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Bartonella Clarridgeiae: Invasion of Human Microvascular Endothelial Cells and Role of Flagella in Virulence

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BARTONELLA CLARRIDGEIAE: INVASION OF HUMAN MICROVASCULAR ENDOTHELIAL CELLS AND ROLE OF FLAGELLA IN VIRULENCE

by

ANNE M. WHITNEY

Under the Direction of Barbara R. Baumstark, Ph.D.

ABSTRACT

B. henselae, *B. bacilliformis* and *B. quintana* are capable of causing vasoproliferative diseases in humans by modulating apoptosis and proliferation of endothelial cells. *Bartonella clarridgeiae*, a close relative of the pathogenic *Bartonellae*, has been implicated in human disease but has not yet been isolated from a human patient. Both *B. bacilliformis* and *B. clarridgeiae* have flagella and a flagellar type 3 secretion system, while *B. henselae* and *B. quintana* do not. We created 2 non-motile mutants of *B. clarridgeiae* by interrupting the flagellin gene, *fla*A, or the flagellar motor genes, *mot*BC. We investigated whether *B. clarridgeiae* could invade human endothelial cells (HMECs) and if functional flagella were important for invasion. The non-motile mutants and the wild-type strain were capable of entering HMECs *in vitro*. The *fla*A mutant was deficient in attachment, but the HMECs in culture with the *fla*A mutant demonstrated increased proliferation. The *mot*BC mutant showed enhanced invasion. Differential secretion of proteins was

revealed by 2-D electrophoresis and MALDI-TOF analysis of secretomes from the co-cultures compared to uninfected HMECs. HMECS infected with wild-type *B. clarridgeiae* secreted proteins indicative of proliferation. The *fla*A mutant induced the secretion of proteins involved in cytoskeletal rearrangement, cell migration, and proliferation. The *mot*BC-infected HMECs showed signs of hypoxia. The co-chaperonin GroES was found in higher concentration in the supernatant of the hyper-invasive *mot*BC strain/HMEC co-culture than the wild-type co-culture and was found at a very low concentration in the *fla*A culture supernatant. Cross-talk between secretion systems is suggested.

INDEX WORDS: Vasoproliferation, 2-Dimensional gel electrophoresis, Angiogenesis, Hypoxia

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You Raise Me Up, To More Than I Can Be. My heartfelt thanks to my family and friends to whom I owe a huge debt of gratitude for their love and support over this long journey. To my Buds Gloria and Vicki, thanks for the purple lab coat and all the love that came with it. Thanks Becky, Lynne, Cindy and Wendy for always encouraging me and getting excited about flagella. Mom and Dad, you've been a great inspiration. Lee and Kat, thanks for understanding my crazy schedule; it is time to plan a trip! To Jill, I cannot say enough. Thanks for your love, support, encouragement, patience, understanding, and numerous sacrifices. I could not and would not have done this without you.

I Love You All.

In Loving Memory of Dudley E. Whitney

Dad, you are always with me.

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INTRODUCTION

The Genus *Bartonella*

Bartonella clarridgeiae is one of more than 19 bacterial species belonging to the genus *Bartonella*. From 1909 until 1993, this genus contained only one species, *B. bacilliformis*. Brenner *et al*. determined from the results of 16S rRNA sequence analysis, DNA-DNA hybridization, guanosine plus cytosine content and phenotypic characteristics that several species in the genus *Rochalimaea* were very closely related to *B. bacilliformis* (Brenner *et al.*,1993). In 1993 the two genera were combined, and *B. quintana, B. vinsonii, B. henselae* and *B. elizabethae* were included with *B. bacilliformis* in the genus. A similar event took place in 1995 when the genus *Grahamella* was combined with *Bartonella*, and *B. talpae, B. peromysci, B. grahamii, B. taylorii* and *B. doshiae* joined the genus. This brought the number of *Bartonella* species to 10, although the original strains of *B. talpae* and *B. peromysci* were found to be no longer viable (Birtles *et al.*,1995). Further studies culturing blood from wild and domestic animals brought about the discoveries of *B. schoenbuchensis* and *B. capreoli* from deer, *B. alsatica* from wild rabbits, *B. tribocorum* from wild rats, *B. vinsonii* subspecies *berkhoffii* from domestic dogs, *B. koehlerae* from cats, *B. chomelii* and *B. bovis* Bermond et al from cattle, *B. birtlesii* and *B. vinsonii* subspecies *arupensis* from mice. *B. weissi* and *B. washoensis* are unofficial names of two additional species. *B. weissi* was recovered from cats and later was found to be identical by 16S rRNA gene sequence to *B. bovis* Bermond et al (Bermond *et al.*,2002), and *B. washoensis* was reported in ground squirrels and a dog (Chomel *et al.*,2003; Kosoy *et al.*,2003). Other species with no official taxonomic acceptance are *B. australis, B. phoceenis, B. rattimassiliensis*, and *B. tamiae*. The Family *Bartonellaceae* is one of 10 Families in the Order *Rhizobiales*. Other

Families include *Brucellaceae* and *Rhizobiaceae* (Figure 1). The genus *Bartonella* is characterized as Gram negative, rod-shaped bacteria that grow best in aerobic conditions on media that contains 5% sheep, horse or rabbit blood (Birtles *et al.*,1995). *B. bacilliformis* grows best at 25° while many other species grow at 37° (Birtles *et al.*,1995). The G+C content of the genomic DNA is 38.5 to 41 mol% (Birtles *et al.*,1995). The type strain of the genus is *B. bacilliformis*.

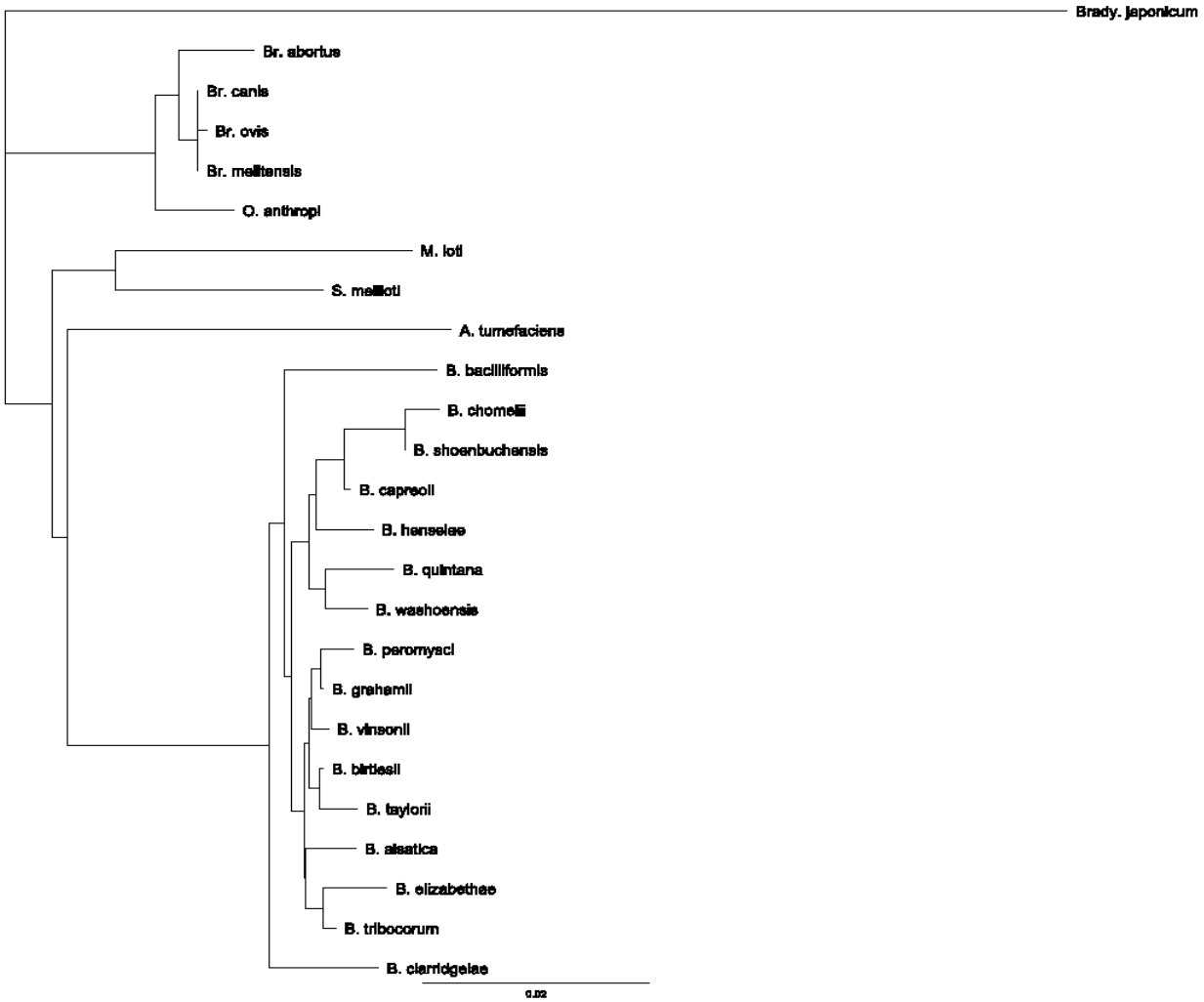


Figure 1. Neighbor-joining phylogenetic tree of some members of the Order Rhizobiales. Phylogeny based on 16S rRNA gene sequence alignment of *Bradyrhizobium japonicum*,

Brucella (Br.) species, *Ochrobactrum anthropi*, *Mesorhizobium loti*, *Sinorhizobium meliloti*, *Agrobacterium tumefaciens*, and 16 *Bartonella* species.

Several *Bartonella* species have been implicated in human disease, and the spectrum of disease that they cause is vast. *B. bacilliformis* is the causative agent of bartonellosis, also known as Carrion's disease, and was first identified in 1905 by Peruvian microbiologist Alberto Barton. *B. bacilliformis* is transmitted by the bite of an infected sand fly, *Lutzomyia* spp., that live at an altitude between 2000 and 9000 feet in the Andes Mountains of Peru, Columbia and Ecuador (Garcia-Caceres and Garcia,1991; Maurin *et al.*,1997). In the initial acute hematic phase of bartonellosis known as Oroya fever, *B. bacilliformis* invades and lyses red blood cells causing severe haemolytic anemia and a high fever (Chamberlin *et al.*,2002). In many cases of untreated bartonellosis, more than 50% of the erythrocytes are lysed, and the fatality rate can be as high as 90% (Garcia-Caceres and Garcia,1991; Maguina *et al.*,2001). In the study of Maguina et al, 65% of the patients in the acute phase of bartonellosis presented with a hematocrit <19% (normal values are 38-46%) (Maguina *et al.*,2001). Patients who survive the first phase often experience verruga peruana, the chronic eruptive phase of the disease, that begins 2 to 8 weeks after the acute phase and is characterized by nodular skin lesions (Maguina *et al.*,2001). The papules often resemble hemangiomas, a benign tumor caused by rapid proliferation of vascular endothelial cells. However, some verruga peruana lesions resemble malignant tumors histologically, and only the detection of *B. bacilliformis* can support a definitive diagnosis of Bartonellosis (Arias-Stella *et al.*,1986; Arias-Stella *et al.*,1987; Bhutto *et al.*,1994).

The species *B. quintana* historically has been associated with trench fever during the two World Wars and more recently has been isolated from homeless individuals (Drancourt *et al.*,1995; Brouqui *et al.*,1996; Jackson and Spach,1996; Anderson and Neuman,1997; Maurin *et*

al.,1997; Brouqui *et al.*,1999; Rydkina *et al.*,1999; Guibal *et al.*,2001; Foucault *et al.*,2002; Sasaki *et al.*,2002; Capo *et al.*,2003; Levy *et al.*,2003; Rolain *et al.*,2003). The vector for *B. quintana* is the human body louse. Symptoms of *B. quintana* infection can include leg pain, fever that usually lasts approximately 5 days (quintane fever) and headache (Maurin and Raoult,1996; Maurin *et al.*,1997) but symptoms vary by individual. In a study of 42 homeless individuals in Marseille, France, who had *B. quintana* bacteremia as evidenced by positive blood culture, there was no universal symptom although 12 had leg pain, 10 had fever, 8 had headache, and18 had lice and skin lesions that itched (Foucault *et al.*,2002). *B. quintana* has been confirmed to be a major cause of endocarditis (Spach *et al.*,1995; Spach *et al.*,1995; Mainardi *et al.*,1996; Raoult *et al.*,1996; Jourdain *et al.*,1998; Guyot *et al.*,1999; Simon-Vermont *et al.*,1999; Lepidi *et al.*,2000; Posfay Barbe *et al.*,2000; Klein *et al.*,2002; Thiam *et al.*,2002). In a study published in 2005, 75% of cases of endocarditis without positive blood cultures were attributed to *B. quintana* by serology and PCR (Houpikian and Raoult,2005). *B. quintana* has also been associated with bacillary angiomatosis (BA), a condition characterized by subcutaneous and cutaneous nodules and papules usually affecting immunocompromised patients such as those with HIV/AIDS. (Cockerell *et al.*,1987; Koehler *et al.*,1992; Santos *et al.*,2000).

In addition to *B. quintana*, *B. elizabethae* has been implicated in one case of endocarditis in a human. *B. vinsonii* subspecies *berkhoffii* has been isolated from dogs with heart disease and has recently been isolated from an afebrile human patient with endocarditis (Anderson and Neuman,1997; Maurin *et al.*,1997; Roux *et al.*,2000). *B. washoensis* was originally isolated from a human patient with cardiac disease (Kosoy *et al.*,2003).

B. henselae has been detected in patients with endocarditis, but it is most well-known as the major cause of cat scratch disease (CSD) in humans. In immunocompetent individuals, the

most common symptoms of CSD are regional lymphadenopathy near the bite or scratch from a cat, low-grade fever, and malaise (Jones,1993; Chen and Gilbert,1994; Hughes and Faragher,1994; Flexman *et al.*,1995). *B. henselae* also has been reported to cause a variety of systemic infections in both immunocompetent and immunocompromised individuals including eye infections such as neuroretinitis, Parinaud's syndrome and karatitis, encephalopathy, osteomyelitis, BA, and peliosis hepatitis in adults as well as hepatosplenic lesions and/or prolonged fever in children (Holmes *et al.*,1995; Marra,1995; Wong *et al.*,1995; Hayem *et al.*,1996; Lamps *et al.*,1996; Liston and Koehler,1996; Raoult *et al.*,1996; Schwartzman,1996; Yamashita *et al.*,1996; Breathnach *et al.*,1997; Cunningham *et al.*,1997; Jones and Cunningham,1997; Sander and Frank,1997; Ahsan *et al.*,1998; Baorto *et al.*,1998; Berdague *et al.*,1998; Jacobs and Schutze,1998; Reed *et al.*,1998; Maggiore *et al.*,1999; Wade *et al.*,1999; Lohmann *et al.*,2000; Massei *et al.*,2000; Dai *et al.*,2001; De La Rosa *et al.*,2001; Fournier *et al.*,2001; Meininger *et al.*,2001; Messina *et al.*,2001; Numazaki *et al.*,2001; van Tooren *et al.*,2001; Al-Matar *et al.*,2002; Besada *et al.*,2002; Verdon *et al.*,2002; Komitova *et al.*,2003; Oman *et al.*,2003; Woestyn *et al.*,2003; Bookman *et al.*,2004; Ledina *et al.*,2004; Rodrick *et al.*,2004). *B. shoenbuchensis, B. chomelii* and *B. capreoli* have not been implicated in human disease but their presence in cattle and deer pose a potential means of transmission to humans (Maillard *et al.*,2004).

B. clarridgeaie was first described in 1995 when the organism was isolated from a kitten belonging to a *B. henselae*-positive patient (Clarridge *et al.*,1995; Collins,1996). Several studies in all four quarters of the world have demonstrated that cats serve as a reservoir for *B. clarridgeiae* and *B. henselae* and are even coinfected with both species in a small percentage of cases (Gurfield *et al.*,1997; Marston *et al.*,1999). Therefore, infection by one species does not

confer protection against the other. *B. clarridgeiae* has been isolated from dogs with infective vegetative valvular endocarditis and in liver specimens from dogs with hepatic disease (Chomel *et al.*,2001; Gillespie *et al.*,2003). The number of human infections by *B. clarridgeiae* is unknown. It has been estimated that there are 24,000 cases of CSD yearly in the US (Jackson *et al.*,1996) and that up to 60% of CSD cases are negative for *B. henselae* by serology (Yoshida *et al.*,1996). Because serologic tests for *B. clarridgeiae* are not usually performed, and because *Bartonella* are difficult to culture, the percentage of CSD cases caused by *B. clarridgeiae* has not been determined. *B. clarridgeiae* was associated with CSD in a veterinarian bitten by a kitten (Kordick *et al.*,1997) and a CSD case in which a patient developed a "massive chest-wall abscess" (Margileth and Baehren,1998). However, much like the difficulty in the late 1980's in obtaining data to support *B. henselae*'s role in CSD, Koch's postulates for *B. clarridgeiae* as the etiologic agent of CSD have not been met. Kordick et al. reported that no bacteria grew in culture from the veterinarian's blood, but *B. clarridgeiae* grew from the blood of the cat that bit the man (Kordick *et al.*,1997). The veterinarian's blood was reactive with the organism cultured from the cat in an immunofluorescence assay, and the cat's blood gave significant reaction with this organism (1:2048), with *B. henselae* (1:1024) and with *B. quintana* (1:1280) (Kordick *et al.*,1997). However, the organism was not isolated from the human, and *B. clarridgeiae*-specific PCR was not attempted from any clinical samples. Likewise, in the CSD case described by Margileth and Baehren, immunofluorescent assay tests using the patient's convalescent serum were positive for *B. clarridgeiae*, but no organism was cultured (Margileth and Baehren,1998).

Cockerell et al.described an HIV-positive patient in 1991 who had reddish or purple papules and nodules on the skin of his face, arms, legs and trunk as well as some subcutaneous nodules (Cockerell *et al.*,1991). A second patient was HIV-negative and had papules on his right

forearm. Neither patient had had exposure to cats, but the second patient had a pet parakeet that he let perch on his arm. Histologic examination of biopsied papules from both patients revealed proliferations of capillaries that were lined with cuboidal endothelial cells. Inflammatory cell types were seen (neutrophils, lymphocytes and histiocytes), and Warthin-Starry staining showed bacterial aggregates between blood vessels. Bacteria grew after 10 days in Trypticase Soy Broth supplemented with 5% horse serum and 5% Fildes reagent. The bacteria stained faintly Gram-negative. Some of the bacteria had a single polar flagellum while others had multiple unipolar flagella when viewed by electron microscopy. The authors concluded from histologic, microscopic and fatty-acid analysis that the organism was closely related to *B. bacilliformis*. While the fatty acid results are characteristic of *B. clarridgeiae* or *B. henselae, B. henselae* does not have flagella (Boone *et al.*,2001). At that time, *B. clarridgeiae* had not been described. There is a possibility, therefore, that these 2 cases represent bacillary angiomatosis caused by *B. clarridgeiae.*

Vector-Borne Transmission

All *Bartonella* species are presumed to be transmitted to their primary reservoirs by arthropod vectors including fleas, sandflies and lice, as identical organisms have been found in the insects and the mammals they use as a blood source (Parola *et al.*,2002; Parola *et al.*,2003; Rolain *et al.*,2003; Sanogo *et al.*,2003; Stevenson *et al.*,2003; Bown *et al.*,2004; Kelly *et al.*,2005; Reeves *et al.*,2005; Sreter-Lancz *et al.*,2006; Li *et al.*,2007; Podsiadly *et al.*,2007; Vorou *et al.*,2007; Tabar *et al.*,2008). In a study conducted in France, *B. quintana* (17%), *B. koehlerae* (4%), *B. henselae* (11%) and *B. clarridgeiae* (68%) were detected by PCR in 89 of 309 cat fleas collected in different regions of the country (Rolain *et al.*,2003). *Rickettsia felis, B.*

henselae or *B. clarridgeiae* were detected by PCR in all 114 cat fleas (*Ctenocephalides felis*) sampled from 3 veterinary clinics in New Zealand, and some fleas had 2 or all 3 of the bacterial species present (Kelly *et al.*,2005). However, Billeter et al. emphasized that detection of *Bartonella* in a vector does not definitively prove that the arthropod is the mode of transmission; replication of the bacteria in the vector and experimental proof of transmission to a host by an infected vector is required (Billeter *et al.*,2008). The confirmed vectors for *Bartonella* transmission are the sandfly for *B. bacilliformis*, the human body lousefor *B. quintana*, the cat flea for *B. henselae*, and the rodent flea *Ctenophthalmus nobilis nobilis* for *B. grahamii* and *B. taylorii* (Billeter *et al.*,2008).

Pathogenesis

A common trait of *Bartonella* species is their affinity for blood (Dehio,2001). Once *Bartonella* are transmitted to their specific reservoir host(s), the haemotropism results in intraerythrocytic bacteremia. However, while *Bartonella*e may persist intraerythrocytically for months or years, they usually cause no or only transient symptoms in the animals they infect other than humans (Kordick and Breitschwerdt,1995). The survival of *Bartonella*e within red blood cells may be an adaptation necessary for successful transmission from host to host by the arthropod vector (Schulein *et al.*,2001). In the human reservoir host, the most extreme result of erythrocyte invasion is the severe and often fatal hemolytic anemia caused by *B. bacilliformis*. Other *Bartonella* spp. do not cause lysis of red blood cells to any significant degree and may use the cells as a means of transportation to a primary but still unknown niche. This niche may be the vascular endothelium or perhaps also the reticuloendothelial system including lymph nodes and the spleen (Ventura *et al.*,1999; Durupt *et al.*,2004; Henriquez *et al.*,2004; Sykes *et al.*,2007;

Breitschwerdt,2008; Lydy *et al.*,2008; Scolfaro *et al.*,2008; van der Veer-Meerkerk and van Zaanen,2008). *B. quintana* is known to enter human erythrocytes but anemia is not a symptom of Trench Fever (Rolain *et al.*,2002). Trench fever is also known as fievre quintane due to the 5-day long fever that may recur multiple times during the illness and for which the causative organism is named (Bass *et al.*,1997). The recurrence may be the periodicity between the invasion of red blood cells and the primary niche (Dehio,2001). A similar recurrent intraerythrocytic bacteremia was seen in rats experimentally infected with *B. tribocorum* (Schulein *et al.*,2001).

Unlike, *B. bacilliformis* and *B. quintana, B. henselae* uses humans only as incidental hosts. The reservoir for *B. henselae* is the cat, and the bacteria have been detected in feline erythrocytes *in vivo* and *in vitro* (Kordick and Breitschwerdt,1995; Mehock *et al.*,1998; Rolain *et al.*,2001). Cats are usually asymptomatic, but histopathologic lesions have been reported in the spleen, liver and lymph nodes of experimentally infected cats (Guptill *et al.*,1997). *B. henselae* may be transmitted to humans via the cat flea or a cat scratch or bite. Recently, *B. henselae* was detected in human erythrocytes after experimental inoculation raising concerns of possible transmission of cat scratch disease through blood transfusion (Pitassi *et al.*,2007).

The invasion of erythrocytes by *B. bacilliformis* has been the subject of many studies designed to investigate the severe hemolysis seen during Oroya fever. While the bacterial adhesins responsible for attachment to erythrocytes are unknown, adhesion is energy-dependent and is correlated with motility (Benson *et al.*,1986; Scherer *et al.*,1993). The flagella of *B. bacilliformis* were reported to be a virulence factor based on the observation that the number of erythrocytes with associated *Bartonella*e was reduced by 52% after the bacteria were treated with anti-flagellar antibodies (Scherer *et al.*,1993). The number of bacteria inside of the

erythrocytes decreased by almost 100% when the bacteria were pre-treated with anti-flagellar antiserum (Scherer *et al.*,1993). Non-motile *B. bacilliformis* showed decreased invasiveness (Benson *et al.*,1986) and *B. bacilliformis* without flagella were significantly hindered from binding to erythrocytes (Battisti and Minnick,1999; Minnick and Anderson,2000). It is unknown whether the motility function is key and/or the flagella themselves participate in adhesion. In addition to *B. bacilliformis, B. clarridgeiae, B. schoenbuchensis, B. chomelii, and B. capreoli* express one to three unipolar flagella (Dehio *et al.*,2001; Bermond *et al.*,2002; Maillard *et al.*,2004). The recently described species *B. rochalimeae* has multiple flagella at one pole (Eremeeva *et al.*,2007). Of these flagellated species, only *B. bacilliformis, B. rochalimeae* and *B. clarridgeiae* are known or thought to cause human disease.

Other virulence factors of *B. bacilliformis* include the two-gene invasion associated locus *ial*A and *ia*lB which encode a nucleoside polyphosphate hydrolase and an outer membrane protein, respectively. Disruption of *ial*B caused a decrease in association and invasion of human erythrocytes by 47 and 53% (Coleman and Minnick,2001). Red blood cells do not have an active cytoskeleton and therefore cannot phagocytize bacteria. A secreted protein (or perhaps water-soluble molecule) called deformin has been found in *B. bacilliformis* and *B. henselae* supernatants and may cause invaginations in the blood cell membrane which are then used as entry portals by the bacteria (Xu *et al.*,1995; Iwaki-Egawa and Ihler,1997; Derrick and Ihler,2001; Hendrix and Kiss,2003). As previously mentioned, *B. bacilliformis* causes hemolysis. The factors involved in hemolysis appear to be contact-dependent and different from deformin (Hendrix,2000). Further evidence that hemolysin and deforming factor are different substances comes from the fact that *B. henselae* did not cause hemolysis but does produce deformin (Iwaki-Egawa and Ihler,1997; Hendrix,2000).

