was added (giving a 1:8 sample to WR ratio). The solutions were mixed by shaking for 30 seconds and the plate was incubated for 30 minutes at 37°C before being cooled to ambient temperature. The absorbance was measured at 562 nm on a plate reader (EnSpire 2300 Multilabel Reader [Perkin Elmer]). The average value for the blank was subtracted from the standards and unknowns and a standard curve was prepared to determine the protein concentration of the unknown samples. All standards and unknowns were analysed in triplicate.

2.6 Cell Culturing

2.6.1 Initiation and Maintenance of HUVEC Cell Culture

Human Umbilical Vein Endothelial Cells (HUVEC; Life Technologies) were stored in liquid nitrogen until use. Cells were thawed by dipping the lower half of the vial into a 37°C water bath. From the vial 1/3rd of the contents was pipetted into 25 mL of Medium 200 (Life Technologies) supplemented with Low Serum Growth Supplement (Life Technologies) in a T75 (250 mL) culture flask. This step was repeated two more times for the remaining HUVEC solution. Cultures were incubated in a 37°C, 5% CO₂ humidified cell culture incubator. Culture media was changed every 2 – 3 days by removing the media, and pipetting fresh supplemented Medium 200 (25 mL) into the culture flask.

2.6.2 Sub-culturing HUVECs

Once HUVEC growth had reached 80 – 90% confluence, cells were sub-cultured by first removing the culture media from the flask. The cells were treated with trypsin by adding 4 mL trypsin-EDTA, spreading it over all cells, and then removing 3 mL of trypsin. After 1 – 2 minutes of incubation, flasks were rapped to dislodge the HUVECs from the flask. Once the cells were dislodged, supplemented Medium 200 (10 mL) was added to the solution. From this a 1 mL sample was removed and added to supplemented Medium 200 (24 mL) in a new T75 flask resulting in a 1:10 dilution of cells.

2.6.3 Initiation and Maintenance of HeLa Culture

Liquid nitrogen stocks of HeLa cells were thawed and cultured in T75 flasks in RPMI 1640 media (Life Technologies) supplemented with 10% foetal calf serum and 1% penicillin-streptomycin solution (10,000 U/mL, Life Technologies). Cultures were incubated in a 37°C, 5% CO₂ humidified cell culture incubator. Culture medium was changed every 2 – 3 days by removing the media and pipetting fresh supplemented RPMI (25 mL) into the culture flask.

2.6.4 Sub-culturing HeLas

Once HeLa growth had reached 90 – 100% confluence, cells were sub-cultured by first removing the culture media from the flask. Cells were rinsed with EDTA/Trypsin solution, before treating with 2 – 3 mL EDTA/Trypsin solution. Once the cells had rounded (after roughly 5 minutes), flasks were rapped to dislodge attached cells from the flask. Supplemented RPMI 1640 medium (8 mL) was added to resuspend the cells; 1 mL of this suspension was transferred to 24 mL supplemented RPMI 1640 in a new T75 flask.

2.6.5 Whole Cell Extract Preparation

Whole cell extracts were prepared for both HUVEC and HeLa cells using a method adapted from Lewallen et al. (2012). Cells were grown in 75 mL flasks to 90% confluence, with roughly 1×10^7 cells per flask.

Adherent cells were removed from the flask with a cell scraper in 10 mL sterile PBS. Suspended cells were transferred to two 50 mL centrifuge tubes (three flasks per tube). The cells were centrifuged at 2500 rpm for 3 minutes to pellet, before being washed with 10 mL of PBS. This step was repeated once more. The cells were then re-suspended gently in 500 µL RIPA buffer (50 mM Tris-HCl pH 8.0 (HUVEC) or 20 mM HEPES pH 7.4 (HeLa), 150 mM NaCl, 1% Triton X-100, 0.05% deoxycholate, 10 µL/mL Halt Protease Inhibitor Cocktail [Thermo Scientific]) and incubated on ice for 30 minutes at 4°C on a orbital shaker. The resulting solution was then transferred to a 1.5 mL centrifuge tube and centrifuged at 13,400 x *g* for 20 minutes. The supernatant, which contained the whole cell extract, was transferred to a separate tube. HUVEC whole cell extracts (500 µL) were concentrated using Microcon-10kDa centrifugal filter columns (Millipore), by spinning for 10 minutes at 14,000 x *g*. Extracts were eluted by spinning the column upside down in a fresh tube for 2 minutes at 1,000 x *g*. This concentration step was omitted for HeLa whole cell extracts. Protein concentration of all cell extracts was quantified as previously described.

2.7 Labelling Reactions

2.7.1 F1-ATP Methodology

Whole cell extracts were immediately combined with purified recombinant Bep proteins, as well as a labelled ATP analogue, following their purification, to identify AMPylated host cell proteins. Purified recombinant *Bartonella* Bep proteins (100 µg/mL final concentration) were combined with F1-ATP (N6-(6-amino)-hexyl-ATP-5-FAM; Jena Bioscience), a commercially available fluorescently labelled ATP derivative, and added to roughly 400 µg of HUVEC cell extracts in Reaction buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 1 mM DTT, 5 mM MgCl₂) in a total volume of 100 µL to give a final concentration of 60 µM F1-ATP. A negative control containing no recombinant protein was also included. The reactions were incubated at 37°C for 90 minutes. Samples were run on pre-cast 8 – 16% Novex Tris-Glycine SDS-PAGE gels (Invitrogen) alongside a BenchMark Fluorescent Protein Standard (Life Technologies), a fluorescent protein marker. Gels were visualised using a Fuji fluorescent gel scanner with a FITC setting, followed by Coomassie staining.

2.7.2 Click Chemistry Methodology

The purified bacterial protein of interest (100 µg/mL final concentration) was combined with N⁶pATP (N⁶-propargyl-adenosine-5'-triphosphate; Jena Bioscience; 100 µM final concentration), a commercially available ATP derivative, and incubated with 200 – 500 µg of HeLa cell lysates for 1 hour at 30°C, in a total volume of 100 µL of 20 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM MgCl₂ and 1 mM DTT. Purified Fic domain proteins, *V. parahaemolyticus* VopS and *B. rochalimae* Bep2, were included as positive controls. A mutant of *B. henselae* BepA, with the critical Fic domain histidine mutated (BhBepA_H159A-Fic) was included as a negative control. A sample with no added recombinant bacterial protein was also included. Reactions were stopped by addition of methanol (400 µL) and chloroform (100 µL), and the samples vortexed. Water (300 µL) was added, and the samples vortexed again. Samples were centrifuged at 16,100 x *g* for 5 minutes at ambient temperature. Following centrifugation, the upper phase was removed, -20°C acetone (1 mL) was added, and the samples were vortexed. Samples were centrifuged at 23,000 x *g* for 10 minutes at 4°C to pellet protein. The supernatant was removed, and pellets allowed to air dry for 5 – 10 minutes. Dried pellets were re-suspended in 100 µL 4% SDS in 20 mM HEPES.

A Click Chemistry mastermix was made up as follows: 22.3 μL of 100 mM CuSO_4 (Jena Bioscience) was added to 877.35 μL of 4% SDS in 20 mM HEPES, followed by 55.75 μL 200 mM THPTA (Tris(3-hydroxypropyltriazolylmethyl)amine; Jena Bioscience). To the resulting solution, 44.6 μL 10 mM 6-FAM-azide (N-(3-azidopropyl)-3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxamide; Jena Bioscience) in DMSO was added, resulting in a final volume of 1 mL. The resulting click chemistry Mastermix (90 μL) was added to the samples. Click chemistry was initiated with the addition of 10 μL of 100 mM sodium ascorbate (Jena Bioscience). This resulted in a final reaction concentration of 1 mM CuSO_4 , 5 mM THPTA, 200 μM 6-FAM azide and 5 mM ascorbate. The reaction mixtures were incubated at 37°C for 1 hour in the dark. Reactions were stopped by addition of SDS sample buffer, with remainder being stored at 4°C. SDS-PAGE samples were loaded onto pre-cast 8 – 16% Novex Tris-Glycine SDS-PAGE gels (Life Technologies) and run for 90 minutes at 125 V alongside a BenchMark Fluorescent Protein Standard (Life Technologies). Gels were visualised on a Fuji fluorescent gel scanner with a FITC setting, before Coomassie staining.

2.8 Vimentin Polymerisation Assay

Vimentin polymerisation was performed using a method adapted from Icenogle et al. (2012). Purified His-Bep protein (100 $\mu\text{g}/\text{mL}$ final concentration) was combined with ATP (1 mM final concentration; ThermoFisher Scientific), and added to 0.05 $\mu\text{g}/\mu\text{L}$ of recombinant Syrian hamster vimentin (Cytoskeleton, Inc.) in 20 mM HEPES, 100 mM NaCl, 1 mM DTT, 5 mM MgCl_2 (500 μL). The reaction was incubated at 37°C for 3 hours. Negative controls were run with BhBepA_H159A-Fic mutant and no Bep protein respectively. Following incubation, samples were reserved for liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) and pyrophosphate assay analysis.

The reactions were split into two equal aliquots. Vimentin polymerisation was induced in one sample by adding NaCl to a final concentration of 150 mM, while ddH₂O was added to the other. Samples were incubated at ambient temperature for 60 minutes. Polymerised filaments were separated from soluble monomer subunits by ultracentrifugation (Airfuge; Beckman Coulter) at 80,000 $\times g$, at 20°C for 90 minutes. The supernatant (100 μL) was collected and each sample was run on pre-cast 8 – 16% Novex Tris-Glycine SDS-PAGE gels (Life Technologies) for 90 minutes at 125 V, before being subjected to western blotting.

2.8.1 Western Blotting (WB)

2.8.1.1 Recipes

Transfer Buffer

- 25 mM Tris Base
- 192 mM Glycine
- 20 % (v/v) Methanol
- 0.01% (w/v) SDS

TBS (Tris-Buffered Saline) 10X

- 200 mM Tris Base
- 1.5 M NaCl
- Adjust pH to 7.6

TBS-T 1X

- 100 mL TBS 10X
- 900 mL ddH₂O
- 0.1% (v/v) Tween 20

Blocking Buffer

- 8% (w/v) milk powder in TBS-T

2.8.1.2 Protocol

Protein gels were removed from electrophoresis equipment and soaked in cold transfer buffer for 10 – 30 minutes. Nitrocellulose membranes (pore size 0.2 µm; Thermo Scientific) were cut to match gel size, then soaked in cold transfer buffer until use. Filter paper was cut to size, then briefly soaked in cold transfer buffer before assembling western blot sandwich.

Protein samples were transferred to a nitrocellulose membrane at 30 V for 90 minutes on ice. Membranes were washed with blocking buffer for 2 – 3 hours at ambient temperature on a shaker. Following blocking, membranes were transferred to a 5 mL solution of diluted primary antibody in TBS-T (1:500; mouse monoclonal [RV202] anti-vimentin antibody [Abcam]) and allowed to shake overnight at 4°C. Membranes were washed four times for 7 minutes each with TBS-T buffer. TBS-T was discarded and 5 mL secondary antibody solution in TBS-T was added (1:2000; Goat anti-mouse IgG H&L [Alexa Fluor® 647; Abcam]) and allowed to shake in the dark for 1 hour at ambient temperature.

Membranes were washed again as above and visualised on a Typhoon FLA 9500 Scanner (GE Healthcare Life Sciences) using an Alexa Fluor® 647 setting.

2.9 Pyrophosphate Detection/Bep activity assay

To assess the recombinant Bep proteins for activity, sub-samples of the vimentin polymerisation assay were taken and analysed for pyrophosphate levels. Similarly, repeat experiments based on the F1-ATP labelling experiments were performed, with regular ATP (1 mM; Thermo Scientific) substituting for F1-ATP, and with the reaction length being extended to 3 hours. To detect pyrophosphate production, the ab112155 Fluorometric Pyrophosphate Assay Kit (Abcam) was utilised as per manufacturer's instructions, summarised below:

PPi sensor stock (200X) solution was prepared by adding 50 µL DMSO to lyophilised sensor. Assay sensor (1X) was prepared by diluting 10 µL PPi sensor in 2 mL Assay Buffer. Pyrophosphate assays were performed by adding Assay Sensor (50 µL) to 50 µL of pyrophosphate standards or AMPylation samples diluted (1:10) in Assay buffer in a fluorescence 96 well plate and mixed thoroughly. The plate was then incubated at ambient temperature for 10 – 30 minutes. Fluorescence was measured on a plate reader (EnSpire 2300 Multilabel Reader [Perkin Elmer]) using excitation/emission wavelengths of 316/456 nm respectively. The resulting data was imported into and analysed by Microsoft Excel.

2.10 LC-MS/MS Preparation

Protein samples were taken from previous experiments and were precipitated using the ProteoExtract® Protein Precipitation kit (Merck Millipore) as per the manufacturer's instructions for samples less than 200 µL. Resulting protein pellets were dissolved in 50 µL 8 M Urea/0.1 M Tris, pH 8.5, before being reduced by 0.2 M DTT (1.25 µL) and incubated at 56°C for 35 minutes. Samples were cooled to ambient temperature and subsequently alkylated with 500 mM iodoacetamide (1 µL) in the dark for 30 minutes. Proteins were diluted 3-fold with 0.1 M Tris, pH 8.5 to bring total urea concentration down to 2 M. CaCl₂ solution was added (1 mM final concentration), and resulting solutions were added to 1 µg of trypsin each. Samples were digested overnight at 37°C and 200 rpm. The reactions were quenched with 90% formic acid and stored at -20°C until ready for further purification with Bond Elut OMIX tips.