Agrobacterium tumefaciens, a plant pathogen, is a member of the *Rhizobiaceae* and is related to the *Bartonella*e (Figure 1). *A. tumefaciens* induces tumor formation on plants by injecting DNA into plant cells. The DNA encodes proteins which cause the induction of cytokines resulting in proliferation of plant cells and formation of a tumor called Crown Gall disease (Backert and Meyer,2006). The tumor-inducing DNA is injected into the plant by the bacteria's Type IV secretion system (T4SS) whichis made up of 11 *vir*B/*vir*D proteins. The proteins are assembled into a bacterial inner and outer membrane-spanning channel and an extracellular pilus that contacts the plant cell (Zupan *et al.*,1998). The T-DNA is translocated into the plant cells as a complex with VirD2 protein (Guo *et al.*,2007). Homologues of the *A. tumefaciens* virB genes were first identified in *Bartonella* by our laboratory during an investigation of an immunodominant 17-kDa protein encoding gene in *B. henselae,* and we have confirmed the presence of the entire operon in *B. clarridgeiae* ((Padmalayam *et al.*,2000) and unpublished, respectively). The majority of the *Bartonella* species, including *B. tribocorum, B. elizabethae, B. vinsonii* and *B. quintana* also possess the *vir*B operon, while it is not present in *B. bacilliformis, B. schoenbuchensis, B. chomelii, B. capreoli* or *B. bovis* (Saenz *et al.*,2007). Of the five species without *vir*B, all except *B. bacilliformis* possess a *vir*B-like operon (*vbh*) (Saenz *et al.*,2007), and, interestingly, all except *B. bovis* are flagellated. The ancestral *B. bacilliformis* does not have any *vir*B-like genes nor remnants thereof, so apparently the T4SS were acquired by the modern-day *Bartonella* spp. through lateral transfer from other genera (Saenz *et al.*,2007).

The role of the VirB system in *Bartonella* species' pathogenesis has been partially elucidated. *B. tribocorum vir*B4 and *vir*D4 mutants failed to cause bacteremia in the rat model. It is not clear whether the failure to cause bacteremia was due to the fact that the mutants could not invade erythrocytes or because they could not invade the primary niche which seeds the

blood invasion (Schulein and Dehio,2002). The latter explanation appears to be supported by the *in vitro* demonstration that the VirB/VirD4 system is involved in endothelial cell cytoskeletal rearrangements leading to internalization of *B. henselae*, activation of a proinflammatory response through NFκB, and inhibition of apoptosis (Schmid *et al.*,2004). Unlike *A. tumefaciens*, however, proliferation of host cells is independent of VirB/VirD4 in *B. henselae* infections; a Δ*vir*B4 *B. henselae* mutant caused even greater proliferation of human umbilical vein endothelial cells (HUVECs) than the vascular endothelial growth factor (VEGF) control (Schmid *et al.*,2004). High titers of VirB-positive *B. henselae* interfered with proliferation and were cytotoxic, while Δ*vir*B4 mutants remained mitogenic at high titers (Schmid *et al.*,2004).

The vasoproliferative manifestations of *B. bacilliformis*, *B. henselae* or *B. quintana* infection such as veruga peruana and bacillary angiomatosis occur in immunosuppressed or immunocompromised individuals. In the case of *B. bacilliformis*, the immunosuppression is an effect of the first stage of bacterial infection (Bass *et al.*,1997). The skin lesions caused by *Bartonella* spp. erupt after a complex series of events beginning with the bacteria coming into contact with and entering vascular endothelial cells (Garcia *et al.*,1992; Koehler,1994; Brouqui and Raoult,1996; Dehio *et al.*,1997; Dehio,1999; Anderson,2001). Invasion of endothelial cells involves different mechanisms than that promoting erythrocyte invasion. Unlike red blood cells, endothelial cells have a dynamic cytoskeleton. *B. bacilliformis* causes the rearrangement of microfilaments in HUVECs which engulf the bacteria as single cells (Hill *et al.*,1992). The rearrangement of actin filaments is dependent on Rho GTPase, and activation of Rac and Cdc42 results in formation of filopodia and membrane ruffles quickly after infection (Verma *et al.*,2000; Verma *et al.*,2001; Verma and Ihler,2002). Clumps of *B. bacilliformis* were seen in close contact with the filopodia and lamellipodia. Inactivation of Rac and Cdc42 results in

dramatic decreases in *B. bacilliformis* internalization (Verma and Ihler,2002). *B. henselae* cells, on the other hand, are taken up by two methods: en masse in a cytoskeletal structure known as an invasome and as individual bacteria by induced phagocytosis (Dehio *et al.*,1997; Fuhrmann *et al.*,2001; Schmid *et al.*,2004). The VirB/VirD4 system is involved in the invasome formation (Schmid *et al.*,2004), but *B. henselae* is the only known species to enter using that mechanism even though other *Bartonella* spp. have the VirB T4SS. *B. bacilliformis* and *B. henselae* localize to the perinuclear region once they enter the endothelial cell and avoid lysosomal degradation (Dehio *et al.*,1997; Verma *et al.*,2000; Fuhrmann *et al.*,2001).

A proinflammatory response mediated by NFκB is induced by *B. henselae* after endothelial cell invasion (Fuhrmann *et al.*,2001; Dehio *et al.*,2005). The subsequent secretion of chemokine IL8/CXCL8 and its receptor on endothelial cells suggests that *B. henselae* can cause endothelial cell proliferation in an autocrine manner (McCord *et al.*,2006). In addition, IL8 secretion potentially causes the influx of lymphocytes and macrophages *in vivo* (Fuhrmann *et al.*,2001; Schmid *et al.*,2004) making it possible for *B. henselae* to come in contact with the recruited macrophages. It has been shown *in vitro* that vascular endothelial growth factor (VEGF) and interleukin-1β production are induced in THP-1 macrophages after exposure to *B. henselae*, and conditioned culture medium from infected macrophages can trigger proliferation of human microvascular endothelial cells (HMECs) (Resto-Ruiz *et al.*,2002). Also, the up-regulation of surface adhesion molecules E-selectin and ICAM-1expression by endothelial cells is induced by live *B. henselae* or purified OMPs, an observation indicative of a proinflammatory phenotype (Fuhrmann *et al.*,2001), In this model, the proliferation of infected endothelial cells is part of a paracrine loop in which the angiogenic factors are produced by macrophages rather than directly by infected endothelial cells, and the bacteria cause receptors for leukocytes to be

produced on the vascular endothelial cell surface. The study of verruga peruana lesions may give more insight into the *in vivo* process of angiogenesis after infection with *Bartonella*. Angiopoietin-2 (Ang-2) is expressed in endothelial cells in verruga peruana lesions, as are VEGF receptor-1 and -2, but VEGF is not (Cerimele *et al.*,2003). Angiopoietin-2 expression without VEGF expression leads to vessel regression, but Ang-2 together with VEGF form an angiogenic signal (Lobov *et al.*,2002). The VEGF production necessary for verruga peruana lesions appears to come from epidermal cells *in vivo*, rather than from the endothelial cells (Cerimele *et al.*,2003). Thus, the two cell types cooperate in angiogenesis.

B. bacilliformis, *B. quintana* and *B. henselae* are capable of stimulating the proliferation of endothelial cells directly after contact or through the use of a secreted factor (Garcia *et al.*,1992; Conley *et al.*,1994; Maeno *et al.*,1999). *B. bacilliformis* cell lysates were found to be mitogenic for HUVECs, but *B. henselae* cell lysates were much less mitogenic (Minnick *et al.*,2003). Proliferation was inhibited when *B. bacilliformis* lysates were added at high doses without pretreatment. In contrast, proliferation was induced when the high dose of cell lysate was heat-treated before addition to the HUVEC culture (Smitherman and Minnick,2005). These experiments led investigators to conclude that two factors may be involved in *B. bacilliformis* stimulation of endothelial cell proliferation, one of which appears to be a protein. Further experiments with *B. bacilliformis* suggest that GroEL may be a mitogenic factor or the chaperonin for a mitogen (Minnick *et al.*,2003; Smitherman and Minnick,2005). In addition to proliferation, other characteristics of angiogenesis are endothelial cell migration and formation of tubules which then become new vessels. Both *B. henselae* and *B. quintana* stimulate HUVEC migration and tube formation in vitro (Conley *et al.*,1994; Kirby,2004; Dehio *et al.*,2005).

The relative increase in endothelial cell number after infection with *Bartonella* may be due to increased cell division or the inhibition of programmed cell death (apoptosis) or both. *B. henselae* suppresses activation of the apoptotic proteases caspase 3 and caspase 8 (Kirby and Nekorchuk,2002). The anti-apoptotic activity requires bacterial protein synthesis. The bacteria are not required for inhibition of apoptosis, since conditioned media itself is anti-apoptotic (Kirby and Nekorchuk,2002). This indicates that the anti-apoptotic factor(s) are produced by *B. henselae* in co-culture with HUVECs and that the factor(s) are secreted (Kirby and Nekorchuk,2002). The anti-apoptotic activity of *B. henselae* requires a functional VirB/VirD type 4 secretion system (Schmid *et al.*,2004).

Seven effector protein genes designated *bep*A-G have been identified downstream of the *vir*B operon in *B. henselae,* and the proteins encoded by these genes have been shown to be translocated into human endothelial cells via the T4SS (Schulein *et al.*,2005). A *bep*A-*bep*G deletion mutant was unable to invade endothelial cells, did not activate NFκB, and did not inhibit apoptosis (Schulein *et al.*,2005). BepA was found to be the protein responsible for inhibiting apoptosis by inducing the production of a second messenger cAMP (Schmid *et al.*,2006). *B. quintana*, *B. henselae,* and several other *Bartonella* species have an additional T4SS called Trw (Dehio,2008; Nystedt *et al.*,2008). A Trw system mutant of *B. tribocorum* was apparently able to colonize the primary niche in the rat model but unable to infect erythrocytes; five days post-inoculation, the bacteria were found in the blood but no long-lasting bacteremia was established (Seubert *et al.*,2003). The Trw operon appears to be a recent lateral transfer and duplication of genes from a conjugation system of the IncW broad host range plasmid R388 (Frank *et al.*,2005). As Dehio points out, it is interesting to speculate that the Trw system replaced flagella as a virulence factor; with the exception of *B. bovis*, the species with no Trw genes retained the

flagellar system (Dehio,2008). A third T4SS, the VirB-homologous (*vbh*) system was subsequently reported. *B. tribocorum, B. elizabethae, B. grahamii, B. birtlesii* and *B. doshiae* have all three T4SSs (Saenz *et al.*,2007; Dehio,2008). *B. clarridgeiae* has only VirB, and *B. bacilliformis* has no T4SS (Dehio,2008). The acquisition of T4SSs and the loss of flagella may indicate host adaptation during radial speciation from the ancestral *B. bacilliformis* to the modern *Bartonella* spp. (Saenz *et al.*,2007).

Flagella

B. bacilliformis, *B. clarridgeiae*, *B. schoenbuchensis*, *B. chomelii*, *B. rochalimae* and *B. capreoli* are currently the only identified *Bartonella* species that are known to have polar flagella. It is estimated that more than 80% of bacterial species have flagella and use them to move through their environment towards or away from particular chemical factors. These factors are sensed by a chemotaxis network which then transmits a signal to the flagellar apparatus (Moens and Vanderleyden,1996; Armitage,1999; Thomas *et al.*,2001). Flagella are helical arrangements of one or more types of flagellin protein that form a flexible, hollow rod. Flagella may be arranged peritrichously (several flagella projecting from the entire surface), monotrichously (one flagellum at one end; polar) or lophotrichously (two or more flagella at one or both ends) on the bacterial cell. The flagellar motor is driven in some bacteria by a proton gradient, and in other genera by a sodium ion gradient. Although flagella were described before 1900, most of the knowledge regarding the many parts of the apparatus has been gleaned and published since 1970 (Macnab,1999). Over the past 30 years, investigators have found more than 50 genes involved in the biosynthesis of flagella (Macnab,2004). The major parts of the flagellar structure are the basal body that spans the cytoplasmic and outer membranes, the rod,

the exterior hook and the flagellar filament (Macnab and DeRosier,1988; Macnab,1999; Bardy *et al.*,2003; Macnab,2003). The sequential expression and assembly of the flagellar apparatus begins with the formation of the proximal basal body with its integral cytoplasmic membrane M and S rings and ends with the distal filament (Macnab and DeRosier,1988; Macnab,2003; Macnab,2004). In order to assemble the structure in the proper sequence, the flagellar biosynthesis genes are expressed in a hierarchical manner that proceeds through three or four classes of genes, depending on the organism. In the 4-class system (represented by *Caulobacter crescentus*), class I consists of *ctr*A, a gene encoding a transcription factor that is essential for expression of class II genes (Champer *et al.*,1985; Champer *et al.*,1987). Class II genes include the components of the basal body (MS ring, motor, switch, and flagellar Type III secretion system) and transcription factors *flb*D and *rpo*N which are required for expression of the next class of genes (Boyd and Gober,2001). Once the basal body is synthesized, Class III genes are the next to be expressed and include the rod and hook (Ohta *et al.*,1985). The flagellin genes are the last to be expressed, and their transcription depends on the prior assembly of the hook structure (Anderson and Gober,2000). In *E. coli*, there are 3 classes of genes in the flagellar hierarchy. Class I consists of the master regulator operon *flh*DC. $FlhD_2C_2$ is a transcriptional activator of Class II genes which includes an operon containing a flagellum-specific sigma factor σ^{28}encoded by the *fli*A gene, the *flh*B operon (part of the type III secretion system) and the *fli*L operon (which encodes cytoplasmic membrane proteins of the basal body) (Liu and Matsumura,1994).

E. coli and *Salmonella* flagellar biosynthesic mechanisms have been particularly well studied. In an elegant set of experiments, Jones and Macnab deduced much of the assembly order in *Salmonella enteric* serovar *Typhimurium* by use of temperature sensitive mutants (Jones

and Macnab,1990). The assembly of the flagellum begins with the most proximal structure known as the basal body. The basal body consists of the MS ring which has two parts but is made of 24-26 subunits of one protein, FliF(Thomas *et al.*,2006). FliG, the switch protein of the cytoplasmic C-ring is assembled after FliF is integrated into the cytoplasmic membrane(Francis *et al.*,1992) followed by FliM and FliN.(Kubori *et al.*,1997). FliE has been described as both a basal body protein and a rod protein because it probably lies on the distal face of the MS ring at the proximal end of the rod (Macnab,2003). The MS ring is assembled in the bacterial cytoplasmic membrane by the Sec general export pathway probably at the same time that the flagellar type 3 export apparatus is assembled within the MS ring (Macnab,2003). The flagellar export apparatus, which is involved in the translocation of many of the flagellar proteins to the outside of the cytoplasmic membrane, has at least 6 proteins integrated into the membrane including FlhA, FlhB, FliO, FliP, FliQ and FliR (Macnab,2003). Substrates to be exported dock on the inner surface where the ATPase (FliI), its regulator (FliH), and chaperone protein (FliJ) are located (Thomas *et al.*,2004; Stafford *et al.*,2007). The substrates are sorted so that early rod and hook proteins are exported before the later hook and filament proteins(Stafford *et al.*,2007). The sorting and export depend on N-terminal signals on the exported proteins (Stafford *et al.*,2007). All proteins residing outside of the cytoplasmic membrane, except the P and L rings, are exported via the flagellar T3SS (Macnab,2004).

The rod spans the cytoplasmic and outer membranes, and the proximal portion of it is made of a helical arrangement of protein subunits FlgB, FlgC, and FlgF (Homma *et al.*,1990). Another protein, FlgJ is essential for rod formation (Kubori *et al.*,1992). At the C-terminus of FlgJ is a muraminidase domain that may be involved in digesting the peptidoglycan layer for the protruding rod (Macnab,2003). A second proposed function of FlgJ is as a rod capping protein

(Hirano *et al.*,2001). Experiments indicate that both of the two possible functions of FlgJ could be correct and may be attributable to the C- and N-termini, respectively (Hirano *et al.*,2001). The last protein of the rod structure is FlgG, and it lies at the rod-hook junction. It is not known how the rod length is determined.

The L and P ring proteins are exported via the Sec pathway and assemble around the rod‘s circumference (Homma *et al.*,1987; Jones *et al.*,1987; Jones *et al.*,1989). The P ring surrounds the rod in the periplasmic space and is made of FlgI. The L ring, FlgH, a lipoprotein, is located in the outer membrane.

The next substructure to be assembled is the hook. FlgE is the single hook protein in *Salmonella*, and the protein subunits are exported through the lumen of the rod and growing hook. As the hook assembly begins, the rod capping protein is replaced by a hook cap, FlgD (Ohnishi *et al.*,1994). The hook is not correctly assembled unless the hook capping protein, FlgD, is present (Suzuki *et al.*,1978; Suzuki and Komeda,1981). A *flg*D mutant was found to secrete FlgE in to the culture media (Ohnishi *et al.*,1994). When the length of the hook reaches about 55nm, hook assembly stops. FliK is the hook length control protein and it is exported during hook assembly (Minamino *et al.*,1999). The mechanism for producing a hook of the proper length is still unknown. Makishima et al. proposed a model in which the C ring acts as a measuring cup that holds 120 hook subunits(Makishima *et al.*,2001). FliK mutants produce elongated hooks with no filament (Muramoto *et al.*,1999). Recently, Moriya et al. proposed that FliK acts as a “ruler” with proper hook length being dependent on FliK and on the hook polymerization rate (Moriya *et al.*,2006). When the hook reaches the desired length, the flagellar T3SS switches to export late flagellar substrates including hook-associated proteins (HAPs) FlgK, FliD, and FlgL (Williams *et al.*,1996; Minamino and Macnab,1999; Hirano *et*

al.,2003). The change of substrate specificity is dependent on FlhB which is some how associated with the hook length protein FliK (Ferris and Minamino,2006). FliD replaces FlgD and remains with the growing filament as its cap protein.

The final structure to be assembled is the filament which is composed of flagellin protein. Some flagella filaments contain over 20,000 flagellin subunits. The flagellin proteins are exported through the hollow filament and are added one by one to the distal end of the growing structure. The filament cap, FliD, may act as a scaffolding to orient the incoming flagellin subunits, but it also acts more like a filter than a stopper, since some proteins such as FlgM, the anti-sigma factor, continue to be exported from the flagellum (Chilcott and Hughes,2000).

The flagellar motor in *E. coli* consists of a stator made of MotA and MotB, perhaps which is believed to contain 4 MotA subunits and 2 MotB subunits (Khan *et al.*,1988). MotA has four transmembrane domains, and the cytoplasmic domain has two charged residues that are important for rotation (Zhou and Blair,1997). Most of MotB is in the periplasmic space and the C-terminal region attaches to the cell wall (Muramoto and Macnab,1998). The rotor/switch complex is made of FliG, FliM and FliN which controls rotation and the direction of rotation (Yamaguchi *et al.*,1986; Yamaguchi *et al.*,1986). In *E. coli* and some other bacteria, the flagella turn in both the clockwise and counter-clockwise direction (Khan and Macnab,1980; Macnab and Han,1983; Yamaguchi *et al.*,1986; Gotz and Schmitt,1987; Cohen-Ben-Lulu *et al.*,2008). Counter-clockwise rotation results in swimming and clockwise rotation causes tumbling (Macnab and Han,1983). The default direction is counter-clockwise (Harshey and Toguchi,1996). When the bacterial cell encounters attractants or repellents that bind to methyl-accepting chemotaxis proteins on the cell membrane, a signal is recognized by CheA-CheW (Armitage,1999). The response regulator CheY is then phosphorylated (Armitage,1999).

CheY, in its phosphorylated state, binds to FliM and causes a change in rotational bias from counter-clockwise to clockwise (Sanders *et al.*,1989; Bren and Eisenbach,2001). Other bacteria have unidirectional flagellar rotation, usually counter-clockwise. However, alpha-proteobacteria with polar flagella such as *Sinorhizobium meliloti* display clockwise rotation and have two CheY regulators (Scharf and Schmitt,2002). CheY2 regulates the speed of the motor, and CheY1 acts as a sink for phosphoryl groups from CheY2 (Scharf and Schmitt,2002). The speed variation depends on two additional motor proteins MotC and MotE (Platzer *et al.*,1997; Eggenhofer *et al.*,2004). Both MotC and MotE are periplasmic. MotC binds MotB and regulates the proton channel (Scharf and Schmitt,2002). MotE is the chaperone for MotC (Eggenhofer *et al.*,2004). The MotD protein was originally believed to be part of the motor mechanism because mutations in motD resulted in a non-motile cell(Platzer *et al.*,1997). However, later studies indicated that MotD was the hook length control protein and as a consequence was renamed as FliK (Eggenhofer *et al.*,2006). Several models have been proposed for the mechanism of torque generation, but none has been confirmed (Kojima and Blair,2004).

The regulation of flagellar gene expression and protein assembly is as complex as the structure itself. In all bacterial flagellar systems examined, production of flagellar components is regulated by a hierarchical cascade of gene expression in which expression of one class of genes does not take place until expression of a preceding class. The expression of the first class, the master regulator, is induced by environmental factors as well as internal signals. Expression of *flh*DC has been shown to be linked to the cell cycle with expression at its lowest point during cell division (Pruss and Matsumura,1996; Pruss *et al.*,1997; Pruss and Matsumura,1997). Likewise, activity of CtrA in *C. crescentus* is regulated by the cell cycle (Quon *et al.*,1996; Domian *et al.*,1999) Cyclic AMP-catabolite activator protein has been shown to activate *flh*DC expression,

and histone-like nucleoid-structuring protein H-NS represses *flh*DC expression in *E. coli* (Soutourina *et al.*,1999). FlhDC production is also influenced by temperature, pH and oxygen levels (Li *et al.*,1993; Shi *et al.*,1993; Liu *et al.*,2000; Soutourina *et al.*,2001). In one study, acetyl-phosphate levels in the cell increased during increased osmolarity as did phosphorylated OmpR, a protein involved in osmoregulation (Shin and Park,1995). The authors found that OmpR-P bound to the *flh*DC promoter and inhibited transcription (Shin and Park,1995). However, subsequent studies did not confirm that finding (Kutsukake,1997). Particular mutations in heat shock protein genes *dna*K, *dna*J or *grp*E resulted in non-motile *E. coli* due to a lack of flagellin production (Shi *et al.*,1992). Specific mutations in other heat shock proteins, namely *gro*EL140 and *gro*ES30, did not cause a loss of motility (Shi *et al.*,1992). Transcriptional fusions indicated that the *dna*K mutant strain had decreased transcription of *flh*D and *fli*A, which encodes σ^{28}, the flagellar sigma factor (Shi *et al.*,1992). When σ^{28} is low, *flh*DC expression is low (Kutsukake,1997). It may be that feedback mechanisms exist in bacteria to turn off the energy-expensive production of flagella during times of stress.

Sigma-28 RNA polymerase is also a critical part of the regulatory system for the expression of class III flagellar genes (Ohnishi *et al.*,1990). FlgM is an anti-sigma factor that binds sigma-28 during the assembly of the basal body and hook (Hughes *et al.*,1993). Once the basal body and hook are completed, FlgM is exported through that structure and σ^{28} is free to promote the transcription of the flagellin, motor and chemotaxis protein genes in class III. Master regulators and flagellar-specific sigma factors have been found in many bacterial genera possessing lateral or polar flagella and some organisms such as *V. alginolyticus* possess both types of flagella with their respective regulatory systems and flagellar motors (Atsumi *et al.*,1992; Kawagishi *et al.*,1996).

Two-component regulators have been found to regulate flagellar production. A transcriptional regulator, VjbR, a protein involved in quorum sensing in *Brucella melitensis* regulates expression of *vir*B and flagellar genes (Delrue *et al.*,2005). A *vjb*R mutant was greatly attenuated in the mouse model (Delrue *et al.*,2005).

Flagella and Virulence

The degree of association between flagella and virulence is controversial and may be species- or even strain-specific. Motility helps bacteria reach the area or organ they ultimately colonize. For example, motility was required in *Burkholderia cepacia* for invasion of cultured epithelial cells (Tomich *et al.*,2002); In addition, *Campylobacter jejuni* need flagella (specifically FlaA) to pass the gastrointestinal tract, and anti-flagellar antibodies reduced colonization (Ueki *et al.*,1987; Wassenaar *et al.*,1993) A *fla*A*fla*B double mutant of *Helicobacter mustelae* was unable to colonize a ferret model of infection and a *flb*A mutant of *H. pylori* was non-motile and unable to colonize gerbils (McGee *et al.*,2002). In some bacteria, expression of flagellin proteins undergoes phase variation, and the change in flagellin antigen expression aids in evading host immune responses (Nuijten *et al.*,1995; Ikeda *et al.*,2001; Bonifield and Hughes,2003). Flagella have also been shown to be involved in adherence of bacteria to mammalian cells. For example, adherence to mouse cecum was 10-fold lower in non-flagellated *Clostridium deficile* strain (Tasteyre *et al.*,2001) while *Aeromonas* strains lacking polar flagella failed to adhere to enterocytes in culture and showed a decreased ability to form biofilms (Kirov *et al.*,2004). Further involvement in virulence has been proposed for the flagellar systems of *Campylobacter jejuni*, *Yersinia enterocolitica* and *Helicobacter pylori*. In *C. jejuni*, secretion of the virulence factor CiaB is dependent on a functional flagellar apparatus (Konkel *et al.*,2004).

FlaC secretion requires a minimal flagellar structure, and invasion of a *C. jejuni fla*C mutant was reduced to 14% of the parental strain (Song *et al.*,2004). The phospholipase YplA was found to be exported by the flagellar T3SS in *Y. enterocolitica* (Young *et al.*,1999; Young and Young,2002). And in *H. pylori*, FlbA, a protein involved in flagellar biosynthesis, has been shown to modulate urease activity and is required for motility and virulence(McGee *et al.*,2002).

Bartonella with Flagella

The flagellum of *B. bacilliformis* is a virulence factor for the organism as indicated by the observation that antiflagellar antibodies reduced attachment and invasion of *B. bacilliformis* into human erythrocytes (Scherer *et al.*,1993). The hallmark of *B. bacilliformis* infection in humans is severe hemolytic anemia (Anderson and Neuman,1997). Even though *B. clarridgeiae* possess flagella and has been isolated from cat blood, most cats infected with *B. clarridgeiae* do not show many overt signs or symptoms of disease and none has been documented with severe anemia (Kordick *et al.*,1995; Kordick *et al.*,1999). Therefore, it seems likely that if *B. clarridgeiae* invades red blood cells, it does not damage them and may reside intracellularly while replicating in other types of cells. While phenotypically similar to *B. bacilliformis*, the route of infection and the symptoms of *B. clarridgeiae* infection in humans more closely resemble those of *B. henselae*, a non-flagellated species. These findings prompted us to ask: is *B. clarridgeiae* capable of invading human endothelial cells and is motility a requirement for invasion of host cells by *B. clarridgeiae*?

To investigate the role of flagella in *B. clarridgeiae* pathogenesis, we sought to separate motility from any other function flagella may have. Depending on the hierarchy of *B. clarridgeiae's* flagellar genes, a site-specific mutation in a gene(s) encoding a flagellar motor

protein(s) may render the organism non-motile while still allowing flagellar biosynthesis. The flagellin gene *flaA* in *B. clarridgeiae* has been sequenced (Sander *et al.*,2000), but other genes in the flagellar regulon of *B. clarridgeiae* have notbeen published or released by GenBank. In this study we determined the sequence of the flagellar motor genes *motBC* of *B. clarridgeiae* and created a single site-specific mutation interrupting both *mot*B and *mot*C. A similar method was used to create a mutation in *fla*A. The *mot*BC mutant was non-motile but flagella were seen by electron microscopy. The *fla*A mutant was void of flagella and was non-motile. The *fla*A and *mot*BC mutants and wild-type *B. clarridgeiae* were tested for their ability to invade HMECs *in vitro,* and the proteins secreted by the three strains and the HMECs were analyzed. We report that *B. clarridgeiae* 1) does not require a flagellar structure to invade endothelial cells; 2) enters endothelial cells as single bacteria rather than in a large mass; and 3) causes proliferation of endothelial cells. In addition, the wild-type bacteria, *fla*A and *mot*BC mutants have different effects on HMECs as demonstrated by protein secretion.

CHAPTER I

Site-specific Mutagenesis of Flagellar Genes in *B. clarridgeiae*

Introduction

Many species of bacteria possess flagella and use them as propellers to move towards favorable environmental conditions (Macnab and Aizawa,1984). In *E. coli*, the concentration of attractant or repellent in the environment is sensed by the bacterium through exposed methyl-accepting chemotaxis proteins which transduce a signal to internal chemotaxis proteins. The signal is then relayed to the flagellar motor which drives the helical flagellar filament (Scharf and Schmitt,2002). The flagellar motor in most bacteria is fueled by a protonmotive force created by a proton gradient across the inner membrane (Macnab,1986). The stator of the flagellar motor appears to be made of a ring of only two proteins, MotA and MotB, in *E. coli*, with a ratio of MotA to MotB reported to be $MotA_4MotB_2$ (Berg,2003). The MotB protein is predominantly in the periplasm and has a peptidoglycan binding domain. Therefore, MotB serves as an anchor as well as proton channel (Kojima *et al.*,2008). Exactly how the motor works is undetermined, but one proposed mechanism hypothesizes that the protonmotive force induces conformational changes in MotB which applies force to the rotor protein FliG(Blair,2003). The rotor turns the rod and the hook which causes the filament to spin.

In a close relative of *B. clarridgeiae*, *Sinorhizobium meliloti*, two additional *mot* genes were discovered downstream from *mot*B (Platzer *et al.*,1997). MotC encodes a putative 434 amino acid protein, and MotD is predicted to be 475 amino acids in length (Platzer *et al.*,1997). MotC was found in the periplasm and MotD was thought to be located in the cytoplasm. Mutants in *mot*B, *mot*C or *mot*D were non-motile but had intact flagella (Platzer *et al.*,1997).

Complementing *mot*B or *mot*C did not completely restore motility, but the *mot*D deletion mutant regained complete motility when *mot*D was provided in trans (Platzer *et al.*,1997). The authors postulated that the correct stoichiometry of MotB and MotC was necessary for controlling the speed of flagellum rotation (Platzer *et al.*,1997). Originally, no known function was attributed to MotD; however, several years after the original description, other investigators concluded that MotD was actually FliK, the hook-length control protein (Eggenhofer *et al.*,2006). A knock-out mutant in *mot*D resulted in variable flagellar hook lengths, typical of *fli*K mutations, which cause the cell to be paralyzed.

B. clarridgeiae has been shown to produce polar flagella (Clarridge *et al.*,1995; Collins,1996). Antibodies made against *B. bacilliformis* flagellar filaments inhibited the bacteria from invading erythrocytes (Scherer *et al.*,1993). Similarly, whole cell antibody raised against a crude cell wall preparation from *B. bacilliformis* blocked the organism's entry into HUVECs (Hill *et al.*,1992). It is not known whether *B. clarridgeiae* is able to invade human endothelial cells, but invasion would be a likely requirement in order to produce the Cat Scratch disease-like symptoms that have been attributed to the organism. Also, if *B. clarridgeiae* is capable of invading endothelial cells, are flagella required? Do the flagella of *B. clarridgeiae* function only as a means of locomotion? In order to address these questions, we sought to separate the function of motility from any other function flagella may have by creating non-motile mutants with and without intact flagella by insertional mutagenesis of flagellar motor genes and the flagellin gene, respectively. We then compared the ability of these mutants to invade human endothelial cells with that of the wild-type *B. clarridgeiae*.

Materials and Methods

Bacteria. *B. clarridgeiae* type strain ATCC 51734 was grown for 5 days on heart infusion agar with 5% rabbit blood (HIA-RB) at 37°C and 5% CO_2. *B. clarridgeiae* mutants were grown on HIA supplemented with kanamycin (50μg/ml). *E. coli* was grown overnight at 37°C on Luria Bertani (LB) agar. Bacterial strains and plasmids used are listed in Table 1.

Table 1. Bacterial Strains and Plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Bartonella clarridgeiae ATCC 51734 ^{T}	a.k.a.,Houston-2 cat	ATCC
E. coli XL2 Blue	Host strain for cloning	Stratagene
pBBR1MCS-2	Rep_{EC},Rep_{Bb},Km^r	ME Kovach (Kovach *et al.*,1995)
pPCRscript	Cloning vector, Rep_{EC},Ap^r	Stratagene
pBCmotBAmp	pPCRscript containing ~480bp SmaI-SacI fragment of *mot*BC	This study
pBCmotBKan	pBCmotBAmp cut with BspHI and *bla* (Amp^r) removed and replaced with ~1700bp nptI Km^r fragment from pBBR1MCS-2.	This study
pBCflaAAmp	pPCRscript containing ~1000bp BamHI-SacI fragment of *fla*A	This study
pBCflaAKan	pBCflaAAmp cut with BspHI and *bla* (Amp^r) removed and replaced with ~1700bp nptI Km^r fragment from pBBR1MCS-2.	This study

Transposon mutagenesis. *B. clarridgeiae* was harvested from HIA-RB plates and suspended in 1ml heart infusion broth at room temperature. The suspension was centrifuged at 2900 x g for

5min at 4°C. The pellet was washed in ice-cold 10% glycerol and centrifuged again. The loose pellet was resuspended in 1ml 10% glycerol. One microliter of EZ::TN <Kan-2> Tnp transposome (Epicentre Biotechnologies, Madison, WI) was added to 40μl *B. clarridgeiae* suspension undiluted, diluted 1:1, diluted 1:3 or diluted 1:7 in 10% glycerol. The reactions were iced for 15min then transferred to iced cuvettes (2cm, Bio-Rad). The cells were electroporated at 12.5kV/cm (2.5kV), 25μF capacitance, and 200 ohms. One milliliter of trypticase soy broth was added to each cuvette, and then the suspension was poured on an HIA-RB slant and incubated overnight at 37°C, 5% CO_2. After 23hr the broth appeared cloudy. Aliquots of the culture were plated on HIA-RB plates containing kanamycin at 50μg/ml. The plates were incubated overnight. After 12 hours of incubation, the plates were examined for growth.

Test for motility. Transparent agar was prepared by a modification of the method of Benson et al. (Benson *et al.*,1986). Briefly, rabbit blood was lysed by mixing 1 volume of outdated rabbit blood with 3 volumes of deionized water. The mixture was shaken gently to lyse the cells and then centrifuged at 3000 x g for 15 min at 4°C to remove debris. The supernatant was retained and added to Heart Infusion broth at a final concentration of 5% vol/vol. Motility agar plates were prepared by adding agar to HIB-RB lysate at a final concentration of 0.35%. Single colonies of bacteria were picked from plated media with a sterile toothpick and stabbed into the motility agar. The motility agar plates were incubated without inversion for 5 days at 37°C with 5% CO_2.

DNA purification. DNA was purified from bacteria using a Qiagen QIAampDNA mini kit (Valencia, CA) as per the kit protocol and eluted with 200μl PCR grade water (APEX Bioresearch Products distributed through DocFrugal Scientific, San Diego, CA).

Sequencing. DNA sequencing was performed on *B. clarridgeiae* transformant genomic DNA using an ABI Prism Big Dye dye terminator kit (Foster City, CA) and primers supplied with the EZ::TN <Kan-2> transposon kit. Sequencing reactions were purified through Centri-Sep columns (Princeton Separations, Princeton, NJ) and electrophoresed through a 50cm capillary array filled with POP-6 polymer using an ABI 3100 automated capillary sequencer. Sequences were analyzed using a UNIX operating system and Wisconsin Package version 10.2 (Genetics Computer Group, Madison, WI).

Genomic DNA library construction. Four genomic DNA libraries were made from *B. clarridgeiae* wild-type DNA according to the instructions included in the Genome Walker Universal Kit (Clontech, Mountain View, CA). The *fla*A region was amplified using primers supplied in the kit and flaF122 or flaR1125 (Table 2). Amplicons were sequenced using flaF122 and flaR1125 and additional primers that were designed as sequence was elucidated (see Table 2).

Site-directed mutations in *fla*A and *mot*BC. To generate a suicide vector, a 1025-bp fragment of the 1200-bp *fla*A gene was amplified using primers using primer SmaflaAF and SacflaAR and ligated into the PCRscript plasmid previously digested with restriction enzymes Sma and Sac and purified. PCR script contains the pMB1 replication origin which functions in *E. coli* but does not function in *Bartonella*. A selectable marker, the kanamycin-resistance gene (*npt*I), was amplified from plasmid pBBR1MCS-2 and ligated into the *fla*A suicide plasmid. A single crossover recombination event would produce a Kan-resistant strain with an interrupted *fla*A gene.

A similar plasmid was constructed to disrupt flagellar motor genes *mot*BC. The DNA sequences of the *mot*B and *mot*C genes were determined in this study. The PCRscript plasmid with the *npt*I gene was ligated to a 460-bp DNA fragment that comprises the 3' terminus of *mot*B and the 5' end of *mot*C from *B. clarridgeiae.*

In order to make large scale plasmid stocks, the suicide vectors were first electroporated into *E. coli*. Plasmid preparations were made from individual kanamycin-resistant colonies subsequently grown in LB broth. *B. clarridgeiae* was electroporated with the purified plasmid DNA and plated on HIA-RB containing 50mg/ml kanamycin. Individual kanamycin-resistant colonies were picked and passed to fresh HIA-RB-Kan plates.

DNA and protein sequence analysis. DNA sequences were analyzed using the GCG (Accelrys, Transmembrane regions and signal peptides were predicted by the program Phobius (Kall *et al.*,2007).

HMEC invasion assay. CDC.HMEC-1 (CDC, Atlanta, GA.) human microvascular endothelial cells were grown at 37^{o}C and 5% CO_2 using MCDB-131 (GIBCO, Invitrogen, Carlsbad, CA) media supplemented with 10% fetal bovine serum (Hyclone, Ogden, UT) and 2mM L-glutamine (GIBCO, Invitrogen, Carlsbad, CA). Cells were seeded at 1.5x10^{5}/mL. HMECS with passage number below 16 were used in invasion assays. HMECs were grown to 80% confluency and inoculated with *Bartonella* at HMEC cell to bacteria ratios of 1:10, 1:30 or 1:100.

Transmission electron microscopy. Human microvascular endothelial cells (1.5x10^{5}/ml) were infected with *B. clarridgeiae* wild-type, the *fla*A mutant or the *mot*BC mutant at multiplicity of

infection (MOI) of 100. After 5 days, the medium was removed and the cells were washed once with phosphate buffered saline, pH 7.4 (PBS). The cells were fixed with Karnovsky's fixative (Electron Microscopy Sciences, Hatfield, PA) for 1 hour before a second wash with PBS. The flasks were stored at 4°C in PBS until processed for transmission electron microscopy.

Phase contrast microscopy. Human microvascular endothelial cells ($1.5x10^5$/ml) in 6-well tissue culture dishes were infected with *B. clarridgeiae* wild-type, the *fla*A mutant or the *mot*BC mutant at MOIs of 10, 30 or 100 and viewed under a phase contrast microscope (Zeiss) at 1 to 6 days post-infection to assess endothelial cell proliferation and bacterial attachment.

Confocal microscopy. HMECs grown on sterile coverslips and infected with *Bartonella* or uninfected were permeabilized at 1 to 5 days post-infection with TritonX100 and then stained. HMECS were treated with propidium iodide to stain dead bacteria red (Baclight kit, Invitrogen, Carlsbad, CA) and Alexa 488 phalloidin (Invitrogen, Carlsbad, CA) which stains F-actin green.

Gentamicin protection assay. HMECS were inoculated with *Bartonella* at an MOI of 30. After 1, 3, or 5 days, the cultures were treated with gentamicin (250µg/ml) to kill extracellular *Bartonella*. The cells were washed twice with media to remove the gentamicin, and the HMECs were lysed with 1% saponin in PBS to release the intracellular organisms. Cell lysates were serially diluted and plated on HIB-Rb agar. The number of intracellular bacteria was compared to lysed cells that had not been treated with gentamicin (intracellular and extracellular bacteria). Medium from gentamicin-treated cultures was also plated on agar to test for sensitivity of the bacteria to the antibiotic. Each experiment was conducted three or more separate times.

Table 2. PCR and Sequencing Primers

Name	Sequence 5' to 3'	Gene target
flaF122	TGT TTC GAC AGG TTT GCG TAT CAG	*fla*A
flaR1125	GCA AGG CAG CCA ACT TAG CAG	*fla*A
flaA1452R	GCC GAT TGA ACT GCA TTA GAA	*fla*A
flaA1516R	ATA CCC TGC TGC CAT ACC AAC	*fla*A
flaA1977F	TGG CGA GTT ACA TTC AGA CAT	*fla*A
flaA2074F	ATT GGA GCC GCT GTT AAT TTG	*fla*A
flaA3144F	GGG TAA GAG CAG TGG CGA TTT	*fla*A
BspHI-U23751F[a]	AAA TCA TGA CCG GAA GCA TAA AGT GTA AAG C	*npt*I
BspHI-U23751R	AAA TCA TGA GCC TGA AGC CCG TTC TG	*npt*I
SMAflaAF	AAA CCC GGG TGT TTC GAC AGG TTT GCG TAT CAG	*fla*A
SACflaAR	AAT GAG CTC GCA AGG CAG CCA ACT TAG CAG	*fla*A
SMAMOTBF	AAA CCC GGG CTA TTC CGA CGG CGC AGA C	*mot*BC
SACMOTBR	AAT GAG CTC GAA GCC CTT CCT TGT TGC ATA AGA	*mot*BC
MotB21F	TAT GAG TTT CCG AAA GA	*mot*BC
MotB639F	GGA GGA CAT GGC GAT CA	*mot*BC
MotB639R	TGA TCG CCA TGT CCT CC	*mot*BC
MotB1255F	ATA TAT TGG CCC ATG TG	*mot*BC
MotB1255R	CAC ATG GGC GAA TAT AT	*mot*BC
MotB1809F	AAC AAG GAA GGG CTT CT	*mot*BC
MotB1809R	AGA AGC CCT TCC TTG TT	*mot*BC
MotB2443F	TTT GGC CTT ATT AGT TC	*mot*BC
MotB2515R	CTG TAA TAT AGC GGG AGT AAA T	*mot*BC
MotB3037R	GTG CGA GAG AAT GTT TGT CTA C	*mot*BC

[a] restriction enzyme recognition sequence

Proliferation assay. HMECs in flat-bottom culture plates were not infected, infected with *B. clarridgeiae* wild-type, the *B. clarridgeiae fla*A mutant or the *B. clarridgeiae mot*BC mutant. Proliferation was assessed using the MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay according to the manufacturer's instructions (Roche). In similar experiments, to eliminate the possibility of bacteria interfering with the MTT assay, the HMECs were treated with gentamicin (250µg/ml) to kill extracellular bacteria, washed and then treated with MTT for 4 hours at 37°C with 5% CO_2. After the MTT treatment, solubilization solution was added to the cells and the plates were incubated overnight at 37°C with 5% CO2. The plates were read in a scanning spectrophotometer at 530nm. Each sample parameter was tested in 8 wells, and three or more experiments were run separately.

Cytotoxicity assay. The cytotoxic effect of *Bartonella* infection was quantitated based on the measurement of lactate dehydrogenase activity in the supernatant of microtiter plate wells containing HMECs and *Bartonella*. The HMECs were inoculated with *Bartonella* at an MOI of 10, 30 or 100. At certain time points, 100µl of each supernatant was combined with 100µl of assay buffer, and the absorbance was measured. Triton X-100 treated HMECs were used as the positive high control, and cell culture medium alone was used as the negative low control. Each sample parameter was tested in 8 wells, and three or more experiments were run separately.

Results

***B. clarridgeiae fla*A gene region sequence analysis.** The *fla*A gene sequence of *B. clarridgeiae* has been previously published (Sander *et al.*,2000). Primers designed from that sequence (Table

2) were used to sequence the DNA surrounding the *fla*A gene to determine if flagellar genes were in a flagellar operon such as is found in *A. tumefaciens*. A 3415-bp region surrounding and including the 1260-bp *fla*A gene was sequenced. In *B. clarridgeiae*, *fla*A was found to be located between proC (pyrroline-5-carboxylate reductase) and *etf*D (electron transfer flavoprotein-ubiquinone dehydrogenase). This gene arrangement is identical to that of *B. bacilliformis* (GenBank accession number for genome sequence NC_008783). In *B. henselae* the gene between *pro*C and etfD is a phage-related protein (GenBank accession number NC_005956), and *B. quintana* has neither a phage related gene nor *fla*A between *pro*C and *etf*D (NC_005955). In *A. tumefaciens*, the genes surrounding *fla*A are all flagellar related (NC_003062) (Figure 2).

Non-motile mutants created by transposon mutagenesis. Attempts to amplify the *B. clarridgeiae mot*B gene using primers derived from the *A. tumefaciens* or *S. meliloti mot*B sequence were unsuccessful. At the time, the *B. bacilliformis mot*B and genome sequences were not available. In order to find the flagellar motor genes, *B. clarridgeiae* strain ATCC 51734^{T} was mutagenized by random transposon mutagenesis, and non-motile mutants were identified on semi-soft agar. The Tn5 insertion site for each of 18 non-motile transformants was determined by sequencing the surrounding DNA using the transposon-specific primers supplied with the transposon kit. The sequences of three flagellar-related genes had been interrupted by transposons in one or more mutants: *mot*B, *mot*C and *fli*F. FliF is the protein that makes up the entire MS ring of the flagellar basal body and is in the second class of genes in the flagellar assembly hierarchy of most bacteria. Because a *fli*F mutant would interrupt the flagellar assembly at an early stage, it was not investigated further. A *mot*B or *mot*C motor mutant,

however, should have an intact flagellum and be paralyzed because of the faulty motor. Primers derived from the *motB* and *motC* sequences around the transposon were used to amplify a larger portion of *B. clarridgeiae* genomic DNA in genome walker libraries. Through primer walking, 3792 base pairs of DNA sequence including the full length gene sequences of *mot*B and *mot*C and the partial sequence of another flagellar gene (m*ot*D) were determined.

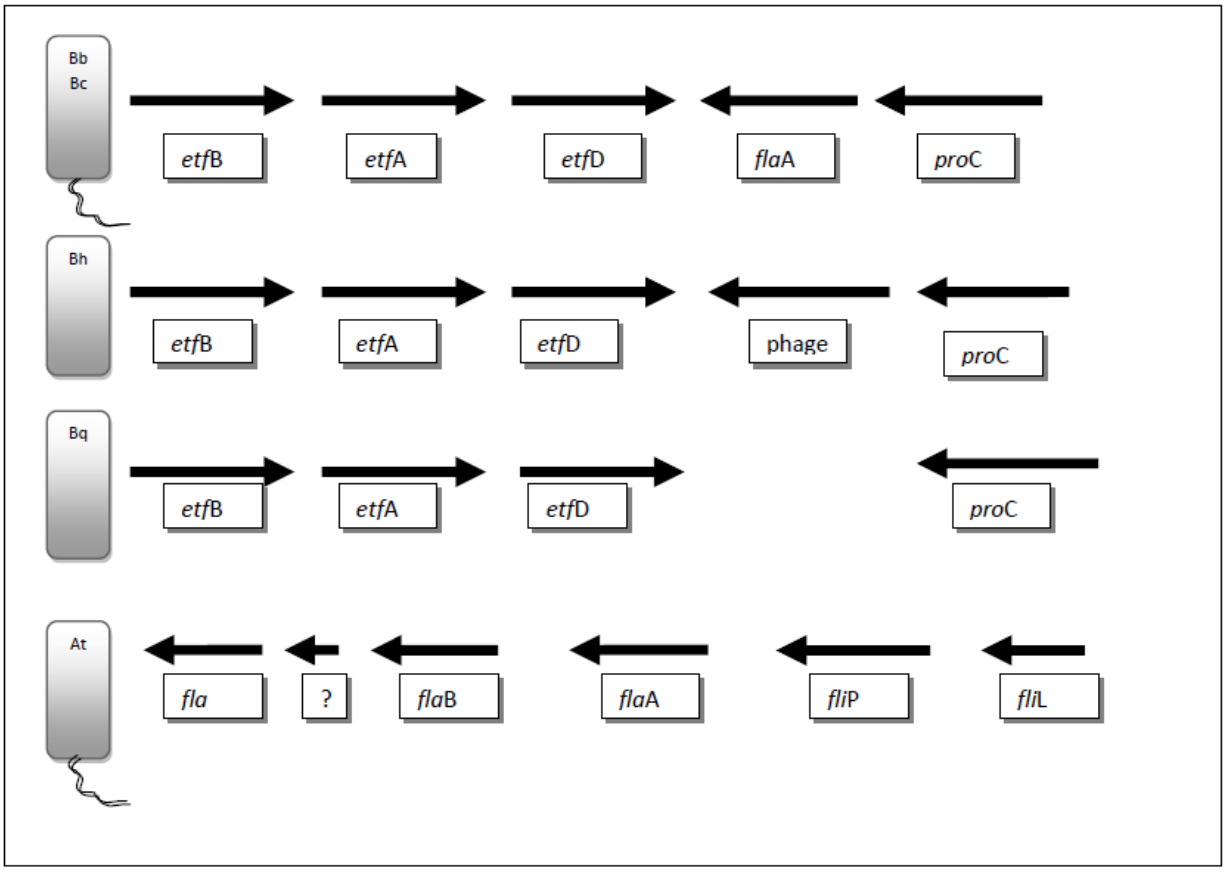


Figure 2. Gene arrangement surrounding *fla*A in *B. bacilliformis*, *B. clarridgeiae*, *B. henselae*, *B. quintana* and *A. tumefaciens*. Order of genes in *B. clarridgeiae* (Bc), *B. bacilliformis* (Bb) *B. henselae* (Bh), *B. quintana* (Bq), and *A. tumefaciens* (At) determined from genome sequence.