2.10.1 Sample Preparation with Bond Elut OMIX Tips

Bond Elut OMIX micro extraction tips (Agilent) were pre-wet with a 1:1 solution of 0.4% trifluoroacetic acid (TFA)/acetonitrile (ACN) by aspirating three times, one tip per protein sample. OMIX tips were then equilibrated with a 1:1 solution of 0.4% v/v TFA/H₂O. To bind protein to OMIX tips, protein solutions were aspirated 20 times, before rinsing the OMIX tip with 0.4% TFA/H₂O. Protein was eluted into 20 µL 0.1% formic acid in 70% ACN/H₂O. Resulting elutions were concentrated to 10 µL volume in a SpeedVac vacuum centrifuge (Labconco), before storing at 4°C until use.

To prepare samples for loading onto the LC-MS, samples were mixed with 70 µL of Buffer A (0.1% formic acid in H₂O), vortexed, then centrifuged for 2 minutes. Most of the supernatant was pipetted into a GC vial with an insert.

2.11 Liquid Chromatography and Mass Spectrometry

LC-MS/MS experiments were performed by Dr Lifeng Peng using a Dionex UltiMate® 3000 RSLCnano System, coupled to a LTQ Orbitrap XL mass spectrometer, via a nanospray ion source (Thermo Fisher Scientific, USA). Peptides were separated on a 75 µm × 15 cm PepMap C18 column (3 µm, 300 Å Dionex) at a flow rate of 300 nL/min. The LC gradient was constructed from Buffer A (0.1% formic acid) and Buffer B (0.1% formic acid in 80% ACN): 2% Buffer B to start, followed by a linear gradient from 2 – 22% B for over 65 minutes, followed by a linear gradient from 22 – 45% B for over 35 minutes, followed by a linear gradient from 45 – 95% B for 20 minutes, before being held at 95% for 5 minutes.

The spray voltage was set at 2.4 kV, and the temperature of the heated capillary was set at 200°C. Full MS scan (m/z 200 – 1850) in profile mode was acquired in the Orbitrap with 30,000 resolution. The six most intense peptide ions from the full scan were selected and fragmented using CID (normalised collision energy, 35%; activation Q, 0.250; and activation time, 30 milliseconds). Dynamic exclusion was enabled with the following settings: repeat count, 2; repeat duration, 30 seconds; exclusion list size, 500; exclusion duration, 90 seconds. The spectra were acquired using Xcalibur (version 2.1.0 SP1, Thermo Fisher Scientific).

2.11.1 Data Analysis

Protein identification was conducted using the search algorithm SEQUEST under Proteome Discoverer (version 1.4.1.14, Thermo Scientific). All searches were performed with two databases; one containing only vimentin, another containing the UniProt all human database as of 12/02/16. The database was indexed with trypsin as the proteolytic enzyme, 'fully enzymatic' as the enzyme limit, with a molecular weight range of 300 – 5000 kDa, and 2 missed cleavage sites. SEQUEST searches were conducted for B and Y ions only, with dynamic protein modifications of 329.052 and 330.060 monoisotopic masses for AMP de-protonated and protonated forms respectively. AMPylation was analysed for serine, threonine, tyrosine, and histidine residues. Oxidation, carbamyl, deamidated, and carbamidomethyl dynamic modifications were also included in the analysis.

3 Labelling Whole Cell Extract with F1-ATP

3.1 Introduction

3.1.1 Bacterial Effectors

Pathogenic bacteria have evolved a wide range of toxins and effector proteins that are able to subvert host cell processes and cause disease. By studying these toxins and effector proteins, a greater understanding of disease progression may be obtained. This can lead to a wide range of treatments and interventions including potential vaccine antigens and novel antibiotic targets. Similarly, bacterial toxins and effectors can serve as tools for studying normal cellular processes including signalling, cytoskeleton rearrangements, and plasma membrane permeability^{90,91}. Many bacterial effector proteins mimic natural host cell proteins such as the GEF (guanine nucleotide exchange factor), and GAP (GTPase activating protein) motif effectors, which regulate host GTPase protein activity^{91,92}.

Increasingly, AMPylation has been shown to be a post-translational modification utilised by bacterial effectors to manipulate host cell processes^{66,71,75,84}. In particular, several Fic domain-containing bacterial effectors have been shown to AMPylate host cell proteins, including the Rho GTPases and vimentin. AMPylation often leads to interference with the normal function of the target protein. Many *Bartonella* species contain several Fic domain-containing proteins, although their precise roles in disease or transmission remain unknown.

3.1.2 Previous AMPylation Studies

Several groups working on Fic domain effector proteins from other bacterial pathogens have successfully identified their host AMPylation targets^{66,71,75,84}. Published work identifying targets of Fic domain effectors have used a number of approaches, the most common being the use of radiolabelled [α -³²P] ATP^{71,75,80,84}. While this method is very effective it has drawbacks, requiring special training and disposal of the radioactive material, and presenting difficulties in identification of the AMPylated (i.e. radiolabelled) protein⁸⁸. Another published method makes use of antibodies against AMPylated threonine or AMPylated tyrosine⁹³. While this approach does not require radioactivity and allows for immunoprecipitation of labelled protein to enable easier identification, the downside is that there are currently no commercially available antibodies to AMPylated serine. This approach could potentially miss novel targets, such as the BiP chaperone, which is AMPylated on a serine residue by the endogenous Fic domain protein, HYPE⁷⁸. Problems can also arise with non-specific binding⁹⁴.

An alternative method proposed by Lewallen et al. (2012) uses an ATP derivative with a covalently linked fluorophore. One such molecule is F1-ATP (Figure 3.1). Experiments involving the *Vibrio parahaemolyticus* VopS Type III effector and whole cell extracts demonstrated that VopS could label known substrates with this ATP analogue, and that target proteins could be easily identified and excised from a SDS-PAGE when viewed under a FITC setting. The benefit of this type of probe is that it is less toxic than radiolabelling and enables immunoprecipitation using anti-FITC antibodies. A potential drawback to this method is that the incorporation of a bulky fluorophore on the ATP substrate may potentially interfere with the Fic proteins ability to catalyse the AMPylation reaction. Despite the potential drawbacks, this method was selected to identify the host cell target proteins of the *Bartonella* Beps studied here.

Figure 3.1: Structural comparison of F1-ATP, FITC, and ATP.

3.1.3 Construct Design

To identify the host target proteins, fragments of the *Bartonella* Fic domain proteins were expressed and purified in an exogenous host (*E. coli*). Recombinant proteins were truncated to omit the BID domain, and contained an N-terminal His₆ tag to enable purification by nickel-affinity chromatography. An N-terminal, over a C-terminal His₆-tag, was chosen to retain the option of removing the His₆-tag by thrombin cleavage.

Plasmids were constructed to enable expression and purification of the Fic domains of *B. henselae* proteins BepA (NCBI – BH13370), BepB (NCBI – BH13390), and BepC (NCBI – BH13400), originally described by Schulein et al. (2005), as well as the *B. quintana* proteins BepA1 (NCBI – BQ10630) and BepC (NCBI – BQ10650), originally described by Alsmark et al. (2004). BepA primers were designed to amplify the first 909 base pairs of the *bepA* gene, as this corresponds with the first 303 amino acids of the protein. Palanivelu et al. (2011) have shown that a similarly truncated BepA protein (10-303) had functional Fic protein activity, and was capable of auto-AMPylation, as well as AMPylation of two unidentified HeLa cell proteins. The BepA fragment length was designed to exclude expression of the *Bartonella* Intracellular Delivery (BID) domain of the C-terminal of the protein. BID domains are highly conserved in many *Bartonella* species Bep proteins and have several of their own functions. For example, the BepA BID domain enables T4SS translocation of BepA into the host cell, where it inhibits host cell apoptosis⁶⁰. By omitting the BID domain, any enzymatic activity detected is likely attributable to the Fic domain of the protein.

A similar methodology was applied for the *bepB* and *bepC* genes. Pulliainen et al. (2012) described the starting point of the BID domain in *B. henselae* Beps A, B, and C and this was used as a reference when constructing the primers. Primers were designed to amplify only the first 894 base pairs of *bepB*, and the first 855 base pairs of *bepC*. Both BepB and BepC have BID domains that occur earlier in the protein than BepA, with BepC being the shortest (Figure 3.2).

Figure 3.2: *Bartonella* effector proteins studied. The arrows show the ends of the fragments expressed in this study. In the case of *B. quintana* BepA1, the whole gene was cloned (*B. quintana* BepA2 is included for completeness).

In the case of the *Bartonella quintana* genes, the primers for *bepA1* were designed to amplify the whole gene as the BID domain is expressed as a separate ORF (*bepA2*)⁶⁰. For *B. quintana* *bepC*, the primers were designed to give as much coverage of the gene as possible while still excluding the BID domain. To do this, similarities to the *B. henselae* *bepC* were used as a reference, with the first 864 base pairs being cloned.

All of the plasmid sequences were confirmed to be correct by aligning with NCBI sequences, using the Geneious 8.1.7 Multiple Alignment function.

3.1.4 Protein Expression

The plasmid constructs were transformed into the BL21 *E. coli* host strain. Following induction of protein expression by IPTG, cells were lysed and recombinant proteins were purified by nickel affinity chromatography. Of the 5 protein constructs described, all were shown to be induced in BL21 cells, but only BhBepA-Fic, and BhBepB-Fic proteins could be successfully isolated (Appendix Section 9.2). Comparison of the soluble and insoluble fractions from cell lysis revealed that the BhBepC-Fic, BqBepA1, and BqBepC-Fic protein fragments were largely insoluble. Unfortunately, time constraints precluded resolution of these insolubility issues.

3.2 Results

3.2.1 Optimisation of Protein Recovery from Whole Cell Extract

Before F1-ATP labelling experiments could be undertaken, optimisation of whole cell extract recovery was required as initial attempts resulted in very low levels of protein (150 $\mu\text{g}/\text{mL}$). In order to increase protein concentration of whole cell extract, three flasks of HUVEC cultures were scraped and combined into a single Falcon tube before lysing with RIPA buffer. The resulting lysate was further concentrated using a 10 kDa Microcon column (Millipore). To further improve protein extraction, protease inhibitor was added to RIPA buffer. The results are shown in Figure 3.3 below. These results clearly indicate that both techniques were able to significantly increase protein concentration obtained in the whole cell extract.

Optimisation of Protein Extraction from HUVEC Cells

Figure 3.3: Optimisation of protein extraction from HUVEC cells.

3.2.2 F1-ATP Labelling

Figure 3.4: Simplified methodology of the F1-ATP labelling experiments. First, protein is extracted from the HUVEC cells (whole cell extract). This whole extract is treated with recombinant Fic-containing protein (BepA) and F1-ATP resulting in fluorescently labelled protein in the whole cell extract. The reaction sample is then run on a SDS-PAGE gel and analysed under a FITC setting. Novel bands are detected by comparison of sample (middle lane) with negative samples (right lane). Fluorescent marker is also included (left lane).

Whole cell extracts obtained from HUVECs were incubated at 37°C with purified recombinant Bep proteins, with F1-ATP added as a substrate, according to a method adapted from Lewallen et al. (2012). A negative control lacking recombinant proteins was included. Following incubation, proteins in the reaction mixture were run on an SDS-PAGE gel and visualised with a fluorescence gel scanner (Figure 3.4). The results are shown in Figure 3.5 below. Each reaction lane contains 3 bands, but no difference was observed between the BhBepA-Fic and BhBepB-Fic reactions and the negative control. This result indicates whole cell extract was not labelled by the recombinant Bep proteins.

Figure 3.5: F1-ATP labelling results. No protein control indicated with a (-). Results show no difference between samples, indicating no AMPylation by recombinant Bep proteins. M = Fluorescent marker, gel used was Novex 8-16% (Life Technologies).

3.3 Discussion

The goal of this part of the project was to label whole cell extract from HUVECs and identify potential AMPylation target proteins of recombinant BepA and BepB from *B. henselae*. Unfortunately, the results from the F1-ATP labelling experiment were inconclusive, as there was no difference between the reactions with the recombinant Bep proteins and the negative control. The bands observed are likely the result of endogenous AMPylation protein activity in the whole cell extract.

There could be a number of factors to explain why no additional bands were observed in the reactions containing recombinant Bep proteins including steric hindrance from the large fluorophore attached to the ATP analogue. As stated by Lewallen et al. (2012), this ATP analogue decreased the rate of AMP transfer by a factor of 10 for VopS. It may also be the case that this substrate results in an even greater decrease in the rate of AMP transfer for BepA and BepB Fic domains. Another factor may be that the target proteins of these Beps are expressed at low levels in the whole cell extracts and was below the level of detection. Finally, the possibility that the recombinant protein fragments were catalytically inactive cannot be excluded.

In order to optimise the experiment, an alternative labelling experiment was employed using a less sterically bulky ATP derivative, as well as positive control recombinant proteins.

3.3.1 Protein Solubility

Three of the proteins expressed in this study, *B. henselae* BhBepC-Fic, *B. quintana* BqBepA1, and *B. quintana* BqBepC-Fic, were unable to be purified successfully due to solubility issues. Despite a large amount of protein being expressed during the preliminary induction, the induced protein was only present in the insoluble fraction during full-scale induction. This indicates that the protein was likely present in inclusion bodies, large spherical particles made up of insoluble protein aggregates⁹⁵. Inclusion body formation is usually the result of the cell's quality control system being unable to repair or remove misfolded or unfolded protein that rapidly accumulations during protein induction⁹⁵. There are several potential factors for why these proteins were insoluble, some of which are described below.

One possible factor is the difference in codon usage between *Bartonella* species and *E. coli*. These two species vary significantly in their relative synonymous codon usage (RSCU)^{96,97}. In particular, the only amino acids with similar RSCU between species are alanine, valine, lysine, glutamine and arginine, along with methionine and tryptophan, which have only a single codon. Target genes containing significant numbers of rare codons particularly in the initiator sequence recognised by the ribosome, are more likely to experience translational stalling in *E. coli*, which could result in low level expression or truncated protein^{95,98}. However, preliminary analysis suggests that all proteins expressed had similar levels of rare codons (around 15 – 21% of codons). The protein constructs also contained an N-terminal His₆ tag, resulting in an initiator sequence that is designed to suit *E. coli*. For these reasons it seems unlikely this is the cause of the solubility issues.