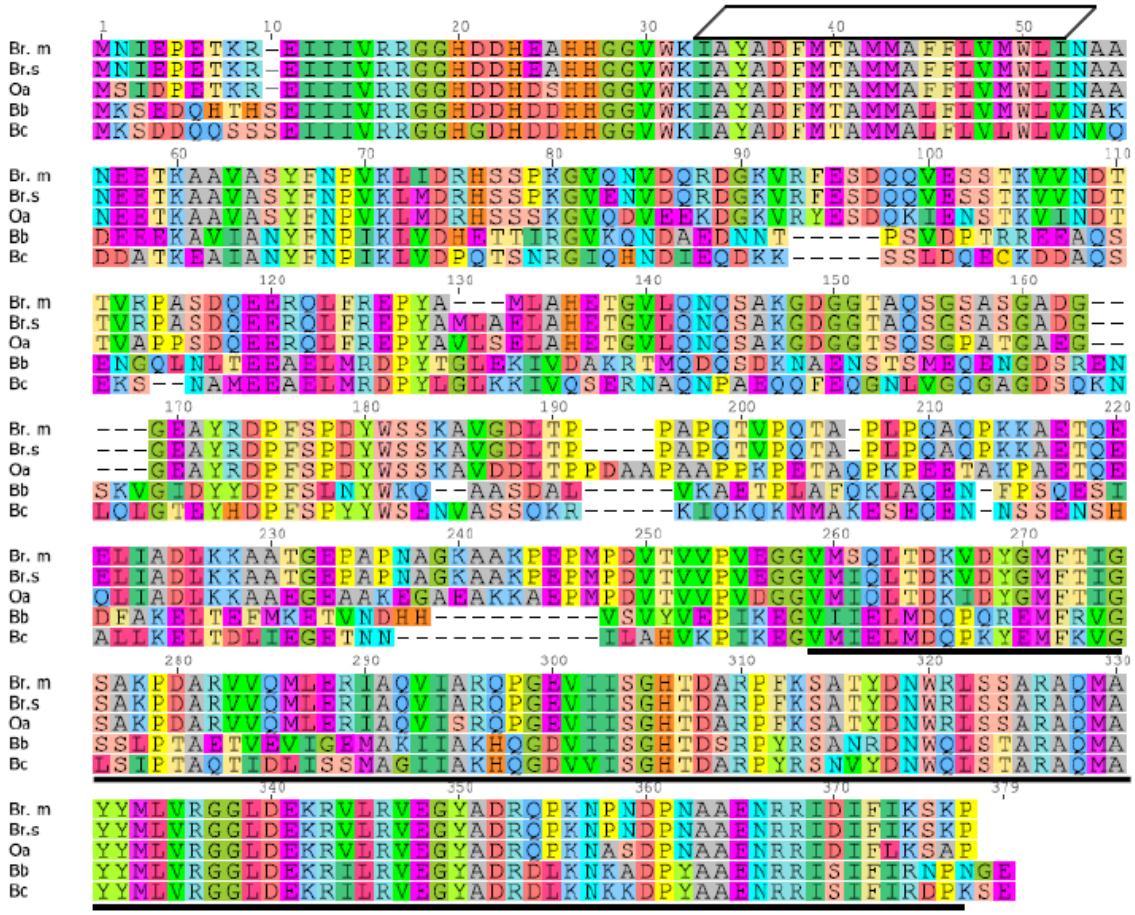


Figure 3. Alignment of MotB protein sequences. *Brucella melitensis* (Br.m) GenBank accession number AAL53395, *Brucella suis* (Br.s) GenBank accession number AAN34303, *Ohrobacter anthropi* (Oa) GenBank accession number ABS16898, *B. bacilliformis* (Bb) ABM44931 and *B. clarridgeiae* (Bc). The transmembrane region is indicated by a parallelogram above the sequence, and the OmpA-like region is underlined.

The *mot*B gene was found to be 1065bp long and encoded a 355 amino acid protein. The N-terminal region was predicted to reside in the cytoplasm, and one transmembrane region with an alpha helix was predicted (Geneious). The final third of the polypeptide contained a 97-aa OmpA-like sequence which corresponds to the putative polysaccharide-binding portion of the

protein. There was 60% similarity over the 357aa shared by *B. bacilliformis* and *B. clarridgeiae mot*B, and *B. clarridgeiae* and *Brucella melitensis mot*B proteins were 42% similar. The OmpA-like domain of the *B. clarridgeiae mot*B sequence was 83% and 65% similar to the corresponding domain in the MotB of *B. bacilliformis* and *B. melitensis*, respectively. When the MotB proteins of *B. clarridgeiae*, *B. bacilliformis*, *B. melitensis*, *B. suis* and *Ochrobacter anthropi* were aligned, 17 of the 21 amino acids in the predicted transmembrane region were completely conserved, and 61 of the 97 residues of the OmpA-like region were identical (Figure 3).

The gene identified as *mot*C in *B. clarridgeiae* was 1350 bp long and was 67% identical to the homologous gene in *B. bacilliformis*. The *B. clarridgeiae* gene encoded a 450-aa polypeptide, and both the *B. clarridgeiae* and *B. bacilliformis* MotC proteins had no predicted transmembrane region and no predicted signal peptide. Therefore MotC is most likely cytoplasmic. The MotC proteins of *B. clarridgeiae*, *B. bacilliformis, A. tumefaciens, B. melitensis, B.ovis, O. anthropi* and *S. meliloti* were not well conserved and shared only 59 identical amino acids, while *B. melitensis, B. ovis* and *O. anthropi* MotC proteins were 74.5% identical to each other. Three signal sequence prediction programs indicated that the MotC proteins from *Bartonella*, and *Brucella ovis* did not have a signal sequence while *S. meliloti*, *A. tumefaciens, Brucella melitensis* and *O. anthropi* MotC proteins included a signal sequence.

The sequence of a 717-bp fragment of a gene downstream of *mot*C was determined, and BLAST results indicated homology to a putative flagellar motor protein gene (motD) of *B. bacilliformis*. No function or location has been attributed to MotD in *B. bacilliformis*. The MotD in *S. meliloti* has been identified as homologous to FliK, the hook-length protein. However, the

B. bacilliformis (and likely *B. clarridgeiae*) MotD has only a 53-aa similarity at the carboxy-terminus to the MotD from *S. meliloti*.

Site-directed mutations of *B. clarridgeiae.* To construct a strain of *B. clarridgeiae* with no flagellum but with the other flagellar structures intact, a site-directed mutation in the *fla*A gene was generated. Plasmid pPCRscript was used for this purpose because it replicates in E. coli but not in *Bartonella*. The plasmid construct containing a 1025-bp region of the *fla*A gene was electroporated into *B. clarridgeiae*. Thirty-two kanamycin-resistant colonies were tested for motility in semi-soft agar. Six appeared to be deficient in motility. Insertion of the suicide plasmid DNA into *fla*A was confirmed in two strains by PCR and sequencing.

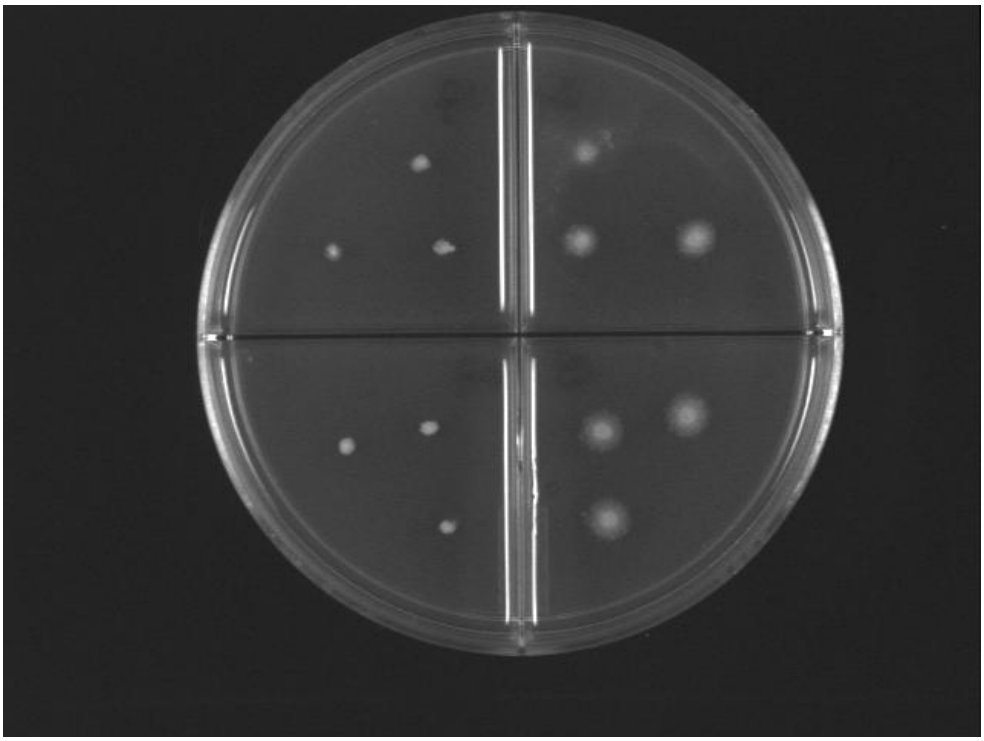


Figure 4. Migration of *B. clarridgeiae* in motility agar. Left upper and lower quadrants: *fla*A mutant. Right upper and lower quadrants: wild-type *B. clarridgeiae*.

The non-motile mutants previously determined to contain transposons in *mot*B and *mot*C became non-viable after freezing. In order to make a new mutant with a flagellum that did not turn, we decided to interrupt both the *mot*B and *mot*C genes. A suicide plasmid containing a 460-bp region encompassing the 3' end of motB and the 5' end of motC was constructed and electroporated into *B. clarridgeiae*. A total of 74 kanamycin-resistant, non-motile mutants were recovered, and interruption of *mot*BC was confirmed in 4 strains by PCR and sequencing. One *fla*A mutant, one *mot*BC mutant and the *B. clarridgeiae* type strain were re-tested for motility and used in subsequent studies. The photo of the *fla*A mutant in motility agar is shown in Figure 4. Like the *fla*A mutant, the *mot*BC mutant also showed no expansion from the initial inoculation site (photo not shown). The presence or absence of flagella was confirmed by transmission electron microscopy of *B. clarridgeiae* wild-type, *fla*A mutant and *mot*BC mutant (Figure 5).

Invasion of HMECs by *B. clarridgeiae.* HMECs inoculated with *B. clarridgeiae* wild-type, the *fla*A mutant or *mot*BC mutant were examined by transmission electron microscopy after 5 days. All three strains were able to enter HMECs. Finger-like projections (filipodia) were seen surrounding individual bacteria in micrographs of all three strains (Figure 6,7,8). No groups of bacteria were seen being engulfed, but several intact bacteria were seen together inside vacuoles.

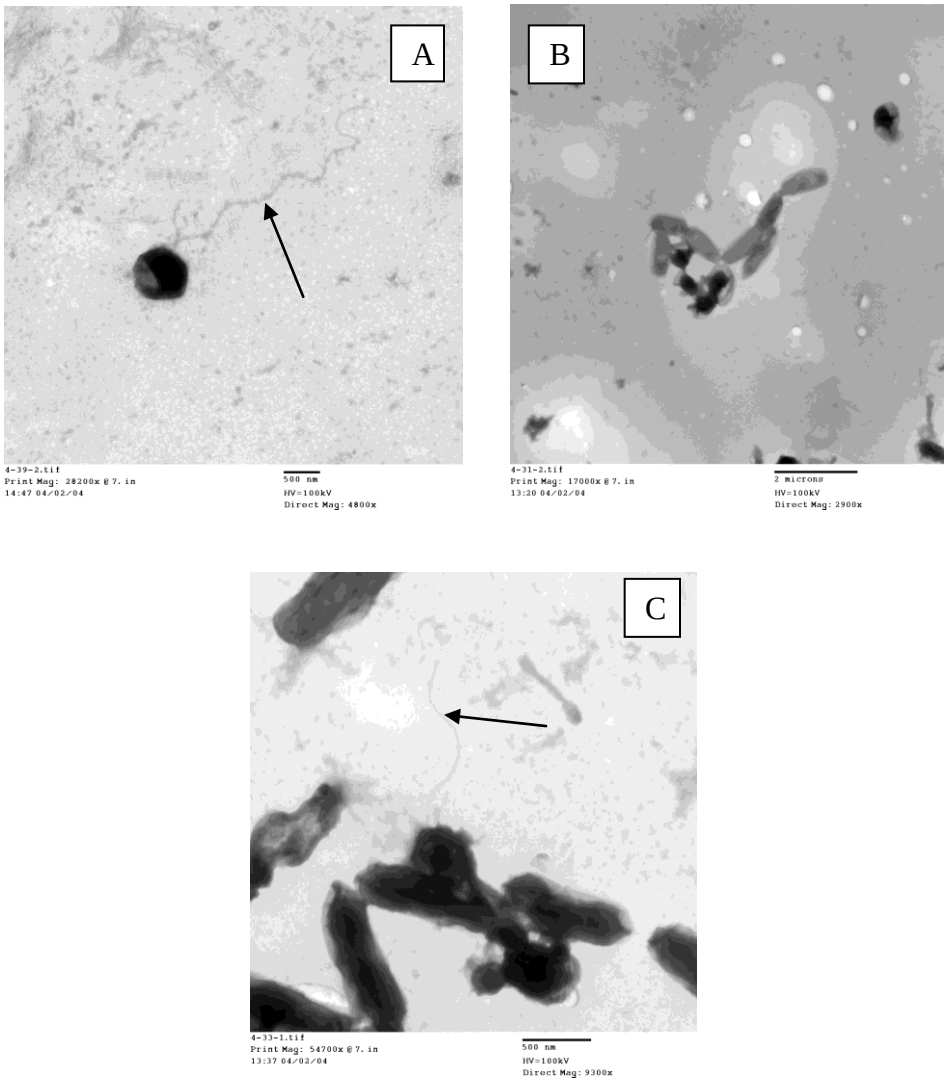


Figure 5. Transmission electron microscopy of *B. clarridgeiae*. A) *B. clarridgeiae* wild-type, B) *B. clarridgeiae fla*A mutant, and C) *B. clarridgeiae mot*BC mutant. Flagella are indicated by arrows.

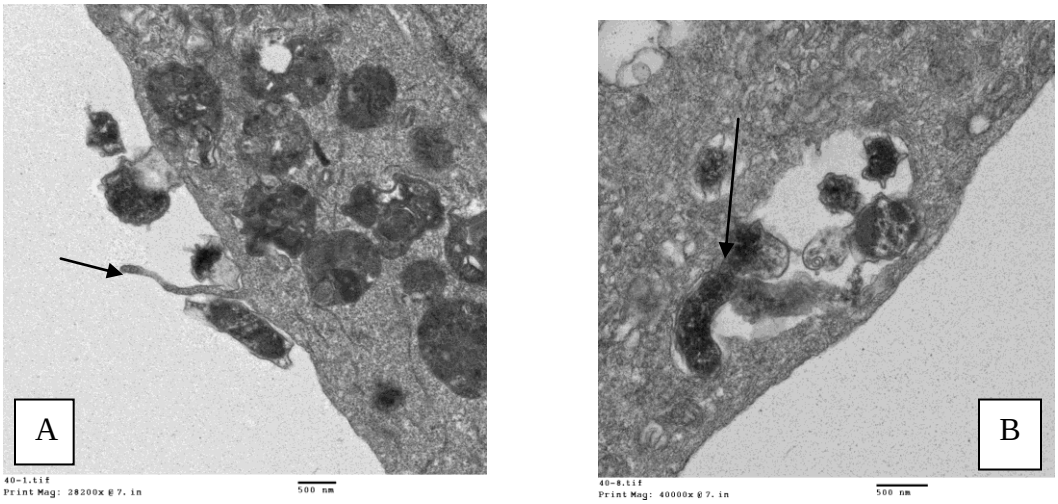


Figure 6. Transmission electron microscopy of HMECs 5 days after inoculation with *B. clarridgeiae* wild-type. A) Filipodium extending from HMEC, B) *B. clarridgeiae* inside HMEC vacuole.

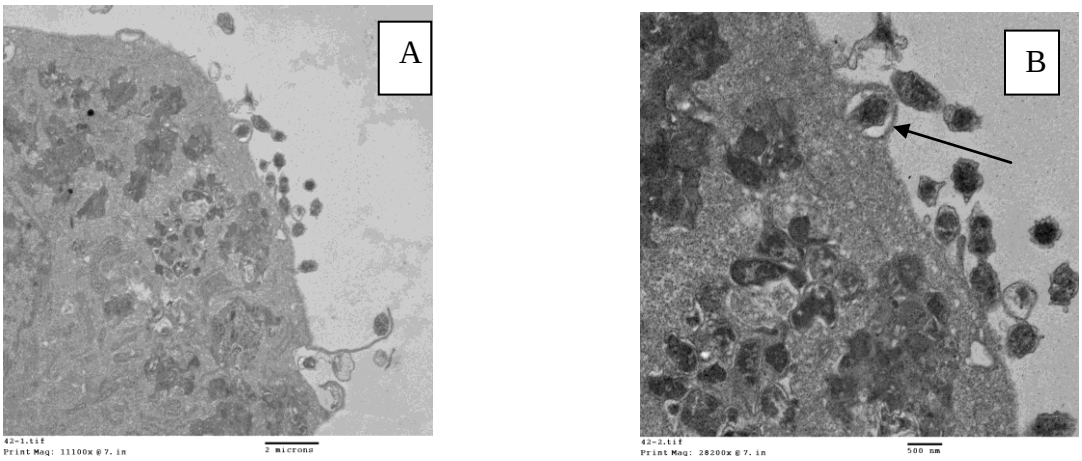


Figure 7. Transmission electron microscopy of HMECs 5 days after inoculation with *B. clarridgeiae fla*A mutant. A) Filipodium extending from HMEC, surrounding bacterium, B) intact *B. clarridgeiae fla*A mutant inside HMEC.

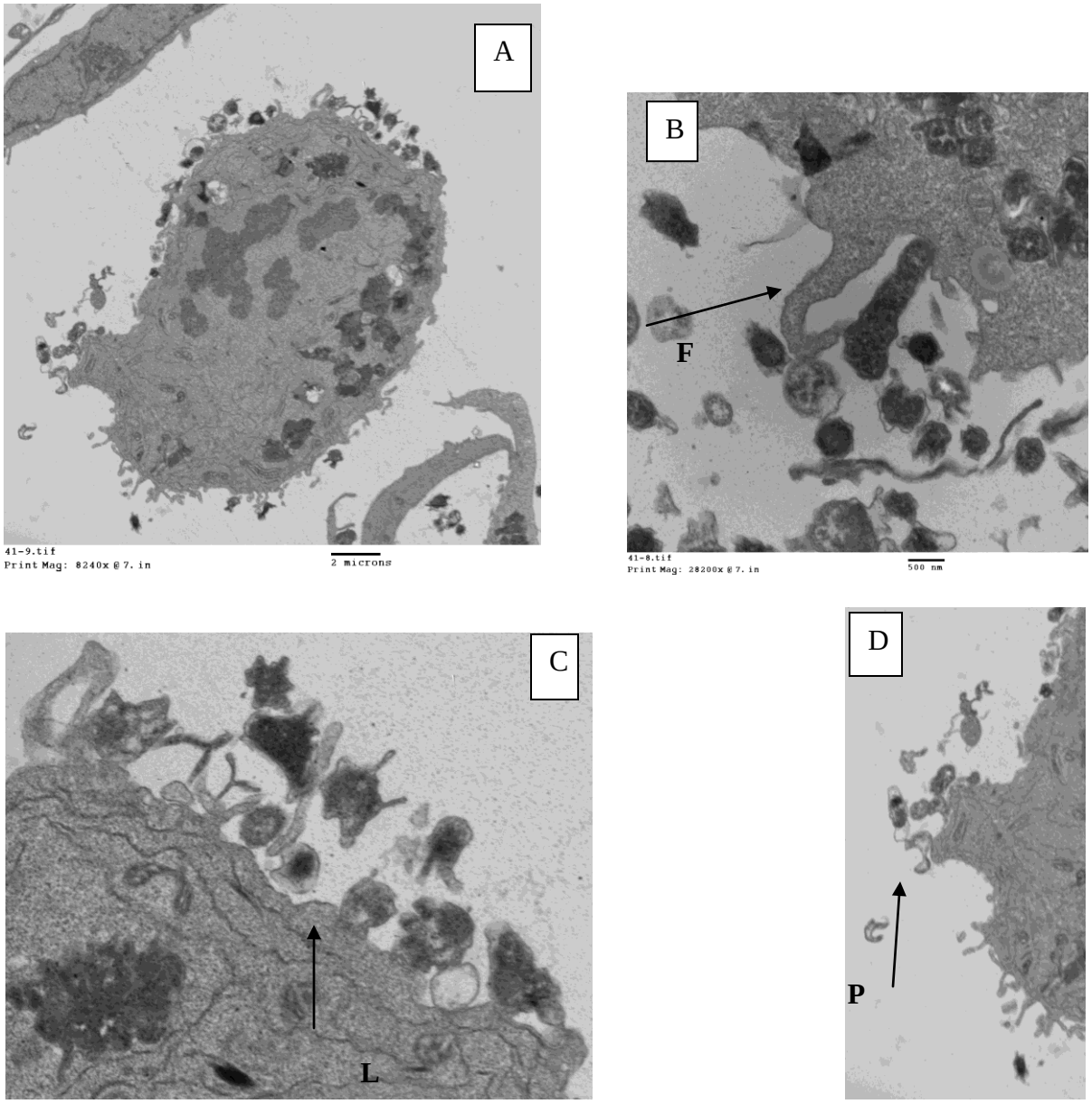


Figure 8. Transmission electron microscopy of HMECs 5 days after inoculation with *B. clarridgeiae mot*BC mutant. A) Many **F**ilipodia extending from HMEC or surrounding bacterium; distinct **L**amellipodia, **P**rotrusion; B) *B. clarridgeiae mot*BC cell at HMEC membrane. C) Distinct lamellipodia and D).a protrusion that resembles a pedestal.

The HMECs were also examined by phase contrast microscopy every 12 hours after the bacteria were added to the culture. At 24 hours post infection, the mock infected cells showed

some signs of migration with long protrusions extending from the cell body (Figure 9A). In Figure 9B, bacteria were seen in a mass on the surface of the HMEC.

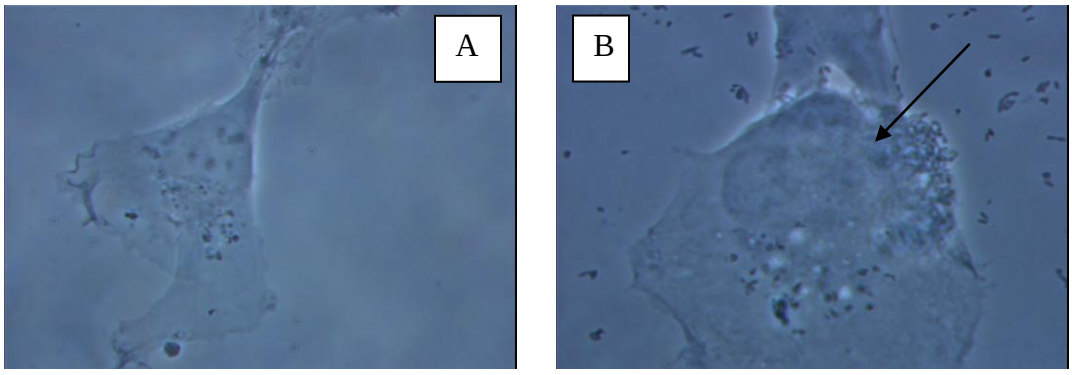


Figure 9. Phase contrast microscopy after 24 hours. A) uninfected HMECs, and B) HMECs infected with *B. clarridgeiae* wild-type. Bacteria seen in mass attached to cell (arrow).

The *fla*A mutant was also attached to cells as a group (Figure 10, A). The *mot*BC mutant attached to cells, but in this case, the bacteria were not in a large mass but covering the length of a membranous extension of the cell (Figure 10, B).

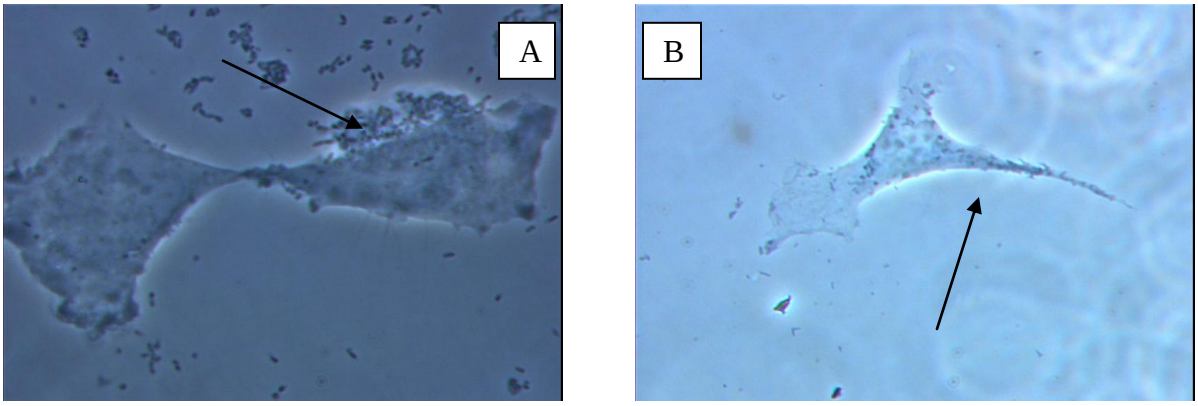


Figure 10. Phase contrast microscopy after 24 hours. A) HMECs infected with *B. clarridgeiae* *fla*A mutant, and B) HMECs infected with *mot*BC mutant. In 10A, only few bacteria can be seen near or on left cell while a mass of bacteria can be seen at the surface of the right-hand cell (arrow). In 10B, bacteria are attached to a long extension of the cell (arrow).