Another factor that could contribute to protein solubility is the presence of large regions of contiguous hydrophobic residues, particularly at the ends of proteins⁹⁸. Although all of the expressed proteins lacked contiguous hydrophobic regions at the N and C termini, previous studies have found that shifting either the N or C terminus of the recombinant protein fragment by as little as 4 amino acids, can have a large effect on protein solubility^{98,99}. As our experiments used recombinant protein fragments, shifting the C-terminus may have resulted in increased solubility.

For future experiments, it may be beneficial to test recombinant protein fragments of several lengths for solubility. There is also the possibility that omission of the BID domain in both BepC-Fic proteins, and the removal of the oligonucleotide/oligosaccharide binding fold in BqBepA1, contributed to loss of solubility. In particular, the full *B. henselae* BepC protein has previously been expressed, and shown to induce invasome formation along with BepF⁵¹, indicating that the protein is soluble, and active, when expressed at full length. Future experiments could look at several methods for increasing solubility of the recombinant proteins before trialling new constructs with the C-terminus shifted, such as those discussed in Chapter 6.

4 Click Chemistry

4.1 Introduction

4.1.1 Copper-catalysed azide-alkyne cycloaddition (CuAAC)

As the F1-ATP labelling experiment did not result in identification of host cell target proteins, an alternative methodology was required. The copper-catalysed azide-alkyne cycloaddition (CuAAC) is the most widely recognised example of click chemistry¹⁰⁰, and provides an alternative to F1-ATP labelling. First described in 2002, it has been used in an expanding range of applications across several different fields. The selective reactivity of azides and alkynes with each other allows CuAAC to be used for the manipulation and labelling of biomolecules of interest, including proteins¹⁰⁰. This high selectivity of CuAAC avoids side reactions, and has the potential to aid in detection of AMPylated substrates.

Grammel et al. (2011) utilised CuAAC to develop a novel alkyne substrate for identifying AMPylated protein targets. N⁶pATP is a derivative of ATP, coupling the NH₂ at the 6 position of adenosine with a propargyl group (Figure 4.1). This N⁶ position was selected based on structural data available for a number of Fido domain-containing proteins, including VopS, IbpA, and BepA¹⁰², thus making it an appropriate ATP analogue for this experiment. The propargyl group can be coupled to various azide molecules, including fluorescent azides and streptavidin-coupled azides, allowing for labelling proteins post AMPylation reaction (Figure 4.2). This has the benefit of omitting large, bulky fluorophores from the AMPylation reaction, as these may have inhibited the F1-ATP labelling reactions. Click chemistry has been used to demonstrate that both VopS and IbpA label distinct proteins in HeLa whole cell extracts, which are consistent with the molecular weight of small GTPases (roughly 21 kDa)^{94,101}.

While Grammel et al. (2011) describe a click chemistry methodology, other groups have published improvements to the basic copper-catalysed azide alkyne cycloaddition, including optimised reagents^{100,103}. Originally TCEP (tris(2-carboxyethyl)phosphine) was described as the reducing agent of choice; however, recent evidence has found that Cu-binding and azide-reducing properties of phosphines can inhibit the CuAAC reaction¹⁰³. For this reason, ascorbate has been proposed as an alternative reducing agent. Another advance was the incorporation of the accelerating ligand THPTA. Previously, the non-water soluble ligand TBTA (Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine) had been used, requiring co-solvents such as DMSO and n-butanol^{100,101,103}.

The amount of THPTA required for a successful CuAAC reaction must be 5 equivalents relative to copper¹⁰⁰. The reason is to intercept and quickly reduce reactive oxygen species that may be generated by ascorbate driven reduction of oxygen present in the reaction mixture. As ascorbate can undergo side reactions with dissolved oxygen, it is important an excess is used relative to copper. Presolski et al. (2011) describe the use of 5 mM ascorbate, which was also used here

Another important factor to consider when performing the CuAAC reaction is the presence of interfering compounds in the reaction buffer. Tris buffer must be avoided during the click chemistry reaction, as Tris will compete with THPTA for copper binding^{100,103}. Similarly, chloride ions above 0.2 M also compete for copper binding¹⁰³. For these reasons Tris-HCl was replaced with HEPES in the RIPA buffer, and salt concentration during the reactions was strictly controlled.

Figure 4.1: Structural comparison of N⁶pATP and ATP. A propargyl group has been added to the N⁶ position of the regular ATP molecule.

Figure 4.2: Basic reaction scheme for the CuAAC click chemistry reaction. The reaction involves a 1,3-dipolar cycloaddition forming a 1,2,3 triazole structure. In this example, the reagents used in the experiments are pictured with 6-FAM azide representing the azide, and N⁶pATP representing the alkyne. The R group represents the labelled protein.

4.1.2 VopS and Bep2-Fic Control Constructs

In order to ensure the labelling reaction was working correctly, positive control proteins were purified. VopS from *Vibrio parahaemolyticus* is the best studied Fic-containing protein and has previously been shown to react with N⁶pATP and label a target protein in whole cell extracts¹⁰¹. Similarly, Bep2 from *B. rochalimae* has also demonstrated AMPylation activity⁸⁴. In particular, Bep2 is related to the Beps studied here, making it an ideal control protein. To produce recombinant proteins without needing to import and culture *Vibrio parahaemolyticus* or *Bartonella rochalimae*, gBlocks from IDT-DNA were designed and ordered, with the resulting DNA fragments cloned into the pET28a vector.

VopS and BrBep2-Fic sequences were confirmed to be correct by aligning with NCBI sequences, using the Geneious 8.1.7 Multiple Alignment function.

4.1.2.1 BepA H159A Mutant

A catalytically inactive *B. henselae* BhBepA-Fic mutant protein was also constructed to include as a negative control for labelling assays. This mutant had the critical and invariant histidine residue of the Fic domain (His159) substituted by an alanine. The site-directed mutation was confirmed by sequencing (Appendix Figure 9.1).

4.2 Results

Figure 4.3: Simplified methodology of the click chemistry labelling experiments. First, protein is extracted from the HeLa cells (whole cell extract). This whole cell extract is treated with recombinant Fic-containing protein (BepA) and N⁶pATP, before subsequently coupling a fluorescent azide to the AMPylated proteins using click chemistry. This results in labelled protein in the whole cell extract. The reaction sample was then run on a SDS-PAGE gel and analysed under a FITC setting. Novel bands were detected by comparison of sample (middle lane) with negative samples (right lane). Fluorescent marker is also included (left lane).

Purified recombinant *B. henselae* Bep proteins, along with recombinant VopS and BrBep2-Fic positive controls, were added to a solution containing whole cell extracts and N⁶pATP and incubated at 30°C. Following incubation, the total protein was precipitated with methanol/chloroform/water, and subjected to treatment with click chemistry reagents. Samples were run on an SDS-PAGE gel and visualised on a gel scanner.

A sample without any added purified protein was included as a negative control. To confirm the role of the Fic enzymatic domain, a Fic catalytic mutant, BhBepA_H159A-Fic, was also included as a control.

Figure 4.4: Results from the Click Chemistry Experiment. No protein control indicated with a (-). Results show no difference between samples, indicating no labelling by recombinant Fic-containing proteins. Two bands appear at the bottom of the gel, one just above the other. M = Fluorescent marker, gel used was Novex 8-16% (Life Technologies).

The results (Figure 4.4) show that similar sized bands were obtained for each of the samples, including both positive and negative controls. All of the lanes had two fluorescent bands of similar size at the bottom of the gel. The bands are likely the unreacted azide, as well as azide that has coupled to any N^6p ATP that remained during the protein purification step. This suggests that the click chemistry reaction did occur, although no labelled protein bands were observed, and would explain why the band occurs at a slightly higher molecular weight than unreacted azide. Unlike the results observed for the F1-ATP reactions, no background proteins were labelled by endogenous AMPylation enzymes present in the whole cell extracts. This could be due to differences between the N^6p ATP and F1-ATP substrates, although this seems unlikely as N^6p ATP is less sterically bulky than F1-ATP.

A more likely explanation is the difference in endogenous proteins expressed in HUVEC and HeLa cells, which represent endothelial and epithelioid cells, respectively.

4.3 Discussion

The goal of this experiment was to develop an improved methodology over the experiments using F1-ATP as a substrate, to label HeLa whole cell extracts and identify protein targets of the Fic domain *B. henselae* Bep recombinant proteins. This click chemistry methodology was adapted from the work by Grammel et al. (2011), while incorporating the advances in click chemistry^{100,101,103}. This methodology was developed to reduce the potential impact of a bulky fluorophore on the ATP analogue, and to determine whether the negative results were due to inactive recombinant proteins. To optimise the experiment, different concentrations of CuSO₄ (0.25 mM or 1 mM) and THPTA (1.25 mM or 5 mM) were trialled, along with differing incubation lengths for both the AMPylation and subsequent CuAAC experiment. Similarly, the AMPylation reaction was optimised by incubation 30°C, rather than 37°C, as used with the F1-ATP experiment. Previous studies of Fic domain proteins VopS, Bep2, and BepA have described reaction incubations at 30°C^{66,84,101}.

Unfortunately, no bands were identified in either the VopS or BrBep2-Fic controls, or the BhBepA-Fic and BhBepB-Fic samples, despite optimising reaction conditions. Click chemistry reactions were trialled for several incubation times, from 60 minutes up to overnight, while reactions were carried out at temperatures ranging from ambient to 37°C. AMPylation reactions with N⁶pATP were also trialled for 60 and 90 minutes at 30°C. No differences were observed for any of these reactions, suggesting additional time does not benefit either the click chemistry or AMPylation reactions. While the concentrations reported appear to give an effective click reaction, experiments utilising lower concentrations of CuSO₄ (0.25 mM) and THPTA (1.25 mM) also gave similar results. Based on these results and those obtained from the F1-ATP labelling experiments, we concluded that the purified protein constructs were inactive. The limited time allowed for this research project precluded extensive optimisation of protein purification to retain activity.

5 Vimentin, Pyrophosphate, and MS Assays.

5.1 Introduction

5.1.1 *B. rochalimae* Bep2 and Vimentin

While the functions of many of the *Bartonella* species effector proteins are unknown, the host cell target protein of Bep2, from the recently discovered human pathogen *B. rochalimae*⁸³, has been identified by Pieleles et al. (2014). Unlike *B. henselae* and *B. quintana*, *B. rochalimae* is a member of the Group B *Bartonella* species³⁷. While there are significant differences between Groups B and C, such as the Trw T4SS in Group C, and the flagella in Group B, there are also some similarities. Both Groups contain a least one VirB/D4 T4SS and associated Bep effector proteins. Furthermore, there is increasing evidence of gene transfers occurring between these two *Bartonella* Groups³⁷. The phylogenetic relationship between *B. rochalimae* Bep2, the *B. henselae* Beps, and other *Bartonella* Fic-containing proteins, discussed in Chapter 1, is illustrated in Figure 5.1. From the phylogenetic tree it is clear that the *B. henselae* Beps separate into two distinct categories: those containing Fic domains, and those without. The tree also demonstrates that both BepC proteins from *B. henselae* and *B. quintana* are more distantly related to the other Fic-containing Beps, including Bep2 from *B. rochalimae*. Interestingly, *B. rochalimae* Bep2 clusters closely with BepA1 from *B. quintana*, which consists solely of the Fic domain, and lacks a BID domain. Pairwise alignment of BepA from *B. henselae* with Bep2 from *B. rochalimae* reveals that the largest difference between these two proteins is in the BID domains of the proteins. Similar results are observed between BepB from *B. henselae* and Bep2 from *B. rochalimae*.

Using a mass spectrometry based approach, with stable isotopically labelled ATP molecules, Bep2 was confirmed to target vimentin, a 50 kDa type III intermediate filament protein of the cytoskeleton, in mouse macrophage cells⁸⁴. Vimentin is also found in human mesenchymal-derived cells and may also be a target protein of BepA⁸⁵.

This hypothesis was supported by Palanivelu et al. (2011) who found that a BepA Fic fragment radioactively labelled an unknown 50 kDa protein in S100 HeLa cell lysate. Vimentin shows a high degree of sequence morphology between species, and has a role in several cellular functions, including maintenance of the integrity of the vascular endothelium⁸⁵.

This suggests that vimentin may play an important role in the ability of *Bartonella* to colonise endothelial cells, and could be a potential target of *B. henselae* and *B. quintana* Fic-containing Beps.

Figure 5.1: Cladogram of protein sequences for major *Bartonella* Bep proteins. The cladogram contains Beps used in this study and other previous studies. A clear distinction is observed between Beps that contain a Fic domain, and those that don't. VbhT from *B. schoenbuchensis* was used as the outgroup. Cladogram was created using Geneious 8.1.7 with Jukes-Cantor Genetic Distance Method using a Global Alignment Setting.

5.1.2 SpyA and Vimentin Polymerisation

Interestingly, Bep2 is not the only bacterial toxin shown to modify host vimentin by transferring a chemical group. SpyA, a toxin produced by the gram-positive human pathogen, *Streptococcus pyogenes*, modifies and inactivates host vimentin by ADP-ribosylation at the head domain^{89,104}. Actin is also modified by SpyA, though to a lesser extent. ADP-ribosylation of vimentin by SpyA interferes in the polymerisation of vimentin monomers, leading to collapse of the vimentin cytoskeleton and defects in wound repair^{89,104}.