After 96 hours in culture without bacteria, the HMECs still appeared healthy (Figure 11A). Many of the HMECs in culture with *B. clarridgeiae* wild-type were covered in clumps of bacteria, and some dark intercellular objects were seen surrounding the nucleus (Figure 11B, arrow).

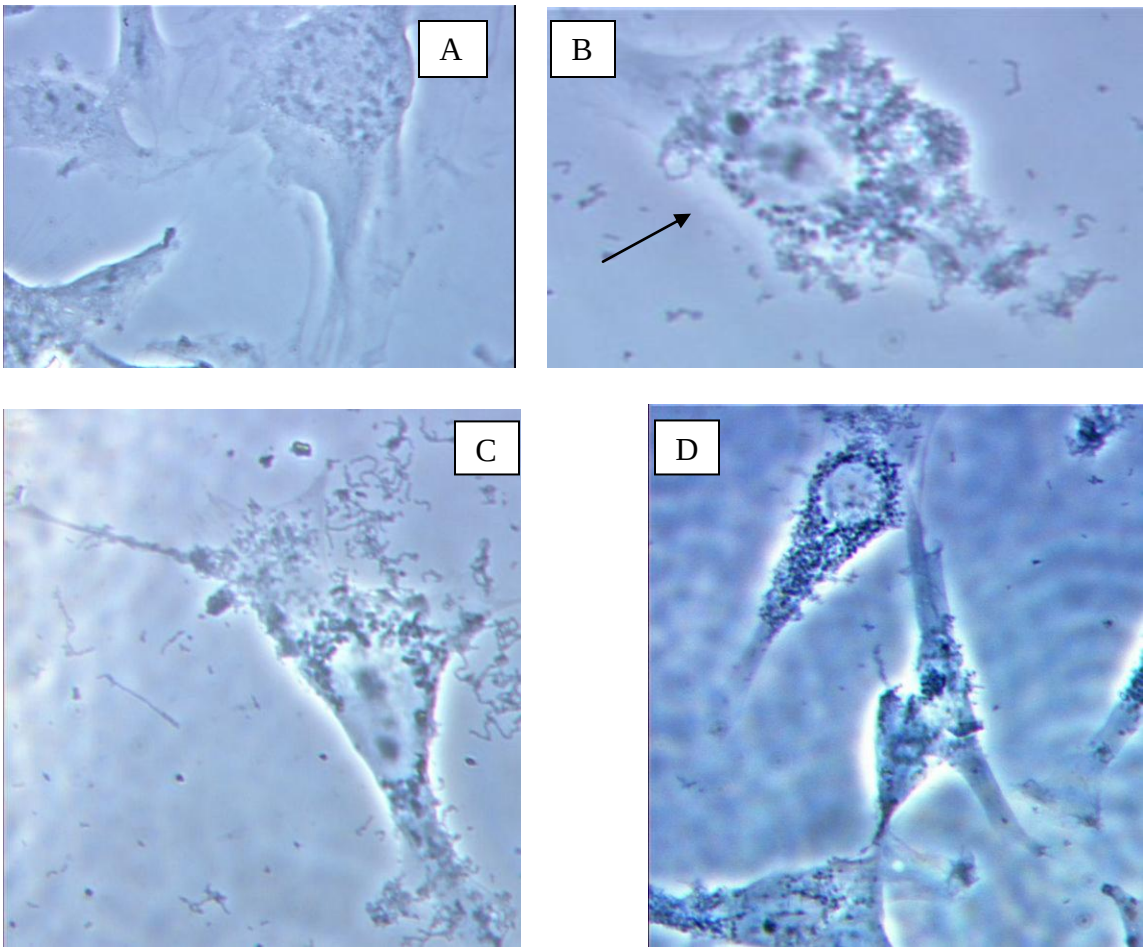
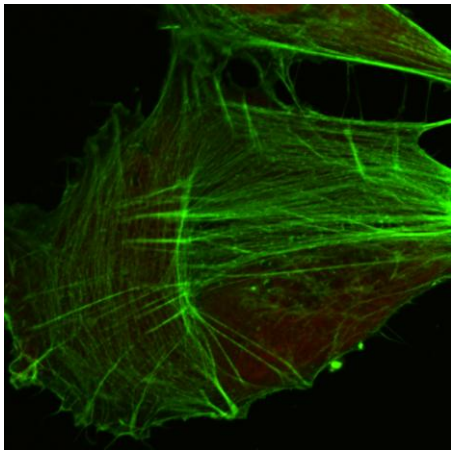
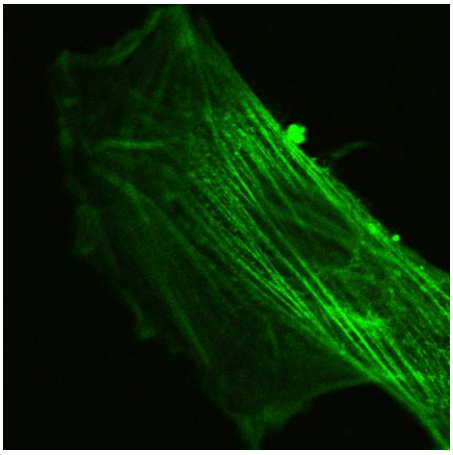


Figure 11. Phase contrast microscopy after 96 hours. A) uninfected HMECs, and B) HMECs infected with *B. clarridgeiae* wild-type. Bacteria can be seen intracellularly surrounding nucleus (arrow). After 96 hours, the *fla*A mutant was attached to some cells and many dark perinuclear spots were evident (C). Some cells were covered in *mot*BC mutant bacteria (D).

No overall difference could be seen between the strains. In all infected cultures, at all time points, some cells were seen with few if any attached bacteria and no intracellular organisms, while other cells had attached and intracellular organisms.

In previous studies, endothelial cells infected with *B. henselae* have been shown engulfing the bacteria *en masse* in a structure called an invasome (Dehio *et al.*,1997). No invasome structure was noted in our TEM or phase contrast micrographs of HMECs infected with *B. clarridgeiae*, so we used confocal microscopy to attempt to view an invasome of *B. clarridgeiae* if present. In Dehio et al's article, the actin filaments were visualized forming the invasome structure (Dehio *et al.*,1997)

In our study, HMECs inoculated and cultured with *B. clarridgeiae* were treated with F-actin phalloidin and propidium iodide to stain actin filaments green and bacteria and nuclear DNA red (Figure 12).

A.  B. 

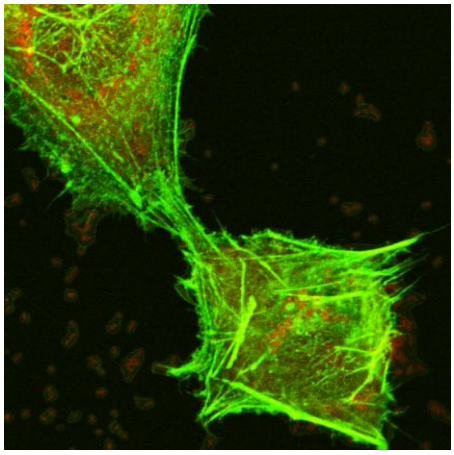
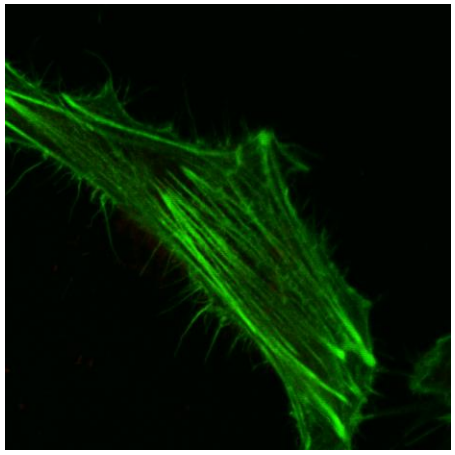
C.  D. 

Figure 12. Confocal transmission electron microscopy of HMECs stained with F-actin phalloidin and propidium iodide. F-actin appears green, and bacteria and nuclear DNA appear red. A) uninfected HMECs, B) HMEC infected with *B. clarridgeiae* wild-type, C) HMEC infected with *fla*A mutant, D) HMEC infected with *mot*BC mutant. Perinuclear bacteria (red) in C indicated with arrow.

Bacteria were clearly visible surrounding the nucleus in the *fla*A mutant cells, and the wild-type and *mot*BC mutant were also seen in perinuclear locations (photos not shown). Some parallel actin fibers could be seen in the wild-type and *mot*BC mutant-infected cells, but no invasome structure was present in any cells. The parallel actin fibers did not resemble the bundles of stress fibers seen in endothelial cells infected with *B. bacilliformis* (Verma *et al.*,2001).

The transmission electron microscopy, phase contrast microscopy and confocal fluorescence microscopy showed that all three strains of *B. clarridgeiae* were able to enter HMECs, and no invasomes or stress fiber bundles were seen. We interpret these results to mean that *B. clarridgeiae, B. henselae* and *B. bacilliformis* have different means to enter and/or cause different effects on HMECs once inside the endothelial cell. Qualitatively, the *mot*BC mutant-infected HMECs appeared to have more bacteria intracellularly than the wild-type or *fla*A

mutant-infected cells (see 96 hour photos, Figure 11). Some cells in each co-culture appeared to be uninfected for reasons unknown, and it was difficult to ascertain the ratio of infected and uninfected HMECs. Therefore, to gain a more quantitative perspective, we used the gentamicin protection assay.

Gentamicin protection assay. We determined that 100% of *B. clarridgeiae* are killed by gentamicin at 250μg/ml (data not shown). We used gentamicin to kill extracellular bacteria in HMEC-*Bartonella* co-cultures and lysed the HMECs to determine the number of intracellular bacteria. The number of bacteria attached to the HMEC surface was also determined by lysing HMECs without gentamicin treatment. Results shown in Figure 13 indicate that the intracellular percentage of the *mot*BC mutant inocula was higher than the other strains at all time points. After 5 days, 72% of the *mot*BC inoculum was either attached to (50%) or had entered (22%) the endothelial cells. While the *fla*A mutant did not attach as well as the other two strains, it exhibited the highest percentage of cell-associated bacteria that had entered the cell at 5 days (37% compared to 19% wild-type and 30% *mot*BC). These observations suggest that the lack of flagella influences attachment more so than invasion. When the flagellum was present but paralyzed (*mot*BC), there was no decrease in the attachment and invasion ability of the bacteria at 3 and 5 days after inoculation. In fact, attachment and invasion were enhanced in the *mot*BC mutant compared to wild-type.

Proliferation assay. Some *Bartonella* species are known for their mitogenicity (Dehio,2005). *B. bacilliformis*, *B. henselae* and *B. quintana* have all been shown to cause vasoproliferative diseases. To investigate *B. clarridgeiae*'s mitogenicity potential, we co-cultured HMECs with *B.*

clarridgeiae wild-type, the *fla*A mutant or the *mot*BC mutant strain for 2 or 4 days. We then treated the cultures with MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a dye that is reduced to formazan in the mitochondria of living cells giving off a purple color when solubilized.

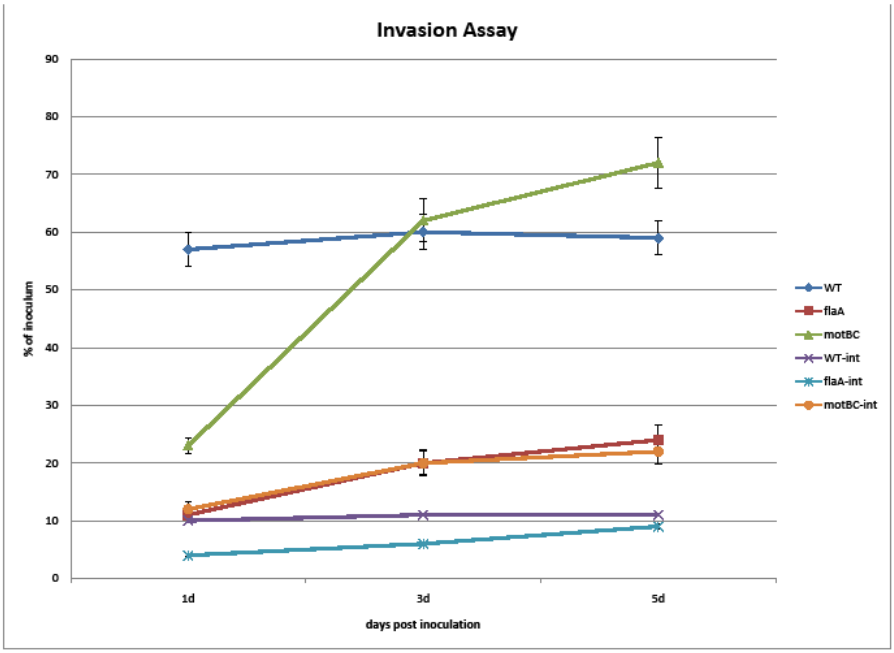


Figure 13. Gentamicin protection assay. HMECS were inoculated with *B. clarridgeiae* at a ratio of 30 bacteria per cell. After 1, 3 and 5 days, the cells were washed to remove unattached bacteria and lysed without or after gentamicin treatment. WT, wild-type extracellular and intracellular; flaA, *fla*A mutant extracellular and intracellular; motBC, *mot*BC extracellular and intracellular; WT-int, wild-type intracellular only; flaA-int, *fla*A mutant intracellular only; motBC-int, *mot*BC mutant intracellular only.

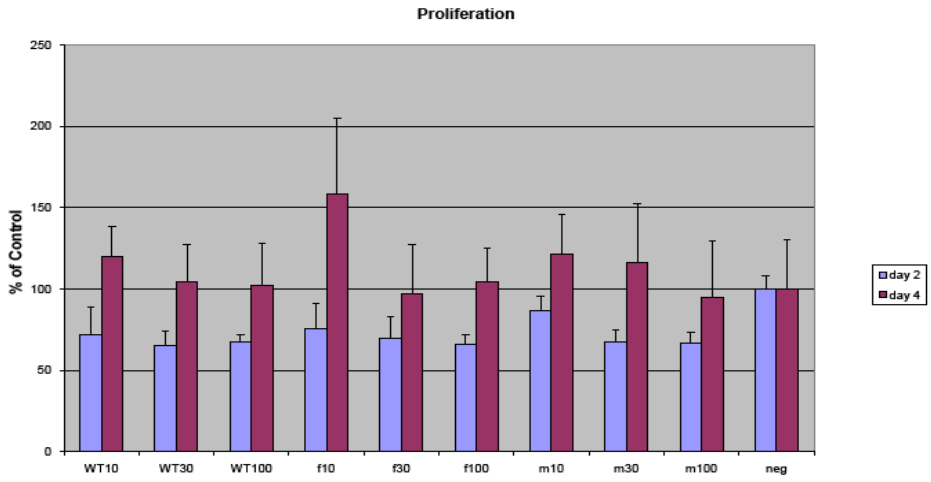


Figure 14. Proliferation assay. HMECs were inoculated with 10, 30 or 100 bacteria per cell. After 2 or 4 days, MTT was added to each well. After 4 hours, a solubilizer was added and incubated overnight. Increased incorporation of MTT is indicative of increased cell number. WT= wild-type *B. clarridgeiae*, f= *fla*A mutant, m= *mot*BC mutant. The values are expressed as the percentage of the negative control values.

At 2 days post infection, all infected HMECs had a lower proliferative index than the negative control. However, after 4 days, the WT10, f10, m10 and m30 had significantly increased proliferative values compared to the negative control, $p<0.05$. Furthermore, all reactions showed significantly less proliferation than the f10 infected cells after 4 days, $p<0.02$.

Taken together, the gentamicin protection assay and MTT assay results suggest that the *fla*A mutant attaches significantly less well than the *mot*BC mutant or wild-type *B. clarridgeiae* but has a greater proliferative effect.

The apparent proliferative effect of the *fla*A mutant may be due to increased cytotoxicity of HMECs by the wild-type or the *mot*BC mutant. To determine if the strains were cytotoxic to HMECs, we performed lactase dehydrogenase cytotoxicity assays on the HMEC co-cultures.

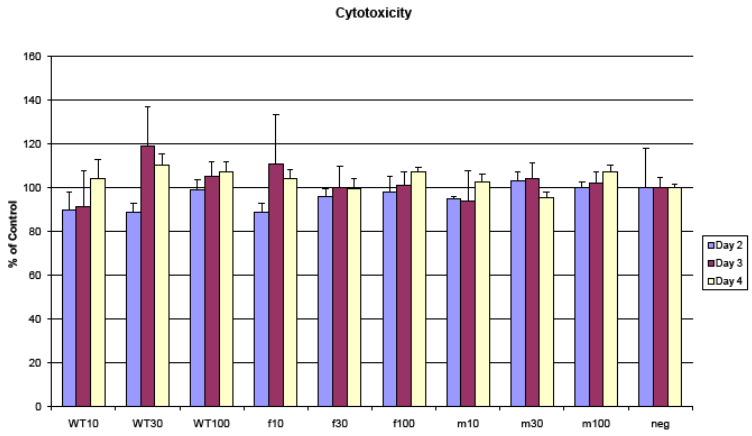


Figure 15. Cytotoxicity assay. HMECs inoculated with *B. clarridgeiae* or uninfected. After 2, 3 or 4 days, the HMECs were assayed for lactose dehydrogenase release indicating cell damage or lysis. Values are expressed as percent of control. Control = uninfected HMECs. WT= wild-type *B. clarridgeiae* , f= *fla*A mutant, m=*mot*BC mutant. 10, 30 and 100 are bacteria per endothelial cell ratio. Students t-test was used to determine significant differences.

After 2 days, WT10, WT30, f10, f30, and m10 had lower LDH values than the negative control ($p<0.02$). M30 was 103% of the control value ($p=0.04$). At 3 days post inoculation, the WT30 had a higher LDH than the control ($p=0.01$). After 4 days, WT30, WT100, f100, and m100 displayed more cytotoxicity than the uninfected control ($p<0.05$), but they were not significantly different from one another.

Lower concentrations of bacteria did not have a cytotoxic effect on the HMECs at the 2-day time point and may actually have protected cells from damage. Higher bacteria to cell ratios resulted in cell death. Over the 4-day time span, an increase in cell cytotoxicity in cultures containing the lower concentrations of bacteria became apparent. This may have been due to bacterial growth in the culture, resulting in a higher bacteria to cell ratio.

Overall, these experiments suggest that wild-type *B. clarridgeiae* is able to enter HMECs and cause significant proliferation if the bacteria to cell ratio is less than 1:100. *B. clarridgeiae* without flagella (*fla*A) induces more of a proliferative effect, and the paralyzed but intact flagellar mutant (*mot*BC) appears to be able to enter the HMECs in greater number than the wild-type or the mutant that lacks flagella.

Discussion

Bartonella species are emerging zoonotic pathogens. *Bartonella henselae* is the major causative agent of Cat Scratch disease in humans (Scott *et al.*,1996). *B. clarridgeiae* has been found with *B. henselae* in cats and cat fleas in many regions of the world (Rolain *et al.*,2003; Kelly *et al.*,2004; Li *et al.*,2007), and has been associated with endocarditis and hepatic lesions in dogs (Chomel *et al.*,2001; Gillespie *et al.*,2003). Although *B. clarridgeiae* has not been isolated from humans, molecular and serologic results have implicated *B. clarridgeiae* in Cat Scratch Disease and a chest wall abcess (Kordick *et al.*,1997; Margileth and Baehren,1998). In immunocompetent humans, infection with *Bartonella*e typically results in a self-limiting benign erythrocyte parasitism from a primary niche thought to be the vascular endothelium. *B. bacilliformis* is the exception and causes major hemolysis. In immunocompromised individuals, and those individuals who survive the initial phase of *B. bacilliformis* infection, *B. bacilliformis*,

B. henselae and *B. quintana* can cause angiogenesis manifesting as vasoproliferative lesions such as bacillary angiomatosis, peliosis or verruga peruana. Until now, it was not known whether *B. clarridgeiae* had the capacity to invade human cells and/or cause proliferation, both prerequisites for angiogenesis. Our experiments sought to determine whether 1) *B. clarridgeiae* could invade HMECs, and 2) if yes, to what extent flagella and motility were involved in the *B. clarridgeiae* invasion process.

Bacillary angiomatosis caused by *Bartonella*e is similar to angiogenesis in embryonic development and in the spouting of vessels feeding cancerous tumors. These conditions are all complicated processes of morphogenesis resulting in the formation of new vasculature from pre-existing vessels (Carmeliet,2003). The first step in *Bartonella*e-induced angiogenesis is the colonization of endothelial cells. *B. bacilliformis* has been shown to subvert Rho GTPases to rearrange the endothelial cell cytoskeleton and induce bacterial uptake *in vitro* (Verma *et al.*,2000; Verma *et al.*,2001; Verma and Ihler,2002). *B. henselae* also induces major cytoskeletal rearrangement to form the invasome that engulfs a mass of bacteria (Dehio *et al.*,1997). In our experiments, *B. clarridgeiae* entered HMECs as single bacteria and no invasome structure was seen. Additionally, stress fiber bundles shown to be present during *B. bacilliformis* invasion were not visible when HMECs infected with *B. clarridgeiae* were viewed under confocal microscopy. The uptake of *B. clarridgeiae* is most likely actin-dependent, and evidence of that was seen in the TEM photographs of filipodia reaching out towards and surrounding individual *B. clarridgeiae* cells (Figures 6-8). However, the massive rearrangements of the cytoskeleton seen in cells infected with *B. henselae* and *B. bacilliformis* were not evident in *B. clarridgeiae*-infected cells.

What could account for the different invasion processes of *B. clarridgeiae*, *B. henselae* and *B. bacilliformis*? In *B. henselae*, the VirB type 4 secretion system causes massive cytoskeletal rearrangement and formation of the invasome most likely through the translocation of one or more Bep proteins (Dehio,2004; Schmid *et al.*,2004). *B. clarridgeiae* has homologues of genes *vir*B2-*vir*B11 (D. Kohlhorst, dissertation, Georgia State University), but it is unknown whether there is a VirD4, the type 4 secretion coupling protein essential for substrate translocation. Given what is known about the arrangement of *vir*B genes in *B. clarridgeiae*, *B. henselae*, *B. tribocorum* and *B. quintana*, it is likely that *B. clarridgeiae* has the same general *vir*B/*vir*D4/*bep* pathogenicity island arrangement as the other 3 *Bartonella* species in which the Bep protein genes are contiguous with the VirB protein genes (Schroder and Dehio,2005)).

Evidence that the Bep proteins translocated by *B. henselae* are involved in the formation of the invasome stems from a report that a deletion of all of the *bep* genes *bep*A-*bep*G results in the same phenotype as a Δ*vir*D4 or Δ*vir*B4 mutant; there is (a) no massive rearrangements of the actin cytoskeleton, (b) no inhibition of apoptosis, and (c) no activation of NF-κB proinflammatory response (Schulein *et al.*,2005). If the VirB T4SS is functional in *B. clarridgeiae*, and if the VirB system secretes Bep proteins, then perhaps 1) the Bep proteins in *B. clarridgeiae* do not function in exactly the same manner as the Bep proteins in *B. henselae*, e.g., massive cytoskeletal rearrangement, or 2) the *B. clarridgeiae* Bep protein that is involved in cytoskeletal rearrangements causes only small cytoskeletal changes, or 3) the Bep protein that is involved in cytoskeletal rearrangements in *B. henselae* and *B. clarridgeiae* (and others) is only indirectly involved and the other protein(s) necessary for the massive rearrangement is not functional or present in *B. clarridgeiae*; or 4) the full complement of Bep protein genes is incomplete in *B. clarridgeiae*. The last option may be the case for *B. quintana* which appears to

have only 4 of the 8 Bep protein genes. Since *B. quintana* does not induce invasome formation, the Bep protein responsible for the massive cytoskeletal rearrangement may be missing. Sequencing of the region downstream of *vir*B11 in *B. clarridgeiae* to determine the presence of the *bep* genes, and testing a *bep* deletion mutant in HMEC culture would be informative.