Icenogle et al. (2012) describe an *in vitro* experiment to detect inhibition of vimentin polymerisation. This method involves incubating recombinant hamster vimentin with SpyA and the enzyme substrate, NAD⁺. This was followed by splitting the reaction in half and inducing polymerisation in one sample with NaCl, and leaving the other sample unpolymerised by adding only water. Samples were subjected to ultracentrifugation to separate polymerised vimentin from soluble vimentin monomers. Supernatants from all samples were then run on an SDS-PAGE gel before being subjected to western blotting using anti-vimentin antibodies. Samples treated with water show a single band that acts as a positive control for antibody binding. The presence of a band in the NaCl treated samples indicates that vimentin polymerisation was inhibited by the ADP-ribosylation reaction from SpyA. This assay could be adapted for the *B. henselae* recombinant Bep proteins.

5.1.3 Pyrophosphate Analysis and Mass Spectrometry.

The vimentin polymerisation assay allowed for analysis of recombinant *B. henselae* proteins, without competing reactions for ATP or interfering compounds from whole cell extract, and utilised ATP, rather than an analogue. However, there were several reasons why the assay might not work. For example, vimentin may not be a target of AMPylation, or, if it is, the AMPylation may not occur on a part of the protein that would result in inhibition of vimentin polymerisation. For these reasons other experiments were also carried out.

An alternative approach used was the detection of the by-products from the AMPylation reaction carried out by recombinant Fic proteins. Based on catalytic mechanisms (Figure 1.3, Figure 1.4), the AMPylation reaction also produces pyrophosphate. Measuring pyrophosphate levels in the buffer, following the AMPylation reaction represents an indirect way to detect AMPylation activity against vimentin in the reaction mixture.

There is also the possibility that the recombinant proteins had AMPylation activity against host cell extract proteins, but that this wasn't observed during the labelling experiments due to problems with the substrates. In order to test this, pyrophosphate assays were also performed against whole cell extract obtained from HUVECs.

For these reactions, the same method was used as for the F1-ATP experiments, except that F1-ATP was substituted with ATP, and the reaction was run for 3 hours at 37°C.

In addition, a mass spectrometry analysis of vimentin, and whole cell extract samples was also performed on reactions for pyrophosphate analysis. Mass spectrometry analysis was previously used to identify AMPylation of Cdc42 by VopS⁷¹. A similar technique could be used for vimentin, or even possibly whole cell extract samples, by detecting an increase in the mass of peptide fragments that corresponds with the mass of AMP.

5.2 Results

5.2.1 Vimentin Polymerisation

Figure 5.2: Vimentin Polymerisation Results. Polymerisation was induced by addition of 150 mM NaCl (+) or not induced by addition of water (-). No difference was observed for any of the four reaction samples (the fainter band for H159A was due to a dilution before running on the gel due to low solution volume remaining).

Recombinant Syrian hamster vimentin was combined in a reaction buffer with recombinant BhBepA-Fic and BhBepB-Fic proteins, along with ATP for 3 hours at 37°C. Both the BhBepA_H159A-Fic catalytic mutant, and a sample without any added recombinant Fic protein, were included as negative controls. Following the reaction, half of each mixture was treated with NaCl to induce polymerisation. Polymerised vimentin was pelleted by ultracentrifugation. The supernatant was applied to an SDS-PAGE gel and subjected to western blot analysis. The presence of a band indicates that (non-polymerised) vimentin monomers were present; absence of a band indicates that the vimentin monomers were incorporated into polymerised vimentin, and removed by ultracentrifugation.

Figure 5.2 illustrates the results from western blot analysis. For all samples, polymerisation occurred when NaCl was added to the reactions, indicating that none of the recombinant Bep proteins were able to inhibit vimentin polymerisation.

Although the BhBepA_H159A-Fic non-polymerisation sample contains a fainter band than the other samples, this was likely due to a dilution that occurred during the preparation, and was not considered significant. This result indicates neither the Fic domain of BepA or BepB inhibited vimentin polymerisation.

The click chemistry results indicated that no host cell proteins were labelled by BrBep2-Fic, suggesting that the purified recombinant protein may have been inactive. Therefore, the vimentin polymerisation assay was not carried out with this protein.

5.2.2 Pyrophosphate Assay

In order to test the recombinant *B. henselae* proteins BhBepA-Fic and BhBepB-Fic for activity, the amount of pyrophosphate present in these samples was quantified using the ab112155 fluorometric pyrophosphate kit (Abcam). This assay provides sensitive detection of pyrophosphate, detecting between 0.1 – 100 μ M pyrophosphate. BepA_H159A-Fic, as well as a sample with no added recombinant Fic protein, were included as negative controls. AMPylation reactions were carried out against vimentin or whole cell extract using ATP (Thermo Scientific) for 3 hours at 37°C. Pyrophosphate levels were measured post AMPylation reaction, and the difference in *B. henselae* BhBepA-Fic and BhBepB-Fic samples was compared against the negative controls to determine if these proteins displayed any AMPylation activity.

The assay results showed no significant change in pyrophosphate levels between BhBepA-Fic, BhBepB-Fic and negative controls for either the vimentin samples or the whole cell extract samples (Appendix Figure 9.16, Appendix Figure 9.17). This is consistent with the results from the polymerisation assay, and the previous labelling experiments, which did not detect AMPylation.

5.2.3 LC-MS/MS

There was a possibility that vimentin was AMPylated by one or both recombinant proteins, but that the AMPylation did not inhibit polymerisation. Similarly, it was possible that the pyrophosphate assay was not sensitive enough to detect minor differences in levels of pyrophosphate production. Both the vimentin and whole cell extract samples from the pyrophosphate analysis were, therefore, analysed by LC-MS/MS, and selected for peptides that had been covalently modified by an AMP moiety.

Results from the LC-MS/MS analysis showed no evidence of AMPylation modification for any of the samples (results not shown). For the vimentin only samples, both databases did find top peptide matches to vimentin, confirming that MS analysis was correctly detecting and analysing peptide fragments.

The complexity of the whole cell extract samples made data analysis difficult to draw conclusions from, and suggests that the LC-MS/MS analysis is better suited for a smaller scale system, such as that from extracted bands in a gel, or a single protein assay, like that for vimentin, or Cdc42, as described previously⁷¹. These results are consistent with those from previous experiments. In summary no evidence of vimentin modification, or AMPylation activity, by either BhBepA-Fic or BhBepB-Fic was detected.

5.3 Discussion

The goal of this part of the project was to determine if the *B. henselae* BhBepA-Fic and BhBepB-Fic recombinant proteins were able to AMPylate vimentin, and if this modification prevented polymerisation. The second goal was to determine if the recombinant Fic proteins displayed any AMPylation activity. The experimental results observed here all support those found by the F1-ATP and click chemistry labelling experiments, and support the hypothesis that the protein purification did not result in enzymatically active recombinant proteins.

5.3.1 Vimentin Polymerisation

The vimentin polymerisation reactions were initially performed without a positive control. BrBep2-Fic was selected as a positive control, however there were delays with purifying this protein. For this reason, a follow up experiment using BrBep2-Fic was planned. However, once the negative result from the click chemistry experiment was observed (section 4.2), the vimentin experiments were not repeated with BrBep2-Fic.

If solubility and purification of recombinant proteins can be improved to give active Fic domain proteins, including BrBep2-Fic, the experiment could be revisited. However, the effect of the AMPylation of vimentin by BrBep2-Fic on vimentin polymerisation is not known, so an alternative positive control may also be required. Previously SpyA has been shown to inhibit vimentin polymerisation by ADP-ribosylating the head region⁸⁹. For this reason, a SpyA control reaction, with NAD⁺ instead of ATP as the substrate, should also be included.

In order to obtain recombinant SpyA, a gBlock for *spyA* could be constructed, as was done for *vopS* and *bep2*.

5.3.2 Pyrophosphate assay

The negative results from the pyrophosphate assay are consistent with the evidence suggesting the recombinant proteins are enzymatically inactive. There was however, a limitation with this assay. While the level of detection of pyrophosphate was sensitive to low levels of pyrophosphate, there was significant variation in fluorescence between replicates for both the standards and the samples. This variability made it difficult to draw conclusions from the results. Despite this limitation, detection of pyrophosphate production could be revisited if recombinant proteins can be purified in an active state. In particular, tests using recombinant vimentin and BrBep2-Fic or Cdc42 and VopS could be undertaken to test improved purification methods. Using these proteins with recombinant substrates would allow for testing in the absence of interfering factors that may be present in whole cell extract, and could give a good indication of activity for the other recombinant proteins studied here. Once protein purification is optimised, and target proteins identified by click chemistry, future experiments looking at kinetic studies of Fic-containing proteins using pyrophosphate assays could also be undertaken. This could potentially involve a similar set up to the vimentin and pyrophosphate experiments, with vimentin being substituted for the relevant target protein. This assay could then be performed over a 30 minute period, with the pyrophosphate levels being measured every five minutes in a 96 well plate reader.

6 Research Motivation, Key Findings, and Future Directions

6.1 Research Motivation

With the recent discovery of the Fic domain function, there have been an increasing number of studies looking at the significance of this domain in bacterial proteins. In particular, Fic domains have been characterised in a number of secreted toxins from several bacterial pathogens including; *Vibrio parahaemolyticus* (VopS), *Legionella pneumophila* (Ank), *Histophilus somni* (IbpA), and *B. rochalimae* (Bep2)^{71,74-76,84}. For these proteins, both the covalent modification and the host interaction target have been described. These proteins all catalyse post-translational modifications, most commonly AMPylation of the Rho GTPase central signalling molecules, preventing them from binding downstream effectors and resulting in cytoskeletal collapse. In the case of VopS and IbpA, the catalytic mechanism has also been proposed^{79,80}. Both *B. henselae* and *B. quintana* express Fic domain-containing proteins that are directly secreted into the host cell by a Type IV secretion system, although these haven't been as extensively studied. *B. henselae* expresses seven secreted effector proteins, three of which contain a Fic domain (BepA – BepC)^{15,66}, with BepA being the best studied example. This protein has already been shown to possess AMPylation activity⁶⁶. Unlike the proteins described above, the BepA host target is not known, but it does not appear to AMPylate Rho GTPases, nor does it cause cytoskeletal collapse.

In this study we set out to identify the host cell target proteins that the Fic domains of the *Bartonella henselae* BepA, BepB, BepC, as well as the *B. quintana* BepA1 and BepC proteins are acting on. Due to issues around solubility, this was limited to BepA and BepB from *B. henselae*. In order to accomplish this, fluorescent probes were utilised as labels to identify AMPylated host cell protein targets. These fluorescent probes are preferable to radioactive probes, which have been utilised in previous studies as they allow for easier detection, and don't require specialised disposal^{88,94,101}. The fluorescent probes contain a FITC moiety that can be used to purify labelled proteins by immunoprecipitation with anti-FITC antibodies. This allows for purification and enrichment of the samples before running on an SDS-PAGE gel and excising for MS analysis if required.

Another approach was to research related *Bartonella* proteins with reported Fic domain activity and known host cell protein targets. Currently, Bep2 is the only *Bartonella* effector studied that contains a Fic domain and that has a known host cell protein target. Similarities between Bep2 and the *B. henselae* Beps studied suggested they could have the same host cell target, vimentin. This formed the basis for the vimentin assays.

6.2 Key Findings

All experiments performed resulted in negative results. This included experiments using purified control proteins that had previously been shown to be active. Taken together, the results suggest that the purified proteins were enzymatically inactive. This is supported by the lack of host cell protein labelling using both F1-ATP and N⁶pATP, despite using VopS, a positive control Fic domain protein that had previously been shown to AMPylate target protein in whole cell extract with both ATP analogues used here. This conclusion was also supported by lack of AMPylation activity detected in the pyrophosphate assay and LC-MS/MS analysis.

Finally, while both *B. henselae* and *B. quintana* BepC-Fic proteins and *B. quintana* BqBepA1 were successfully induced with IPTG, they could not be isolated during protein extraction.

6.3 Future Directions

6.3.1 Improving Recombinant Protein Purification

Before future experiments can be designed in order to determine the host cell protein targets of the *B. henselae* and *B. quintana* Fic domain-containing Bep proteins, the purification methods must be improved.

In these experiments, bacterial cell lysis was performed with B-PER reagent (Pierce). This system contains Tris-HCl (20 mM) and a proprietary, non-ionic, detergent to lyse the bacteria. While this reagent is described as mild, using it without additives could result in destabilised protein. One way that this problem could be avoided is by adding NaCl to the lysis buffer. High ionic strength is recommended in protein extraction buffer, as it enhances protein solubility and stability⁹⁹. Previous extractions involving VopS, IbpA, and Bep2 have all been described with the addition of between 0.1 – 0.2 M NaCl^{75,79,84,88}. In the case of Bep2, MgCl₂ (10 mM) was also added to the lysis buffer. This could be helpful in this instance as Mg²⁺ has been described as a component of the active site for both VopS and BepA^{66,79}.

Similarly, many of the published lysis buffers for Fic-containing protein purification include either β -mercaptoethanol (2.5 – 5 mM)^{84,88} or DTT (1 mM)^{79,101} to prevent oxidation of the expressed proteins. Other additives to the lysis buffer could include DNase and lysozyme to aid in breakdown of nucleic acid and cell wall material to remove particularly viscous material before adding extracted protein to the nickel affinity columns⁹⁹. Finally, addition of EDTA-free protease inhibitors could also be beneficial.

While the method we used has the benefit of being a simple and inexpensive method, it does involve the use of chemical additives to lyse the cell. In particular, Tris inhibits the click chemistry reaction by competing with the THPTA ligand for copper binding¹⁰³. For these reasons, a buffer containing HEPES or phosphate is recommended. It is also not known what the detergent is in B-PER, or how it could be interacting with the expressed proteins. Previous studies have used NP-40⁷⁵, or Triton X-100 as mild detergents. For these reasons, it is recommended in future protein extractions to use a lysis buffer containing 0.5 – 1% of either of these detergents, as well as the additives described.

Alternatively, mechanical lysis methods such as a French press or sonication could be utilised. Mechanical lysis has the benefit of avoiding chemicals that could potentially denature or inactive expressed proteins, but does require costlier equipment to perform. These methods have previously been used to purify several of the Bep and Fic-containing proteins, including Bep2 and VopS^{79,84}.

6.3.2 Protein Solubility

As the *B. henselae* and *B. quintana* recombinant proteins BhBepC-Fic, BqBepA1, and BqBepC-Fic were insoluble and therefore not purified, a potential area to further explore are improvements in protein solubility.

6.3.2.1 Altering Induction Conditions

One of the most common methods to increase protein solubility is to decrease the temperature during the protein induction. Lower temperatures slow down all cell processes, resulting in lowered protein aggregation in inclusion bodies, as well as reduced activity of proteases^{95,98,99}. This approach was attempted for BqBepA1, by inducing at 18°C for 24 hours without success; however, due to time constraints with the project, this was not attempted for either of the BepC-Fic proteins which may have more success.

Another approach would be to reduce the concentration of IPTG used for induction. Several studies have observed increased solubility and activity using a lower IPTG concentration than recommended⁹⁸. Previous studies of VopS, Bep2, BepA, IbpA, along with other Bep proteins all describe using between 0.05 – 0.5 mM IPTG, with many recommending 0.4 mM, rather than 1 mM, as was used here^{23,40,51,60,62,66,71,75,79,84,88,101}. Another factor to consider is the pH of the media, as fluctuation may denature proteins depending on their isoelectric points. To overcome this, a buffered media may be appropriate. Similarly, pH alteration of the media may also aid in solubilisation if the isoelectric point of the induced protein is particularly high or low⁹⁵. Along these lines, an alternative media such as Terrific Broth (TB), could also aid in solubilisation. TB is a widely used media that is specifically formulated to increase protein solubility, as it contains both glycerol and phosphate buffer^{84,98,99}. Alternatively, 2X YT media has also previously been described^{75,99}. Supplementing the media with co-factors of the recombinant Fic proteins could also benefit solubility, as several proteins require co-factors to fold correctly^{95,99}. Crystal structure studies with BepA and VopS have shown that Mg^{2+} ions appear to act as a co-factor in the binding site, and it is likely that the other Fic-containing proteins would also require magnesium ions^{66,79}. While yeast extract does contain Mg^{2+} , there is the possibility that increasing the amount of Mg^{2+} ions in the media could aid in protein solubility. Both TB and 2X YT media contain significantly more yeast extract, so these media should also be trialled in future experiments.

Other additives to the media include chemical chaperones such as sugars and amino acids that inhibit protein aggregation during refolding of misfolded or unfolded protein, can also be effective¹⁰⁵. Some examples of useful sugars include sorbitol, a sugar alcohol formed by reduction of glucose, and trehalose, a disaccharide of glucose. For example, sorbitol has previously been shown to increase fluorescence of GFP 12 fold, an indication of increased solubility¹⁰⁵.

Some examples of helpful amino acid additives include arginine and glycylglycine. The latter is a dipeptide of glycine that has previously been shown to increase solubility of PPE proteins by 170-fold¹⁰⁶

These strategies could also be applied to the enzymatically inactive recombinant proteins successfully purified in this study. While these proteins were soluble, it is possible that the inactivity observed was due to misfolding during protein induction. For this reason, many of these suggestions may also aid in purifying enzymatically active proteins. In particular, inductions should be trialled using a lower induction temperature, using an alternative media such as TB, and including additives that aid in protein folding such as sorbitol, arginine, or additional MgCl_2 in the media.

6.3.2.2 Fusion Tags

If these strategies were not able to increase protein solubility, alternative fusion tags could be employed, although this would require creating new expression constructs. There are several fusion tags that can improve protein solubility or promote folding, some of which are described below.

The two most common fusion tags that enhance solubility are thioredoxin (Trx) and maltose-binding protein (MBP). Trx aids in both solubilisation of proteins and in disulfide bond formation, although it is not sufficient for protein purification without a second tag⁹⁸. Trx also typically works best at the N-terminus, so if a His_6 tag were to be used in purification, it would need to be C-terminal. MBP is an alternative fusion tag that has the benefit of passively promoting folding of its fused protein partner⁹⁸. MBP also has the benefit of use as a selection agent during purification, as it can bind sugars bound to agarose or sepharose beads⁹⁸. The downside to MBP is that it is a large protein (42 kDa) and this size is more likely to interfere with protein activity. For this reason MBP is usually cleaved before assays are performed. It can also solubilise misfolded proteins, resulting in time wasted on purifying inactive proteins^{98,99}.

Other fusion tags include N-utilisation substance A (NusA), a transcription termination/anti-termination factor that was shown to be effective at solubilising proteins⁹⁸. However, NusA is a large protein (55 kDa) and requires a purification tag. Solubility enhancing tags (SETs) consist of small, highly acidic peptides and help promote soluble expression of folded proteins that are prone to aggregation could also be utilised⁹⁸. The main benefit of these tags is their small size, typically <40 amino acids.

Finally the small ubiquitin like modifier (SUMO) tag, is small (11.2 kDa) and has a specific protease for cleavage, therefore avoiding the risk of cleavage of target protein⁹⁸. The only downside is it must be N-terminal and can't precede a proline moiety.

It is worth noting that most fusion tags benefit solubility by being in the N-terminal position. N-terminal tags also have the benefit of exposing the ribosome to an initiator sequence that is suitable for *E. coli*⁹⁹. If a second purification tag, such as His₆ or GST, is required, it is recommended that it be at the C-terminus position. In particular, the His₆ tag can benefit from being C-terminal as when purification occurs, only fully translated proteins will be purified.

6.3.2.3 Molecular Chaperones

Finally, co-expression with molecular chaperones could enhance protein solubility by aiding in protein folding^{95,98,99}. For example both the DnaK-DnaJ-GrpE and GroEL-GroES systems bind-solvent exposed hydrophobic domains, preventing peptide aggregation⁹⁸. One study has shown that by overexpressing DnaK-DnaJ-GrpE, either by a plasmid system, or by adding 3% v/v ethanol to the media, the solubility of preS2-S'- β -galactosidase was increased 2-fold¹⁰⁷. Most recombinant proteins favour one of these two chaperone systems, so it is best to test each system separately. The downside to using one of these two chaperone systems however, is that they have reduced activity when cultured at lower temperatures. When lower temperatures are desired, the cold-adapted Cpn10-Cpn60 system, can be utilised instead⁹⁸.

When using molecular chaperones, it is important to overexpress them before expressing the recombinant protein, as insoluble proteins are unable to re-fold once in inclusion bodies¹⁰⁷.

6.3.3 Follow-up Experiments

As discussed, the click chemistry assay appears to work in terms of coupling the azide and alkyne, but future efforts must be concentrated on expressing soluble, active proteins. This is an essential prerequisite for subsequent experiments, such as whole cell extract labelling, click chemistry experiments, or mass spectrometry analysis of potential targets. If purification of labelled proteins is required before mass spectrometry analysis, an alternative azide tag can also be utilised along with the 6-FAM-azide. For example, Grammel et al. (2011) utilised a biotin tagged azide for mass spectrometry analysis. This allowed for purification of labelled protein on streptavidin columns.

There are several biotin-tagged azide derivatives available, and this method could be used in future experiments over anti-FITC antibodies, which could have issues with non-specific binding.

Alternatively, before click chemistry experiments are undertaken, both recombinant vimentin and Cdc42 could be used in pyrophosphate assays and mass spectrometry analysis to test protein purification methodology for enzymatically active recombinant Fic proteins. Similarly, if BrBep2-Fic can be shown to have activity during whole cell extract labelling, then vimentin polymerisation assays could also be revisited with this enzyme, as well as SpyA from *S. pyogenes* to act as a positive control⁸⁹.

If these experiments were to successfully identify target protein, additional experiments could also be performed.

6.3.4 Host Cell Protein Analysis

Analysis of any identified AMPylated host cell proteins could be undertaken. This could include changes in expression or localisation in the presence and absence of recombinant Fic protein. For proteins of known function, changes in activity could be further studied, such as how downstream effectors of the protein may be affected. The effects of target protein modification on host cell morphology and integrity could also be analysed. This could involve fluorescence microscopy techniques, to identify changes to host cell morphology, as well as analysis of markers that indicate cellular health.

During this project, both BhBepA-Fic and BhBepA_H159A-Fic were cloned into a eukaryotic expression system, with a GFP C-terminal fusion tag, for transfection and expression in cultured human cells. However, due to time constraints, these plasmids were not further developed. With this system, analysis of wound repair, intracellular localisation of Beps using fluorescence microscopy, as well as staining for effects on potential intracellular protein targets, could be looked at. Similarly, changes in mRNA expression could also be analysed to determine the effect of BhBepA-Fic expression on host cell protein expression. Similar constructs could also be made for the other Beps studied if required.

6.3.5 Fic Activity Inhibitors

Future work could also look at inhibitors of the recombinant Bep proteins. This could involve creating an assay to screen a compound library for potential molecules that inhibit the activity of the Fic domain in these proteins. Those identified in an initial screen could be tested under a number of different assays such as an internalisation assay for *Bartonella henselae* Houston-1 strain. If such an inhibitor was effective this could be further characterised as a potential *Bartonella*-specific antibiotic. Similar work has already been investigated for VopS. Lewallen et al. (2014) have described an assay using F1-ATP and Cdc42 to test for inhibition of VopS. This assay resulted in identification of several compounds with moderate efficacy in inhibiting VopS AMPylation of Cdc42¹⁰⁸. This suggests that a similar assay could be developed for the recombinant Fic proteins studied here.

7 Concluding Remarks

This work built on previous studies investigating novel ATP analogues for successful labelling and identification of host cell protein targets in whole cell extract^{88,101}. The key goal of this project was to identify novel targets of the *B. henselae* and *B. quintana* Fic domain-containing Bep proteins. While potential protein targets of BhBepA-Fic and BhBepB-Fic were not identified, significant progress was made in optimising whole cell extract recovery and labelling reaction conditions. This will provide a good starting point for future experiments once active Fic domain proteins can be expressed and purified.

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9 Appendix

9.1 Sequence Alignments

The following sequence alignments were performed using the multiple sequence alignment tool ClustalOmega (Goujon et al., 2010; Sievers et al., 2011) (available online at www.ebi.ac.uk/Tools/msa/clustalo/ - 23/5/16).

9.1.1 *Bartonella henselae* BepA-Fic H159A mutant design

BhBepA Fic fragment	ATGCCAAAGGCAAAAGCAAAAACGAAAAATACAGAAATTATTTCCCCCATCATTAT
BhBepAFicH159A_40	ATGCCAAAGGCAAAAGCAAAAACGAAAAATACAGAAATTATTTCCCCCATCATTAT
consensus	*****
BhBepA Fic fragment	GTCTATCCTAACACCACAACACTCAAAAAATAATATGGAATAAAAACTTAAATGCT
BhBepAFicH159A_40	GTCTATCCTAACACCACAACACTCAAAAAATAATATGGAATAAAAACTTAAATGCT
consensus	*****
BhBepA Fic fragment	TTTCTGGAAAAATGCTCGCATGATACAGCCAAAGCAATGATCAATCTGCGTGAAGAA
BhBepAFicH159A_40	TTTCTGGAAAAATGCTCGCATGATACAGCCAAAGCAATGATCAATCTGCGTGAAGAA
consensus	*****
BhBepA Fic fragment	TCCTTACCAGAATATTTTGATACCGCCTATCTATGCCATATTCATCAACAGTTGTTT
BhBepAFicH159A_40	TCCTTACCAGAATATTTTGATACCGCCTATCTATGCCATATTCATCAACAGTTGTTT
consensus	*****
BhBepA Fic fragment	AAAAATACATTTGAATGGGCTGGATACCTTCGCCATATCCCCTTCACATTTGCAGAT
BhBepAFicH159A_40	AAAAATACATTTGAATGGGCTGGATACCTTCGCCATATCCCCTTCACATTTGCAGAT
consensus	*****
BhBepA Fic fragment	GGCACCACAGCCGCCATGCCAGAAATGAAAAGAACGGGTGGAAAAATGCTTTTGCA
BhBepAFicH159A_40	GGCACCACAGCCGCCATGCCAGAAATGAAAAGAACGGGTGGAAAAATGCTTTTGCA
consensus	*****
BhBepA Fic fragment	ATTGGTGATGAGATCCAAGAAGGCTTGCAAAGATTAGATCAAACACTTGCCGAAAAA
BhBepAFicH159A_40	ATTGGTGATGAGATCCAAGAAGGCTTGCAAAGATTAGATCAAACACTTGCCGAAAAA
consensus	*****
BhBepA Fic fragment	AACAATTTACAAGGTTTAACGCGCAAGAATTTAACTCTGAAGCAATAGAAGTGT
BhBepAFicH159A_40	AACAATTTACAAGGTTTAACGCGCAAGAATTTAACTCTGAAGCAATAGAAGTGT
consensus	*****
BhBepA Fic fragment	AATTCTCTCAACCAACTCCACCCATTAGAGAGAGGAATGGACGAACACAACGGCTA
BhBepAFicH159A_40	AATTCTCTCAACCAACTCCACCCATTAGAGAGAGGAATGGACGAACACAACGGCTA
consensus	*****
BhBepA Fic fragment	TTTTTTGAAAAATCTTGCCAAAGCAGCAGGACATCAGCTTAACTTCTCGCTTATCACA
BhBepAFicH159A_40	TTTTTTGAAAAATCTTGCCAAAGCAGCAGGACATCAGCTTAACTTCTCGCTTATCACA
consensus	*****
BhBepA Fic fragment	AAAGAACGCATGATGGTTGCCAGTGTGCGGTAGCAGAAAATGGTGACCTAGAACCT
BhBepAFicH159A_40	AAAGAACGCATGATGGTTGCCAGTGTGCGGTAGCAGAAAATGGTGACCTAGAACCT
consensus	*****
BhBepA Fic fragment	ATGCAACATTTGTTTGAGGATATCTCCAATCCAGAAAAAATTCGTCTTTTAAAGAA
BhBepAFicH159A_40	ATGCAACATTTGTTTGAGGATATCTCCAATCCAGAAAAAATTCGTCTTTTAAAGAA
consensus	*****