B. bacilliformis does not possess a VirB T4SS or *bep* genes but induces its own phagocytosis through cytoskeletal rearrangement involving activation of small GTPases in the host cell (Rho, Rac and Cdc42) by an undetermined mechanism (Verma and Ihler,2002). Cytochalasin D which inhibits actin polymerization, reduced the intracellular numbers of *B. bacilliformis* by only 75% indicating that additional factors from *B. bacilliformis* are responsible for part (25%) of the endocytic process (Hill *et al.*,1992). The small GTPases Rac and Cdc42 are known to be involved in formation of lamellipodia and filopodia, respectively, which engulf *B. bacilliformis* (Verma and Ihler,2002). Inactivation of endothelial Rac, Rho and Cdc42 GTPases reduced the entry of *B. bacilliformis* by 80-90% (Verma and Ihler,2002). However, the stress fibers caused by activation of Rho are not involved in the internalization process and are considered an effect of internalization (Verma *et al.*,2001). The mechanism used by *B. bacilliformis* to gain entry into endothelial cells may be very similar to that used by *B. clarridgeiae*. Therefore, the lack of stress fibers when *B. clarridgeiae* invade may be a result of different bacterial factors released after the bacteria enter the endothelial cell.

Other bacteria have been found to influence the actin cytoskeleton for their own micropinocytosis. Shigella protein IpaC is secreted by the Mxi-Spa type 3 secretion system and leads to entry of Shigella into HeLa cells via lamellipodia and filipodial extensions (Tran Van Nhieu *et al.*,1999). Actin rearrangement at the site of bacterial attachment is induced by IpaC and involves Rac and Cdc42 (Tran Van Nhieu *et al.*,1999). Rho, Rac and Cdc42 are required for

internalization of *B. abortus*, and a BvrR/BvrS mutant was reported to be unable to recruit these GTPases (Guzman-Verri *et al.*,2001). BvrR/BvrS is a two-component regulatory system highly homologous to systems in other alpha-proteobacteria, including the ChvI/ChvG of *A. tumefaciens* and BatR/BatS in *Bartonella*. We discovered *bat*R/*bat*S genes in *B. clarridgeiae* (data not shown), and they have been sequenced from *B. bacilliformis*, *B. tribocorum*, *B. quintana*, *B. taylorii*, and *B. henselae*. The role of BatR/BatS in *Bartonella* is unknown; however, the *Brucella abortus* BvrR/BvrS system regulates expression of outer membrane proteins (Lamontagne *et al.*,2007). No outer membrane proteins have been identified in *B. clarridgeiae* yet, but variable outer membrane proteins (Vomps) of *B. quintana* have been shown to be involved in induction of VEGF from macrophages and epithelial cells (Schulte *et al.*,2006). Other outer membrane proteins described as virulence factors in *Bartonella*e include the adhesin BadA, an outer membrane protein found in *B. bacilliformis*, *B. henselae*, and *B. quintana*, and GroEL, a chaperonin protein localized to the outer membrane of *B. bacilliformis* and *B. henselae* (Minnick *et al.*,2003; Boonjakuakul *et al.*,2007). We discuss GroEL in more detail in Chapter II.

B. bacilliformis and *B. clarridgeiae* are 2 of 5 *Bartonella* species that have a flagellar system. Flagella are assembled using a flagellar-specific type III secretion system (T3SS) that is very similar to the T3SS called the injectisome found in many other bacteria. No trace of either the flagellar or injectisome system is present in *B. henselae* or *B. quintana*. Instead, *B. henselae* and *B. quintana* have two T4SS, VirB and Trw. The ancestral species *B. bacilliformis*, *B. clarridgeiae*, and the ruminant-specific sub-lineage *B. bovis*, *B. capreoli*, *B. chomelii* and *B. schoenbuchensis* have flagella and no Trw T4SS (Schroder and Dehio,2005; Dehio,2008). It has been postulated that *Bartonella*e have replaced the flagellar system with the Trw T4SS during adaptive evolution (Saenz *et al.*,2007). Evidence points to flagella as more than just a

means of propulsion in *B. bacilliformis*, however. Anti-flagellin antibodies inhibited *B. bacilliformis* from entering erythrocytes by 99% and whole-cell antibodies to *B. bacilliformis* reduced the bacteria from entering HUVECs by 50% (Hill *et al.*,1992; Scherer *et al.*,1993). Our results using a *fla*A mutant void of flagella indicated that flagella are important for attachment to HMECs, but that motility was much less important, as the *mot*BC mutant that had intact flagella but was non-motile was able to attach and invade at even higher numbers than the wild-type strain at days 3 and 5 post-inoculation (Figure 13). The *B. clarridgeiae fla*A mutant was able to enter the HMECs once it attached to the cell. This finding suggests that the external flagellar filament is not necessary for invasion *per se*, although we have not ruled out that it participates in the invasion process at the attachment stage. Another possible explanation is that the bacteria that entered the HMECs were revertants or had a second site compensatory mutation that aided the invasion process.

Wild-type *B. clarridgeiae*, the *fla*A mutant and the *mot*BC mutant were mitogenic at low doses after 4 days of culture with HMECs, but proliferation was negligible at an MOI of 100 compared to uninfected HMECs (Figure 14). The latter effects were due, at least in part, to cytotoxicity of the 3 strains at higher doses after 4 days' incubation with the host cells (Figure 15). Proliferation was significantly increased in the *fla*A mutant cultures at the lowest dose (MOI=10) at day 4 compared to that of the wild-type or *mot*BC mutant cultures. These results are similar to those seen in with a *B. henselae* ΔvirB4 mutant except that the ΔvirB4 mutant cultures showed enhanced proliferation even at the highest MOI (Schmid *et al.*,2004). Schmid et al. (2004) reasoned that VirB is involved in a cytostatic effect that interferes with proliferation and is cytotoxic at high concentrations. When VirB is deleted, the cytostatic and cytotoxic effects are not seen and proliferation, caused by an elusive mitogen that is not a substrate of

VirB, increases significantly (Schmid *et al.*,2004). The cytotoxicity seen with high concentrations of *B. clarridgeiae* may also be VirB-dependent.

A link between the flagellar system and the VirB T4SS has not been postulated previously. However, negative crosstalk between the flagellar T3SS and the non-flagellar T3SS has been documented in *Pseudomonas aeruginosa* (Soscia *et al.*,2007). Secretion of effector proteins from the T3SS was enhanced in the non-flagellated mutant (Soscia *et al.*,2007). Perhaps the enhanced proliferation of HMECs by the *B. clarridgeiae fla*A mutant was not due to a decrease in cytostasis but rather an increase in the production and/or secretion of a mitogenic factor. At low concentrations, the mitogen expressed by *B. clarridgeiae* wild-type and the *mot*BC mutant may overcome a cytostatic effect of a possible VirB-dependent substance, resulting in the proliferation that is seen at day 4 post infection (Figure 14). The *fla*A mutant, however, may express a higher concentration of the mitogen while the concentration of the cytostatic substance remains the same. At high concentrations of all three strains, the cytostatic substance is presumed to have a greater effect than the mitogen. GroEL is found in *B. bacilliformis* culture supernatants and has been implicated as a mitogenic factor for HUVECs (Minnick *et al.*,2003; Callison *et al.*,2005; Smitherman and Minnick,2005). The secretion mechanism for GroEL in *B. bacilliformis* is unknown, but *B. bacilliformis* does not have a VirB system. It is tempting to speculate that *B. clarridgeiae* might secrete GroEL and that this substance may be mitogenic for HMECs. If so, this raises the possibility that the *fla*A mutant secretes more GroEL than the wild-type strain

Although the percentages of the *mot*BC mutant and wild-type *B. clarridgeiae* associated with endothelial cells during the invasion assay (Figure 13) were similar, the *mot*BC mutant was able to enter the cells in higher numbers than the wild-type (22% vs. 11% of the inoculum was

intracellular, respectively). These results were comparable to those reported for a Δ*vir*B4 mutant of *B. henselae*. Schmid et al. (2004) proposed that the increased numbers of intracellular Δ*vir*B4 bacteria relative to the wild-type may have been due to increased endocytosis (since the invasome was not formed) and that endocytosis occurs at a much faster rate than the invasome-mediated invasion. Therefore, according to this model, wild-type *B. henselae* were slower to appear intracellularly because of their dependence on invasome formation. This appears not to be the explanation for wild-type *B. clarridgeiae*, as no invasomes were seen by transmission or confocal microscopy.

Why would a non-motile mutant invade better than wild-type? The *mot*BC mutant has a complete flagellar filament, and experiments with our *fla*A mutant suggested that the filament aids in attachment but is perhaps only minimally involved, if at all, in invasion. Is the energy normally used for rotation used for another function if the motor is not complete? Komoriya et al. (1999) reported the isolation of a Δ*mot*AB mutant of *Salmonella typhimurium* that secreted more flagellin and less virulence protein than the wild-type strain. They reasoned that kinetics for export systems, either ATP or proton motive force, may have been disrupted by the *mot*AB mutation. Antibodies made against *B. bacilliformis* flagellar filaments inhibited the bacteria from iinvading erythrocytes (Scherer *et al.*,1993). Similarly, whole cell antibody raised against a crude cell wall preparation from *B. bacilliformis* blocked the organism's entry into HUVECs (Hill *et al.*,1992).

If *B. clarridgeiae mot*BC mutant, an increased secretion of flagellin protein would be predicted to block attachment of *B. clarridgeiae* if the similarities with *B. bacilliformis* hold true, so that may not be what was occurring in our experiments. Perhaps a mutation in *mot*B and *mot*C rather than *mot*A leads to decreased flagellin secretion and increased secretion of virulence

factors through an as yet undetermined mechanism. Rajagopala et al recently determined that there are over 100 protein-protein interactions involved in a complex chemotaxis and motility network, so unraveling the link between the flagellar system in *B. clarridgeiae* and *B. bacilliformis* and invasion may be very complex (Rajagopala *et al.*,2007).

In the present study, wild-type *B. clarridgeiae*, a mutant with no flagella (*fla*A) and a mutant with paralyzed flagella (*mot*BC) were analyzed for their ability to enter endothelial cells and their effects on the endothelial cells in co-culture. All three strains were able to enter human microvascular endothelial cells, and all appeared to enter as single bacterial cells rather than in groups. The *mot*BC mutant entered the HMECs in greater total numbers than the other two strains; however, the *fla*A mutant caused the most proliferation of the HMECs. All three strains caused cytotoxicity when the MOI was high.

In order to determine if secretion of a mitogen or other factor was increased or decreased in the *fla*A mutant or *mot*BC mutant culture compared to the wild-type infected HMECs or uninfected HMECs, we next examine culture supernatants for differential protein secretion (Chapter II).

CHAPTER II

The Secretome of *B. clarridgeiae* and Human Microvascular Endothelial Cells in Co-Culture

Introduction

The ability of *B. henselae*, *B. bacilliformis* and *B. quintana* to cause the development of new blood vessels from existing vessels in humans, a process called angiogenesis, is a unique feature of these bacteria. During angiogenesis, activated endothelial cells degrade the extracellular matrix, proliferate and migrate to form new capillaries (Risau,1997). While the exact mechanism is unknown, proliferation of endothelial cells by *B. henselae* is independent of the type 4 secretion system (T4SS) VirB. However, the VirB system is responsible for several changes in the endothelial cells necessary for angiogenesis. The VirB system translocates proteins into the endothelial cells resulting in cytoskeletal rearrangements, the induction of a pro-inflammatory response through NFκB, and inhibition of apoptosis (Schmid *et al.*,2004). The cytoskeletal rearrangements enable the engulfment of large aggregates of *B. henselae* (Schmid *et al.*,2004). *B. henselae* may directly stimulate endothelial cell proliferation and also trigger a paracrine proangiogenic stimulus through NFκB activation (Dehio,2003). NFκB induces IL-8 secretion and results in the chemoattraction of macrophages which then secrete vascular endothelial growth factor (Dehio,2003). The anti-apoptotic effect of *B. henselae* infection is mediated by the VirB-translocated protein BepA (Schmid *et al.*,2006).

Kirby and Nekorchuk reported that the increase in endothelial cell numbers in culture with *B. henselae* was not due to proliferation, *per se*, but to the inhibition of apoptosis (Kirby and Nekorchuk,2002). An anti-apoptotic factor is released by *B. henselae* in culture media in the

presence or absence of endothelial cells (Kirby and Nekorchuk,2002). *B. bacilliformis*, on the other hand, releases a proteinaceous factor(s) in culture supernatants that is mitogenic, not anti-apoptotic (Minnick *et al.*,2003). At least one of the *B. bacilliformis* factors was reported to be the chaperonin GroEL (Minnick *et al.*,2003).

In the preceding experiments, we have shown that *B. clarridgeiae* iss able to invade human endothelial cells *in vitro* and cause proliferation. Additional information is needed to determine if *B. clarridgeiae* has effects on endothelial cells similar to those of the proangiogenic *Bartonella*e and whether the effects are due to bacterial secreted proteins such as GroEL. The mechanism by which GroEL is secreted is unknown, but *B. bacilliformis* does not have a VirB or Trw secretion system. Both *B. clarridgeiae* and *B. bacilliformis* have flagella, and the Trw secretion system has been postulated to be a replacement for the flagellar system during radial speciation of *Bartonella* (Dehio,2008). Secretion of virulence factors by the flagellar export system has been demonstrated in *C. jejuni* and *Y. enterocolitica* (Young *et al.*,1999; Konkel *et al.*,2004). We have shown that *B. clarridgeiae* is motile but does not form the large diameter rings that *A. tumefaciens* does in motility agar (Merritt *et al.*,2007). Perhaps the primary function of flagella and the flagellar type three secretion system (T3SS) in *B. bacilliformis* and *B. clarridgeiae* is protein secretion not motility.

In the following sections, we report the construction of a non-motile *fla*A mutant strain of *B. clarridgeiae* lacking the flagellar filament and a *mot*BC mutant strain with paralyzed flagella. We examined the proteins released into the media when wild-type and mutant *B. clarridgeiae* and HMECs were co-cultured in order to characterize the secreted bacterial proteins and the proteins secreted by the HMECs in response to *B. clarridgeiae*.

Materials and Methods

Bacteria. *B. clarridgeiae* type strain ATCC 51734 was grown for 5 days on heart infusion agar with 5% rabbit blood (HIA-RB) at 37°C and 5% CO_2. Two non-motile strains of *B. clarridgeiae* were created by interrupting the *fla*A flagellin gene and the flagellar motor genes *mot*BC, as previously described (Chapter I). *B. clarridgeiae* mutants were grown on HIA supplemented with Kanamycin 50μg/ml.

Protein collected from HMEC and *B. clarridgeiae* co-culture. HMECs at $1x10^5$/ml were seeded into 150-cm^2 culture flasks with MCDB-131 media containing 10% fetal calf serum and 2mM L-glutamine and allowed to grow to confluency at 37°C. The medium was removed, and the cells were washed with media without serum, followed by a 30-minute incubation in media without serum. This procedure was followed to remove as much serum as possible from the cells so that serum proteins would not mask bacterial and HMEC-secreted proteins when electrophoresed. One set of 10 flasks served as the uninfected control. One set was inoculated with *B. clarridgeiae* wild-type ATCC 51734, one with the *fla*A mutant and one set with the *mot*BC mutant strain. Bacteria were added to 10 flasks at a MOI of 100 in fresh media with no serum but containing L-glutamine and fungizone and incubated for 2-5 days at 37°C with 5% CO_2. The medium was collected and proteinase inhibitors were added. The medium was then filter-sterilized through 0.2μM filter units and lyophilized in 10-ml aliquots.

2-D gel electrophoresis. Sets of like samples (e.g. media from uninfected controls) were dissolved in minimal amounts of SDS Boiling Buffer (5% SDS, 10% glycerol and 60 mM Tris, pH 6.8), combined and lyophilized a second time. The lyophilized material was redissolved in

ultrapure water and dialyzed for 2 days against 5 mM tris, pH 6.8 using 6-8,000 molecular weight cutoff membranes at 4°C. After dialysis, the samples were lyophilized again, redissolved in 1200 µl of 1:4 diluted SDS Boiling Buffer :water and heated in a boiling water bath for 3 minutes. The protein concentrations of the samples were determined using the BCA Assay (Smith *et al.*,1985). The samples were then diluted to 1 mg/ml in 1:1 diluted SDS Boiling Buffer (5% SDS, 5% BME, 10% glycerol and 60 mM Tris, pH 6.8) Urea Sample Buffer (9.5 M urea, 2% w/v IGEPAL CA-630 (a non-ionic detergent, or Nonidet P-40), 5% beta-mercaptoethanol (BME), and 2% ampholines consisting of 1.6% pH 5-7 and 0.4% pH 3.5-10 (Amersham Biosciences, Piscataway, NJ), centrifuged at 2500xg for 10 seconds, and the supernatants were loaded on polyacrylamide tube gels.

For the first dimension of the 2-dimensional polyacrylamide electrophoresis, 100µg of total protein was separated by isoelectric focusing in glass tubes of inner diameter 3.5 mm, using 2% pH 3.5-10 (GE Healthcare, Piscataway, NJ) for 20,000 volt-hrs. An IEF internal standard, 50ng Tropomyosin, was added to each sample prior to loading. The tube gels were equilibrated after the first dimension run in SDS sample buffer (10% glycerol, 50 mM dithiothreitol, 2.3% SDS and 0.0625 M tris, pH 6.8) for 10min, then each tube gel was sealed to the top of a stacking gel that overlays a 10% acrylamide slab gel (1.0 mm thick). SDS slab gel electrophoresis was carried out for about 5 hrs at 25 mA/gel. The following proteins (Sigma Chemical Co, St. Louis, MO) were added as molecular weight markers: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000) carbonic anhydrase (29,000) and lysozyme (14,000). The gels were stained with Coomassie brilliant blue R-250 overnight, destained in 50% methanol/10% acetic acid and dried between sheets of cellophane. At least two gels were run with each sample. Spot density was determined by Progenesis SameSpots software (Non-Linear Dynamics,

Durham, NC). Spot density values were normalized by determining a single spot density value as the percentage of total density in all spots analyzed in that sample.

Mass spectrometry. Protein spots excised from Coomassie Blue-stained 2D gels were subjected to in-gel digestion followed by micro-purification using a ZipPlate micro-SPE according to manufacturer's In-Gel Digestion Protocol (Millipore) with minor modifications. Briefly, gel spots were destained in buffer 1 (25 mM ammonium bicarbonate and 5% acetonitrile) for 30 min and then in buffer 2 (25 mM ammonium bicarbonate and 50% acetonitrile) foran additional 30 min and the steps were repeated until the blue color disappeared. After reducing with 10 mM DTT and alkylating with 55 mM iodoacetamide, the gel pieces were digested in a 15-µL reaction solution (25 mM ammonium bicarbonate) containing 0.1 – 0.2 µg of trypsin and the reaction was incubated overnight at 37ºC. The solution containing the peptide digest obtained from extraction and micro-purification was dried in a vacuum centrifuge and resuspended in 1% formic acid and 2% acetonitrile for mass spectrometric analysis. Nano LC-MS/MS analysis was performed in a Micromass Q-TOF Ultima mass spectrometer equipped with a nanospray ion source and coupled with an nanoAcquity ultraperformance liquid chromatography system (UPLC) (Waters, Milford, MA). The acquisition of data was performed on a MassLynx data system (version 4.0) using a data-dependent mode where three most intense precursors in a survey scan were isolated for collision-induced dissociation. Resulting MS/MS data were used to search for protein candidates by automated database searching against the NCBInr database using MASCOT Daemon software (Matrix Sciences). An additional search was conducted against a database of proteins predicted from genome sequences of *Bartonella* species and closely related bacteria in the Order *Rhizobiales*.

Results

Four sets of flasks with confluent monolayers of HMECS were inoculated with *B. clarridgeiae* wild-type, the *fla*A mutant, the *mot*BC mutant or were grown without bacteria. After 5 days, the culture supernatants were recovered and the proteins were separated on 2-D gels. Over 400 protein spots were detected in each gel.

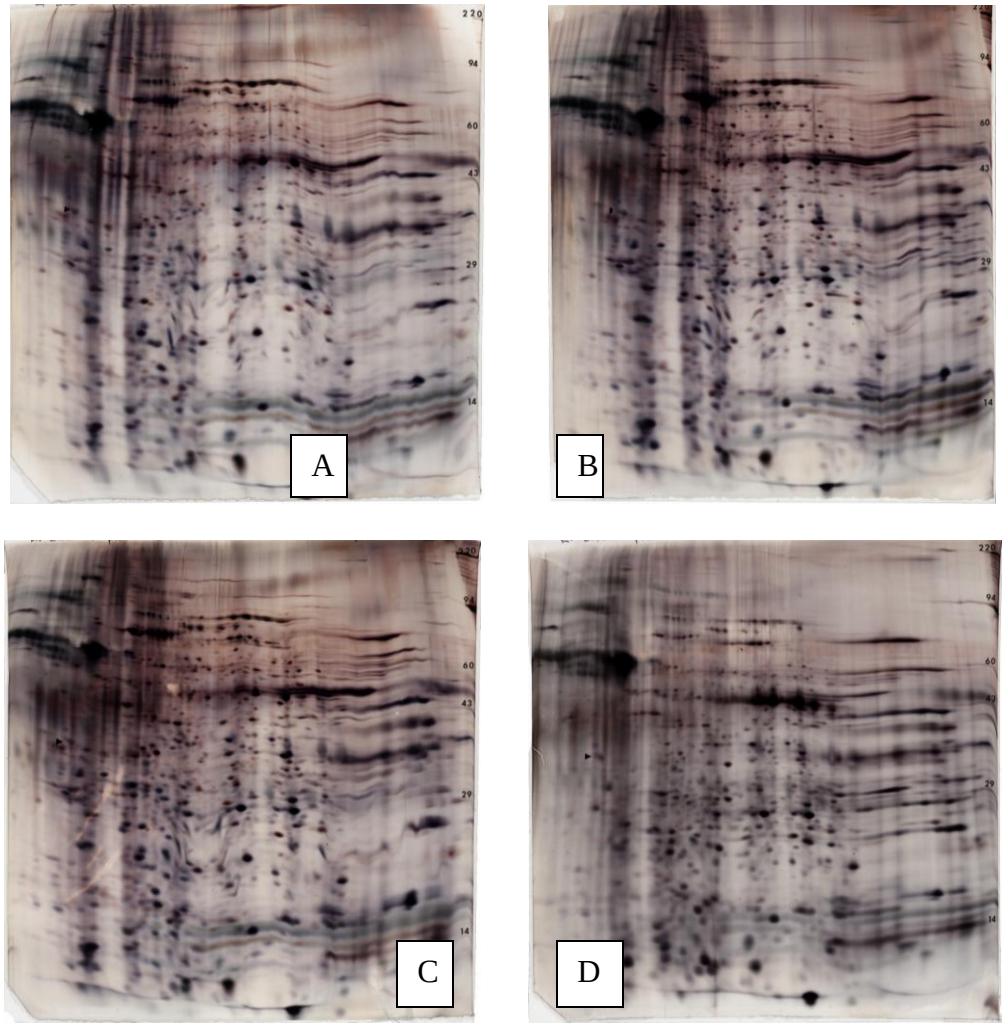


Figure 16. 2-D gel electrophoresis of proteins from culture media. A) uninfected HMECs; B) HMECs infected with *B. clarridgeiae* wild-type; C) HMECS infected with *fla*A mutant; D) HMECs infected with *mot*BC mutant.

Spot densities were determined and the percentage of total protein that each spot represented was calculated. To identify proteins that were common to all 4 gels, and therefore not secreted by HMECs because of bacterial infection or secreted by bacteria, 10 spots with the same approximate density in all 4 gels were cut out of the Coomassie stained gels and analyzed by mass spectrometry. More than one identification was made for 4 of the spots. This result could be due to 2 or more proteins having the same apparent molecular weight and pI, or the peptides identified were found in 2 or more proteins in the database.

Table 3. Identification of Proteins Found at Equivalent Densities in All HMEC Cultures

Spot	# of Potential Identities	Protein Name[1]	Accession ID. [2]	M.W. (kDa)	pI	Score [3]	Matched Peptides [4]	Sequence Coverage (%)
71	1	Glutathione synthetase	GSHB_HUMAN	52352	5.67	403	9	21
88	1	Plasminogen activator inhibitor 1 precursor	PAI1_HUMAN	45031	6.68	1146	51	64
	2	Fumarate hydratase	FUMH_HUMAN	54602	8.85	134	5	13
	3	Alpha-enolase	ENOA_HUMAN	47139	7.01	133	3	11
165	1	Transaldolase	TALDO_HUMAN	37516	6.36	229	6	16
227	1	Purine nucleoside phosphorylase	PNPH_HUMAN	32097	6.45	433	16	41
243	1	Nicotinamide N-methyltransferase	NNMT_HUMAN	29555	5.56	148	7	27
260	1	3,2-trans-enoyl-CoA isomerase	D3D2_HUMAN	32795	8.80	360	9	29
	2	Enoyl-CoA hydratase	ECHM_HUMAN	31367	8.34	223	10	26
307	1	Proteasome subunit beta type 2	PSB2_HUMAN	22822	6.51	330	9	38
	2	Peroxiredoxin-1	PRDX1_HUMAN	22096	8.27	110	3	15
317	1	Protein DJ-1	PARK7_HUMAN	19878	6.33	332	8	46
	2	Peroxiredoxin-2	PRDX2_HUMAN	21878	5.66	227	8	32
	3	Heme-binding protein 1	HEBP1_HUMAN	21084	5.71	163	6	39
	4	Ras-related protein Rab-11A	RB11A_HUMAN	24378	6.12	119	3	15
368	1	Hippocalcin-like protein 1	HPCL1_HUMAN	22299	5.21	132	5	24
395	1	Elongation factor 1-gamma	EF1G_HUMAN	50087	6.25	422	8	15

Many of the proteins found in all of the culture supernatants are involved in normal homeostasis and metabolism. Glutathione synthetase and peroxiredoxins are involved in protection from oxidative stress and regulation of cell proliferation. Transaldolase is an enzyme in the pentose phosphate pathway, and purine nucleoside phosphorylase acts in the metabolism of purine. Nicotinamide N-methlytransferase is an enzyme that recycles nicotinamide back to

NAD. Fatty acid oxidation uses 3,2-trans-enoyl-CoA isomerase. Hippocalcin-like protein 1 may inhibit apoptosis (Mercer *et al.*,2000; Korhonen *et al.*,2005). Elongation factor 1-gamma is a subunit of the multimeric human Elongation factor responsible for transferring aminoacyl-tRNAs to the ribosome during translation of mRNA to polypeptides.

We next examined spots on each 2-D gel that were differentially secreted. Sixty-seven spots were selected and subjected to trypsin digestion and LC-MS/MS spectrometry. Peptide fingerprint analysis and comparison to the NCBI non-redundant database and *Rhizobiales*-specific database revealed possible, plausible identifications for the majority of the proteins. MASCOT scores of more than 50 with 2 or more peptide matches are presented in Table 4.

When compared with uninfected HMECs, the culture supernatant from the HMECs in co-culture with wild-type *B. clarridgeiae* contained an increased amount of glycolytic proteins such as pyruvate kinase and malate dehydrogenase. During glycolysis, pyruvate kinase acts on phosphoenolpyruvate to produce pyruvate and ATP (Mazurek *et al.*,2005). Malate dehydrogenase is an enzyme necessary for carbohydrate catabolism that catalyzes the transition from malate to oxaloacetate in the Krebs cycle. The enzyme thioredoxin reductase (TrxR) is part of the thioredoxin (Trx) redox system which is found in virtually all organisms (Arner and Holmgren,2000). The Trx system has many functions including working with peroxiredoxins to reduce hydrogen peroxide, reducing ribonucleotides during DNA synthesis, regulating of transcription factors such as NFκB, and inhibiting of apoptosis (Arner and Holmgren,2000).

Other regulatory proteins were found in the wild-type *B. clarridgeiae* supernatant. Protein-L-isoaspartate O-methyltransferase (PIMT), which repairs damaged proteins, also protects cells against apoptosis (Huebscher *et al.*,1999). Heat shock proteins such as Hsp70 are found in most organisms and protect cells against stress including heat and can also act as

chaperones for other proteins. F-actin capping protein regulates the actin cytoskeleton distribution in cells and is essential for motility (Wear *et al.*,2003). The proteasome activator 28α subunit plays a role in degradation of proteins by stimulating the activity of the proteasome (Tanahashi *et al.*,1997). The proteasome degrades misfolded or damaged proteins that have been modified by ubiquitinylation and is involved in many, if not most, of the functions that take place in mammalian cells (Hilt and Wolf,2004).

Table 4. Identification of Proteins Found in Spots at Different Densities in the 4 Cultures

Protein	Source	Peptides	Score	Bc WT vs. NC	Bc flaA vs. NC	Bc motBC vs. NC
Pyruvate kinase M1/M2	Human	27	799	Up[a]	Up	Down
Thioredoxin reductase	Human	20	734	Up	Up	Down
Malate dehydrogenase	Human	3	122	Up	Nc	Up
Protein-L-isoaspartate O-methyltransferase	Human	14	413	Up[b]	Up	Up
HSP70	human	6	192	Up	Up	NC
Proteasome activator 28-alpha subunit	human	6	118	Up	Up	NC
Aldoketo reductase family 1	human	6	179	Up	Up	Up
Ubiquitin conjugating enzyme E2	human	3	73	NC	Up	NC
Galectin 1	human	4	90	NC	Up	NC
Tissue inhibitor of metalloproteinase 1	human	2	52	Up	Up	NC
Triose phosphate isomerase	human	7	161	Up	Up	NC
Stathmin	human	5	183	NC	Up	NC
Proteasome subunit beta type 3	human	17	544	Down	NC	NC
Vimentin	human	11	503	NC	Up	Down
Peroxiredoxin 1	human	6	222	NC	Nc	Up
SH3 domain binding glutamic acid rich protein	human	6	288	NC	Nc	Up
Plasminogen activator inhibitor 1	human	31	951	NC	Nc	Up
Dimethylarginine dimethylaminohydrolase	human	11	379	NC	Nc	Down
Lamin A/C	human	9	363	NC	Nc	Down
Insulin-like growth factor binding protein 7	human	3	127	NC	Nc	Down
Acetyl-Coenzyme A acetyltransferase	human	5	161	Up	Up	Down
PPIC-type PPIase domain protein	bacteria	4	266	NC	NC	Up
GroEL	bacteria	7	298	Up	NF	NF
GroES	bacteria	5	135	Up	Up	Up

NC, no change. Up, increased. Down, decreased. NF, not found. [a] medium blue, Up 2-5-fold. [b]Dark blue, Up 5-40-fold.

Tissue inhibitor of metalloproteinase-1 was also found in the *B. clarridgeiae* cell culture media. TIMP-1 is normally secreted and regulates extracellular matrix remodeling by binding matrix

metalloproteinases (Brew *et al.*,2000). However, conflicting roles of TIMP-1 have been published in which TIMP-1 blocks tumor angiogenesis but is also associated with accelerated tumor progression (Loboda *et al.*,2006).

The wild-type *B. clarridgeiae*-HMEC culture supernatant also contained proteins secreted by the bacteria. Acetyl-coenzymeA acetyltransferase catalyzes the first step in the mevalonate metabolic pathway which results in energy production. Most proteins need to be folded into a particular 3-dimensional structure in order to function, and GroEL and GroES are chaperonins, together with ATP, which cause the folding of many proteins (Lin and Rye,2006). Peptides matching *B. henselae* GroEL were identified in the wild-type culture media when the *Rhizobiales*-specific database was queried.

The *fla*A mutant-infected HMEC culture media and the wild-type culture media contained 8 of the same proteins at higher concentrations than in the uninfected HMEC media. The significant differences were that the *flaA* mutant culture media had at least a 1.5-fold decrease in malate dehydrogenase. A spot corresponding to GroEL was not detected. Proteins identified in the *fla*A mutant culture media that were more concentrated than in the gels of the wild-type and *mot*BC mutant media were galectin-1, ubiquitin conjugating enzyme E2, tissue inhibitor of metalloproteinase-1, stathmin, and triose phosphate isomerase. In addition, a cluster of spots were all identified as vimentin, and some of the spots were significantly increased in the *fla*A mutant culture (Figure 16).

Galectin-1 has intercellular and extracellular functions and is involved in integrin trafficking, adhesion, proliferation, and motility. It binds many types of molecules including carbohydrates and proteins (Horiguchi *et al.*,2003; Scott and Weinberg,2004; Camby *et al.*,2006; Fortin *et al.*,2008). Stathmin depolymerizes microtubules and is a critical component in

regulating the mitotic spindle of replicating cells as well as in remodeling microtubules at the leading edge of migrating cells (Wittmann *et al.*,2004). Ubiquitin conjugating enzyme E2 is part of the ubiquitin-proteasome system that degrades unneeded or misfolded proteins as mentioned earlier (Hilt and Wolf,2004). Ubiquitin is a 76 amino acid protein that is attached to proteins by the ubiquitin conjugating enzymes and results in a target for the proteasome (Hilt and Wolf,2004). Triosephosphate isomerase catalyzes the formation of glyceraldehyde-3 phosphate. And vimentin is an intermediate filament in the endothelial cytoskeleton (Gonzales *et al.*,2001).

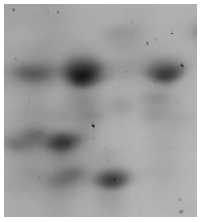
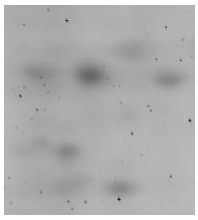
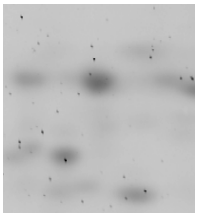
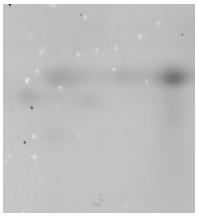
A.  B.  C.  D. 

Figure 17. Vimentin. 2-D gel electrophoresis of culture supernatants, silver stained. A. *fla*A mutant, B. uninfected control, C. wild-type *B. clarridgeiae*, D. *mot*BC mutant. Similar results seen in Coomassie stained gels (not shown).

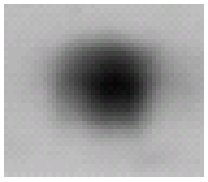
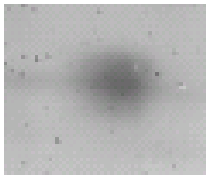
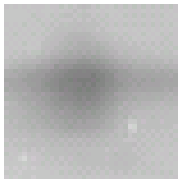
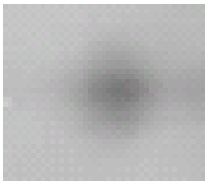
A.  B.  C.  D. 

Figure 18. Galectin. 2-D gel electrophoresis of culture supernatants, silver stained. A. *fla*A mutant, B. uninfected control, C. wild-type *B. clarridgeiae*, D. *motBC* mutant.

Pyruvate kinase, thioredoxin reductase, vimentin, DDAH, lamin A/C, insulin-like growth factor binding protein 7, as well as the bacterial protein acetyl-coA acetyltransferase were all decreased in the *mot*BC mutant-HMEC co-culture media. Proteins that were increased in the *mot*BC mutant media compared to the uninfected control culture media included peroxiredoxin

1, SH3 domain binding glutamic acid rich protein and plasminogen activator inhibitor 1 from the endothelial cells and GroES and PPIC-type PPIase domain protein secreted by the *B. clarridgeiae*. The GroES protein was 3-fold, 12-fold and 25-fold more concentrated than the uninfected culture in the wild-type, *fla*A, and *mot*BC mutant cultures, respectively. However, as with that of *fla*A, the *mot*BC culture media did not produce detectable amounts of GroEL.

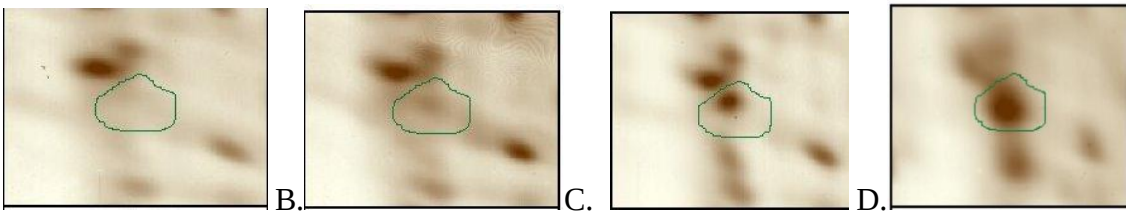


Figure 19. GroES. A. Uninfected control HMECs. HMECs infected with *B. clarridgeiae*: B. *fla*A mutant; C. wild-type; D. *mot*BC mutant.

Discussion

Angiogenesis, the formation of new capillaries from existing blood vessels, is a key component of bacillary angiomatosis and other vasoproliferative diseases caused by *B. bacilliformis*, *B. henselae* and *B. quintana*. Angiogenesis is also an important process during embryogenesis, development, wound healing and cancerous tumor progression. The formation of new capillaries involves the degradation of the surrounding extracellular matrix, proliferation and migration of endothelial cells, and formation of tubules that then become new capillaries. Several molecules are capable of directly or indirectly stimulating angiogenesis including vascular endothelial growth factor (VEGF), nitric oxide (NO), hypoxia inducible factor (HIF), and angiopoietin-1 (Ang-1).

We tested *B. clarridgeiae* wild-type, and two non-motile mutants that were devoid of flagella (*fla*A) or had flagella that were paralyzed (*mot*BC) for their ability to invade human

microvascular endothelial cells (HMECs) and cause proliferation (Chapter 1). All three strains were able to enter human endothelial cells but at different rates. The *fla*A mutant was deficient in attachment, and the *mot*BC mutant had the greatest percentage of attached and intercellular bacteria at later time points. All three strains caused some cytotoxicity at an MOI of 100, but induced proliferation of HMECs at an MOI of 10. The *fla*A mutant, however, induced more proliferation than the wild-type or *mot*BC strains at an MOI of 10. The mitogen responsible for *Bartonella*-induced angiogenesis remains elusive, although GroEL of *B. bacilliformis* has been recently implicated (Minnick *et al.*,2003; Smitherman and Minnick,2005). We sought to determine if secreted proteins from the endothelial cells and/or bacteria were responsible for the different characteristics seen among the three *B. clarridgeiae*-HMEC co-cultures. While there were proteins in common in the culture media of the 3 sets of infected HMECs, the wild-type, *fla*A and *mot*BC protein profiles were different from one another.

To better understand the significance of each protein identified in 2-D gel electrophoresis and MS/MS, the first question to address was whether the proteins found in culture supernatants are normally secreted by HMECs or other endothelial cells in culture. Gene expression and total cellular protein content of normal and stimulated human endothelial cells has been studied previously (Bruneel *et al.*,2003; Scheurer *et al.*,2004; Gonzalez-Cabrero *et al.*,2007; Katanasaka *et al.*,2007). However, the identity of proteins secreted (secretome) by human endothelial cells has not been well characterized to date. Some conclusions may be drawn from data for the rat endothelial secretome (Pellitteri-Hahn *et al.*,2006). Vimentin, plasminogen activator inhibitor-1, heat shock protein 70kDa protein 1 (Hsp70p1), pyruvate kinase, tissue inhibitor of metalloproteinase-1 (TIMP1), peroxiredoxins, alpha enolase and elongation factors were among the 225 proteins secreted by rat endothelial cells under normal conditions (Pellitteri-Hahn *et*

al.,2006). Several of these proteins were identified in our study in all 4 cultures at equal concentrations and others were differentially secreted in the HMECs infected with *B. clarridgeiae* (Tables 3 and 4). Therefore, it appears likely that many of the proteins we identified are normally secreted by HMECs and were not present due to cell lysis.

The culture supernatants from both the HMECs infected with the *B. clarridgeiae* wild-type and those infected with the *fla*A mutant strain exhibited an increase in pyruvate kinase, thioredoxin reductase, protein-L-isoaspartate-O-methyltransferase, Hsp70, triosephosphate isomerase and TIMP-1. Pyruvate kinase is upregulated in proliferating cells, especially tumor cells, and is under the control of Ras, and transcription factors hypoxia inducible factor-1 (HIF-1), SP1 and SP3 (Mazurek *et al.*,2005). Up-regulation of the pyruvate kinase isoenzyme PK-M2 is one of the hallmarks of cancer cells and is thought to help cells at the interior of a tumor survive in a low oxygen environment (hypoxia) (Mazurek *et al.*,2005). PK-M2 was detected at 3-fold higher concentration in hypoxic rat brain endothelial cells compared to normoxic conditions (Haseloff *et al.*,2006). Thioredoxin reductase (TrxR), a selenoprotein, reduces thioredoxin which is an essential anti-oxidant in humans (Cox *et al.*,2008; Peng *et al.*,2008). Intracellularly, thioredoxin reduces diverse proteins including peroxiredoxin and transcription factors such as NFκB and AP-1 (Cox *et al.*,2008; Peng *et al.*,2008). NFκB is only able to bind DNA in its reduced state. Thioredoxin also works with peroxiredoxin to increase HIF-1α expression and has been shown to do so under normoxic conditions in transfected breast cancer cells (Welsh *et al.*,2002). During oxidative stress and inflammation, thioredoxin is secreted by an unknown mechanism and is a co-cytokine and chemotactic factor (Arner and Holmgren,2000). TrxR was found to be actively secreted by peripheral blood mononuclear cells after stimulation by IFN-γ, LPS or IL-1α (Soderberg *et al.*,2000). Because inhibition of TrxR results in inhibition

of cancer cell growth (Yoo *et al.*,2007), induction of apoptosis (Cox *et al.*,2008; Peng *et al.*,2008), and decreased HIF-1α and VEGF expression (Powis *et al.*,2006),TrxR is an attractive therapeutic cancer treatment target. The increase in TrxR and PK seen in the wild-type and *fla*A supernatants may indicate hypoxia, oxidative stress and/or the presence of stimulated proliferating endothelial cells.

Protein-L-isoaspartyl-O-methyltransferase, one of the proteins whose concentration was increased in wild-type and flaA-infected cells, is an enzyme that repairs damaged proteins. Synthesis of PIMT is significantly upregulated when bovine aortic endothelial cells become detached from their substratum (Lanthier and Desrosiers,2006). Antibody to integrin αvβ3 significantly reduced the upregulation of PIMT expression in human umbilical vein endothelial cells (HUVECs) (Lanthier and Desrosiers,2006). However, Lanthier and Desrosiers point out that PIMT levels in attached and detached HMECs were equal to or higher than the level of upregulated PIMT seen in bovine cells or HUVECs (Lanthier and Desrosiers,2006). Therefore, the up-regulation of PIMT seen in our HMECs infected with wild-type and *fla*A mutant strains of *B. clarridgeiae* compared to the uninfected cells may be due to a factor other than detachment from the substratum (plastic). Recently, PIMT overexpression in pig aortic endothelial cells was found to prevent apoptosis induced by oxidative stress (Cimmino *et al.*,2008). In addition, substrates for PIMT during oxidative stress include Hsp70 and Hsp90, actin and Bcl-xl (Cimmino *et al.*,2008). Repair of deamidation sites on these proteins appears to be part of the anti-apoptotic response (Cimmino *et al.*,2008).

Heat shock protein 70 (Hsp70) was found in the culture supernatants of the wild-type and *fla*A mutant-infected HMECs at a higher concentration than in the uninfected or *mot*BC-infected media. Hsp70 and other heat shock proteins are constitutively expressed and play an important

role in protecting newly synthesized proteins from degradation. Hsp70 is overexpressed in some cancers (Aghdassi *et al.*,2007) and is often up-regulated during oxidative stress and after heat shock (Morano,2007). In two separate proteomic studies, HUVECs stimulated with VEGF expressed increased amounts of Hsp70 protein (Pawlowska *et al.*,2005; Katanasaka *et al.*,2007). In addition, unlike in many cell types, Hsp70 is downregulated in HMECs and HUVECs under hypoxic conditions (Oehler *et al.*,2000; Eguchi *et al.*,2008). Therefore, our data suggest that the wild-type and *fla*A-infected cells were stimulated but not hypoxic.

Triosephosphate isomerase, an enzyme up-regulated in wild-type and *fla*A mutant cultures, has been shown to be up-regulated in VEGF- or low dose ouabain-treated HUVECs (Qiu *et al.*,2007). Ouabain is a poison from the foxglove seed that blocks the sodium-potassium pump, and the pharmaceutical version of it, digitalis, has been used to treat cardiomyopathy since 1785. Recent studies have shown that at low doses, ouabain is mitogenic for HUVECs, but induces apoptosis at high doses (Qiu *et al.*,2008). Because triosephosphate isomerase is important in glycolysis and energy production, it is reasonable to find it and other glycolytic enzymes up-regulated in proliferating cells. Glycolytic enzyme expression is also upregulated by HIF-1, and an autocrine loop involves the end products of glycolysis, lactate and pyruvate, which promote HIF-1α protein stability (Lu *et al.*,2002; Milovanova *et al.*,2008).

Tissue inhibitor of metalloproteinase-1 (TIMP-1) is normally secreted from cells to regulate the matrix-degrading metalloproteinases. Matrix metalloproteinases are important for angiogenesis because they disrupt the extracellular matrix allowing endothelial cells to detach and migrate (Reed *et al.*,2003). TIMP-1, therefore, negatively regulates angiogenesis. In contradiction to this, up-regulation of TIMP-1 has been linked with several types of cancers, and high concentrations of TIMP in cancer patient plasma is correlated with increased mortality and

poor outcome (Porter *et al.*,2005). The function of TIMP as anti-angiogenic or anti-apoptotic may be tissue specific (Hornebeck *et al.*,2005). In HMECs, TIMP was down-regulated during hypoxia (Loboda *et al.*,2006). We found increased levels of TIMP-1 in the HMEC cultures infected with *B. clarridgeiae* wild-type and *fla*A mutant strains but not with the *mot*BC strain. The protein profile showing increased pyruvate kinase, thioredoxin reductase, PIMT and Hsp70 in the HMECs infected with the *B. clarridgeiae* wild-type and *fla*A mutant strains is most similar to the pro-angiogenic, anti-apoptotic scenario seen with HUVECs stimulated by exogenous VEGF (Pawlowska *et al.*,2005; Loboda *et al.*,2006; Katanasaka *et al.*,2007).

Proteasome activator PA28 was found in the supernatants of the wild-type and *fla*A mutant co-cultures, but the proteasome subunit beta type 3 was at a lower concentration in the wild-type media compared to the uninfected and other two infected cultures. The proteasome has an important role in cell cycle progression, apoptosis and signal transduction (Hershko and Ciechanover,1998). Inhibition of the proteasome results in apoptosis, and a proteasome inhibitor is being evaluated in clinical trials as an anti-cancer treatment to induce apoptosis in tumor cells (Sterz *et al.*,2008). However, other experiments indicate an important role of the proteasome in angiogenesis. Lactacystin, a proteasome inhibitor, suppressed the formation of tubules in HMEC cultures and prevented the production and secretion of plasminogen activator, a known component of the angiogenic process (Oikawa *et al.*,1998). The proteasome is made of 7 α and 7 β subunits, and all are necessary for proteasomal function. The significance of the β3 subunit in the media is unknown. However, HepG2 cells secrete proteasome α 4 subunit (Higa *et al.*,2008), and proteasome subunit α type 6 is increased in VEGF-stimulated HUVECs (Pawlowska *et al.*,2005).

The *fla*A mutant-infected culture supernatant contained several proteins that were more concentrated in the 2D gels than the same spots in the other 3 gels including ubiquitin conjugating enzyme E2, galectin 1, stathmin and vimentin. Ubiquitin conjugating enzyme E2 is part of the ubiquitin-proteasome system and is involved in labeling proteins for degradation by the proteasome. It has been found to be down-regulated in pulmonary artery endothelial cells in hypoxic conditions and increased in gastric, breast and lung cancer cell lines (Manalo *et al.*,2005; Chen *et al.*,2006; Tedesco *et al.*,2007; Hao *et al.*,2008).

Stathmin, galectin 1 and vimentin are all found at focal adhesions between endothelial cells and the extracellular matrix. By destabilizing microtubules, stathmin is involved in the cytoskeletal remodeling that takes place in cell migration and it also regulates the mitotic spindle during cell proliferation (Wittmann *et al.*,2004). Stathmin is up-regulated in many types of cancers, and inhibition of stathmin inhibits HUVEC proliferation, migration and tube formation (Mistry *et al.*,2007). Galectin-1 is a carbohydrate binding protein with many functions. It is secreted by endothelial cells and plays a role in adhesion to the extracellular matrix by cross-linking integrins to laminin and fibronectin, as well as in cell motility through its reorganization of the actin cytoskeleton in conjunction with RhoA (Camby *et al.*,2006). Galectin-1 expression was correlated with migration and invasiveness of glioblastoma cell lines (Jung *et al.*,2008). Galectin-1 is also mitogenic, and the concentration of galectin is associated with increased tumor progression (Camby *et al.*,2006). Vimentin is an intermediate filament of the cytoskeleton and is linked to angiogenesis through an interaction with the αvβ3 integrins (Gonzales *et al.*,2001). The αvβ3 integrins are expressed on endothelial and glial cells and mediate attachment to several ligands in the extracellular matrix during angiogenesis (Silva *et al.*,2008). Ligation of αvβ3 activates VEGF receptor signaling, activates the NFκB inflammatory reponse, suppresses

apoptosis, and induces cell migration (Ruegg *et al.*,2004). Anti-vimentin antibodies inhibit angiogenesis (van Beijnum *et al.*,2006), and vimentin protein levels were shown to increase in whole cell lysates of hypoxic endothelial cells compared to cells in normoxic conditions (Scheurer *et al.*,2004). Genes coding for vimentin, however, have not been shown to be upregulated by HIF-1 to date. Taken together, the presence of increased concentrations of stathmin, galectin and vimentin in the *fla*A mutant media is indicative of early stages of angiogenesis in which endothelial cells proliferate and migrate. The *fla*A protein profile correlates well with the increased mitogenicity of the *fla*A strain in MTT assays (Chapter I). Further tests are necessary to determine if the *fla*A mutant-infected cells have increased migration rates compared to the wild-type or *mot*BC mutant-infected HMECs.