BhBepA Fic fragment	TTTATGCACACCATGAAAAATACTGGGCGCAATGTTAATGATCGTCCTGTTATGGTT
BhBepAFicH159A_40	TTTATGCACACCATGAAAAATACTGGGCGCAATGTTAATGATCGTCCTGTTATGGTT
consensus	*****
BhBepA Fic fragment	GCAAAGGAAGGGGAACTTACACGGGCACCTATAGAGGTGCTGGTCTTGAAGGTTTT
BhBepAFicH159A_40	GCAAAGGAAGGGGAACTTACACGGGCACCTATAGAGGTGCTGGTCTTGAAGGTTTT
consensus	*****
BhBepA Fic fragment	GCACTCAATGTAAAAGGTGCTTACATTATCGGCAATATAGACCACCTCCCACCAGAA
BhBepAFicH159A_40	GCACTCAATGTAAAAGGTGCTTACATTATCGGCAATATAGACCACCTCCCACCAGAA
consensus	*****
BhBepA Fic fragment	CAACTAAAAATATTAAAACTGGTGATAAAATTACCTTTACAGCTCCAAAAGCCTGA
BhBepAFicH159A_40	CAACTAAAAATATTAAAACTGGTGATAAAATTACCTTTACAGCTCCAAAAGCCTGA
consensus	*****

Appendix Figure 9.1: BhBepA-Fic H159A mutant sequence alignments. BepA sequence data was obtained from NCBI for *B. henselae* Houston-1 strain for the gene fragment expressed in this study and the additional stop codon. Blue indicates base changes introduced in the H159A Fic domain catalytic mutant

9.1.2 *B. henselae* BepB-Fic Alignment

pET28a+BepBFic_insert BhBepBFic_19	GACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTA GACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTA *****
pET28a+BepBFic_insert BhBepBFic_19	ACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCACAGCAGCGGC ACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCACAGCAGCGGC *****
pET28a+BepBFic_insert BhBepBFic_19	CTGGTGCCGCGCGGCAGCCATATGCCAAAAGCAAAAGCAAAAAATATCTCCACCGCTTCT CTGGTGCCGCGCGGCAGCCATATGCCAAAAGCAAAAGCAAAAAATATCTCCACCGCTTCT *****
pET28a+BepBFic_insert BhBepBFic_19	CCCCATAATTATATCTATCCTGGTACCCAAATACTCAAAAATAAATATGGAGAAACAGAT CCCCATAATTATATCTATCCTGGTACCCAAATACTCAAAAATAAATATGGAGAAACAGAT *****
pET28a+BepBFic_insert BhBepBFic_19	TTAAAACTCCTTCTGGAAAAATGCTTACATGATAGGGAACAAGCAATGATGAATCTGCGT TTAAAACTCCTTCTGGAAAAATGCTTACATGATAGGGAACAAGCAATGATGAATCTGCGT *****
pET28a+BepBFic_insert BhBepBFic_19	GCAGAATCCCTACCAGAATATTTTGATTGCGCTTATCTATGCTATATCCACCAACAATTG GCAGAATCCCTACCAGAATATTTTGATTGCGCTTATCTATGCTATATCCACCAACAATTG *****
pET28a+BepBFic_insert BhBepBFic_19	TTTCAAAAAACCTTTGAATGGGCTGGCCATCTCCGCCACACTCCCTTCACATTTGCAGAT TTTCAAAAAACCTTTGAATGGGCTGGCCATCTCCGCCACACTCCCTTCACATTTGCAGAT *****
pET28a+BepBFic_insert BhBepBFic_19	GGTAGCATTGCCGCCATGCCAGAAATGAAAAGAACAGAAATGGGGCAAAGATTTTGCAAAA GGTAGCATTGCCGCCATGCCAGAAATGAAAAGAACAGAAATGGGGCAAAGATTTTGCAAAA *****
pET28a+BepBFic_insert BhBepBFic_19	AGTGAAAAAATCCCCGAACCTTTTACAAAAATTAGATCAAGAGCTTGCCGAAAAAGACAAT AGTGAAAAAATCCCCGAACCTTTTACAAAAATTAGATCAAGAGCTTGCCGAAAAAGACAAT *****
pET28a+BepBFic_insert BhBepBFic_19	TTGCAAGGCTTAACACGCGAAGAGTTTATCAAGCAAGCAACGGAGCTATTTTATTCTCTT TTGCAAGGCTTAACACGCGAAGAGTTTATCAAGCAAGCAACGGAGCTATTTTATTCTCTT *****
pET28a+BepBFic_insert BhBepBFic_19	CACAAAATCCACCCATTCATAGATGGCAATGAACACACAGAACAATTCCTTTTTGAAAAAT CACAAAATCCACCCATTCATAGATGGCAATGAACACACAGAACAATTCCTTTTTGAAAAAT *****
pET28a+BepBFic_insert BhBepBFic_19	CTCGCCAAAGCCGCCGACATCAACTTGACTTTTCGCTTGTCACACAAAAACGCATGATG CTCGCCAAAGCCGCCGACATCAACTTGACTTTTCGCTTGTCACACAAAAACGCATGATG *****
pET28a+BepBFic_insert BhBepBFic_19	GCAGTCTGTAGCGAAGCAATGCAATATGGCGATACACAACTGATGAAAGATCTTTTTGAA GCAGTCTGTAGCGAAGCAATGCAATATGGCGATACACAACTGATGAAAGATCTTTTTGAA *****
pET28a+BepBFic_insert BhBepBFic_19	GATATCTCCAATCCAGAAAAAATACGTCTTTTAAAAGACTTTATGAACAACATCAACAAT GATATCTCCAATCCAGAAAAAATACGTCTTTTAAAAGACTTTATGAACAACATCAACAAT *****

pET28a+BepBFic_insert BhBepBFic_19	ACAGGACGCAACGTGAATGATTGCCTTGTTATGGTGACAAAGGCAGGAGAAACCTACAGA ACAGGACGCAACGTGAATGATTGCCTTGTTATGGTGACAAAGGCAGGAGAAACCTACAGA *****
pET28a+BepBFic_insert BhBepBFic_19	GGAACCTATCAAGGTTGTTATTCAGAAGCTTTTGTGCTCGATATGCAAGGGACTTACATC GGAACCTATCAAGGTTGTTATTCAGAAGCTTTTGTGCTCGATATGCAAGGGACTTACATC *****
pET28a+BepBFic_insert BhBepBFic_19	ATCGGCAAAAAAGATGATCTCACACCAGAACGATTAAAATCATTAAAACCTGGTGATACA ATCGGCAAAAAAGATGATCTCACACCAGAACGATTAAAATCATTAAAACCTGGTGATACA *****
pET28a+BepBFic_insert BhBepBFic_19	ATTACCTTTATCGCCTGACTCGAGCACCACCACCACCACCAGATCCGGCTGCTAAC ATTACCTTTATCGCCTGACTCGAGCACCACCACCACCACCAGATCCGGCTGCTAAC *****
pET28a+BepBFic_insert BhBepBFic_19	AAAGCCCGAAAG AAAGCCCGAAAG *****

Appendix Figure 9.2: BhBepB-Fic sequence alignment. pET28a sequence data was obtained from Invitrogen, BepB sequence data was obtained from NCBI for *B. henselae* Houston-1 strain. pET28a insert sequence was generated by inserting the target BepB sequence between the NdeI and XhoI cut sites, along with an additional stop codon preceding the XhoI cut site.

9.1.3 *B. henselae* BepC Alignment

```

pET28a+BhBepCFic_insert  TAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGA
BhBepCFic_10              TAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGA
*****

pET28a+BhBepCFic_insert  AGGAGATATACCATGGGCAGCAGCCATCATCATCATCACAGCAGCGGCCTGGTGCCG
BhBepCFic_10              AGGAGATATACCATGGGCAGCAGCCATCATCATCATCACAGCAGCGGCCTGGTGCCG
*****

pET28a+BhBepCFic_insert  CGCGGCAGCCATATGTTAGAGCATAATTATCTGTATAAAAAACAGCAACACACTGAAAAAT
BhBepCFic_10              CGCGGCAGCCATATGTTAGAGCATAATTATCTGTATAAAAAACAGCAACACACTGAAAAAT
*****

pET28a+BhBepCFic_insert  AAATATGGCATAAAAGACCCGCAAAGACTGTATGAGCGCTGTGCCCATGATACAGCCAGA
BhBepCFic_10              AAATATGGCATAAAAGACCCGCAAAGACTGTATGAGCGCTGTGCCCATGATACAGCCAGA
*****

pET28a+BhBepCFic_insert  GAGGTTATGAATCTTCGCTTTGAACCAACCCCAACAAAGATTTGGTCTCGCTTATTTGAAA
BhBepCFic_10              GAGGTTATGAATCTTCGCTTTGAACCAACCCCAACAAAGATTTGGTCTCGCTTATTTGAAA
*****

pET28a+BhBepCFic_insert  TTGATCCATTGGACCTCTTCCATAGGAGTTTGAATGGGCGGGTCATACTCGTGATGAG
BhBepCFic_10              TTGATCCATTGGACCTCTTCCATAGGAGTTTGAATGGGCGGGTCATACTCGTGATGAG
*****

pET28a+BhBepCFic_insert  CCCTTTACATTTGAAGATGGTAGCACCGCCCGTATGCCAGCTATGCGCCCAAAGGTCAT
BhBepCFic_10              CCCTTTACATTTGAAGATGGTAGCACCGCCCGTATGCCAGCTATGCGCCCAAAGGTCAT
*****

pET28a+BhBepCFic_insert  AGGGTTCCTTTTGCCGTTGGTCCACAGATTCAAAAAGAACTTAAACAATTAGAGAGAAAA
BhBepCFic_10              AGGGTTCCTTTTGCCGTTGGTCCACAGATTCAAAAAGAACTTAAACAATTAGAGAGAAAA
*****

pET28a+BhBepCFic_insert  CTTACCGCAAAGAATAACTTACAAGGCTTATCACGCCAAGAGTTTGCTGAAAGTGCTGCG
BhBepCFic_10              CTTACCGCAAAGAATAACTTACAAGGCTTATCACGCCAAGAGTTTGCTGAAAGTGCTGCG
*****

pET28a+BhBepCFic_insert  GAAGTTTTTATGACTCTCGACCACGCGCATCCTTTCAGAAAAGGCAATGGGCGCGCGCAA
BhBepCFic_10              GAAGTTTTTATGACTCTCGACCACGCGCATCCTTTCAGAAAAGGCAATGGGCGCGCGCAA
*****

pET28a+BhBepCFic_insert  AGAATATTTATGGAAAAGCTTGGACAAGCAGCAGGCTATAAGATTGACTTTTCTTTTATC
BhBepCFic_10              AGAATATTTATGGAAAAGCTTGGACAAGCAGCAGGCTATAAGATTGACTTTTCTTTTATC
*****

pET28a+BhBepCFic_insert  ACAAAGAGCGCCTCACCCAAGCTAGTATTGCCGCAATGCAACATGGCAACACACAACCC
BhBepCFic_10              ACAAAGAGCGCCTCACCCAAGCTAGTATTGCCGCAATGCAACATGGCAACACACAACCC
*****

pET28a+BhBepCFic_insert  ATGAAAGATCTTTTGTGAGGATATCACCCACCCGCAAAAATTCCTTCTTTTAAAGGAATTC
BhBepCFic_10              ATGAAAGATCTTTTGTGAGGATATCACCCACCCGCAAAAATTCCTTCTTTTAAAGGAATTC
*****

pET28a+BhBepCFic_insert  ATCTCTCAAATGAGAAATAGCGGACTTGAGGAAATCAACGAACATATTGTTGTCGTAGCA
BhBepCFic_10              ATCTCTCAAATGAGAAATAGCGGACTTGAGGAAATCAACGAACATATTGTTGTCGTAGCA
*****

```

```

pET28a+BhBepCFic_insert  AAAGAAGGTGTAAAGTATGATGGCATCTATAAAGGTTGTGCTGCAGAAGGTTTTGTCATA
BhBepCFic_10              AAAGAAGGTGTAAAGTATGATGGCATCTATAAAGGTTGTGCTGCAGAAGGTTTTGTCATA
                          *****

pET28a+BhBepCFic_insert  GAATTAGAAGATGGTTTTGTTGTTCGGCCACAAAGATGATCTTACGCCAGAACAAGTCAAA
BhBepCFic_10              GAATTAGAAGATGGTTTTGTTGTTCGGCCACAAAGATGATCTTACGCCAGAACAAGTCAAA
                          *****

pET28a+BhBepCFic_insert  ACATTACAGAACGGTGATCGCATAGTCTGACTCGAGCACCACCACCACCACCAGAT
BhBepCFic_10              ACATTACAGAACGGTGATCGCATAGTCTGACTCGAGCACCACCACCACCACCAGAT
                          *****

pET28a+BhBepCFic_insert  CCGGCTGCTAACAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA
BhBepCFic_10              CCGGCTGCTAACAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA
                          *****

pET28a+BhBepCFic_insert  CTAGCATAACCCCTTGGGGCCTCTAAACG
BhBepCFic_10              CTAGCATAACCCCTTGGGGCCTCTAAACG
                          *****

```

Appendix Figure 9.3: Bh BepC-Fic sequence alignment. pET28a sequence data was obtained from Invitrogen, BepC sequence data was obtained from NCBI for *B. henselae* Houston-1 strain. pET28a insert sequence was generated by inserting the target BepC sequence between the NdeI and XhoI cut sites, along with an additional stop preceding the XhoI cut site.

9.1.4 *B. quintana* BepA1 Alignment

```

pET28a+BqBepA1_insert  ATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTG
BqBepA1_1               ATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTG
*****

pET28a+BqBepA1_insert  TTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCACAGCAG
BqBepA1_1               TTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCACAGCAG
*****

pET28a+BqBepA1_insert  CGGCCTGGTGCCGCGCGGCAGCCATATGCCAAAAGCAAAGTCAAAGAAACGCAAACACC
BqBepA1_1               CGGCCTGGTGCCGCGCGGCAGCCATATGCCAAAAGCAAAGTCAAAGAAACGCAAACACC
*****

pET28a+BqBepA1_insert  ATCCTCTACCCGCACCTCCTTACCTTATCATTATCTTTATTCTGATAGCAACATCCTGAA
BqBepA1_1               ATCCTCTACCCGCACCTCCTTACCTTATCATTATCTTTATTCTGATAGCAACATCCTGAA
*****

pET28a+BqBepA1_insert  AAATAAATATAGAATAAAAAATTTGGATGTTTTCTTAAGAAATGTTGCGATGATACGAC
BqBepA1_1               AAATAAATATAGAATAAAAAATTTGGATGTTTTCTTAAGAAATGTTGCGATGATACGAC
*****

pET28a+BqBepA1_insert  AAAAGCAATGGTCAATCTGTGCCAAGCATCCTTGCCAGAAAAGCTTGATTCTTCCTATCT
BqBepA1_1               AAAAGCAATGGTCAATCTGTGCCAAGCATCCTTGCCAGAAAAGCTTGATTCTTCCTATCT
*****

pET28a+BqBepA1_insert  AAGAGATCTTCATCAACAGTTATTTCAAATACGTTTGAATGGGCTGAAACTACCCGTGA
BqBepA1_1               AAGAGATCTTCATCAACAGTTATTTCAAATACGTTTGAATGGGCTGAAACTACCCGTGA
*****

pET28a+BqBepA1_insert  TACAGCCTTTAAATTTGAAGATGGCACCACCTGCTGTCATGCCAGAAATGAAAAGAACAGG
BqBepA1_1               TACAGCCTTTAAATTTGAAGATGGCACCACCTGCTGTCATGCCAGAAATGAAAAGAACAGG
*****

pET28a+BqBepA1_insert  GTGGAAAAATCCCTTTTCAATCGATGATGAAATCCAAAAGGCTTACAAAAATTAGATCG
BqBepA1_1               GTGGAAAAATCCCTTTTCAATCGATGATGAAATCCAAAAGGCTTACAAAAATTAGATCG
*****

pET28a+BqBepA1_insert  CACACTTGCTGAAAAAAACAATTTGCAAGGCTTATCACGTGAAGCGTTTATCTGTGAATC
BqBepA1_1               CACACTTGCTGAAAAAAACAATTTGCAAGGCTTATCACGTGAAGCGTTTATCTGTGAATC
*****

pET28a+BqBepA1_insert  AGTGGAATTTTTATCTCTTTTAATCATACCCACCCTTTCATAGAAGGCAATGAGCGCAC
BqBepA1_1               AGTGGAATTTTTATCTCTTTTAATCATACCCACCCTTTCATAGAAGGCAATGAGCGCAC
*****

pET28a+BqBepA1_insert  CCAACGGTTGTTTTTTCAACAATTTGCGCAAATAGCGGGCCATCAGCTGGATTTTTTACT
BqBepA1_1               CCAACGGTTGTTTTTTCAACAATTTGCGCAAATAGCGGGCCATCAGCTGGATTTTTTACT
*****

pET28a+BqBepA1_insert  CGTAACAAAAGAACGCATGCTAGTTGCCAGTCTTGACGCTGCACAAGATAGTCAATCTAG
BqBepA1_1               CGTAACAAAAGAACGCATGCTAGTTGCCAGTCTTGACGCTGCACAAGATAGTCAATCTAG
*****

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pET28a+BqBepA1_insert  AGCCTATGAAACATCTGTTTGA
BqBepA1_1               AGCCTATGAAACATCTGTTTGA
                        *****

pET28a+BqBepA1_insert  TAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATA
BqBepA1_1               TAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATA
                        *****

pET28a+BqBepA1_insert  ACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTT
BqBepA1_1               ACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTT
                        *****

```

Appendix Figure 9.4: BqBepA1 sequence alignment. pET28a sequence data was obtained from Invitrogen, BepA1 sequence data was obtained from NCBI for *B. quintana* JK-31 strain. pET28a insert sequence was generated by inserting the target BepA1 sequence between the NdeI and XhoI cut sites.

9.1.5 *B. quintana* BepC Alignment

```

pET28a+BqBepCFic_insert TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTC CCTCTAGAAATAATTT
BqBepCFic_2 TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTC CCTCTAGAAATAATTT
*****

pET28a+BqBepCFic_insert TGTTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCACAGC
BqBepCFic_2 TGTTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCACAGC
*****

pET28a+BqBepCFic_insert AGCGGCCTGGTGCCGCGCGGCAGCCATATGGCTAGCATGTTAGAGCATAATTATTTTAT
BqBepCFic_2 AGCGGCCTGGTGCCGCGCGGCAGCCATATGGCTAGCATGTTAGAGCATAATTATTTTAT
*****

pET28a+BqBepCFic_insert AAAAAACAGCGCAACACTGAAGAATAAACATGGCATAAAAAACCCGCGAAAACTGTATGAA
BqBepCFic_2 AAAAAACAGCGCAACACTGAAGAATAAACATGGCATAAAAAACCCGCGAAAACTGTATGAA
*****

pET28a+BqBepCFic_insert CGCTGTGCTCATGAGACAGCCAGAGAGGCTGTAAATTTTCGCCTTGAACCGCCACCAGGG
BqBepCFic_2 CGCTGTGCTCATGAGACAGCCAGAGAGGCTGTAAATTTTCGCCTTGAACCGCCACCAGGG
*****

pET28a+BqBepCFic_insert AAATTTGATGCCGCTTATCTAAGGACAATTCACTGGTGCCTTTTCCATAAAACTTTTGAA
BqBepCFic_2 AAATTTGATGCCGCTTATCTAAGGACAATTCACTGGTGCCTTTTCCATAAAACTTTTGAA
*****

pET28a+BqBepCFic_insert TGGGCCGGTGTTACCCGAGATCAGCCCTTTACATTTGAAGATGGCAGCACTGCATGTATG
BqBepCFic_2 TGGGCCGGTGTTACCCGAGATCAGCCCTTTACATTTGAAGATGGCAGCACTGCATGTATG
*****

pET28a+BqBepCFic_insert CCAGCTATGCGACCAAAAGGTTATAAGGTTTCCTTTTGCTGTGCGGTTCAAAATTCAAAGA
BqBepCFic_2 CCAGCTATGCGACCAAAAGGTTATAAGGTTTCCTTTTGCTGTGCGGTTCAAAATTCAAAGA
*****

pET28a+BqBepCFic_insert GAGCTTAAAAAATTAGAACAAAGACTAACCGCGAAGAATAATTTACAAGGCTTATCGCGC
BqBepCFic_2 GAGCTTAAAAAATTAGAACAAAGACTAACCGCGAAGAATAATTTACAAGGCTTATCGCGC
*****

pET28a+BqBepCFic_insert CAAGAATTTGCTGCAAATGCTGCTGAAGTTTTTACAGCTCTCGACCACGCGCATCCTTTC
BqBepCFic_2 CAAGAATTTGCTGCAAATGCTGCTGAAGTTTTTACAGCTCTCGACCACGCGCATCCTTTC
*****

pET28a+BqBepCFic_insert AGAAAAGGCAATGGGCGCACACAACGAATGTTTATGGAAAAACTCGGACAAGCGGCAGGC
BqBepCFic_2 AGAAAAGGCAATGGGCGCACACAACGAATGTTTATGGAAAAACTCGGACAAGCGGCAGGC
*****

pET28a+BqBepCFic_insert TATAAGATTGATTTTTCTTTGATCACAAAAGAACGCATGACATATGCCAGCATTGAAGCA
BqBepCFic_2 TATAAGATTGATTTTTCTTTGATCACAAAAGAACGCATGACATATGCCAGCATTGAAGCA
*****

pET28a+BqBepCFic_insert ATGCAACATAACAAATCCAGAACCCATGAAAGATCTTTTTGAGGATATCACTCACCCTCAA
BqBepCFic_2 ATGCAACATAACAAATCCAGAACCCATGAAAGATCTTTTTGAGGATATCACTCACCCTCAA
*****

```

```

pET28a+BqBepCFic_insert  AAATCCCTTCTTTTAAAGGAATTTATCTCTCAGATGAGAAGCGCTAGACTTGATGAAATT
BqBepCFic_2              AAATCCCTTCTTTTAAAGGAATTTATCTCTCAGATGAGAAGCGCTAGACTTGATGAAATT
                          *****

pET28a+BqBepCFic_insert  AACAAATCATATTGTTTTGGCAGCAAAAGAAGGTGTGACCTATGATGGCATTATATAAAGGT
BqBepCFic_2              AACAAATCATATTGTTTTGGCAGCAAAAGAAGGTGTGACCTATGATGGCATTATATAAAGGT
                          *****

pET28a+BqBepCFic_insert  TCTTCAGCTGAAGGTTTTGTTATAGAAGTAGAAGGTGGCACTTTCATCGTCGGGCACAAA
BqBepCFic_2              TCTTCAGCTGAAGGTTTTGTTATAGAAGTAGAAGGTGGCACTTTCATCGTCGGGCACAAA
                          *****

pET28a+BqBepCFic_insert  GATGATCTTAAGCCAGAGCAAGTAAAAATATTACAGAATGGTGATTCATCTCCTTTCAG
BqBepCFic_2              GATGATCTTAAGCCAGAGCAAGTAAAAATATTACAGAATGGTGATTCATCTCCTTTCAG
                          *****

pET28a+BqBepCFic_insert  TGACTCGAGCACCACCACCACCACCCTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAA
BqBepCFic_2              TGACTCGAGCACCACCACCACCACCCTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAA
                          *****

pET28a+BqBepCFic_insert  GCTGAGTTGGCTGCT
BqBepCFic_2              GCTGAGTTGGCTGCT
                          *****

```

Appendix Figure 9.5: BqBepC-Fic sequence alignment. pET28a sequence data was obtained from Invitrogen, BepC sequence data was obtained from NCBI for *B. quintana* JK-31 strain. pET28a insert sequence was generated by inserting the target BepC sequence between the NheI and XhoI cut sites, along with an additional stop codon preceding the XhoI cut site.

9.1.6 *B. rochalimae* Bep2

```

pET28a+BrBep2Fic_insert  TTTGTTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCACA
BrBep2Fic_5                TTTGTTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCACA
                             *****

pET28a+BrBep2Fic_insert  GCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGCTAGCATGACTGGTGGACAGCAAATGG
BrBep2Fic_5                GCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGCTAGCATGACTGGTGGACAGCAAATGG
                             *****

pET28a+BrBep2Fic_insert  GTCGCGGATCCATGAAGAAAAGTGAAATGATGATAAAGTTGGAGGATATTAACATCCCTT
BrBep2Fic_5                GTCGCGGATCCATGAAGAAAAGTGAAATGATGATAAAGTTGGAGGATATTAACATCCCTT
                             *****

pET28a+BrBep2Fic_insert  CTCCTAAAAATTATGTCTATCCTGACAGTAGAGTGTGAAGAATAAATATGGAATCATGG
BrBep2Fic_5                CTCCTAAAAATTATGTCTATCCTGACAGTAGAGTGTGAAGAATAAATATGGAATCATGG
                             *****

pET28a+BrBep2Fic_insert  AGCTTCAAAAGTTCGAGGAGAGAATGACGCATGATGCATTAAGAAAGAACTGTAAAGGTGC
BrBep2Fic_5                AGCTTCAAAAGTTCGAGGAGAGAATGACGCATGATGCATTAAGAAAGAACTGTAAAGGTGC
                             *****

pET28a+BrBep2Fic_insert  TAGAAGAACCTGCTCCAGAGCGGTTTGATTCTTCTTATCTAAAGTATTTGCATAAACGTT
BrBep2Fic_5                TAGAAGAACCTGCTCCAGAGCGGTTTGATTCTTCTTATCTAAAGTATTTGCATAAACGTT
                             *****

pET28a+BrBep2Fic_insert  TATTTTCAGCATGCATTGGAATGGGCTGGGTGCACCCGTGATGTTCCATTTACATTTTGTAG
BrBep2Fic_5                TATTTTCAGCATGCATTGGAATGGGCTGGGTGCACCCGTGATGTTCCATTTACATTTTGTAG
                             *****

pET28a+BrBep2Fic_insert  ATGGTACAAGGGCTGTGATGCCCTCCATGGCAAAGTGTGATTCCGATTTTGGTTTTGCAT
BrBep2Fic_5                ATGGTACAAGGGCTGTGATGCCCTCCATGGCAAAGTGTGATTCCGATTTTGGTTTTGCAT
                             *****

pET28a+BrBep2Fic_insert  TTGGTTCAGAGATTAAGGAAGGTTTAGATTTTCATCGATCAAAACTCTCTGAGAAGAATT
BrBep2Fic_5                TTGGTTCAGAGATTAAGGAAGGTTTAGATTTTCATCGATCAAAACTCTCTGAGAAGAATT
                             *****

pET28a+BrBep2Fic_insert  ATTTGAGAGGCTTATCGCGTGAAGAATTTGTTTCATAATGCAGCAAAAATATTTGGCTCTA
BrBep2Fic_5                ATTTGAGAGGCTTATCGCGTGAAGAATTTGTTTCATAATGCAGCAAAAATATTTGGCTCTA
                             *****

pET28a+BrBep2Fic_insert  TCAATTATATTCATCCTTTTAGGGAAGGTAATGGACGCGTACAAAGATTGTTTTTGTAA
BrBep2Fic_5                TCAATTATATTCATCCTTTTAGGGAAGGTAATGGACGCGTACAAAGATTGTTTTTGTAA
                             *****

pET28a+BrBep2Fic_insert  AGCTAGCCAGATCTGTAGGCTATAAACTTGACTTTTCCGCTACAACAACAAAGCGCATGA
BrBep2Fic_5                AGCTAGCCAGATCTGTAGGCTATAAACTTGACTTTTCCGCTACAACAACAAAGCGCATGA
                             *****

pET28a+BrBep2Fic_insert  CATATGTGAGTGTTGAGGTTACAAAAACGCATATATGAAGCCTTTGCAACATTTGTTTG
BrBep2Fic_5                CATATGTGAGTGTTGAGGTTACAAAAACGCATATATGAAGCCTTTGCAACATTTGTTTG
                             *****

pET28a+BrBep2Fic_insert  AAGATATTTCCAATCCAGAAAAGGTCGGTATTTTAAAGCAATTCATAAGCAATGCCAAAG
BrBep2Fic_5                AAGATATTTCCAATCCAGAAAAGGTCGGTATTTTAAAGCAATTCATAAGCAATGCCAAAG
                             *****