In contrast to that of the wild-type or *fla*A mutant, the *mot*BC-infected HMEC culture media exhibited a decrease in secretion of pyruvate kinase, thioredoxin reductase, vimentin, dimethylarginine dimethylaminohydrolase 1 (DDAH-1), lamin A/C and insulin-like growth factor binding protein 7 (IGFBP7). Gastric cancer cells exhibit decreased expression of pyruvate kinase and thioredoxin reductase but show an increase in lamin A/C (Chen *et al.*,2006). Other dissimilarities with angiogenic cancer cells include observations of colon cancer tumor endothelial cells in which both vimentin and IGFBP7 were reported to be up-regulated (van Beijnum *et al.*,2006). IGFBP-7 is a secreted protein and has been found to be up-regulated in colorectal cancer cells and glioma cell lines and regulates glioma cell migration (Jiang *et al.*,2008). Conversely, IGFBP-7 is down-regulated in prostate and breast cancer cells (Unterman *et al.*,1991), and loss of IGFBP-7 has been implicated in melanoma formation (Wajapeyee *et al.*,2008). DDAH-1 and DDAH-2 metabolize asymmetric dimethyl-L-arginine which is a NO synthase inhibitor. Enhanced expression of DDAH-1 in a glioma cell line increases angiogenesis

by increasing nitric oxide synthesis and secretion of VEGF (Kostourou *et al.*,2002). However, in endothelial cells, DDAH-2 predominates, not DDAH-1 (Tran *et al.*,2000). VEGF expression and secretion are up-regulated by over-expression of DDAH-2, not DDAH-1, in bovine aortic endothelial cells independently of the NO/NOS pathway (Hasegawa *et al.*,2006). VEGF is upregulated when DDAH-2 binds to protein kinase A and phophorylates the transciption factor SP-1 which then binds to the VEGF promoter (Hasegawa *et al.*,2006). DDAH-2 is also up-regulated by low amounts of NO in a positive feedback loop (Sakurada *et al.*,2008). DDAH-2 was not identified in the culture supernatants in our study, but many spots subjected to MS/MS analysis did not yield positive identifications. Also, we did not measure the transcription or translation rates of any genes. Therefore, the expression and secretion levels of DDAH-2 in our study are unknown.

The media from the HMECs infected with the *mot*BC mutant strain had an increase, when compared to the uninfected control, in protein concentrations of malate dehydrogenase and PIMT similar to levels seen in the wild-type media. However, the *mot*BC mutant media had a 24-fold increase in the protein identified as aldo-keto reductase family 1 B1 (aldose reductase), while both the wild-type and *fla*A media had a more modest 3-fold increase. Aldose reductase is the rate-limiting enzyme in the polyl pathway in which glucose is reduced to sorbitol and then to fructose. When glucose concentrations are high, excess glucose enters the polyl pathway and is reduced by aldose reductase. The polyl pathway is also utilized for energy when oxidative phoshorylation is decreased due to low oxygen concentrations (Favaro *et al.*,2008). A by-product of the polyl pathway is ROS, reactive oxygen species, which cause oxidative stress if too concentrated. ROS stimulate HIF-1 up-regulated VEGF expression and angiogenesis (Xia *et al.*,2007). In diabetes, this angiogenesis can lead to retinopathy, nephropathy and other

microvascular complications (Chung and Chung,2005). Microvascular changes caused by hyperglycemia and diabetes include endothelial cell junction disruption, increased vascular permeability and blocked retinal capillaries (Caldwell *et al.*,2005). The blockage of blood flow then leads to hypoxia, HIF-1 activation, VEGF expression and proliferation. (Caldwell *et al.*,2005). Significantly, more than 50 articles have been published describing cases of neuroretinitis and other ocular manifestations caused by infection with *B. henselae, B. quintana* or *B. grahamii* have been reported (Kerkhoff *et al.*,1999; Michau *et al.*,2003; Mason,2004). To date, only *B. henselae* has been shown to cause hypoxia, but the other species may do the same or trigger angiogenesis in another manner causing symptoms similar to diabetic retinopathy.

The *mot*BC mutant media had an 8-, 11- and 40-fold increase in the concentrations of peroxiredoxin 1, plasminogen activator inhibitor 1 (PAI-1), and SH3-domain binding glutamic acid rich protein 3 (SH3BGRL3), respectively. Peroxiredoxin 1 expression increases in HUVECs stimulated with VEGF (Katanasaka *et al.*,2007) and in cells undergoing oxidative stress (Immenschuh and Baumgart-Vogt,2005). Patients with diffuse systemic sclerosis have lesions in which angiogenesis of dermal HMECs is impaired but the pattern of gene expression is pro-angiogenic and includes an increase in peroxiredoxin 1 (Giusti *et al.*,2006). SH3BGRL3 is a member of the thioredoxin super family but its function is unknown (Xu *et al.*,2005). Both SH3BGRL3 and PAI-1 are markers of poor prognosis in some cancers (Aviel-Ronen *et al.*,2008) even though PAI-1 has been shown to inhibit angiogenesis (Deng *et al.*,1996). PAI-1 is a secreted serine protease inhibitor (SERPINE1) and directly inhibits tissue plasminogen activator. When not inhibited, plasminogen activator converts plasminogen in the extracellular matrix to plasmin which then cleaves FasL and induces apoptosis (Bajou *et al.*,2008). The effect of PAI-1 appears to be dose-dependent, and low doses promote angiogenesis by inhibiting FasL-mediated

apoptosis (Bajou *et al.*,2008). HIF-1α is the major transcription factor involved in PAI-1 expression during hypoxia (Fink *et al.*,2002). PAI-1 is up-regulated in cysteine-rich 61 (Cyr61)-induced gastric cancer cell invasion in an HIF-1-dependent manner (Lin *et al.*,2008). HUVECs infected with *Rickettsia rickettsii* show an increase in PAI-1 mRNA stability, protein expression, and secretion (Shi *et al.*,1998), while bovine aortic endothelial cells demonstrate an increase in PAI-1 transcription rate following exposure to LPS, TNF-α or TGF-β (Sawdey *et al.*,1989). The gene for PAI-1 also has binding sites for transcription factors NFκB, SP-1, Smad and AP-1. Given that PAI-1 secretion was increased 10-fold in the *mot*BC-infected HMEC supernatant, aldose reductase was increased 24-fold, TIMP was not increased, and thioredoxin reductase was decreased, the *mot*BC-infected HMECs appear to be undergoing hypoxia and oxidative stress (Scheurer *et al.*,2004; Loboda *et al.*,2006).

B. henselae causes hypoxia and activation of HIF-1 in HUVECs. In addition, several HIF-1 regulated genes have been shown to be up-regulated in *B. henselae*-infected cells including VEGF, adrenomedullin, insulin-like growth factor binding protein 3, and alpha enolase (Kempf *et al.*,2005). We did not find any of those proteins in the culture supernatant of the HMECs infected with *B. clarridgeiae*. However, many of the protein spots did not generate conclusive identifications after MS peptide mass fingerprinting. It is not known whether *B. bacilliformis* causes hypoxia, but *B. bacilliformis* infected HMECs up-regulate HIF-1α 6 hours after inoculation, and HIF-2α and and HIF-3α after 1 hour (D. Kohlhorst, PhD dissertation). HIFs regulate over 100 other genes including VEGF receptors, erythropoietin, plasminogen activator inhibitor 1 (PAI-1), nitric oxide synthase 2, proteasome activator, proteasome subunits, ubiquitin conjugating enzymes, transcription factors, oxidoreductases, and cell surface receptors (Manalo *et al.*,2005; Hirota and Semenza,2006). In hypoxic conditions, VEGF is up-regulated

by HIF-1, is secreted and causes proliferation in an autocrine fashion. VEGF is also secreted by other cells *in vivo*, including macrophages, and stimulates endothelial cells in a paracrine angiogenic loop. VEGF-stimulated HUVECs *in vitro* have increased concentrations of heat shock 70 protein 8, heat shock 90 protein 1, peroxiredoxin 1, and many other proteins involved in signal transduction (integrins), glycolysis (pyruvate kinase), cytoskeletal regulation (vimentin) and metabolism (aldehyde dehydrogenase) (Pawlowska *et al.*,2005; Katanasaka *et al.*,2007). Most of these proteins have been found in the extracellular milieu in our study. The HMECs were not tested for hypoxia in our experiments, but the pH of the culture media remained neutral indicating that hypoxia, if it did occur, was not caused by bacterial overgrowth.

The different profiles of secreted human proteins identified in the culture supernatants of HMECs infected with the wild-type, the *fla*A mutant or the *mot*BC mutant of *B. clarridgeiae* indicate that the 3 strains induced different responses by the HMECs. The wild-type strain caused a pro-angiogenic response, and the *fla*A mutant-infected HMECs secreted proteins involved in cytoskeletal rearrangements indicative of cell migration. Proteins known to be expressed under hypoxic conditions and/or by HIF-1 up-regulation were secreted by the *mot*BC mutant-infected cells. We next examined the spots on 2-D gels that were identified as homologous to predicted proteins of *B. bacilliformis*, *B. henselae* and other close relatives to get clues as to the manner in which the different HMEC responses were induced. The bacterial proteins identified in the culture supernatants were peptidyl prolyl cis/trans isomerase C (PPIC)-type PPIase domain protein, GroEL, and GroES.

A PPIC-type PPIase domain protein was found at a higher concentration in the *mot*BC mutant-infected culture supernatant than the uninfected HMECs or the HMECs infected with either the wild-type *B. clarridgeiae* or the *fla*A mutant strain. Proteins that contain a PPIC-type

PPIase domain have been found in human cells (cyclophilins) as well as bacteria, fungi and plants and are involved in protein folding. Some PPIases are found in the bacterial periplasm and some, such as the Mip protein in *Legionella*, are outer membrane proteins (Debroy *et al.*,2006). The peptidyl prolyl isomerase SurA is a virulence determinant in *Proteus mirabilis* (Himpsl *et al.*,2008). Homologues of SurA have been found in *E. coli*, *Brucella* spp. and *Shigella flexneri* and are critical for folding of outer membrane proteins (Lazar and Kolter,1996; Dartigalongue and Raina,1998; Delpino *et al.*,2007; Purdy *et al.*,2007; Watts and Hunstad,2008). *B. tribocorum* has an identified *sur*A gene (BT_0824), and hypothetical proteins in the *B. quintana*, *B. henselae* and *B. bacilliformis* genomes are 81% identical to the SurA of *B. tribocorum*. *B. clarridgeiae* most likely also has a *sur*A homologue. *Helicobacter pylori* secretes a PPIase HP0175 which binds TLR4 in human gastric epithelial cells and activates apoptosis signal regulating kinase 1 (Basak *et al.*,2005). A human peptidyl-prolyl isomerase, Pin1, is over-expressed in many types of cancer cells and has recently been found to stimulate VEGF expression by activating HIF-1α and AP-1(Kim *et al.*,2008). It would be interesting to determine if the *B. clarridgeiae* PPIase has a similar effect on HMECs.

A GroEL homologue was identified in the wild-type *B. clarridgeiae* culture supernatant but was not found in the other two bacterial co-cultures or the uninfected control. GroES was found in all three supernatants from the infected HMECs, but was most concentrated in the *mot*BC gel and was least concentrated in the *fla*A gel (Figure 19). Given that the inocula and growth curves were similar for all three strains, a higher concentration of one strain over another cannot account for these differences. GroEL is actively secreted by *B. bacilliformis* and participates in the bacterial lystate's mitogenic effect on HUVECs (Minnick *et al.*,2003). Antibodies against GroEL or GroES reduced the mitogenicity of *B. bacilliformis* cell lysates by

40% suggesting that these proteins are involved in but are not solely responsible for the proliferative effect of the lysate (Minnick *et al.*,2003). The genes *gro*EL and *gro*ES are co-transcribed in *B. bacilliformis* (Callison *et al.*,2005), but it is unknown by what mechanism GroEL is secreted and whether GroES is secreted by the same mechanism. What is known, however, is that *B. bacilliformis* does not have a VirB T4SS, and the VirB system of other *Bartonella* species has been ruled out as a participant in the proliferative effect on human cells (Schmid *et al.*,2004). We have confirmed that both GroEL and GroES were secreted by the wild-type *B. clarridgeiae*, but only GroES was secreted by the *fla*A or *mot*BC mutant. It appears that the secretion of GroEL, but not GroES, requires a complete flagellar system.

Secretion of virulence factors by the flagellar T3SS has been demonstrated in *Campylobacter jejuni*, *Yersinia enterocolitica* and *Serratia liquifaciens*. In these organisms, secretion requires a minimal flagellar apparatus including the hook and basal body and flagellar T3SS (Givskov *et al.*,1995; Young *et al.*,1999; Young and Young,2002; Konkel *et al.*,2004; Guerry,2007). If the gene expression hierarchy for *B. clarridgeiae* flagellar assembly is similar to that elucidated for other bacteria, the *fla*A and *mot*BC mutants are predicted to have a minimal flagellar structure and flagellar T3SS. This appears not to be sufficient for GroEL secretion, or the *fla*A and *mot*BC mutations affect another necessary and as yet unknown component for secretion of GroEL.

The increased secretion of GroES by the *mot*BC mutant is similar to virulence factor secretion by a *Vibrio cholerae* flagellar motor mutant. The flagellar T3SS of *Vibrio cholerae* and the type 2 secretion system (T2SS) appear to interact. *Vibrio cholerae* secretes hemagglutinin/protease HapA and cholera toxin (CT) by a T2SS. Secretion of CT and HapA were significantly increased in a mutant with an interrupted gene encoding the flagellar motor

stator MotY (Silva *et al.*,2006). The polar flagellum of *V. cholerae* is driven by a sodium pump rather than a proton pump, and a sodium channel inhibitor decreased the secretion of CT in a wild-type strain and the *mot*Y mutant indicating that the sodium channel was still functioning in the untreated mutant (Silva *et al.*,2006). The authors hypothesized that the energy not used to produce flagellar torque in the *mot*Y mutant was used for secretion.

Crosstalk between the flagellar system and the general Type III secretion system in *Y. enterocolitica* has been reported. The Yop (yersinia outer proteins) virulence factor secretion system is negatively regulated by the flagellar master regulator FlhDC (Bleves *et al.*,2002). Expression of the *Y. enterocolitica* flagellar T3SS is inversely proportional to the expression of the Yops T3SS (Wilharm *et al.*,2004). Furthermore, the Yops T3SS is dependent on the proton motive force of the flagellar motor but Yops were secreted in a *mot*AB mutant (Wilharm *et al.*,2004). If the flagellar system in *B. clarridgeiae* and *B. bacilliformis* is constructed like that of their relative *Sinorhizobium meliloti*, the motor consists of MotAB (Platzer *et al.*,1997). MotC, which binds MotB, has been proposed as an energy transducer for the flagellar motor (Platzer *et al.*,1997). Flagellin and MotC also appear to be important for crosstalk between the flagellar system and the general Type III secretion system in *P. aeruginosa*. Gene expression of the T3SS regulon, secretion of the ExoS effector, and cytotoxicity were all decreased in *mot*AB and *mot*Y mutants but increased in the *mot*BC mutant and a *fliC* (flagellin) mutant (Soscia *et al.*,2007). There may be crosstalk between secretion systems in *B. clarridgeiae*, as the *mot*BC mutation caused a marked increase in GroES and PPIC PPIase secretion. However, the levels of GroES, as determined by 2-D gel electrophoresis, in the culture supernatant of the *fla*A mutant-infected cells were less than in the wild-type strain which may indicate a requirement for contact between the bacteria and HMECs. More work is needed to determine the gene expression hierarchy in

Bartonella flagellar assembly as well as the molecular mechanisms and pathways involved in regulating the secretion systems.

The master regulator for *Bartonella* flagellum protein synthesis and assembly has not been determined. FtcR has been identified in *Brucella melitensis*, a close relative of *Bartonella*, as a master regulator of flagellar gene expression (Leonard *et al.*,2007). A DNA-binding response regulator in the *B. bacilliformis* genome (BARBAKC583_1123) has 78% amino acid similarity to FtcR and is a potential candidate for the master regulator role. Other regulators have been shown to control flagellar and general T3SSs directly or indirectly. In *Bordetella bronchiseptica,* expression of the sensor kinase and response regulator BvgAS negatively regulates flagellar gene transcription and motility but positively regulates T3SS secretion of virulence factors (Akerley and Miller,1993; Han *et al.*,1999; Mattoo *et al.*,2004). The two component system BarA/SirA in *Salmonella* indirectly represses the flagellar system upstream of the flhDC master regulator genes and directly activates the HilA virulence regulon (Teplitski *et al.*,2003). The genomes of *B. bacilliformis*, *B. henselae*, *B. quintana* and *B. tribocorum* contain several genes for sensor histidine kinases/response regulator two-component systems including BatRS which are homologous to Bvg. The *bat*R and *bat*S genes were partially sequenced from *B. clarridgeiae* during this study when non-motile transposon mutants were initially screened, (data not shown). The role of BatRS in *Bartonella* virulence, and specifically in regulation of the flagellar system, is undetermined.

The profile of proteins secreted by *mot*BC-infected HMECs is indicative of cells that are experiencing hypoxia. Proliferation of HUVECs and HMECs is triggered, at least in part, by hypoxia and the subsequent activation of HIF-1 after *B. henselae* infection (Kempf *et al.*,2005). Other bacteria, however, activate and/or stabilize HIF-1 without hypoxia, as do some viruses

(Nasimuzzaman *et al.*,2007). *Y. enterocolitica*, *S. enterica* subspecies *enterica* and *E. aerogenes* co-cultured with HMECs activated HIF-1 at a higher level than *B. henselae* and in an oxygen-independent manner (Hartmann *et al.*,2008). In addition, it was determined that the Enterobacteriaceae's siderophores (yersiniabactin, salmochelin and aerobactin) were critical for HIF-1 activation and VEGF secretion, and bacteria-host cell contact was not necessary (Hartmann *et al.*,2008). Siderophore-dependent induction of HIF-1-regulated adrenomedullin and hexokinase were also reported (Hartmann *et al.*,2008). The siderophores may compete with the host cells for iron. Iron deprivation would then cause the prolyl hydroxylases, which require iron, to be unable to hyroxylate the HIF-1α molecules and target them for degradation (Hartmann *et al.*,2008). The siderophore enterobactin has recently been shown to induce a pro-inflammatory response in respiratory epithelial cells *in vitro* resulting in secretion of IL-8, the product of an NFκB-regulated gene (Nelson *et al.*,2007). Siderophores have not been described for *Bartonella* species; however, they are produced by close relatives of *Bartonellae* such as *Brucellae* and *S. meliloti*. In addition, the *B. henselae*, *B. bacilliformis* and *B. tribocorum* genomes contain ferric anguibactin (siderophore) transport system gene homologues. Siderophores may be an additional virulence factor for *Bartonella*.

If secretion of proteins is an indication of an intracellular increase in production of those proteins, given previous results, the presence of increased Hsp70 and TIMP-1 in the culture supernatants of the wild-type and *fla*A-infected HMECs suggests that proliferation is occurring without hypoxia (Oehler *et al.*,2000; Loboda *et al.*,2006). Minick and co-workers reported that GroEL has a role in mitogenicity induced by *B. bacilliformis* perhaps by acting as a chaperone for an unidentified protein or by directly triggering signal transduction and cytokine secretion (Minnick *et al.*,2003). Recently, incubation of purified *B. bacilliformis* GroEL with HMECs

revealed a significant increase in the tubule formation that is characteristic of angiogenesis (D. Kohlhorst, Ph.D. Dissertation). We found that only the wild-type strain of *B. clarridgeiae* secreted GroEL suggesting that the flagellar complex has a direct or indirect role in GroEL secretion. The *fla*A mutant induced more proliferation than the other strains at an MOI of 10, and the galectin, stathmin and vimentin identified in the supernatant are indicative of cytoskeletal rearrangement and cell migration. The bacterial factors secreted by the *fla*A mutant that could account for the cytoskeletal changes and proliferative effect were not identified, but GroEL was not found. The *mot*BC strain attached and entered the HMECs better than the wild-type strain, and proteins found in the culture supernatant suggest that the HMECs were undergoing hypoxia. A high concentration of GroES in the culture media may indicate that a non-flagellar T3SS or other type of secretion system was up-regulated in the *mot*BC mutant. Crosstalk between secretion systems may be occurring in a similar fashion to that found in *P. aeruginosa* in which a flagellin mutant and a *mot*CD mutant each demonstrated increased secretion of virulence factors through the general T3SS (Chapter I, (Soscia *et al.*,2007)).

If GroEL is secreted by all *Bartonella* species, it may be secreted by the flagellar T3SS in *B. bacilliformis*, *B. clarridgeiae* and the other flagellated *Bartonella*, and secreted by the Trw secretion system in the *Bartonella* lacking flagella. It has been proposed that the Trw system has replaced flagella during radial speciation (Dehio,2008). *B. clarridgeiae* is motile but does not swim particularly well, and the ancestral species *B. bacilliformis* does not have genes encoding typical chemotaxis proteins such as CheW or CheY. This may indicate that the flagellar system in Bartonella in addition to or other than motility. We found that the non-motile flagellated *mot*BC mutant attached to and entered the HMECs better than the wild-type strain. The *Bartonella* flagellar system, therefore, may be used more as a T3SS than for motility.

SUMMARY

B.bacilliformis, *B. henselae* and *B. quintana* are pathogenic for humans and cause vasoproliferative diseases in immunosuppressed or immunocompromised individuals. *B. clarridgeiae* has been implicated in human infections such as cat scratch disease, yet the bacteria have never been isolated directly from human clinical specimens. *B. clarridgeiae* has been found in cats and has been postulated to be the etiologic agent of endocarditis and hepatic disease in dogs (Chomel *et al.*,2001; Gillespie *et al.*,2003). If *B. clarridgeiae* is capable of causing vasoproliferative disease in humans characterized by angiogenesis, one of the first steps would most likely be invasion of endothelial cells. We tested *B. clarridgeiae* with human microvascular endothelial cells *in vitro* and found that the bacteria formed clumps on the endothelial cell surface but entered the cell as individual bacteria. The endothelial cells proliferated in the presence of *B. clarridgeiae,* particularly at a low inoculum of MOI 10. A high MOI of 100 was cytotoxic.

Two non-motile mutants of *B. clarridgeiae* were created to evaluate the role that the flagellar system may play in virulence. The *fla*A gene was disrupted in one mutant and no flagella were visible by microscopy. A flagellar motor mutant with a disruption of the *mot*B and *mot*C genes had paralyzed flagella. Both mutants entered endothelial cells as single bacteria, and the *mot*BC mutant attached to and entered the endothelial cells significantly better than the *fla*A mutant. One of the *mot*BC-infected cells showed signs of increased cytoskeletal involvement (lamellipodia and filopodia) by electron microscopy, but a qualitative assessment of changes in cytoskeletal features was not performed. Both mutants caused proliferation of the endothelial cells, but the *fla*A mutant had a more dramatic increase in proliferation than the

wild-type or *mot*BC mutant. The *fla*A and *mot*BC mutants caused cytotoxicity at high doses similar to that of the wild-type.

The proliferative effect of the *fla*A mutant and the increased invasion by the *mot*BC mutant were similar to the effects of a *vir*B4 deletion in *B. henselae*. The increased proliferation by the *vir*B mutant was postulated to be due to the elimination of cytostatic and cytotoxic effects of VirB (Schmid *et al.*,2004). The increased invasion was thought to be due to increased endocytosis which would engulf *B. henselae* faster than the VirB-dependent invasome formation would (Schmid *et al.*,2004). *B. clarridgeiae* has *vir*B genes but it is not known whether the VirB system is functional. A relationship between the flagellar system and VirB has not been postulated previously, perhaps because the well-studied *Bartonella* species do not possess both systems. *B. bacilliformis* does not have a VirB system, and *B. henselae* and *B. quintana* do not have a flagellar system. However, crosstalk between the flagellar system and the general T3SS in *P. aeruginosa* and *Y. enterocolitica* have been reported (Wilharm *et al.*,2004; Soscia *et al.*,2007), as well as crosstalk between the flagellar system and the T2SS in *V. cholerae* (Silva *et al.*,2006). *B. clarridgeiae* offers the opportunity to investigate an interaction between the flagellar T3SS and the VirB T4SS in the future.

The chaperonin GroEL was identified in the culture supernatant of the HMECs infected with the wild-type *B. clarridgeiae* but not in the *fla*A or *mot*BC cultures. This was surprising considering that GroEL was thought to be involved in the mitogenicity of *B. bacilliformis* (Minnick *et al.*,2003), and the *B. clarridgeiae fla*A mutant in our study induced more proliferation than the wild-type strain. We conclude that GroEL secretion requires a complete flagellar system. GroES, a co-chaperonin, was found at a higher concentration in the *mot*BC mutant culture than in the culture supernatants of the *fla*A mutant or wild-type. This result may

indicate that another export system was up-regulated due to the flagellar motor mutation, as seen in other bacteria. The low concentration of GroES was inversely proportional to the degree of proliferation induced by the *fla*A mutant. The high concentration of GroES in the supernatant was associated with the strain that was highly invasive (*mot*BC). Studies with *B. bacilliformis* by Minnick et al., and Kohlhorst (GSU) have examined the effect of GroEL levels on HUVECs and found that low concentrations of GroEL are mitogenic and high concentrations are inhibitory (Minnick *et al.*,2003; Smitherman and Minnick,2005)(D. Kohlhorst, PhD Dissertation 2008). However, GroES levels were not measured in those studies. Determining the role of GroES and GroEL in proliferation of endothelial cells and invasion by *B. clarridgeiae* and other *Bartonella* species will require further investigation.

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