```

```

pET28a+BrBep2Fic_insert  AATATAATATCTTTGATTTCGAATGAAAAGTTGATTACAGTAGCGAGAGAAGGTGAAACCT
BrBep2Fic_5              AATATAATATCTTTGATTTCGAATGAAAAGTTGATTACAGTAGCGAGAGAAGGTGAAACCT
                          *****

pET28a+BrBep2Fic_insert  ATAAGGGGATTGGTAAATTCCTTGCTTCAGATTTTATTGTAATAGAAGCGGGAGATACTT
BrBep2Fic_5              ATAAGGGGATTGGTAAATTCCTTGCTTCAGATTTTATTGTAATAGAAGCGGGAGATACTT
                          *****

pET28a+BrBep2Fic_insert  GTATCCTCTCCAATAAAGATTCCCTTACACCAGAACAATTAAGGACATTCAAACCTGGTA
BrBep2Fic_5              GTATCCTCTCCAATAAAGATTCCCTTACACCAGAACAATTAAGGACATTCAAACCTGGTA
                          *****

pET28a+BrBep2Fic_insert  GTGATTTTCATTTTACAGCTTTAGATACTAAAGATCTTAAAGAAATTTTATCCCAGAAG
BrBep2Fic_5              GTGATTTTCATTTTACAGCTTTAGATACTAAAGATCTTAAAGAAATTTTATCCCAGAAG
                          *****

pET28a+BrBep2Fic_insert  AAAAAATTAGCTGCTTTGAGTGAAGATGAAATGATTGAAAAAATTTTAGAATATAGTTCTG
BrBep2Fic_5              AAAAAATTAGCTGCTTTGAGTGAAGATGAAATGATTGAAAAAATTTTAGAATATAGTTCTG
                          *****

```

Appendix Figure 9.6: Bep2-Fic sequence alignment. pET28a sequence data was obtained from Invitrogen, Bep2 sequence data was obtained from NCBI based of sequence data from Pieleles et al. (2014) for *B. rochalimae*. pET28a insert sequence was generated by inserting the target Bep2 sequence between the BamHI and XhoI cut sites, along with an additional stop codon before the XhoI cut site..

9.1.7 *Vibrio parahaemolyticus* VopS

pET28a+VpVopS_10 VpVopS_10	TTTGTTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCACA TTTGTTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCACA *****
pET28a+VpVopS_10 VpVopS_10	GCAGCGGCCTGGTGCCGCGCGGCAGCCATATGATCAGTTTGGAAAGTGTGAGTGCATTGC GCAGCGGCCTGGTGCCGCGCGGCAGCCATATGATCAGTTTGGAAAGTGTGAGTGCATTGC *****
pET28a+VpVopS_10 VpVopS_10	AAGCGGCGATGCCACAAGCTCGTAATGAGATTCTAAATGAAGGCAAACCTCAGCATTGGTG AAGCGGCGATGCCACAAGCTCGTAATGAGATTCTAAATGAAGGCAAACCTCAGCATTGGTG *****
pET28a+VpVopS_10 VpVopS_10	GTAAGGAATATACGATCAATGCTGCTACTCAAGAGTTTACACGCGCAAACCTACAAGTG GTAAGGAATATACGATCAATGCTGCTACTCAAGAGTTTACACGCGCAAACCTACAAGTG *****
pET28a+VpVopS_10 VpVopS_10	GTGCAGTCGCGCGCTTCTTTGAAGCTACTGGGAAATTATTTCTGTAAGGCAGCACGCAGT GTGCAGTCGCGCGCTTCTTTGAAGCTACTGGGAAATTATTTCTGTAAGGCAGCACGCAGT *****
pET28a+VpVopS_10 VpVopS_10	CTGTGGCAAAAGCGATCACAAAAGCAGTGTTTGACAACGAACAGGGACAAGCTCAGCGTT CTGTGGCAAAAGCGATCACAAAAGCAGTGTTTGACAACGAACAGGGACAAGCTCAGCGTT *****
pET28a+VpVopS_10 VpVopS_10	TGCAAACATCGTCTTCTGTCGAGCATGGGCAGATGTTGTTTAAAGACGCGAACCTCAAAA TGCAAACATCGTCTTCTGTCGAGCATGGGCAGATGTTGTTTAAAGACGCGAACCTCAAAA *****
pET28a+VpVopS_10 VpVopS_10	CGCCTTCAGATGTTCTGAATGCGTTTGCGAAGTTGGACAGCAAAATGGTGAAGTCTCACG CGCCTTCAGATGTTCTGAATGCGTTTGCGAAGTTGGACAGCAAAATGGTGAAGTCTCACG *****
pET28a+VpVopS_10 VpVopS_10	CTGCTGAGCTGAGCCAACCTTGCCGAACGAGCCATGACAGAAGTGATGCTAGAGACGGATT CTGCTGAGCTGAGCCAACCTTGCCGAACGAGCCATGACAGAAGTGATGCTAGAGACGGATT *****
pET28a+VpVopS_10 VpVopS_10	CTGGCAAGAACCTCAAAGCGCTGATTGGTGATGATGCAGTTAAATCGCTCGCGGTGCGTG CTGGCAAGAACCTCAAAGCGCTGATTGGTGATGATGCAGTTAAATCGCTCGCGGTGCGTG *****
pET28a+VpVopS_10 VpVopS_10	TGGTGAAAGATTACGGCGGTGGCGTGGCGGCTGCTCAGAAAAATCCAGAAGTACGCATCA TGGTGAAAGATTACGGCGGTGGCGTGGCGGCTGCTCAGAAAAATCCAGAAGTACGCATCA *****
pET28a+VpVopS_10 VpVopS_10	ACCAAATGCAAGCTGTCTTTGACATGGAGGTGATGCACCTGAAAGCAGCGCAGCGACATA ACCAAATGCAAGCTGTCTTTGACATGGAGGTGATGCACCTGAAAGCAGCGCAGCGACATA *****
pET28a+VpVopS_10 VpVopS_10	TTGAAGGGTTAGCAAGTACGATTTAGACCAAGGCGTGATGCAGAAGGTCTTCCTGAAG TTGAAGGGTTAGCAAGTACGATTTAGACCAAGGCGTGATGCAGAAGGTCTTCCTGAAG *****

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pET28a+VpVopS_10  ACGCTTTCAATAAAGCTGGCGTGACCAAAAATGTTGAACGCGCAGCGGCTTGGATCATCA
VpVopS_10          ACGCTTTCAATAAAGCTGGCGTGACCAAAAATGTTGAACGCGCAGCGGCTTGGATCATCA
*****

pET28a+VpVopS_10  ACGCTTCAAACCTCAAAGGGAACGATGCGGAAAACATCACGTCATTATTGAAAGAGTACG
VpVopS_10          ACGCTTCAAACCTCAAAGGGAACGATGCGGAAAACATCACGTCATTATTGAAAGAGTACG
*****

pET28a+VpVopS_10  CGACCAACGGTAAAGACTTGCTGAATATGGACAACCTAAAAGAGTTGCATGCTCGTTTGG
VpVopS_10          CGACCAACGGTAAAGACTTGCTGAATATGGACAACCTAAAAGAGTTGCATGCTCGTTTGG
*****

pET28a+VpVopS_10  TACCAAATGTAGAACGCGATTACCGTGGGCCAAATATCTCTGGCGGCACATTACCATCAA
VpVopS_10          TACCAAATGTAGAACGCGATTACCGTGGGCCAAATATCTCTGGCGGCACATTACCATCAA
*****

pET28a+VpVopS_10  GCATTGGTGGGGAAGGCATGTTGAAGCAACACATTGAAGGCTTTTGAAGAGAATCCAG
VpVopS_10          GCATTGGTGGGGAAGGCATGTTGAAGCAACACATTGAAGGCTTTTGAAGAGAATCCAG
*****

pET28a+VpVopS_10  TTGCGGACAAAGACCTCGGTAAACATTTGTTTGCTGGTGTGATTGGTTATCACGGCTTTA
VpVopS_10          TTGCGGACAAAGACCTCGGTAAACATTTGTTTGCTGGTGTGATTGGTTATCACGGCTTTA
*****

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Appendix Figure 9.7: VopS sequence alignment. pET28a sequence data was obtained from Invitrogen, VopS sequence data was obtained from NCBI for *Vibrio parahaemolyticus* serotype O4:K12.. pET28a insert sequence was generated by inserting the target VopS sequence between the NdeI and XhoI cut sites.

9.2 Protein Induction Gels

9.2.1 *B. henselae* BhBepA-Fic

Appendix Figure 9.8: Nickel column affinity purification of BhBepA-Fic protein. The dark bands in the elution samples correspond with the predicted weight for the BhBepA-Fic protein. M = Marker, UI = Uninduced protein, I = Induced total protein, Eq = Equilibration sample.

9.2.2 *B. henselae* BhBepA_H159A-Fic

Appendix Figure 9.9: Nickel column affinity purification of BhBepA_H159A-Fic. The dark bands in the elution samples correspond with the predicted weight for the BhBepA_H159A-Fic protein. M = Marker, UI = Uninduced protein, I = Induced protein, Eq = Equilibration sample.

9.2.3 *B. henselae* BhBepB-Fic

Appendix Figure 9.10: Nickel affinity purification of BhBepB-Fic protein. The dark bands in the elution samples correspond with the predicted weight for the BhBepB-Fic protein. M = Marker, UI = Uninduced protein, I = Induced protein, Eq = Equilibration sample.

9.2.4 *B. henselae* BhBepC-Fic

Appendix Figure 9.11: Nickel affinity purification of BhBepC-Fic protein. (a) Induction samples with dark band in the Induced sample indicating a successful BhBepC-Fic induction. (b) Purification samples of BhBepC-Fic. The lack of bands in the elution samples corresponding to the molecular weight of BhBepC-Fic, suggests the protein is insoluble. M = Marker, UI = Uninduced protein, I = Induced protein, Eq = Equilibration sample.

9.2.5 *B. quintana* BqBepA1

Appendix Figure 9.12: Nickel affinity purification of BqBepA1 protein. The dark bands in the induced and insoluble samples correspond with the predicted weight for the BqBepA1 protein. While there is clear evidence of protein induction, it was not purified in the elution samples and remained in the insoluble samples. Insoluble samples were redissolved in 8 M Urea. M = Marker, UI = Uninduced protein, I = Induced protein, Eq = Equilibration sample, IS = Insoluble protein.

9.2.6 *B. quintana* BqBepC-Fic

Appendix Figure 9.13: Nickel affinity purification of BqBepC-Fic protein. The dark bands in the induced and insoluble samples correspond with the predicted weight for the BqBepC-Fic protein. While there is clear evidence of protein induction, it was not purified in the elution samples and remained in the insoluble samples. Insoluble samples were redissolved in 8 M Urea. M = Marker, UI = Uninduced protein, I = Induced protein, Eq = Equilibration sample, IS = Insoluble protein.

9.2.7 *B. rochalimae* Bep2-Fic

Appendix Figure 9.14: Nickel affinity purification of BrBep2-Fic protein. The bands in the elution samples correspond with the predicted weight for the BrBep2-Fic protein. M = Marker, UI = Uninduced protein, I = Induced protein, Eq = Equilibration sample.

9.2.8 *Vibrio parahaemolyticus* VopS

Appendix Figure 9.15: Nickel affinity purification of VopS protein. The bands in the elution samples correspond with the predicted weight for the VopS, although there is a large number of protein still in the Insoluble fraction. M = Marker, UI = Uninduced protein, I = Induced protein, IS = insoluble protein.

9.3 Pyrophosphate Analysis

Pyrophosphate analysis of Bep-Fic treated vimentin samples

Appendix Figure 9.16: Pyrophosphate reactions for *B. henselae* Fic proteins on vimentin samples. The graph indicates no significant difference in the levels of pyrophosphate between samples and controls, suggesting no detectable change in pyrophosphate occurred during the reaction. Standard curve $R^2 = 0.9608$.

Pyrophosphate analysis of Bep-Fic treated whole cell extract samples

Appendix Figure 9.17: Pyrophosphate reactions for *B. henselae* Fic proteins on WCE samples. The graph indicates no significant difference in the levels of pyrophosphate between samples and controls. Data obtained was significantly more varied than initial vimentin experiments above, indicating the freeze-thawing of the pyrophosphate reagent may have decreased accuracy. Standard curve $R^2 = 0.796$.